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Morfologia do sistema reprodutivo masculino e dos espermatozóides de Ephemeroptera (Insecta) e análise do seu potencial filogenético.

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) *Pedro Vale de Azevedo Brito*

e aprovada pela Comissão Julgadora.

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Orientadora: Profa. Dra. Mary Anne Heidi Dolder

Co-Orientador: Prof. Dr. Frederico Falcão Salles

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Índice

Resumo

Entre as ordens de insetos alados com representantes vivos, os membros da ordem Ephemeroptera estão entre os mais antigos que existem. Suas ninfas são aquáticas e os adultos, alados, sobrevivem por pouco tempo, morrendo logo após o acasalamento. Ainda existem algumas dúvidas sobre a relação dos Ephemeroptera com os demais Pterygota, bem como algumas famílias dentro da ordem são atualmente consideradas parafiléticas. A morfologia do sistema reprodutivo masculino e dos espermatozoides dos insetos pode fornecer informações úteis para estudos filogenéticos. No entanto, tais estudos envolvendo espécies de Ephemeroptera são escassos. O objetivo deste trabalho foi estudar a morfologia do sistema reprodutivo masculino e dos espermatozoides de espécies de Ephemeroptera existentes no Brasil, analisando a variabilidade morfológica encontrada nessas espécies. No Brasil são encontradas espécies pertencentes a dez famílias de Ephemeroptera e analisamos a morfologia do sistema reprodutor masculino de seis espécies pertencentes a cinco famílias e os espermatozoides de 17 espécies pertencentes a nove famílias. Nas seis espécies a morfologia do sistema reprodutivo foi muito constante sem glândulas acessórias ou órgãos especializados no armazenamento de espermatozoides. No entanto, observamos diferentes padrões de organização da musculatura intrínseca dos ductos espermáticos, provavelmente refletindo diferenças na fisiologia reprodutiva de cada espécie. A morfologia dos espermatozoides se mostrou mais variável. As espécies da família Leptophlebiidae possuem espermatozoides aflagelados e imóveis. Nas demais famílias, os espermatozoides são flagelados e móveis. A organização do axonema se mostrou constante nas diferentes espécies com o padrão 9+9+0 típico para esses insetos. Apenas os microtúbulos acessórios mostraram variação na estrutura, podendo assumir o padrão de subunidades 13+7 ou 13+0. Os flagelos são caracterizados por apenas uma mitocôndria que se alonga por quase todo flagelo. A morfologia dos corpos acessórios dos flagelos varia entre as espécies. Parece haver correlação entre a organização das cristas mitocondriais e os corpos acessórios. A morfologia da vesícula acrossomal é variável podendo estar relacionada com diferenças na espessura do corion dos ovos. No início dos flagelos observamos o adjunto do centríolo, que acreditava-se estar ausente nos espermatozoides dos Ephemeroptera. Em uma espécie estudada o núcleo dos espermatozoides está associado paralelamente ao flagelo. Nossos resultados sugerem que os espermatozoides dos Ephemeroptera possuem variabilidade morfológica suficiente para fornecer dados para futuros estudos filogenéticos. No entanto, é preciso que mais espécies sejam estudadas aumentando a abrangência dentro do grupo. Alem disso, alguns pontos como a origem dos corpos acessórios dos espermatozoides dos Ephemeroptera precisam ser melhor estudados.

Abstract

Ephemeroptera species are the oldest living winged insects. Their nymphs are aquatic and the adults are short living, dying just after mating. At the present, there are still some doubts about the phylogenetic relationships between Ephemeroptera and the other Pterygota. The morphology of the male reproductive systems and of the spermatozoa is useful to furnish data for phylogenetic studies. However, there are few studies on this subject for Ephemeroptera. This study analyzes the morphology of the male reproductive system and of the spermatozoa of Brazilian Ephemeroptera species.. Species from ten Ephemeroptera families are found in Brazil. In the present study we analyzed the male reproductive system of six species from five families. We also analyzed the sperm morphology of 17 species from nine families. The male reproductive systems analyzed were very similar in the different species, with no accessory glands or specialized organs for sperm storage. However, the intrinsic musculatures of the sperm ducts have different organization patterns, probably related to differences in the reproductive physiology of each species. Greater morphological variation was observed among the spermatozoa. Species from Leptophlebiidae family have aflagellate and immotile spermatozoa. Species from the other families have mobile and flagellate spermatozoa. The organization of the axoneme was the same in all species, with the 9+9+0 microtubule pattern, typical for this insect group. Only the accessory microtubules vary between the 13+7 and the 13+0 subunit patterns. The flagella are characterized by the presence of only one mitochondrion along the flagellum. The accessory bodies morphology may vary between the species and it seems to be correlated to the organization of the mitochondrial cristae and the accessory bodies morphology. The acrosomal vesicles have morphological variations that must be related to differences in the egg chorion thickness. A centriolar adjunct is observed at the flagellum anterior region of the spermatozoa. This structure was thought to be absent in the Ephemeroptera spermatozoa. One species studied has its nucleus laterally associated to the flagellum. Our results suggest that the spermatozoa of Ephemeroptera have enough morphological variation to furnish useful data for future phylogenetic studies. However, more species, representing different groups of the order must be studied, increasing the scope of these studies. Also some questions, such as the origin of the accessory bodies of Ephemeroptera must be further studied.

Introdução

A ordem Ephemeroptera possui mais de 3.000 espécies distribuídas em 42 famílias (Barber-James et al. 2008), representando o grupo mais antigo de insetos alados existente. Seus indivíduos são caracterizados pelas ninfas aquáticas e os adultos terrestres (Fig. 1). As ninfas podem apresentar grande diversidade de estratégias alimentares, podem ser filtradoras, raspadoras, fragmentadoras, coletoras ou predadoras e podem viver desde algumas semanas a alguns meses (Fig. 2A). Já os adultos possuem as peças bucais atrofiadas, não se alimentam e podem viver desde algumas horas até poucos dias (Fig. 2B). Além disso, os Ephemeroptera possuem uma peculiaridade que é a presença de um estágio alado intermediário entre ninfa e adulto, denominado subimago (Fig. 1). As espécies de Ephemeroptera se distribuem por todos os continentes, exceto a Antártida, mas sua maior diversidade é observada em rios e córregos das zonas tropicais e temperadas (Brittain 1982; Brittain & Sartori 2003).

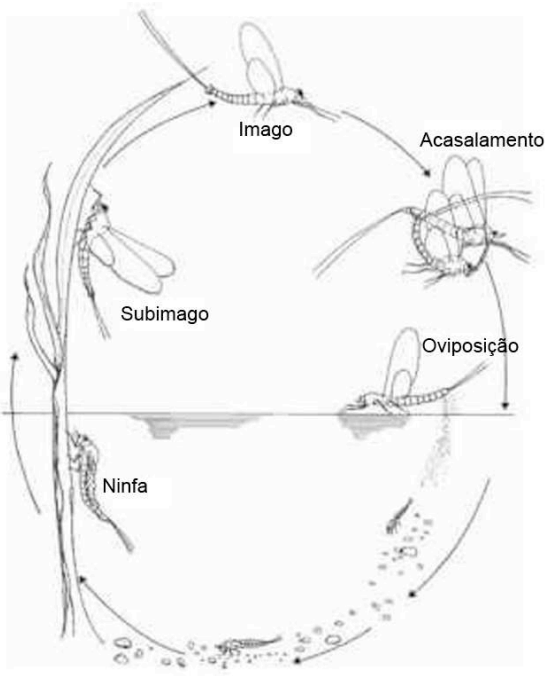

Fig. 1 – Representação do ciclo de vida dos Ephemeroptera. Modificado a partir da Internet.

No Brasil foram registradas até o momento 233 espécies em 68 gêneros, distribuídos em 10 famílias da ordem Ephemeroptera: Melanemerellidae, Coryphoridae, Ephemeridae, Baetidae, Leptohyphidae, Leptophlebiidae, Euthyplociidae, Oligoneuriidae, Polymitarcyidae e Caenidae (Salles et al. 2011).

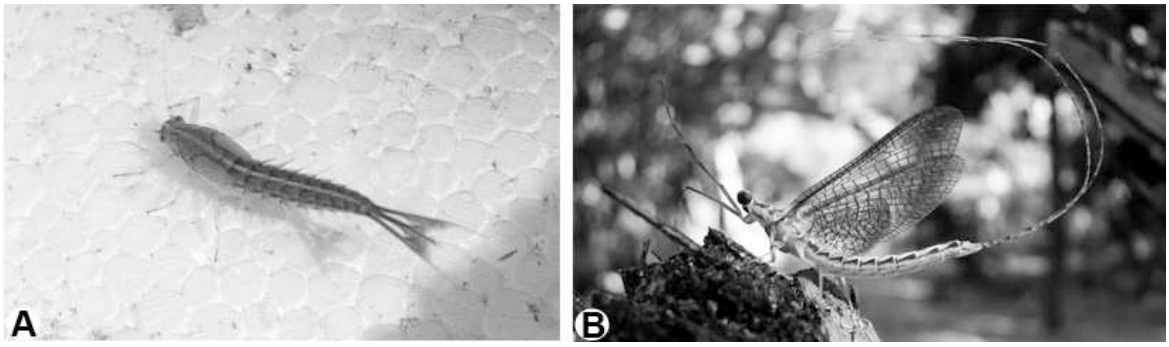

Fig. 2 – A) Ninfa de *Americabaetis longetron* Lugo-Ortiz & McCafferty, 1996. B) Imago de *Hexagenia albivitta* Walker, 1853. Fotografias por Frederico F. Salles.

Os macroinvertebrados bentônicos, incluindo os Ephemeroptera, são responsáveis pela maior parte da produção secundária em ecossistemas aquáticos continentais. Em Ephemeroptera, essa produção pode atingir até 25% daquela atribuída aos macroinvertebrados bentônicos (Elliot et al. 1988). Dessa forma, eles asseguram grande disponibilidade de biomassa e representam a principal via de transferência de energia e matéria para níveis tróficos superiores (Robertson 1995; Grubaugh et al. 1997), constituindo importante fonte de alimento para peixes (Nyströn et al. 1996; Pierce & Hinrichs 1997, Rosenfeld 1997).

Devido à sua longa associação com ambientes aquáticos e à sensibilidade de algumas espécies a distúrbios ecológicos (Merrit & Cummins 1984; Da Silva 1994), os Ephemeroptera vêm sendo amplamente estudados e utilizados como bioindicadores (Leal & Esteves, 2000; Buffagni & Comin, 2000; Brittain & Sartori, 2003).

Apesar de haver muitos estudos sobre a biologia, a taxonomia e a importância ecológica dos Ephemeroptera, o estudo da filogenia da ordem ainda apresenta problemas. A ordem Ephemeroptera forma, juntamente com a ordem Odonata (libélulas), a infraclasse Paleoptera, que corresponde aos insetos alados mais antigos que existem. Porém, não existe um consenso sobre a relação entre "Ephemeroptera + Odonata", com os demais insetos (Neoptera), com Paleoptera algumas vezes considerado um grupo parafilético (Kristensen 1981; Ogden & Whiting 2003; Regier et al. 2010). Sendo Ephemeroptera e Odonata os dois grupos mais antigos de insetos alados que existem, a resolução dessa questão pode ajudar inclusive a esclarecer a origem das asas nos insetos (Ogden & Whiting 2005).

Existem dois principais sistemas de classificação para as famílias de Ephemeroptera (Fig. 3) estabelecidos por McCafferty (1991) e Kluge (2004). Estes dois sistemas baseiam-se principalmente em caracteres morfológicos externos e, apesar de bastante parecidos entre si, eles estabelecem diversos grupos (desde subordens até famílias) que foram considerados parafiléticos em estudos moleculares recentes (Fig. 4) (Ogden & Whiting 2005; Ogden et al. 2009). A classificação de espécies em agrupamentos artificiais pode levar a conclusões equivocadas em estudos sobre ecologia, diversidade biológica e impacto ambiental envolvendo esses organismos. Dessa forma, é essencial aperfeiçoar o conhecimento acerca da filogenia do grupo.

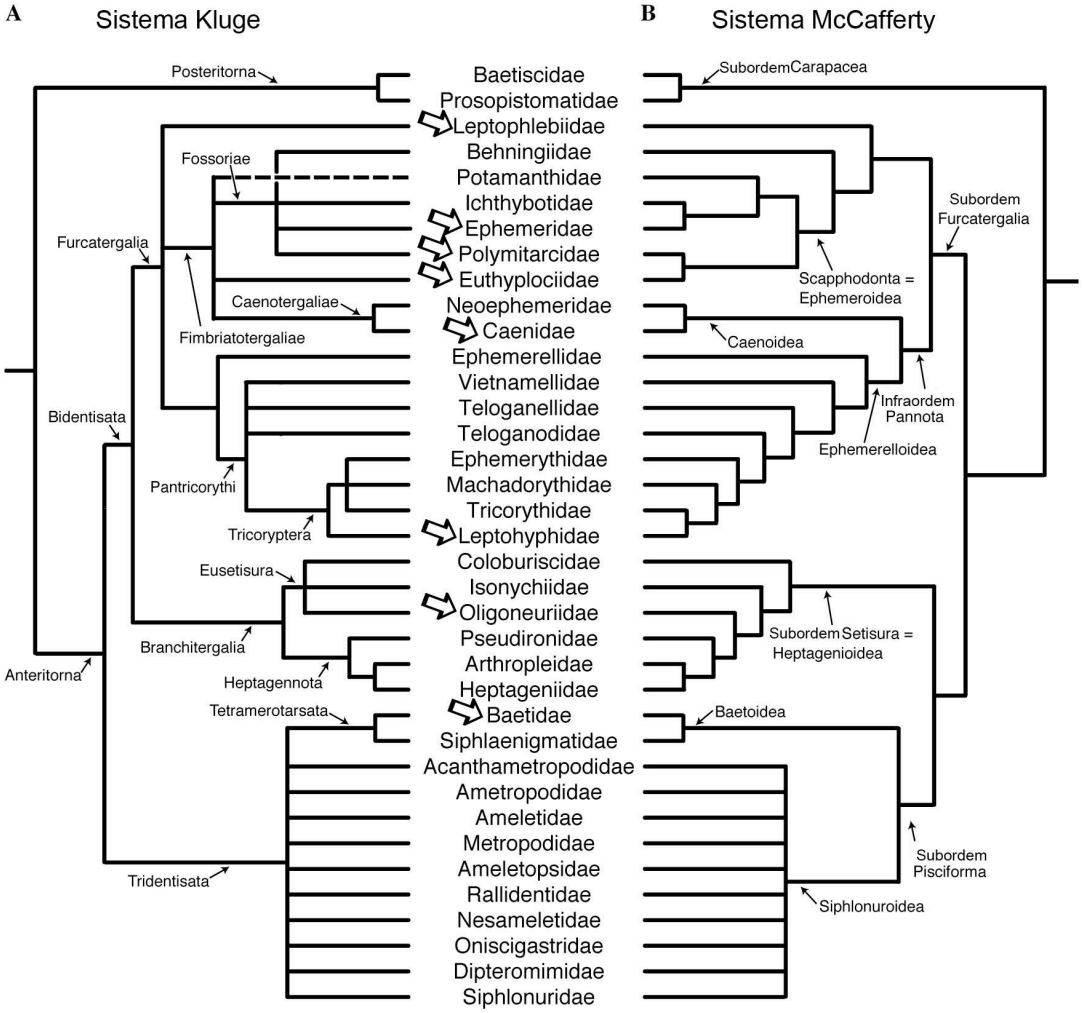

Fig. 3 – Comparação entre os sistemas de classificação de Kluge (2004) e McCafferty (1991), modificado a partir de Ogden & Whiting (2005). As setas abertas indicam as famílias de Ephemetoptera encontradas no Brasil, exceto Melanemerellidae e Coryphoridae não representadas no esquema.

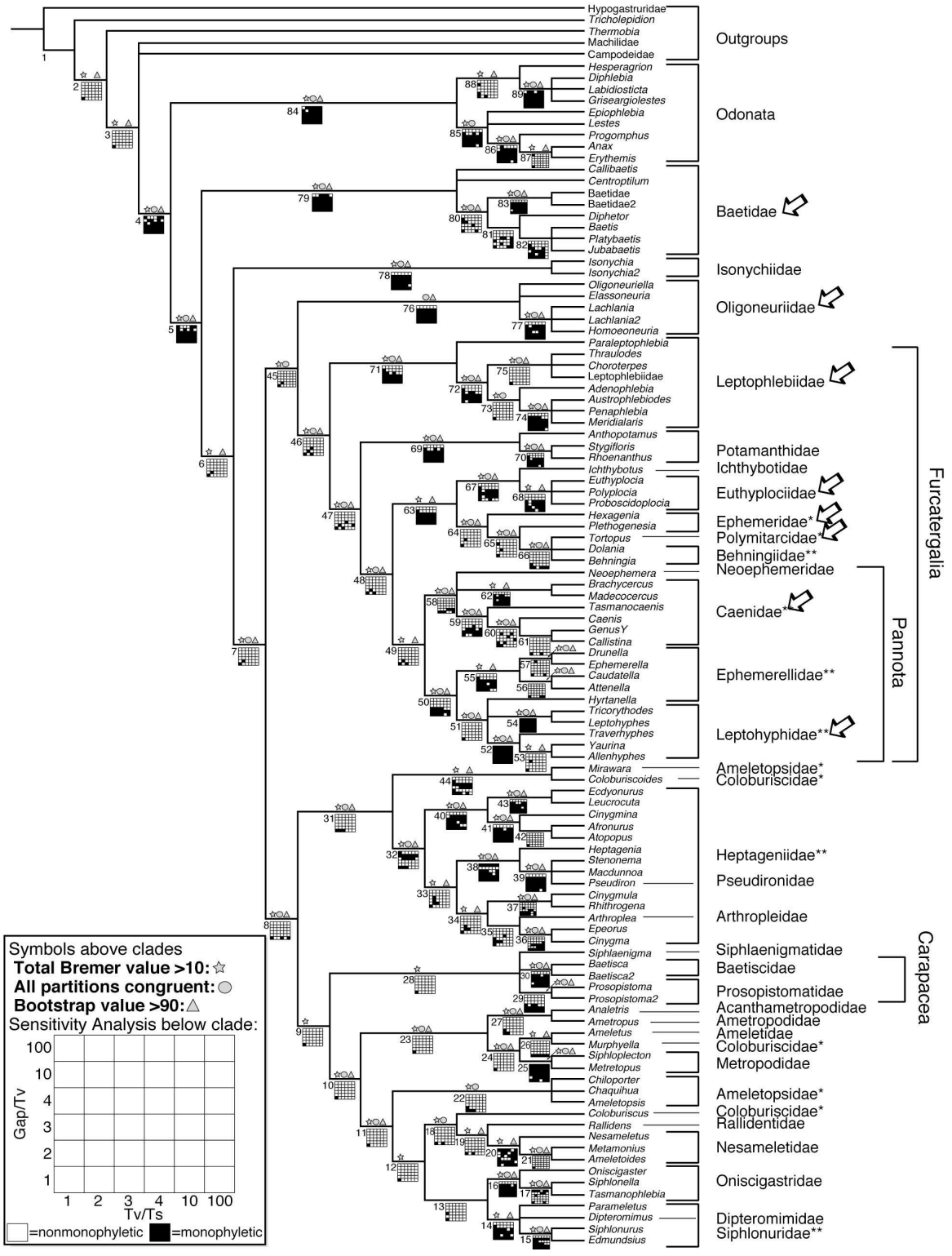

Fig. 4 – Figura modificada a partir de Ogden & Whiting (2005). Filogenia proposta para as famílias de Ephemetoptera. As setas abertas indicam as famílias de Ephemetoptera encontradas no Brasil, exceto Melanemerellidae e Coryphoridae não representadas no esquema.

Morfologia do Sistema Reprodutivo e dos Espermatozoides

A morfologia do sistema reprodutivo masculino e dos espermatozoides tem sido utilizada em estudos filogenéticos em quase todos os grupos de insetos (Callahan & Chapin 1960; Lai-Fook 1981; Gotwald & Burdette 1981; Wheeler & Krutzsch 1992; Jamieson et al. 1999; Lino-Neto & Dolder 2001; Zama et al. 2005; Mancini et al. 2006), colaborando para a resolução de diversas questões ainda pouco esclarecidas. Porém, até o momento na ordem Ephemeroptera esses estudos são escassos.

O sistema reprodutivo masculino dos Ephemeroptera é constituído por um par de testículos que pode se estender desde o metatórax até o VI segmento abdominal. Cada testículo é formado por um número variado de folículos, entre 140 e 290, dependendo da espécie e inclusive do indivíduo (Fig. 5). Cada testículo desemboca os espermatozóides em um ducto espermático, que funciona também para armazenagem dos espermatozoides depois que esses deixam os testículos (Fig. 5). Os ductos espermáticos encontram-se dilatados nas ninfas de último ínstar (com teca alar escura) e desembocam nos gonoporos entre os segmentos IX e X (Brinck 1957; Soldán 1979a). Embora o sistema reprodutivo masculino dos Ephemeroptera siga o plano básico descrito acima, extensas observações realizadas por Grimm (1985) permitiram a identificação de diferentes padrões de organização da musculatura intrínseca dos ductos espermaticos.

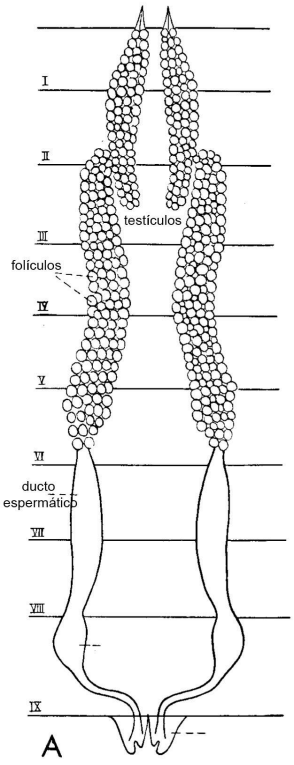

Fig. 5 – Esquema da morfologia do sistema reprodutivo masculino de *Siphlonurus* sp. (Ephemeroptera), modificado a partir de Brinck (1957). Os algarismos romanos correspondem aos segmentos abdominais.

A morfologia dos espermatozoides de espécies representando onze famílias foi estudada em microscopia de luz (Soldán 1979b). Apesar disso, a ultraestrutura dos espermatozoides de apenas doze espécies havia sido estudada, sendo que alguns desses trabalhos apresentam apenas descrições parciais dos espermatozoides (Baccetti et al. 1969; Phillips 1969; Grimm 1985; Fink & Yasui 1988; Dallai & Afzelius 1990; Gaino &

Mazzini 1991a,b; Lupetti et al., 2011). Ainda assim, algumas características foram consideradas típicas dos espermatozoides de Ephemeroptera: acrossomo em monocamada (constituído apenas pela vesícula acrossomal) (Fig. 6A), ausência do adjunto do centríolo (Fig. 6B), apenas uma mitocôndria sem material cristalino na matriz (Fig. 6C), axonema com padrão 9 + 9 + 0 (sem o par central de microtúbulos), ausência do braço externo de dineína nas duplas de microtúbulos e microtúbulos acessórios com padrão 13 + 7 com 13 protofilamentos de tubulina envolvendo sete pequenas unidades de natureza desconhecida (Fig. 6D) (Jamieson et al. 1999). Corpos cristalinos observados externamente à mitocôndria dos espermatozoides de Ephemeroptera tem sido relacionados ao paracristalino da matriz mitocondrial da maioria dos insetos (Baccetti et al. 1969; Gaino & Mazzini 1991b; Jamieson et al. 1999; Jamieson 2011).

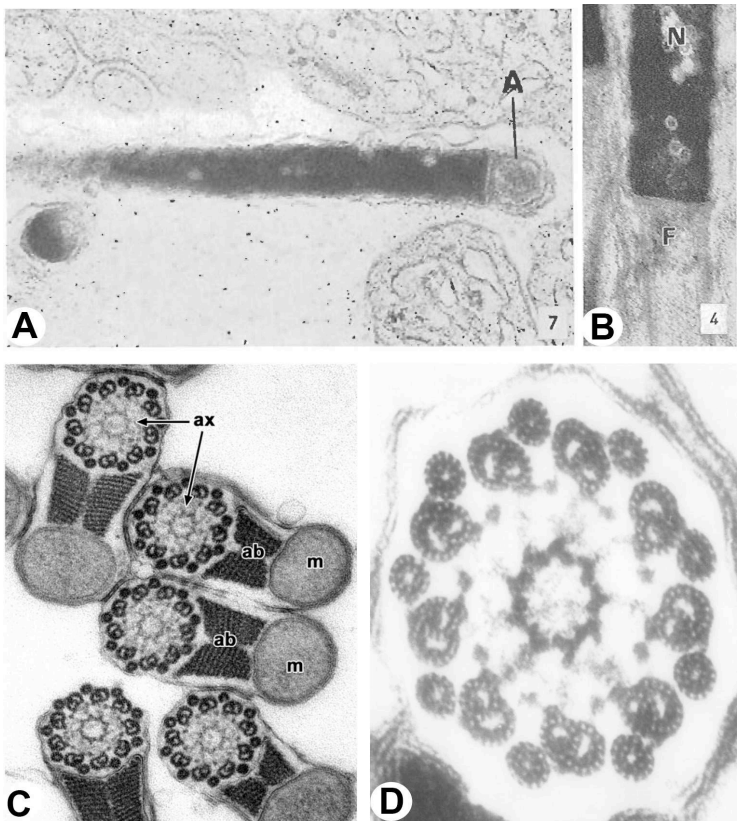

Fig. 6 – Espermatozóide de *Cloeon dipterum* Linnaeus, 1761. A) Corte longitudinal da porção anterior do espermatozóide, (A) acrossomo. B) Corte longitudinal da região de transição entre o núcleo (N) e o flagelo (F). C) Corte transversal do flagelo, (ax) axonema, (ab) corpo acessório, (m) mitocôndria. D) Grande aumento do axonema. A,B) modificado de Baccetti et al. (1969). C) modificado de Luppetti et al. (2011). D) modificado de Dallai & Afzelius (1990).

Dentro da ordem Ephemeroptera, as espécies da família Leptophlebiidae são uma exceção por possuírem espermatozoides aflagelados e aproximadamente esféricos, constituídos pelo núcleo, vesícula acrossomal e um número variado de pequenas mitocôndrias (Fig. 7) (Soldán 1979b; Gaino & Mazzini 1991a).

Acreditamos que aumentando o numero de espécies e famílias com a morfologia do sistema reprodutivo e dos espermatozoides descrita, novos padrões de organização podem ser encontrados, aumentando o conhecimento a respeito desses insetos.

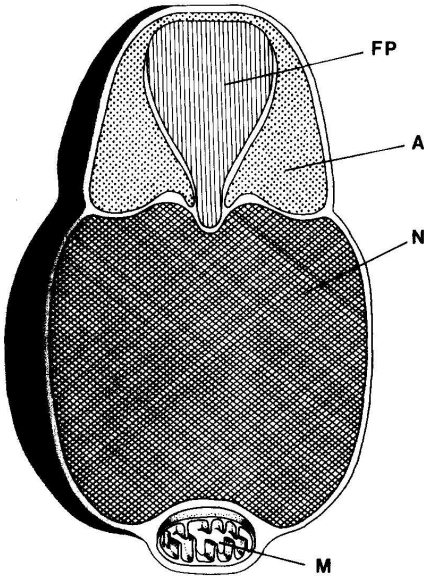

Fig. 7 – Esquema dos espermatozóides de *Habroleptoides umbratilis* Eaton, 1884, retirado de Gaino & Mazzini (1991a). (FP) perforatorium, (A) acrossomo, (N) núcleo, (M) mitocôndria.

Objetivos

Este trabalho tem como objetivo descrever a morfologia (estrutura e ultraestrutura) do sistema reprodutivo masculino e dos espermatozoides de representantes das famílias de Ephemeroptera encontrados no Brasil. Dessa forma, além de aumentar a disponibilidade de informações sobre o assunto, buscamos verificar se existe variabilidade morfológica entre os diferentes grupos de Ephemeroptera, quanto à morfologia do sistema reprodutivo masculino e dos espermatozoides.

Objetivos específicos:

1) Descrever a morfologia do sistema reprodutivo masculino dos Ephemeroptera
2) Descrever a ultraestrutura dos espermatozoides dos Ephemeroptera

Resultados

Neste trabalho apresentamos a descrição de aspectos da morfologia e da organização da musculatura intrínseca do sistema reprodutivo de seis espécies, representando cinco famílias de Ephemeroptera. Além disso, apresentamos a descrição morfológica dos espermatozoides de 17 espécies, representando nove famílias de Ephemeroptera. Das famílias de Ephemeroptera encontradas no Brasil, apenas Melanemerellidae não foi incluída nesse trabalho pois adultos desse grupo não foram coletados. Os espécimes coletados encontram-se depositados no Museu de Zoologia da Unicamp e no Museu de Entomologia do INPA.

Os resultados encontram-se organizados na forma de dez capítulos, constituídos por um artigo já publicado, dois manuscritos submetidos para publicação, cinco manuscritos que encontram-se prontos para serem submetidos e dois em estágio final de preparação.

Capítulo 1 – Morphology of Male Reproductive System in Ephemeroptera: Intrinsic Musculature – Submetido para publicação na revista Neotropical Entomology

Capítulo 2 - Characteristics of the Male Reproductive System and Spermatozoa of Leptophlebiidae (Ephemeroptera). Artigo publicado na revista Neotropical Entomology, 2011, 40: 103-107.

Capítulo 3 - New Data on Mayfly Spermatozoa (Ephemeroptera). Submetido para publicação na revista Micron.

Capítulo 4 - The sperm morphology of two Baetidae species (Ephemeroptera)

Capítulo 5 - Sperm morphology of *Asthenopus curtus* (Hagen, 1861) and *Campsurus* sp. (Ephemeroptera: Polymitarcyidae); a comparative analysis.

Capítulo 6 - What does Sperm Morphology says about the relationship of Coryphoridae and Leptohyphidae?

Capítulo 7 - The unusual morphology of the spermatozoa of *Lachlania* sp. (Ephemeroptera: Oligoneuriidae).

Capítulo 8 - Morphological study of *Campylocia anceps* Eaton 1883 (Euthyplociidae) spermatozoa, and comparisons with other Ephemeroptera.

Capítulo 9 - Estudo comparativo entre os espermatozoides de Caenidae (Ephemeroptera).

Capítulo 10 - Morfologia dos espermatozoides de *Hexagenia (Pseudeatonica) albivitta* Walker (Ephemeroptera: Ephemeridae).

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Capítulo 1

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Morphology of Male Reproductive Systems in Ephemeroptera: Intrinsic Musculature

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Running title: Ephemeroptera male reproductive system

Abstract- Although the Ephemeroptera have been studied over a long period of time, even today, there are few studies on the morphology of male reproductive system. The spermatic ducts are considered conserved among different Ephemeroptera groups. However, previous studies distinguished different organizational patterns of the spermatic duct intrinsic musculature. This study describes the morphology of the spermatic ducts, especially their musculature, in six species of Ephemeroptera, representing five families. We have observed variations in the organizational pattern of the spermatic ducts, even between species from the same family. Moreover, all species studied had intrinsic musculature in the spermatic ducts although with different organizational patterns. Thus, we believe that this musculature is important to move the spermatozoa along the ducts of all Ephemeroptera and not only of those with aflagellated spermatozoa (Leptophlebiidae). The variations in musculature organization must be related to differences in reproductive physiology (i.e. copula duration) and not only with spermatozoa characteristics.

Key Words: Mayfly, spermatic duct, Scanning and Transmission Electron Microscopy.

Introduction

Although Ephemeroptera internal morphology has been studied over a long period of time (e.g. Swammerdan 1681), even today, there are few studies of the male reproductive system morphology for this group. While taxonomists extensively study external genitalia, the internal seminal ducts are almost unknown. Moreover, these ducts are considered conserved among different Ephemeroptera groups (Brinck 1957).

The Ephemeroptera male reproductive system ground plan is a pair of testis that elongate from the metatorax to the VI abdomnal segment. Depending on the species and the specimen, each testicle may be made up of a variable number of follicles, from 140 to 290. Each testicle empties its content into a spermatic duct, which acts also as a storage organ for sperm, after these leave the testes. Last instar nymphs have dilated spermatic ducts that empty into the gonopores between segments IX and X (Brinck 1957, Soldán 1979a).

Although the male reproductive system of Ephemeroptera follows the basic plan described above, extensive observations made by Grimm (1985) allowed the identification of different patterns of the spermatic duct intrinsic musculature organization. The major conclusions obtained in the above study are summarized below:

1) Species from Leptophlebiidae have strong ring musculature surrounding the ducts in order to pump their aflagellate and immotile spermatozoa;
2) Some species from Baetidae do not present enough intrinsic duct musculature to pump the spermatozoa.
3) The intrinsic musculature organization pattern is variable even between species from the same family.

The present study provides a collection of observations on the morphology of the male reproductive system of some Ephemeroptera. Special attention was directed to the musculature associated to the duct epithelium. Thus we intend to complement the data available in the literature, providing new information about the subject.

Material and Methods

Males from six species representing five families, at imago, subimago or last instar nymph stages were collected and processed for morphological analysis, as described below.

- *Miroculis (Atroari) amazonicus* Savage & Peters (Leptophlebiidae)

Last instar nymphs were collected in Presidente Figueiredo, state of Amazonas, Brazil (2° 1' 5" S; 59° 49' 25.70" O), and maintained in river water up to subimago stage. Their reproductive systems were dissected in phosphate buffer 0.1M, pH 7.2, some being processed with routine Scanning Electron Microscopy techniques. Other reproductive systems were fixed in 1% tannic acid, 2.5% glutaraldehyde solution in the same buffer. The material was then processed by routine Transmission Electron Microscopy techniques.

- *Farrodes carioca* Dominguez *et al.* (Leptophlebiidae)

Subimagoes were attracted by a light trap near a river in Santa Teresa, state of Espírito Santo, Brazil (19° 52' 31.60" S; 40° 31' 49.10" O). The male reproductive systems were dissected and processed as described above.

- *Asthenopus curtus* Hagen (Polymitarcyidae)

Imagos were collected by a light trap at Lake Catalão, Manaus, Amazonas (3° 9' 13.16" S; 59° 54' 56.40" O). The male reproductive systems were dissected in phosphate buffer 0.1M, pH 7.2 and fixed in a 2.5% glutaraldehyde solution in the same buffer. The spermatic ducts were photographed with an Olympus BX41 photomicroscope and then processed by routine Scanning Electron Microscopy techniques.

- *Lachlania* sp. (Oligoneuriidae)

Last instar nymphs were collected at Dores do Rio Preto, Espírito Santo (20° 37' 30.37" S; 41° 49' 26.46" O). Their reproductive systems were dissected in phosphate buffer 0.1M, pH 7.2 and then processed by Scanning Electron Microscopy.

- *Callibaetis jocosus* Navás (Baetidae)

Last instar nymphs were collected in a lake at "Serra do Japi", Jundiaí, State of São Paulo, Brazil (23° 14' 18.30" S; 46° 56' 27.25" O), and maintained in the laboratory up to imago stage. Their reproductive systems were dissected in phosphate buffer 0.1M, pH 7.2. The material was fixed in 1% tannic acid and 2.5% glutaraldehyde solution and processed by routine Transmission Electron Microscopy techniques.

- *Traverhyphes (Mocoihyphes) yuati* Molineri (Leptohyphidae)

Subimagoes were attracted by a light trap near a river in Santa Teresa, Espírito Santo, Brazil (19° 52' 31.60" S; 40° 31' 49.10" O). Their reproductive systems were dissected in phosphate buffer 0.1M, pH 7.2. The material was fixed in 1% tannic acid and 2.5% glutaraldehyde solution and processed by routine Transmission Electron Microscopy techniques.

A ZEISS LEO 906 Transmission Electron Microscope and a JEOL JSM5800LV Scanning Electron Microscope were used in the analyses, both belonging to the Biology Institute of Unicamp.

Results

The male reproductive system of *M. amazonicus* features an approximately cylindrical, dilated spermatic duct (Fig 1A), with reduced testicle vestiges (not shown). At the scanning electron microscope, parallel grooves forming rings are observed along the entire spermatic ducts (Fig 1B). A duct was broken showing that the grooves represent muscle fibers of the spermatic duct intrinsic musculature (Fig 1C). The muscle fibers are strongly stained by tannic acid and are not uniformly distributed, externally to the epithelium, as seen in ultrathin sections (Fig 1D).

The spermatic duct of *F. carioca* is pear shaped, dilated at the proximal portion, juxtaposed to the testes (Fig 1E). The testes are reduced and globular (Fig 1E, F), coated by a thin conjunctive layer. Some grooves are observed in both the dilated and the tapered portion, near the penis (Fig 1F, G). However, these grooves do not seem to follow an organized pattern. Observed in ultrathin sections, the muscle fibers make up a thicker layer, weakly stained by the tannic acid, covered by a thin epithelium that lines the lumen and a complex external conjunctive coat (Fig 1H).

