UNIVERSIDADE ESTADUAL DE CAMPINAS

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ESTRUTURA E ULTRA-ESTRUTURA DOS ESPERMATOZÓIDES DE ALGUNS HYMENOPTERA (INSECTA)

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

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Co-orientadora: Profa. Dra. Sônia Nair Báo

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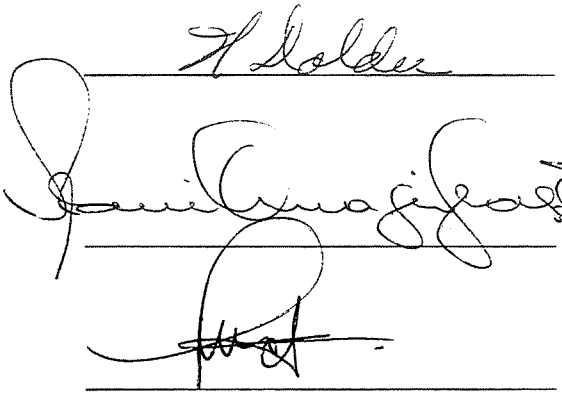

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Índice

1. Resumo

A enorme diversidade morfológica dos espermatozóides tem levado a um extensivo número de estudos taxonômicos e filogenéticos feitos a partir da ultra-estrutura dessas células em diversos grupos animais. Os insetos constituem o maior e mais diverso grupo do reino animal. Sua diversidade está também expressa na morfologia de seus espermatozóides, os quais apresentam um número de variações ultra-estruturais não observado em nenhum outro grupo. Por isso a análise filogenética a partir da morfologia dessas células tem despertado o interesse também de entomologistas sistematas e muitos trabalhos têm sido produzidos em vários grupos de insetos usando esta nova fonte de caracteres. Além da alta diversidade, dados morfológicos a partir dos espermatozóides assumem uma maior importância em estudos filogenéticos e taxonômicos por serem de natureza mais conservativa do que aqueles considerados tradicionais. O presente trabalho tem como objetivo fornecer informações morfológicas dos espermatozóides de algumas espécies dessa ordem. Para tanto usamos a microscopia de luz e eletrônica para descrever a estrutura e ultra-estrutura dos espermatozóides de três espécies de Trichogrammatidae, uma de Eurytomidae e duas de Scelionidae. Também fornecemos novos dados ultra-estruturais da região de transição núcleo-flagelo do espermatozóide de *Apis Mellifera*. Embora, o total de famílias de Hymenoptera cujos espermatozóides estão descritos ainda seja muito pequeno para se fazer uma análise filogenética, nós acreditamos que este estudo confirma a presença de uma variedade de caracteres nestas células que poderão contribuir para o entendimento das relações evolutivas dos Hymenoptera.

2. Abstract

The almost unlimited morphological diversity of the spermatozoa has led to many taxonomic and phylogenetic studies based on the ultrastructure of these cells in various animal groups. The insects constitute the largest and most diverse group of the animal kingdom and their diversity is also very well expressed by their spermatozoa, which present ultrastructural variations not found in any other group. For this reason, a phylogenetic analysis based on the morphology of these cells has called the attention of systematic entomologists and many studies have been produced in the various insect groups, using this new source of characters. Besides their great diversity, the morphologic data obtained from spermatozoa is particularly important in phylogenetic and taxonomic studies, since in these cells their ultrastructure is more highly conserved than the morphologic characters traditionally considered. In an attempt to contribute to the understanding of hymenopteran phylogeny, the present thesis aims to furnish morphological information of the spermatozoa of some species of this order. Considering this objective, we used the light and electron microscopes to describe the sperm structure and ultrastructure of three species of Trichogrammatidae, one of Eurytomidae and two of Scelionidae. We also furnish new ultrastructural data of the nucleus-flagellum transition zone of *Apis mellifera* spermatozoa. Although the total number of hymenopteran spermatozoa as yet described is still too small to permit a phylogenetic study, we believe that this research demonstrates the large variety of characters found in these cells, which will surely contribute towards the understanding of evolutionary relationships of the Hymenoptera.

3. Introdução

Os Hymenoptera, com pouco mais de 115.000 espécies descritas (Gauld & Hanson, 1995), constituem uma das quatro maiores ordens de insetos. Embora haja divergência, atualmente são reconhecidas por volta de 80 famílias, divididas nas subordens Symphyta e Apocrita (LaSalle & Gauld, 1992). Os Symphyta são considerados mais basais e contém pouco mais de 5% das espécies já descritas, distribuídas nas superfamílias: Cephoidea, Megalogontoidea, Orussoidea, Siricoidea, Tenthredinoidea e Xyeloidea. Os Apocrita contém a grande maioria das espécies e são, ainda, divididos em Aculeata e Parasitica. Os Aculeata, representando pouco mais de 45% das espécies descritas (Gaston, 1992), são considerados o grupo mais derivado dos Hymenoptera, no qual o ovipositor foi modificado em ferrão. Eles compreendem 19 famílias (nas superfamílias Chrysidoidea, Vespoidea e Apoidea), onde Apidae (abelhas), Formicidae (formigas) e Sphecidae contêm o maior número de espécies (LaSalle & Gauld, 1992). O grupo Parasitica é definido como sendo formado por todos os Apocrita que não tiveram o ovipositor modificado em ferrão. Este é o maior grupo de Hymenoptera, com aproximadamente 50% das espécies descritas, entretanto ele pode, eventualmente, conter 75% de todos os Hymenoptera. Os Parasitica são classificados em 48 famílias distribuídas em 11 superfamílias (Mason & Huber, 1993), sendo a maioria das espécies pertencentes às superfamílias Ichneumonoidea e Chalcidoidea (Gaston, 1992).

Hymenoptera, além de ser uma das ordens mais ricas em espécies, é também uma das que apresentam maior diversidade biológica (Gaston, 1991). Os Hymenoptera podem ser encontrados em grande número em quase todos os ecossistemas terrestres e, mais importante, se interagem com outras espécies do ecossistema mais do que qualquer outro grupo de insetos. Seus membros podem ser fitófagos, entomófagos ou uma combinação de ambos. Os entomófagos podem, ainda, ser predadores ou parasíticos, com uma variedade de hábitos intermediários tão grande que, às vezes, dificulta a sua classificação. Também foi nesta ordem que o comportamento social alcançou seu auge. Nos insetos, com exceção dos Isoptera, a eusociabilidade está presente apenas nos Hymenoptera. Contudo, somente nesta ordem existem grupos (abelhas e vespas) que exibem todas as gradações de organização social, de solitária a eusocial avançada.

Segundo Hanson (1995), os Hymenoptera contém mais espécies benéficas do que qualquer outra ordem de insetos. Como a maioria é parasitóide de outros insetos, muitas espécies desempenham papel importante no equilíbrio natural das populações de seus hospedeiros. Por esta mesma razão, os Hymenoptera são de longe os insetos mais usados em programas de controle biológico, tanto de pragas agrícolas como florestais. Entre os membros fitófagos da ordem, as abelhas constituem o grupo mais

importante de polinizadores, sendo absolutamente essenciais à manutenção da diversidade das angiospermas (Hanson, 1995). Qualquer redução na diversidade deste grupo de insetos causará seguramente um impacto ambiental pelo desaparecimento de várias espécies vegetais. Entretanto, impacto mais direto será sobre a alimentação humana, pois aproximadamente 30% desta vem de espécies vegetais polinizadas por abelhas (O'Toole, 1992).

Embora seja uma pequena minoria, entre os Hymenoptera há também espécies que podem causar grandes prejuízos econômicos. Dentre estas, as formigas cortadeiras (*Atta* e *Acromyrmex*) são consideradas as mais sérias. Por exemplo, no Estado de São Paulo a redução das pastagens, por elas, é equivalente àquela consumida por um milhão de cabeças de gado (Cherrett, 1986).

Apesar da inquestionável importância econômica e ecológica dos Hymenoptera, ainda existem muitas dúvidas ou controvérsias sobre as relações evolutivas desta ordem de insetos (Rasnitsyn, 1988; Gauld & Bolton, 1988; Dowton & Austin, 1994; Dowton *et al.*, 1997; Ronquist *et al.*, 1999). A primeira e mais completa hipótese filogenética a respeito dos Hymenoptera foi proposta por Rasnitsyn (1988). A maioria dos estudos feitos posteriormente tem abrangido apenas grupos específicos dentro da ordem, praticamente não alterando as relações filogenéticas propostas inicialmente por este autor. Ainda, vários estudos utilizando dados moleculares em geral não têm chegado às mesmas hipóteses filogenéticas propostas a partir da morfologia somática (Cameron, 1993; Dowton & Austin, 1994; Dowton *et al.*, 1997; Whitfield, 1998).

Nós acreditamos que provavelmente a maioria das controvérsias ou dúvidas a respeito da filogenia dos Hymenoptera, como um todo ou de grupos específicos, somente será resolvida com análises conjuntas usando dados moleculares e morfológicos, incluindo nestes, novos sistemas de caracteres, especialmente aqueles obtidos a partir de caacterísticas menos susceptíveis às condições ambientais.

Como os aspectos morfológicos do espermatozóide são definitivamente característicos da espécie que o produz, constituindo, portanto, um caracter único de identidade para a espécie, e como, obviamente, a sua evolução acompanha a evolução da espécie, a ultra-estrutura dessa célula vem sendo largamente usada em estudos taxonômicos e filogenéticos de vários grupos de animais, incluindo os insetos (Baccetti, 1972; Dallai, 1979; Dallai & Afzelius, 1990, 1995; Carcupino *et al.*, 1995; Jamieson *et al.*, 1999). Em Hymenoptera, cada vez mais se tem demonstrado que a diversidade morfológica dos espermatozóides é suficiente para compor um sistema de caracteres (Quicke *et al.*, 1992; Lino-Neto *et al.*, 1999, 2000a, b; Lino-Neto & Dolder, 2001a). Associado a outros, esse sistema poderá ser usado em estudos filogenéticos para resolver vários pontos controvertidos das relações evolutivas dos Hymenoptera. Entretanto, até então se encontravam apenas 9 trabalhos na literatura tratando da ultra-

estrutura de espermatozóides maduros destes insetos. Ainda destes, cinco tratam da morfologia dos espermatozóides de apenas uma espécie, *Apis mellifera*, e em alguns outros as descrições apresentadas não mostram detalhes suficientes para se estabelecer comparações. Portanto, se considerarmos que os Hymenoptera constituem uma das maiores ordens de insetos, com aproximadamente 80 famílias, fica evidente que este número de trabalhos é extremamente pequeno e, portanto, o conhecimento da morfologia dos espermatozóides destes insetos está apenas começando.

3.1. Morfologia geral dos espermatozóides de Hymenoptera

De modo geral, os espermatozóides dos Hymenoptera são semelhantes àqueles considerados típicos para os Pterygota (Baccetti, 1972). Portanto, eles são finos, geralmente muito longos e apresentam a região de cabeça formada, anteriormente, por um acrossomo que é seguido pelo núcleo (Quicke *et al.*, 1992; Jamieson *et al.*, 1999). O acrossomo, em geral, é formado pela vesícula acrossomal e pelo perforatorium, o qual tem a base inserida em uma cavidade na ponta do núcleo. O núcleo também é geralmente bastante longo e com a cromatina muito elétron densa e bastante compacta.

Na maioria dos Hymenoptera, o flagelo é formado por um axonema, dois derivados mitocondriais e dois corpos acessórios. O axonema apresenta 9 + 9 + 2 microtúbulos: sendo nove túbulos acessórios simples, nove duplas periféricas e dois microtúbulos centrais simples. Os dois derivados mitocondriais geralmente apresentam cristas e podem, ou não, conter material paracristalino. Eles também podem, ou não, ser iguais em diâmetro e comprimento (Cruz-Hölfing *et al.*, 1970; Lensky *et al.*, 1979; Cruz-Landim & Beig, 1980; Cruz-Landim & Silva de Moraes, 1980; Wheeler *et al.*, 1990; Quicke *et al.*, 1992; Peng *et al.*, 1992, 1993; Newman & Quicke, 1998). Os dois corpos acessórios (Baccetti, 1972) são estruturas longas, situadas entre os derivados mitocondriais e o axonema e, geralmente, possuem formato aproximadamente triangular, em corte transversal. Nos espermatozóides dos Chalcidoidea já estudados, diferente da maioria dos Hymenoptera, o núcleo, os derivados mitocondriais e o axonema apresentam um curso em espiral (Lee & Wilkes, 1965; Wilkes & Lee, 1965; Hogge & King, 1975; Quicke *et al.*, 1992).

3.2. Objetivos

Este trabalho tem como objetivo geral aumentar as informações sobre a morfologia dos espermatozóides dos Hymenoptera, procurando descrever elementos que sirvam como caracteres em estudos filogenéticos deste grupo de insetos. Como objetivo específico, descrever a estrutura e ultra-estrutura dos espermatozóides dos Chalcidoidea: *Bephratelloides pomorum* (Eurytomidae), *Trichogramma pretiosum*, *T. atopovirilia* e *T. dendrolimi* (Trichogrammatidae), dos Platygastroidea: *Trissolcus basalis* e *Telenomus podisi* (Scelionidae) e do Apoidea: *Apis mellifera* (Apidae).

4. Artigos publicados ou aceitos para publicação

Durante a realização desta tese foram produzidos cinco trabalhos, dos quais três já foram publicados e dois estão aceitos para publicação. São eles:

4.1. **Lino-Neto, J.**, Báo, S.N. & Dolder, H. (1999). Structure and ultrastructure of the spermatozoa of *Bephratelloides pomorum* (Fabricius) (Hymenoptera: Eurytomidae). *International Journal of Insect Morphology and Embryology* **28**: 253-259.

4.2. **Lino-Neto, J.**, Báo, S.N. & Dolder, H. (2000a). Structure and Ultrastructure of the Spermatozoa of *Trichogramma pretiosum* Riley and *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae). *Acta Zoologica* **81**: 205-211.

4.3. **Lino-Neto, J.**, Báo, S.N. & Dolder, H. (2000b). Sperm ultrastructure of the honey bee (*Apis mellifera*) (L) (Hymenoptera, Apidae) with emphasis on the nucleus-flagellum transition region. *Tissue & Cell* **32**(4): 322-327.

4.4. **Lino-Neto, J.** & Dolder, H. (2001a). Ultrastructural characteristics of the spermatozoa of Scelionidae (Hymenoptera; Platygastroidea) with phylogenetic considerations. *Zoologica Scripta* **30**(1): (in press).

4.5. **Lino-Neto, J.** & Dolder, H. (2001b). Redescription of sperm structure and ultrastructure of *Trichogramma dendrolimi* (Hymenoptera: Chalcidoidea: Trichogrammatidae). *Acta Zoologica* **82**: (in press).

4.1. **Lino-Neto, J.**, Báo, S.N. & Dolder, H. (1999). Structure and ultrastructure of the spermatozoa of *Bephratelloides pomorum* (Fabricius) (Hymenoptera: Eurytomidae). *International Journal of Insect Morphology and Embryology* **28**: 253-259.

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Structure and ultrastructure of the spermatozoa of *Bephratelloides pomorum* (Fabricius) (Hymenoptera: Eurytomidae)

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Abstract

The spermatozoa of *Bephratelloides pomorum* are very long and fine. Each spermatozoon measures about 620 μm in length by 0.38 μm in diameter and, when seen under the light microscope, appears to be wavy along its entire length. The head, which is approximately 105 μm, comprises a small acrosome and a nucleus. The acrosome is made up of a cone-shaped acrosomal vesicle surrounding the perforatorium and the anterior end of the nucleus. Innumerable filaments radiate from it. The perforatorium has a diameter equal to that of the nucleus at their junction, where it fits with a concave base onto the rounded nuclear tip. The nucleus is helicoidal and completely filled with homogeneous compact chromatin. It is attached to the tail by a very long and quite electron-dense centriolar adjunct that extends anteriorly from the centriole in a spiral around the nucleus for approximately 8.5 μm. The tail consists of an axoneme with the 9+9+2 microtubule arrangement pitched in a long helix, as well as a pair of spiraling mitochondrial derivatives (with regularly arranged cristae) that coil around the axoneme, and two small accessory bodies. As well as the spiraling of the nucleus, mitochondrial derivatives and axonemal microtubules, the sperm of *B. pomorum* present other very different morphological features. These features include the acrosome and centriolar adjunct, both of which differentiate the spermatozoa from the majority of sperm found in other Hymenoptera. In addition these structural variations demonstrate that the sperm of chalcidoids provide characteristics that can certainly prove useful for future phylogenetic analysis at the subfamily level and, possibly, the genus too.

Keywords: Chalcidoids; Wasps; Parasitoids; DAPI; Centriolar adjunct

1. Introduction

The Hymenoptera constitutes one of the largest insect groups, when considering the total number of specimens, and their members are ecologically and economically very important. In this group, the chalcidoids or chalcid wasps comprise the family with the most numerous and diverse species in relation to structure and biology. Some chalcidoids are economically harmful to man in that they are phytophagous (particularly the seed-feeding Eurytomidae) or can be hyperparasites. However, the large majority prove to be beneficial since they are parasites of other insects. As a consequence, chalcidoids carry out an important role in the natural equilibrium of the numbers of host insects, and for this reason they have been used widely in biological pest control. In spite of their importance, the chalcidoids are not well known and their taxonomy and phylogeny are still incomplete.

Gamete structure and ultrastructure have been extensively used for solving various systematic and phylogenetic problems in many animal groups, including insects (Baccetti, 1972; Dallai, 1979; Jamieson, 1987; Carcupino et al., 1995; Dallai and Afzelius, 1995).

With the exception of studies on the sperm of bees,

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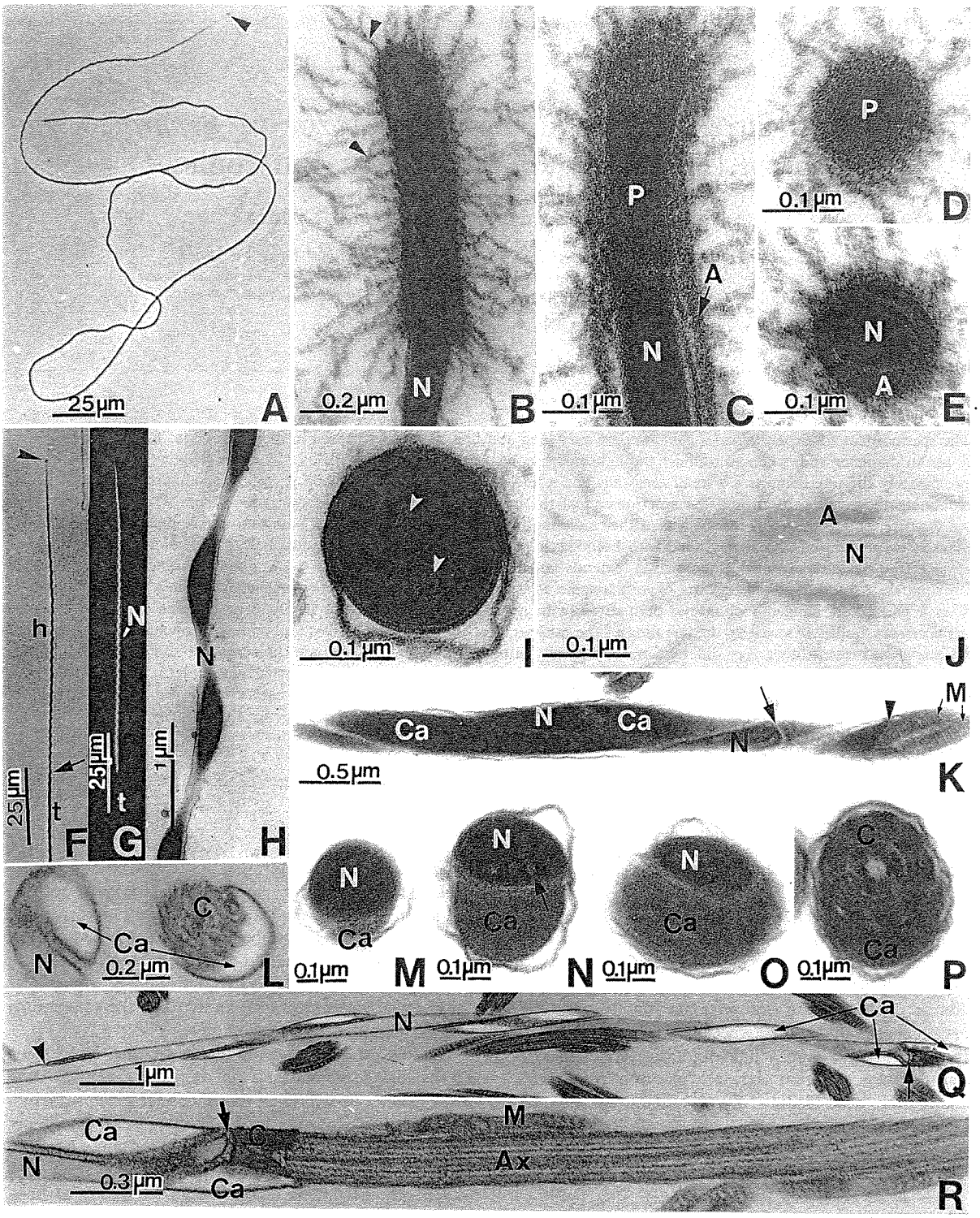

Fig. 1. Caption on facing page.

especially *Apis mellifera*, and ants, very little research has been done on the structure and ultrastructure of hymenopteran spermatozoa. The most detailed publications to date are those by Quicke et al. (1992), who studied various species of hymenopterans; Newman and Quicke (1998) on the spermiogenesis of a braconid, *Aleiodes coxalis*; Chauvin et al. (1988) dealing with the ichneumonid, *Diadromus pulchelles*, including sperm from diploid males; and the work of Hogge and King (1975) on the spermatogenesis of the chalcidoid, *Nasonia vitripennis*. Still concerning a chalcidoid, although with fewer ultrastructural details, mention can be made of the articles by Wilkes and Lee (1965) on the eulophid, *Dahlbominus fuscipennis*, and Lingmei and Dunsu (1987) about a trichogrammatid.

