ALINE BEATRIZ REIS SANTOS

MORFOLOGIA ESPERMÁTICA DE DUAS ESPÉCIES DE ANTHOCORIDAE (HETEROPTERA: CIMICOMORPHA) COM CONSIDERAÇÕES FILOGENÉTICAS E TAXONÔMICAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Magister Scientiae*.

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SUMÁRIO

RESUMO

SANTOS, Aline Beatriz Reis, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Morfologia espermática de duas espécies de Anthocoridae (Heteroptera: Cimicomorpha) com considerações filogenéticas e taxonômicas.** Orientador: José Lino Neto

A ordem Hemiptera é dividida nas subordens: Sternorryncha, Coleorryncha, Heteroptera e Auchenorryncha. Heteroptera constitui um dos maiores grupos de insetos formado por 75 famílias, entre elas Anthocoridae. A família Anthocoridae possui cerca de 600 espécies em todo o mundo, sendo que *Amphiareus constrictus* e *Blaptostethus pallescens* são espécies predadoras que podem ser usadas no controle biológico de pragas agrícolas. O entendimento das relações filogenéticas, bem como da biologia reprodutiva dessas espécies podem contribuir para o manejo e uso desses insetos no controle de pragas. Considerando que desde 1950 os espermatozoides têm sido utilizados como uma importante ferramenta em várias áreas da biologia, incluindo a taxonomia e filogenia. Nós apresentamos neste trabalho informações sobre a estrutrura e ultraestrutura dos espermatozoides de *A. constrictus e B. pallescens*, usando técnicas de microscopia de luz e eletrônica de transmissão. Os espermatozóides de *A. constrictus* e *B. pallescens* são finos e longos, medindo 220 µm e 185 µm de comprimento, dos quais 36 µm e 17 µm correspondem aos núcleos, respectivamente. Quando corados com DAPI (para DNA), a metade posterior do núcleo em *B. pallescens* apresentou pouca fluorescência. Enquanto que em *A. constrictus* esta característica foi observada somente nos últimos 5 µm. Em ambas as espécies, como em Heteroptera em geral, os espermatozóides têm, na região da cabeça, um acrossomo e núcleo, e na região flagelar, um axonema com 9 + 9 + 2 microtúbulos, dois derivados mitocondriais (DMs) e um adjunto centríolo na transição núcleo-flagelo. No entanto, ao contrário da maioria dos Heteroptera, nessas espécies, os DMs são assimétricos e o adjunto centríolo é bastante longo e circunda completamente a extremidade nuclear posterior e as pontas anteriores dos DMs. Essas características podem ser consideradas como derivadas, apoiando assim a condição derivada de Anthocoridae dentro de Cimicomorpha. Além disso, vários traços dos espermatozóides dessas duas espécies distinguem facilmente uma espécie (e provavelmente uma tribo) da outra, por exemplo, a diferença de formatos nos DMs e a longa projeção anterior do adjunto centríolo paralelo ao núcleo em *B. pallescens*.

ABSTRACT

SANTOS, Aline Beatriz Reis, M.Sc., Universidade Federal de Viçosa, July, 2017. **Sperm morphology of two species of Anthocoridae (Heteroptera: Cimicomorpha) with phylogenetic and taxonomic considerations.** Adviser: José Lino Neto

The order Hemiptera is divided into four suborders: Sternorryncha, Coleorryncha, Heteroptera and Auchenorryncha. Heteropterais one of the largest groups of insects, is made up of 75 families, among them Anthocoridae. The Anthocoridae family has about 600 species worldwide, and *Amphiareus constrictus* and *Blaptostethus pallescens* are predatory species that can be used in the biological control of agricultural pests. The understanding of the phylogenetic relationships, as well as the reproductive biology of these species can contribute to the management and use of these insects in pest control. Considering that since 1950 spermatozoa have been used as an important tool in several areas of biology, including taxonomy and philogeny. We present in this work information on the structure and ultrastructure of spermatozoa of *A. constrictus* and *B. pallescens* using microscopy techniques of light and transmission electronics. The spermatozoa of *A. constrictus* and *B. pallescens* are fine and long, measuring 220 µm and 185 µm in length, of which 36 µm and 17 µm correspond to the nuclei, respectively. When stained with DAPI (for DNA), the posterior half of the nucleus in *B. pallescens* exhibited low fluorescence. While in *A. constrictus* this feature was observed only in the last 5µm. In both species, as in Heteroptera in general, the spermatozoa have in the head region an acrosome and nucleus, and in the flagellar region, an axoneme with 9 + 9 + 2 microtubules, two mitochondrial derivatives (MDs), and a centriole adjunct in the nucleus-flagellum transition. However, unlike most Heteroptera, in these species, the MDs are asymmetric and the centriole adjunct is quite long, and circulates completely the posterior nuclear end, and the anterior tips of the MDs. These features can be are considered as derived, thus supporting the condition derived of Anthocoridae within Cimicomorpha. In addition, several traits of the spermatozoa of these two species easily distinguish one species (and probably a tribe) from the other, for example, the difference of formats in the MDs, and the long anterior projection of the centriole adjunct parallel to the nucleus in *B. pallescens.*