The male reproductive system of *A. curtus* is long (Fig 2A), extending from thorax, where testes vestiges are observed, leading to the spermatic ducts that run along the abdomen. The spermatic ducts are thinner near the testes, increasing their volume near the penis, where a constriction is observed (Fig 2A). At the scanning electron microscope few grooves are observed (Fig 2B), while near the penis many grooves are observed ringing the duct (Fig 2C).

Spermatic ducts of *Lachlania* sp. can be divided into three distinct regions (Fig 2D). The proximal region is thicker and has several grooves forming rings around the duct. The thinner region of the duct is divided in two portions, where the first portion, has the same groove pattern (Fig 2E), while the portion near the gonopore is characterized by strong muscle bundles running parallel to the duct (Fig 2F). This region is also identified by the brownish color in fresh specimens, due to a chitin cuticle at the epithelial surface (not shown).

The spermatic ducts of *C. jocosus* have a 4.6 μm thick epithelial layer with many degenerative vesicles in the cytoplasm. This pseudostratified epithelium has nuclei in

different positions, varying from basal to apical. A thin basal lamina separates a uniform muscle layer, which is approximately 1.9 μm thick (Fig 3A).

The spermatic ducts of *T. yuati* have an epithelial layer, approximately 2 μm thick, with basal nuclei. Externally, a fibrous basal lamina separates the well developed muscle layer, which is approximately 11 μm thick (Fig 3B). However, this muscle layer surrounds only half of the duct's perimeter, while the other half has no musculature (not shown).

Discussion

Previous studies describe the spermatozoa of Ephemeroptera species as flagellated and motile (Baccetti *et al* 1969, Phillipps 1969, Soldán 1979b, Grimm 1985, Fink & Yasui 1988, Dallai & Afzelius 1990, Gaino & Mazzini 1991), with exception of the species from Leptophlebiidae, which has aflagellate and immotile spermatozoa (Soldán 1979b, Gaino & Mazzini 1991, Brito *et al* 2011).

According to Grimm (1985), Leptophlebiidae species should have ringged musculature surrounding the spermatic ducts. This characteristic would be important to move the sperm, since their spermatozoa are aflagellated (Soldán 1979b, Grimm 1985, Gaino & Mazzini 1991, Brito *et al* 2011). The morphology observed in the present study for *M. amazonicus* confirms Grimm's (1985) conclusions. On the other hand, although *F. carioca* spermatic ducts have a well-developed musculature, it is not organized in rings. Thus, this characteristic is not common to all Leptophlebiidae.

The intrinsic musculature of the *A. curtus* spermatic ducts is well developed and organized, especially near the penis, which should be related to sperm ejaculation. Muscular sphincters were described along the spermatic ducts of different species of Ephemeroptera (Grimm 1985), therefore the constrictions observed near the penis of *A. curtus* are probably sphincters.

Ephemeroptera ejaculatory ducts are ectodermic invaginations with a chitin cuticle covering the epithelium (Quadri 1940). Thus, the caudal portion of the spermatic ducts of *Lachlania* sp. with longitudinal muscle bundles is the ejaculatory duct. These muscles are responsible for the duct eversion described during copulation of Oligoneuriidae species (Pescador & Peters 1980).

The well-organized and evenly distributed musculature along the spermatic ducts of *C. jocosus* does not support the conclusions of Grimm (1985), since this species is flagellated and should not need, according to this author, a strongly muscular duct. Another unusual occurrance is the thick muscle layer surrounding only half of the spermatic duct in *T. yuati*, which has not been previously observed in Ephemeroptera.

Conclusions

We have observed variations in the organizational pattern of the spermatic ducts, even between species from the same family (i.e. *M. amazonicus* and *F. carioca*). Moreover, even with different patterns, all species studied showed intrinsic musculature in the spermatic ducts. According to Werner & Simmons (2008) the contraction of the spermatic duct musculature seems to be essential to move spermatozoa along the ducts in major insect groups. Thus, our hipothesis is that this musculature is important to move the spermatozoa along the ducts of all Ephemeroptera and not only those with aflagellated spermatozoa (Leptophlebiidae). The diverse organization patterns of this musculature must be related to differences in reproductive physiology (i.e. copula duration) and not only with the spermatozoa characteristics.

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Figure Legends

Fig 1: A-D) *Miroculis amazonicus;* E-H) *Farrodes carioca*. A,E) Reproductive systems visualized with Scanning Electron Microscopy (SEM). (d) spermatic duct, (tt) reduced testes, (p) penis, (f) forceps. **B, C, F, G)** higher magnification of boxed regions, (arrows) broken muscle fibers, (z) spermatozoa. **D, H)** Transmission Electron Microscopy of the spermatic duct wall, (ms) muscles, (e) epithelium, (b) basal lamina, (n) epithelial cell nuclei, (c) conjunctive capsule, (lu) lumen.

Fig 2: A-C) *Asthenopus curtus*; D-F) *Lachlania* sp. A) Male reproductive system, with reduced testes (tt) and a pair of long spermatic ducts with constrictions (open arrow), (p) penis. **B, C)** SEM of indicated regions, (fb) fat body. **D)** SEM of a spermatic duct (d) associated to the subgenital plate. **E, F)** Higher magnifications of boxed regions, (arrows) longitudinal muscular fibers.

Fig 3: Transmission Electron Microscopy. A) Spermatic duct wall of *Callibaetis jocosus*. **B)** Spermatic duct wall of *Traverhyphes yuati*. (e) epithelium, (m) muscle layer, (bl) basal lamina, (z) spermatozoa, (n) epithelial cell nuclei.

Figure 1

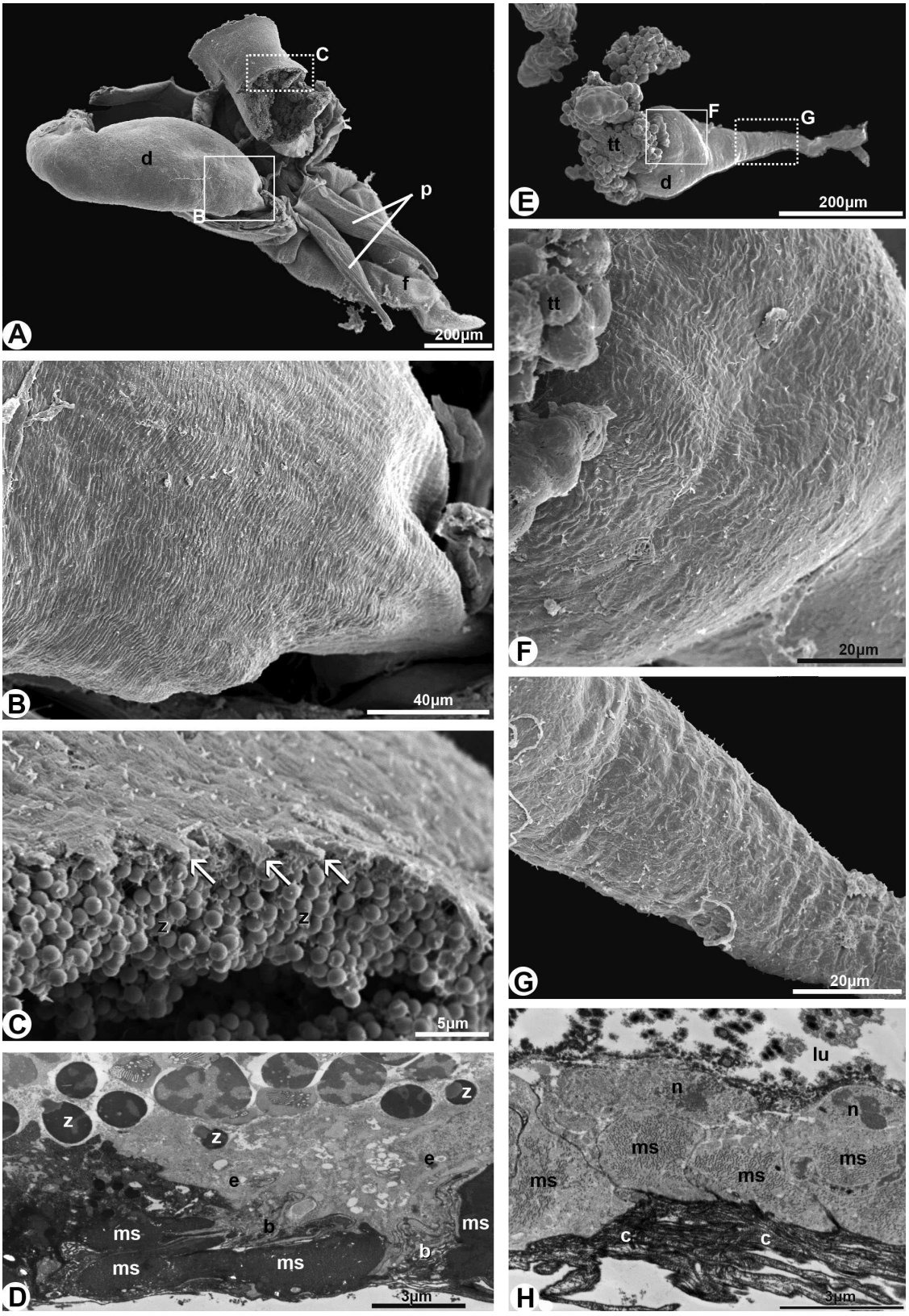
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Figure 2

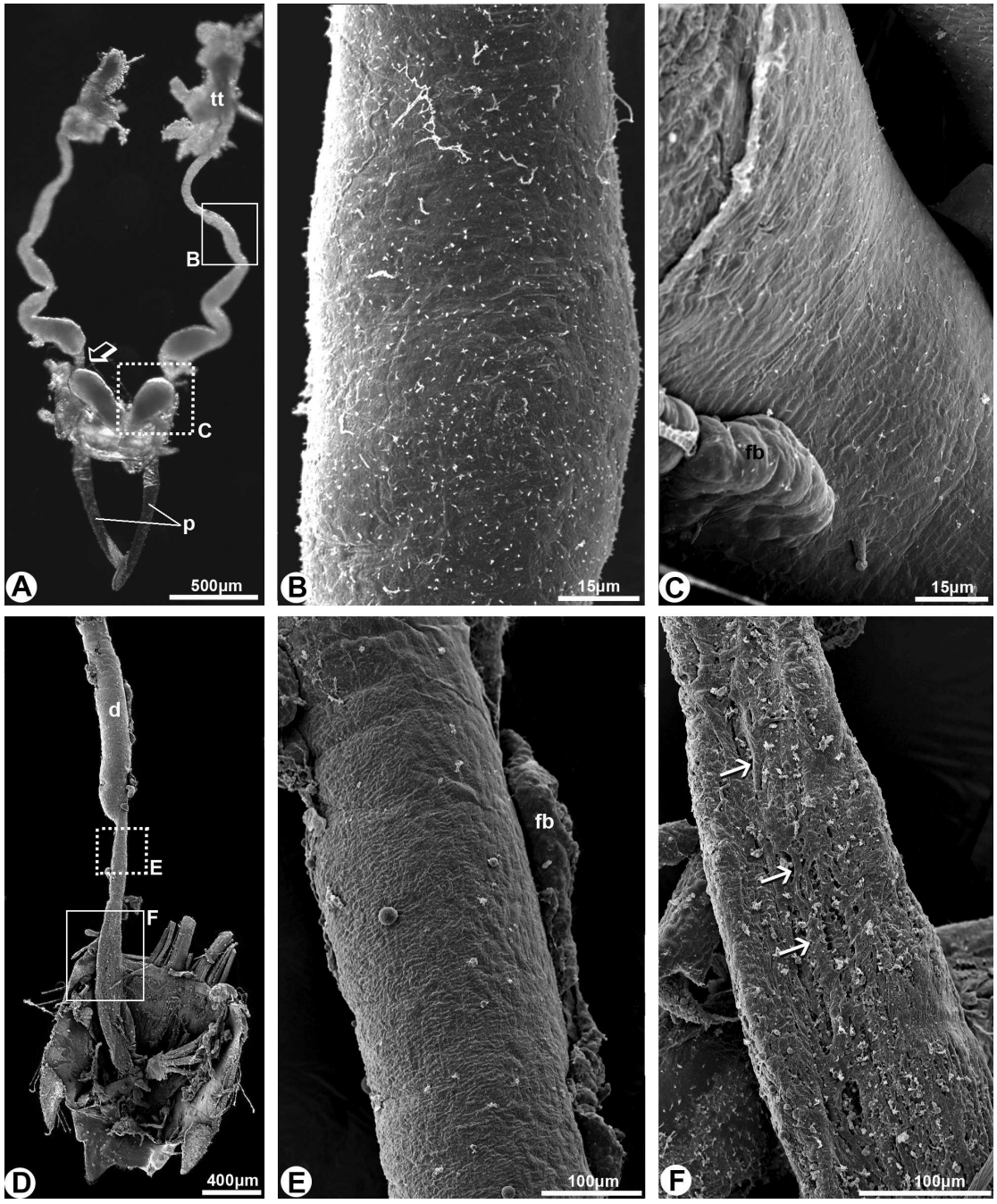
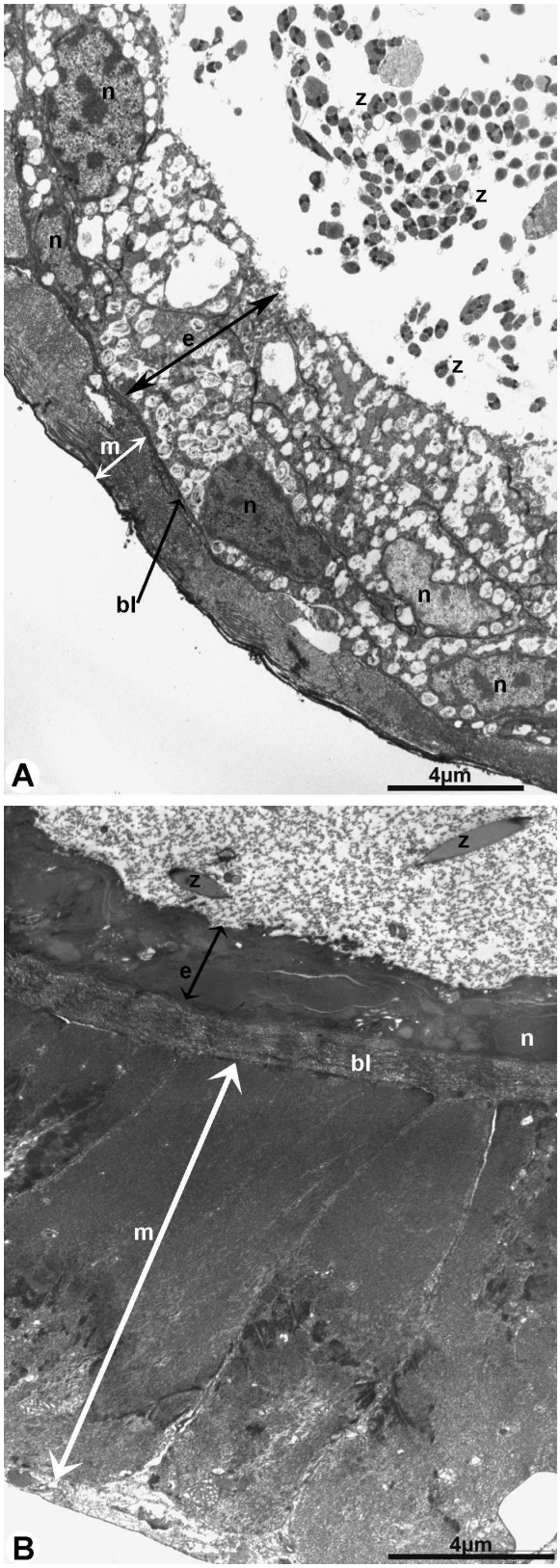


Figure 3



Capítulo 2

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SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Characteristics of the Male Reproductive System and Spermatozoa of Leptophlebiidae (Ephemeroptera)

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Keywords
Morphology, sperm, aflagellate, sexual maturation

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Abstract

This study describes morphological changes in the male reproductive system of *Miroculis amazonicus* (Savage & Peters) from mature nymphs to subimago stages. The sperm ultrastructure of *Massartela brieni* (Lestage), *Farrodes carioca* (Domínguez *et al*) and *Miroculis mourei* (Savage & Peters), as well as aspects of cell fragments observed in these species' subimagos deferent ducts were described. Sperm from the three species studied are aflagellated and immotile, while those from *F. carioca* and *Ma. brieni* are approximately spherical with a homogenous nucleus and acrosome. Sperm of *F. carioca* present two or three mitochondria located between the nucleus and the acrosome. In *Ma. brieni*, only one lateral mitochondria was found. Sperm from *Mi. mourei* are shaped as a number 'eight', with electron lucent spots inside the nucleus and two mitochondria above the acrosome. Large cell fragments containing degenerative vesicles and some sperm were observed in the deferent duct lumen of the three species. Testes of *Mi. amazonicus* are extremely reduced in the subimago stage, which suggests that these cell fragments originated from testes degeneration.

Introduction

Species from Leptophlebiidae are distributed around the world and can be found in very diverse aquatic environments. Recent studies show approximately 130 genera and 610 species described in this family (Barber-James *et al* 2008). In South America, it represents one of the two major families of Ephemeroptera (Pescador *et al* 2001), being represented by 40 genera and 150 species (Domínguez *et al* 2006). Species of Leptophlebiidae are grouped in three subfamilies: Leptophlebiinae, Atalophlebiinae and Habrophlebiinae. Only species from Atalophlebiinae are found in South America.

Sperm from Leptophlebiidae males are notably aflagellate (Soldán 1979a, Grimm 1985, Gaino & Mazzini 1991a), which differentiates them from other Ephemeroptera. However, only four species have been ultrastructurally described: *Habrophlebia lauta* (Eaton) (Grimm 1985), *Habroleptoides umbratilis* (Eaton), *Habrophlebia eldae* (Jacob & Sartori) and *Choroterpes picteti* (Eaton) (Gaino & Mazzini 1991a). Furthermore, little is known of the morphology of male reproductive system in Ephemeroptera, but it is considered simple. Male reproductive system is composed of a pair of testes where the sperm cells develop and the seminal ducts which also store the spermatozoa, without accessory glands or other specializations (Soldán 1979b). Secretions produced by accessory glands are usually reported to play

a role in the reproductive success of most insects (Chen 1984, Gillott 2003), but nothing is known about the possible consequences of the absence of these glands in the physiology of Ephemeroptera.

To provide new information on the Leptophlebiidae biology, this study describes morphological changes in the male reproductive system of *Miroculis* (*Atroari*) *amazonicus* (Savage & Peters) from their last instar to the subimago stage. The sperm ultrastructure of *Massartela brieni* (Lestage), *Farrodes carioca* (Domínguez *et al*) and *Miroculis* (*Ommaethus*) *mourei* (Savage & Peters) was also described, as well as aspects of cell fragments observed in these species' deferent ducts.

Material and Methods

Light microscopy

Miroculis amazonicus at the last nymph stage were collected from a river in Presidente Figueiredo, state of Amazonas, Brazil. Some specimens were dissected as nymphs and others were maintained until the first winged stage (subimago) and then dissected. Their reproductive systems were fixed in a 2.5% glutaraldehyde and 4% paraformaldehyde solution. They were placed on a histological slide and photographed unstained with an Olympus BX41 light microscope.

Transmission electron microscopy

Farrodes carioca, *Ma. brieni* and *Mi. mourei* male subimagos were collected near a river in Santa Teresa, state of Espirito Santo, Brazil. The deferent ducts of the specimens were dissected and fixed in a 2% glutaraldehyde and 1% tannic acid solution, and post-fixed in 1% uranyl acetate solution. The material was dehydrated and embedded in Epon resin; ultrathin sections were contrasted with 3% uranyl acetate and lead citrate and analyzed in a Zeiss Leo 906 transmission electron microscope.

Results

The spermatozoa of *F. carioca* and *Ma. brieni* are approximately spherical and consist of a nucleus with uniformly condensed chromatin and an acrosome with median electron density (Fig 1a, b). Two or three spherical mitochondria are observed between acrosome and nucleus in *F. carioca* sperm (Fig 1a). *Massartela brieni* sperm cells generally present one spherical mitochondrion laterally observed between the nucleus and the acrosome (Fig 1b). Spermatozoa of *Mi. mourei* consist of a nucleus and an acrosome, but their shape is similar to a number 'eight', since they have a constriction at the acrosome base, near the nucleus. The nucleus is filled with compact chromatin containing electron lucent regions (Fig 1c). In general, spermatozoa of *Mi. mourei* present two spherical mitochondria located above the acrosome (Fig 1c). The longer axis measured from the acrosome tip to the nuclear base of the sperm is approximately: 1.4 μm in *F. carioca* and 1.6 μm in *Ma. brieni* and *Mi. mourei*.

The sperm are stored for copula in the deferent duct lumens of these species. Many cellular fragments were observed filling a large part of the volume of these ducts. These fragments are different among the species studied, but all three species have vesicles or degenerative vacuoles in their cellular fragments (Fig 1d-f). The cellular fragments observed in *F. carioca* presented a large vesicle partially filled with electron dense material and many small clear vacuoles in their cytoplasm (Fig 1d). Nuclei with partially condensed chromatin can still be recognized among these fragments. The cellular fragments observed in *Ma. brieni* made up most of the content of the deferent ducts. They presented vesicles partially filled with electron dense material and some interspersed spermatozoa (Fig 1e). There are two types of cellular fragments in *Mi. mourei*: one with variable electron density (Fig 1f, lower part of the figure), while the other type contained complex membranous structures derived from partially reabsorbed organelles (Fig 1f).

The male reproductive system of *Mi. amazonicus* undergoes profound changes from mature nymphs to subimagos. In mature nymphs, the testes were well developed (Fig 2a) while the deferent ducts were thin, approximately 52 μm in diameter, with empty lumens (Fig 2b). In subimagos, the testes were degenerated and the deferent ducts were dilated to approximately 124 μm in diameter, and their lumens were completely filled with sperm and cell fragments, as described earlier (Fig 2c).

Discussion

Data presented in this manuscript confirm aflagellate and non-motile sperm as an autapomorphy of Leptophlebiidae in the Order Ephemeroptera. Despite the apparent simplicity, the sperm morphology in this family allows us to distinguish each of the genera analyzed.

The presence of a *perforatorium* in the acrosome is a plesiomorphic characteristic of insects (Baccetti 1972, Jamieson *et al* 1999), which was lost in the main Ephemeroptera lineages (Baccetti *et al* 1969, Fink & Yasui 1988). This fibrous structure was described in the acrosome of *H. umbralitis* (Habrophlebiinae) and *C. picteti* (Atalophlebiinae) (Gaino & Mazzini 1991a). In *H. eldae* (Habrophlebiinae) (Gaino & Mazzini 1991a). However, in *F. carioca*, *Ma. brieni* and *Mi. mourei* (Atalophlebiinae) sperm, no *perforatorium* was observed. Probably, the presence of a *perforatorium* is related to the thickness of the egg chorion

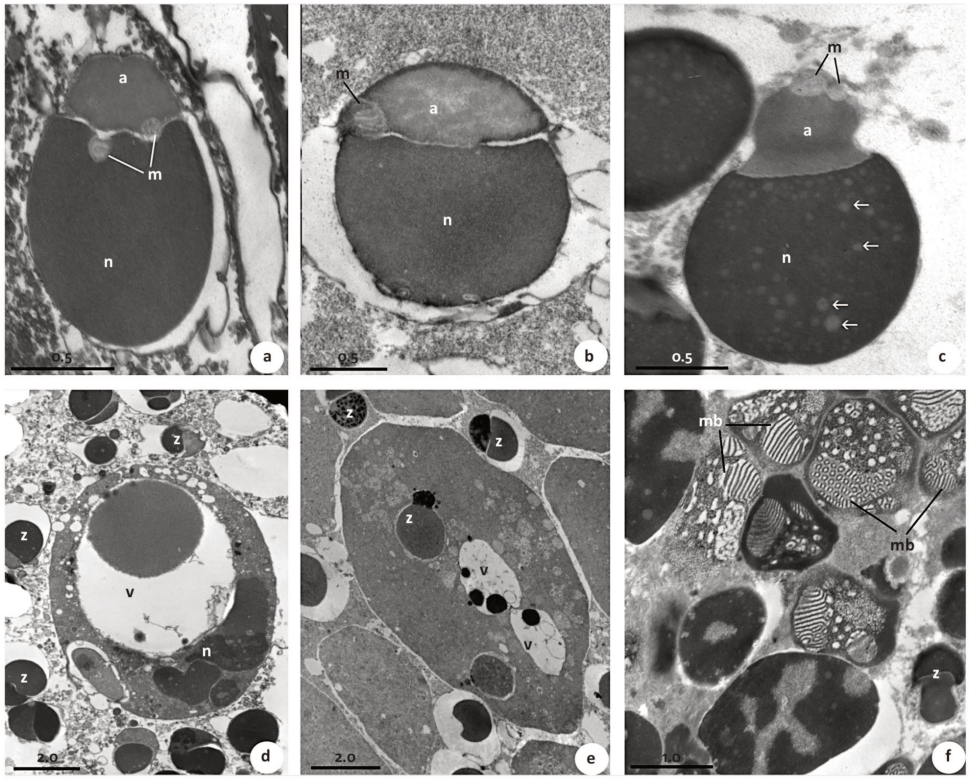

Fig 1 Transmission electron microscopy of subimagos. Sperm and cell fragments with degenerative vesicles from deferent duct lumens of *Farrodes carioca* (a, d), *Massartela brieni* (b, e), *Miroculis mourei* (c, f), respectively. (n) nucleus, (m) mitochondria, (a) acrosome, (z) sperm, (v) vacuole, (mb) membranous structures, (arrows) electron lucid chromatin regions in *Mi. mourei* spermatozoa. All bars are in μm.

for these different species as suggested by Gaino and Mazzini (1991b), and does not seem to have a phylogenetic significance in Leptophlebiidae, since this characteristic is not shared among species of the same subfamily.

Mitochondrion position is another variable characteristic among Leptophlebiidae sperm. A mitochondrion was observed beneath the nucleus in *H. umbralitis* (Gaino & Mazzini 1991a), laterally located between the nucleus and the acrosome in *C. picteti* (Gaino & Mazzini 1991a) as well as in *F. carioca*, but different from *Ma. brieni*, where it occurs laterally in the nucleus/acrosome contact region, and from *Mi. mourei,* where it is found above the acrosome. Interestingly, no visible mitochondrion was observed in *H. eldae* sperm (Gaino & Mazzini 1991a). Most Ephemeroptera sperm present a single, enlarged mitochondrial derivative with a separated paracrystalline body that extends along the tail (Baccetti *et al* 1969, Phillips 1969, Fink & Yasui 1988, Gaino & Mazzini 1991b).

The presence of small, simple mitochondria in Leptophlebiidae sperm is a derivation of the typical pattern described for this order. Since the mitochondrial derivative is associated with the flagellum and its movement, these immotile species would have very different energetic needs and therefore their mitochondria were not modified in the same manner. To confirm a probable phylogenetic significance of different localizations of the mitochondria in the spermatozoa of Leptophlebiidae, more species must be studied. A phylogenetic inference based on the available data would be premature.

The presence of large cell fragments in the deferent duct lumens has not been previously reported in Ephemeroptera. Since the testes of *Miroculis amazonicus* were greatly reduced and the enlarged seminal duct in the subimago stage, the cell fragments observed in this duct probably originated from the degenerated testes. The presence of some spermatozoa (as observed in *Ma. brieni*) and of degenerative vesicles inside these

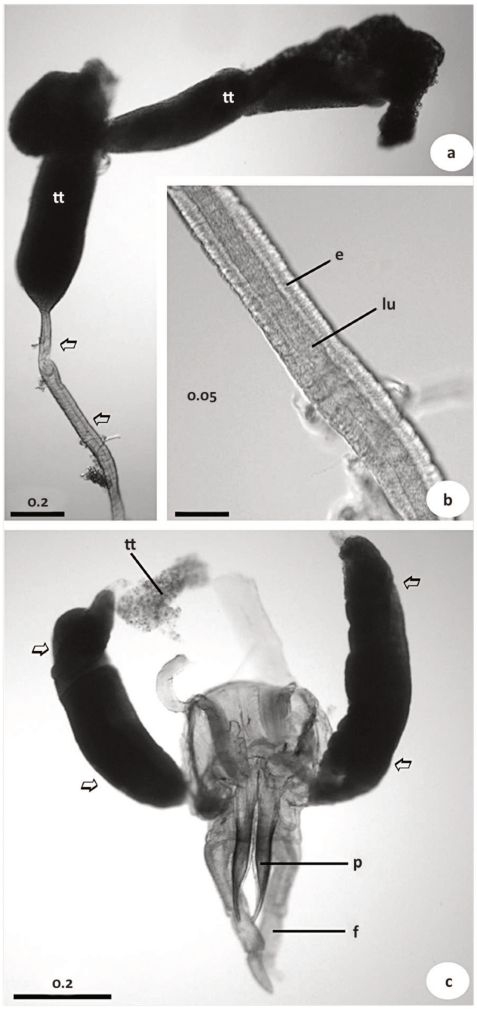

Fig 2 a, b) Male reproductive system of a *Miroculis amazonicus* mature nymph, (tt) testes, (open arrows) still empty deferent ducts; b) High magnification of the deferent duct, (e) epithelium, (lu) lumen; c) Male reproductive system of a *Mi. amazonicus* subimago, (tt) degenerated testes, (open arrows) deferent ducts full of spermatozoa and cell fragments, (p) penises, (f) forceps. All bars are in mm.

cell fragments seem to confirm this hypothesis. Some studies have reported the presence of holocrine and apocrine secretion in the ducts of male reproductive systems of different insects (Leopold 1970, Perotti 1971, Riemann 1973, Almadoss 1990, Brito *et al* 2010). Since Leptophlebiidae, as all other Ephemeroptera, do not present accessory glands in the male reproductive system, the cell fragments observed in this study could act as functional secretions inside the deferent ducts or in the female reproductive tract. They could also provide nutrients for sperm nutrition or function as a protective medium for sperm storage, or modulate post copula behavior in females. However, this hypothesis must be confirmed by future investigations.

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Capítulo 3

New Data on Mayfly Spermatozoa (Ephemeroptera)

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Abstract

Because of its position among winged insects, Ephemeroptera is a key study group for research intending to understand the natural history of the insects. Previous studies pointed several autopomorphic characteristics in Ephemeroptera spermatozoa, which suggest their placement as a sister-group of "Odonata + Neoptera". The present study focused attention on two regions in which autopomorphic characteristics have been described (nucleus-flagellum transition and mitochondrion + accessory body structure). Our observations included 12 species from six families and showed at least four different organizational patterns for the mitochondrion + accessory bodies complex. Some patterns seem to be consistent in some species groups and must have a phylogenetic significance. At the nucleus-flagellum region an electron-dense material was observed associated to the centriole in all species analyzed. Our results strongly suggested that this material is the centriolar adjunct, although the absence of this structure has been considered one of the Ephemeroptera autopomorphies.

Keywords: Centriolar adjunct, Accessory body, Mitochondria, Phylogeny.

1. Introduction

The position of the order Ephemeroptera (mayfly) among other Pterygota insects makes them an interesting study group in order to understand insect evolution. Research on insect sperm morphology revealed that Ephemeroptera spermatozoa possess several characteristics that suggest, as in other recent studies (e.g. Willkommen, 2008), their placement as the sister-group of "Odonata + Neoptera" (Jamieson et al., 1999; Lupetti et al., 2011).

Historically, spermatozoa of mayflies have been considered to have autopomorphic characteristics in the nucleus-flagellum transition region and in the mitochondrial structure.

In many insects, the nucleus-flagellum transition region includes a centriolar adjunct, which is considered an insect plesiomorphic characteristic (Baccetti, 1972; Jamieson et al., 1999), and is supposed to give mechanical resistance to the region. However, in previously studied species of Ephemeroptera, this structure has not been found (Baccetti et al., 1969; Jamieson et al., 1999; Jamieson, 2011).

Also unique among in Ephemeroptera spermatozoa are the mitochondrial alterations. For most insect groups, the flagellum has two mitochondrial derivatives with crystalline material inside them, while in Ephemeroptera only a single mitochondrion is observed along the flagellum (Baccetti et al., 1969; Phillips, 1969; Fink and Yasui, 1988; Gaino and Mazzini, 1991; Jamieson et al., 1999; Jamieson, 2011). Besides, this mitochondrion does not have any crystalline material inside but is closely associated with the accessory bodies. The accessory bodies of Ephemeroptera sperm can be amorphous or crystalline (Baccetti et al., 1969; Fink and Yasui, 1988) and accessory bodies with different shapes have been described in the present study.

After analyzing specimens from almost all families reported from Brazil, pertaining to 11 genera and 12 species, we were able to find new organizational patterns for the structures mentioned above. The present paper was designed to discuss the results found for these species and to review the literature in light of the new evidence.

2. Material and Methods

All species examined were collected in the Brazilian states of São Paulo and Espírito Santo (Southeastern Region) and Amazonas (Northern Region) in Brazil. The list of species with their families and collection locations is given below:

- *Tupiara ibirapitanga* Salles et al., 2003, Baetidae (20°28'57.10"S; 41°49'50.40"W);
- *Callibaetis jocosus* Navás, 1912, Baetidae (23°14'18.30"S; 46°56'27.25"W);
- *Waltzoyphius fasciatus* Lugo-Ortiz & McCafferty, 1995, Baetidae (19°02'9.72.2"S; 40°10'68.0"W);
- *Hexagenia albivitta* Walker, 1853, Ephemeridae (19°10'9.70"S; 40°11'25.20"W);
- *Campylocia anceps* Eaton, 1883, Euthyplociidae (02º 55'46.7"S; 59º58'22.0"W);
- *Caenis* sp., Caenidae, (19°3'55.92"S; 40°42'45.40"W);
- *Caenis fittkaui* Malzacher, 1986, Caenidae, (19°3'55.92"S; 40°42'45.40"W);
- *Brasilocaenis renata* Malzacher, 1986, Caenidae, (3°9'13.16"S; 59°54'56.40"W);
- *Campsurus* sp., Polymitarcyidae, (2°49'22.52"S; 60°2'5.17"W);
- *Asthenopus curtus* Hagen, 1861, Polymitarcyidae, (3°9'13.16"S; 59°54'56.40"W);
- *Traverhyphes (Mocoihyphes) yuati* Molineri, 2004, Leptohyphidae, (19°52'31.60"S; 40°31'49.10"W);
- *Coryphorus aquilus* Peters, 1981, Coryphoridae, (2°49'22.52"S; 60°2'5.17"W).

Transmission Electron Microscopy

All specimens were dissected in 0.1M sodium phosphate buffer, pH 7.2. The seminal ducts were fixed with a 2.5% glutaraldehyde and 1% tannic acid solution in the same buffer for 5 days. The material was contrasted “en-bloc” with 1% uranyl acetate aqueous solution for 2 hours, dehydrated in an acetone series and embedded in Epon (Dallai and Afzelius, 1990). Ultra-thin sections were contrasted with 3% uranyl acetate followed by 3% lead citrate and analyzed with a Zeiss-Leo 906 Transmission Electron Microscope.

3. Results

3.1 Mitochondria

The mitochondrion + accessory body complex of the spermatozoa studied presented great variability in the organization of the accessory bodies. At least four patterns could be identified.

1) In cross sections, two large triangular bodies with crystalline organization make up the accessory bodies. They are laterally located between the mitochondrion and the axoneme. A central element may be observed between these bodies (Fig. 1A). This pattern was observed for all the Baetidae species studied and sometimes the crystalline organization can also be observed in longitudinal sections (Fig. 4C, E, F).

2) The accessory bodies consist of two bodies with variable shapes, laterally located between the mitochondrion and the axoneme, however, without a crystalline organization (Fig. 1B). This pattern was observed in the Ephemerelloidea, *C. aquilus* and *T. yuati.*

3) A single rectangular accessory body without crystalline organization, located between the mitochondrion and the axoneme was observed in *H. albivitta* and in *C. anceps* (Fig. 1C, D). In *H. albivitta* cross sections, the accessory body is wider, measuring approximately 0.4 μm in the "mitochondrion – axoneme" axis, in contrast to 0.15 μm in *C. anceps*.

4) The accessory body consists of a sheath with no crystalline arrangement that surrounds the mitochondrion (Fig. 2A, B). This characteristic was observed in *Caenis* sp., *Campsurus* sp. and *A. curtus*. The sheath thickness varies from 0.04 to 0.08 μm.