The typical hymenopteran sperm, as in most insects (Phillips, 1970), is quite long, ranging approximately from 40 to 250 μm (Quicke, 1997). The anterior region, called the head, is occasionally distinguished clearly, while the posterior region is the flagellum. The head includes an anterior acrosome, followed by the nucleus. The acrosome is constituted generally of a conical acrosomal vesicle surrounding a cavity filled by an electron-dense rod—the perforatorium. The nucleus is, in general, quite long with a tapering anterior portion and a truncated posterior end. The chromatin is usually homogeneous and densely compacted.

In the majority of hymenopterans, the flagellum is formed by an axoneme, two mitochondrial derivatives and two accessory bodies. The axoneme has the typical arrangement of 9+9+2 microtubules. The mitochondrial derivatives may or may not contain paracrystalline structures, and usually they are of unequal length and occasionally of unequal width (Cruz-Hölfing et al., 1970; Lensky et al., 1979; Cruz-Landim and Beig, 1980; Cruz-Landim and Silva de Morais, 1980; Wheeler et al., 1990; Quicke et al., 1992; Peng et al., 1992, 1993; Newman and Quicke, 1998). The accessory bodies (Baccetti, 1972) are elongated structures situated between the axoneme and the mitochondrial derivatives. Their origin and function have not yet been determined. In the spermatozoa from the majority of the chalcidoids already studied, the mitochondrial derivatives, the nucleus and the axoneme present a spiraling arrangement (Lee and Wilkes, 1965; Wilkes and Lee, 1965; Hogge and King, 1975; Quicke et al., 1992).

In the present study, the structure and ultrastructure of the spermatozoa of *B. pomorum* (Chalcidoidea: Eurytomidae) are described, which may provide additional information for future comparisons. The spermatozoa of this species have ultrastructural characteristics similar to those observed in other chalcid wasps. Spermatozoa structures that have not yet been described in other insects were also found.

2. Materials and methods

The spermatozoa were obtained from the seminal vesicle of living male wasps of the adult *B. pomorum*, collected in a soursop orchard in the state of Minas Gerais, Brazil, in 1997.

2.1. Light microscopy

Drops of sperm suspension were spread on clean glass microscope slides and fixed with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, at room temperature. Preparations were then stained with Giemsa for 15 min and washed with running water. After drying at room temperature, the preparations were observed with a photomicroscope (Olympus, BX60), equipped with phase contrast.

To measure the nucleus, some slides were stained for 15 min with 0.2 μg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS, washed, and mounted with Vectrashield. These slides were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360–370 nm excitation filter.

2.2. Transmission electron microscopy

Seminal vesicles were fixed for 4 h in a solution containing 2.5% glutaraldehyde, 3% sucrose and 5 mM $CaCl_2$ in 0.1 M phosphate buffer, pH 7.2. Specimens, after rinsing in buffer, were post-fixed with 1%

Fig. 1. Light micrographs (A), (F), (G) and transmission electron micrographs showing various features of the sperm of *B. pomorum*. Phase contrast micrographs of spermatozoa are shown in (A) and (F). The arrowheads indicate the acrosome and the arrows the transitional region between the head (h) and tail (t). Longitudinal (B), (C) and transverse (D), (E) sections of the acrosome showing the innumerable filaments (arrowheads) radiating from the acrosomal vesicle (A), nucleus (N) and perforatorium (P). DAPI-stained fluorescence (G) and electron micrographs (H) of the head region showing the helicoidal nucleus (N) and tail (t). (I) Transverse section of the nucleus. The arrowheads indicate the less electron-dense areas. (J) Longitudinal section of the acrosome stained with alcoholic PTA. Longitudinal (K) and transverse (M)–(P) sections of the transition region of nucleus–flagellum showing the centriolar adjunct (Ca), which extends anteriorly beyond the centriole (C) in a spiral around the nucleus (N). In (K), (Q) and (R), the arrows indicate the abutting nuclear base and centriole. In (N), the arrow indicates less electron-dense areas in the chromatin. The arrowhead in (Q) shows the anterior extremity of the centriolar adjunct. Observe in (K) that the two mitochondrial derivatives (M) begin together at a short distance from the nucleus (arrowhead). The axoneme (Ax) is shown. The spermatozoa in (J), (L), (Q) and (R) were stained with alcoholic PTA.

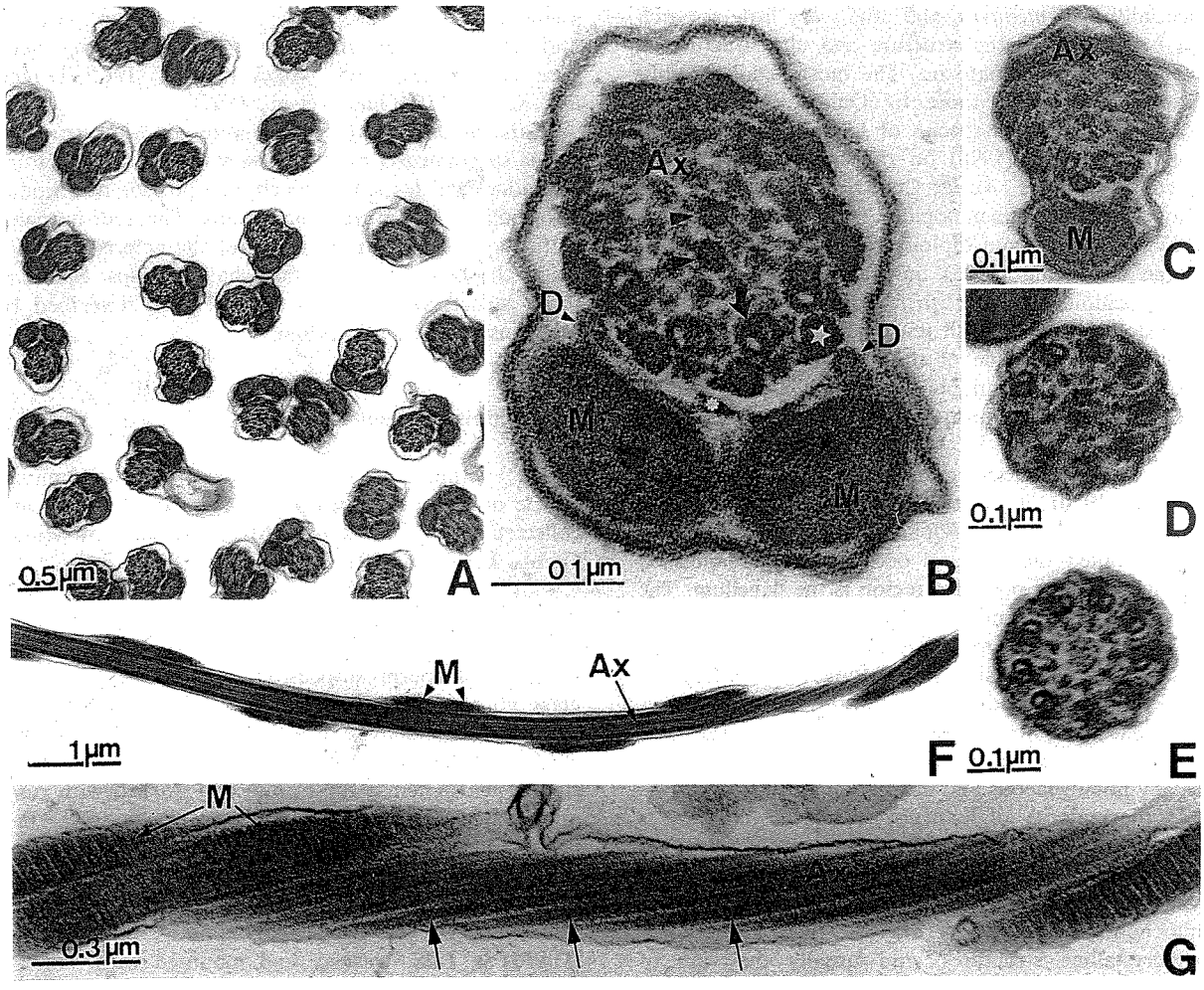

Fig. 2. Ultrastructural features of the flagellum. (A) Cross-section of various flagella. (B) Cross-section of a flagellum with high magnification showing the axoneme (Ax) made up of nine doublets (curved arrow), a central pair (arrowheads), and accessory tubules (star). Also illustrated are the mitochondrial derivatives (M), dense accessory bodies (D), and a central rod (asterisk). (C)–(E) Cross-sections of flagella showing the tip where one mitochondrial derivative terminates before the other one and both before the axoneme. Observe also that, in the axoneme, the accessory tubules finish first, followed by the central pairs and, finally, the nine doublets. (F) and (G) are longitudinal sections of flagella showing the mitochondrial derivatives (M) coiling regularly around the axoneme (Ax), which is twisted in a long pitched helix (arrowheads).

osmium tetroxide in the same buffer. Dehydration was carried out in acetone and the specimens were embedded in Epon 812 resin. Ultrathin sections were stained with uranil acetate and lead citrate. For the detection of basic proteins, the ethanolic phosphotungstic acid (E-PTA) method, modified from Bloom and Aghajanian (1968), was applied. Thus, the seminal vesicles were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C. After washing in the same buffer and dehydrating in alcohol, the fixed material was stained "en bloc" with a solution of 2% PTA in absolute ethanol for 2 h at room temperature and then embedded in Epon 812 resin. Ultrathin sections, stained or unstained, were observed by transmission electron microscopy (Zeiss, Leo 906), operating at 60 and 80 kV.

3. Results

The spermatozoon of the *B. pomorum* is very long and fine, measuring approximately 620 µm (Fig. 1(A)). With the light microscope it appears wavy along its entire length, with a distinct head, gradually tapering anteriorly and measuring about 105 µm (Fig. 1(F),(G)). The acrosome is very small (Fig. 1(A),(F)), approximately 0.6 µm in length, where the acrosomal vesicle has a cone shape, surrounding the perforator-

ium for 0.3 μm and the anterior end of the nucleus for another 0.3 μm (Fig. 1(B),(C)). Innumerable filaments radiate from this vesicle (Fig. 1(B)–(E)). The acrosomal vesicle and the filaments are lightly E-PTA-positive (Fig. 1(J)). The perforatorium measures about 0.3 μm in length, and has a diameter equal to that of the nucleus at their junction. It has a concave base, fitting onto the rounded nuclear tip (Fig. 1(C)). The nucleus is helicoidal (Fig. 1(F)–(H)) and, in transverse section, it is circular (Fig. 1(I)). The nucleus measures about 105 μm in length (Fig. 1(F),(G)) and is 0.3 μm in diameter at its base. It is completely filled with homogeneous compact chromatin (Fig. 1(I)), though in the basal region it has small, less electron-dense areas (see arrow in Fig. 1(N)). The nucleus is attached to the tail by a very long and quite electron-dense centriolar adjunct, which extends anteriorly from the centriole in a spiral around the nucleus for approximately 8.5 μm (Fig. 1(K),(Q)). This adjunct tapers gradually anteriorly and, in transverse section, it is concave where it first fits around the centriole and then the nucleus (Fig. 1(M)–(P)). The centriolar adjunct is totally E-PTA-negative (Fig. 1(L),(Q),(R)). The tail is about 520 μm in length and 0.38 μm in diameter and consists of the axoneme, a pair of mitochondrial derivatives and two small accessory bodies (Fig. 2(A)–(G)). The axoneme follows the 9+9+2 microtubule arrangement (Fig. 2(B)): nine outer single accessory tubules, nine doublets and two central single microtubules. In longitudinal sections, spirally twisted microtubules can be observed clearly (Fig. 2(G)). In transverse sections, spiraling is also evident, since not all of the doublets can be sectioned at perfect right angles (Fig. 2(A)–(C)). The centriole is quite evident, measures approximately 0.27 μm in length and is in direct contact with the nuclear base (Fig. 1(R)). The mitochondrial derivatives are alike in cross-section, more or less oval, measuring about 0.12 by 0.18 μm at their widest and are positioned very close to the axoneme (Fig. 2(A),(B),(F),(G)). In longitudinal sections, the mitochondrial derivatives coil regularly around the axoneme and their parallel, equidistant (42 nm) cristae are clearly visible (Fig. 2(F),(G)). Anteriorly, the mitochondrial derivatives originate together, in contact with the posterior extremity of the centriolar adjunct, approximately 0.5 μm from the nuclear base (Fig. 1(K)). In the final tail region, one mitochondrial derivative terminates just before the other and this one is immediately above the axoneme tip (Fig. 2(C),(D)). In this last structure, the accessory microtubules finish first, followed by the two central ones and, finally, the nine doublets (Fig. 2(C)–(E)). Between each mitochondrial derivative and the axoneme there is an accessory body (Fig. 2(B)), while a dense rod can be found between the three flagellar organelles (refer to the asterisk in Fig. 2(B)).

4. Discussion

The basic structure of the spermatozoon of *B. pomorum* is similar to that of most Hymenoptera (Quicke et al., 1992). It contains: (1) a small acrosome; (2) an elongate, condensed nucleus; (3) a centriole adjunct; (4) two elongate mitochondrial derivatives of unequal length with cristae; (5) two accessory bodies in the tail and; (6) an axonemal arrangement of 9+9+2. As in the majority of the chalcidoids studied to date (Wilkes and Lee, 1965; Lee and Wilkes, 1965; Hogge and King, 1975; Quicke et al., 1992), this spermatozoon is helically twisted along its entire length (including the head), the mitochondrial derivatives coiling around the axoneme and the axoneme itself. However, the sperm of *B. pomorum* present some structural characteristics not yet described for any other Hymenoptera, including Chalcidoidea. With a length of 620 μm, this species has the longest sperm of all the known Hymenoptera (Quicke et al., 1992). Length is variable in eurytomids, since in *Eurytoma* sp. the sperm measures 219 μm in length (Quicke et al., 1992). However, this value is only a little more than one-third that of the length of the *B. pomorum* sperm.

The acrosome covering the anterior extremity of the nucleus is, in general, similar to those already described for some Hymenoptera (Hogge and King, 1975; Quicke et al., 1992). However, a few differences should be considered. In *A. mellifera* (Cruz-Hölfing et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993), in ants (Wheeler et al., 1990) and in the ichneumonids *D. pulchellus* (Chauvin et al., 1988), *Encarsia* (Quicke, 1997) and *A. coxalis* (Newman and Quicke, 1998), the acrosome, or acrosomal vesicle, is in front of the nucleus. In these species, the perforatorium is located in the subacrosomal space; it is thin and its base is inserted into a cavity in the anterior nuclear tip. In the ichneumonids, *Encarsia* (Quicke, 1997) and *A. coxalis* (Newman and Quicke, 1998), there is a third layer, called the extracellular sheath, which covers the whole acrosomal complex, extending posteriorly over the tip of the nucleus (see Fig. 4.2c of Quicke, 1997). It is possible that this "extracellular sheath" is also present in *D. pulchellus*, but the electron micrographs (Fig. 2(A)) provided by Chauvin et al. (1988) show spermatids, from which the mature spermatozoon structures cannot be deduced. However, in *B. pomorum*, as in the pteromalid, *N. vitripennis* (Hogge and King, 1975), it is the acrosome itself (or acrosomal vesicle) that extends posteriorly over the anterior end of the nucleus. In addition, the perforatorium, with a diameter equal to that of the nucleus in this region, has a concave base, fitting onto the rounded nuclear tip. In the species studied here, the extremely reduced acrosome (0.6 μm) is noteworthy, especially when compared with the nuclear length (105 μm). This reduced size may be a

characteristic of the Eurytomidae family, since Quicke et al. (1992) did not find the acrosome in *Eurytoma* sp. However, this very small acrosome is not common to all chalcidoids. In *N. vitripennis* (Pteromalidae) the acrosome measures about 1.8 μm in length (Hogge and King, 1975). Another unusual feature, not yet described for any other Hymenoptera, is the presence of innumerable filaments extending from the acrosomal vesicle (probably corresponding to a well-developed glycocalix). However, various electron micrographs, presented by Quicke et al. (1992), illustrate fine extracellular filaments that might be part of such a glycocalix, possibly associated with an acrosomal vesicle.

The presence of a very long centriolar adjunct, which extends anteriorly beyond the centriole in a spiral around the nucleus, also distinguishes the eurytomid spermatozoa from those of other Hymenoptera. Even in the pteromalid, *N. vitripennis* (Hogge and King, 1975), the only chalcidoid in which this structure was in fact observed, it lies parallel to the nucleus for only a short distance. The centriolar adjunct is therefore quite different from that found in spermatozoa of *B. pomorum*. Quicke et al. (1992) did not observe any obvious centriolar adjunct in the species they investigated. However, we believe that a structure which appears on one side of, and closely opposed to, the nucleus in the electron micrograph (see Fig. 7A of Quicke et al., 1992) of *Eurytoma* sp. may be the same as was observed in *B. pomorum* and identified as the centriolar adjunct. The authors interpreted that structure as "a thin anterior extension of at least one of the mitochondrial derivatives".

Although the centriolar adjunct has already been described or mentioned for many insects (Cantacuzene, 1970), until recently no structure had been identified as a centriolar adjunct in mature hymenopteran sperm (Breland et al., 1966; Cruz-Landim and Beig, 1980; Jamieson, 1987). Exception should be made for honey bees, where Lensky et al. (1979) described a small "triangular body" in mature sperm as being a centriolar adjunct. More recently, Wheeler et al. (1990), studying ant sperm, and Newman and Quicke (1998), in the ichneumonid, *A. coxalis*, observed the presence of a long, electron-dense, approximately cylindrical structure, which they called the centriolar adjunct, located between the nucleus and one of the mitochondrial derivatives. However, owing to this structure's shape and location, earlier studies interpreted it as being the dense anterior tip of one of the mitochondrial derivatives (Quicke et al., 1992) or a region where the axoneme, nucleus and mitochondrial derivatives overlap (Chauvin et al., 1988). Therefore, it is possible that the centriolar adjunct may be present in various other groups of Hymenoptera and this should be further investigated.