1. Introdução

1.1. Hemiptera: Heteroptera

A ordem Hemiptera possui cerca de 89.000 espécies descritas (Rafael et al., 2012), as quais exibem hábitos alimentares variáveis, podendo ser fitófagas, zoófagas e hematófagas (Forero, 2008). Em decorrência desses hábitos, muitas espécies são pragas importantes de diversos cultivares, algumas são vetores de sérias doenças humanas (Forero, 2008) e, ainda, aquelas que são predadoras podem ser utilizadas no controle de insetos praga (Desneux et al., 2006; Pereira et al., 2014). Esta ordem é considerada monofilética (Cryan e Urban, 2012) e possui quatro subordens: Sternorryncha, Coleorryncha, Auchenorryncha e Heteroptera (Bourgoin et al., 1997). Esta última, com 75 famílias, é um dos maiores e mais diversos grupo de insetos com metamorfose incompleta e ocorrem em todos os continentes (exceto Antártida) e em algumas ilhas (Schuh e Slater, 1995).Os Heteroptera são agrupados em sete infraordens: Enicocephalomorpha, Dipsocoromorpha, Gerromorpha, Nepomorpha, Leptopodomorpha, Pentatomorpha e Cimicomorpha (Wheeler et al., 1993; Schuh et al., 2009).

1.2. Família Anthocoridae (Cimicomorpha)

Como nos Heteroptera em geral, as relações filogenéticas entre as famílias de Cimicomorpha, bem como a classificação taxonômica para os anthocorídeos, ainda estão abertas à discussão. Por exemplo, Kerzhner (1981), o primeiro a considerar as famílias de Cimicomorpha em um contexto filogenético, propôs a ocorrência de seis superfamílias, com o clado Reduviidae e Pachynomidae (Reduvioidea) irmão de Nabidae e Prostemmatinae. Por outro lado, Schuh e Štys (1991) classificaram Cimicomorpha em sete superfamílias, e colocaram Reduvioidea irmão dos demais Cimicomorpha. Quanto aos anthocorídeos, Carayon (1972) os agrupa nas subfamílias Lasiochilinae, Lyctocorinae, Anthocorinae. Nestas duas últimas se encontram os gêneros *Amphiareus* (Cardiastethini) e *Blaptostethus* (Blasptostethini), respectivamente. Então Cassis e Gross (1995) renomearam Cardiastethini para Dufouriellini, e transferiu quatro das cinco tribos de Lyctocorinae para Anthocorinae. Finalmente Schuh e Slater (1995), seguindo a análise filogenética de Schuh e Štys (1991), elevaram Lasiochilinae Lyctocorinae à família (ver Horton, 2008). Nesse trabalho seguimos essa classificação.

A família Anthocoridae compreende 500 a 600 espécies em todo o mundo (Shapiro et al., 2010) e, como todas são predadoras, elas certamente desempenham importante papel no controle de outros insetos em sistemas naturais e, ainda, algumas espécies apresentam potencial para serem usadas no controle biológico em vários sistemas cultivados. Contudo, o conhecimento sobre esses anthocorídeos predadores se restringem apenas a alguns gêneros como *Anthocoris*, *Orius* e *Xylocoris*,muito provavelmente por isso apenas estes têm sido utilizados com sucesso em programas de controle biológico (Lattin, 1999).

Tem sido descrito que as espécies do gênero *Amphiareus* Distant 1904 habitam pilhas de material vegetal morto, como lenha e casca de árvores em decomposição (Yamada e Hirowatari, 2003). SegundoPéricart (1996), *Amphiareus constrictus* Stal 1860é encontrada nos trópicos do Velho e Novo Mundo e são predadoras de pequenos artrópodes. Já sobre *Blaptostethus pallescens* Poppius 1909 Tawfik e El-Husseini (1971) observou que predam ovos e larvas jovens de lepidópteros, e Sobhy et al.(2014) afirmou que indivíduos dessa espécie são encontrados com abundância em pomares de manga e outros sistemas de cultivo no Egito. Contudo, essas duas espécies também foram encontradas em plantações de tomates no Brasil onde são predadoras de muitos insetos praga, incluindo a mariposa *Tuta absoluta* Meyrick 1917 (Lepidoptera: Gelechiidae) (Pereira et al.,2014), uma das mais importantes pragas do tomateiro.

1.3. Espermatozoide em insetos

Os espermatozoides são células altamente especializadas destituídas da maioria das organelas associadas com o metabolismo celular. Em geral mantendo apenas aqueles elementos essenciais para o seu papel na fertilização que consiste no carreamento do material genético paterno e sua introdução no óvulo durante o processo de fecundação (Phillips, 1974). Contudo, nos animais em geral, esses relativamente poucos elementos exibem diversidade morfológica que não tem paralelo em nenhum outro tipo celular. Em inseto, os espermatozoides geralmente são compostos pelas regiões de cabeça e flagelar. Na maioria dos Pterygota, na cabeça são encontrados o núcleo e o acrossomo e, no flagelo, um axonema, dois derivados mitocondriais, dois corpos acessórios e um adjunto do centríolo na região de transição núcleo-flagelo.