A membrane surrounding the mitochondrion and the accessory bodies was observed in several species (Figs. 1A, C, D, 2, 3D).

3.2 Centriole region

The nucleus often has basal projections that can surround the flagellar anterior extremity completely (i.e. *H. albivitta*, Fig. 3A, C) or partially, this latter condition being observed more often (Figs. 4B, C, D, G; 5A-D). The nuclear base with no projections was observed in *Caenis* sp., *B. renata* (Fig. 6A, C) and *Campsurus* sp. (not shown).

The centriole represents the initial portion of the axoneme and, in all species analyzed in this study, it is associated to, or surrounded by, an electron-dense material here

called the centriolar adjunct. This centriolar adjunct has some morphological differences between the species. In *H. albivitta*, it is observed in the basal nuclear cavity (Fig. 3A) and extends for at least 2 μm along the flagellum (Fig. 3C). It can be distinguished from the accessory body in longitudinal and cross sections, because of the differences in electron density of the two structures (Fig. 3B).

All other species analyzed, showed a reduced centriolar adjunct, which was restricted only to the nucleus-flagellum contact area, approximately 0.4 μm along the flagellum. In *W. fasciatus*, *C. jocosus* and *T. yuati* the centriolar adjunct is very dense, making it very difficult to identify the centriolar microtubules (Fig. 4B, G; 5D), usually seen as doublets plus a single microtubule, but very occasionally may be seen in the arrangement of triplets (Fig. 4B). In species such as *T. ibirapitanga* and *A. curtus* the centriolar adjunct is organized in a less compact manner which permits the observation of centriolar microtubules (Fig. 4D, 5B). Sometimes, longitudinal sections of the centriolar region do not distinguish between the accessory bodies and the dense material associated with the centriole (Fig. 4A; 5A, C). These structures can be distinguished when the crystalline pattern of the accessory body is evident, as in Baetidae species (Fig. 4C, E).

The species from the Caenidae family in this study showed two distinct organizational patterns of the centriolar adjunct. *Caenis* sp. has a large amount of dense material concentrated at one side of the centriole, only partially surrounding the centriole (Fig. 6A). An electron-lucid space is also observed between the centriole and its adjunct (Fig. 6B). The other two species, *B. renata* and *C. fittkaui*, do not have a fused layer of centriolar adjunct, but nine tufts, each directly associated with one of the accessory microtubules (Fig. 6C-E).

4. Discussion

4.1 Accessory bodies

This study identified four organizational patterns for the accessory bodies. Another pattern was described in *Dolania americana* (Behningiidae) spermatozoa, which have a central, bilobed and homogeneous accessory body (Fink and Yasui, 1988). In the spermatozoa of *Cloeon dipterum* (Baetidae) (Baccetti et al., 1969; Jamieson et al., 1999; Lupetti et al., 2011; Jamieson 2011) the same morphology was described as that we

encountered in species from the same family, in which there are two crystalline bodies laterally located between the axoneme and the mitochondrion. However, this characteristic is not exclusive for this family, since the spermatozoa of *Electrogena grandiae* and *Ecdyonurus venosus* (Heptageniidae) also possess accessory bodies in the form of two crystalline bodies (Gaino and Mazzini, 1991). Two accessory bodies without a crystalline organization, as described here in *C. aquilus* and *T. yuati*, were also observed in *Siphlonurus croaticus* (Siphlonuridae) (Grimm, 1985).

Interpreting and assigning phylogenetic value to the accessory bodies morphological variation should be done with caution, since the studies to date include few species, representing a small amount of families, when considering the Ephemeroptera diversity. However, some preliminary deductions can be suggested:

i) Enough species from Baetidae have already been studied to suggest that the accessory bodies organized as two triangular crystalline bodies (in cross section) are a pattern for species of this family. This same characteristic was also observed in some Heptageniidae species (Gaino and Mazzini, 1991). Phylogenetic studies do not suggest any proximity between Baetidae and Heptageniidae and the family Baetidae is being considered sister-group of the other Ephemeroptera (Ogden and Whiting, 2005; Ogden et al., 2009). So, it is possible that this characteristic of crystalline accessory bodies is plesiomorphic in Ephemeroptera, or it appeared independently twice in the two groups. Species representing more families of Ephemeroptera must have their spermatozoa studied to confirm or reject these hypotheses.

ii) Both *C. aquilus* (Coryphoridae) and *T. yuati* (Leptohyphidae) showed the same organization of the accessory bodies, which is in agreement with the statement that Coryphoridae and Leptohyphidae are sister-groups (Molineri, 2006). More species from the Ephemerelloidea group, where Coryphoridae and Leptohyphidae are included, should be studied to confirm if this characteristic is shared by all families of this group or only by "Coryphoridae + Leptohyphidae".

iii) Only one accessory body organized as a sheath surrounding the mitochondrion (in *Campsurus* sp. and *A. curtus*) seems to be a Polymitarcyidae characteristic. An eventual phylogenetic proximity with Caenidae is improbable (Ogden and Whiting, 2005;

Ogden et al., 2009) and this characteristic of the mitochondrial adjunct must have evolved independently in the two families.

4.2 Centriolar adjunct

All species of this study have electron-dense material associated with the axoneme centriole, although it is hard to distinguish whether this material is continuous with the accessory bodies. The observation of the spermatozoa of some species allows easy distinction between the accessory bodies and the dense material around the centriole (*H. albivitta, T. ibirapitanga, C. jocosus*). The complex shape and organization of the dense material associated with the centriole in *B. renata* and *C. fittkaui* clearly show that it is not some kind of technical artifact, but a true spermatozoan characteristic. The consistent occurrence of this material in close association with the centriole of the spermatozoa of species from different families allows the establishment of this structure as a centriolar adjunct.

The centriolar adjunct has not previously been observed in Ephemeroptera spermatozoa and its absence was considered a group apomorphy (Baccetti et al., 1969, Jamieson et al., 1999). Indeed, this statement can be explained because the centriolar adjunct is small and difficult to distinguish in some species, as discussed above.

In the present study, 12 species from six different families of mayflies (not necessarily with phylogenetic proximity) were analyzed and all of them had the centriolar adjunct. This evidence strongly suggests that the presence of a centriolar adjunct is a common characteristic in Ephemeroptera spermatozoa.

5. Conclusions

To completely understand the origin of the accessory bodies in Ephemeroptera sperm and to understand if they are isolated structures or just a centriolar adjunct continuation, the spermatogenesis of these insects should be studied in the future. This study will also help to understand why sometimes only one accessory body is present in the Ephemeroptera sperm.

Different studies should be performed to clarify the presence or not of a membrane enclosing the mitochondrion and the accessory bodies. Such a membrane was previously

observed in other studies (Baccetti et al., 1969; Gaino and Mazzinni, 1991; Jamieson et al., 1999; Jamieson, 2011), but a strong confirmation is still lacking. This investigation could provide different phylogenetic interpretations for some of the results presented in this manuscript.

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Figure Legends

Fig 1: Flagella cross-sections from: **A)** *Callibaetis jocosus*; **B)** *Coryphorus aquilus*; **C)** *Hexagenia albivitta*; **D)** *Campylocia anceps*. (ab) accessory body, (m) mitochondrion, (ax) axoneme, (pm) plasma membrane, (*) central element, (open arrow) membrane enclosing mitochondrion and accessory bodies. All bars are in μm.

Fig 2: Flagella cross-sections from: **A)** *Caenis* sp.; **B)** *Campsurus* sp. (ab) accessory body, (m) mitochondrion, (ax) axoneme, (pm) plasma membrane, (open arrow) membrane enclosing mitochondrion and accessory body. All bars are in μm.

Fig 3: Basal portion of the flagellum of *Hexagenia albivitta*. **A)** Cross section of the centriole inserted in the nucleus basal cavity; **B, C)** Longitudinal sections of the nucleus-flagellum transition. Note the electron density difference between the accessory body and the centriolar adjunct in B, and the extension of the centriolar adjunct along the flagellum in C; **D)** Cross section of the flagellum where the centriolar adjunct is separated from the accessory body by a membrane. (c) centriole, (ca) centriolar adjunct, (n) nucleus, (pm) plasma membrane, (m) mitochondrion, (ab) accessory body, (ax) axoneme, (open arrow) membrane enclosing the mitochondrion and the accessory body. All bars are in μm.

Fig 4: Longitudinal (A, C, E, F) and cross (B, D, G) sections of the nucleus-flagellum transition of: **A, B)** *Waltzoyphius fasciatus*; **C, D)** *Tupiara ibirapitanga*; **E-G)** *Callibaetis jocosus*. Note the crystalline organization of the accessory body (ab) in C, E and F. In F, note that, although both the mitochondrion and the accessory body have a striped appearance, they can hardly be confused. (n) nucleus, (c) centriole, (ca) centriolar adjunct, (ax) axoneme, (pm) plasma membrane, (m) mitochondrion, (t) microtubules. All bars are in μm.

Fig 5: Longitudinal (A, C) and cross (B, D) sections of the nucleus-flagellum transition of: **A, B)** *Asthenopus curtus*; **C, D)** *Traverhyphes yuati*. Note that, in longitudinal sections it is hard to distinguish the centriolar adjunct. (n) nucleus, (c) centriole, (ca) centriolar adjunct, (ax) axoneme, (m) mitochondrion, (pm) plasma membrane. All bars are in μm.

Fig 6: Longitudinal (A, C) and cross (B, D, E) sections of the nucleus-flagellum transition of: **A, B)** *Caenis* sp., **C, D)** *Brasilocaenis renata*, **E)** *Caenis fittkaui*. Note that the centriolar adjunct (ca) can be distinguished from the accessory microtubules (a) in figure E. (n) nucleus, (c) centriole, (ca) centriolar adjunct, (ax) axoneme, (pm) plasma membrane, (d) microtubule doublets. All bars are in μm.

Figure 1

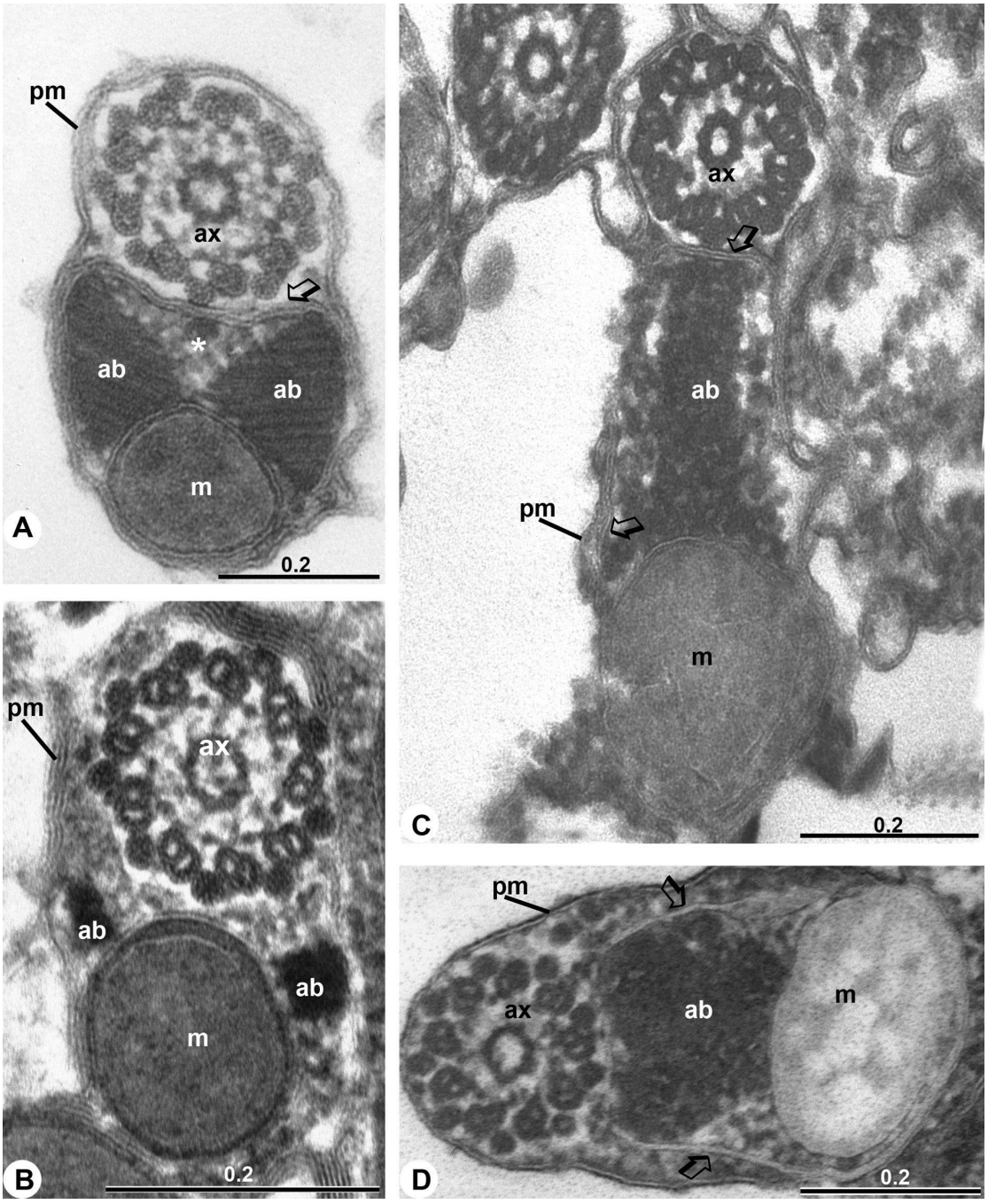
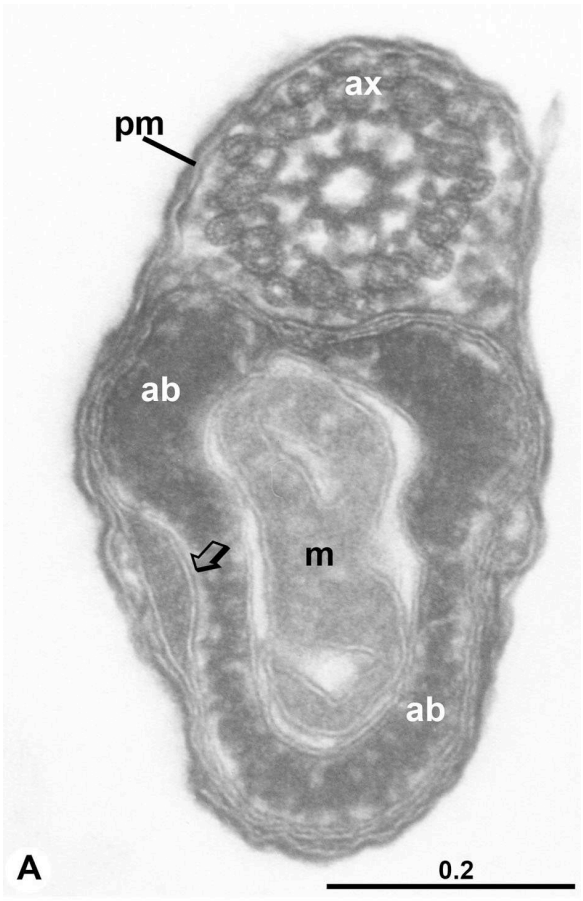
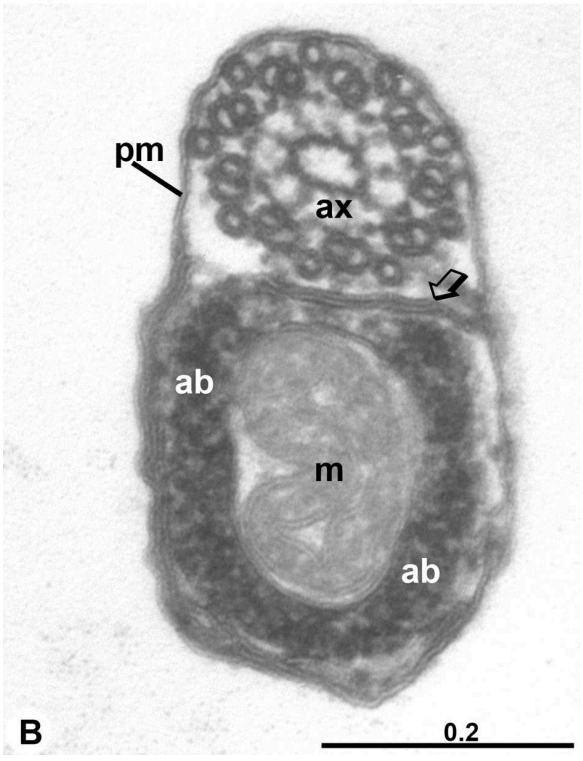


Figure 2





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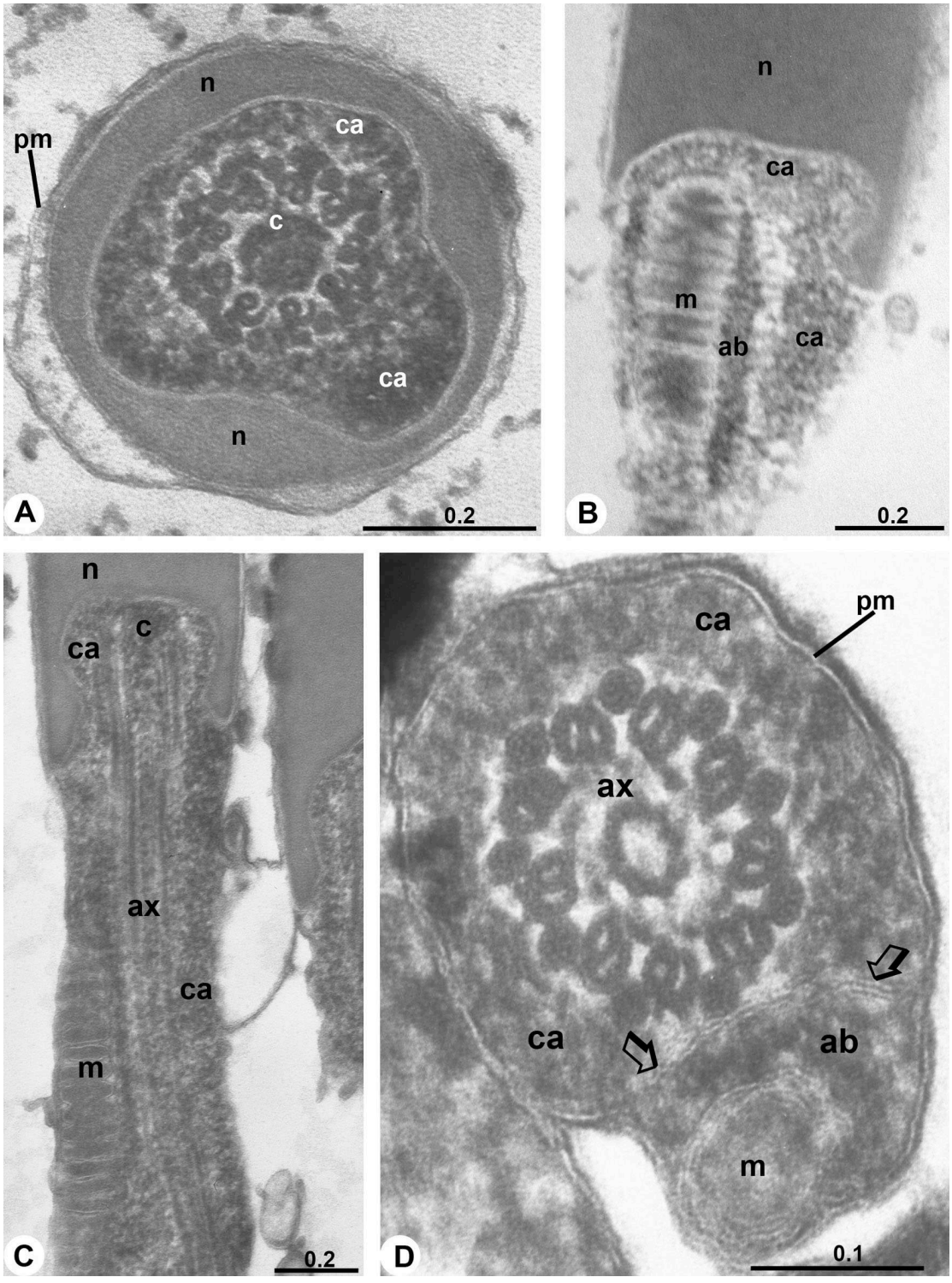

Figure 4

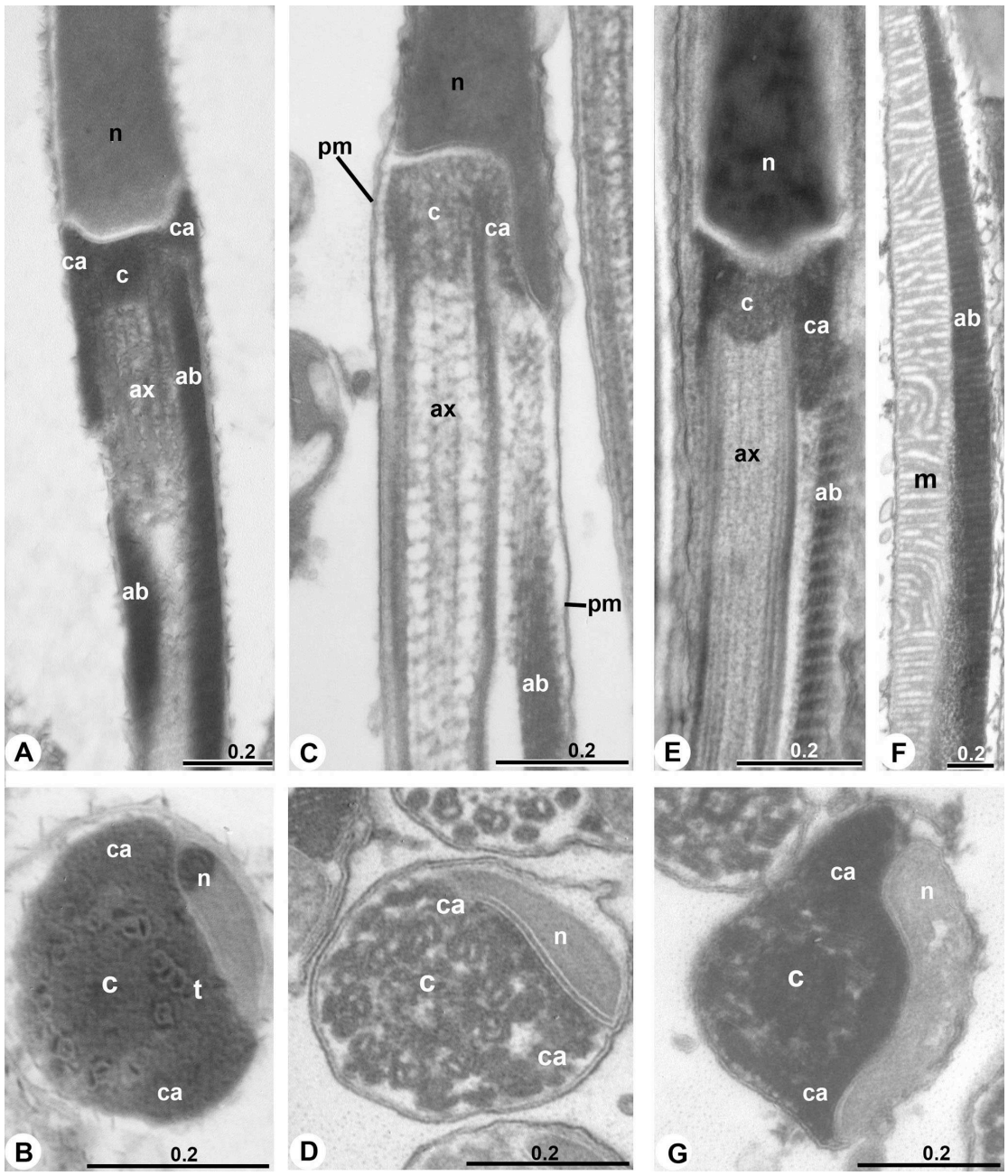
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Figure 5

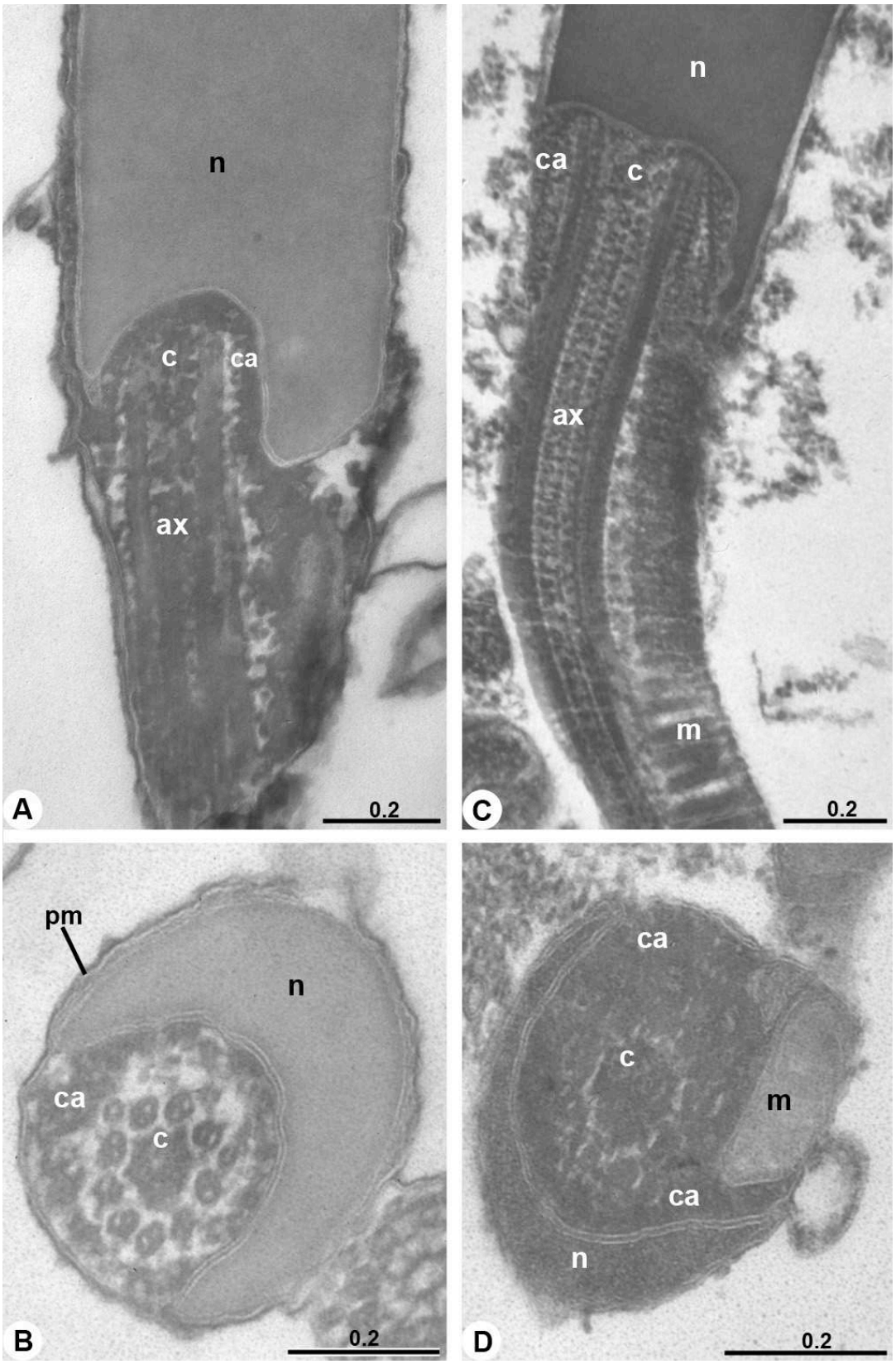
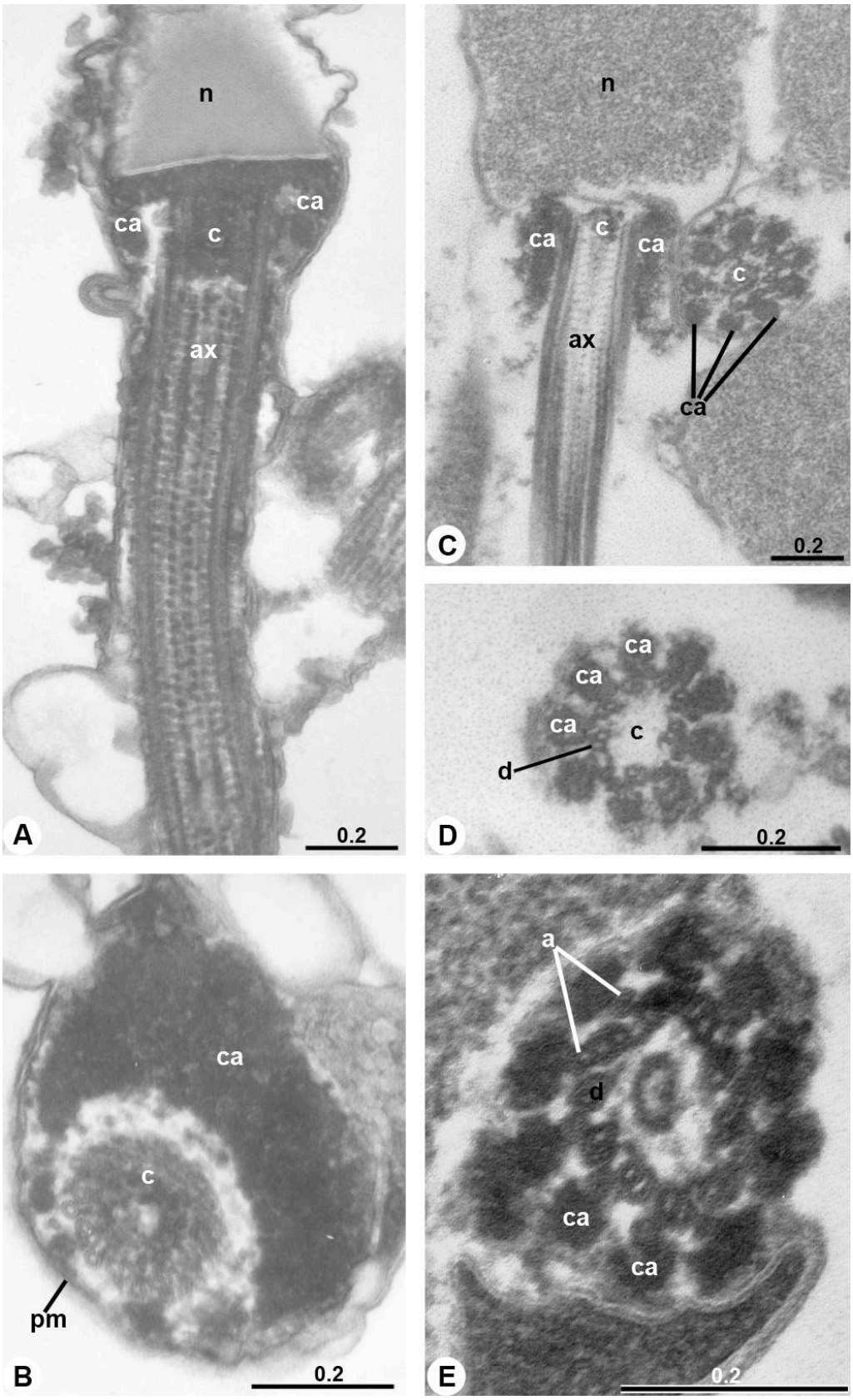


Figure 6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Capítulo 4

The sperm morphology of two Baetidae species (Ephemeroptera)

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Abstract

Insects of the order Ephemeroptera are aquatic and represent the oldest existing winged insects. Improving the phylogeny of this order is important to understand insects' relations. Spermatozoa ultrastructure has been used in phylogenetic studies together with traditional morphological data in many insect groups, helping to explain many unresolved questions. However, these studies are few in Ephemeroptera. This study describes sperm morphology of two Baetidae species: *Callibaetis jocosus* and *Tupiara ibirapitanga* in order to provide new data for the group. The present study confirmed some apomorphies of Ephemeroptra spermatozoa, such as: a single mitochondrion in the flagellum without paracrystalline material inside, axoneme with the 9+9+0 microtubule pattern, lack of outer dynein arms in the microtubule doublets and the accessory microtubule with the 13+7 organization pattern. This manuscript is also the first record for a bilayered acrosome for Ephemeroptera species and it confirms the presence of a centriolar adjunct in both species. These data will be useful for future phylogenetic studies and suggest that Ephemeroptera spermatozoa morphology is more variable than was previously assumed.

Keywords

Callibaetis jocosus, *Tupiara ibirapitanga*, ultrastructure, acrosome

Introduction

The order Ephemeroptera consists of more than 3.000 species, distributed in 42 families (Barber-James et al. 2008), representing the oldest existing winged insects. Their nymphs are aquatic and this stage represents the major part of their life span. Only the two last stages of their lives are winged (subimago and imago), when they leave the aquatic environment. The specimens at the winged stages do not feed and can live for some hours up to a few days, in order to reproduce. They are distributed over all continents, except Antarctica, but their greatest diversity is observed in rivers of tropical and temperate zones (Brittain 1982; Brittain and Sartori 2003).

Family Baetidae presents almost a worldwide distribution, being absent only in New Zealand and some oceanic islands (Dominguez et al. 2006). With approximately 100 genera, this is the second largest family in species number in South America, with species exploring almost every habitat and microhabitat (Dominguez et al. 2006). Baetidae is traditionally grouped in the suborder Pisciforma (McCafferty 1991), however, recent phylogenetic studies suggest that this suborder is parafiletic and Baetidae is the sister group of the remaining Ephemeroptera, except for Siphluruscidae (Ogden and Whiting 2005; Ogden et al. 2009).

The use of new morphological characters together with those traditionally used may be useful to improve the phylogeny of Ephemeroptera. Spermatozoa ultrastructure has been used in phylogenetic studies of many insect groups (Jamieson et al. 1999; Lino-Neto and Dolder 2001; Zama et al. 2005; Mancini et al. 2006), helping to explain many unresolved questions. These studies are scarce in Ephemeroptera, and furthermore, some of them do not present complete descriptions of the spermatozoa (Baccetti et al. 1969; Phillips 1969; Grimm 1985; Fink and Yasui 1988; Dallai and Afzelius 1990; Gaino and Mazzini 1991a,b; Brito et al. 2011). However, some apomorphies have been pointed out for Ephemeroptera spermatozoa: a monolayered acrosome (with only the acrosomic vesicle), a single mitochondrion without paracrystalline inclusions, the axoneme with the 9+9+0 microtubule pattern (without the central pair), lack of the outer dynein arm on the microtubule doublet and 13 protofilaments containing 7 small internal units in the accessory microtubules (Jamieson et al. 1999). The absence of a centriolar adjunct was previously considered another apomorphy for the group, but its existence has already been

demonstrated (Brito et al. submitted). In Baetidae, the spermatozoa ultrastructure has previously been described for only one species: *Cloeon dipterum* Linnaeus (Baccetti et al. 1969).

The present study analyses spermatozoa ultrastructure of two Baetidae species: *Callibaetis jocosus* Navás and *Tupiara ibirapitanga* Salles et al., with the intention of improving the available information for Ephemeroptera and furnishing new data, useful for future phylogenetic studies.

Material and Methods

Transmission Electron Microscopy

Last instar male nymphs of *Callibaetis jocosus* were collected in the marginal vegetation of a lake at "Serra do Japi", Jundiaí, SP, Brazil (S 23º 14' 18,3"; W 46º 56' 27,25"), and at a lake in the campus of the State University of Campinas, Unicamp, Campinas, SP, Brazil (S 22^{o} 49'23.4"; O 47^{o} 03'40.0). The specimens were maintained in the laboratory until they reached subimago and imago stages.

A last instar male nymph of *Tupiara ibirapitanga* was collected at the "Sete Pilões" waterfall in the Caparaó National Park, ES, Brazil (S 20º 28' 57.1"; W 41º 49' 50.4").

The deferent ducts of the specimens were dissected and fixed in a 2% glutaraldehyde and 1% tannic acid solution, and contrasted 'en-bloc' in 1% uranyl acetate solution. The material was dehydrated and embedded in Epon resin; ultrathin sections were contrasted with 3% uranyl acetate and 3% lead citrate and analyzed in a Zeiss Leo 906 transmission electron microscope.

Immunocytochemistry for Actin

Imagoes of *C. jocosus* were dissected and their deferent ducts were fixed in a Karnovsky solution with 4% of paraformaldehyde and 0.5% of glutaraldehyde in 0.1M Cacodylate buffer, pH 7.2, for 12 hours at 4^{o}C. The material was dehydrated in a alcoholic series and embedded in LR-White Resin, polymerized with UV at -20^{o}C.