If it is confirmed that the centriolar adjunct, as described for ants and ichneumonids, is common to the majority of the Hymenoptera, then this structure may be another characteristic that can be used to distinguish the chalcidoids from various other Hymenoptera.

Another difference between the spermatozoa of *B. pomorum* and those of some other Hymenoptera, is the fact that, in *B. pomorum*, both mitochondrial derivatives begin together, a small distance from the nucleus. For example, in *A. mellifera* (Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992, 1993) and in Diapriidae (Quicke et al., 1992) one of the mitochondrial derivatives runs parallel to the nucleus, for a variable length. In an ichneumonid (Newman and Quicke, 1998), ants (Wheeler et al., 1990) and probably in the majority of Hymenoptera, the mitochondrial derivatives begin one ahead of the other, since the centriolar adjunct is located between one of them and the nucleus. Even in the chalcidoids, there is at least one family, Trichommatidae, that can be distinguished by the position of the derivatives, which extend as far as the acrosome (Lingmei and Dunsu, 1987).

It is believed that it is possible to differentiate between two types of hymenopteran spermatozoa, as proposed by Hogge and King (1975): that is, those of the Chalcidoidea, which have a spiraled nucleus, mitochondrial derivatives and axonemal microtubules, and the sperm of the majority of Hymenoptera, where all these structures are straight. These two types can be further subdivided if other ultrastructural characteristics are taken into consideration, such as those of the acrosome, and especially the centriolar adjunct. The present study corroborates the claims of Quicke et al. (1992) in that the structural diversity of hymenopteran spermatozoa is sufficient to furnish useful characteristics for phylogenetical analyses at the subfamily level and, possibly, of the genus.

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4.2. **Lino-Neto, J.**, Báo, S.N. & Dolder, H. (2000a). Structure and Ultrastructure of the Spermatozoa of *Trichogramma pretiosum* Riley and *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae). *Acta Zoologica* **81**: 205-211.

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Structure and ultrastructure of the spermatozoa of *Trichogramma pretiosum* Riley and *Trichogramma atopovirilia* Oatman and Platner (Hymenoptera: Trichogrammatidae)

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Abstract

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Spermatozoa of the *Trichogramma pretiosum* and *T. atopovirilia* are very slender and long, about 0.35 µm in diameter and 283 µm and 106 µm in length, respectively. Under light microscopy, they appear wavy along their entire length. The head contains a small acrosome which, together with the initial nuclear region is surrounded by an 'extracellular sheath', from which innumerable filaments irradiate. The nucleus is filled with homogeneous, compact chromatin and is attached to the flagellum by an electron dense centriolar adjunct, which extends anteriorly from the nuclear base. The flagellum consists of an axoneme with the 9 + 9 + 2 microtubule arrangement pitched in a long helix, as well as a pair of spiralling mitochondrial derivatives which coil around the axoneme. Based on these characteristics, the sperm of these *Trichogramma* are very similar to the chalcidoids studied to date and differ from non-chalcidoid Hymenoptera. They differ widely from the sperm of *T. dendrolimi* and *T. ostriniae* studied, where no helically twisted structure is shown. However, based on these results we argue that the spiralling of the flagellar structures is a synapomorphy for Trichogrammatidae as well as for Eulophidae + Eurytomidae + Pteromalidae.

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Introduction

The superfamily Chalcidoidea is one of the most speciose and biologically diverse group of insects (Grissell and Schauff 1997). It accounts for roughly one-third of the world's parasitic species of Hymenoptera (LaSalle and Gauld 1991). Though some chalcidoids are phytophagous (particularly seed-feeding Eurytomidae) or hyperparasites, the large majority is parasitic on other insects and play a major role in regulating populations of terrestrial insects (Greathead 1986).

In spite of the considerable importance to applied entomology, the knowledge of evolutionary relationships among chalcidoids is still in its infancy. Until recently, the families have been defined on the basis on similarities or differences rather than on shared apomorphies (Heraty *et al.* 1997). There is still disagreement over the placement of several subfamilies and genera. In agreement with Grissell and Schauff (1997), it is necessary to have clear definitions of all families based on characters which would withstand any deep analysis, cladistic or otherwise. Heraty *et al.* (1997) also drew attention to the need of a new character system to resolve the relationships among families and subfamilies of Chalcidoidea. In this respect, however, very little has so far been done and the pattern of relationships is still vague for most groups (Grissell and Schauff 1997).

Structure and ultrastructure of the spermatozoa has been

extensively used for solving various taxonomic and phylogenetic problems in many animal groups, including the insects (see Dallai 1979; Jamieson 1987; Carcupino *et al.* 1995; Dallai and Afzelius 1995; Jamieson *et al.* 1999). We believe that the structural diversity of the spermatozoa in Chalcidoidea is sufficient to furnish a character system, and that this, associated to other character systems, may be used as a basis for phylogeny, as well as resolving some uncertainly about the relationships among families and genera.

With the exception of studies concerning bees, specially *Apis mellifera*, and ants, very little research has been done on the structure and ultrastructure of hymenopteran spermatozoa. The most detailed publications to date are those of Quicke *et al.* (1992), with various species of hymenopterans, Newman and Quicke (1998) on the spermiogenesis of *Aleiodes coxalis* (Braconidae) and Chauvin *et al.* (1988) dealing with the ichneumonid, *Diadromus pulchelles*, including sperm from diploid males. In relation to chalcidoids, we can be cite the work of Hogge and King (1975) on the spermatogenesis in *Nasonia vitripennis* and of Lino Neto *et al.* (1999) on the structure and ultrastructure of the sperm of the eurytomid, *Bephratelloides pomorum*. Also concerning chalcidoids, although with fewer ultrastructural details, there are the articles of Wilkes and Lee (1965) on the eulophid, *Dahlbominus fuscipennis*, and Lingmei and Dunsu (1987) on trichogrammatids.

The typical hymenopteran sperm, as in most insects (Phillips 1970), is long, ranging from approximately 40 µm to 250 µm in length (Quicke 1997). It is made up of an anterior region, called the head and a posterior region, the flagellum. The head includes an anterior acrosome, followed by the nucleus. The flagellum, in most of the hymenopterans, is formed by an axoneme, two mitochondrial derivatives and two accessory bodies. The basic structure of the spermatozoa known for chalcidoids is similar to that of the rest of the hymenopterans. Nevertheless, in the majority, the mitochondrial derivatives follow a spiral course around a similarly twisted axoneme. The nucleus is also often spirally twisted, though not always in the same direction (Lee and Wilkes 1965; Wilkes and Lee 1965; Hogge and King 1975; Quicke *et al.* 1992; Lino Neto *et al.* 1999).

In the present work we describe the structure and ultrastructure of the spermatozoa of two species of Trichogrammatidae, which contrast with the information known to date for this family (Lingmei and Dunsu 1987). Here we show that the sperm ultrastructure of the two species is basically the same as other Chalcidoidea and widely different from the ultrastructure presented by those authors.

Materials and Methods

Adult virgin males of Trichogramma pretiosum and T. atopovirilia were obtained from of colonies maintained in the Insect Biology Laboratory, Department of Entomology of the Agricultural College 'Luiz de Queiroz', University of São Paulo in Piracicaba, S.P., Brazil.

Light microscopy

Seminal vesicles were dissected and broken open on clean glass microscope slides, where the sperm were spread and fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparations were observed with an Olympus photomicroscope BX60 (Olympus Optical Co. LTD, Japan), equipped with phase contrast.

To measure the nucleus, some of these preparations were stained for 15 min with 0.2 µg/mL 4,6-diamino-2-phenylindole (DAPI) in PBS, washed, and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360–370 nm excitation filter.

Scanning electron microscopy

Spermatozoa from the seminal vesicle were spread on a coverglass slip, fixed in 2.5% glutaraldehyde, dehydrated in acetone, critical point dried and sputter-coated with gold. They were observed with a scanning electron microscope, JEOL JSM5800LV.

Transmission electron microscopy

Seminal vesicles were treated as follows: (a) fixed for 2–4 h in a solution containing 2.5% glutaraldehyde, 3% sucrose, 0.2% picric acid and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer at pH 7.2. After rinsing in buffer, they were postfixed with 1% osmium tetroxide in the same buffer for 1–2 h. Dehydration was carried out in acetone and embedding in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate. (b) For the detection of basic proteins, the ethanolic phosphotungstic acid method (EPTA), modified from Bloom and Aghajanian (1968), was applied. Seminal vesicles were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2, for 24 h at 4 °C. After washing in the same buffer and dehydrating in alcohol, the material was treated 'en bloc' with a solution of 2% PTA in absolute ethanol for 2 h at room temperature and embedded in Epon 812 resin. Ultrathin sections, stained (a) or unstained (b), were observed by transmission electron microscopy (Zeiss, Leo 906), operating at 40 and 80 kV.

Results

The spermatozoa of *T. pretiosum* and *T. atopovirilia* are slender and long, measuring about 0.35 µm in diameter and 283 µm and 106 µm in length, respectively (Fig. 1A,B). In phase contrast microscopy, they are wavy along their entire length, especially in *T. atopovirilia* (Fig. 1B), and in this latter chalcidoid, it is possible to distinguish the head region from the flagellum. The acrosome is small, measuring about 1 µm in length in *T. atopovirilia* (Fig. 2K), and, in both

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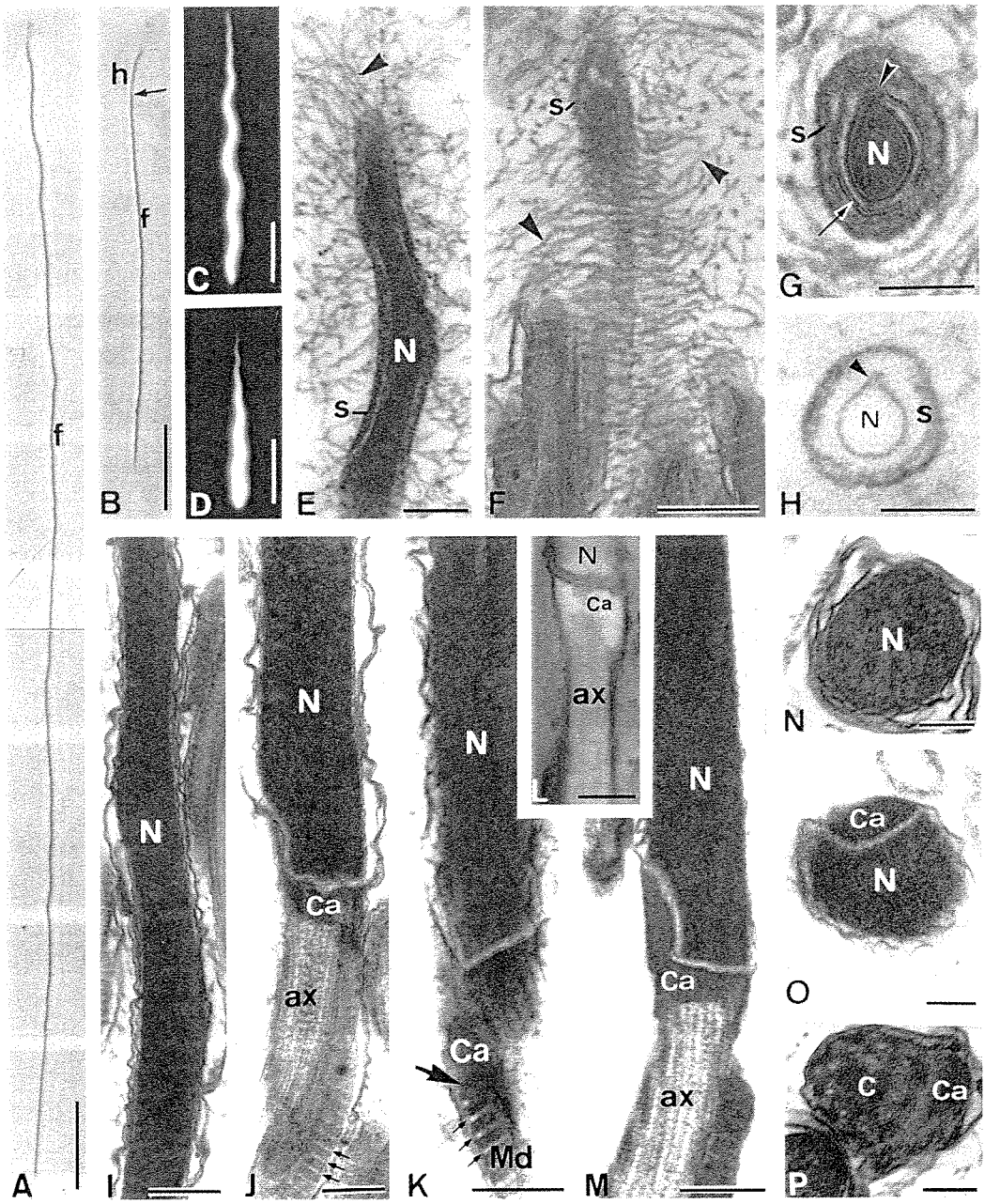

Fig. 1—**A-D**, Light micrographs of spermatozoa. The arrow indicates the head (h) and flagellum (f) limit. —**C-D**, DAPI stained fluorescence of the helicoidal nucleus. —**E,F**, Longitudinal sections of the acrosomal region showing the filaments irradiating (arrowheads) from the extracellular sheath (s). —**G,H**, Cross-sections of the acrosomal region showing the cellular membrane (arrow) and the region of cellular and nuclear membrane fusion (arrowhead). (H) treated with ethanolic PTA. —**I-M**, Longitudinal sections of a nuclear region and the nucleus-flagellum transition. The small arrows indicate mitochondrial cristae and the large one, the beginning of a mitochondrial derivative (Md). In figure L ethanolic PTA was used. —**N-P**, Cross-sections of the nucleus, nuclear base and centriolar adjunct region. Abbreviations: N = nucleus; Ca = centriolar adjunct; ax = axoneme; c = centriole. Figures A,C,E,H,K,M-P are of *T. pretiosum*; B,D,F-G,I-J,L are of *T. atopovirilia*. Scale bars: A-B = 20 μm, C-D = 4 μm, E-F,J-M = 0.2 μm, I = 0.3 μm, G-H,N-*P* = 0.1 μm.

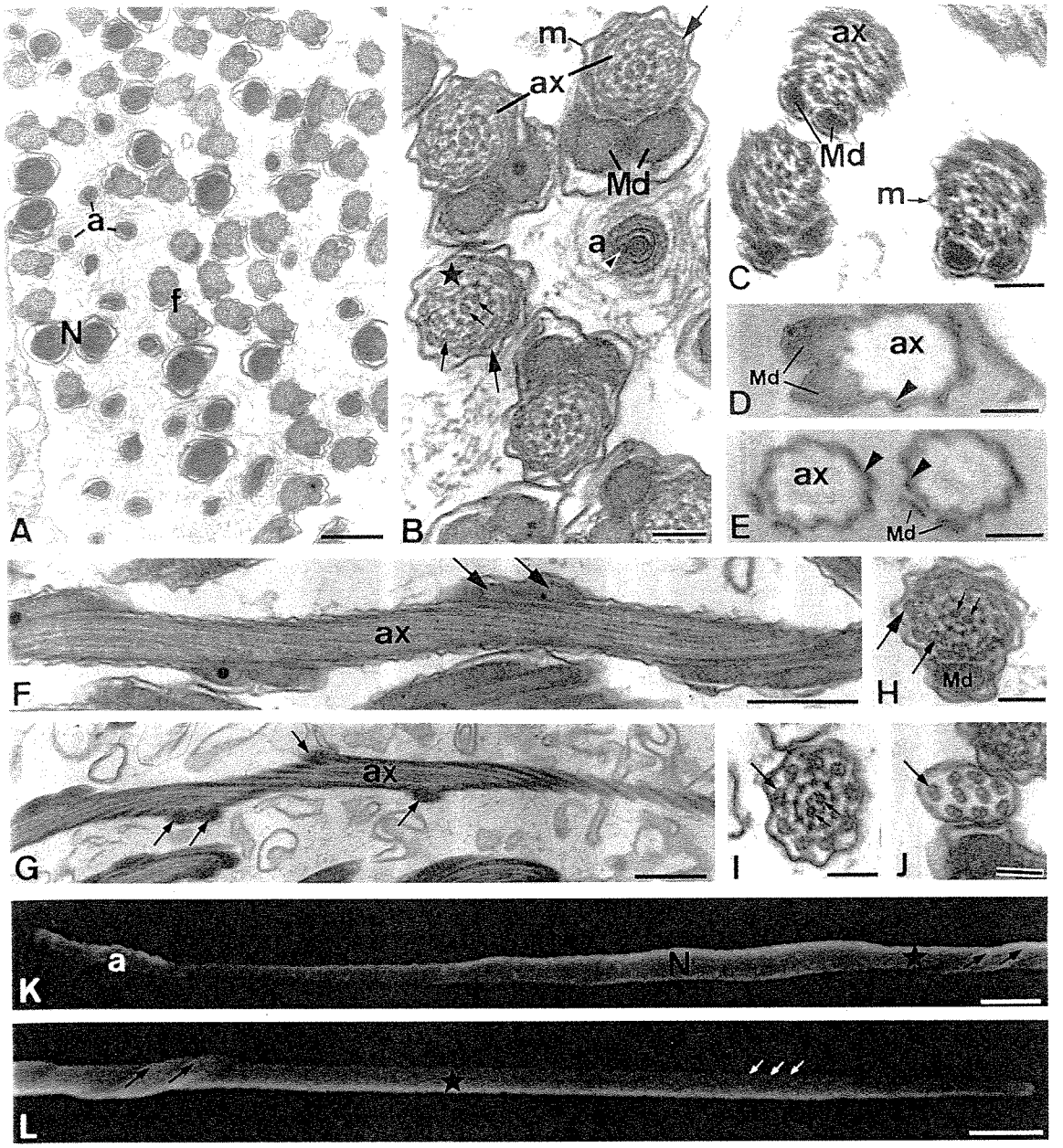

Fig. 2—**A**, Cross section of sperm at different levels. —**B-E**, Cross sections flagella showing the axoneme made up of 9 doublets (medium arrow), a central pair (small arrows), and 9 accessory tubules (large arrows). Star indicates a flagellum sectioned just below the end of the mitochondrial derivatives. (D-E) the arrowheads indicate EPTA-positive material coating the axoneme. —**F,G**, Longitudinal sections of flagella showing the mitochondrial derivatives (arrows) coiling around the axoneme (ax). —**H-J**, Cross sections showing the flagella tips where one mitochondrial derivative (Md) terminates before the other (H) and both before the axoneme. Also, the accessory tubules (large arrow) finish first, followed by the two central ones (small arrows), finally, the nine doublets (medium arrows). —**K,L**, Scanning electron micrographs of the head and the flagellar extremity, respectively. The axoneme (stars), mitochondrial derivatives (black arrows) and spirally twisted axonemal microtubules (white arrows) can be observed. Abbreviations: N = nucleus; a = acrosome; m = cellular membrane. Figures A,B,D,F,H-L are of *T. atopovirilia*; C,E,G are of *T. pretiosum*. Scale bars: A,F-G,L-M = 0.5 μm, **B-E,H**-J = 0.1 μm.

species, this structure, as well as the initial nuclear region, is surrounded by an 'extracellular sheath', from which innumerable filaments irradiate (Fig. 1E-G). This sheath is EPTA-positive (Fig. 1H). In the sections we obtained of this region, it was not possible, optically, to clearly distinguish the presence of an acrosomal vesicle and a perforatorium. The nucleus is helicoidially twisted (Fig. 1C,D,I) and circular in cross section (Figs 1N, 2A), but in the anterior region, covered by the extracellular sheath, it has a pear-shaped cross section. At the pointed portion of the nucleus, a close association of nuclear and plasma membranes can be observed (Figs 1G,H,2B, arrowheads). In both species, the nucleus is completely filled with homogeneous, compact chromatin (Figs 1G,I-O,2 A). Its posterior truncated region has a short longitudinal groove, in which an anterior projection of the centriolar adjunct fits tightly (Fig. 1 J-M,O). In *T. pretiosum*, the nucleus measures about 18 μm in length (Fig. 1C) and 0.28 μm in diameter at its base, while in *T. atopovirilia* it is about 12 μm in length (Fig. 1D) and 0.33 μm in diameter at the base. In *T. pretiosum* the nucleus gradually tapers anteriorly from the mid-region toward the apex (Fig. 1C). On the other hand, in *T. atopovirilia*, it tapers strongly from base to apex (Fig. 1D). In both species, the nucleus is attached to the flagellum by a centriolar adjunct that is electron dense, forming a 83-nm high disk between nucleus and axoneme with an anterior projection which extends for 200 nm, penetrating into the baso-lateral nuclear groove (Fig. 1 J-M). The centriolar adjunct is EPTA-negative (Fig. 1 L). The flagellum consists of an axoneme and a pair of mitochondrial derivatives (Fig. 2A-G). It was not possible to distinguish the presence of accessory bodies (Fig. 2B,C), perhaps because they have a very small diameter and also because the mitochondrial derivatives are very closely adpressed to the axoneme. The axoneme follows the 9 + 9 + 2 microtubule arrangement; including 9 outer single accessory tubules, 9 doublets and 2 central single microtubules (Fig. 2B,C,H). In transverse sections, a spiral twisting of the microtubules is clearly demonstrated, since not all of the doublets can be sectioned at perfectly right angles (Fig. 2B,C,H). In longitudinal sections as well as in scanning electron micrographs, they can be seen to coil regularly around the axoneme (Fig. 2F,G,L, white arrows). Anteriorly the axoneme begins just below the nuclear base with the microtubules inserted in the centriolar adjunct (Fig. 1 J-M,P). The mitochondrial derivatives are alike in cross section, changing from circular to oval, measuring about 0.10 μm in diameter and are placed very close to the axoneme (Fig. 2A-H). In longitudinal sections as well as scanning electron micrographs, they can be seen to coil regularly around of the axoneme (Fig. 2F,G,K,L). Longitudinal sections, show the cristae as clearly parallel with a periodicity about 37 nm (Fig. 1 J,K). Anteriorly, the mitochondrial derivatives begin in contact with the posterior extremity of the centriolar adjunct, approximately 0.25 μm from the nuclear base (Fig. 1K). In the final flagellar region, one mitochondrial derivative terminates shortly before the other (Fig. 2G,H) and this one immediately above of the axoneme tip (Fig. 2B,L, stars). In this latter structure, the accessory microtubules finish first, followed by the two central ones and, finally, the nine doublets (Fig. 2 H-J).

The plasma membrane, in cross sections of the flagellum, show an aspect pleated (Fig. 2B,C,H,I). When treated with ethanolic PTA, an EPTA-positive material is clearly observed inside this membrane and coating the axoneme, but not the mitochondrial derivatives (Fig. 2D,E).

Discussion

The basic structure of the spermatozoa of *Trichogramma pretiosum* and *T. atopovirilia* is similar to that in most Hymenoptera (Quicke *et al.* 1992). They consist in: (1) a small acrosome; (2) an elongate, condensed nucleus; (3) a centriolar adjunct; (4) two elongate mitochondrial derivatives of unequal length, with clearly distinguishable cristae; (5) an axoneme with the typical 9 + 9 + 2 arrangement of microtubules. As in the majority of chalcidoid spermatozoa studied to date (Lee and Wilkes 1965; Wilkes and Lee 1965; Hogge and King 1975; Quicke *et al.* 1992; Lino Neto *et al.* 1999), in these two species of *Trichogramma*, the nucleus is helicoidially twisted and the mitochondrial derivatives coil around the twisted axoneme.

In these *Trichogramma*, as well as in the chalcidoids, *Nasonia vitripennis* (Hogge and King 1975) and *Bephratelloides pomorum* (Lino Neto *et al.* 1999), and many other wasp groups (Quicke *et al.* 1992), an anterior region of the nucleus is surrounded by part of the acrosomal complex. Quicke (1997) expressed some doubt whether this layer is intra or extracellular. However, in these species, we observed it is extracellular (Fig. 1G, arrow) and therefore we call it the 'extracellular sheath'. In these species, as in Eurytomidae (Lino Neto *et al.* 1999), the amount of filaments that irradiate from this sheath is remarkable. We believe that both this feature and the filamentous structures should be common to chalcidoids, and eventually that these filaments may also exist for all wasp species that present the extracellular sheath (Quicke *et al.* 1992), but they may not yet have been observed due to unfavourable sections. These structures are obviously restricted to certain subgroups of the Apocrita (Quicke *et al.* 1992; Lino Neto *et al.* 1999), since in the species of bees (Cruz-Höfling *et al.* 1970; Hoage and Kessel, 1968; Lensky *et al.* 1979; Peng *et al.* 1992, 1993) and ants observed to date, they are absent (Wheeler *et al.* 1990).

The mitochondrial derivatives in *Trichogramma* are also very similar to those of the rest of the chalcidoids that have been examined with the transmission electron microscope. They are oval, alike in diameter and have a compressed appearance, lying very close to the axoneme (Quicke *et al.* 1992; Lino Neto *et al.* 1999). Differing from other Hymenoptera examined to date, the mitochondrial derivatives are more or less circular or, when oval, extend outward

from the axoneme (Cruz-Höfling *et al.* 1970; Cruz-Landim and Silva de Moraes 1980; Wheeler *et al.* 1990; Quicke *et al.* 1992; Newman and Quicke 1998).

In these species of *Trichogramma*, the mitochondrial derivatives begin together, a small distance from the nucleus and in contact with the basal region of the centriolar adjunct. This same characteristic was observed in the eurytomid *Bephratelloides pomorum* (Lino Neto *et al.* 1999). Quicke *et al.* (1992) believe that in the eurytomid *Eurytoma* sp. at least one of the mitochondrial derivatives runs anteriorly beyond the axoneme in parallel to the nucleus. However, Lino Neto *et al.* (1999) have shown in *B. pomorum* that the structure along the side of the nucleus is the centriolar adjunct. In the rest of the Hymenoptera already investigated, only Diapriidae (Proctotrupoidea) presented one of the mitochondrial derivatives extending parallel to the nucleus for a considerable length (Quicke *et al.* 1992). Also the axoneme begins at the nuclear base in all those hymenopterans examined and, in the chalcidoids, including *Trichogramma*, the anterior tips of the microtubules are inserted in the centriolar adjunct (Hogge and King 1975; Lino Neto *et al.* 1999).

The centriolar adjunct in the chalcidoids, considering its location and relationship with the nucleus, axoneme and mitochondrial derivatives (Hogge and King 1975; Lino Neto *et al.* 1999), is deduced to have the role of holding these structures together, as proposed by Breland *et al.* (1966) and Fawcett and Phillips (1969). It is important to note that the nucleus, centriolar adjunct and axoneme in these species, as in the other chalcidoids (Hogge and King 1975; Lino Neto *et al.* 1999), are perfectly aligned while the mitochondrial derivatives are laterally attached, next to the axoneme. In *Trichogramma*, as in the pteromalid, *Nasonia vitripennis* (Hogge and King 1975), only a short projection of the adjunct advances laterally to nucleus, while in the eurytomid, *B. pomorum*, it extends anteriorly and in a spiral around the nucleus for approximately 8.5 µm (Lino Neto *et al.* 1999). In the ant (Wheeler *et al.* 1990) and in the braconid, *Aleiodes coxalis* (Newman and Quicke 1998), the structure identified as a centriolar adjunct is very different from that present in the chalcidoids. In these hymenopterans, this structure is elongated, located laterally to the axoneme and interposed between the nuclear base and the tip of one of the mitochondrial derivatives. Although the homology between the above structure and the centriolar adjunct present in chalcidoids has yet to be established, we believe that the absence of a structure interposed between the nucleus and one of the mitochondrial derivatives, as described in ants and the braconid, will prove to be a synapomorphy for chalcidoids.

In spite of the similarity of spermatozoa of *Trichogramma pretiosum* and *T. atopovirilia* to that of the rest of Chalcidoidea, the former present at least two ultrastructural characteristics not yet observed in other chalcidoids. The lateral projection of the nucleus, in transverse sections, near the acrosome is one if them. In this region, because of this projection, the nuclear and plasma membranes are very close together. The other characteristic is the pleated aspect of the cellular membrane observed in transverse sections of the flagellum and/or the presence of EPTA-positive material coating the axoneme. As these two characteristics are observed in both species, we believe they may represent a synapomorphy for the genus *Trichogramma*.

According to Pinto (1998) the taxonomy and systematic classification of *Trichogramma* is based almost exclusively on male genitalia morphology and, possibly for this reason, they are still confusing. According to this author, the difficulty is due to their small size and absence of discriminating characters. In this study, we found clearly noticeable differences between the spermatozoa of the two species, mainly, as regards the nuclear and total length, characters which can be verified even with the light microscope. Therefore, we believe that in *Trichogramma* the spermatozoa can also contribute towards the discrimination and classification of similar species.

Based on the data presented above we can affirm that the structure and ultrastructure of the spermatozoa of these two species of *Trichogramma* are different from that presented by Lingmei and Dunsu (1987) for *T. dendrolimi* and *T. ostriniae*. According to those authors, the mitochondrial derivatives and axoneme lie parallel to the nucleus. Also, these derivatives extend as far as the acrosome and, in cross sections, are approximately triangular with different diameters. As seen above, in all the rest of the chalcidoids, including the species of *Trichogramma* studied here, the mitochondrial derivatives have approximately equal diameters and are more or less oval or circular. Other differences include the posterior part of the acrosome which lies parallel to the nucleus, and the lack of a centriolar adjunct. Also, in the micrographs presented in the article, no helicoidially twisted structure is visible.

In the genealogical tree presented by Voegelé and Pintureau (1982), based on male genitalia morphology, *T. pretiosum* is more derived then *T. ostriniae* and more primitive then *T. dedrolime*. Considering the monophyly of the genus and even of the superfamily Chalcidoidea (Hanson and LaSalle 1995) it is difficult to reconcile the structural and ultrastructural differences between the spermatozoa of *T. pretiosum*, for example, and those of the two species analysed by Lingmei and Dunsu (1987).

Considering these aspects, we suggest that the structure and ultrastructure of *T. dendrolimi* and/or *T. ostriniae* spermatozoa should be revised, to determine whether the spiralling structure is a synapomorphy, plesiomorphy or character reversal for Trichogrammatidae. Unfortunately neither wasp occurs here in South America.

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4.3. **Lino-Neto, J.**, Báo, S.N. & Dolder, H. (2000b). Sperm ultrastructure of the honey bee (*Apis mellifera*) (L) (Hymenoptera, Apidae) with emphasis on the nucleus-flagellum transition region. *Tissue & Cell* **32**(4): 322-327.

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Tissue & Cell

Sperm ultrastructure of the honey bee (*Apis mellifera*) (L) (Hymenoptera, Apidae) with emphasis on the nucleus-flagellum transition region

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Keywords: sperm, ultrastructure, centriolar adjunct, honey bee

Abstract. The flagellum of *Apis mellifera* (Hymenoptera, Apidae) consists of two mitochondrial derivatives, an axoneme and two accessory bodies. The mitochondrial derivatives are of unequal size and lie parallel to the axoneme. In the larger derivative four regions can be distinguished while in the smaller, only three. The region occurring only in the larger derivative consists of paracystalline material. The smaller mitochondrial derivative terminates anterior to the larger one. An extremely long centriolar adjunct is observed between the nucleus and the smaller mitochondrial derivative. This adjunct is compact, very electron dense and gradually tapers from base toward apex, finishing at the anterior extremity of the axonemal microtubules. In this flagellar region, there is only one accessory body present between the larger mitochondrial derivative and the axoneme. Anteriorly, the tips of the axonemal microtubules are inserted in a well developed mass of granular appearance. This material surrounds the nuclear base, separating it from the anterior end of the larger mitochondrial derivative. We believe that the structure identified here as a centriolar adjunct is homologous to that observed in Formicidae, Ichneumonoidea and Symphyta. Therefore, very probably, it is common to most Hymenoptera. © 2000 Harcourt Publishers Ltd

Introduction

The ultrastructure of the spermatozoa has been extensively used in taxonomic and phylogenetic studies of various animal groups, including the insects (Baccetti, 1972; Dallai, 1979; Dallai & Afzelius, 1990; 1995; Carcupino, et al., 1995; Jamieson et al., 1999). In Hymenoptera, the structural diversity of the spermatozoa seems to be sufficient to furnish character sets, which could be used in phylogenetic studies (Quicke et al., 1992). In the hymenopteran sperm, the nucleus-flagellum transition is a complex region and there are still many uncertainties in relation to its structural organization. Recently the possibility has been raised that this region might provide new phylogenetic indicators (Newman & Quicke, 1999a). However, to apply these indicators to phylogenetic studies, the structures must be positively identified so that the homology can be correctly established.

In the hymenopterans, the honey been is, surely, the species in which the spermatozoa have best been studied.

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They were briefly described by Rothschild (1955), Hoage and Kessel (1968) studied their spermiogenesis and Cruz-Höfling et al. (1970) and Lensky et al. (1979) examined in detail the mature sperm ultrastructure. Woyke (1984) compared the ultrastructural differences between haploid and diploid sperm. Peng et al. (1992; 1993) studied the ultrastructure of the sperm, specially the acrosomal complex, submitted to high-pressure freezing fixation, and the integrity of these cells after rapid freezing and thawing. Also, an excellent revision of this subject can be found in Jamieson et al. (1999).

The honey bee sperm, as in most insects (Phillips, 1970), are quite long and filamentous and about 250–270 µm long. The acrosomal complex is formed by a conical acrosomal vesicle and internally, the perforatorium which extends from a deep fossa in the anterior nuclear tip. The total length of the acrosomal complex is 5 µm (Lensky et al., 1979). The nucleus is homogeneous and strongly electron dense, measuring 5 µm in length. Its posteriorly tapering nuclear cone is eccentric, where the anterior extremities of the axoneme and the two mitochondrial derivatives are attached. The tail is formed by an axoneme, two mitochondrial derivatives and two accessory bodies. The axoneme, as is the rule for insects, has the typical 9 + 9 + 2 arrangement of microtubules. The mitochondrial derivatives are of unequal diameter and length and lie parallel to the axoneme. Their matrix is composed of amorphous and paracrystalline materials (Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992; 1993). Two accessory bodies (deltoid structures in Cruz-Höfling et al., [1970] or triangular rods in Lensky et al., [1979]) are situated between the axoneme and each mitochondrial derivative.

The present study provides some additional information about the ultrastructure of mature spermatozoa of *Apis mellifera*, specially of the nuclear-flagellar transitional region.

Materials and Methods

The adult honey bee drones used in this study were obtained from colonies maintained in the Apiary of the Federal University of Viçosa, MG, Brazil.

Seminal vesicles were dissected and treated as follows: a. fixed in a mixture of 2.5% glutaraldehyde, 1% tannic acid, 1.8% sucrose in 0.1 M phosphate buffer followed by block staining in 1% uranyl acetate in distilled water (Afzelius, 1988). The specimens were dehydrated in acetone. b. For the detection of basic proteins, seminal vesicles were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in alcohol and treated 'en bloc' with a solution of 2% phosphotungstic acid in absolute ethanol (E-PTA). The dehydrated samples were embedded in Epon 812 resin and the ultrathin sections, stained with uranyl acetate and lead citrate a. or unstained b., observed by transmission electron microscopy (Zeiss, Leo 906), operating at 40 or 80 kV.

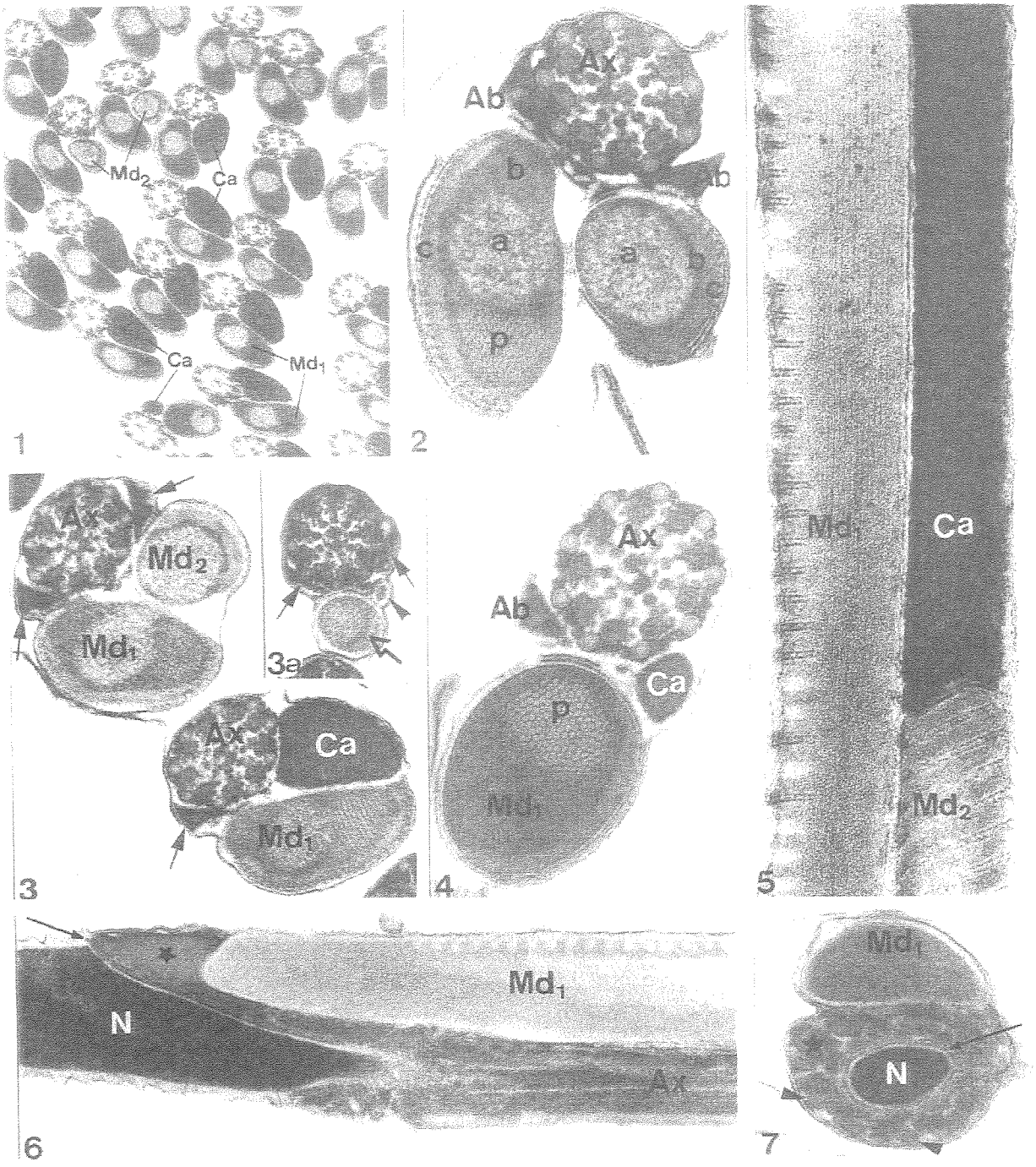
To help compare transverse sections of flagella, the micrographs were always reproduced with the dynein arms oriented counterclockwise.