Ultrathin sections collected on nickel grids, pre-incubated in 0.05M Tris-HCl buffer pH 7.2, containing 0.05 glycin and 1% bovine serum albumin for 10 minutes at room temperature. Unspecific binding was blocked with 0.05M Tris-HCl containing 1% bovine serum albumin and 0.05% Tween 20, for 30 minutes. Subsequently incubated for 1

hour with an antibody (Monoclonal Anti-Actin, produced in mouse, Ref. A2228, Sigma-Aldrich), diluted 1:100. After washing with 0.05M Tris-HCl, containing 0.5% bovine serum albumin, the grids were incubated for 1 hour with the respective labeled secondary antibody, Anti-Mouse IgG (whole molecule) # gold, produced in goat, Ref. G7777, Sigma-Aldrich, diluted 1:100. After incubation, the grids were washed with 0.05M Tris-HCl and distilled water. The grids were contrasted with routine methodology for TEM and analyzed in a Zeiss LEO 906. Negative control was performed omitting the primary antibody.

Results

Spermatozoa of *Callibaetis jocosus* and *Tupiara ibirapitanga* are very similar; they are long, slender and composed of a head and a flagellum. The head consists of an acrosome and a nucleus. The flagellum includes four long structures: an axoneme, a mitochondrion and a pair of accessory bodies. The initial region of the flagellum is characterized by the presence of the centriolar adjunct, a dense proteic agglomerate that involves the initial portion of the axoneme, the centriolar region.

Callibaetis jocosus –The nucleus is approximately cylindrical, varying from 0.5 to 0.1 μm in diameter from base to apex (Fig. 1A, C, D), with a total length of approximately 7.5 μm. The nucleus is full of compacted chromatin. Some immunocytochemical labels for actin were observed in the nucleus (Fig. 1F). The acrosome is above the nucleus, separated from it by a clear layer, the acrosome has a conical shape that is approximately 0.5 μm in its long axis and is bilayered. The acrosomal vesicle is conical and is composed of a homogeneous electron dense material; a thin layer composed of median electron dense material covers the acrosomal vesicle (Fig. 1A). Neither the acrosomal vesicle, nor the outer layer showed gold particles from the immunocytochemical test for actin (Fig. 1B).

The nucleus base is separated from the flagellar structures by a clear layer. The centriolar adjunct extends along the flagellum for approximately 0.35 μm from the nuclear base (Fig. 1C, D) and is observed mainly between the centriolar region and the basal nuclear projection (Fig. 1E, G). The mitochondrion is a long structure that begins at the nuclear base, just below its projection and the centriolar adjunct (Fig. 1E), and extends

along the flagellum (Fig 1E, M). In cross sections, it is elliptical, measuring approximately 0.20 x 0.15 μm along its long and short axes respectively (Fig. 1H). At the end of the flagellum, the mitochondrion tapers and finishes before the axoneme (Fig. 1J, K). The mitochondrial cristae are organized perpendicular to the mitochondrion long axis, with some cristae organized in a variable way (Fig. 1M). The accessory body is observed between the mitochondrion and the axoneme, extending along the flagellum (Fig. 1E, H, J). In some sections, a membrane seems to surround both the mitochondrion and the accessory body (Fig. 1H). The accessory bodies begin just below the nuclear base or just below the centriolar adjunct, with no clear limit between the accessory body and the centriolar adjunct (Fig. 1D, E). The accessory bodies are observed in cross sections as two large, approximately triangular portions with a small central element near the flagellar base (Fig. 1H). Below the flagellar base, the central element disappears and the accessory bodies diminish in diameter. They extend along the flagellum, until they disappear together with the mitochondrion (Fig. 1K). Some immunocytochemical labeling for actin was observed on the axoneme and the accessory bodies (Fig. 1I)

The axoneme is the longest structure of the flagellum; it begins at the nuclear base (Fig. 1C-E) and is the last to disorganize at the flagellum tip, being observed alone in cross sections of this final region (Fig. 1L). The axoneme base, or centriolar region, is characterized by the presence of dense proteins in the center of the "axoneme" and by their association with the centriolar adjunct (Figs. 1C-E, G). The axoneme follows the 9+9+0 arrangement, with nine outer accessory microtubules, nine doublets and the absence of the central pair (Fig. 1L). The accessory microtubules are characterized by 13 subunits of tubulin surrounding 7 smaller subunits of unknown chemical composition. The "A" tubule of the doublet consists of 13 subunits of tubulin and the "B" tubule of 10 subunits forming an arc and 2 or 3 inner subunits. Only the inner dynein arm was observed on the doublets; the center of the axoneme is occupied by a central sheath, from which the radial spokes emerge (Fig. 1L). The accessory microtubules are the first axoneme elements disorganized at the flagellar terminal portion and the central sheath, the last (Fig. 1K).

Tupiara ibirapitanga – The nucleus is cylindrical, measuring approximately 8.5 μm in length and varying in diameter from 0.3 to 0.15, from base to apex (Fig. 2A-C). It presents a projection at the apex, fitting into the acrosome, which is separated from the nucleus by

a 0.15 μm clear layer (Fig. 2A). The acrosome is approximately conical, with 0.5 μm in length and consists of two layers. The acrosomal vesicle is the most internal component and is formed by homogeneous electron dense material; the outer component is formed by a granular, 0.02 μm thick layer that surrounds the acrosomal vesicle (Fig. 2A).

The nucleus – flagellum transition region is characterized by the presence of a centriolar adjunct that surrounds the centriolar region of the axoneme and is observed mainly at the junction of the axoneme and the basal nuclear projection (Fig 2B, D, E). The mitochondrion begins below the basal nuclear projection and extends along the flagellum (Fig. 2F, G, J). In cross sections, its shape is approximately circular with 0.15 μm in diameter (Fig 2F, G). Its cristae have a parallel arrangement that is perpendicular to the long axis of the mitochondrial derivative (Fig. 2J). The accessory bodies begin below the centriolar adjunct (Fig. 2B) and extend along the flagellum between the axoneme and the mitochondrion (Fig. 2F, G). The accessory bodies are paracrystalline and, observed in cross sections are approximately rectangular, measuring 0.10 x 0.07 μm along their larger and smaller sides (Fig. 2F). Near the flagellum extremity, they thin down until they disappear together with the mitochondrion (Fig. 2G, H). The axoneme is the longest flagellar structure; it begins at the nuclear base as the centriolar region, characterized by the presence of dense proteins, centrally located, and by the association with the centriolar adjunct (Fig. 2B, D, E). The axoneme follows the 9+9+0 microtubule pattern, with nine outer accessory microtubules and nine doublets but without a central pair of microtubules (Fig. 2F). Only the inner dynein arms are observed on the doublets and the center of the axoneme is occupied by a central sheath from which the radial spokes emerge. The accessory microtubules have lumens filled by electron dense material, but no subunits could be distinguished in this case (Fig. 2E-G). The axoneme is the last element disorganized at the flagellum tip and can be observed alone in cross sections. The accessory tubules are the first elements to disorganize, then the doublets and finally the central sheath is the last element to disorganize (Fig. 2H, I).

Discussion

Sperm morphology of *C. jocosus* and *T. ibirapitanga* is similar to that previously known for Ephemeroptera, reinforcing some apomorphies: a single mitochondrion without paracrystalline material inside, the axoneme with a 9+9+0 microtubule pattern and the lack of an outer dynein arm. The accessory microtubules consisting of 13 tubulin molecules surrounding 7 smaller subunits is also considered an apomorphic characteristic of Ephemeroptera. In this study the 13+7 pattern was observed in *C. jocosus* accessory microtubules, confirming this apomorphy. The microtubule subunits could not be observed in *T. ibirapitanga* spermatozoa, but the presence of dense material in the accessory microtubules of this species, suggests that this characteristic is also present. Recently, spermatozoa organized in a spermatodesm were registered for *Cloeon dipterum* (Lupetti et al., 2011). However, no evidence of this kind of organization was observed in both species presented of this study.

The bilayered acrosome is considered basal among insects (Baccetti 1972; Jamieson et al. 1999). The spherical and monolayered acrosome observed in *Cloeon dipterum* (Baccetti et al. 1969), *Dolania americana* (Behningiidae) (Fink and Yasui 1988), *Electrogena grandiae* and *Ecdyonurus venosus* (Heptageniidae) (Gaino and Mazzini 1991b) spermatozoa, have been assumed as an apomorphy for the Ephemeroptera order. Some exceptions have already been observed in the order: *Habroleptoides umbralitis* and *Choroterps picteti* (Leptophlebiidae) (Gaino and Mazzini 1991a, b) which presented a perforatorium surrounded by the acrosomic vesicle. However, both species are from the Leptophlebiidae family, in which spermatozoa are characterized as aflagellate and very autopomorphic. Some Leptophlebiidae species presented a monolayered acrosome: *Habrophlebia eldae* (Gaino and Mazzini 1991a), *Farrodes carioca*, *Massartela brieni* and *Miroculis mourei* (Brito et al. 2011). In the Leptophlebiidae family, the acrosome pattern variation is observed even in the same subfamily, and may reflect specific variations in the egg chorion (Gaino and Mazzini 1991b). Spermatozoa of *C. jocosus* and *T. ibirapitanga* presented in this study, have a conical and bilayered acrosome. These species are from the same family as *C. dipterum* (Baetidae), which have a monolayered acrosome (Baccetti et al. 1969). This data from Baetidae suggest a division in two lineages within the family or may reflect some differences between new world (*C. jocosus* and *T. ibirapitanga*) and

European species (*C. dipterum*). However, for less speculative conclusions more species must be studied.

Immunocytochemical studies revealed the presence of actin in the perforatorium of vertebrate spermatozoa (Courtens et al. 1991; Fouquet et al. 1991, 1992; Paranko et al. 1994, Ferreira et al. 2006). However, our analysis did not reveal the presence of actin in the acrosome of *C. jocosus*. The actin present in the nucleus, axoneme and accessory bodies of these spermatozoa, probably is monomeric, not filamentous, because it is irregularly distributed, observed in very small quantity and no microfilament structures were observed.

As previously observed by Brito et al. (submitted) the spermatozoa of *C. jocosus* and *T. ibirapitanga* present a centriolar adjunct at the flagellum base. It is well developed in *C. jocosus* and can be observed in cross and longitudinal sections. In *T. ibirapitanga* spermatozoa the centriolar adjunct is smaller and observed with some difficulty, in longitudinal sections.

The mitochondrion of *C. jocosus* has areas with disorganized cristae, while those of *T. ibirapitanga* had an organized parallel pattern. The mitochondrion of *C. dipterum* spermatozoa showed round vesicles between parallel cristae (Baccetti et al. 1969). These observations suggest that an irregular organization pattern of mitochondrial cristae must be common, at least in the Baetidae family.

This study demonstrated that the sperm morphology of Ephemeroptera species is more variable than was previously assumed, suggesting that the spermatozoa of more species of this order shoud be studied.

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Figure Legends

Fig 1 Transmission electron microscopy of *Callibaetis jocosus* spermatozoa: **A)** Longitudinal section of the nucleus (n) and acrosome (a), (o) acrosome outer layer, (pm) plasma membrane; **B)** Immunocytochemistry for actin at the acrosome region, notice that no labeling occurred in the acrosome region; **C-E)** Longitudinal section of the nucleus-flagellum transition, (c) centriole, (ca) centriolar adjunct, (ab) accessory body, (ax) axoneme, (m) mitochondrion; **E)** Immunocytochemistry for actin at a cross section of the nucleus, the arrows indicate the gold label; **G, H)** Cross sections of: G) nucleus -flagellum transition, H) Initial portion of the flagellum, (*) central element of the accessory body, (open arrow) accessory body membrane; **I)** Immunocytochemistry for actin at the initial portion of the flagellum, the arrows indicate the gold marks; **J, K)** Sequential cross sections of the flagellum terminal tip, **L)** High magnification of the axoneme, (a) accessory microtubules, (d) doublet microtubules, (s) central sheath, (double arrows) inner dynein arms; **M)** Longitudinal section of the flagellum including the accessory body and the mitochondrion with its cristae (ct). All bars are in μm.

Fig 2 Transmission electron microscopy of *Tupiara ibirapitanga* spermatozoa: **A-B)** Longitudinal sections of: A) Nucleus (n) and acrosome (a), (o) acrosome outer layer, B) Nucleus-flagellum transition, (c) centriole, (ax) axoneme, (ab) accessory body, (pm) plasma membrane, (arrow) centriolar adjunct; **C)** Nucleus cross section; **D-E)** Sequential cross sections of the flagellum initial portion, (ca) centriolar adjunct; **F-I)** Sequential cross sections of the flagellum, from base to end tip, (a) accessory microtubules, (d) doublet microtubules, (s) central sheath, (double arrows) inner dynein arms; **J)** Longitudinal section of the flagellum with accessory body and mitochondrion with its cristae (ct). All bars are in μm.

Figure 1

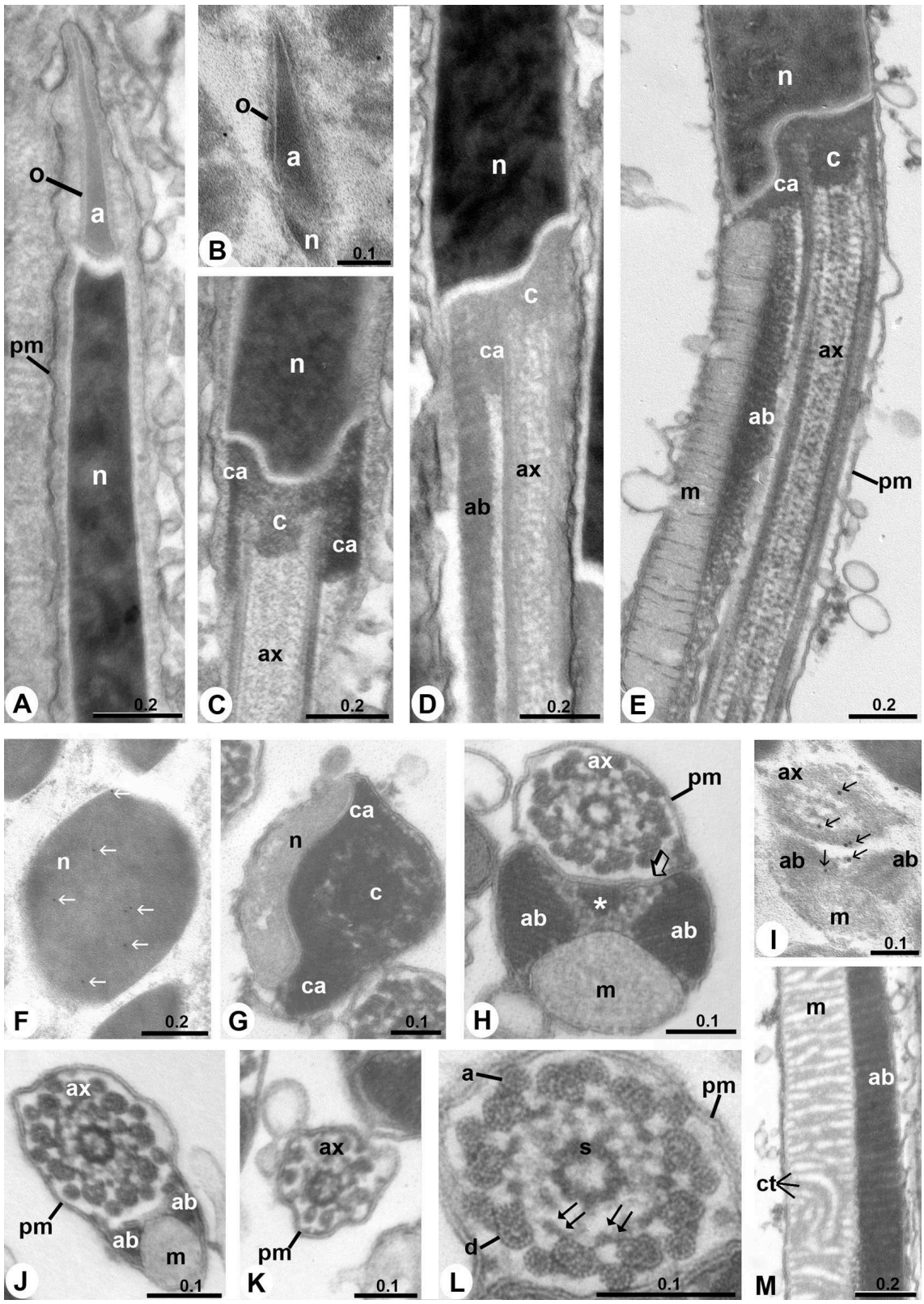
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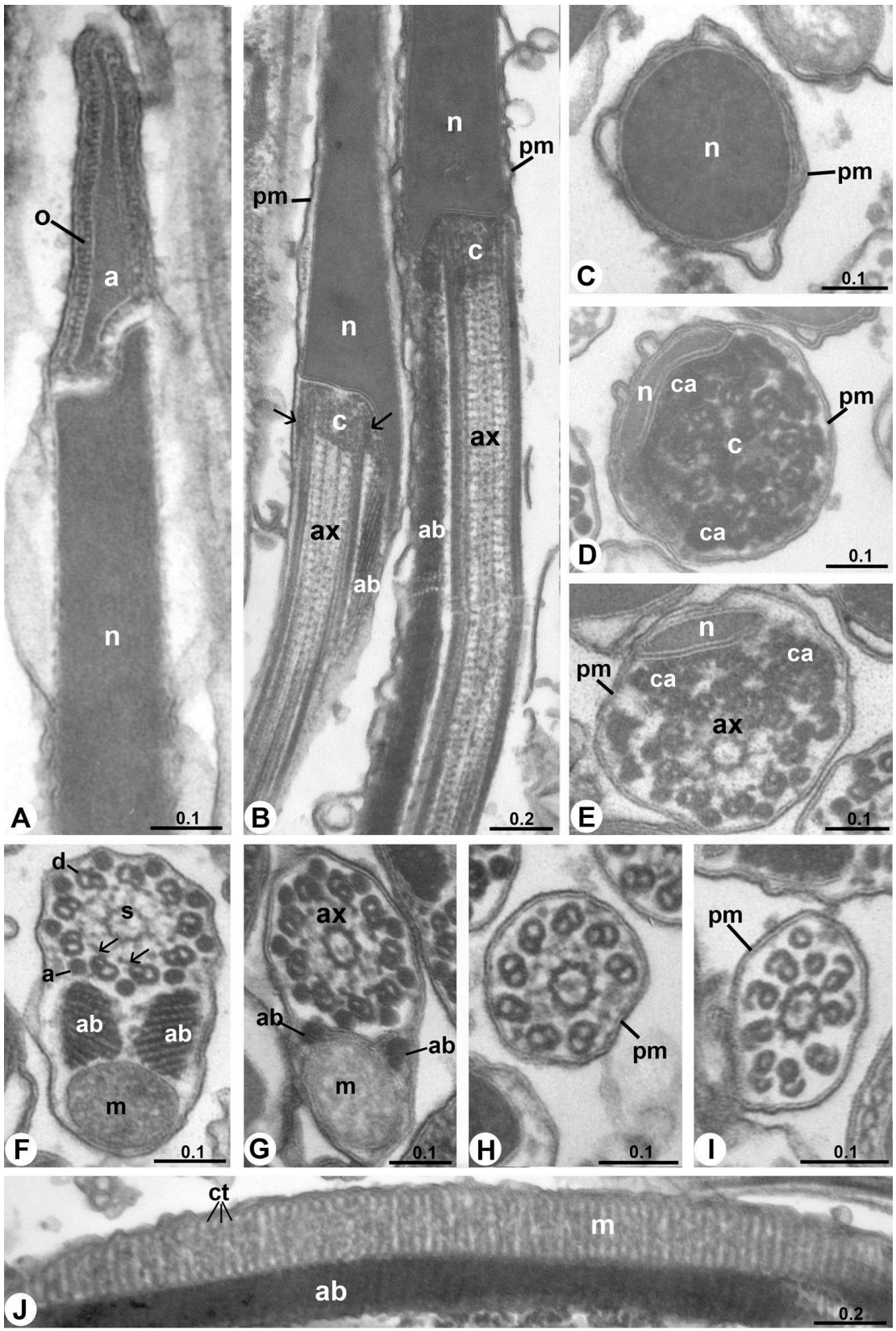
Figure 2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Capítulo 5

Sperm morphology of *Asthenopus curtus* (Hagen, 1861) and *Campsurus* sp. (Ephemeroptera: Polymitarcyidae); a comparative analysis.

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Running Title: Polymitarcyidae sperm

Keywords: Spermatozoa, mitochondria, centriolar adjunct, accessory body.

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Abstract

In order to improve available data about Ephemeroptera spermatozoa, the sperm morphology of *Asthenopus curtus* and *Campsurus* sp. (Polymitarcyidae) is described. These species belong to two of the three Polymitarcyidae subfamilies: Asthenopodinae and Campsurinae. Some of the characteristics observed in this study are common to both species, but are not observed in all Ephemeroptera species: a) accessory body surrounding the mitochondria, b) absence of the 13 + 7 accessory microtubule pattern. Some characteristics observed distinguish the two species of this study from each other: a) morphology of the nucleus base, b) morphology of the mitochondria, c) acrosome content. Furthermore it is the first observation of several aligned mitochondria, as in *Campsurus* sp., instead of a single mitochondrion along the flagellum, as is typical for Ephemeroptera spermatozoa. These data provide new sperm characteristics for Pterygota and may help to understand the evolution of the insects' spermatozoa.

Introduction

The order Ephemeroptera, or mayflies, consists of more than 3.000 species, distributed in 42 families (Barber-James et al. 2008). These insects used to be grouped together with the Odonata order in the Paleoptera group, representing the two most basal extant lineages of winged insects. However, the monophyly of the Paleoptera is controversial (Ogden and Whiting 2003; Regier et al. 2010) and even the phylogenetic relationships among mayflies are questionable (Ogden and Whiting 2005; Ogden et al. 2009). Since Ephemeroptera are likely to represent the sister group to the remaining Pterygota, understanding the phylogenetic relationships among their families is essential to understand the insects as a whole.

The use of new morphological characteristics associated to those traditionally used, as well as molecular data, is important for phylogenetic studies (Jenner 2006). Sperm morphological characteristics of several insect groups have been used for this purpose (Baccetti 1972; Kristensen 1981; Jamieson et al. 1999). However, there are few studies of sperm morphology in the Ephemeroptera order (Baccetti et al. 1969; Phillips 1969; Grimm 1985; Fink and Yasui 1988; Dallai and Afzelius 1990; Gaino and Mazzini 1991a, b; Brito et al. 2011). Some of these studies do not contain complete sperm descriptions. Nevertheless, some apomorphies have been pointed out for Ephemeroptera spermatozoa: an acrosome consisting of only an acrosomic vesicle, a single mitochondrion without paracrystalline material inside, the axoneme with a 9 + 9 + 0 microtubule pattern (with absence of the central microtubule pair), lack of the outer dynein arms in the microtubule doublets, and 13 protofilaments containing seven small internal units in the accessory microtubules (Jamieson et al. 1999). The absence of the centriolar adjunct was also pointed as an apomorphy, but the presence of this structure in the Ephemeroptera spermatozoa has already been demonstrated (Brito et al. submitted).

This study presents the first ultrastructural description of the sperm morphology of species from Polymitarcyidae: *Asthenopus curtus* Hagen, and *Campsurus* sp. These species are characterized by nymphs living in lentic and/or lotic environments, constructing "U" shaped tunnels and galleries in specific substrates. Species of this study represent two of the three subfamilies of this group: Asthenopodinae and Campsurinae.

Material and Methods

Male imagoes of *Asthenopus curtus* were collected with a light trap on Catalão Lake, Manaus, state of Amazonas, Brazil (03º 09' 76.3" S, 59º 54' 47.5" W).

Male imagoes of *Campsurus* sp. were collected with a light trap on the river bank near the BR 174 highway, state of Amazonas, Brazil (02º 49' 2.5" S, 60º 02' 09.6" W).

The seminal ducts of the specimens were dissected and fixed with a 2.5% glutaraldehyde and 1% tannic acid solution in 0.1M phosphate buffer, pH7.2, for 5 days. The material was contrasted en-bloc with 1% uranyl acetate aqueous solution for 2 hours (Dallai and Afzelius 1990). The material was dehydrated in an acetone series end embedded in Epon. Ultrathin sections were contrasted with 3% uranyl acetate followed by 3% lead citrate and analyzed with a Zeiss-Leo 906 Transmission Electron Microscope.

Results

Asthenopus curtus – Spermatozoa of *Asthenopus curtus* are divided in two regions, head and flagellum. The head consists of an approximately cylindrical nucleus and a rounded acrosome at the nucleus tip (Fig 1A). The acrosome is composed of a vesicle, with approximately 0.4 μm in diameter, heterogeneously filled with medium electron dense material, interspersed with electron lucid material (Fig 1A). Depending on the region there is more electron lucid than electron dense material (Fig 1B). A narrow electron dense subacrosomal layer is observed surrounding the acrosome base (Fig 1A, B). The nucleus is homogenously filled with electron dense chromatin and extends in a basal projection that partially surrounds the initial portion of the axoneme (Fig 1A, D, E, F). The length of the projection varies from approximately 0.3 to 0.5 μm (Fig 1A, E, F). In cross sections of its medial portion, the nucleus is approximately circular with 0.6 m in diameter (Fig 1C).

The flagellum of *A. curtus* spermatozoa is composed of an axoneme, a mitochondrion and the accessory body. Furthermore, the nucleus-flagellum region is characterized by the presence of a centriolar adjunct, consisting of an agglomerate of electron dense material that surrounds the centriolar region (Fig 1A, E, D). The centriolar region is the anterior portion of the axoneme. Besides its association with the centriolar adjunct, it is also characterized by electron dense material filling the centriole core and by a reduction of the intertubular material (Fig 1D, E). Below the centriolar region is the

axoneme with a 9 + 9 + 0 microtubule pattern with nine external accessory tubules, nine doublets and no central pair of microtubules (Fig 2A). The accessory microtubules have clear lumens (Fig 2A). The doublet microtubules have only the innermost dynein arms and connecting rays that reach the central sheath (radial spokes). This sheath begins just below the centriole and occupies the innermost region of the axoneme; it is composed of electron dense material and may have its lumen irregularly filled with electron dense material (Fig 2A-E). The axoneme is the longest tail structure and the last one to disorganize at the distal flagellum tip, where it is observed in cross sections (Fig 2E). The accessory microtubules are shorter than the other axoneme elements and are not observed in cross sections of the distal tail region (Fig 2D, E).

The accessory body is a 0.04 m thick electron dense layer that surrounds the mitochondrion almost completely, with exception of the portion near the axoneme (Fig 2A – C). Sometimes two membranes seems to limit the accessory body, an external one entirely surrounding the accessory body and the mitochondrion (Fig 2B) and an internal one that is in close contact with the external mitochondrial membrane (Fig 2A). It is hard to distinguish the separation between the accessory body and the centriolar adjunct as they come in contact, at the flagellum base (Fig 1A, F).

The mitochondrion is a long organelle that extends parallel to the axoneme. It begins below the basal nuclear projection (Fig 1A, F) and is elliptical in cross sections, with approximately 0.25 x 0.15 μm for its larger and smaller axes, respectively (Fig 2A, B). Mitochondrial cristae could be observed in longitudinal and cross sections and they do not follow an organized pattern (Figs 1F, 2A, F). The mitochondrial intermembrane space is filled with electron dense material while the mitochondrial matrix appears clear (Figs 1F, 2A, F). The mitochondrion tapers gradually, as the accessory body, at the distal tail region (Fig 2C, D) and ends after the accessory microtubules, but before the other axoneme elements (Fig 2D, E).

Campsurus sp. – Spermatozoa of *Campsurus* sp. are divided in two regions, head and flagellum. The head is composed of a cylindrical nucleus homogenously filled with compact chromatin and an approximately round acrosome, homogenously filled with medium electron dense material (Fig 3A). The acrosome is approximately 0.45 μm in

diameter (Fig 3A). The nucleus is circular, in cross sections, with approximately 0.5 μm in diameter (not shown).

The flagellum components are the axoneme, the centriolar adjunct, the mitochondria and the accessory body. The nucleus-flagellum junction is characterized by the presence of a well-developed centriolar adjunct that attaches the flagellum components at the nucleus base (Fig 3B, C). Notice the regular shape of the nucleus base with no projections or fossae.

The accessory body surrounds several aligned mitochondria in the flagellum (Fig 3B-H). In cross sections, one (Fig 3E), two (Fig 3F) or even more mitochondria (not shown) can be observed side by side. These mitochondria vary from approximately 0.4 to 0.7 μm in length. Their, approximately 0.04 m thick, inter-membrane space is filled with electron dense material (Fig 3B-F). The accessory body consists in a 0.07 μm thick amorphous layer and surrounds all the mitochondria. Due to their density, it is hard to distinguish the limit between the centriolar adjunct and the accessory body at the flagellum base (Fig 3B, C). In some sections, the accessory body seems to be limited by two membranes, an external and an internal one, both closely associated with their dense amorphous content (Fig 3E). The accessory body's inner membrane and the outer mitochondrial membrane are clearly separated by an electron lucid space (Fig 3B-F). At the terminal flagellum region, the mitochondrial complex tapers gradually and finishes before the axoneme disorganizes completely (Fig 3G-I).

The axoneme is the longest flagellum structure; it begins at the nucleus base, below the centriolar adjunct (Fig 3B) and can be observed alone in cross sections of the distal flagellum (Fig 3I, J). At the axoneme base, the centriole is characterized by its close association with the centriolar adjunct and by the presence of electron dense material in the centriole center (Fig 3B). The axoneme microtubules follow the 9 + 9 + 0 pattern with nine external accessory tubules, nine doublets and no central pair of microtubules (Fig 3G). The accessory microtubules have clear lumens. The microtubule doublets have only the innermost dynein arms and connecting rays that reach the central sheath (radial spokes) (Fig 3G). This sheath is composed of an electron dense layer with a clear lumen (Fig 3E-I). The first axoneme element to disorganize at the flagellum terminal region are the accessory microtubules, they finish before the mitochondria (Fig 3H). Only the doublet

microtubules and the central sheath are observed in cross sections near the flagellum end (Fig 3I) and the doublet microtubules are observed alone at the flagellum extremity (Fig 3J).

Discussion

Structures observed in *A. curtus* and *Campsurus* sp. confirm some of the characteristics considered typical for Ephemeroptera spermatozoa *i.e.,* absence of a perforatorium, axoneme with the 9 + 9 + 0 microtubule pattern and the presence of only the innermost dynein arms associated with microtubule (Baccetti et al., 1969; Phillips, 1969; Grimm, 1985; Fink and Yasui, 1988; Gaino and Mazzini, 1991b; Jamieson et al., 1999). Recently, the presence of spermatozoa organized in a spermatodesm was registred in *Cloeon dipterum* (Lupetti et al., 2011). However, no evidence of this kind of organization was observed in both species presented in this study. It was demonstrated that the centriolar adjunct must be a common characteristic in Ephemeroptera (Brito et al. submitted) and the observations in this manuscript confirm this statement.

Because of the fixation method used in our material (tannic acid), the presence of membranes associated to the internal and external sides of the accessory body cannot be confirmed by our results. The repeated way that these membranes were observed suggests that, it is not a technical artifact. However, to confirm this hypothesis, studies would be necessary using conventional fixation methods and/or descriptions of the spermatogenesis of these species.

The spermatozoa of some insect groups are characterized by two layered acrosomes. These acrosomes may present two different patterns: those composed of an electron dense perforatorium surrounded by the acrosome vesicle; or composed of an acrosomal vesicle surrounded by an external layer, as observed in some Ephemeroptera from Baetidae (Chapter 4). This external layer may show some variation in composition, but it usually surrounds the acrosome vesicle in a concentric manner and is located at the anterior portion of the acrosome (Baccetti, 1972; Jamieson et al., 1999; Brito et al., 2009). The acrosome of *A. curtus* has a subacrosomic layer between the acrosome vesicle and the nucleus. This layer probably represents some cytoplasm residue. On the other hand, the

Campsurus sp. spermatozoa show a typical monolayered acrosome pattern composed only by an acrosomic vesicle.

Moreover, other differences could be observed between the two species: *A. curtus* presented a heterogeneous and *Campsurus* sp. a homogeneous acrosomic content. The insertion of the flagellum structures at the nucleus base also distinguishes the two species of this study: the nuclear base of *Campsurus* sp. spermatozoa does not have a projection similar to that observed in *A. curtus* nucleus base.

The 13 + 7 accessory microtubule pattern, with 13 tubulins surrounding 7 inner units of unknown nature has been considered a typical Ephemeroptera characteristic (Dallai and Afzelius, 1990; Jamieson et al. 1999). It was observed in species of the families Baetidae (Dallai and Afzelius, 1990), Coryphoridae, Leptohyphidae and Oligoneuriidae families (Chapters 6 and 7). However, even using the same fixation method as in the studies mentioned above, both species in this study did not show any element inside the accessory microtubules, which always have clear lumens. This characteristic distinguishes Polymitarcyidae spermatozoa from other known flagellated ephemeropteran sperm. Even species from the Ephemeroidea group, close related to Polymitarcyidae, such as *Hexagenia albivitta* Walker, have the 13+7 pattern in the accessory microtubules (Chapter 10).

Mitochondria as observed here in *Campsurus* sp., are very unusual. A similar characteristic was previously observed in some Protura spermatozoa, with several mitochondria distributed along the spermatozoa (Dallai et al., 1992). However, this is the first description of a Pterygota spermatozoon with several mitochondria aligned along the flagellum. It could be interpreted as a simplification of the mitochondrial derivative's formation process, avoiding the mitochondrial fusion that culminates with the nebenkern structure in the spermatocyte (Tokuyasu, 1975). Or it could be interpreted as a derivation of the mitochondrial derivative's formation process, with fragmentation after the mitochondrial derivative's elongation. Depending on what the real formation process of this mitochondrial complex is, it could be interpreted as a basal or derived characteristic. Thus the correct interpretation of these facts would be useful because of the basal taxonomic position of Ephemeroptera among the extant Pterygota.

In some *Campsurus* nymphs, a mitochondrial fusion process was observed in the spermatocytes (personal observation). Since all spermatocytes observed were at the same maturation stage, these observations were not enough to clarify this question and a description of the entire process is still needed. The mitochondrion of *A. curtus* with irregular cristae is also interesting, once most species studied up to the moment have parallel or regularly organized cristae (Baccetti et al., 1969; Gaino and Mazzini, 1991b).

The 9 + 9 + 0 axoneme pattern, as observed in this study, has been extensively described for many species of Ephemeroptera (Baccetti et al., 1969; Phillipps, 1969; Grimm, 1985; Fink and Yasui, 1988; Dallai and Afzelius, 1990; Gaino and Mazzini, 1991b). Another axoneme pattern (9 + 9 + 1) was described for *Dolania americana*, (Fink and Yasui, 1988). However, the density of the central sheath does vary, even within the same specimen and may or may not have a dark spot inside it (Gaino and Mazzini, 1991b). This characteristic is also supported by the findings in *A. curtus* and therefore it does not constitute a new axoneme pattern.

Although the majority of the Ephemeroptera possess movable spermatozoa, little data about their motility pattern is available in the literature (Phillips, 1983; Werner and Simmons, 2008). This data would be useful to understand how the lack of outer dynein arms and of the central microtubule pair in the axoneme interfere in spermatozoa movements. The lack of these structures in Ephemeroptera axonemes is interpreted as steps in a tendency toward flagellum reduction in Ephemeroptera (Dallai et al., 2006) that culminated with immovable aflagellate spermatozoa in Leptophlebiidae species (Gaino and Mazzini, 1991a; Brito et al., 2011).

Conclusion

Some data of this manuscript contributes to distinguish between the two species of this study: differences in the nucleus base, morphology of the mitochondria and the acrosome content. These characteristics may distinguish Polymitarcyidae subfamilies in general. However, other species from Asthenopodinae and Campsurinae (and, possibly, species from the Holartic subfamily: Polimitarcyinae) must be studied to confirm whether these are really autopomorphies for these subfamilies.

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Figure Legends

Fig 1 Spermatozoa of *Asthenopus curtus*: A) Longitudinal section of head, from acrosome (ac) to nucleus-flagellum region, (n) nucleus, (ax) axoneme, (ca) centriolar adjunct, (ab) accessory body, (*) subacrosomal layer; **B-D)** Cross sections of acrosome, nucleus and nucleus-flagellum region respectively, (pm) plasma membrane, (am) acrosome membrane, (c) centriole; **E-F)** Longitudinal sections of nucleus-flagellum region, (arrows) mitochondrial cristae.