Results

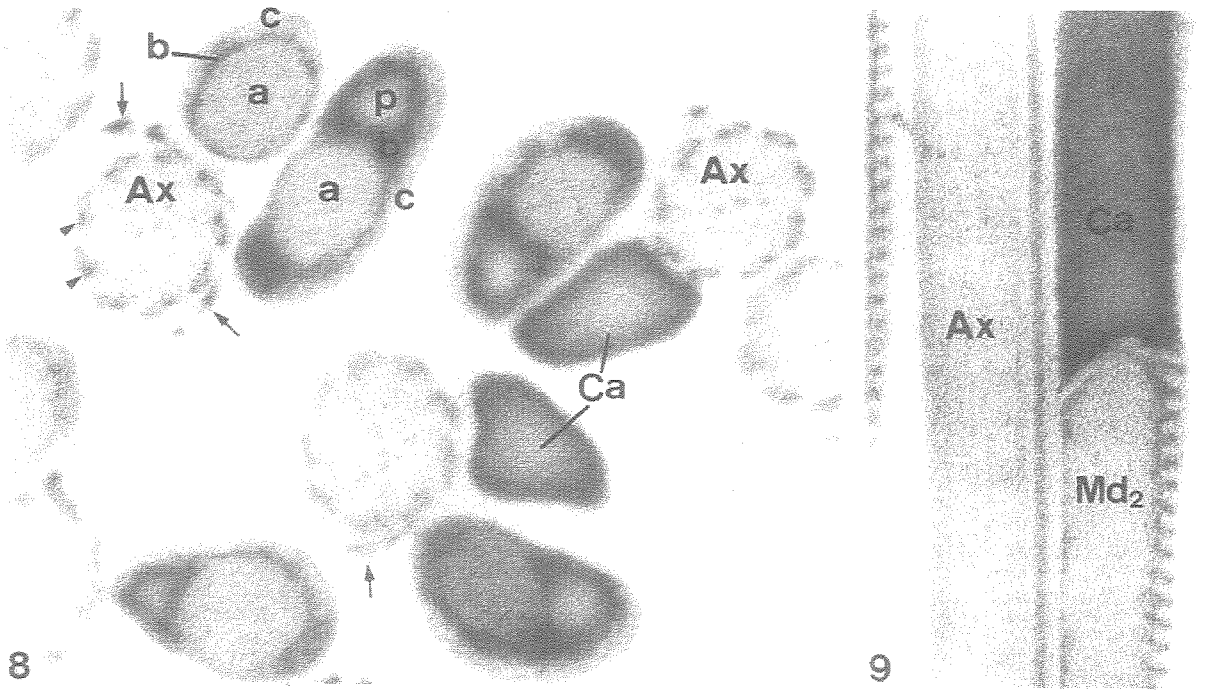
The flagellum of honey bee spermatozoa consists of two mitochondrial derivatives, an axoneme, and two triangular-shaped accessory bodies (Figs. 1–4, 10 D). In cross sections, the larger derivative has an oval form and the smaller is more or less circular (Figs. 2–3, 10 D). In the former, four different regions can be distinguished while only three are found in the smaller one (a, b, c and p in Figs. 2, 8). The region designated a is circular, central, unstructured and less electron dense. It occupies almost all the minor mitochondrial derivative. The p region is only present in the big mitochondrial derivative and is formed by paracrystalline material. The b region is unstructured and quite electron dense. This region is well developed in the larger mitochondrial derivative and reduced in the smaller. The c region is semi-circular, located on opposing faces of the mitochondrial derivatives and contains the mitochondrial cristae. When treated with E–PTA, only the b region is E–PTA–positive (Fig. 8). When the flagellum is sectioned next to the nucleus, the larger mitochondrial derivative becomes more or less circular, the b region making up almost the totality with the a region disappearing (Fig. 4, 10 B). Observing Fig. 3A, it is possible to deduce that, of the two mitochondrial derivatives, it is the smaller that terminates anterior to the larger one.

In most flagella, the smaller mitochondrial derivative (Md_2) is 'replaced' by a more or less triangular structure, which is uniformly compact and very electron dense (Ca in Figs. 1, 3, 10 B–C). In a favourable longitudinal section, this structure can be observed to be long, and to have a truncated and concave posterior extremity, in which the tip of the smaller mitochondrial derivative fits (Fig. 5). We believe that it corresponds to the centriolar adjunct. In negative staining preparations (not shown) we observed that this centriolar adjunct is very long, about 30 µm. It gradually tapers anteriorly from base toward apex (Figs. 1, 4, 10B), and finishes at the level of the anterior extremity of the axonemal microtubules. When treated with E–PTA it appears electron dense (Figs. 8–9). This centriolar adjunct can yet be differentiated from the minor mitochondrial derivative since it does not have membranes, lies very close to the axoneme and, principally, because of the absence of the accessory body between it and the axoneme (Fig. 3, 10B–C).

The axoneme follows the typical pattern of 9 + 9 + 2 arrangement of microtubules: including 9 outer single accessory tubules, 9 doublets and 2 single central microtubules (Figs. 2–4, 10B–D). The presence of electron dense material is also observed in the axoneme between the accessory microtubules (Figs. 2–4). It is E–PTA–positive



Figs. 1–7 1. Various flagella cross sectioned anteriorly. Observe the presence of flagella sectioned through the centriolar adjunct (Ca) and more posteriorly, at the level of the smaller mitochondrial derivative (Md1). × 83,000. 2. A flagellum cross sectioned at the level of two mitochondrial derivatives shows the central region, unstructured and less electron dense **a**, the paracrystalline region **p**, a unstructured, electron dense one **b** and the region of the cristae **c**. × 135,000. 3. A flagellum sectioned through the two mitochondrial derivatives (left upper corner) and another through of the centriolar adjunct. Arrows indicate the accessory body. × 83,000. 3a. Cross section of the flagellar extremity showing the larger and smaller mitochondrial derivatives (⇨ and →, respectively) and the accessory bodies. (→). × 65,000. 4. A flagellum cross sectioned anteriorly. Observe the reduced diameter of the centriolar adjunct. × 142,000. 5. Longitudinal section of a flagellum at the transition of the centriolar adjunct (Ca) and smaller mitochondrial derivative (Md1). × 130,000. 6.–7. Longitudinal and transverse sections, respectively, of the nucleus-flagellum transition showing the posterior nuclear extremity (N) surrounded by a lamellar structure (→) and the material of granular appearance (stars), where the tips of the axonemal microtubules (➤) are inserted. 6: × 83,000; 7: × 110,000. Ab, accessory bodies; Ax, axoneme; Md1, larger mitochondrial derivative.



Figs. 8–9 Transverse and longitudinal sections, respectively, of flagella treated with E-PTA. The arrows indicate the accessory bodies and the ➤, the material between the accessory microtubules. Observe in the mitochondrial derivatives that the b region is E-PTA-positive, while a, c and p are not. Ca, centriolar adjunct; Ax, axoneme; Md., smaller mitochondrial derivative. 8: × 117,000; 9: × 95,000.

(arrowhead in Fig. 8). Anteriorly, the tips of the axonemal microtubules are inserted in a material with a granular appearance (Fig. 7, 10A). This material is abundant and surrounds the posterior nuclear projection (Figs. 6–7, 10A). The anterior extremity of the large mitochondrial derivative, which lies beside this nuclear projection, is separated from the latter by the same material (stars in Figs. 6, 7, 10A). This material also forms a triangular expansion, which fills the space between the large mitochondrial derivative tip and the baso-lateral region of the nucleus (star in Fig. 6). Finally, between the basal nuclear projection and this material there exists another lamellar structure (arrows in Figs. 6, 7, 10A).

In cross section, two accessory bodies are observed between axoneme and mitochondrial derivatives (Figs. 2–4, 10D). These structures, are electron dense using routine staining methods (Figs. 2–3) and have E-PTA–positive regions (Fig. 8). As mentioned above, an accessory body does not exist between the axoneme and the centriolar adjunct; in this region, only one accessory body is located between the axoneme and the larger mitochondrial derivative (Figs. 3–4, 8, 10B–C).

Discussion

Although the centriolar adjunct has already been described for many insects (Cantacuzene, 1970), in hymenopterans only recently a structure positioned between the nucleus and one or both mitochondrial derivatives has been identified as a centriolar adjunct in mature sperm of the ant (Wheeler et al., 1990), braconids (Newman & Quicke, 1998), Symphyta (Newman & Quicke, 1999a) and cynipoids (Newman & Quicke, 1999b). No structure homologous to those of other hymenopterans was identified in bees. In *Apis mellifera*, Lensky et al. (1979) identified an electron dense 'triangular body' located between the anterior tip of the larger mitochondrial derivative and the nucleus, opposite to the axoneme. This triangular structure was tentatively considered to be a centriolar adjunct (Jamieson, 1987; Jamieson et al., 1999). However, when observed in an appropriate section it is, in fact, an antero-lateral expansion of the material that surrounds the tapered nuclear base (see Fig. 6, asterisk). Therefore, it is not homologous to those structures that have been called centriolar adjuncts in other hymenopterans (Wheeler et al., 1990; Quicke, 1997; Newman & Quicke, 1998; 1999a; 1999b).

In spite of not having been previously identified, the drone spermatozoa also have an electron dense structure anteriorly located in relation to the smaller mitochondrial derivative, which we believe to be homologous to the centriolar adjunct observed in other hymenopterans (*q.v.*). However, in *Apis mellifera*, differing from those hymenopterans, this structure is extraordinary long and anteriorly tapered (see Fig. 4). This shape and position

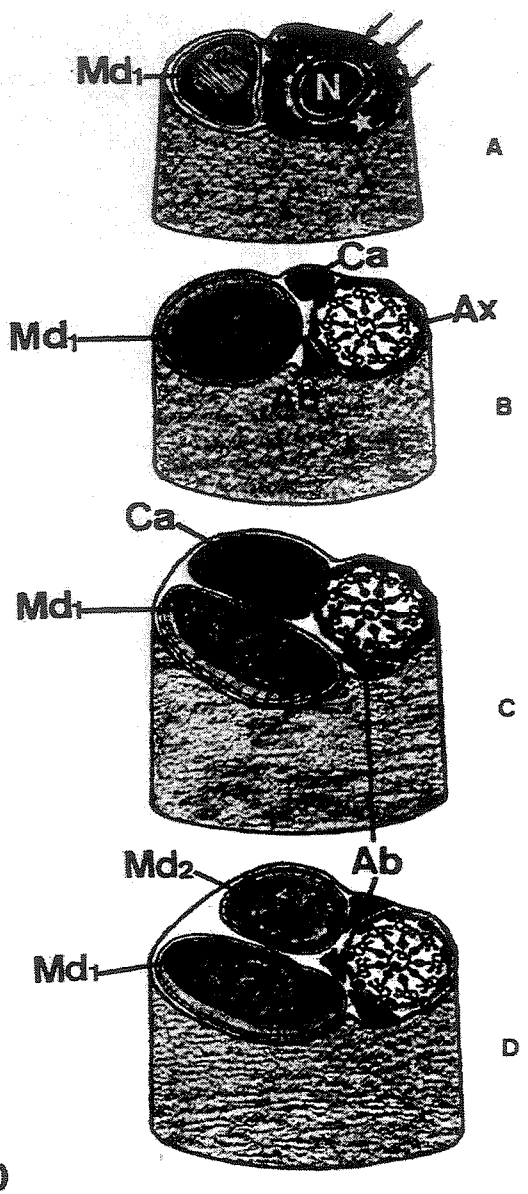


Fig. 10 Schematic diagram of the nucleus-flagellum transition region. The smaller arrows indicate microtubules; and the larger → indicates the lamellar structure. The star, appears showing the material of granular appearance. N, posterior nuclear extremity; Md_1, larger mitochondrial derivative; Ca, centriolar adjunct; Ax, axoneme; Ab, accessory bodies; Md_2, smaller mitochondrial derivative. × 75,000.

probably made the structure difficult to observe or it was misinterpreted (Hoage & Kessel 1968). Also in other hymenopterans, this structure has been initially interpreted as being the anterior tip of one of the mitochondrial derivatives (Quicke et al., 1992) or a region where the axoneme, nucleus and mitochondrial derivatives overlap (Chauvin et al. 1988). In the majority of those species, if not in all, only one accessory body was observed near the nuclear base, associated to one of the mitochondrial derivatives (Quicke et al. 1992); the other structure was interpreted also as a mitochondrial derivative, probably corresponding to the centriolar adjunct. In those chalcidoids, where sperm ultrastructure already has been observed in detail, the centriolar adjunct covers the nuclear base, separating it from the axoneme and mitochondrial derivatives. This adjunct also presents an anterior projection that overlies the basal nuclear region (Lino Neto et al. 1999; 2000). However, the centriolar adjunct in these wasps differs morphologically from most hymenopterans (Wheeler et al., 1990; Newman & Quicke, 1998; 1999a; 1999b), including *Apis*. Further, this structure in honey bee presents a predominance of basic proteins (E–PTA–positive), even though it differs from chalcidoids in which it is E–PTA–negative (Lino Neto et al. 1999; 2000). Based on the various hymenopteran species, from Symphyta to the bees, where a centriolar adjunct has been described, we believe that it is now possible to admit that this is a structure common to most or all species of this order. Moreover, it occurs with strong variations in shape (ex., chalcidoids) and dimensions (ex., *Apis*), and may be a good phylogenetic indicator.

In *Apis mellifera*, the mitochondrial derivatives, which have already been described (Cruz-Höfling et al., 1970; Lensky et al., 1979; Peng et al., 1992; 1993), are unequal in diameter and length and lie parallel to the axoneme. However, only the larger mitochondrial derivative has a paracrystalline region, differing from previous descriptions.

Newman and Quicke (1998; 1999a; 1999b) proposed that the presence of a centriolar adjunct between nucleus and one of the mitochondrial derivatives could explain why, in the final flagellar region, one mitochondrial derivative terminates before the other. However, observing the position of the mitochondrial derivatives in relation to the orientation of the axoneme's dynein arms in figures 1–4, it becomes evident that the smaller derivative terminates first, which is exactly the one located abutting the centriolar adjunct. In the eulophid, *Trichospilus diatraeae*, and in other chalcidoids (Lino Neto et al., 1999, 2000), the two derivatives always begin together a small distance from the nucleus and in contact with the basal region of the centriolar adjunct. Although, in these species a difference in length is always observed for the mitochondrial derivatives, this difference is unusually large in the case of *T. diatraeae* (Lino Neto et al., in preparation). Therefore, contrary to the proposition of Newman and Quicke (1998), we believe that the different lengths of the mitochondrial derivatives observed in the final flagellar region, are not directly related to the centriolar adjunct's position and, therefore, this should be another hymenopteran sperm characteristic to be considered in phylogenetic analyses.

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Ultrastructural characteristics of the spermatozoa of Scelionidae (Hymenoptera; Platygastroidea) with phylogenetic considerations

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The Scelionidae sperm are distinguished from those of all hymenopterans already studied at least by the presence of a single mitochondrial derivative and the absence of a centriolar adjunct. The absence of an acrosome, in *Telenomus podisi*, is also unique. The helical nucleus and mitochondrial derivative spiralling around a twisted axoneme can be considered as synapomorphies shared with the Chalcidoidea, and the mitochondrial derivative running together with the nucleus for a long distance can be considered as a synapomorphy shared with the Diapriidae. Therefore, from a consideration of these features, it is possible to suppose that the Scelionidae, Chalcidoidea and Diapriidae are more closely related between themselves than are any of them to the Cynipoidea, since the latter does not share any of the above-mentioned features. This supposition agrees with phylogenetic analyses that supported the inclusion of Platygastroidea (Scelionidae and Platygastridae) and Chalcidoidea within the Proctotrupomorpha lineage, as well as the close relationship of these to the Diapriidae, and the exclusion of the Cynipoidea from this lineage.

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Introduction

The Hymenoptera, with slightly more than 115 000 species described (Gauld & Hanson 1995), comprises one of the four largest and most biologically diverse order of insects (Gaston 1991). Apart from Isoptera, eusocial behaviour is present only in the Hymenoptera. In this order, there are groups (bees and wasps) which exhibit all gradations of social organization, from solitary to advanced eusocial. In addition, the Hymenoptera contains some very serious pest species, such as the leaf-cutting ants. This order also contains the single most important group of plant pollinators, the bees, and the most important group of biological control agents for insect pests, the parasitic wasps (Hanson 1995). In spite of the unquestionable economic and ecological importance of the Hymenoptera, there are still many controversies about the evolutionary relationships of this order of insects (Gauld & Bolton 1988; Rasnitsyn 1988; Dowton & Austin 1994; Dowton *et al.* 1997; Ronquist *et al.* 1999).

The family Scelionidae has been traditionally included in the Proctotrupoidea (Königsmann 1978; Gauld & Hanson 1995), a paraphyletic superfamily which was a catch-all group for small non-chalcidoid Apocrita not readily assignable to other taxa (Masner 1995). According to Dowton *et al.* (1997), the phylogeny of the Proctotrupoidea is perhaps least understood within the Apocrita. Probably for these reasons, its families have been grouped in several ways (see Gauld & Hanson 1995; Dowton *et al.* 1997). However, Masner (in Goulet & Huber 1993) places Scelionidae and Platygastridae in a superfamily, the Platygastroidea, separate from other proctotrupoids, in spite of believing that a thorough phylogenetic analysis, supported by an in-depth morphological study, would be necessary to justify the superfamily and to recognize it properly. Although there is uniformity of opinion about the very close relationship between these two families, some researchers consider this separation somewhat artificial (Gauld & Hanson 1995).

Spermatozoal ultrastructure has been widely used in taxonomic and phylogenetic studies of various animal groups, including the insects (see Baccetti 1972; Dallai 1979; Dallai & Afzelius 1990, 1995; Carcupino *et al.* 1995; Jamieson *et al.*

1999). In Hymenoptera, the spermatozoa present sufficient ultrastructural diversity to furnish a character system (Quicke *et al.* 1992; Lino-Neto *et al.* 1999, 2000a,b). This system, associated with other character systems, certainly may be used as a basis for phylogeny, to resolve several uncertainties about relationships at different levels, possibly from genera to superfamily.

Dowton & Austin (1994) and Dowton *et al.* (1997) claim that apocritan wasp phylogenies based on morphology are hindered by problems associated with reductional synapomorphies because of the extremely small size of many members of this group. This should not be a problem when using spermatozoal ultrastructure as a basis for phylogenetic relationships, since the spermatozoan's size and consequently its ultrastructural characteristics are independent of wasp body size. In parasitic wasps, the longest spermatozoa are found in species of the superfamilies that constitute the so-called microhymenoptera (Proctotrupomorpha *sensu* Rasnitsyn 1988). For example, in the chalcidoid *Bephratelloides pomorum*, the spermatozoa measure 620 µm in length, these being the largest spermatozoa so far observed in Hymenoptera (Lino-Neto *et al.* 1999). However, very little is yet known about the spermatozoal ultrastructure in the hymenopterans compared with other insect orders (Jamieson *et al.* 1999). While the spermatozoal ultrastructures of a few species pertaining to five of the six Symphyta families have been described (Quicke *et al.* 1992; Newman & Quicke 1999a), this information is known for only six of the approximately 16 apocritan superfamilies, and some descriptions do not show sufficient detail to establish comparisons. Platygastroidea is one of the superfamilies whose spermatozoa have not yet been described; therefore, this is the first study of representatives of this taxon, which may shed new light on the evolutionary relationships between the parasitic wasps and, possibly, hymenopterans in general.

Materials and methods

Adult virgin males of *Trissolcus basalis* and *Telenomus podisi* were obtained from colonies maintained at the National Center for Genetic Resources of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), an institution for agricultural research, in Brasília, Brazil.

Light microscopy

The vas deferens was dissected and broken open on a clean glass microscope slide, where the sperm were spread and fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparation was observed with an Olympus photomicroscope BX60 (Olympus Optical Co., Japan), equipped with phase contrast. To measure the nucleus, some of the preparations were stained for 15 min with 0.2 µg/mL 4,6-diamino-2-phenylindole (DAPI) in phosphate-buffered saline (PBS), washed and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus BX60), equipped with a BP360–370 nm excitation filter.

Scanning electron microscopy

Spermatozoa from the vas deferens were spread on a glass cover slip, fixed in 2.5% glutaraldehyde, dehydrated in acetone, critical point dried and sputter coated with gold. They were observed with a scanning electron microscope, JEOL JSM5800LV.

Transmission electron microscopy

Vasa deferentia were fixed for 2–4 h in a solution containing 2.5% glutaraldehyde, 3% sucrose, 0.2% picric acid in 0.1 M cacodylate buffer at pH 7.2. After rinsing in buffer, they were post-fixed with 1% osmium tetroxide in the same buffer for 1–2 h. Dehydration was carried out in acetone and embedding in Epon 812 resin. Ultrathin sections, stained with uranyl acetate and lead citrate, were observed by transmission electron microscopy (Zeiss, Leo 906), operating at 40 and 80 kV.

Results

The spermatozoa of *Trissolcus basalis* and *Telenomus podisi* are slender and long, measuring about 100 µm and 170 µm in length, respectively (Figs 1A, 2A). In *T. basalis*, the acrosome is very small, measuring about 0.4 µm in length, and is formed by an acrosomal vesicle and a perforatorium, which extends into the nucleus for approximately 0.5 µm (Fig. 1C–E). No acrosome was observed in *T. podisi*, in spite of various spermatozoa having been observed sectioned in the anterior head region (arrows in Fig. 2C; Fig. 2E,F).

The nucleus, when stained with DAPI, is seen to be helicoidal (Figs 1B, 2B). In longitudinal sections as well as in scanning electron micrographs, it can be seen to coil around the single mitochondrial derivative (Figs 1H,J, 2K,M). In *T. basalis*, the nuclear tip extends beyond the mitochondrial derivative (arrowheads in Fig. 1D–F). The nucleus is about 25 µm in length and the diameter is more or less constant, measuring approximately 160 nm at the base and 110 nm at the anterior tip (Fig. 1B–F). Where it runs in contact with the mitochondrial derivative, it is more or less triangular in cross-section (Fig. 1D,F). However, at its base, cross-sections show a crescent shape (Fig. 1I) and, at the anterior extremity, above the mitochondrial derivative, the nucleus assumes a pear shape (arrowheads in Fig. 1D–F). In *T. podisi*, the nucleus terminates shortly below the mitochondrial derivative tip (arrows in Fig. 2C,E). It is about 45 µm in length, gradually tapers anteriorly from the base, where it measures approximately 240 nm (Fig. 2B,C,F,H). Differing from *T. basalis*, in this species the nucleus always presents a circular shape in cross-section (Fig. 2C,G,I). In both species, it is completely filled with homogeneous, compact chromatin (Figs 1B–F,H–L,

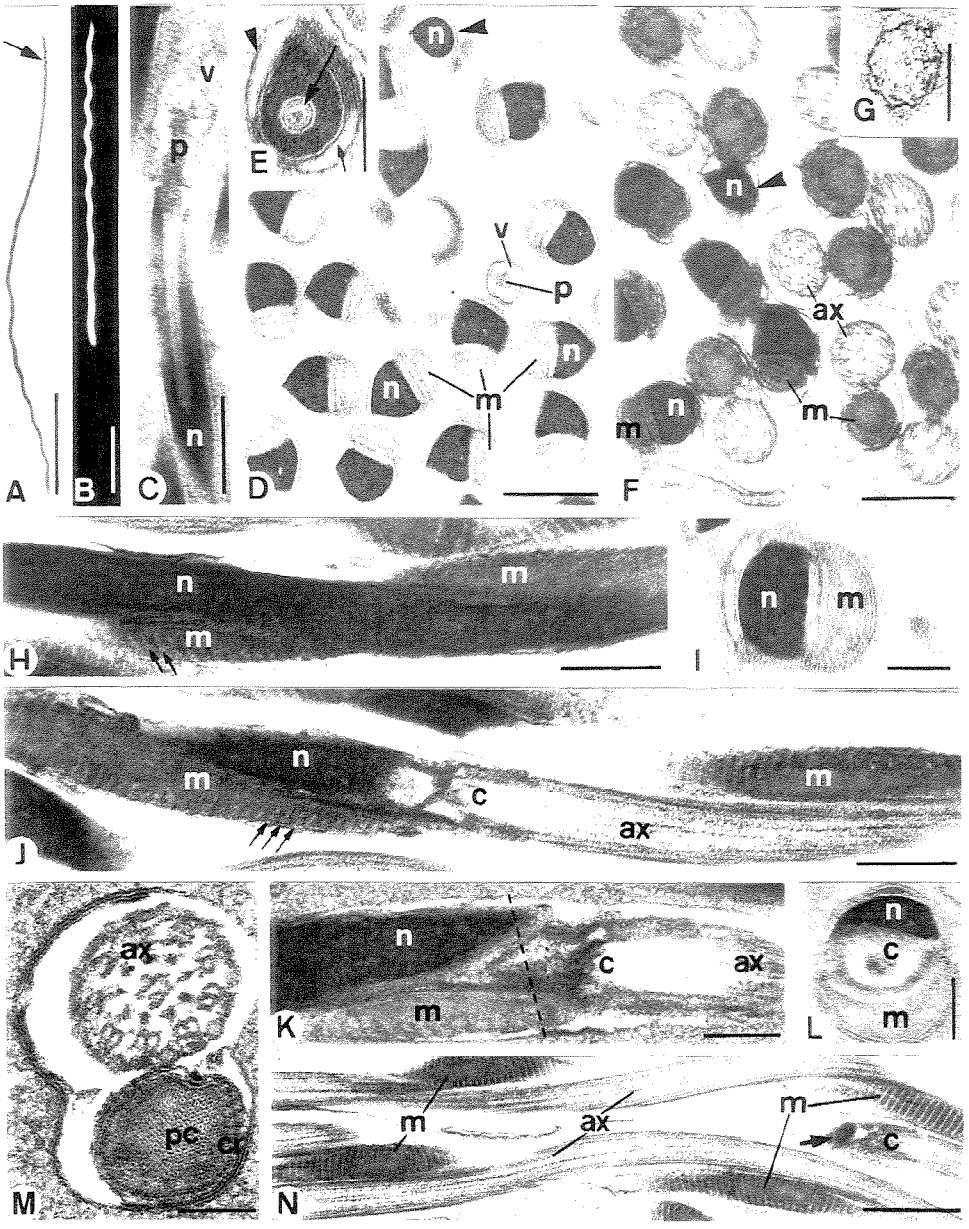

Fig. 1 Light micrographs (*A,B*) and transmission electron micrographs (*C–N*) showing morphological features of the spermatozoa of *Trissolcus basalis*. —*A*. Phase contrast of a spermatozoon. The arrow indicates the head region. —*B*. DAPI-stained fluorescence of the helicoidal nucleus. —*C*. Longitudinal section of the acrosomal region showing the acrosomal vesicle (v) and the perforatorium (p). —*D*. Cross-sections of spermatozoa in the head region. Observe an acrosome (v, p) and the nucleus (n) juxtaposed to the mitochondrial derivative (m). The arrowhead indicates a nucleus sectioned beyond the mitochondrial derivative and below the acrosome. —*E*. Cross-section of a nuclear tip in which the perforatorium fits (larger arrow). The smaller arrow indicates the nuclear membrane, and the arrowhead the cell membrane. —*F*. Cross-section of spermatozoa at different levels. Notice that the mitochondrial derivative is circular in the flagellar region and crescent shaped when juxtaposed with the nucleus. The arrowhead indicates a nucleus sectioned beyond the mitochondrial derivative. —*G*. Cross-section of an axoneme below the mitochondrial derivative. —*H*. Longitudinal section of a nuclear region. The arrows indicate the mitochondrial cristae. —*I*. Cross-section in the nuclear base. Observe that both the mitochondrial derivative and the nucleus are crescent shaped in this region. —*J,K*. Longitudinal sections of the nucleus–flagellum transition region. Notice the perfect fitting of the axonemal tip (c) in the nuclear wedge-shaped base. Arrows indicate the mitochondrial cristae. —*L*. Cross-section of the nucleus–flagellum transition region, probably positioned as indicated by the broken line in the previous figure. —*M*. Cross-section of a flagellum showing the twisted axoneme (ax) with the typical 9 + 9 + 2 arrangement of microtubules, the intertubular material (arrow) and the mitochondrial derivative, consisting basically of cristae (cr) and a paracrystalline region (pc). —*N*. Longitudinal section of flagella. The arrow indicates the posterior nuclear tip. Scale bars: *A* = 20 µm; *B* = 5 µm; *C,G,K* = 0.2 µm; *D,F,H,J* = 0.3 µm; *E,I,L,M* = 0.1 µm; *N* = 0.5µm.

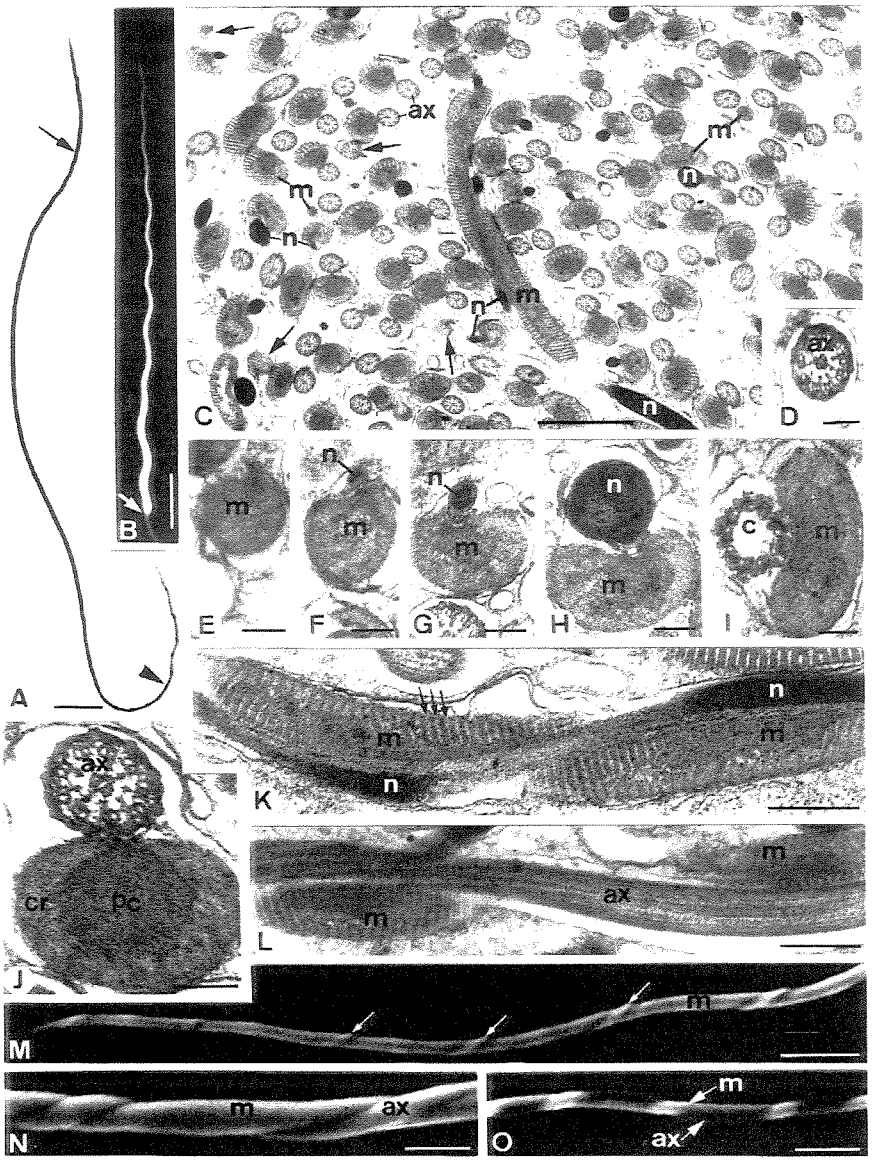

Fig. 2 Light micrographs (*A,B*) and transmission (*C–L*) and scanning (*M–O*) electron micrographs showing various features of the spermatozoa of *Telenomus podisi*. —*A*. Phase contrast of a spermatozoon showing the head region (arrow) and the posterior flagellar extremity (arrowhead). —*B*. DAPI-stained fluorescence of the helicoidal nucleus. The arrow indicates the nuclear base. —*C*. Micrograph showing various spermatozoa cross-sectioned at different levels. The arrows indicate mitochondrial derivatives (m) sectioned above the nucleus (n). —*D*. Cross-section of an axoneme (ax) below the mitochondrial derivative. —*E–H*. Spermatozoan heads cross-sectioned either above the nucleus (*E*), at the anterior (*F,G*) or posterior (*H*) nuclear extremities. —*I*. Flagellum cross-sectioned at the centriole (c). —*J*. Cross-section of a flagellum showing the axoneme (ax) with the typical 9 + 9 + 2 arrangement of microtubules, the intertubular material (arrow) and the mitochondrial derivative, consisting basically of cristae (cr) and a paracrystalline region (pc). —*K,L*. Longitudinal sections of the head and flagellum regions, respectively. Arrows indicate the mitochondrial cristae. —*M–O*. Scanning electron micrographs showing the mitochondrial derivative coiling around the nucleus (arrows in *M*) and the axoneme (*N,O*). Observe the different pattern between the anterior (*M*) and posterior (*O*) extremities, as seen with light microscopy (*A*). Scale bars: *A* = 10 μm; *B* = 5 μm; *C,N* = 1 μm; *D–J* = 0.1 μm; *K,L* = 0.3 μm; *M,O* = 2 μm.

2B,C,G,H,K) and, posteriorly, it is cut off and directly attached to the axoneme. No centriolar adjunct, as found in other hymenopterans, was observed in the nucleus–flagellum transition region (Figs 1J–L, 2I).

The flagellum is formed by the axoneme and the single mitochondrial derivative. It was not possible to clearly distinguish the presence of accessory bodies (Figs 1F,J,M,N, 2C,I,J,L,N,O). The axoneme, in the 9 + 9 + 2 microtubule arrangement, has nine outer single accessory tubules surrounding nine doublets and two central single microtubules, as well as intertubular material (Figs 1F,M, 2J). In transverse sections, a spiral twisting of the microtubules is clearly observed, being more tightly twisted in *T. basalis*, since not all of the doublets can be sectioned at perfect right angles (Fig. 1F,M). In longitudinal sections as well as in scanning electron micrographs, the axoneme can be seen exhibiting a regular coil around the mitochondrial derivative (Figs 1N, 2L,N,O). The anterior axonemal tip fits perfectly into the wedge-shaped nuclear base (Fig. 1J,K). In *T. basalis*, the mitochondrial derivative begins shortly below the acrosome at the nuclear tip (arrowheads in Fig. 1D–F) and terminates just above the posterior axoneme tip (Fig. 1G). In cross-section, it is circular in the flagellar region and has a diameter approximately equal to the axoneme (Fig. 1F,M). Where it runs together with the nucleus, it assumes an approximately crescent shape (Fig. 1D,I,L). In *T. podisi*, the mitochondrial derivative begins above the nuclear tip (arrows in Fig. 2C,E,F), and its more or less oval cross-section is maintained along the entire spermatozoon. In the region of largest diameter, it attains approximately twice or more the diameter of the axoneme in cross-section (Fig. 2C,E–J). In the nucleus–flagellum transition region it is more or less concave, with the nucleus or axoneme (centriole) fitting its cavity (Fig. 2H,I). Here, the cross-section of the mitochondrial derivative measures almost twice the diameter of the nucleus (Fig. 2H). This proportion is not maintained since its diameter diminishes more slowly as the nucleus tapers off at the anterior tip (Fig. 2F,G). In both species, the mitochondrial derivative coils around the nucleus and axoneme (Figs 1N, 2L,N,O), and terminates just above the axoneme tip (Figs 1G, 2D). In cross-section, it is divided into two regions: a well-developed paracrystalline core surrounded by the mitochondrial cristae (Figs 1M, 2J,L). Longitudinal sections show that the cristae are parallel, with a periodicity of about 40 nm in *T. basalis* and 33 nm in *T. podisi* (Figs 1N, 2L).

Discussion

The spermatozoa of *T. basalis* and *T. podisi* present some ultrastructural characteristics which have not yet been observed in other Hymenoptera. Of these, the most notable is the presence of a single mitochondrial derivative, since the presence of two mitochondrial derivatives in the sperm of hymenopterans is considered standard. Two derivatives were observed in all the species already studied; therefore this is a derived characteristic and certainly very important for phylogenetic and taxonomic studies of this group. Also, the extension of the mitochondrial derivative, twisting around the nucleus up to its anterior end, as occurs in *T. podisi*, has not yet been observed in other hymenopterans. To date, the proctotrupoid, *Psilus fuscipennis* (Diapriidae), is the only hymenopteran in which one of the mitochondrial derivatives overlays the nucleus (Quicke *et al.* 1992). However, in this species, as in *T. basalis*, the derivative terminates below the nuclear tip. The compacted form of nucleus and derivative in cross-section is another similarity between *T. basalis* and *P. fuscipennis* (see fig. 6e in Quicke *et al.* 1992).

With regard to nuclear spiralling, to date, it has only been clearly demonstrated in chalcidoids (Lee & Wilkes 1965; Hogge & King 1975; Quicke 1997; Lino-Neto *et al.* 1999, 2000a). However, in these, no mitochondrial derivative overlays the nucleus. In the cynipoid, *Leptopilina heterotoma*, the spermatozoa present a nucleus with a posteriorly directed ridge, spiralling down along its surface (Newman & Quicke 1999b). Although this can be considered a spiralling sperm form, we doubt if this 'spiralling' can be considered homologous to the nuclear spiralling that occurs in the chalcidoids and in the two species studied here. In these species, the nuclear axis follows a spiralling course while, in the cynipoid, the nuclear axis appears to be twisted and therefore its nuclear projection shows a spiralling course.

The acrosome in *T. basalis* can be considered to be the 'simple type', basically formed by an acrosomal vesicle and a perforatorium. Acrosomes with only these structures were also observed in the siricoid, *Tremex* sp. (Symphyta) (Newman & Quicke 1999a), in ants (Wheeler *et al.* 1990) and in bees (J. Lino-Neto & H. Dolder, personal observation). However, in most of the apocritan wasps already examined, there is an extracellular sheath completely covering the acrosome and the anterior nuclear tip (see Quicke *et al.* 1992; Newman & Quicke 1999b). Also, in the chalcidoids, numerous filaments radiate from this sheath (Lino-Neto *et al.* 1999, 2000a).

We recognize the difficulty of affirming that a structure does not exist because it has not been observed with electronic microscopy. However, in *T. podisi*, based on the several sections at different levels of the spermatozoan's anterior end (see arrows in Fig. 2C and Fig. 2E–G), we are convinced that there really is no acrosome. For example, in this species, it is possible to observe the nuclear tip reduced to about 30 nm in diameter (approximately the same diameter as that of the perforatorium of *T. basalis*). As the perforatorium's diameter usually measures about one-third of the nuclear diameter in this region, if *T. podisi* had an acrosome and a perforatorium, this structure could not be more than 10 nm in diameter. None of the hymenopterans in which the acrosome has been described in detail present a perforatorium with such a small diameter.

In most insects, there is usually an electron dense structure which surrounds, often asymmetrically, the bases of the mitochondrial derivatives and the axoneme where these attach to the posterior end of the nucleus (Jamieson *et al.* 1999). This structure is called the centriolar adjunct and in the mature spermatozoa of hymenopterans has only recently been identified (Wheeler *et al.* 1990; Newman & Quicke 1998, 1999a,b; Lino-Neto *et al.* 1999, 2000a,b). Based on the hymenopterans in which it has been described, the centriolar adjunct can be classified morphologically into three types. The first type is more or less cylindrical and located between the nuclear base and the anterior tip of only one of the mitochondrial derivatives. The anterior tip of the other mitochondrial derivative is either in contact with the nuclear base (e.g. in *Cephalcia*, Newman & Quicke 1999a) or lies beside the nucleus in a basolateral position (e.g. in *Apis*, Lino-Neto *et al.* 2000b). This type has already been identified in Ichneumonoidea (Newman & Quicke 1998), Symphyta, except Siricoidea (Newman & Quicke 1999a), Cynipoidea (Newman & Quicke 1999b) and Apoidea (Lino-Neto *et al.* 2000b). In cross-sections of this region in the spermatozoa of these groups, the axoneme, one mitochondrial derivative and the centriolar adjunct are always observed together. This type is apparently the most common, being present, probably, in most bees and wasps. However, initially it was misinterpreted as being one of the mitochondrial derivatives (Quicke *et al.* 1992) or the nucleus (Chauvin *et al.* 1988). The second type differs from the first by its location between the nuclear base and both mitochondrial derivatives. This type has been observed in Siricoidea (Newman & Quicke 1999a) and Formicidae (Wheeler *et al.* 1990). Initially, this type was misinterpreted as being an extension of the nucleus overlying the axoneme. The third type to date has been observed only in chalcidoids, where it reaches partially around the basolateral nuclear region and the axoneme tip. In this type, the two mitochondrial derivatives touch the adjunct's posterior extremity (Lino-Neto *et al.* 1999, 2000a). Cross-sections shortly below the nucleus show only the axoneme and the centriolar adjunct in spermatozoa of those species that present the second and third types.