Fig 2 Spermatozoa of *Asthenopus curtus*: A, B) Cross sections of the flagellum, **C-E)** Sequential cross sections of the final flagellum portion, **F)** Longitudinal section of the mitochondria + accessory body. (d) doublet microtubules, (a) accessory microtubules, (s) axoneme central sheath, (o) accessory outer membrane, (pm) plasma membrane, (m) mitochondrion, (ab) accessory body, (ax) axoneme, (double arrows) dynein arms, (open arrow) inner accessory body and outer mitochondrial membranes, (arrows) mitochondrial cristae.

Fig 3 Spermatozoa of *Campsurus sp.*: A) Longitudinal section of the head, (ac) acrosome, (n) nucleus; **B, C)** Longitudinal sections of the nucleus-flagellum region, (c) centriole, (ca) centriolar adjunct, (ax) axoneme, (ab) accessory body, (m) mitochondria; **D)** Longitudinal section of the mitochondria in the flagellum; **E, F)** Cross sections of the flagellum, (pm) plasma membrane, (i) inner mitochondrial membrane, (o) outer mitochondrial membrane, (open arrow) accessory body inner membrane, (arrow) accessory body outer membrane. Notice the mitochondrial inter-membrane space filled with electron dense material and the clear space between the accessory body and the mitochondria. **G – J)** Sequential cross sections of the flagellum end, (a) accessory microtubules, (d) doublet microtubules, (s) axoneme central sheath, (double arrows) inner dynein arms.

Figure 1

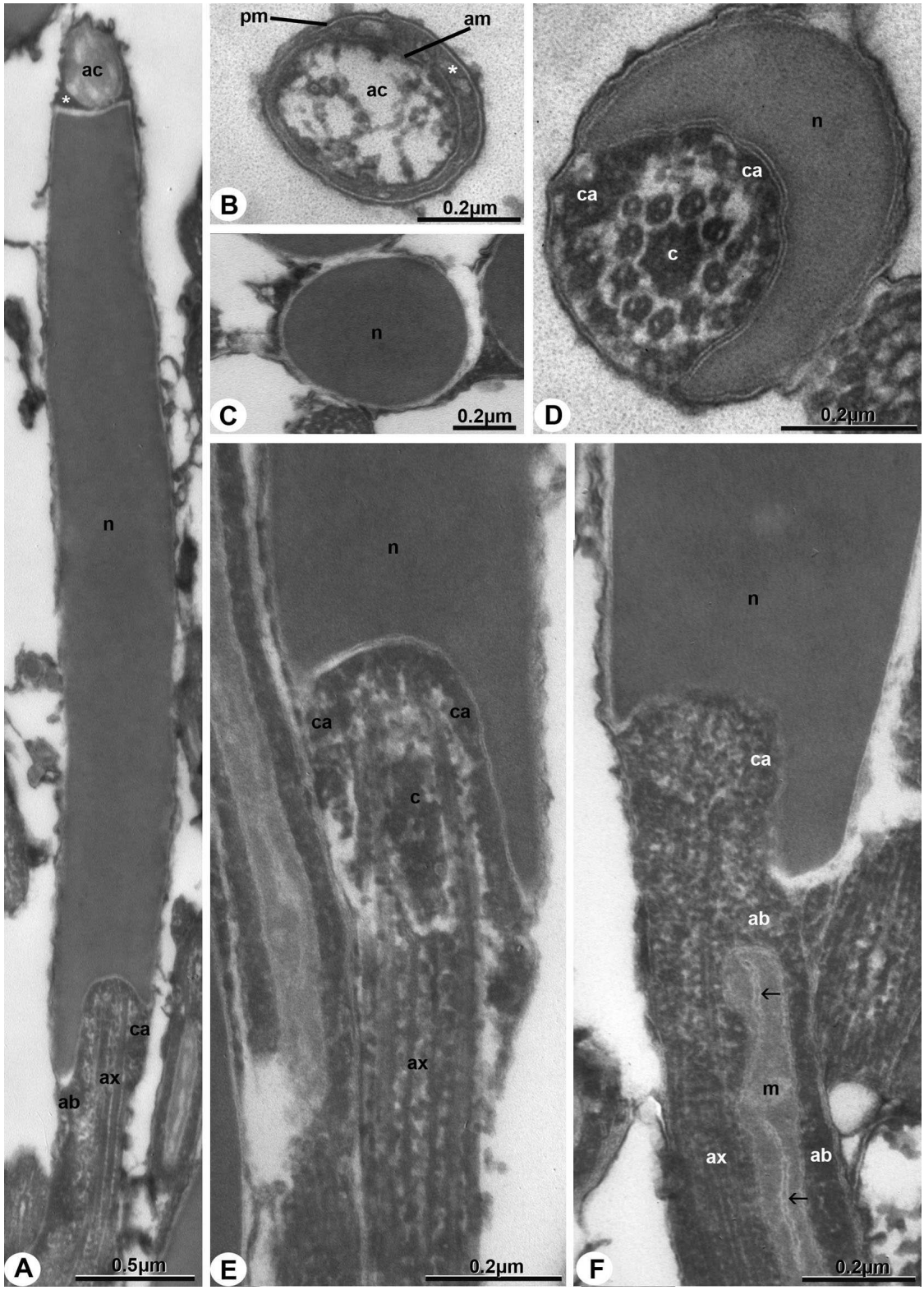
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Figure 2

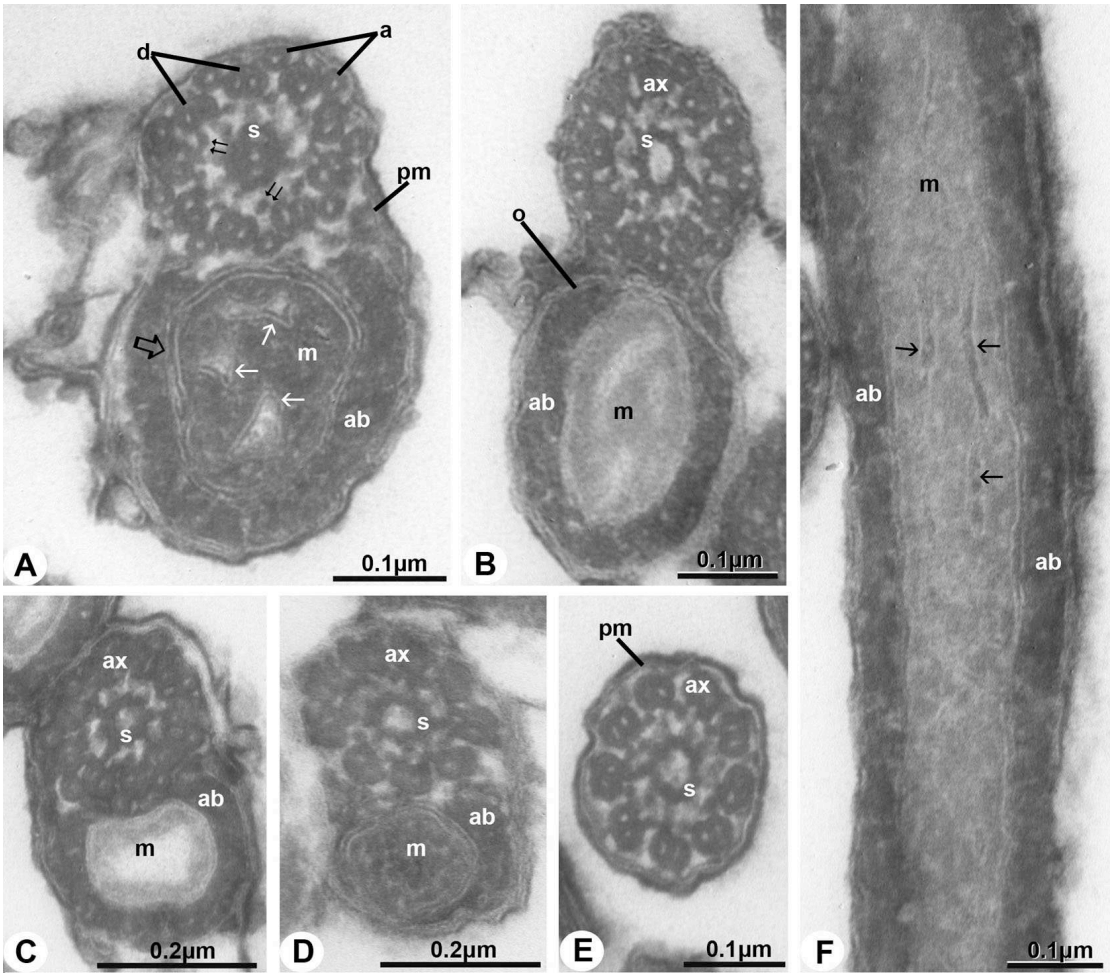
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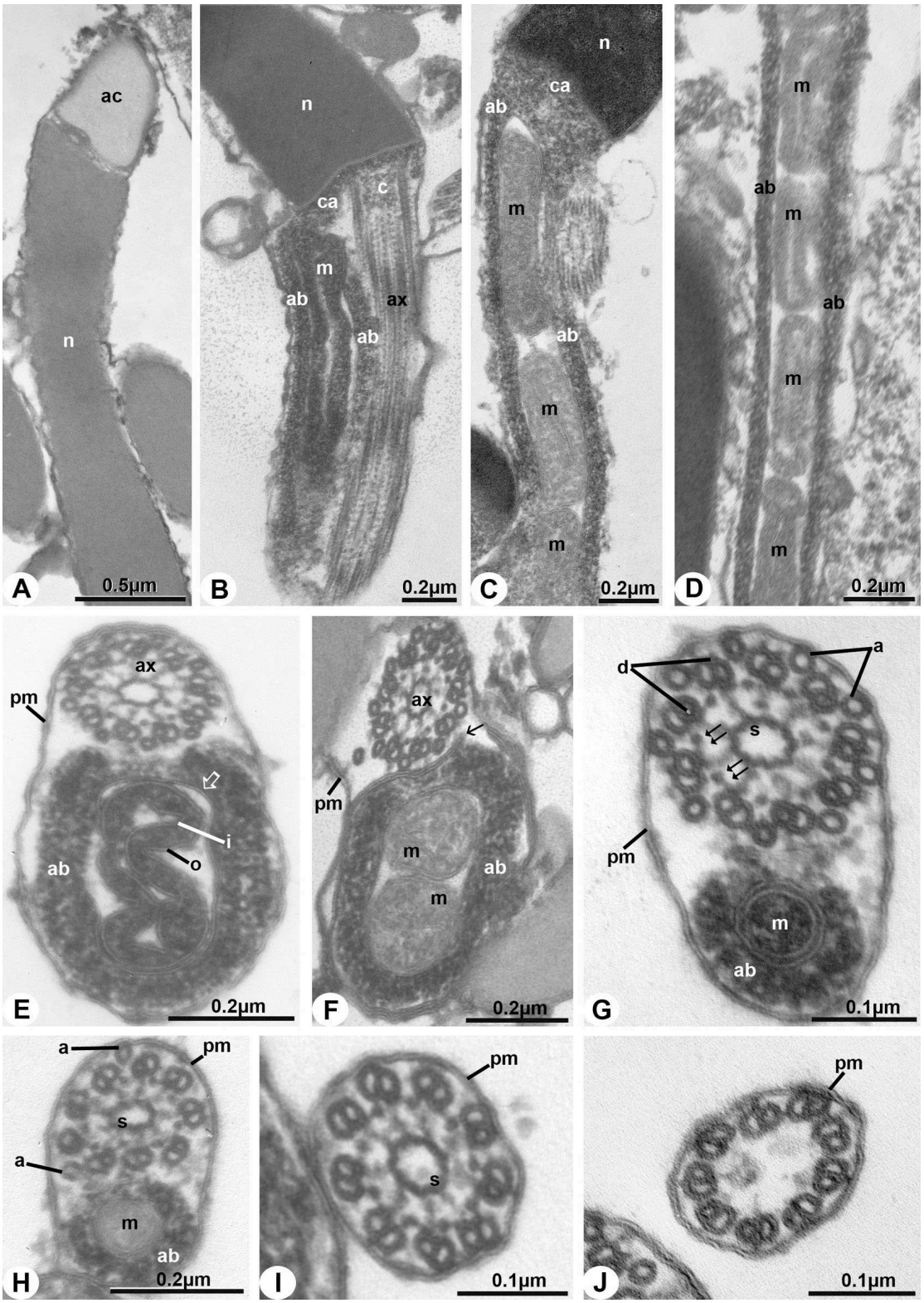
Figure 3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Capítulo 6

What does Sperm Morphology says about the relationship of Coryphoridae and Leptohyphidae?

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Key words: Ephemerelloidea, Pannota, Ephemeroptera, Spermatozoa, Mayfly

Abstract

Insects from the Ephemeroptera order represent the oldest existing winged insects. Leptohyphidae and Coryphoridae are two New-World families and are considered sister-groups. This study describes sperm morphology of *Traverhyphes (Mocoihyphes) yuati* (Leptohyphidae) and *Coryphorus aquilus* (Coryphoridae). Some similarities were observed among these species, such as: 1) monolayered homogeneous acrosome; 2) nucleus with homogeneous compact chromatin; 3) flagellum inserted in an irregular cavity in the nucleus base; 4) divided accessory body without a crystalline arrangement; 5) parallel mitochondrial cristae; 6) axoneme with a dark granule in the central sheath. There are few studies describing the sperm morphology of Ephemeroptera species, but the shared sperm characteristics observed in this study support the close relation suggested for Leptohyphidae and Coryphoridae families.

Introduction

More than 3.000 species distributed in 42 families consists the order Ephemeroptera (Barber-James et al., 2008), representing the oldest existing winged insects. Their nymphs are aquatic and this stage represents the major part of their life span. Only the two last stages of their lives are winged (subimago and imago), when they leave the aquatic environment. The winged stages do not feed and can live for some hours up to a few days, in order to reproduce. They are distributed over all continents, except Antarctica, but their greatest diversity is observed in rivers of tropical and temperate zones (Brittain, 1982; Brittain and Sartori, 2003).

The family Leptohyphidae is an exclusively New-world group that is distributed along both American continents. Approximately 120 species are described for this family, of which 67 are found in South America (Molineri, 2006). Coryphoridae is a monotypic family, represented by *Coryphorus aquilus* Peters, distributed in the Neotropical region and considered the sister-group of Leptohyphidae (Molineri, 2006). Along with Melanemerellidae, these families are the only representatives of Ephemerelloidea in South America.

The use of new morphological characteristics associated to those traditionally used, as well as molecular data, is important for phylogenetic studies (Jenner, 2006). Sperm morphological characteristics of several insect groups have also been used for this purpose (Baccetti, 1972; Kristensen, 1981; Jamieson et al., 1999). However, there are few studies of sperm morphology in the Ephemeroptera order and some of them do not contain complete sperm descriptions (Baccetti et al., 1969; Phillipps, 1969; Grimm, 1985; Fink and Yasui, 1988; Dallai and Afzelius, 1990; Gaino and Mazzini, 1991a, b; Brito et al., 2011).

This is the first sperm ultrastructural study of Ephemerelloidea species and increases the knowledge about Ephemeroptera spermatozoa, providing a morphological comparison between *Traverhyphes (Mocoihyphes) yuati* Molineri (Leptohyphidae) and *Coryphorus aquilus* Peters (Coryphoridae) spermatozoon, with the intention of checking whether sperm morphology also supports this relationship among these families.

Material and Methods

Male imagoes of *Traverhyphes yuati* were collected with a light trap at "Capitel de Santo Antonio" farm Santa Teresa, ES, Brazil (19º 52' 31.6" S, 40º 31' 49.1" W). Male imagoes of *Coryphorus aquilus* were collected with a light trap beside a stream close to the BR174 highway, Brazil (02º 49' 2.5" S, 60º 02' 09.6" W).

Light Microscopy

Spermatic ducts of *C. aquilus* were dissected in sodium phosphate buffer (pH 7.2) and broken open on histological slides. The sperm were spread and fixed with Karnovsky's solution, 2.5% glutaraldehyde and 4% paraformaldehyde. The material was analyzed with a BX41 Olympus, Phase Contrast Microscope.

Scanning Electron Microscopy

Spermatic ducts of *T. yuati* were dissected in sodium phosphate buffer (pH 7.2) and broken open on histological slides. The sperm were spread and fixed with Karnovsky's solution, 2.5% glutaraldehyde and 4% paraformaldehyde. The material was processed with conventional scanning electron microscopy techniques and analyzed in a Jeol JSM5800LV.

Transmission Electron Microscopy

Spermatic ducts of *T. yuati* and *C. aquilus* specimens were dissected in sodium phosphate buffer (pH 7.2), fixed with a 2% glutaraldehyde and 1% tannic acid solution and stained 'en-bloc' with 1% uranyl acetate. The material was dehydrated and embedded in Epon resin. Ultra-thin sections were contrasted with 3% uranyl acetate and 3% lead citrate, and analyzed with a Zeiss Leo 906 Transmission Electron Microscope.

Results

Coryphorus aquilus – The spermatozoa of *C. aquilus* are long and slender, with approximately 32 μm in length, of which approximately 11 μm consist in the head region (Fig. 1A, *inset*). The head is composed of a cylindrical nucleus, with 0.5 μm in diameter, containing compact chromatin (Fig. 1A-F) and an apical acrosome (Fig. 1A). The acrosome is a rounded vesicle with a granular content, measuring 0.4 μm in diameter.

The spermatozoa flagellum consists of three structures: an axoneme, the mitochondrion and the accessory body (Fig. 1G, H). The flagellum initial portion is

located in an irregular cavity of the nuclear base (Fig. 1C-F). The nucleus-flagellum transition region is characterized by the presence of the centriolar adjunct, a dense structure that surrounds the centriole at the initial portion of the axoneme and makes it difficult to distinguish the centriolar microtubules, occasionally seen as triplets (Fig. 1C-E). The axoneme presents the 9+9+0 microtubule pattern, with 9 external accessory microtubules, 9 doublets and without the central pair of microtubules (Fig. 1I). The central region of the axoneme is occupied by a sheath, which may or may not present a dark granule inside it (Fig. 1G-I). Only the innermost dynein arms are present on the microtubule doublets. The microtubule doublets are connected to the central sheath by connecting rays (radial spokes). Accessory microtubules present the 13+7 pattern with 13 outer tubulins surrounding 7 inner units of undetermined nature (Fig. 1I).

The mitochondrion is a long structure that begins at the nucleus base (Fig. 1E,F) and extends parallel to the axoneme (Fig. 1J). In cross sections, the mitochondrion is circular with approximately 0.15 μm in diameter (Fig. 1G, H). The mitochondrial cristae are organized in parallel, but perpendicular to the long axis of the mitochondrion (Fig. 1J). The accessory bodies are always observed between the mitochondrion and the axoneme (Fig. 1G, H). It is composed of two homogeneous parallel portions that gradually taper at the flagellum tip (Fig. 1G, H). A medium electron dense material is also observed associated to the accessory body, between the mitochondrion and the axoneme (Fig. 1G, H).

Traverhyphes yuati – The spermatozoon of *T. yuati* are long and slender with approximately 28 μm in length, of which 8.5 μm represents the head region (Fig. 2A). The head region is composed of a cylindrical nucleus, with approximately 0.45 μm in diameter, filled with compact chromatin (Fig. 2B-D) and an apical acrosome (Fig. 2B). The acrosome consists in a simple rounded acrosomic vesicle, approximately 0.4 μm in diameter, filled with granular material (Fig. 2B).

The flagellum is composed of three long structures: axoneme, mitochondrion and an accessory body (Fig. 2 F, G). The flagellum base is located in an irregular cavity at the nucleus base (Fig. 2C). This region is characterized by the presence of the centriolar adjunct (Fig. 2C, E), a dense structure that surrounds the initial portion of the axoneme,

the centriole, and makes difficult to distinguish the microtubules. The axoneme presents the 9+9+0 microtubule pattern, with 9 external accessory microtubules, 9 doublets and without the central pair of microtubules (Fig. 2H). A sheath occupies the central region of the axoneme with irregularly distributed dense granular material inside (Fig. 2F-I). The radial spokes, connecting the microtubule doublets, are originated from this sheath. The accessory microtubules present the 13+7 pattern, with 13 tubulin subunitis surrounding 7 inner units of unknown nature. Only the innermost dynein arms are observed at the microtubule doublets (Fig. 2H).

The mitochondrion is a long structure following parallel to the axoneme (Fig. 2I), with its apical portion inserted in the basal nuclear cavity (Fig. 2C, E). Mitochondrial cristae are organized in parallel, but perpendicular to the long axis of the mitochondrion (Fig. 2I). Homogeneous accessory bodies are always present between the mitochondrion and the axoneme (Fig. 2F, G). The accessory bodies are composed of two parallel homogeneous portions that, at the flagellum base, are thicker and approximately triangular in cross sections, compressing the mitochondrion into a pear shape (Fig. 2F). Near the flagellum tip, the accessory bodies taper and the mitochondrion becomes elliptical (Fig. 2G). A membrane surrounding the accessory bodies and the mitochondrion is observed in some sections (Fig. 2G).

Discussion

The morphology of *C. aquilus* and *T. yuati* spermatozoa is similar to previous descriptions for Ephemeroptera. The axoneme 9+9+0 pattern, such as observed for both species in this study, has been described as characteristic for the Ephemeroptera order (Baccetti et al., 1969; Phillips, 1969; Grimm, 1985; Dallai and Afzelius, 1990; Gaino and Mazzini, 1991a, Jamieson et al., 1999), with exception of species from the Leptophlebiidae family that possess aflagellate spermatozoa (Gaino and Mazzini, 1991 a, b, Brito et al., 2011). A second axoneme pattern was described for *Dolania americana* (Fink and Yasui, 1988), in which a central spot was observed inside the central cylinder. However, as discussed by Gaino and Mazzini (1991a) and also observed for *C. aquilus* and *T. yuati* in this study, the same species may, or may not, present this central dense granule, so that it cannot be considered a new axoneme pattern for Ephemeroptera sperm.

The axoneme accessory microtubule 13+7 pattern observed in *C. aquilus* and *T. yuati,* was first described in *Cloeon dipterum* (Dallai and Afzelius, 1990) and has been considered an apomorphy for flagellated Ephemeroptera spermatozoa. The microtubule doublets with only the innermost dynein arm, as observed in this study, is considered an apomorphic characteristic of the Ephemeroptera sperm axoneme (Dallai and Afzelius, 1990; Gaino and Mazzini, 1991a; Jamieson et al., 1999).

In the present study, both species presented a monolayered, rounded acrosome, composed of an acrosomic vesicle filled with granular homogeneous material. This is similar to the structure observed in *C. dipterum* (Baetidae) (Baccetti et al., 1969), *Electragena grandiae* and *Ecdyonurus venosus* (Heptageniidae) (Gaino and Mazzini, 1991a). Monolayered acrosomes, with some clear regions inside the acrosomic vesicle were observed in *D. americana* (Behningiidae) sperm (Fink and Yasui, 1988). Acrosomes with a perforatorium surrouded by an acrosomal vesicle were observed in spermatozoa of some Leptophlebiidae species, such as *Habroleptoides umbratilis* and *Choroterpes picteti* (Gaino and Mazzini, 1991 a, b). Conical acrosomes with the acrosomal vesicle surrounded by an external layer were observed in *Callibaetis jocosus*, *Tupiara ibirapitanga* (Baetidae) and *Lachlania* sp. (Chapters 4 and 7).

The cylindrical nucleus, filled with homogenously compact chromatin, as observed in *C. aquilus* and *T. yuati*, is the most common type of nucleus, but it is not present in all Ephemeroptera species. Some species from the Baetidae family presented incompletely compacted chromatin (Baccetti et al., 1969), and *E. grandiae* presented clear vesicles inside the nucleus (Gaino and Mazzini, 1991a). Also in some Oligoneuriidae species the chromatin is granular and not completely compacted (Chapter 7).

Ephemeroptera spermatozoa usually have only one mitochondrion along the flagellum, with one known exception, *Campsurus* sp. (Chapter 5), which has many mitochondria aligned along the flagellum. Some species have the mitochondrial cristae with complex organizational patterns, such as *Asthenopus curtus*, *Callibaetis jocosus* (Chapters 4 and 5) and *Cloeon dipterum* (Baccetti et al., 1969). Both species of the present study have parallel well-organized cristae, perpendicularly oriented in relation to the mitochondrion's long axis.

The presence of a centriolar adjunct in the nucleus-flagellum transition region of *C. aquilus* and *T. yuati* reinforces the statements of Brito et al. (submitted) that the presence of a centriolar adjunct in Ephemeroptera spermatozoa must be considered a common characteristic for the group. The spermatozoa of Ephemeroptera present a great diversity of accessory bodies morphology (Brito et al., submitted). However, both *C. aquilus* and *T. yuati* present accessory body divided in two portions without crystalline organization. In *T. yuati* spermatozoa a membrane surrounding the mitochondrion and the accessory body is sometimes observed. This characteristic was not observed in the spermatozoa of *C. aquilus*, in which membranes were not as well preserved. Based on our observations, we were not able to determine if the presence of such membrane is a real characteristic or some kind of artifact.

Spermatozoa from *C. aquilus* and *T. yuati* showed differences in total and head lengths, but in general, they have very similar characteristics and structural organization when analyzed with the transmission electron microscope. They share six principal characteristics: 1) Monolayered homogeneous acrosome; 2) Nucleus with homogeneous dense chromatin; 3) Flagellum inserted in an irregular cavity of the nucleus base; 4) A divided accessory body, without crystalline organization; 5) Parallel mitochondrial cristae; 6) An axoneme with a central dark granule.

There are few studies of Ephemeroptera spermatozoa and some of them do not give complete descriptions, so that any conclusion about families' relation among Ephemeroptera, based on sperm morphology would be premature. However, the characteristics numbered above suggest that *C. aquilus* and *T. yuati* are closely related, since they share important features. This is in accordance with Molineri (2006) who suggested that Coryphoridae is a sister-group of Leptohyphidae. It is important to notice that this is the first ultrastructural study of the sperm morphology of Ephemerelloidea species and this kind of study must be expanded to more species of this group before major conclusions.

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Figure Legends

Fig 1: Transmission electron microscopy of *C. aquilus* sperm. **A)** Longitudinal section of spermatozoa apex, (ac) acrossomal vesicle, (n) nucleus; ***inset:*** phase contrast light microscopy of *C. aquilus* sperm, (h) head, (t) tail; **B-D)** Cross sections of: B) nucleus, C) nucleus-flagellum transition, D) nucleus-flagellum transition at lower level, observe the arrangement in triplets (arrow), (c) centriole, (ca) centriolar adjunct; **E,F)** Longitudinal sections of nucleus flagellum transition, (m) mitochondrion, (ab) accessory body; **G,H)** Flagellum cross sections at: G) basal region, H) final region, (ax) axoneme, (thick arrows) medium electron dense material between accessory bodies and axoneme; **I)** Axoneme great magnification, (a) accessory microtubules, (d) microtubule doublets, (s) central sheath, (double arrows) dynein arms, (g) central granule; **J)** Longitudinal flagellum section, (ct) mitochondrial cristae. All bars are in μm.

Fig 2: A) Scanning electron microscopy of *T. yuati* spermatozoa, (h) head, (t) tail; **B-J)** Transmission electron microscopy of *T. yuati* spermatozoa; **B)** Longitudinal section of sperm apex, (ac) acrossomal vesicle, (n) nucleus; C) Longitudinal section of nucleus-flagellum transition region, (c) centriole, (ax) axoneme, (ca) centriolar adjunct, (m) mitochondrion; **D-G)** Cross sections of: D) Nucleus (pm) plasma membrane, E) Nucleus-flagellum transition, F) Basal flagellum region, G) Final flagellum region, (ab) accessory body, (open arrow) accessory body membrane; **H)** Higher magnification of an axoneme, (a) accessory microtubules, (d) microtubule doublets, (s) central sheath, (double arrow) dynein arm; **I)** Flagellum in longitudinal section, (ct) mitochondrial cristae, (arrows) irregularly organized granules inside the central sheath. All bars are in μm.

Figure 1

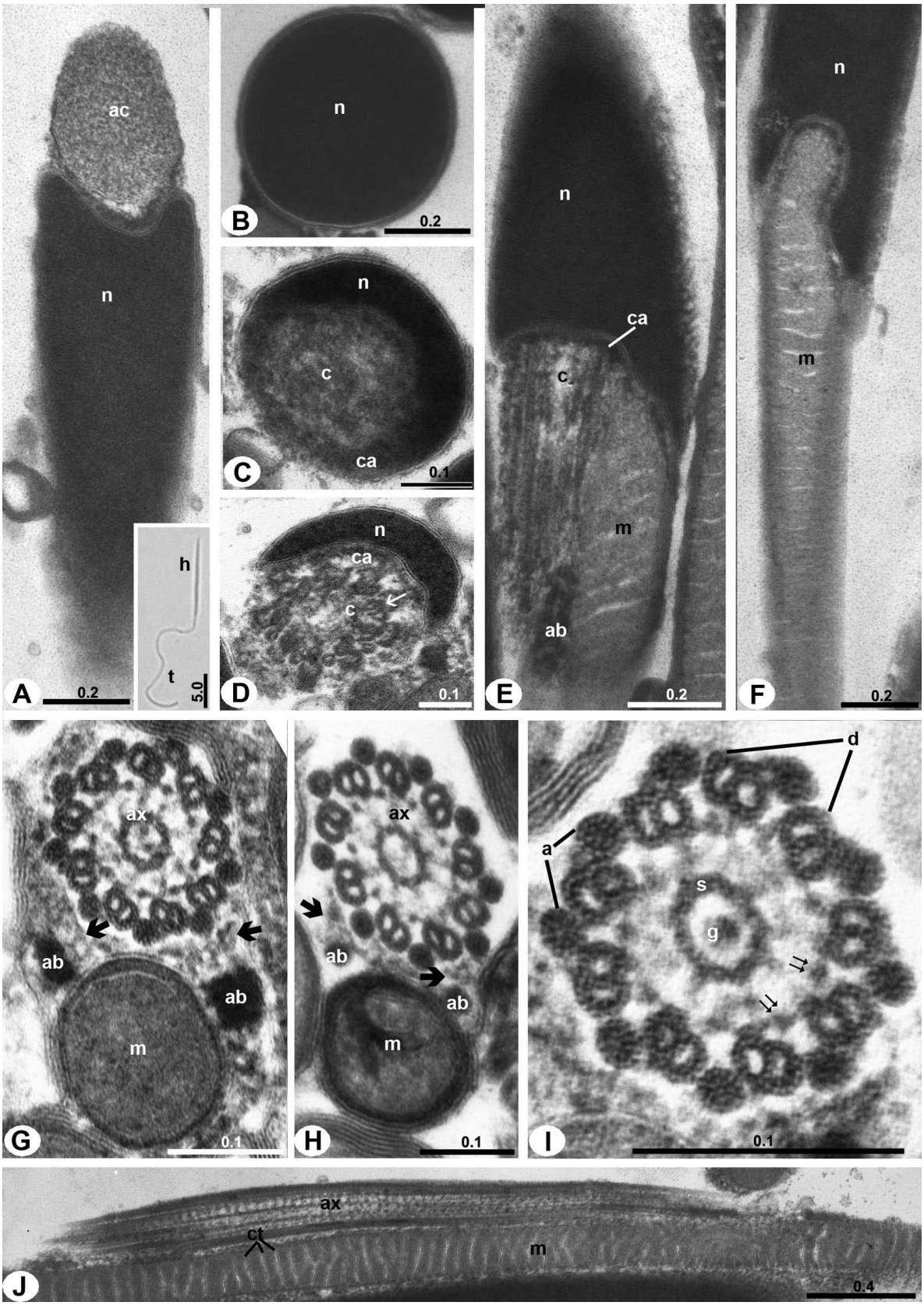
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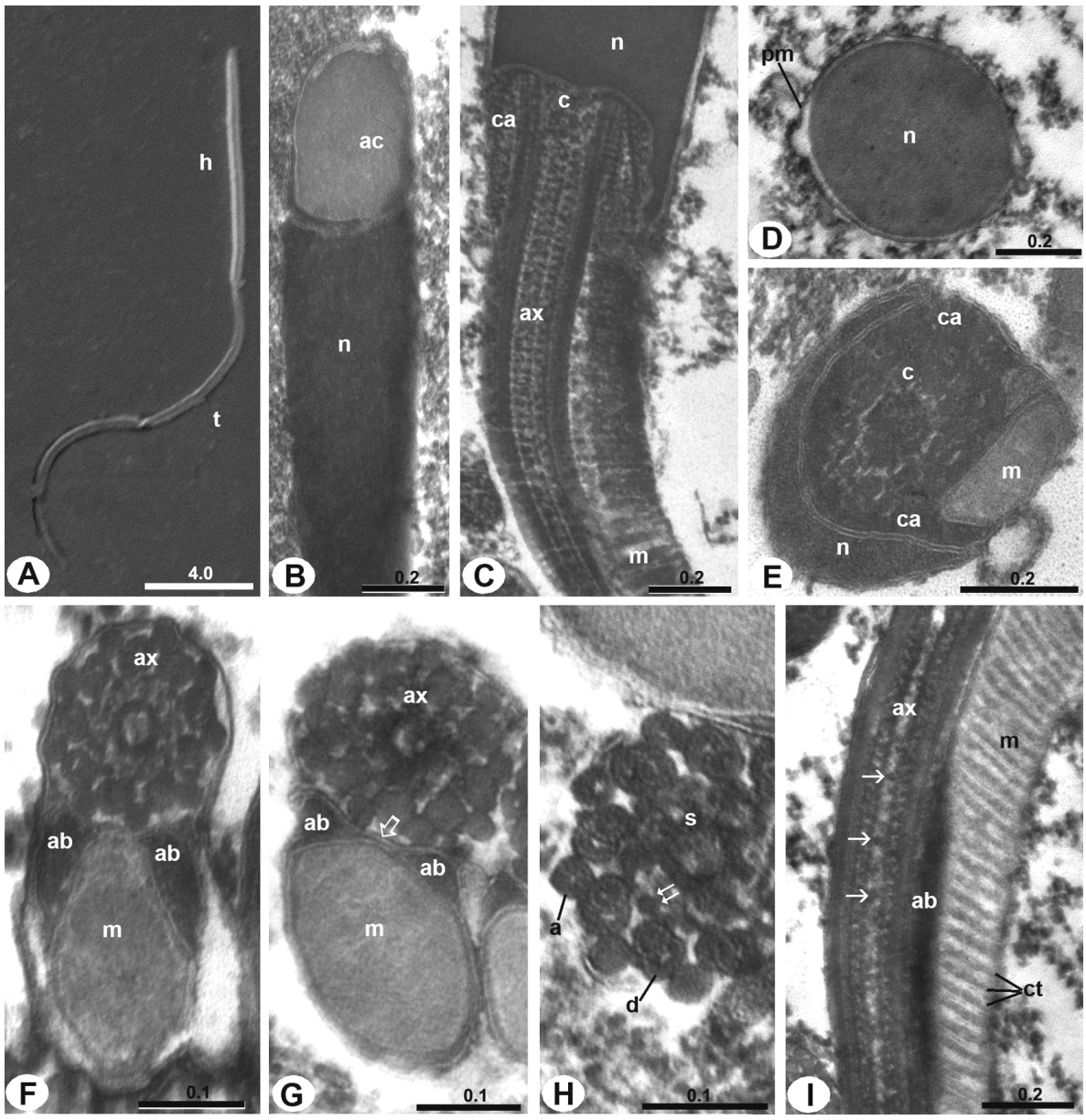
Figure 2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Capítulo 7

The unusual morphology of the spermatozoa of *Lachlania* sp. (Ephemeroptera: Oligoneuriidae)

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Abstract

Insect sperm morphology is being used as a source of useful data for phylogenetic studies. Besides the importance on studying species from Ephemeroptera because of its basal position among Pterygota, there are few studies about the sperm morphology in this group. This manuscript describes the sperm morphology of *Lachlania* sp., which has many differences in relation to other known Ephemeroptera. It is the first report in this group of a nucleus located beside the flagellum and of the acrosome in contact with the centriole. The sperm mitochondrion has cristae with variable organization patterns and the accessory body surrounds it. Possible alterations in the motility pattern of these spermatozoa make them a good model for understanding sperm motility in insects.

Keywords: Mayfly, ultrastructure, accessory body, mitochondria, nucleus.

Introduction

Species of the family Oligoneuriidae (Ephemeroptera) are distributed among three subfamilies: Colocruninae (extinct), Chromarcyinae and Oligoneurinae (including the genus *Lachlania*) (Domínguez et al., 2006). The genera *Lachlania* Hagen is composed by 14 species (Domínguez et al., 2006), of which only two species are registered from Brazil (Salles et al. 2011). The nymphs are commonly found in fast currents, firmly attached to snags and sticks wedged between rocks. Identification of *Lachlania* species often requires the access to the type specimens, since there is no available key to identify South American species (Domínguez et al., 2006).