Probably, the early misinterpretations cited above occurred because the structure and location of the centriolar adjunct in hymenopterans differ widely from that in other insects (see Jamieson *et al.* 1999) and, due to these misinterpretations, this structure was believed, until recently, to be absent in the Hymenoptera. However, considering recent publications, it seems probable that the centriolar adjunct is present in practically all Hymenoptera. Therefore, since this structure, as described in other hymenopterans, does not exist in Scelionidae, this may be an important characteristic to be used in phylogenetic studies of parasitic wasps.

The presence of a single mitochondrial derivative in scelionids differentiates this family, as well as the superfamily Platygastroidea, should platygastrids also possess only one derivative, from all the other hymenopteran groups already studied. The basic type of hymenopteran spermatozoon presents a flagellum consisting of an axoneme, two mitochondrial derivatives and two accessory bodies (Jamieson *et al.* 1999). As regards the coiling of the mitochondrial derivatives around the twisted axoneme, this has been described in detail only in chalcidoids (Lee & Wilkes 1965; Wilkes & Lee 1965; Hogge & King 1975; Quicke *et al.* 1992; Lino-Neto *et al.* 1999, 2000a). However, this group presents two mitochondrial derivatives which have a very small diameter and are closely adpressed to the axoneme. In the chalcidoids, the derivatives begin in contact with the posterior extremity of the centriolar adjunct, a short distance from the nuclear base (Lino-Neto *et al.* 1999, 2000a). Quicke *et al.* (1992) described that, besides the chalcidoids, the spermatozoan spiralling was 'not found in any other superfamily apart from a weak indication in the Diapriidae.' However, this arrangement in these wasps was confirmed recently by D. L. J. Quicke (personal communication).

Rasnitsyn (1988) proposed four major lineages within the suborder Apocrita: the Ichneumonomorpha, the Vespomorpha (Aculeata), the Evaniomorpha and the Proctotrupomorpha. Also, according to this author, the latter lineage contains the Cynipoidea, Proctotrupoidea, Chalcidoidea and Platygastroidea. However, analyses based on molecular data placed the Cynipoidea as a relatively basal apocritan lineage (Dowton *et al.* 1997). These same analyses also supported the inclusion of this superfamily within the Evaniomorpha rather than within the Proctotrupomorpha. On the other hand, it supported the inclusion of the Platygastroidea (Scelionidae and Platygastridae) and Chalcidoidea within the Proctotrupomorpha, and suggested that they were more closely related to the Diapriidae (Proctotrupoidea) than to any other family included in the Proctotrupomorpha (Dowton *et al.* 1997). This proposal agrees with Rasnitsyn (1980) (Diapriidae closely related to Platygastroidea) and Rasnitsyn (1988) (Chalcidoidea closely related to Platygastroidea). Gibson (1999) suggested, based on morphological data, that the Chalcidoidea are a relatively early clade within Apocrita and that this superfamily is, possibly, more closely related to Diapriidae. This author also proposed that 'Platygastroidea forms a monophyletic lineage with Pelecinidae, Proctotrupidae and Vanhorniidae, as with Chalcidoidea'.

Based on the data presented above, we can observe that the ultrastructures of the spermatozoa of those species of Proctotrupomorpha (*sensu* Rasnitsyn 1988) already studied have elements in common, which would lead to a grouping according to that suggested by most of the phylogenetic studies based on morphological and molecular data (Rasnitsyn 1988; Dowton & Austin 1994; Dowton *et al.* 1997). For example, the helicoidal nucleus and the mitochondrial derivatives coiling around the twisted axoneme in Platygastroidea

(Scelionidae) suggest that it is closely related to Chalcidoidea. Likewise, the mitochondrial derivative running together with the nucleus for a long distance suggests that it is also closely related to Diapriidae (Quicke *et al.* 1992). If, in fact, Diapriidae presents spiralling spermatozoa, this would suggest that this family is as closely related to Platygastroidea as to Chalcidoidea. Two characters suggest that the cynipoids are not closely related to the scelionids and chalcidoids. The first is the lack of a spiralling structure in the flagellar elements of *Figites* sp. (Quicke *et al.* 1992) and *L. heterotoma* (Newman & Quicke 1999b). The second is the fact that there is no homology between the type of 'nuclear spiralling' present in spermatozoa of *L. heterotoma* and that of scelionids and chalcidoids.

Also, the spermatozoa of these cynipoids present a centriolar adjunct located between the nuclear base and one of the mitochondrial derivatives (Newman & Quicke 1999b) which differentiate them from those of Chalcidoidea and Scelionidae. All these characteristics appear to agree with the phylogenetic analyses presented by Dowton *et al.* (1997) when the inclusion of this superfamily is considered within other lineages (Evaniomorpha) rather than within the Proctotrupomorpha.

According to Dowton *et al.* (1997), there is still little consensus between the several phylogenetic hypotheses for the Proctotrupomorpha. This is likely to be resolved only by the combined analysis of molecular and morphological data, including newly described character systems. Therefore, we are more and more convinced that, in the hymenopterans, or at least in the parasitic wasps, the structural diversity of the spermatozoa will be sufficient to furnish a character system which could be used, associated with other systems, to resolve some of the uncertainties about the evolutionary relationships of this important insect group.

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4.5. **Lino-Neto, J.** & Dolder, H. (2001b). Redescription of sperm structure and ultrastructure of *Trichogramma dendrolimi* (Hymenoptera: Chalcidoidea: Trichogrammatidae). *Acta Zoologica* **82**: (in press).

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Redescription of sperm structure and ultrastructure of *Trichogramma dendrolimi* (Hymenoptera: Chalcidoidea: Trichogrammatidae)

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Abstract

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To verify the questionable description of sperm structure of *Trichogramma dendrolimi*, in relation to Chalcidoids, a reinvestigation was undertaken. The spermatozoa appear wavy along their entire length. A small acrosome, together with the anterior nuclear region, is surrounded by an extracellular sheath, from which filaments radiate. The nucleus is helicoidal and attached to the flagellum by a centriolar adjunct. The axoneme has the 9 + 9 + 2 microtubule arrangement pitched in a long helix, with the spiralling mitochondrial derivatives coiling around it. Therefore, the spermatozoa of *T. dendrolimi* are very similar to those of other chalcidoids and different from the first description. It is now possible to affirm that the helical sperm structures are an apomorphic homology for Trichogrammatidae as well as Eulophidae, Pteromalidae, Eurytomidae, Torymidae, Mymaridae and for some other chalcidoids, if not all.

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Introduction

The Chalcidoidea is the second most species-rich superfamily of Hymenoptera and biologically it is one of the most diverse (Harrison and LaSalle, 1995). It accounts for roughly one-third of the world's parasitic species of Hymenoptera (LaSalle and Gauld, 1991). Most Chalcidoidea are parasites of other insects and they have been used extensively in the biological control of insect pests (Grissell and Schauff, 1997). According to Greathead (1986), the majority of the successful biological control projects of insect pests have used chalcidoids to achieve substantial or complete control.

In spite of the considerable importance to applied entomology, the knowledge of evolutionary relationships among chalcidoids is in many respects the same today as it was in the early 1900s (Grissell and Schauff, 1997), perhaps because this group includes some of the smallest insects, such as Trichogrammatidae at 0.2–1.5 mm. There is still disagreement as to the placement of several subfamilies and genera. According to Heraty *et al.* (1997) new character systems are needed to resolve the relationships among families and subfamilies of Chalcidoidea. However, very little has so far been done and the pattern of relationships is still vague for most groups (Grissell and Schauff, 1997).

The ultrastructure of the spermatozoa has been extensively used in taxonomic and phylogenetic studies of various animal groups, including the insects (see Baccetti, 1972; Dallai, 1979; Dallai and Afzelius, 1990, 1995; Carcupino *et al.*, 1995; Jamieson *et al.*, 1999). In Chalcidoidea, the spermatozoa present sufficient ultrastructural diversity to furnish a character system (Quicke *et al.*, 1992; Lino-Neto *et al.*, 1999, 2000a). This system, associated with other character systems, may be used as a basis for phylogeny, as well as resolving some uncertainty about the relationships among families and genera. However, to apply these characters to phylogenetic studies, the structures must be positively identified so that homology can be correctly established.

The typical hymenopteran sperm, as in most insects (Phillips, 1970), is long, ranging from approximately 40 μm to 250 μm in length (Quicke, 1997). It is comprises an anterior region, called the head, and a posterior region, the flagellum. The

head includes an anterior acrosomal complex, followed by the nucleus. The flagellum, in most hymenopterans, is formed by an axoneme with a 9 + 9 + 2 microtubule arrangement, two mitochondrial derivatives that are alike or of unequal diameter and two accessory bodies. The basic structure of the spermatozoa known for chalcidoids is similar to that of the rest of the hymenopterans, but can be distinguished by the mitochondrial derivatives following a spiral course around a similarly twisted axoneme. The nucleus is often also spirally twisted (Lee and Wilkes, 1965; Wilkes and Lee, 1965; Hogge and King, 1975; Quicke *et al.*, 1992; Lino-Neto *et al.*, 1999, 2000a).

In the present work we describe the structure and ultrastructure of the spermatozoa of *Trichogramma dendrolimi*, which contrast with the previous description of Lingmei and Dunsu (1987). Here we show that the sperm ultrastructure of this species is basically the same as other Chalcidoidea and widely different from the ultrastructure presented by those authors.

Materials and Methods

Adult virgin males of *Trichogramma dendrolimi* Matsumura 1925 were obtained from colonies maintained in the Laboratoire de Biologie Appliquée, INSA, Institut National de la Recherche Agronomique, Lyon, France. Voucher specimens, mounted on glass microscope slides, have been deposited in the entomological collection of the Entomology Department of the 'Luiz de Queiroz' Agronomic School (ESALQ/USP), Piracicaba, SP, Brazil.

Light microscopy

Seminal vesicles were dissected and broken open on clean glass microscope slides, where spermatozoa were spread and fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparations were observed with a photomicroscope (Olympus, BX60) equipped with phase contrast.

To measure the nucleus, some of these preparations were stained for 15 min with 0.2 µg/mL 4,6-diamino-2-phenylindole (DAPI) in phosphate-buffered saline, washed and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360–370 nm excitation filter.

Scanning electron microscopy

Spermatozoa from the seminal vesicle were spread on a coverslip, fixed in 2.5% glutaraldehyde, dehydrated in acetone, critical-point-dried and sputter-coated with gold. They were observed with a scanning electron microscope, JEOL JSM5800LV.

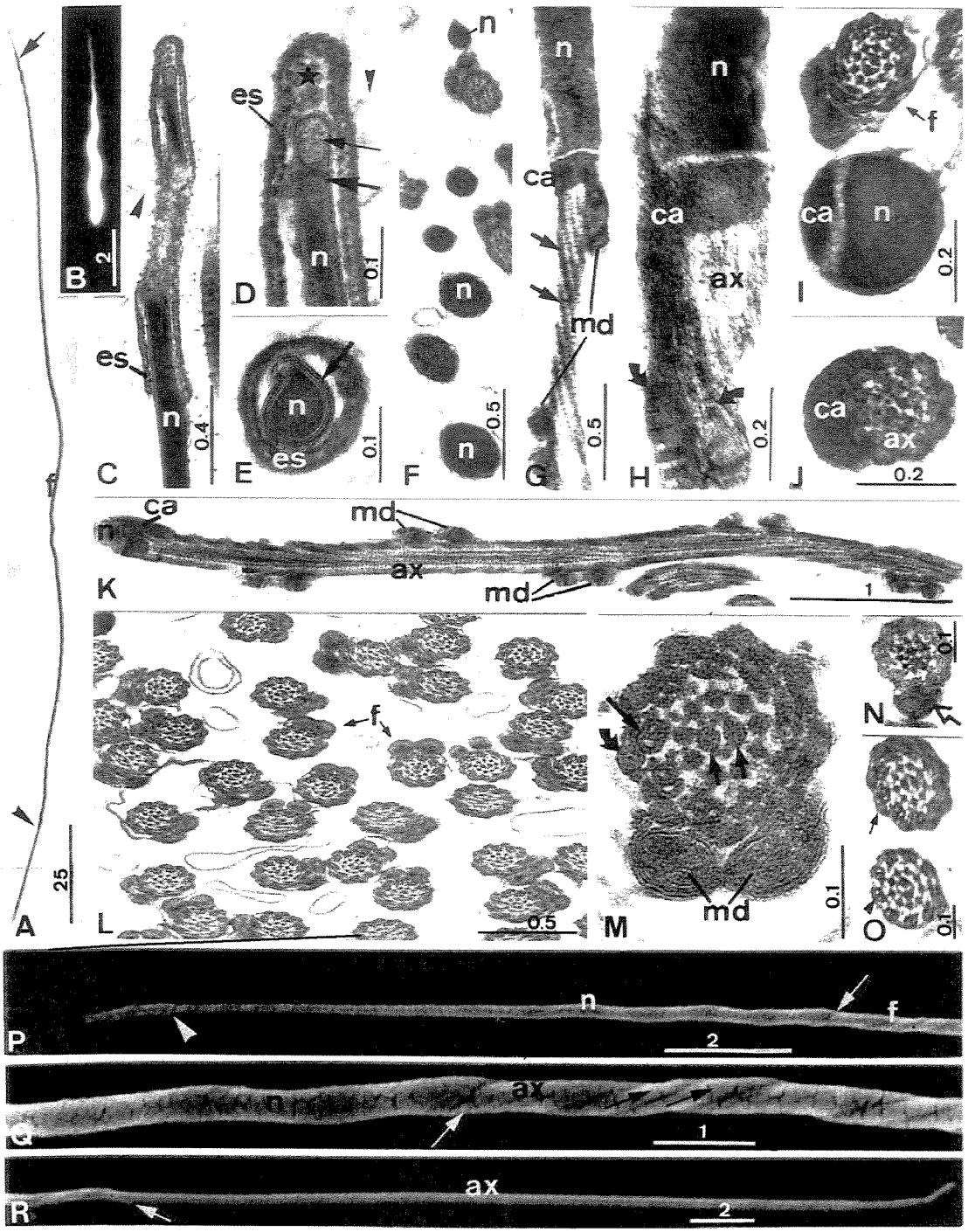
Transmission electron microscopy

Seminal vesicles were dissected and fixed in a mixture of 2.5% glutaraldehyde, 1% tannic acid, 1.8% sucrose in 0.1 M phosphate buffer followed by block staining in 1% uranyl acetate in distilled water (Afzelius, 1988). After dehydrating in acetone and embedding in Epon 812 resin, the specimens were sectioned, the sections were stained with uranyl acetate and lead citrate and were examined in a transmission electron microscope (Zeiss, Leo 906), operating at 40 or 80 kV.

Results

The spermatozoa of *Trichogramma dendrolimi* are long and slender, measuring about 0.32 µm by 238 µm and under phase contrast microscopy they appear wavy along their entire length (Fig. 1A). The acrosomal complex is very small, measuring about 1.5 µm in length (Fig. 1C,P). It is formed by a small structure with a diameter equal to that of the nucleus at their junction (smaller arrow in Fig. 1D), where it fits with a concave base onto the rounded nuclear tip (larger arrow in Fig. 1D). In the sections we obtained of this region, it was not possible to clearly identify this structure as being a perforatorium or an acrosomal vesicle. This structure,

Fig. 1—A, Light micrographs of spermatozoa. The arrow indicates the head and the arrowhead, the posterior end of the mitochondrial derivatives. —B, DAPI stained fluorescence of the helicoidal nucleus. —C, Longitudinal sections of the acrosomal complex showing the filaments irradiating (arrowhead) from the extracellular sheath(s). —D, High power image of a longitudinal section through of the anterior acrosomal region. The larger arrow indicates the junction of nucleus (n) and acrosomal vesicle (or perforatorium) (smaller arrow). The arrowhead indicates the filaments and a star, the inside region of the extracellular sheath tip. —E, Cross-section of the acrosomal complex region. The arrow shows the cellular and nuclear membranes. —F, Cross-section of nuclei at different levels. —G, H, Longitudinal sections of the nucleus–flagellum transition region. Arrows indicate the axonemal microtubules and curved arrows, the anterior tip of the mitochondrial derivatives. —I, Cross-section of the nuclear base. —J, Cross-section of the axonemal anterior extremity at the level of the centriolar adjunct. —K, Anterior tip of a longitudinally sectioned flagellum. —L, Various cross-sectioned flagella. —M, Cross-section of a flagellum with high magnification showing the axoneme (Ax) made up of nine doublets (longer arrow), a central pair (shorter arrows) and accessory tubules (curved arrow). —N, O, Cross-sections of the posterior flagellar region. Observe that one mitochondrial derivative is longer (open arrow) and the accessory microtubules (arrow) finish before the doublets (arrowhead). —P, R, Scanning electron micrographs of the head, of the nucleus–flagellum transition and of the flagellar extremity, respectively. Arrowhead indicates the acrosomal inferior limit, the white arrows indicate the nucleus–flagellum transition, the black arrows, the mitochondrial derivatives and the shorter arrow, the posterior end of the mitochondrial derivatives. Abbreviations: ax = axoneme; c = centriolar adjunct; es = extracellular sheath; f = flagellum; md = mitochondrial derivatives; n = nucleus. Scale bars in µm.



as well as the anterior nuclear region (about 1.3 μm), is surrounded by an extracellular sheath, from which numerous filaments arise (Fig. 1C,D). The extracellular sheath extends anteriorly producing a small space, which is filled by a roughly granular material (star in Fig. 1D). The nucleus measures about 16 μm in length, it is twisted in a helix (Fig. 1B,P) and completely filled with homogeneous, compact chromatin (Fig. 1C–I). It is circular in cross-section (Fig. 1F), but in the anterior region, where it is covered by the extracellular sheath, it has a pear-shaped cross-section (Fig. 1E). The nucleus tapers more strongly from the mid-region towards the apex (Fig. 1B). Its posterior, truncated extremity, with a diameter of approximately 270 nm, is flattened on one side where an anterior projection of the centriolar adjunct fits tightly (Fig. 1G–I). The nucleus is attached to the flagellum by a centriolar adjunct that is electron dense, forming a 160-nm thick disk between nucleus and axoneme with an anterior projection extending 400 nm, juxtaposed basolaterally to the nucleus. A posterior expansion of this adjunct extends approximately 320 nm and is intimately associated with the axoneme's anterior tip (Fig. 1G–K). The flagellum consists of an axoneme and a pair of mitochondrial derivatives (Fig. 1K–M). The axoneme follows the 9 + 9 + 2 microtubule arrangement, including nine outer accessory tubules, nine doublets and two central single microtubules (Fig. 1M). The microtubules are twisted, as can be clearly observed in cross-sections, since not all of the doublets can be sectioned at perfect right angles (Fig. 1L–O), and in longitudinal sections (Fig. 1G,K). Anteriorly, the axoneme begins just below the nuclear base with the microtubules inserted in the centriolar adjunct (Fig. 1G–H,K). Posteriorly, the accessory microtubules terminate before the other microtubules (Fig. 1O). The mitochondrial derivatives are alike in cross-section, oval, measuring on average 100 nm in diameter and are placed very close to the axoneme (Fig. 1K–N,Q). In longitudinal sections as well as in scanning electron micrographs, they can be seen coiling regularly around the axoneme (Fig. 1G,K,Q). Anteriorly, the mitochondrial derivatives begin together in contact with the posterior extremity of the centriolar adjunct, approximately 480 nm from the nuclear base (Fig. 1H). In the final flagellar region, one mitochondrial derivative terminates shortly before the other (Fig. 1N), about 28 μm above the axoneme tip (Fig. 1O,R). Typical accessory bodies, as found in most Hymenoptera, do not occur (Fig. 1L,M).