Aquatic nymphs and short living adults characterize Ephemeroptera. They represent the oldest living winged insects and improving the knowledge of the phylogenetic relations between the Ephemeroptera families should receive special attention because of their basal position among other Pterygota (Kristensen, 1981). Thus, understanding mayfly phylogeny would help the study of all insect systematics in general. Studying sperm morphology of insects has proved to be a good source of characters for phylogenetic studies (Jamieson et al., 1999; Lino-Neto and Dolder 2001; Zama et al., 2005; Mancini et al., 2006). With the objective of improving the available data about sperm morphology of Ephemeroptera, the present study is the first to describe the sperm morphology of an Oligoneuriidae species (*Lachlania* sp.), which present characteristics that have not been previously recorded in other species of Ephemeroptera.

Material and Methods

Last instar nymphs of *Lachlania* sp. were collected from the marginal vegetation of the "Cambucá" waterfall in Dores do Rio Preto – ES, Brazil (20°37'30.37"S; 41°49'26.46"O) and imagoes were collected with a light trap in "Funil" river, Afonso Cláudio – ES, Brazil (20^{o}08'35.1"S; 41^{o}09'02."0).

Immunofluorescence for Tubulin

The seminal ducts of the imagoes were dissected in sodium phosphate buffer 0.1M, pH 7.2; they were broken open on clean microscope slides spreading the spermatozoa. The spermatozoa were fixed with a 2.5% glutaraldehyde solution in sodium phosphate buffer for a few minutes and then washed in running water.

The slides were washed for 10 minutes in a solution of 0.25% Triton X-100 and PBS. The slides were then washed three times for five minutes each in PBS. After this, the slides were incubated for 30 minutes in a solution of PBST + 1% BSA + 0.3M of glycine. The primary antibody used was a monoclonal anti-α-tubulin produced in mouse (Ref. T5168, Sigma-Aldrich). It was diluted 1:1000 in 1% BSA + PBST. The incubation was performed overnight in a humid chamber at 4°C.

The slides were washed three times for five minutes each in PBS and then incubated with the secondary antibody. The secondary antibody (Alexa Fluor® 555 goat anti-mouse IgG monoclonal; Ref. B35131) was used in a dilution of 1:500 in 1% BSA solution for one hour at room temperature in a dark camera. The slides were washed three times for five minutes with PBS in the dark. The slides were counterstained with DAPI to show the nucleus and analyzed with a fluorescent microscope, Leica TM 2500 (Wetzlar, Switzerland).

Transmission electron microscopy

The seminal ducts of the males were dissected in sodium phosphate buffer 0.1M, pH 7.2, fixed with 1% tannic acid and 2.5% glutaraldehyde in the same buffer for 5 days. The material was contrasted "en-bloc" with 1% aqueous uranyl acetate solution for 2 hours, dehydrated in an acetone series and embedded in Epon (Dallai and Afzelius 1990). Ultra-thin sections were contrasted with 3% uranyl acetate followed by 3% lead citrate and analyzed with a Zeiss-Leo 906 Transmission Electron Microscope.

Results

The spermatozoa of *Lachlania* sp. are long, slender and have a wavy shape when spread over glass slides (Fig. 1). With transmission electron microscopy, it is difficult to obtain longitudinal sections of spermatozoa because of this wavy arrangement. The most conspicuous characteristic of these spermatozoa is the location of the nucleus beside the flagellum components, instead of an anterior position. The nucleus is elongated but shorter than the axoneme, which begins first and finishes after the nucleus (Fig. 1).

The anterior part of the spermatozoon corresponds to the acrosome that consists of a rod-like acrosomal vesicle of medium electron density, coated by an electron dense

amorphous layer (Fig. 2A). The acrosomal length varies from approximately 0.4 to 0.7 μm. Just below the acrosome is the centriole where complete microtubule triplets can be found organized around an amorphous electron dense core (Fig. 2B). The centriole is also surrounded by a small amount of electron dense material that could represent a reduced centriolar adjunct (Fig. 2B). This material is not observed in longitudinal sections (Fig. 2A). Below the centriolar region, the axoneme follows the 9 + 9 + 0 microtubule pattern that is characterized by nine external accessory microtubules, nine doublets of microtubules and no central microtubules pair (Fig. 2I). The accessory microtubules are characteriscally made up of 13 protofilaments of tubulin surrounding 7 globular subunits of undetermined chemical composition. The microtubule doublets have only the innermost dynein arm. The central region of the axoneme is characterized by the presence of an electron dense central sheath, from which arise the radial spokes connecting the microtubules doublets (Fig. 2I). The lumen of the central sheath is usually clear, but can be partially filled with medium electron dense material (Fig. 2C-I). At the posterior tip of the spermatozoa, the axoneme is the last structure to disorganize, being observed alone in cross sections of this region (Fig. 2H). The accessory microtubules are the first structure to finish (Fig. 2G) and the central sheath is the last (Fig. 2H). Immunofluorescence for tubulin showed the last portion of the axoneme to taper gradually (Fig. 1B, E)

Only one elongated mitochondrion is observed along the spermatozoon. It is surrounded by the accessory body. The initial portion of the accessory body is observed already in the centriolar region, as a narrow structure (Fig. 2B). Below this region, the accessory body gradually enlarges (Fig. 2C) and surrounds the mitochondrion that has a diameter of 30 nm in its anterior portion (Fig. 2D). Here, the anterior portion of the nucleus is also observed. In some images a membrane can be observed surrounding the accessory body and mitochondrion (Fig. 2D-F). However, our study, using only one kind of fixative, did not allow us to determine whether or not this structure could be some kind of methodological artifact. The nucleus of the spermatozoon is always observed in one side of the spermatozoon, with the mitochondrion and the accessory body separating it from the axoneme (Fig. 2D, E). The nucleus tapers in its anterior and posterior tips (Fig. 1A, D). In the medial portion of the spermatozoon it presents a “half moon” shape when observed in cross sections and is filled with granular electron dense chromatin (Fig. 2E).

The nucleus is shorter than the mitochondrion and the axoneme components and this flagellar portion can be observed without the nucleus in cross sections of the posterior portion of the spermatozoon (Fig. 2F). In the medial portion of the spermatozoon, the diameter of the mitochondrion is about 150 nm and where the nucleus is also present, the accessory body has two main squared portions linked to one another by two narrow bridges (Fig. 2E). When the nucleus is no longer present, these bridges become widen and the accessory body completely surrounds the mitochondrion (Fig. 2F). Some circular structures are also observed inside the accessory body (Fig. 2D, E). At the posterior tip of the spermatozoon, the mitochondrion and the accessory body gradually taper and disappear before the axoneme (Fig. 2G).

When observed in longitudinal sections, the bridges of the accessory body between mitochondrion and nucleus have a striped pattern (Fig. 3A, B). Depending on the region, the mitochondrial cristae may be organized parallel or perpendicular to the longitudinal axis (Fig. 3A-C).

Discussion

Sperm morphology of Ephemeroptera species is characterized by the presence of some apomorphies: only one mitochondrion without internal paracrystalline material (with exception of *Campsurus* sp. that have many aligned mitochondrion in the flagellum; Chapter 5), axoneme with the 9+9+0 microtubule pattern and only the innermost dynein arm on the microtubule doublets (Baccetti et al., 1969; Phillips, 1969; Grimm, 1985; Fink and Yasui, 1988; Gaino and Mazzini, 1991a). Although most Ephemeroptera possess mobile spermatozoa, little data about their motility pattern is available in the literature (Phillips, 1983; Werner and Simmons, 2008). This data would be useful to understand how the lack of the outer dynein arms and of the central microtubule pair in the axoneme could interfere in spermatozoa movements. The lack of these structures in Ephemeroptera axonemes is interpreted as steps in a tendency toward flagellum reduction in Ephemeroptera (Dallai et al., 2006) that culminated with immotile aflagellate spermatozoa in Leptophlebiidae species (Gaino and Mazzini, 1991a, b; Brito et al., 2011).

The spermatozoa of *Lachlania* sp. also have these same characteristics, however, different from the other species, they cannot be divided into head and flagellum. The

unusual condition of the nucleus, laterally associated with the flagellum and the acrosome, in direct contact with the centriole, has never been recorded before in Ephemeroptera.

The nucleus parallel to the flagellum probably modifies the motility pattern of the sperm. Future studies comparing the motility of *Lachlania* sp. and other Ephemeroptera sperm could furnish interesting data and help to understand insect sperm mobility. The granular, loosely compacted chromatin filling the nucleus of *Lachlania* sp. is probably a co-evolution that allows the flagellar movements, since a nucleus with compact chromatin would be very rigid.

The bilayered acrosome is considered basal among insects (Baccetti, 1972; Jamieson et al., 1999). The spherical and monolayered acrosome observed in *Cloeon dipterum* (Baccetti et al., 1969), *Dolania americana* (Fink and Yasui, 1988), *Electrogena grandiae* and *Ecdyonurus venosus* (Gaino and Mazzini, 1991b) spermatozoa, have been assumed to be an apomorphy for the Ephemeroptera order (Jamieson et al., 1999). Some exceptions have already been observed in the order: *Habroleptoides umbralitis* and *Choroterps picteti* (Gaino and Mazzini 1991a, b) which presented a perforatorium surrounded by the acrosomic vesicle. However, both species are from the Leptophlebiidae family, in which spermatozoa are characterized as aflagellate and very autopomorphic. Some Leptophlebiidae species presented a monolayered acrosome: *Habrophlebia eldae* (Gaino and Mazzini 1991a), *Farrodes carioca*, *Massartela brieni* and *Miroculis mourei* (Brito et al. 2011). In the Leptophlebiidae family, the acrosome pattern variation is observed even within the same subfamily, and may reflect specific variation in the egg chorion (Gaino and Mazzini 1991b). A conical acrosomal vesicle coated by another layer was observed in *Callibaetis jocosus* and *Tupiara ibirapitanga* (personal observation). This is similar to that found in *Lachlania* sp., but in this case the acrosomal vesicle has a rod-like shape. These variations in the acrosome morphology indicate that this characteristic is more variable than was previously assumed in Ephemeroptera, suggesting that more species must be studied to confirm whether a monolayered acrosome must be considered as apomorphic for the order.

The presence of a centriolar adjunct was observed in many species of Ephemeroptera and it can vary in size and morphology depending on the species (Brito et al., submitted). It is assumed that the centriolar adjunct helps to maintain the structural

integrity of the spermatozoa, attaching the flagellum components to the nucleus base (Jamieson et al., 1999). As the centriole of *Lachlania* sp. sperm is localized in an apical position, just below the acrosome, it is expected that it must be under less mechanical stress than a typical Ephemeroptera spermatozoa. This may explain the small amount of electron dense material associated to the centriole, as observed in this study. However, there are other species of Ephemeroptera with a "typical" spermatozoa shape that also have a small centriolar adjunct (Brito et al., submitted). Probably the characteristic "small centriolar adjunct" is wide spread in Ephemeroptera with no direct relation with the position of the nucleus or centriole.

Mitochondria in Ephemeroptera spermatozoa seem to be a considerably variable characteristic between the species. The majority of the species have parallel mitochondrial cristae, perpendicular with the mitochondrial long axis (Baccetti et al., 1969; Fink and Yasui, 1988; Gaino and Mazzini, 1991a). Spermatozoa of *Campsurus* sp. have many short mitochondria align in the flagellum and spermatozoa of *Caenis* sp. and *Asthenopus curtus* have cristae with no regular organization pattern (personal observation). The spermatozoa of *Lachlania* sp. have a variable organization pattern of the cristae, with some regions of parallel cristae and some of not parallel.

Accessory body of Ephemeroptera sperm also varies its structural organization. It can be divided in two crystalline or non-crystalline portions, can have only a central and no crystalline portion, or it can surround the mitochondrion as an amorphous layer (Brito et al., submitted). The accessory body of *Lachlania* sp. sperm surrounds the mitochondrion but has some regions with crystalline organization (between the nucleus and the mitochondrion).

It is interesting to note that the accessory body surrounding the mitochondrion is frequently observed in species with non parallel mitochondrial cristae (e.g. *Campsurus* sp., *A. curtus*, *Caenis* sp. and *Lachlania* sp.). A hypothesis that can be formulated is that an accessory body surrounding the mitochondrion would reinforce the flagellar mechanical resistance, compensating that lost with cristae disorganization.

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Figure Legends

Fig. 1: Fluorescent staining of spermatozoa of *Lachlania* sp. **A, D)** nuclear staining with DAPI; **B, E)** immunofluorescence staining of microtubule components; **C, F)** merged figures. The bars measure 10 μm.

Fig. 2: Transmission electron microscopy of *Lachlania* sp. spermatozoa. **A)** longitudinal section of the anterior region of the spermatozoa, (ac) acrosomal vesicle, (e) acrosomic external layer, (c) centriole, (ax) axoneme; **B – H)** sequence of cross sections, from the centriolar region (B) to the posterior tip (H); **I)** higher magnification of the axoneme. (pm) plasma membrane, (t) microtubule triplets, (ab) accessory bodies, (m) mitochondrion, (am) membrane of the accessory bodies, (n) nucleus, (arrows) circular structures inside the accessory bodies, (n) nucleus, (*) electron dense material near the axoneme, (a) accessory microtubules, (d) doublet microtubules, (cs) central sheath, (double arrows) dynein arm.

Fig. 3: Longitudinal sections of different regions of the spermatozoa. Notice the different organization patterns of the mitochondrial cristae (open arrows), which depending on the region can vary from a parallel organization pattern (B, C), to an organization with no clearly repeated pattern (A, C). Notice also the striped organization of the accessory bodies between the mitochondrion and the nucleus (dashed arrows). (ax) axoneme, (m) mitochondrion, (n) nucleus, (ab) accessory bodies.

Figure 1

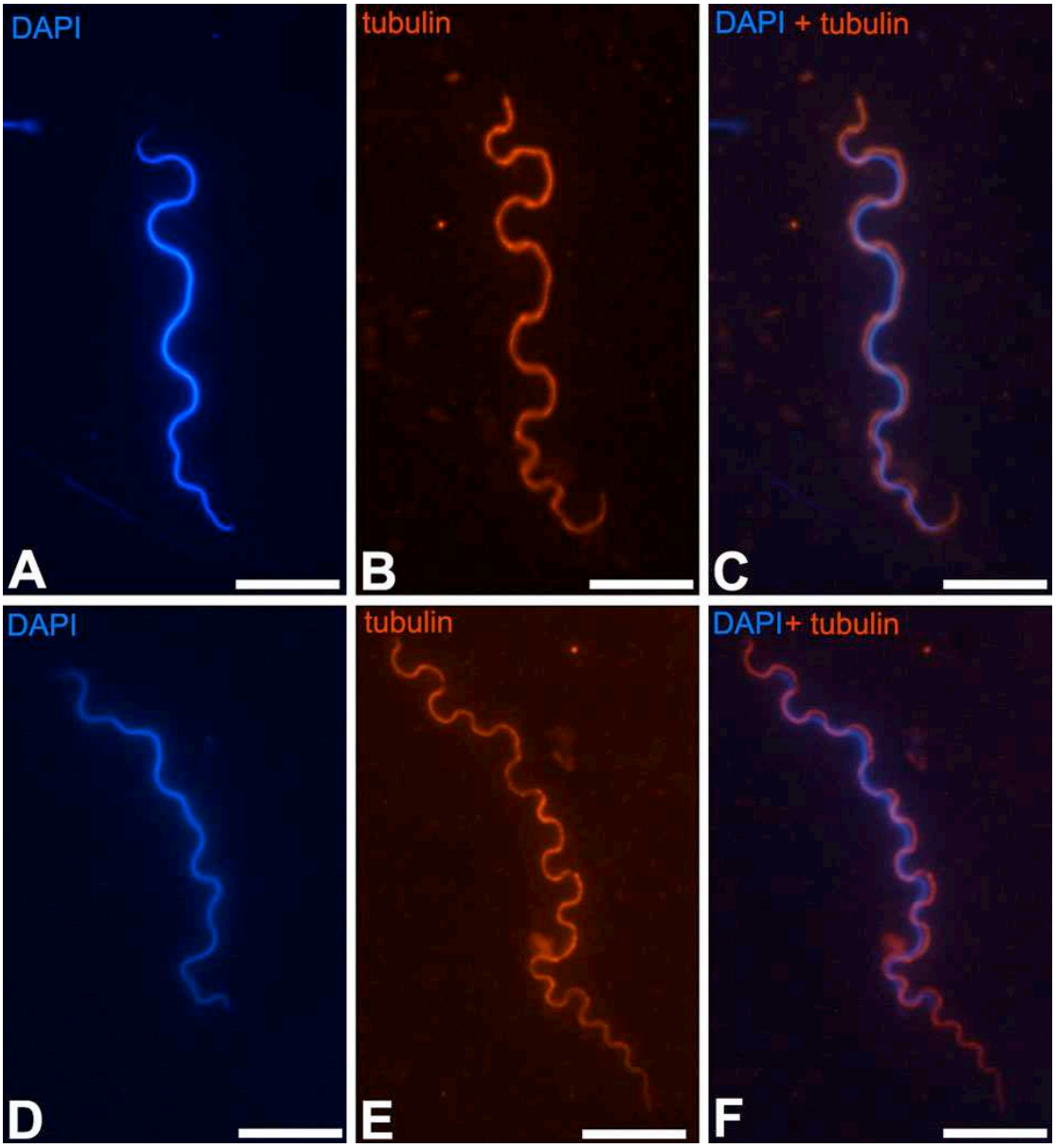
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Figure 2

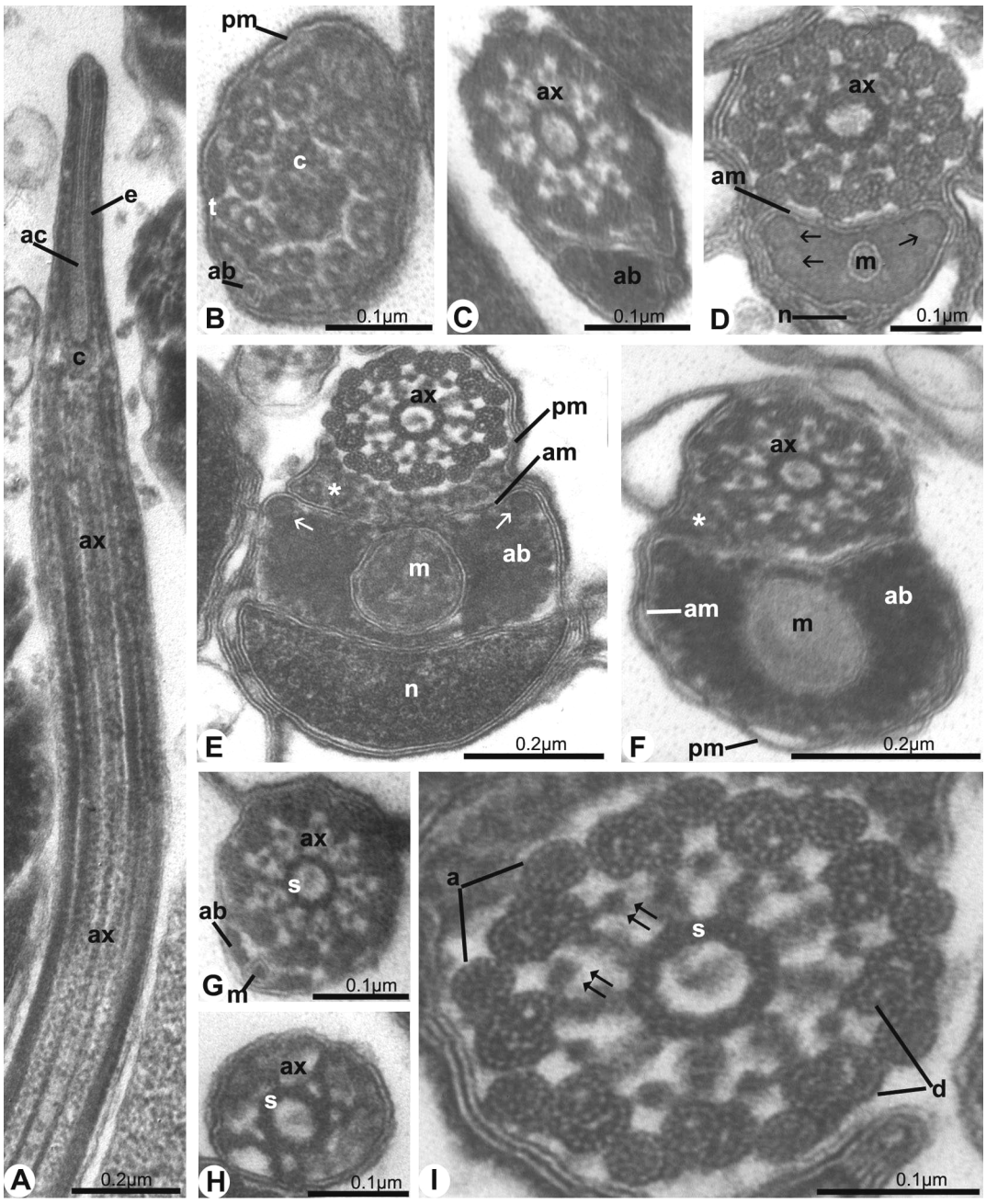
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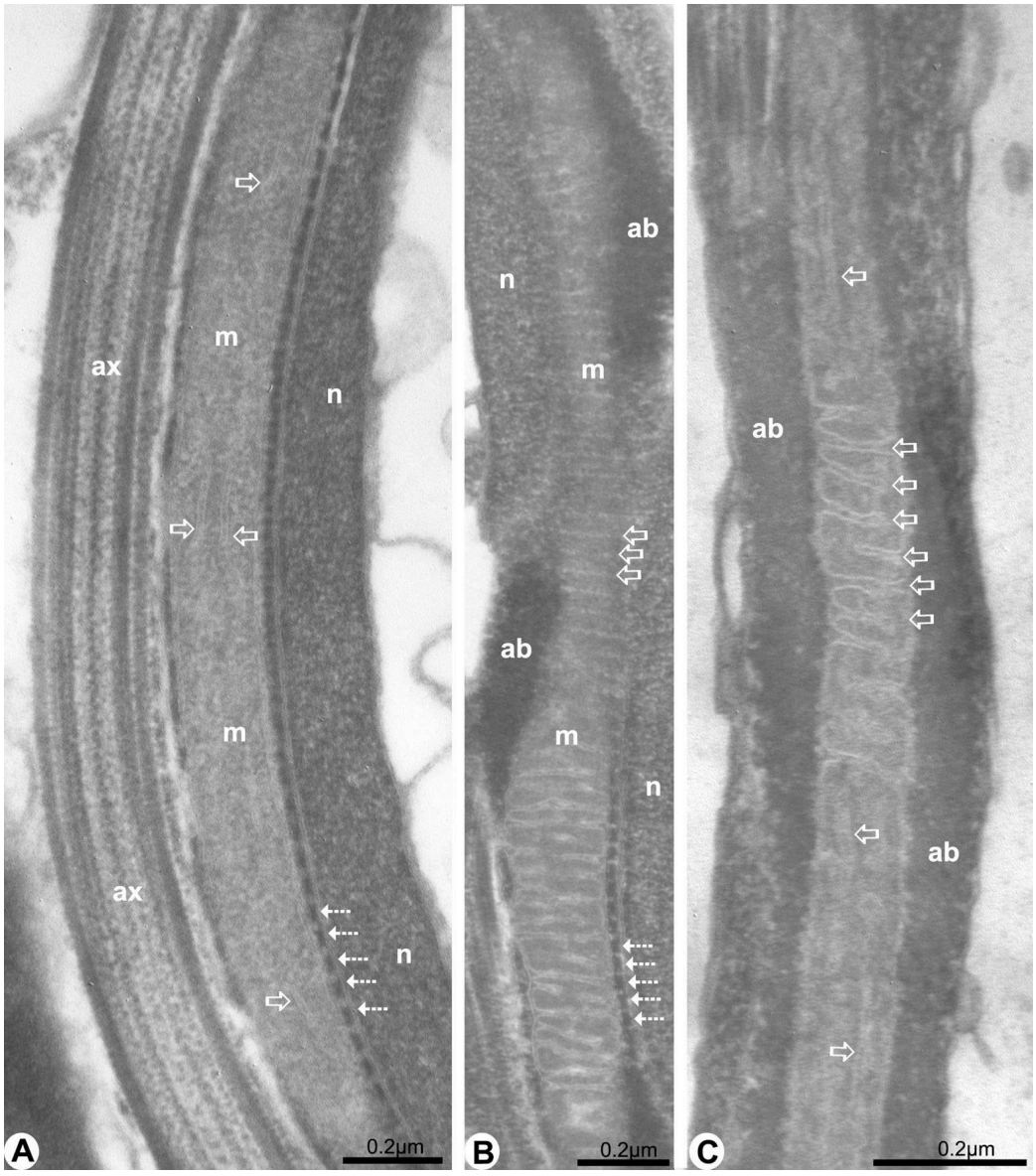
Figure 3



Capítulo 8

Morphological study of *Campylocia anceps* (Eaton, 1883) (Euthyplociidae) spermatozoa, and comparisons with other Ephemeroptera

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Abstract

Comparing spermatozoa morphology has proved to be useful for studies regarding insect phylogeny, however, there is little information about Ephemeroptera sperm morphology. This study provides the morphological description of *Campylocia anceps* spermatozoa. They can be divided in two regions: head and flagellum. The head is composed of a nucleus and a monolayered acrosome at the nucleus tip. The flagellum is composed of three structures: axoneme, mitochondrion and an accessory body. The axoneme is the longest structure and present the 9 + 9 + 0 microtubule pattern. The mitochondrion is parallel to the axoneme and between them there is an accessory body. This is composed of a single and rectangular body in cross sections, with no crystalline organization. An electron dense body is observed beside the centriole and probably corresponds to a modified centriolar adjunct. Comparing this data to that available for Ephemeroptera spermatozoa, the large number of variations found in shape and type of organelles reinforces the importance of these studies, that could furnish characters for taxonomic and phylogenetic studies.

Keywords: Electron microscopy, sperm, Morphology, Phylogeny.

1. Introduction

The order Ephemeroptera consists of more than 3.000 species, distributed in 42 families (Barber-James et al., 2008), representing the oldest existing winged insects. Their nymphs are aquatic and this stage represents the major part of their life span. Only the two last stages of their lives are winged (subimago and imago), when they leave the aquatic environment. The winged stages do not feed and can live for some hours up to a few days, in order to reproduce.

Since members from Ephemeroptera order are considered the oldest existing winged insects, understanding the phylogeny of this group and their relation with other insects is a challenge, and is essential toward understanding all Insecta phylogeny. Although spermatozoan morphology has proved useful for Insect phylogeny (Baccetti, 1972; Kristensen, 1981; Jamieson et al., 1999), the studies describing Ephemeroptera sperm morphology are scarce and some of them present only partial descriptions (Baccetti et al., 1969; Phillips, 1969; Grimm, 1985; Fink and Yasui, 1988; Dallai and Afzelius, 1990; Gaino and Mazzini, 1991a,b; Brito et al., 2011;)

In order to improve the available information on Ephemeroptera sperm morphology, this study describes the morphology of *Campylocia anceps* spermatozoa, a member of Euthyplociidae family that belongs to Furcatergalia suborder (Kluge, 2004; Ogden et al., 2009). This species is largely distributed throughout South and Central America and has the predominating characteristic of long mandibular tusks on the nymphs (Domínguez et al., 2006).

2. Material and Methods

Male nymphs of *Campylocia anceps* with dark wing pads were collected at Ducke's florest, Manaus – AM, Brazil (02º 55'46.7"S; 59º58'22.0"W). Their reproductive systems were dissected with sodium phosphate buffer pH 7.2 and fixed with a 1% tannic acid and 2.5% glutaraldehyde solution. The material was stained 'en-bloc' in 1% uranyl acetate, than dehydrated and embedded in Epon resin. Ultra-thin sections were contrasted with 3% uranyl acetate followed by 3% lead citrate solutions and photographed with a Zeiss Leo 906 transmission electron microscope.

3. Results

Spermatozoa of *Campylocia anceps* are divided in two portions: the flagellum and the head, composed of a nucleus and an acrosome. The nucleus is cylindrical with approximately 4 μm in length filled with compacted chromatin with some clear internal areas (Fig 1A). Cytoplasmic manchette microtubules are observed surrounding some nuclei, indicating that spermatogenesis was in an advanced stage, but not concluded in all spermatozoa (Fig 1B, C). However, some spermatozoa are observed with no manchette (Fig 1D). The acrosome is a round vesicle with approximately 0.5 μm in diameter, located at the nucleus tip and filled with medium electron dense granular material, with clear internal areas (Fig 1A).

The flagellum is composed of three long structures: an axoneme, an accessory body and a mitochondrion. Cross sections at the nucleus-flagellum transition showed the nucleus partially surrounding the initial portions of the tail components (Fig. 1E). The axoneme is the longest structure of the flagellum; it begins at the nucleus base (Fig 1A, B) and is the last structure to disorganize at the flagellum tip, being observed alone in cross sections of this region (Fig. 2C). The axoneme follows the 9 + 9 + 0 microtubule arrangement, with nine external accessory microtubules, nine doublets and without the central pair of microtubules (Fig 2A, B). Only the innermost dynein arm is observed linked to the microtubule doublets and a central sheath with a clear lumen is present at the axoneme center (Fig. 2A). It could not be observed whether the accessory microtubules present the 13 + 7 arrangement, with 13 tubulins subunits surrounding 7 inner subunits. The accessory microtubules are the shortest components of the axoneme, they disorganize before the doublets and the central sheath and are not observed in sections of the flagellum tip (Fig. 2C) The axoneme anterior region, considered the centriole region, is characterized by a protein-rich, electron dense agglomerate in the center of the structure (Fig. 1A, B, E). There is also an electron dense centriolar adjunct, separated from the centriole region by an electron lucid space (Fig. 1E). It is approximately square in longitudinal sections, measuring approximately 0.2 μm (Fig. 1B, E). The centriolar adjunct has a different granularity when compared with the accessory body and is separated from it by an electron lucid space (Fig. 1B).

The mitochondrion is a long organelle, parallel to the axoneme, which begins at the nucleus base (Fig. 1A) and finishes a little before the axoneme at the flagellum tip. In cross sections, the mitochondrion has an elliptical shape, measuring approximately 0.25 x 0.15 μm along its long and short axes respectively (Fig. 2A, B). The mitochondrial cristae are organized perpendicular to the mitochondrion long axis (Fig. 2D) and can be observed in cross sections (Fig. 2B). There is an accessory body between the axoneme and the mitochondrion; it is composed of electron dense granular material with no crystalline organization (Fig. 1A, 2A, B, D). The accessory body begins below the centriolar adjunct (Fig. 1B). In cross sections of the flagellum, the accessory body has an approximately rectangular shape with 0.15 x 0.30 μm along its small and large sides, respectively (Fig. 2B).

Spermatozoa at different maturation stages showed different concentrations of dense proteins in the accessory bodies. The lower concentration of dense proteins in the accessory body of the spermatozoon of Figure 2A and the large amount of cytoplasm still associated with this spermatozoon (not shown in the Figure) indicate that it is less mature than spermatozoon of Figure 2B. A structure similar to a membrane surrounding both accessory body and the mitochondrion can be observed (Fig 2A, B).

4. Discussion

Spermatozoa of *Campylocia anceps* share many characteristics also described for other Ephemeroptera species. The axoneme with the 9 + 9 + 0 microtubule pattern observed in this study is a common characteristic among all known Ephemeroptera species (Baccetti et al., 1969; Phillips, 1969; Grimm, 1985; Fink and Yasui, 1988; Dallai and Afzelius, 1990; Gaino and Mazzini, 1991a), with the exception of species from the Leptophlebiidae family that have aflagellate spermatozoa (Gaino and Mazzini, 1991b; Brito et al., 2011). Fink and Yasui (1988) described a 9 + 9 + 1 axoneme pattern for *Dolania americana*. However, the presence of a dark spot inside the central sheath is a variable characteristic, even in the same specimen (Gaino and Mazzini, 1991a) and this should not be considered a new axoneme pattern for Ephemeroptera. The axoneme doublet with only the innermost dynein arm, as described for *C. anceps* in this study, is also

considered an Ephemeroptera apomorphy (Dallai and Afzelius, 1990; Gaino and Mazzini, 1991; Jamieson et al., 1999).

Clear areas in the condensed chromatin as observed in *C. anceps* spermatozoa, have already been observed in *Cloeon dipterum* (Baccetti et al., 1969) and the spermatozoa of *Lachlania* sp. have granular and not compacted chromatin (personal observation). Similar clear areas, as observed in *C. anceps* acrosome, are also known for *D. americana* acrosomes (Fink and Yasui, 1988) and the spermatozoa acrosome of *Asthenopus curtus* is filled with a heterogeneous material (personal observation). Probably that these characteristics of *C. anceps* do not represent artifacts nor are they due to the slightly immature state of some spermatids observed in this study, but rather represent the degree of chromatin and acrosome condensation typical for this species.

A monolayered acrosome exclusively composed of an acrosome vesicle is a common characteristic among Ephemeroptera species. It was observed in *C. dipterum* (Baccetti et al., 1969), *D. americana* (Fink and Yasui, 1988), *Habrophlebia eldae* (Gaino and Mazzini, 1991a*), Electrogena grandiae, Ecdyonurus venosus* (Gaino and Mazzini, 1991a), *Coryphorus aquilus*, *Traverhyphes yuati, Campsurus sp.* and *Asthenopus curtus* (Chapters 5, 6) and *C. anceps* (this study). This characteristic is usually pointed as typical for Ephemeroptera spermatozoa (Jamieson et al., 1999). However, a different organization was observed in some Leptophlebiidae, with an acrosomal vesicle surrounding a perforatorium (Gaino and Mazzini, 1991a, b). This difference probably reflects some variation in the egg chorion structure (Gaino and Mazzini, 1991a; Brito et al., 2011). In some species of Baetidae the acrosomal vesicle is conical and surrounded by a layer of medium electron density (Chapter 4).

The centriolar adjunct morphology observed in *C. anceps* is different from those species of Ephemeroptera previously studied (Brito et al., submitted). The remainder species usually have a centriolar adjunct closely associated to the axoneme, in most of the cases surrounding its initial portion (Brito et al., submitted). It was also observed that the centriolar adjunct is in close association with the accessory bodies, making it difficult to individualize these structures (Brito et al., submitted). In *C. anceps* spermatozoa the centriolar adjunct is squared and separated from the centriole and the accessory body by an electron lucid space. However, the specimens analyzed in this study were not adults

and it is not clear whether the centriolar adjunct maturation is completed only at the end of spermatogenesis. Therefore, *C. anceps* probably posses a different type of centriolar adjunct, not previously observed in other Ephemeroptera.

5. Conclusion

This is the first study describing the sperm morphology of a Euthyplociidae family specimen. The results confirm the morphological variability among Ephemeroptera spermatozoa, suggesting that more studies should be done in order to provide consistent data for future phylogenetic analyses.

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Figure Legends

Fig 1: A,B) Longitudinal sections of spermatozoa. **A)** A complete head in longitudinal section, (ac) acrosome, (n) nucleus, (m) mitochondrion, (ab) accessory body, (c) centriole, (ax) axoneme (open arrows) clear areas inside the nucleus. **B)** Detail of the nucleus flagellum region, (arrows) "manchette" microtubules, (*) centriolar adjunct. **C)** Cross section of a nucleus with several manchette microtubules (arrows). **D)** Cross section of a nucleus with no manchette microtubules. **E)** Cross section of a nucleus-flagellum region of an immature spermatozoon, with several manchette microtubules (arrows) and a centriolar adjunct (*). (pm) plasma membrane.

Fig 2: A, B) Cross sections of the spermatozoa tail at different maturation stages, notice spermatozoon A with a less developed accessory body (ab). **C)** Cross section at the tail tip, with only some of the axoneme elements. **D)** Longitudinal section of the tail, note the parallel mitochondrial cristae. (m) mitochondrion, (am) accessory body's membrane, (a) accessory microtubules, (d) microtubules doublets, (s) central sheath, (pm) plasma membrane, (ax) axoneme, (arrows) mitochondrial cristae, (open arrow) inner dynein arms.

Figure 1

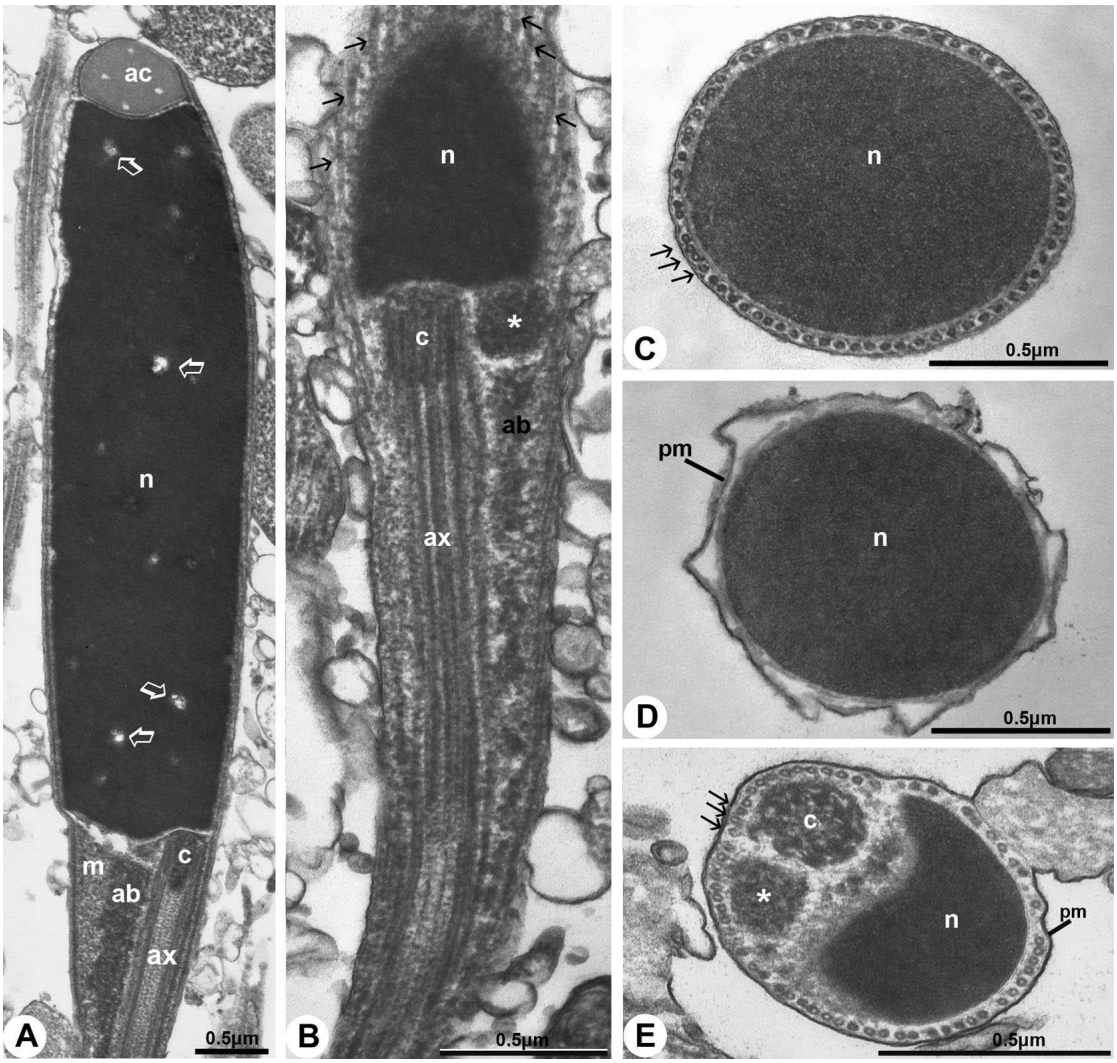
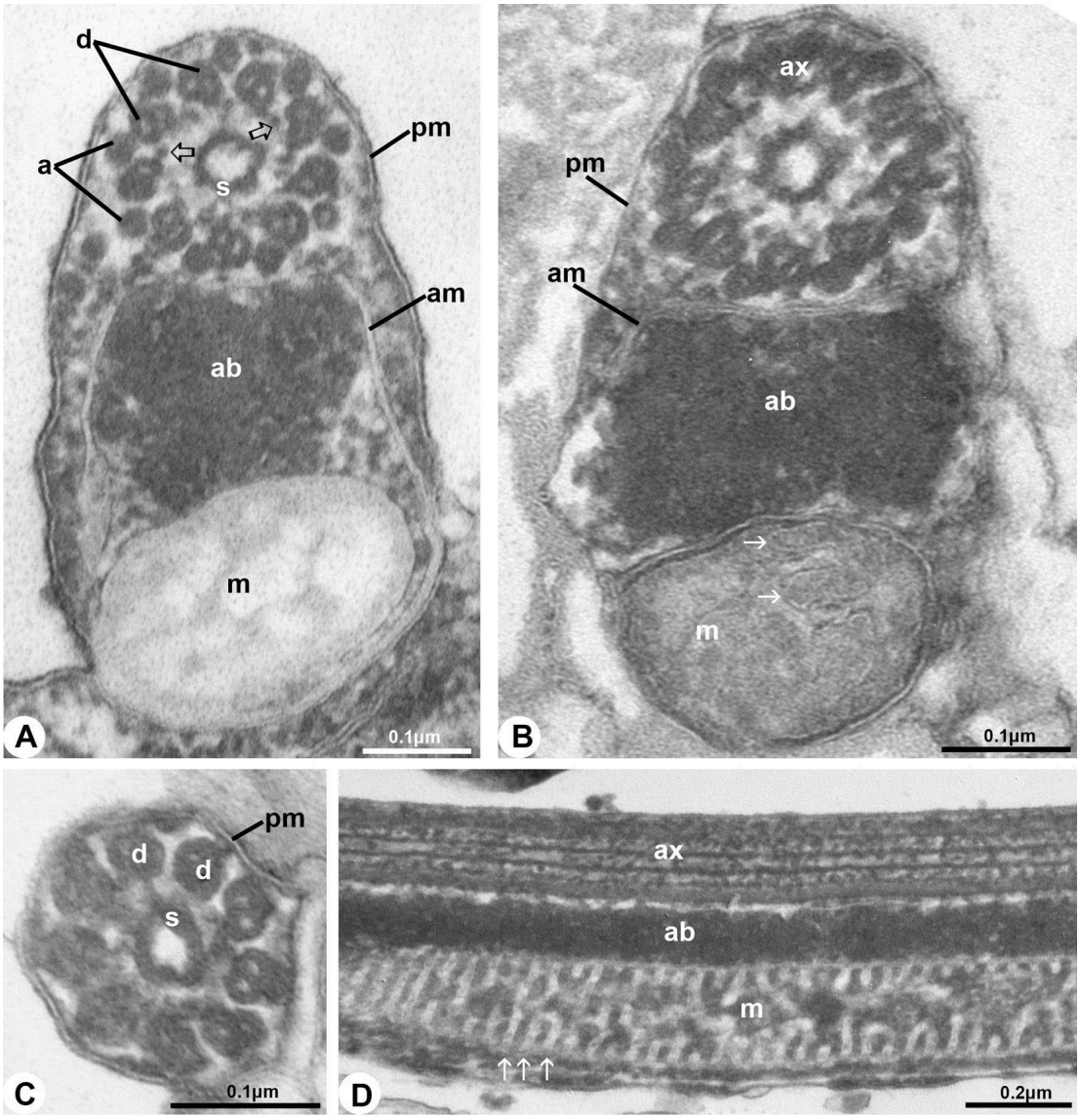


Figure 2



Capítulo 9

Estudo comparativo entre os espermatozoides de Caenidae (Ephemeroptera)

Resumo

O presente estudo fornece uma análise comparativa dos espermatozoides de três espécies de Ephemeroptera da família Caenidae: *Caenis fittkaui* Malzacher, *Caenis* sp. e *Brasilocaenis renata* Malzacher. Alguns autores ainda questionam a validade do gênero *Brasilocaenis*, argumentando que este deveria fazer parte de *Caenis*. Comparando oito estruturas dos espermatozoides das três espécies, observamos que os espermatozoides de *B. renata* e *C. fittkaui* são similares em quatro estruturas, já os espermatozoides de *C. fittkaui* e *Caenis* sp. são similares em três estruturas. Os espermatozoides de *B. renata* e *Caenis* sp. não são similares em nenhuma das oito estruturas comparadas neste trabalho. Assim, os espermatozoides de *B. renata* possuem mais características em comum com os espermatozoides de *C. fittkaui*, do que os espermatozoides de uma espécie do mesmo gênero, *Caenis* sp. Como a morfologia dos espermatozoides parece variar muito entre as espécies de Caenidae, a ampliação desse tipo de estudos pode fornecer dados que ajudem na determinação da validade dos gêneros propostos para a família.

Palavras chave: *Caenis*, *Brasilocaenis*, Microscopia Eletrônica

Introdução

A família Caenidae (Ephemeroptera) é cosmopolita, distribuindo-se por todos os continentes, exceto Nova Zelândia e algumas ilhas oceânicas. Atualmente, na América do Sul, existem cerca de 20 a 30 espécies de Caenidae descritas, além de muitas espécies que ainda não foram descritas e outras com classificação duvidosa. Estas espécies encontram-se distribuídas em quatro gêneros (Domínguez et al. 2006).

O gênero *Caenis* Stephens, possui 22 espécies descritas para a América do Sul, sendo divididas em dois grupos, *fittkaui* e *reissi* (Malzacher 1986, 1990).

Atualmente existem seis espécies descritas para o gênero *Brasilocaenis* Puthz*,* distribuídas no Brasil e na Colômbia (Domínguez et al., 2006). As espécies desse gênero podem ser divididas em dois grupos, *irmlei* (*B. irmlei* Puthz, *B. puthzi* Malzacher e *B. renata* Malzacher) e *septentrionalis* (*B. septentrionalis* Malzacher e *B. mendesi* Malzacher). Além disso, *B. intermediata* possui classificação duvidosa (Malzacher 1998). Ainda existem dúvidas a respeito da validade do gênero *Brasilocaenis,* sendo que este pode na verdade ser parte de *Caenis* (Malzacher 1998).

Estudamos a morfologia dos espermatozoides de três espécies de Caenidae (*Caenis fittkaui* Malzacher, *Caenis* sp. e *Brasilocaenis renata*) e encontramos três padrões bastante distintos, indicando a possibilidade de haver uma grande variabilidade nos padrões de espermatozoides da família. A comparação entre esses padrões pode fornecer informações úteis a respeito da relação entre *Caenis* e *Brasilocaenis*, bem como da relação entre os Caenidae com as demais famílias de Ephemeroptera.

Material e Métodos

- *Coleta de espécimes*

Imagos de *Brasilocaenis renata* foram coletados, atraídos por uma fonte luminosa no lago do Catalão, Manaus, AM (3° 9'13.16"S, 59°54'56.40"O). A revoada aconteceu entre 03:00 e 04:00h.

Imagos de *Caenis fittkaui* foram coletados, atraídos por uma fonte luminosa às margens de uma lagoa em Pedra Torta, Águia Branca, ES (19° 3'55.92"S, 40°42'45.40"O). A revoada aconteceu entre 03:00 e 04:00h.

Apenas uma ninfa de *Caenis* sp. foi coletada na vegetação marginal da mesma lagoa em Pedra Torta, Águia Branca, ES. Este indivíduo foi mantido vivo até emergir a subimago, às 03:00h. Não foi possível estabelecer a espécie desse indivíduo pois este ficou com o abdômen danificado durante a dissecção do sistema reprodutivo. No entanto, algumas características morfológicas (tamanho e coloração), além da morfologia dos espermatozoides indicam se tratar de outra espécie que não *C. fittkaui*.

- *Microscopia Eletrônica de Transmissão*

Os machos tiveram seus sistemas reprodutivos dissecados em tampão fosfato 0,1M, pH7,2. O material foi fixado em solução de glutaraldeído 2,5% e ácido tânico 1% e contrastado 'en-bloc' com solução aquosa de acetato de uranila 1%. O material foi desidratado em série de acetona e incluído em resina Epon. Cortes ultrafinos, contrastados com acetato de uranila 3% e citrato de chumbo 3% foram analisados em microscópio eletrônico de transmissão Zeiss Leo 906.

Resultados

Brasilocaenis renata

Os espermatozoides podem ser divididos entre cabeça e flagelo. A cabeça é volumosa e globular, sendo formada pelo núcleo e o acrossomo (Fig. 1A). O núcleo é aproximadamente elíptico quando observado em cortes longitudinais, medindo aproximadamente 1,4 x 1,1 μm em seus eixos maior e menor, respectivamente. O núcleo é preenchido por cromatina descompactada, com aspecto granuloso (Fig. 1A). O acrossomo é formado apenas pela vesícula acrossomal preenchida por material granuloso e se localiza na porção anterior do núcleo (Fig. 1A). O acrossomo estende-se por aproximadamente 0,4 μm à frente do núcleo (Fig. 1A).

O flagelo, observado na base do núcleo (extremidade oposta ao acrossomo), é formado por três estruturas alongadas: o axonema, uma mitocôndria e o corpo acessório (Fig. 1A, C). O axonema é a estrutura mais longa do flagelo, sua porção inicial, a região do centríolo, é caracterizada inicialmente pela presença de um aglomerado elétron denso no centro do axonema (Fig. 1A), seguida por uma região sem material algum no centro do axonema (Fig. 1A, B). O adjunto do centríolo de *B. renata* é formado por nove corpos alongados, elétron densos, que se estendem acompanhando os microtúbulos na porção

inicial do axonema (Fig. 1A, B). Mais abaixo, o adjunto do centríolo se desorganiza e o axonema assume a conformação 9 + 9 + 0, com nove microtúbulos acessórios mais externos, nove duplas de microtúbulos e sem o par central de microtúbulos (Fig. 1C, E). Os microtúbulos acessórios possuem o interior preenchido por material elétron denso, sugerindo o padrão 13 + 7, com 13 tubulinas envolvendo 7 subunidades de natureza desconhecida mais internas (Fig. 1E). As duplas de microtúbulos possuem apenas o braço mais interno de dineína e o centro do axonema é ocupado por uma bainha elétron densa com centro claro (Fig. 1E). Além disso, raios elétron densos (radial spokes) são observados ligando cada dupla de microtúbulos a essa bainha no centro do axonema (Fig. 1E). O axonema é a última estrutura a se desorganizar na extremidade do flagelo onde pode ser observado sozinho em cortes transversais. As duplas de microtúbulos são os últimos elementos do axonema a se desorganizarem na extremidade do flagelo (Fig. 1D).

A mitocôndria é paralela ao axonema e possui forma aproximadamente elíptica quando observada em cortes transversais, medindo aproximadamente 0,25 x 0,15 μm em seus eixos maior e menor respectivamente (Fig. 1C). A mitocôndria possui cristas paralelas que se organizam perpendiculares ao maior eixo do flagelo (Fig. 1A). O corpo acessório é localizado entre a mitocôndria e o axonema e, em cortes transversais, encontra-se dividido em dois lobos aproximadamente triangulares (Fig. 1C).

Caenis fittkaui

Os espermatozoides dividem-se em duas regiões, cabeça e flagelo. A cabeça é formada por um núcleo cilíndrico homogeneamente preenchido por cromatina densa, ligeiramente granulosa e por um acrossomo localizado no ápice do núcleo (Fig. 2A). O acrossomo é constituído apenas por uma vesícula acrossomal, homogeneamente preenchida por material de elétron densidade mediana e se estende por aproximadamente 1,2 μm além do núcleo. O núcleo e o acrossomo encontram-se separados por uma faixa elétron lúcida (Fig. 2A).

Os flagelos dos espermatozoides de *C. fittkaui* são constituídos por três componentes alongados: o axonema, a mitocôndria e o corpo acessório (Fig. 2F). A porção inicial do axonema, a região do centríolo, é caracterizada pelo acúmulo de material elétron denso na sua região central (Fig. 2B). Além disso, a porção inicial do axonema

encontra-se inserida em uma cavidade na base do núcleo (Fig. 2B, E). Essa porção inicial também é caracterizada pela presença do adjunto do centríolo, organizado na forma de nove estruturas alongadas que se estendem próximas aos microtúbulos acessórios do axonema. No entanto, essas estruturas não se sobrepõem e podem ser individualizadas (Fig. 2B, E). O axonema segue o padrão de organização 9 + 9 + 0, com nove microtúbulos acessórios mais externos, nove duplas de microtúbulos e sem o par central de microtúbulos (Fig. 2D). Os microtúbulos acessórios possuem o centro preenchido por material elétron denso, composto por subunidades morfologicamente semelhantes a tubulina, sugerindo o padrão 13 + 7, com 13 tubulinas envolvendo 7 subunidades de natureza desconhecida mais internas (Fig. 2D). Apenas o braço mais interno de dineína é observado ligando as duplas de microtúbulos. A porção central dos axonemas é ocupada por uma bainha elétron densa com centro claro; desta bainha partem raios, "radial spokes", ligando as duplas de microtúbulos (Fig. 2D). O axonema é a estrutura mais longa do flagelo, podendo ser observado sozinho em cortes transversais da extremidade final do flagelo (Fig. 2G). Nessa região, os microtúbulos acessórios não são mais observados, indicando que estes são os primeiros a se desorganizarem na extremidade do flagelo (Fig. 2G).

A mitocôndria inicia-se abaixo do adjunto do centríolo e se estende paralelo ao axonema. Suas cristas organizam-se paralelamente umas às outras, de forma perpendicular ao maior eixo do flagelo (Fig. 2C). A mitocôndria é aproximadamente circular em cortes transversais, com 0,25 μm de diâmetro (Fig. 2F). O corpo acessório é observado entre a mitocôndria e o axonema (Fig. 2C, F). Em cortes transversais observamos que o corpo acessório encontra-se dividido em dois corpos aproximadamente triangulares. Uma estrutura semelhante a uma membrana parece envolver o corpo acessório e a mitocôndria (Fig. 2F).

Caenis sp.

Os espermatozoides de *Caenis* sp. podem ser divididos em duas regiões, cabeça e flagelo. A cabeça é formada pelo núcleo e pelo acrossomo, o acrossomo é formado por uma vesícula acrossomal aproximadamente cilíndrica, homogeneamente preenchida por material de elétron densidade mediana que se estende por até 2 μm além do núcleo (Fig.

3A-C). O acrossomo é aproximadamente elíptico em cortes transversais, afinando ligeiramente da base para o ápice (Fig. 3C, D). A base do acrossomo é caracterizada por uma invaginação da vesícula acrossomal de aproximadamente 0,5 μm, o espaço formado por essa invaginação encontra-se irregularmente preenchido por material de elétron densidade mediana (Fig. 3B, D). O núcleo dos espermatozoides possui formato cilíndrico com aproximadamente 0,55 μm de diâmetro (Fig. 3E).

O flagelo dos espermatozoides inicia-se na base do núcleo, que não possui nenhuma cavidade ou projeção (Fig. 3F-H). O flagelo dos espermatozoides é constituído por três estruturas: o axonema, a mitocôndria e o corpo acessório. O axonema é a estrutura mais longa do flagelo, sua porção inicial, a região do centríolo, começa abaixo da base do núcleo (Fig. 3F-H) e sua extremidade pode ser observada sozinha em cortes transversais do final do flagelo (Fig. 3L). Nessa região, os microtúbulos acessórios não são mais observados, sendo as primeiras estruturas dos axonemas a se desorganizarem (Fig. 3K, L).

A região do centríolo é caracterizada pela presença de material elétron denso amorfo no interior do axonema (Fig. 3F, G). O adjunto do centríolo é uma estrutura elétron densa associada à região do centríolo, que se organiza como uma fina camada entre a região do centríolo e a base do núcleo e que possui uma porção mais desenvolvida lateralmente ao axonema, acima do derivado mitocondrial (Fig. 3F-I). A porção mais desenvolvida do adjunto do centríolo se estende entre 0,2 e 0,3 μm paralelo ao axonema abaixo da base do núcleo (Fig. 3G, H). O axonema segue o padrão 9 + 9 + 0 com nove microtúbulos acessórios mais externos, nove duplas de microtúbulos e sem a presença do par central de microtúbulos (Fig. 3J). Os microtúbulos acessórios são constituídos apenas por 13 protofilamentos de tubulina e possuem seu interior vazio, sem material elétron denso. Apenas o braço mais interno de dineína é observado associado às duplas de microtúbulos. A região mais interna do axonema é ocupada por uma bainha elétron densa, com centro elétron lúcido, essa estrutura se liga a cada uma das duplas de microtubulos através de um material elétron denso organizado na forma de raios, “radial spokes” (Fig. 3J).

A mitocôndria dos espermatozoides inicia-se abaixo da região mais desenvolvida do adjunto do centríolo e se estende paralelo ao axonema (Fig. 3G, H). A membrana interna da mitocôndria organiza-se formando cristas não paralelas em direção à matriz

mitocondrial (Fig. 3G, J). A matriz mitocondrial encontra-se preenchida por material elétron denso amorfo, enquanto o espaço intermembranas é elétron lúcido (Fig. 3J). A mitocôndria possui formato elíptico quando observada em cortes transversais, com aproximadamente 0,3 x 0,1 μm em seu maior e menor eixo (Fig. 3J).

O corpo acessório desses espermatozoides organiza-se como uma camada variando entre 0,08 e 0,04 μm de espessura que envolve a mitocôndria em todo seu comprimento (Fig. 3G, H, J). É difícil distinguir a região de início do corpo acessório e o final do adjunto do centríolo (Fig. 3G), no entanto, em alguns espermatozoides com a estrutura parcialmente danificada, estes encontram-se separados (Fig. 3H). O corpo acessório dos espermatozoides de *Caenis* sp. parecem ser envolvidos externamente por duas membranas que, algumas vezes, podem se encontrar espaçadas, com material elétron denso acumulado entre elas (Fig. 3J). Nas porções finais do flagelo, a mitocôndria, assim como o corpo acessório diminuem de tamanho (Fig. 3K) até desaparecerem completamente antes do axonema se desorganizar totalmente (Fig. 3L).

Discussão

Observamos entre as espécies de Caenidae aqui estudadas, maior variabilidade morfológica dos espermatozoides do que dentro das demais famílias de Ephemeroptera estudadas até o momento. Descrevemos a morfologia dos espermatozoides de três espécies de Caenidae e encontramos três padrões estruturais distintos. Mesmo com as diferenças observadas entre as espécies, é possível comparar as estruturas dos espermatozoides separadamente. Comparando oito estruturas dos espermatozoides das três espécies, observamos que os espermatozoides de *B. renata* e *C. fittkaui* são similares em quatro estruturas, já os espermatozoides de *C. fittkaui* e *Caenis* sp. são similares em três estruturas (Tabela 1). Os espermatozoides de *B. renata* e *Caenis* sp. não são similares em nenhuma das oito estruturas comparadas neste trabalho (Tabela 1).

A análise dessas características indica que apesar da morfologia pouco comum dos núcleos dos espermatozoides de *B. renata*, esses espermatozoides possuem mais características em comum com os espermatozoides de *C. fittkaui*, do que com os espermatozoides de uma espécie do mesmo gênero, *Caenis* sp. A ampliação desse tipo de estudo para outras espécies de *Caenis* e *Brasilocaenis*, assim como para espécies dos

demais gêneros dentro da família, indicam existir grande potencial para ajudar a esclarecer as relações filogenéticas dentro de Caenidae, assim como para confirmar ou não o status de *Brasilocaenis* como gênero.

Tabela 1 Comparação entre as estruturas dos espermatozoides das três espécies de Caenidae estudadas. As caixas sombreadas indicam semelhança entre as estruturas de diferentes espécies.

	Acrossomo	**Núcleo**	**Cromatina**	**Adj. Centríolo**	**Região Centríolo**	**Microtub. acessórios**	**Mitocôndria**	**Corpo acessório**
Brasilocaenis renata	< 1μm	Elíptico	Descompactada	9 estrut. alongadas	s/ mat. no centro do axo.	13 + 7	Cristas paralelas	Entre mit. e axo.
Caenis fittkaui	> 1μm	Cilíndrico	Granulosa	9 estrut. alongadas	Mat. denso no centro do axo.	13 + 7	Cristas paralelas	Entre mit. e axo.
Caenis sp.	> 1μm	Cilíndrico	Compactada	1 estrut.	Mat. denso no centro do axo	13 + 0	Cristas não paralelas	Envolvendo mit.

O acrossomo dos espermatozoides dos Ephemeroptera normalmente é constituído por uma vesícula acrossomal esférica, cujo diâmetro é normalmente menor que 0,5μm (Baccetti et al., 1969; Fink & Yasui, 1988; Gaino & Mazzini, 1991; observação pessoal). Acrossomos longos, com comprimento acima de 1 μm ainda não haviam sido observados nos espermatozoides de Ephemeroptera. A maior quantidade de enzimas armazenadas nesses acrossomos provavelmente deve refletir modificações na estrutura do córion dos ovos dessas espécies.

Algumas espécies de Ephemeroptera apresentam a cromatina compactada, mas com espaços elétron lúcidos no interior do núcleo dos espermatozoides (Baccetti, et al., 1969), outras apresentam cromatina granular e não compactada (ex. *Lachlania* sp.)

(Capítulo 7). No entanto, uma espécie com o núcleo elíptico/arredondado como em *B. renata* ainda não havia sido descrito.

O corpo acessório dos espermatozoides de *Caenis* sp. envolvendo a mitocôndria já havia sido descrito e se assemelha com o corpo acessório dos espermatozoides das espécies de Polymitarcyidae (Brito et al., submetido). Os espermatozoides de *Lachlania* sp. também possuem o corpo acessório envolvendo a mitocôndria, mas nesse caso o corpo acessório concentra seu maior volume em duas porções laterais (Capítulo 7) diferente do observado em *Caenis* sp. e em Polymitarcyidae. No entanto, é interessante observar que esse tipo de modificação do corpo acessório parece estar sempre associada à presença de cristas não paralelas nas mitocôndrias. Provavelmente, o corpo acessório envolvendo a mitocôndria serve para compensar a perda de resistência mecânica ocasionada pelo padrão de organização das cristas mitocondriais.

Os microtúbulos acessórios dos espermatozoides dos Ephemeroptera normalmente possuem um padrão chamado 13+7 onde 13 protofilamentos de tubulina envolvem sete outras subunidades de natureza desconhecida (Dallai & Afzelius, 1990; Jamieson et al., 1999). O padrão 13 + 0 das tubulinas dos microtúbulos acessórios do axonema de *Caenis* sp. também foi observado nos espermatozoides de espécies de Polymitarcyidae (Capítulo 5).

Características como a organização do corpo acessório e o padrão dos microtúbulos acessórios coincidem entre *Caenis* sp. e as espécies de Polymitarcyidae. Caenidae e Polymitarcyidae estão agrupadas dentro de Furcatergalia (Ogden & Whiting, 2005; Ogden et al., 2009), assim como outras famílias de Ephemeroptera que não possuem essas características nos espermatozoides (ex: Ephemeridae, Leptohyphidae, Coryphoridae e Euthyplociidae) (Capítulos 6, 8 e 10). Assim, é mais provável que as características compartilhadas entre *Caenis* sp. e Polymitarcyidae sejam apenas uma convergência de características.

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Legendas

Fig 1 – Ultra-estrutura do espermatozoide de *B. renata*. A) Corte longitudinal da porção inicial do espermatozoide, observe no canto direito um corte longitudinal de uma mitocôndria de outro espermatozoide. **B-D)** Cortes transversais da região do centríolo, da região mediana do flagelo e da porção final do flagelo, respectivamente. **E)** Maior aumento do axonema de um espermatozoide. (ac) acrossomo, (n) núcleo, (c) região do centríolo, (ca) adjunto do centríolo, (ax) axonema, (m) mitocôndria, (setas) cristas mitocondriais, (d) duplas de microtúbulos, (ab) corpo acessório, (a) microtúbulos acessórios, (s) bainha central, (setas duplas) braços internos de dineína.

Fig 2 – Ultra-estrutura dos espermatozoides de *C. fittkaui*. A) Corte longitudinal da porção anterior do espermatozoide, (ac) acrossomo, (n) núcleo. **B)** Corte longitudinal da região de transição entre o núcleo e o flagelo, (c) centríolo, (ca) adjunto do centríolo, (ax) axonema. **C)** Corte longitudinal da mitocôndria (m), (ct) cristas mitocondriais, (ab) corpo acessório. **D)** Grande aumento de um axonema, (d) duplas de microtúbulos, (s) bainha central, (setas) microtúbulos acessórios, (setas duplas) braço interno de dineína. **E-G)** Cortes transversais da região do centríolo, da porção mediana e da porção final do flagelo, respectivamente. Observe que é possível diferenciar o microtúbulo acessório (seta) e o adjunto do centríolo (ca) na figura E, observe também uma estrutura semelhante a uma membrana (seta aberta) envolvendo o corpo acessório na figura F.

Fig 3 – Ultra-estrutura dos espermaozóides de *Caenis* sp. A, B) Cortes longitudinais da porção apical dos espermatozoides, (ac) acrossomo, (n) núcleo, (seta aberta) invaginação da vesícula acrossomal. **C,D)** Cortes transversais da porção mediana e basal do acrossomo respectivamente, (pm) membrana plasmática, (am) membrana do acrossomo. **E)** Corte transversal do núcleo (n). **F-H)** Cortes longitudinais da região de transição entre o núcleo e o flagelo, note que os elementos do flagelo estão paralelos em G e, na figura H, que o corpo acessório (ab) encontra-se deslocado e separado do adjunto do centríolo (ca), (c) centríolo, (ax) axonema, (m) mitocôndria. **I)** Corte num plano aproximado àquele indicado pela linha tracejada na figura H. **J)** Corte transversal da porção mediana do flagelo, (a)

microtúbulos acessórios, (d) duplas de microtúbulos, (s) bainha central, (setas duplas) braços internos de dineína, (setas) membranas do corpo acessório, (o) membrana externa da mitocôndria, (i) membrana interna da mitocôndria, (x) matriz mitocondrial. **K, L)** Cortes seqüenciais da porção final do flagelo.

Figura 1

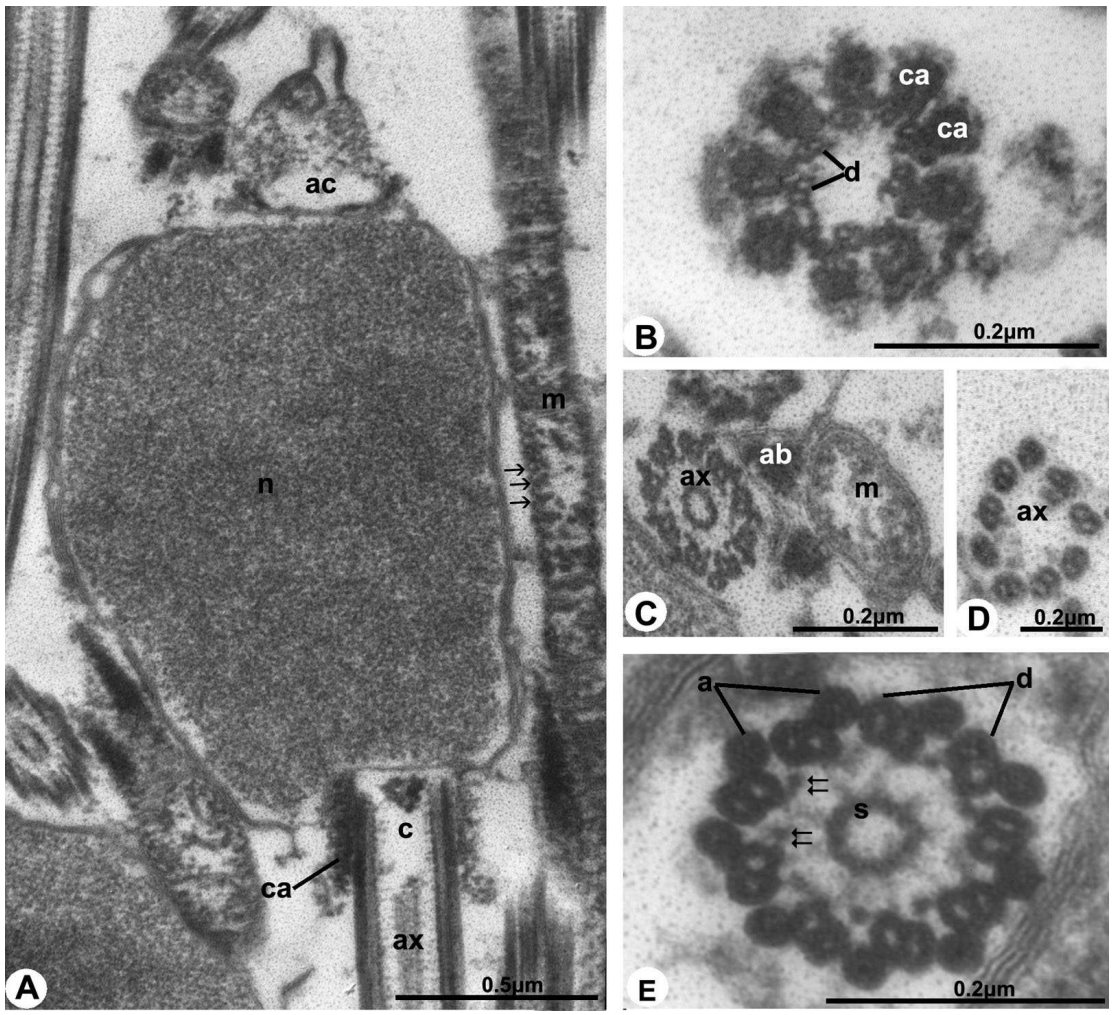
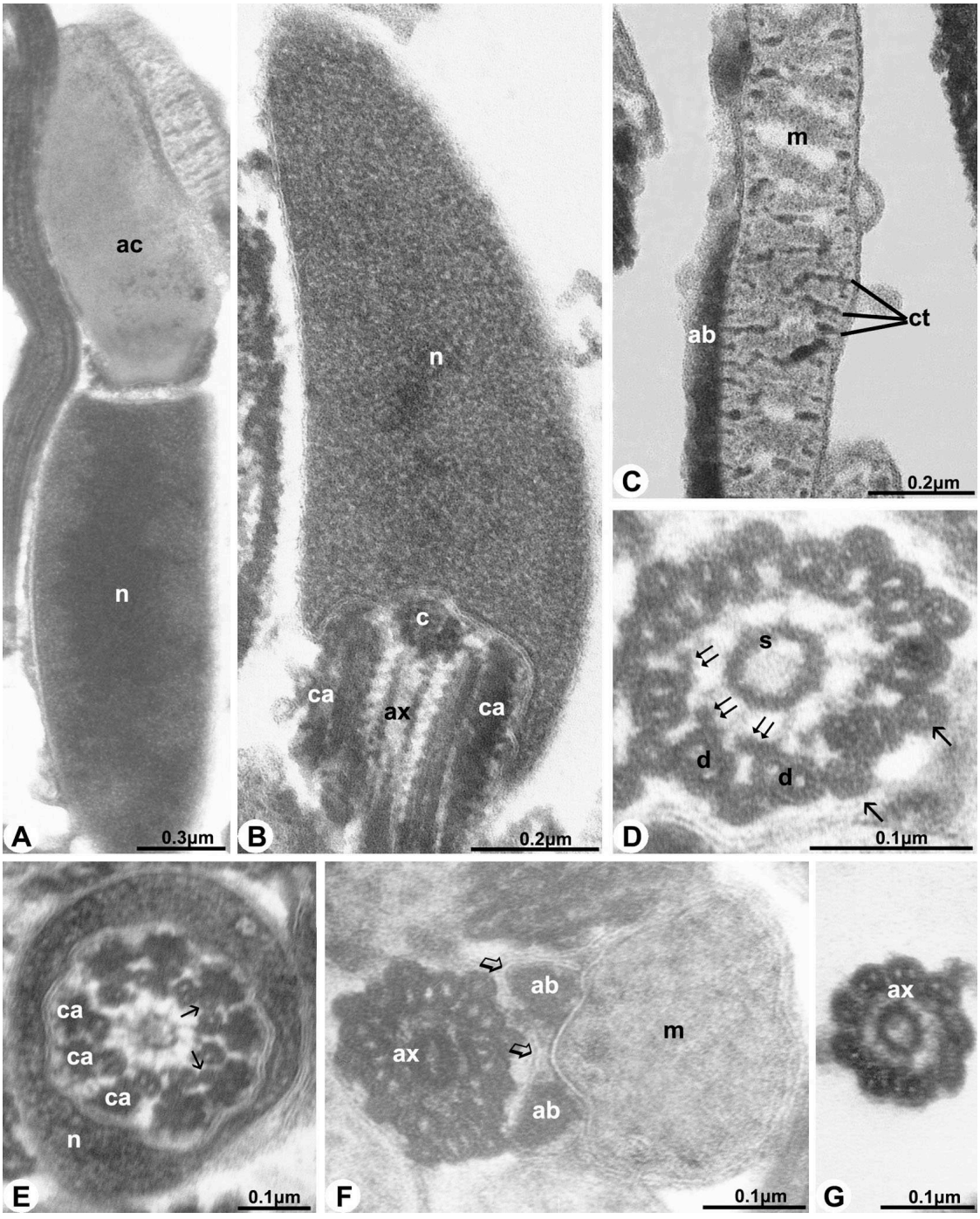


Figura 2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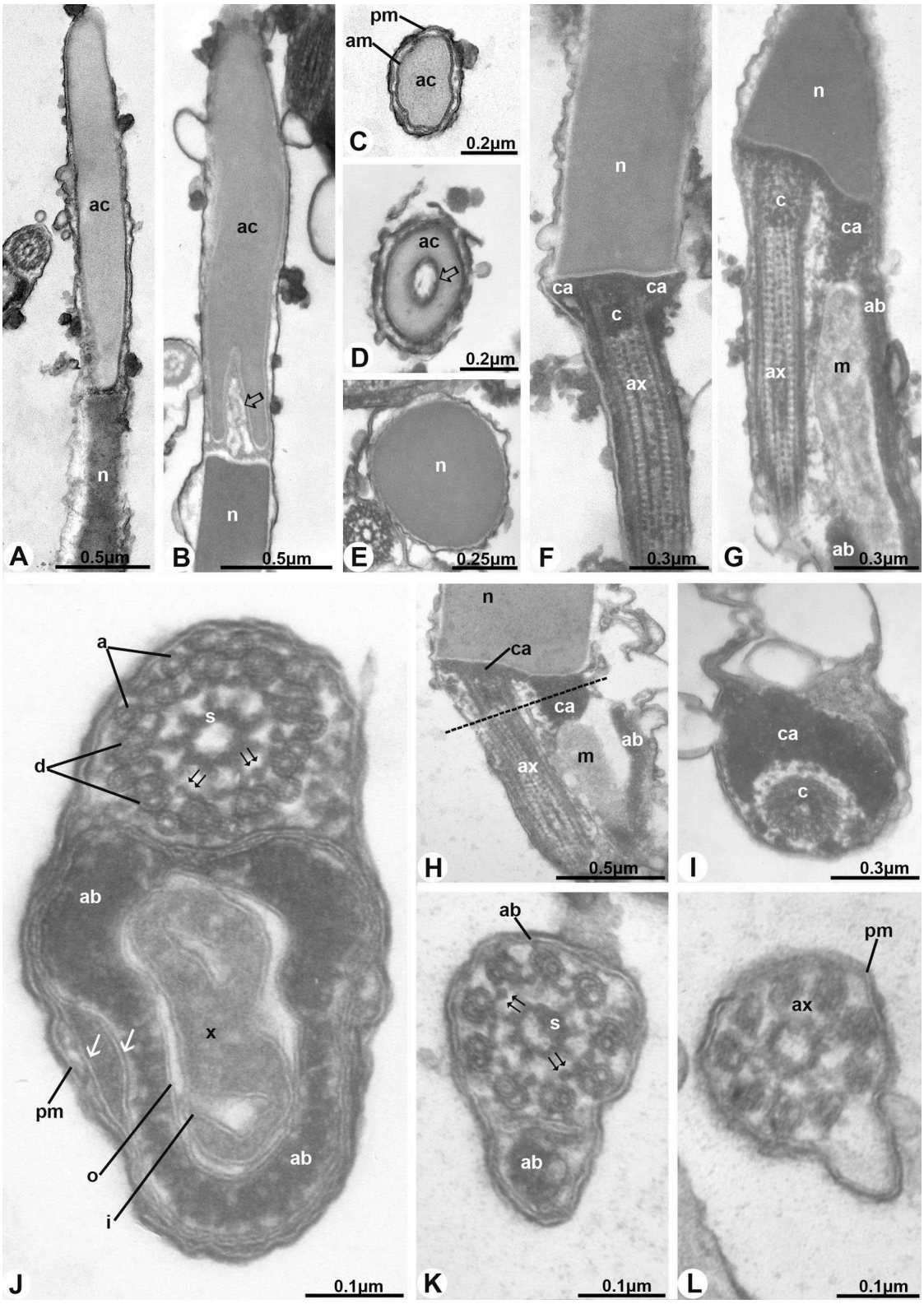
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Capítulo 10

Morfologia dos espermatozoides de *Hexagenia (Pseudeatonica) albivitta* Walker (Ephemeroptera: Ephemeridae)

Resumo

Os espermatozoides de *Hexagenia albivitta* são semelhantes aos observados em outras espécies de Ephemeroptera, mas possuem duas características que os diferem dos demais: adjunto do centríolo longo e mitocôndria com formas complexas no final do flagelo. O adjunto do centríolo de *H. albivitta* é o maior já observado em Ephemeroptera e possivelmente deve alterar o padrão de movimentos do flagelo dos espermatozoides dessa espécie. No final do flagelo a mitocôndria pode assumir a forma de "haltere" e nesses casos o corpo acessório encontra-se difuso ao redor da mitocôndria. Isso reforça a hipótese de que a função do corpo acessório seja fornecer resistência mecânica à mitocôndria.