Discussion

The ultrastructure of the spermatozoon of *Trichogramma dendrolimi* is basically the same as that of the majority of the Hymenoptera (Quicke *et al.*, 1992; Newman and Quicke, 1998; 1999a,b). In all species of chalcidoids in which the sperm ultrastructure has already been described (Lee and Wilkes, 1965; Wilkes and Lee, 1965; Hogge and King, 1975; Quicke *et al.*, 1992; Lino-Neto *et al.*, 1999, 2000a), the nucleus is twisted into a helix and the mitochondrial derivatives coil around the twisted axoneme.

The presence of an extracellular sheath surrounding the true acrosome and an anterior region of the nucleus has also been described for the chalcidoids, *Nasonia vitripennis* (Hogge and King, 1975), *Bephratelloides pomorum* (Lino-Neto *et al.*, 1999), *Trichogramma atopovirilia* and *T. pretiosum* (Lino-Neto *et al.*, 2000a) and many other wasp groups (Quicke *et al.*, 1992; Newman and Quicke, 1998; 1999b). To date, however, only in chalcidoids has the presence of numerous filaments arising from the extracellular sheath been clearly demonstrated (Lino-Neto *et al.*, 1999, 2000a).

In most hymenopterans the acrosomal complex is basically formed by a cone-shaped acrosomal vesicle surrounding a cavity which holds the perforatorium. This structure, as a rule, is inserted in a cavity of the nuclear tip (Quicke, 1997). However, in this species, as in *B. pomorum* (Lino-Neto *et al.*, 1999), besides the extracellular sheath, only one other small structure has been found, which has a concave base fitting onto the rounded nuclear tip. In *B. pomorum* (Lino-Neto *et al.*, 1999), it was identified as perforatorium. In spite of the fact that it is still not possible to define this structure as being a true perforatorium or an acrosomal vesicle, we now believe that it probably corresponds to the latter. In any case, one of the two structures is, apparently, absent and this may be another characteristic that differentiates chalcidoids from most hymenopterans, although Newman and Quicke (1999a) indicate 'the apparent absence of the acrosomal rod' (perforatorium) in the xyeloid, *Xyela julli* (Symphyta).

The mitochondrial derivatives in *T. dendrolimi*, as in the rest of the chalcidoids (Quicke *et al.*, 1992; Lino-Neto *et al.*, 1999; 2000a), are oval, alike in diameter, lie very close to the axoneme and coil regularly around the axoneme. This characteristic differs from other Hymenoptera. In the latter, the mitochondrial derivatives are straight, more or less circular or, when oval, extend outward from the axoneme (Cruz-Höfling *et al.*, 1970; Cruz-Landim and Silva de Moraes, 1980; Wheeler *et al.*, 1990; Quicke *et al.*, 1992; Newman and Quicke, 1998; 1999a,b; Lino-Neto *et al.*, 2000b). The mitochondrial derivatives beginning together at a small distance from the nucleus and in contact with the posterior base of the centriolar adjunct have been observed in chalcidoids (Lino-Neto *et al.*, 1999, 2000a), including this species, as well as in the siricoid, *Tremex* sp. (Newman and Quicke, 1999a) and, apparently, in ants (Wheeler *et al.*, 1990). In the rest of the hymenopterans where this characteristic has been observed the mitochondrial derivatives began one after the other, that is, one in contact with the centriolar adjunct and the other, more anteriorly, in contact with the nuclear base (e.g. the pamphiliid, *Cephalcia arvensis*, Newman and Quicke, 1999a) or above the nuclear base (e.g. *Leptopilina heterotoma*, Newman and Quicke, 1999b).

The axoneme begins in all the hymenopterans examined to date directly below the nuclear base, either juxtaposed to the nucleus, as described in chalcidoids (Lino-Neto *et al.*,

1999, 2000a) including the species studied here, or the initial portion of the axonemal microtubules surrounds the strongly tapered nuclear base, as occurs in *Apis mellifera* (Hoage and Kessel, 1968; Lino-Neto *et al.*, 2000b). No description has been found in which the axoneme begins laterally in relation to the nucleus, as in Lingmei and Dunsu (1987).

According to Jamieson *et al.* (1999), the centriolar adjunct usually surrounds asymmetrically the base of the mitochondrial derivatives and the axoneme where all of these structures attach to the posterior end of the nucleus. In the other hymenopterans where this structure has been observed (Wheeler *et al.*, 1990; Newman and Quicke, 1998; 1999a,b; Lino-Neto *et al.*, 2000b), it is located only between the nuclear base and one or both mitochondrial derivatives. Still, in these hymenopterans, the anterior extremities of the axonemal microtubules are parallel to the adjunct and not inserted into it, as in chalcidoids (Lino-Neto *et al.*, 1999; 2000a).

Lingmei and Dunsu (1987) observed the following structural characteristics in the spermatozoa which they described as *T. dendrolimi*. A total length of approximately 110 µm, of which the nucleus occupied about 7 µm and the flagellum, 100 µm. A well-developed acrosome, lying laterally in relation to the nucleus. The mitochondrial derivatives and axoneme also lie parallel to the nucleus and the first extend as far as the acrosome, although no mitochondrial derivative can be observed in the micrographs of the basal nuclear region viewed in cross section (Fig. 2b,c in Lingmei and Dunsu, 1987). Also, micrographs of flagella in cross-sections show the mitochondrial derivatives as being approximately triangular, not compressed to the axoneme. The accessory bodies are clearly visible and no helicoidally twisted structure was found (see Figs 3, 4 in Lingmei and Dunsu, 1987).

Therefore, as we suggested previously (Lino-Neto *et al.*, 2000a), the first description appears to be mistaken, since spermatozoa with such structural characteristics cannot belong to *T. dendrolimi*, which we describe in this study. It is possible that the specimens used by these authors did not even belong to the order Hymenoptera, since the ultrastructural characteristics observed by the authors are completely different from those observed in all other known hymenopteran species. This is true even for Symphyta (Newman and Quicke, 1999a), which is considered the most primitive hymenopteran taxon.

Quicke *et al.* (1992), considering the article of Lingmei and Dunsu (1987), described the absence of a spiralling structure in Trichogrammatidae as either plesiomorphous or as a character reversal. However, based on these results, as well as those presented by Lino-Neto *et al.* (2000a), we believe that the spiralling of the nucleus and of the mitochondrial derivatives as well as the twisting of the axonemal microtubules are synapomorphies for the Trichogrammatidae as well as for the Eulophidae (Lee and Wilkes, 1965; Wilkes and Lee, 1965), Pteromalidae (Hogge and King, 1975), Eurytomidae (Quicke *et al.*, 1992; Lino-Neto *et al.*, 1999) and Aphelinidae (Quicke, 1997). Besides these five chalcidoid families, this spiralling is probably also a synapomorphy for species of the Chalcididae, Encyrtidae, Agaonidae, Torymidae and Mymaridae families, in which we have observed, with the light microscope, typical twisted spermatozoa.

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5. Discussão e conclusão sobre a diversidade morfológica dos espermatozóides de Hymenoptera

A morfologia básica dos espermatozóides de Hymenoptera é similar àquela considerada padrão para todos os Pterygota (Baccetti, 1972). São espermatozóides móveis, filiformes, com a região de cabeça formada pelo acrossomo e núcleo, e a do flagelo, por um axonema do tipo 9 + 9 + 2 microtúbulos, dois longos derivados mitocondriais, dois corpos acessórios e, na região de transição núcleo-flagelo, um adjunto centriolar (Cruz-Höfling *et al.*, 1970; Wheeler *et al.*, 1990; Quicke *et al.*, 1992; Newman & Quicke, 1998, 1999a, b, 2000; Jamieson *et al.*, 1999; Lino-Neto *et al.*, 1999, 2000a, b). Entretanto, mesmo considerando apenas o tipo básico, os espermatozóides de Hymenoptera apresentam algumas características ultra-estruturais que, quando observadas em conjunto, tornam-os facilmente distintos daqueles de outros insetos. Da mesma forma, se consideradas as variações morfológicas já observadas dentro da própria ordem, é possível ver que neste grupo de insetos os espermatozóides possuem diversidades estruturais capazes de compor um sistema de caracteres que, associado a outros sistemas, certamente será de grande importância em estudos filogenéticos considerando famílias ou até mesmo gêneros como taxa.

Em Hymenoptera, como na maioria dos insetos, os espermatozóides são finos e, em geral, bastante longos. Entretanto, o comprimento pode variar muito, até dentro de uma mesma família. Até o momento, em Hymenoptera, o menor espermatozóide, com 8 μm, foi observado no Braconidae, *Meteorus* sp. (Quicke *et al.*, 1992) e o mais longo, com 620 μm, no Eurytomidae, *Bephratelloides pomorum* (Lino-Neto *et al.*, 1999).

O complexo acrossomal em 'três-camadas' é considerado padrão para os Pterygota (Jamieson *et al.*, 1999). Entretanto, em Hymenoptera o acrossomo padrão apresenta apenas duas camadas (bicamadas): a vesícula acrossomal e o perforatorium (Wheeler *et al.*, 1990; Jamieson *et al.* 1999; Newman & Quicke, 1999a; Zama *et al.*, em preparação). Contudo, em Chalcidoidea (Lino-Neto *et al.*, 1999, 2000a; Lino-Neto & Dolder, 2001b), Cynipoidea (Quicke *et al.*, 1992; Newman & Quicke, 1999a), Ichneumonoidea (Quicke *et. al.*, 1992; Newman & Quicke, 1998) e Bethyloidea (Quicke *et. al.*, 1992) tem sido observada uma terceira camada revestindo externamente todo o complexo acrossomal e a extremidade anterior do núcleo. Entretanto, esta estrutura, aparentemente, não é homologa àquela que constitui a terceira camada do acrossomo da maioria dos outros insetos. Pois naquele acrossomo de 'três-camadas', o material que constitui a terceira camada localiza-se abaixo da membrana celular, portanto ela é intracelular, enquanto que nestes Hymenoptera, a estrutura que

reveste o acrossomo e parte do núcleo localiza-se externamente à membrana celular, sendo assim extracelular (ver Fig. 1G, Lino-Neto *et al.*, 2000a).

Nos espermatozóides de Hymenoptera, como dos insetos em geral, o núcleo é fino, longo, de cromatina elétron densa e, geralmente, compacta. Até o momento, nesta região celular ainda não foi observada nenhuma característica ultra-estrutural capaz de distinguir os espermatozóides de Hymenoptera daqueles das demais ordens de insetos. Entretanto, quando esta região é comparada entre os próprios Hymenoptera, nota-se grandes variações, especialmente, em comprimento, podendo medir de 4 μm (Braconidae, Quicke *et al.*, 1992) a 105 μm (Chalcididae, observação pessoal). Mas, a diferença mais notável é o curso em espiral que este núcleo pode apresentar, como observado em Chalcidoidea (Lee & Wilkes, 1965; Wilkes & Lee, 1965; Hogge & King, 1975; Quicke *et al.*, 1992; Quicke, 1997; Lino-Neto *et al.*, 1999; 2000a; Lino-Neto & Dolder, 2001b), Scelionidae (Lino-Neto & Dolder, 2001a) e Diapriidae (Quicke, informação pessoal).

A transição núcleo-flagelo é uma região relativamente complexa e, provavelmente, por isso a sua organização estrutural tem sido constantemente mal interpretada nos Hymenoptera. Tanto que a estrutura denominada de adjunto do centríolo, a qual faz parte dessa região em quase todos os insetos (Jamieson *et al.*, 1999), só recentemente foi corretamente identificada nos Hymenoptera (Wheeler *et al.*, 1990; Newman & Quicke, 1998, 1999a, b, 2000; Lino-Neto *et al.*, 1999, 2000a, b; Lino-Neto & Dolder, 2001b). Nos insetos em geral o adjunto do centríolo usualmente circunda, assimetricamente, a extremidade anterior dos derivados mitocondriais e do axonema onde estes se prendem ao núcleo (Jamieson *et al.*, 1999). Provavelmente a não identificação, ou identificação incorreta, ocorrida inicialmente nos Hymenoptera seja devido a forma e localização incomum dessa estrutura nestes insetos. Pois na maioria deles, essa estrutura é aproximadamente cilíndrica, longa, ou muito longa, e localiza-se paralelamente ao axonema, entre a base do núcleo e a extremidade anterior de um (ex., em abelhas, Lino-Neto *et al.*, 2000b) ou dos dois (ex., formigas, Wheeler *et al.*, 1990) derivados mitocondriais. Até o momento adjunto do centríolo com estas características não foi descrito em nenhuma outra ordem de insetos, portanto constituindo uma, e até o momento a única, característica autopomórfica nos espermatozóides de Hymenoptera. Embora o tipo de adjunto do centríolo descrito acima esteja presente na maioria dos Hymenoptera, algumas exceções já foram observadas. Por exemplo, nos Chalcidoidea parte do adjunto se projeta anteriormente, além da base nuclear, circundando parcial ou totalmente a extremidade posterior do núcleo por uma distância, algumas vezes, muito longa (ex., *Bephratelloides pomorum*, Lino-Neto *et al.*, 1999). Ainda, em Scelionidae não foi observada nenhuma estrutura que se assemelhe a um adjunto centriolar (Lino-Neto & Dolder, 2001a).

Também o flagelo, nos espermatozóides da maioria dos Hymenoptera, tem basicamente as mesmas estruturas que tem aquele considerado convencional para todos os insetos. Portanto, constituído por um axonema do tipo 9 + 9 + 2 microtúbulos, dois corpos acessórios e dois derivados mitocondriais com cristas e, às vezes, material paracristalino. Entretanto, mais uma vez, quando esta região é comparada entre os próprios Hymenoptera, observa-se diferenças estruturais que certamente constituirão caracteres importantes para estudos filogenéticos do grupo. Por exemplo, o axonema pode ser de dois tipos: aquele em que os microtúbulos são dispostos paralelamente entre si e aquele onde os microtúbulos apresentam um curso em espiral. O primeiro tipo está presente na maioria dos Hymenoptera e o segundo tem sido observado, até o momento, em Chalcidoidea (Lee & Wilkes, 1965; Wilkes & Lee, 1965; Hogge & King, 1975; Quicke *et al.*, 1992; Quicke, 1997; Lino-Neto *et al.*, 1999; 2000a; Lino-Neto & Dolder, 2001b), Scelionidae (Lino-Neto & Dolder, 2001a) e Diapriidae (Quicke, informação pessoal). A extremidade posterior dessa estrutura tem também nos chamado a atenção, pois em Chalcidoidea (Lino-Neto *et al.*, 1999; Lino-Neto & Dolder, 2000a, 2001b) e Ichneumonoidea (Newman & Quicke, 1998) os microtúbulos acessórios são os primeiros a terminarem, já em Apoidea (Wheeler *et al.*, 1990; Zama *et al.*, em preparação) estes microtúbulos terminam por último. Nós acreditamos que, possivelmente, a característica observada em Apoidea seja comum a todos os Aculeata, da mesma forma que aquela observada em Chalcidoidea e Ichneumonoidea seja a todos os Parasitica (Apocrita não-Aculeata) e, quiçá, aos Symphyta. Se assim for, isso estaria de acordo com a hipótese de que os Symphyta constituem um grupo-irmão dos Parasitica e, como na maioria dos insetos os microtúbulos acessórios são os primeiros a terminarem, os Aculeata, dentre os Hymenoptera, seriam mais derivados. Ainda, poderia ser mais uma evidencia para a hipótese de monofilia dos Aculeata. Mas infelizmente, informações sobre a terminação dos microtúbulos do axonema só existem, até o momento, para representantes das três superfamílias citadas acima, portanto ainda é cedo para qualquer sugestão.

Quanto aos corpos acessórios, na maioria dos Hymenoptera eles têm diâmetro, geralmente, triangular e bastante conspícuo (Cruz-Höfling *et al.*, 1970; Wheeler *et al.*, 1990; Quicke *et al.* 1992; Newman & Quicke, 1999a; Lino-Neto *et al.*, 2000b). Entretanto, em alguns grupos, como por exemplo Chalcidoidea (Lino-Neto et al., 1999, 2000a; Lino-Neto & Dolder, 2001b) e Scelionidae (Lino-Neto & Dolder, 2001a), eles são tão reduzidos que é até difícil assegurar sua presença em algumas espécies.

Conforme podemos observar, o axonema e os corpos acessórios apresentam poucas diferenças estruturais, embora importantes. Entretanto, o maior número de variações existentes no flagelo é observado nos dois derivados mitocondriais. Por exemplo, em corte transversal, eles podem ter diâmetros iguais entre si (ex., formigas, Wheeler *et al.*, 1990) ou diferentes (ex., abelhas, Lino-Neto *et*

al., 2000b), apresentarem formato aproximadamente circular (ex., Chalcidoidea, Lino-Neto *et al.*, 1999, 2000a; Lino-Neto & Dolder, 2001b), muito ovalado, onde o eixo maior tem a até três ou mais vezes o menor (ex. abelhas, Lino-Neto *et al.*, 2000b; Zama *et al.*, em preparação) ou, ainda, de lua crescente, onde circundam quase totalmente o axonema (ex., *Ageniaspis citricola* (Encyrtidae: Chalcidoidea), observação pessoal). Ainda com relação ao diâmetro dos derivados, este pode variar entre, aproximadamente, três vezes maior (ex., Scelionidae, Lino-Neto & Dolder, 2001a) a três vezes menor (ex., Heloridae, Quicke *et al.*, 1992) o diâmetro do axonema. Anteriormente, os dois derivados mitocondriais podem começar juntos, associados ao adjunto centriolar, e imediatamente abaixo da base nuclear, como ocorre em Chalcidoidea (Lino-Neto *et al.*, 1999, 2000a; Lino-Neto & Dolder, 2001b), em formigas (Wheeler *et al.*, 1990) e no Symphyta, *Tremex* sp. (Siricoidea) (Newman & Quicke, 1999a). Em outros casos um dos derivados pode começar antes ou, às vezes, muito antes do outro. Neste caso, o derivado que começa antes pode, ainda, iniciar junto à base nuclear (ex., no Symphyta *Cephalcia arvensis* (Siricoidea), Newman & Quicke, 1999a) ou acima desta, portanto correndo em paralelo com o núcleo por uma distância pequena (ex., abelhas, Lino-Neto *et al.*, 2000b; Zama *et al.*, em preparação) ou, às vezes, iniciar à uma grande distância afrente da base do núcleo (ex., no Megalyroidea, *Megalyra fasciatipennis*, Newman & Quicke, 2000). Entretanto, as diferenças mais marcantes nos derivados mitocondriais que ocorrem entre os Hymenoptera são a sua disposição em espiral em volta do axonema, como observada em Chalcidoidea (*q.v.*), Scelionidae (Lino-Neto & Dolder, 2001a) e Diapriidae (Quicke, informação pessoal), e a presença de apenas um derivado, como observado em Scelionidae (Lino-Neto & Dolder, 2001a).

As diferenças estruturais citadas acima certamente são as mais representativas, ou evidentes, entretanto há várias outras que, embora menos conspícuas, com certeza constituirão caracteres importantes a serem considerados em uma análise filogenética.

Com base em todo o exposto, nós achamos que é possível, conforme já observado por Quicke *et al.*, (1992), afirmar que o conhecimento da estrutura e ultra-estrutura dos espermatozóides dos Hymenoptera com certeza contribuirá para elucidar várias das muitas dúvidas que ainda permanecem sobre as relações filogenéticas desse grupo de insetos. Entretanto, para que estudos filogenéticos usando estas informações possam ser realizados, ainda é necessário que um grande número de espécies, representando muito mais famílias ou, no mínimo, superfamílias, tenham seus espermatozóides estudados. Também com esse trabalho, esperamos estimular mais pesquisadores a investigar a morfologia deste tipo celular em mais Hymenoptera, para que em um tempo não muito longo possa ser demonstrado, na prática, que aí há realmente um conjunto de informações que,

juntamente com as informações moleculares e da morfologia somática, sejam capazes de indicar, com grande consenso, uma historia evolutiva para os Hymenoptera.

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