Palavras chave: Corpo acessório, Microscopia Eletrônica, mitocôndria

Introdução

As ninfas das espécies da família Ephemeridae são caracterizadas por construírem túneis em forma de "U" na lama ou sedimento fino no fundo de lagos ou rios com pouca correnteza. Seis gêneros compõem a família, mas apenas o gênero *Hexagenia* Walsh, é encontrado na América do Sul (Domínguez et al., 2006). Alguns estudos vêm utilizando ninfas de espécies de *Hexagenia* como indicadores biológicos para o monitoramento da contaminação de corpos d'água (Bartsch et al., 1999).

O gênero *Hexagenia* pode ser dividido em dois subgêneros (*Hexagenia* e *Pseudeatonica*), sendo que o subgênero *Hexagenia* é encontrado do México até o Canadá e o subgênero *Pseudeatonica* encontrado do México até a Argentina (Domínguez et al. 2006). Apenas a espécie *Hexagenia (Pseudeatonica) albivitta* Walker é encontrada no Brasil (Salles et al., 2011).

Este estudo fornece a primeira descrição da ultraestrutura dos espermatozoides de uma espécie de Ephemeridae (*H. albivitta*), com o objetivo de ampliar o conhecimento disponível sobre os espermatozoides em Ephemeroptera.

Material e Métodos

Imagos de *Hexagenia albivitta* foram atraídos por uma fonte luminosa na margem da Lagoa Juparanã, Patrimônio da Lagoa, Sooretama, ES (19°10'9.70"S; 40°11'25.20"O).

Medida dos Espermatozoides

Indivíduos machos tiveram seus sistemas reprodutivos dissecados em tampão fosfato 0.1M, pH7.2. Alguns indivíduos tiveram os ductos seminíferos rompidos, os espermatozoides espalhados sobre lâminas histológicas e fixados em solução de glutaraldeído 2.5% em tampão fosfato. As lâminas foram examinadas em microscópio de contraste de fase Olympus BX41 e os espermatozoides tiveram sua medida total e da cabeça obtida com auxílio do programa "Image Pro Plus".

Microscopia Eletrônica de Transmissão

Indivíduos machos tiveram seus sistemas reprodutivos dissecados em tampão fosfato 0.1M, pH 7,2. Seus ductos seminíferos foram fixados em solução de ácido tânico 1% e glutaraldeído 2,5% em tampão fosfato. Posteriormente o material foi contrastado em bloco com solução aquosa de acetato de uranila 1%. O material foi então desidratado em

série de acetona e incluído em resina Epon. Cortes ultrafinos foram contrastados com acetato de uranila 3% e citrato de chumbo 3% e analisados em microscópio eletrônico de transmissão Zeiss Leo 906.

Resultados

Os espermatozoides de *Hexagenia albivitta* medem aproximadamente 30 μm dos quais aproximadamente 9,5 μm correspondem à região da cabeça. A região da cabeça é composta pelo núcleo e pelo acrossomo. O núcleo é homogeneamente preenchido por cromatina condensada (Fig. 1A-F) e possui formato cilíndrico. Em cortes transversais, o núcleo é elíptico com aproximadamente 0,6 x 0,4 μm em seus eixos maior e menor respectivamente (Fig. 1B). O acrossomo é constituído por uma vesícula acrossomal aproximadamente esférica com 0,3 μm de diâmetro homogeneamente preenchida por material elétron denso (Fig. 1A). A vesícula acrossomal localiza-se inserida sobre uma pequena depressão no ápice do núcleo, com uma faixa elétron lúcida separando o acrossomo do núcleo (Fig. 1A).

O flagelo dos espermatozoides é constituído por três estruturas alongadas: o axonema, uma mitocôndria e um corpo acessório. Além disso, observamos um longo adjunto do centríolo que se estende, paralelo ao flagelo, a partir da base do núcleo (Fig. 1C-E). A porção inicial do axonema, região do centríolo, encontra-se inserida numa cavidade de aproximadamente 0,4 μm de profundidade na base do núcleo (Fig. 1C-E, G). A região do centríolo é caracterizada pela presença de material elétron denso amorfo no centro (Fig. 1C, D, G). Essa região encontra-se intimamente envolvida pelo adjunto do centríolo, constituído de material elétron denso granulado (Fig. 1G). O adjunto do centríolo prolonga-se no flagelo por mais de 2 μm, envolvendo quase completamente o axonema (Fig 1H, I). Abaixo desse ponto, o adjunto do centríolo não é mais observado envolvendo o axonema (Fig. 2A-H).

O axonema dos espermatozoides segue o padrão 9 + 9 + 0, com nove microtúbulos acessórios mais externos, nove duplas de microtúbulos e sem o par central de microtúbulos (Fig. 2H). Os microtúbulos acessórios possuem o interior preenchido por sete subunidades, sugerindo o padrão 13 + 7, onde 13 protofilamentos de tubulina envolvem outras sete subunidades de natureza desconhecida. Apenas o braço interno de

dineína é observado unindo uma dupla de microtúbulo à outra (Fig. 2H). O centro do axonema é ocupado por uma bainha de material elétron denso com o centro claro (Fig. 2A-F, H). Cada dupla de microtúbulo encontra-se ligada a essa bainha por um raio de material elétron denso, "radial spokes" (Fig. 2A-F, H).

O axonema é a estrutura mais longa do flagelo dos espermatozoides, iniciando-se na cavidade basal do núcleo (Fig. 1C-E) e se estendendo até a extremidade do flagelo, onde pode ser observado sozinho em cortes transversais (Fig. 2F,G). Na porção final do flagelo, observamos que os microtúbulos acessórios são os primeiros a se desorganizar e já não são mais observados antes da mitocôndria ou do corpo acessório se desorganizarem (Fig. 2E). A bainha central dos axonemas desorganiza-se próximo do fim do flagelo, mas antes que as duplas de microtúbulos que são as ultimas estruturas a se desorganizarem no final do flagelo (Fig. 2G).

A mitocôndria inicia-se abaixo da cavidade basal do núcleo e de uma camada do adjunto do centríolo (Fig. 1C-F). Em cortes transversais da porção inicial do flagelo, a mitocôndria é circular com aproximadamente 0,2 μm de diâmetro (Fig. 1H, I). Na porção mediana do flagelo a mitocôndria assume um formato elíptico em cortes transversais, com aproximadamente 0,3 x 0,2 μm em seus eixos maior e menor respectivamente (Fig. 2A). Próximo à porção final do flagelo, a mitocôndria pode assumir formas complexas, com a matriz mitocondrial dividida em duas porções (Fig. 2B) ou com a mitocôndria, vista em corte transversal, em forma de "haltere" (Fig. 2C). Essas características nem sempre são observadas em todos os flagelos (Fig. 2D) e nas porções terminais do flagelo apenas uma mitocôndria simples é observada (Fig. 2E). A mitocôndria diminui de tamanho gradativamente na porção final do flagelo (Fig. 2D, E) até se desorganizar antes do fim do flagelo (Fig. 2F, G). Em cortes longitudinais do flagelo dos espermatozoides, observamos que as cristas mitocondriais organizam-se perpendicularmente ao maior eixo das mitocôndrias (Figs. 1C, F; 2 I).

Entre a mitocôndria e o axonema observamos um corpo acessório, que se inicia próximo à base do núcleo e estende ao longo do flagelo (Fig. 1E, F). O corpo acessório é formado por material elétron denso granuloso, circundado por uma membrana que envolve também a mitocôndria (Figs. 1I; 2A-D). Na porção inicial do flagelo, o corpo acessório corresponde a uma fina camada que envolve parcialmente a mitocôndria (Fig. 1H), e

aumenta de espessura gradativamente (Fig. 1I). Em cortes transversais das porções medianas do flagelo, o corpo acessório assume um formato aproximadamente retangular com seu lado maior variando de 0,3 a 0,4 μm e seu lado menor com aproximadamente 0,15 μm (Fig. 2A-C), conferindo ao flagelo uma forma alongada no eixo axonema-mitocôndria. Nas regiões onde a mitocôndria assume forma de "haltere", o corpo acessório encontra-se difuso entre as porções da mitocôndria (Fig. 2C). O corpo acessório diminui gradativamente de espessura nas porções finais do flagelo (Fig. 2D, E) até se desorganizar junto com a mitocôndria.

Discussão

Os espermatozoides de *H. albivitta* apresentam o plano básico semelhante ao dos demais Ephemeroptera, porém duas características diferenciam os espermatozoides dessa espécie dos demais: (1) adjunto do centríolo longo e (2) porção final da mitocôndria com formas complexas.

A maioria das espécies de Ephemeroptera possui o adjunto do centríolo pequeno, raramente ultrapassando 0,5μm de comprimento, sendo que em algumas espécies essa estrutura é reduzida ao ponto de dificultar sua visualização (Brito et al., submetido). O adjunto do centríolo de *H. albivitta* é, até o momento, o maior entre as espécies de Ephemeroptera estudadas.

Muito pouco se conhece sobre o padrão de movimentação dos espermatozoides dos insetos, que difere bastante daquele observado em mamíferos devido às várias diferenças morfológicas entre os espermatozoides dos dois grupos (Phillips, 1983; Wernner & Simmons, 2008). Devido às apomorfias observadas nos espermatozoides dos Ephemeroptera (ex: ausência do par central de microtúbulos no axonema, ausência do braço externo de dineína nas duplas de microtúbulos, ausência de material cristalino na matriz mitocondrial), os espermatozoides dessas espécies são considerados um modelo interessante para o estudo da mobilidade dos espermatozoides e sobre como a ausência dessas estruturas interfere no padrão do movimento (Wernner & Simmons, 2008). A presença de um adjunto do centríolo longo como observado em *H. albivitta* torna essa espécie um modelo interessante para futuros estudos sobre como essa estrutura, estendendo-se ao longo do flagelo, altera os movimentos espermáticos.

Mitocôndria com morfologia complexa, como a observada em *H. albivitta,* ainda não havia sido descrito em Ephemeroptera. Os espermatozoides de *Campsurus* sp. apresentam várias mitocôndrias distribuídas ao longo de todo o flagelo, sendo possível observá-las em cortes longitudinais e transversais do flagelo (Capítulo 5). Nesses espermatozoides, observamos também que as cristas mitocondriais não se organizam de forma paralela. Já nos espermatozoides de *H. albivitta* mitocôndrias individualizadas não são observadas em cortes longitudinais e mesmo nos cortes transversais, observamos uma ligação entre as duas porções da mitocôndria. Em *H. albivitta* a mitocôndria apresenta as cristas paralelas, mas é interessante observar que nas regiões onde as mitocôndrias assumem formato de "haltere", o corpo acessório encontra-se difuso entre as porções da mitocôndria. Essa característica poderia ser considerada como intermediária àquela onde o corpo acessório envolve completamente a mitocôndria que não possui cristas paralelas como observado em *Lachlania* sp., *Campsurus* sp., *Asthenopus curtus* e *Caenis* sp. (Capítulos 5, 7 e 9), reforçando a hipótese de que a função dos corpos acessórios seja de fornecer resistência mecânica à mitocôndria.

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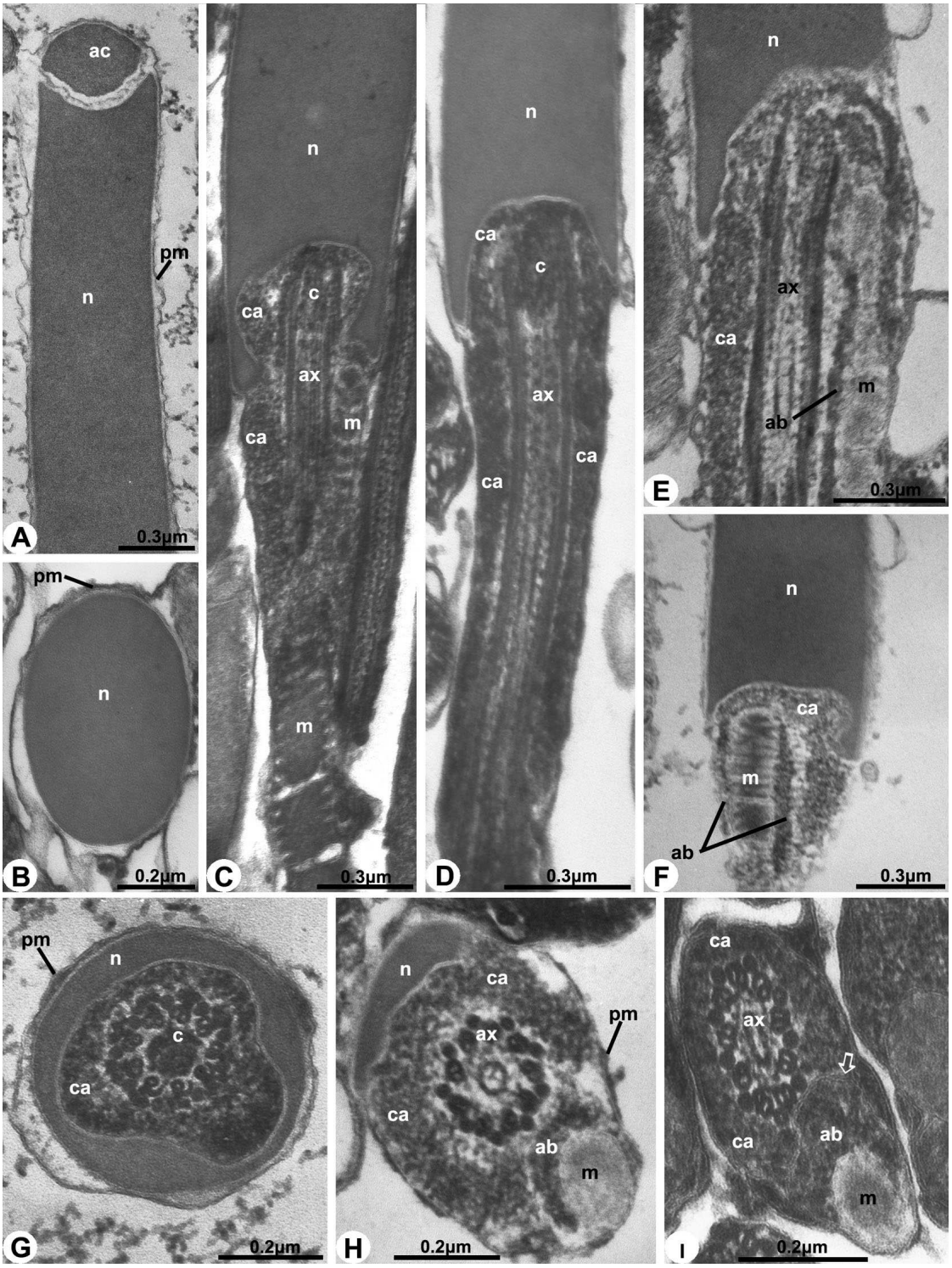
Legendas

Fig 1 – Ultra-estrutura dos espermatozoides de *H. albivitta*. A) Corte longitudinal da região anterior do espermatozoide, (ac) acrossomo, (n) núcleo, (pm) membrana plasmática. **B)** Corte transversal do núcleo. **C-F)** Cortes longitudinais da região de transição entre o núcleo e o flagelo. D) Observe o comprimento do adjunto do centríolo, paralelo ao axonema. F) Observe a diferença de densidade entre o adjunto do centríolo e o corpo acessório. (c) centríolo, (ca) adjunto do centríolo, (ax) axonema, (ab) corpo acessório. **G)** Corte transversal do centríolo envolvido pelo adjunto do centríolo, inserido na cavidade basal do núcleo. **H)** Porção inicial do flagelo; observe o início do corpo acessório entre a mitocôndria e o axonema. **I)** Corte do flagelo em região mais afastada da porção anterior, observe o corpo acessório maior e separado por uma estrutura semelhante a uma membrana (seta aberta).

Fig 2 – Ultra-estrutura dos espermatozoides de *H. albivitta*. A-C) Seqüência de cortes transversais do flagelo. Observe que na porção final do flagelo (C, D) a mitocôndria (m) possui morfologia complexa e o corpo acessório (ab) encontra-se difuso ao seu redor, (ax) axonema, (pm) membrana plasmática, (seta aberta) membrana envolvendo o corpo acessório. **D-G)** Seqüência de cortes transversais da porção final do flagelo mostrando a diminuição da mitocôndria e a desorganização do axonema. **H)** Grande aumento de um axonema, (a) microtúbulos acessórios, (d) duplas de microtúbulos, (s) bainha central, (setas duplas) braço interno de dineína. **I)** Corte longitudinal do flagelo.

Figura 1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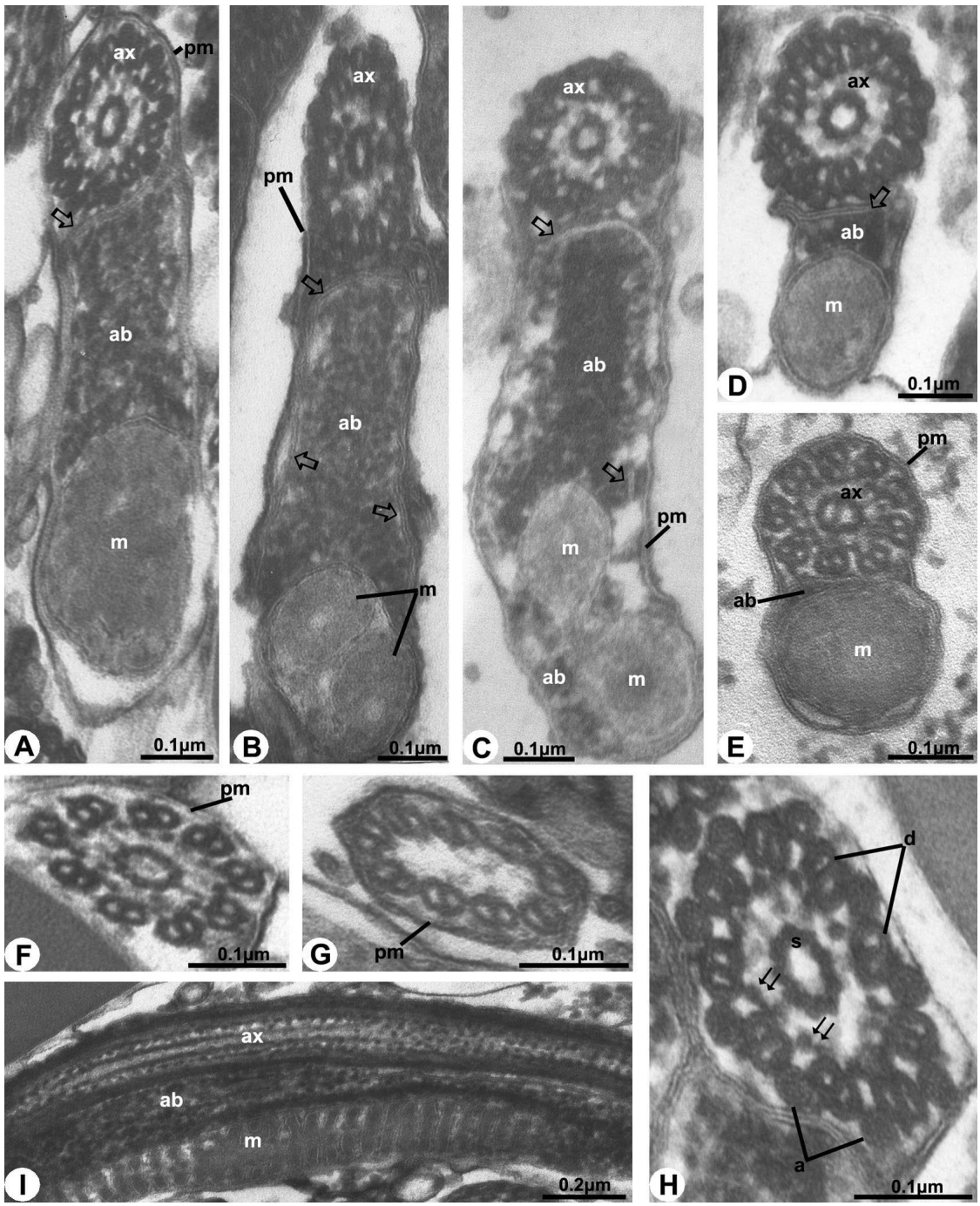

Tabela comparativa entre os espermatozoides flagelados estudados nesse trabalho

Com o objetivo de facilitar a comparação entre os espermatozoides das espécies analisadas nesse estudo, montamos uma tabela onde estão relacionadas suas principais características (Tabela 1). As espécies de Leptophlebiidae estudadas foram omitidas por possuírem espermatozoides aflagelados, ou seja, sem a maioria das características aqui comparadas.

Tabela 1 Em cada coluna as características iguais foram destacadas com a mesma cor. ? – Morfologia desconhecida. [1] – Acrossomo > 1μm. [2] – Aproximadamente 2 μm

Família	Espécie	Acrossomo	Ad. Centríolo	Corpos acessórios	Mitocôndria	Mic. Acessórios	Nucleo
Baetidae	*W. fasciatus*	?	1 estrutura	2 crist.	Cristas paralelas	?	?
	C. jocosus	Bicamada	1 estrutura	2 crist.	Cristas paralelas	13+7	Cromat. compactada
	T. ibirapitanga	Bicamada	1 estrutura	2 crist.	Cristas paralelas	13+7	Cromat. Compactada
Polymitarcyidae	*Campsurus* sp.	Monocamada	1 estrutura	1 envolvendo mit.	Várias mitocond.	13+0	Cromat. Compactada
	A. curtus	Monocamada hetero.	1 estrutura	1 envolvendo mit.	Cristas não paralelas	13+0	Cromat. Compactada
Leptohyphidae	*T. yuati*	Monocamada	1 estrutura	2 não Crist.	Cristas paralelas	13+7	Cromat. Compactada
Coryphoridae	*C. aquilus*	Monocamada	1 estrutura	2 não crist.	Cristas paralelas	13+7	Cromat. compactada
Oligoneuriidae	*Lachlania sp.*	Bicamada	1 estrutura	1 envolvendo mit.	Padrão de cristas variável	13+7	Cromat. granulosa
Euthyplociidae	*C. anceps*	Monocamada hetero.	1 estrutura	1 central	Cristas paralelas	13+7	Cromat. compactada
Caenidae	*B. renata*	Monocamada	9 est. Alongadas	2 não crist.	Cristas paralelas	13+7	Cromat. descompactada
	C. fittkaui	Longo[1]	9 est. Alongadas	2 não crist.	Cristas paralelas	13+7	Cromat. granulosa
	Caenis sp.	Longo[1]	1 estrutura	1 envolvendo mit.	Cristas não paralelas	13+0	Cromat. compactada
Ephemeridae	*H. albivitta*	Monocamada	1 estrutura longa[2]	1 central	Cristas paralelas	13+7	Cromat. compactada

Conclusões

1) Sistemas Reprodutivos Masculinos

A morfologia dos sistemas reprodutivos masculinos dos Ephemeroptera parece ser bastante conservada e simplificada nas diferentes famílias da ordem. Não são observadas glândulas acessórias ou estruturas especializadas para o armazenamento de espermatozoides. A ausência dessas estruturas em parte pode ser explicada pela curta vida dos adultos de Ephemeroptera, assim os espermatozoides não ficam estocados por muito tempo nos machos. No entanto, os fragmentos celulares observados em meio aos espermatozoides das espécies de Leptophlebiidae podem possuir alguma função fisiológica, seja na nutrição espermática ou na modulação de algum comportamento pós-cópula.

As constrições observadas externamente nos ductos seminíferos de *Asthenopus curtus* provavelmente correspondem a esfíncteres nos ductos. Estudos da histologia desses órgãos devem ser realizados para confirmar essa suposição, já que a presença de esfíncteres nos ductos seminíferos normalmente refletem modificações na fisiologia reprodutiva das espécies.

Todas as espécies analisadas possuíam fibras musculares adjacentes ao epitélio dos ductos. Algumas espécies possuem a musculatura mais desenvolvida que em outras e a forma como as fibras musculares estão organizadas também é variável em diferentes espécies. Em *Lachlania* sp., as fibras musculares longitudinais dos ductos ejaculatórios são os responsáveis pela eversão desses ductos durante a cópula.

Nosso estudo com a morfologia dos sistemas reprodutivos masculinos dos Ephemeroptera encontrou pouca diversidade morfológica nessas estruturas. Talvez aumentando a diversidade de espécies analisadas, sejam encontrados padrões de organização diferentes. Existem indícios de que o estudo da morfologia dos sistemas reprodutivos pode ajudar na compreensão de aspectos da fisiologia reprodutiva de algumas espécies.

2.1) Espermatozoides aflagelados

As espécies da família Leptophlebiidae analisadas apresentaram espermatozoides aflagelados e imóveis, assim como o esperado para as espécies desse grupo. No entanto, mesmo espermatozoides com morfologia simplificada como esses apresentaram variação na composição do acrossomo e na posição das mitocôndrias. Algumas espécies apresentam um perforatorium envolvido pela vesícula acrossomal, enquanto outras espécies possuem apenas a vesícula acrossomal. Essa variação parece não estar ligada com as relações filogenéticas entre as espécies, já que dentro de uma mesma subfamília encontramos espécies com, e outras sem, o perforatorium. O número e a localização das mitocôndrias nos espermatozoides é constante dentro de uma espécie mas bastante variável entre diferentes espécies. As variações dos espermatozoides dentro de Leptophlebiidae não parecem capazes de fornecer informações sobre a relação entre as subfamílias. A morfologia dos espermatozoides indica que este é um grupo monofilético e derivado dentro da ordem Ephemeroptera.

2.2) Espermatozoides flagelados

2.2.1) Adjunto do centríolo

Aparentemente, o restante das famílias de Ephemeroptera é formada por espécies com espermatozoides flagelados. Acreditava-se que não havia adjunto do centríolo associado ao início do flagelo nos espermatozoides dos Ephemeroptera, no entanto mostramos que tal estrutura é presente na maioria das espécies estudadas. A exceção é *Lachlania* sp. onde não conseguimos determinar se o material associado ao centríolo dos espermatozoides corresponde realmente a um adjunto. No entanto, *Lachlania* sp. possui um espermatozoide altamente modificado, onde talvez a função do adjunto do centríolo em fornecer resistência mecânica não seja necessária. O adjunto do centríolo observado em *Hexagenia albivitta* foi o maior dentre as espécies estudadas. Outras espécies dentro da mesma família (Ephemeridae) precisam ser estudadas para confirmar se essa é uma característica típica do grupo.

A morfologia do adjunto do centríolo varia entre as espécies e pode ajudar fornecendo informações sobre a relação entre as espécies. Por exemplo, nas espécies de

Caenidae analisadas a morfologia do adjunto do centríolo parece fornecer um bom indicativo sobre a relação entre gêneros diferentes.

2.2.2) Acrossomo

Excetuando algumas espécies de Leptophlebiidae que possuem perforatorium, o acrossomo da maioria das espécies é formado apenas por uma vesícula acrossomal aproximadamente esférica, com diâmetro de aproximadamente 0,5 μm. No entanto, dois padrões diferentes foram observados: 1) duas espécies de Caenidae possuem acrossomo alongado, medindo mais de 1 μm; 2) as duas espécies de Baetidae estudadas e a de Oligoneuriidae (*Lachlania* sp.) possuem a vesícula acrossomal em forma de cone ou de bastão e envolvida por uma camada externa.

Estudos recentes sobre a filogenia dos Ephemeroptera têm apontado a família Baetidae como a mais basal da ordem, considerada grupo-irmão das demais famílias. Interessante observar que o acrossomo bicamada, como observado em Baetidae, é considerada uma plesiomorfia para os insetos (Jamieson et al. 1999; Jamieson 2011). Assim, o acrossomo bicamada observado em Oligoneuriidae provavelvente representa uma plesiomorfia mantida nesses dois grupos de Ephemeroptera.

2.2.3) Corpos acessórios

Nas espécies analisadas, os corpos acessórios certamente são as estruturas que apresentaram maior variabilidade e também as que levantaram mais questões que merecem ser abordadas em estudos futuros. Estudos da espermatogênese dos Ephemeroptera possivelmente esclarecerão a origem dos corpos acessórios e também as diferenças no processo que levam algumas espécies a possuírem dois corpos acessórios e outras apenas um. Estudos também são necessários para esclarecer se a presença de uma membrana envolvendo os corpos acessórios, juntamente com a mitocôndria é um artefato devido à fixação com ácido tânico, ou realmente uma estrutura dos espermatozoides. Caso seja confirmada a presença dessa membrana, cabe uma reavaliação do termo "corpos acessórios", bem como sua homologia com os corpos acessórios dos espermatozoides dos demais insetos. Foi levantada a hipótese dos corpos acessórios dos Ephemeroptera serem homólogos às inclusões paracristalinas das mitocôndrias da maioria dos insetos. No entanto, ainda existe carência de estudos bioquímicos que corroborem essa hipótese.

Como demonstrado nesse estudo, a maioria dos grupos de Ephemeroptera parecem possuir corpos acessórios constituídos por material amorfo, não cristalino. Estudos que confirmem se os corpos acessórios dos Ephemeroptera são homólogos a alguma estrutura presente nos espermatozoides dos demais insetos, podem responder o questionamento que avalia se os espermatozoides nessa ordem de insetos são apenas uma derivação do plano básico encontrado nos demais Pterygota, ou se os espermatozoides dos Ephemeroptera seguiram uma linhagem evolutiva diferente. Essa informação pode ajudar a esclarecer a relação entre Ephemeroptera, Odonata e Neoptera.

Parece haver uma co-evolução entre a morfologia dos corpos acessórios e das mitocôndrias nos espermatozoides dos Ephemeroptera. Nas quatro espécies deste estudo em que a mitocôndria dos espermatozoides apresentava padrão irregular de organização das cristas, o corpo acessório organizava-se de maneira singular, como uma camada contínua envolvendo a mitocôndria. Nos espermatozoides de *Hexagenia albivitta*, na porção do flagelo onde a mitocôndria apresenta morfologia complexa (em haltere), o corpo acessório encontra-se difuso envolvendo parcialmente a mitocôndria.

No momento, poucas inferências filogenéticas podem ser feitas na ordem Ephemeroptera com base na morfologia dos corpos acessórios. No entanto, a diversidade estrutural observada nesse estudo indica que estudos da morfologia dos corpos acessórios podem fornecer dados úteis para estudos sobre a filogenia dessa ordem de insetos.

2.2.4) Núcleo

Os espermatozoides da maioria das espécies de Ephemeroptera possui o núcleo com cromatina uniformemente compactada. No entanto, algumas espécies de Ephemeroptera apresentam a cromatina compactada espassada por regiões elétron lúcidas, ex: *C. dipterum* (Baccetti et al. 1969). A cromatina do núcleo dos espermatozoides de *Lachlania* sp. apresentam cromatina granular e não compactada, provavelmente para permitir o movimento dos espermatozoides, já que o núcleo desses espermatozoides é paralelo ao flagelo. Dentre as espécies estudadas nesse trabalho, *B. renata* é a única que não possui o núcleo alongado, sendo elíptico/arredondado e com a cromatina descompactada, demonstrando a variabilidade de características encontradas nas espécies de Caenidae.

2.2.5) Microtúbulos acessórios

A organização dos microtubulos acessórios observada nas espécies de Polymitarcyidae e em *Caenis* sp. difere do padrão 13+7 da maioria dos Ephemeroptera estudados, sendo este padrão considerado uma característica da ordem. Possivelmente trata-se de uma característica convergente, já que famílias mais próximas de Polymitarcyidae (Ephemeridae e Euthyplociidae) não possuem essa característica. No entanto, os espermatozoides de *Caenis* sp. e de Polymitarcyidae possuem outra característica em comum, o corpo acessório envolvendo a mitocôndria. Essas características podem sugerir uma proximidade evolutiva entre os dois grupos, mas no momento, qualquer conclusão a esse respeito seria precipitada e mais espécies precisam ser estudadas, verificando a freqüência dessas características entre as espécies do grupo.

A família Caenidae foi a que apresentou maior diversidade morfológica dentre os grupos estudados. Analisando as três espécies incluídas nesse trabalho, a morfologia dos espermatozoides de Caenidae demonstrou potencial de fornecer dados úteis inclusive para o estudo das relações entre os gêneros.