O tamanho, especialmente o comprimento, e a ultraestrutura dos espermatozoides mostram considerável variação entre os diferentes grupos de insetos. Por exemplo, os espermatozoides do térmita *Reticulitermes lucifugus* Rossi 1792 (Isoptera:

Rhinotermitidae) medem por volta de 1,7 µm de comprimento (Baccetti et al., 1981), enquanto que os espermatozoides de *Drosophila bifurca* Patterson e Wheeler, 1942 (Diptera: Drosophilidae) 58 mm (Pitnick et al., 1995). Entretanto, a morfologia dos espermatozoides de uma dada espécie é bastante constante e característica daquela espécie que o produz, assim, eles são espécie específica (Phillips, 1974). Portanto, considerando que a diversidade morfológica dos espermatozoides reflete mudanças acumuladas ao longo do processo evolutivo e, ainda, que pode constituir uma característica de identidade para cada espécie de inseto, trabalhos sobre essas células têm sido conduzido em vários grupos de insetos com o objetivo auxiliar a compreensão de várias áreas como reprodução e, especialmente, taxonomia e sistemática (Jamieson, 1999; Dallai 2014; Dallai et al., 2016).

2. Objetivo

Descrever a estrutura e ultraestrutura dos espermatozoides de *A. constrictus* e *B. pallescens* buscando informações que possam auxiliar na compreensão da filogenia e sistemática dos Anthocoridae.

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Capítulo I

Sperm morphology of predatory pirate bugs *Amphiareus constrictus* and *Blaptostethus pallescens* (Heteroptera: Anthocoridae) with phylogenetic inferences

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Sperm morphology of predatory pirate bugs *Amphiareus constrictus* and *Blaptostethus pallescens* (Heteroptera: Anthocoridae) with phylogenetic inferences

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Sperm morphology of Anthocoridae

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Keywords: Hemiptera; Cimicomorpha; Systematic; Microscopy; Sperm Structure

Santos, A.B.R. & Lino-Neto, J. (2017) Sperm morphology of predatory pirate bugs *Amphiareus constrictus* and *Blaptostethus pallescens* (Heteroptera: Anthocoridae) with phylogenetic inferences.

The sperm morphology of two predatory bugs *Amphiareus constrictus* and *Blaptostethus pallescens*, representatives the tribes Dufouriellini and Blasptostethini, respectively, was described using light and transmission electron microscopy. The spermatozoa of *A. constrictus* and *B. pallescens* are filliform and long, each measuring 220 µm and 185 µm in length, of which 36 µm and 17 µm, respectively, comprise the nuclei. When stained with DAPI (for DNA), the posterior half of the nucleus in *B. pallescens* exhibited low fluorescence,while in *A. constrictus* this feature was observed only in the last 5 µm. In both species the spermatozoa have, in the head region, an acrosome and nucleus, and in the flagellar region, an axoneme with 9 accessory tubules, 9 peripheral doublets and 2 central microtubules (9 + 9 + 2 microtubules), 2 mitochondrial derivatives (MDs), and a centriole adjunct in the nucleus-flagellum transition. However, unlike some Heteroptera, in these species, the MDs are asymmetric, and the centriole adjunct is quite long and encompasses completely the posterior nuclear end and the anterior tips of the MDs. These features are considered as derived, thus supporting the condition derived for Anthocoridae within Cimicomorpha. In addition distinguish the species, the differences in the MDs, and the long anterior projection of the centriole adjunct parallel to the nucleus in *B. pallescens*..

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Introduction

The Hemiptera, with about 89,000 described species (Rafael et al., 2012), comprises four main suborders: Sternorrhyncha, Auchenorrhyncha, Coleorrhyncha and Heteroptera (Wheeler et al., 1993). The last mentioned is one of the greater groups of insects with incomplete metamorphosis (Schuh &Slater, 1995), and inhabit different ecological niches (Goodchild, 1966). The Cimicomorpha comprises about 19,400 species, distributed in 16 families (Schuh & Slater, 1995) divided into: Reduvioidea, Velocipedoidea, Microphysoidea, Joppeicoidea, Miroidea, Naboidea, Cimicoidea (Schuh &Štys, 1991). The Cimicomorpha phylogenetic relationships, as well as the taxonomic rank for the anthocorids, is still open to discussion. For instance, Kerzhner (1981), the first to consider the cimicomorphan families in a phylogenetic context, proposed the existence of six superfamilies with the clade Reduviidae and Pachynomidae (Reduvioidea) sister of the Nabidae and Prostemmatinae.superfamilies. On the other hand, Schuh & Štys (1991) classified the Cimicomorpha into seven superfamilies, and placed Reduvioidea as sister-group of the other cimicomorphan. Regarding anthocorids, Carayon (1972) distributes them in Lasiochilinae, Lyctocorinae and Anthocorinae. In these last two are the genera *Amphiareus* (Cardiastethini) and *Blaptostethus* (Blasptostethini), respectively. Then Cassis & Gross (1995) renamed Cardiastethini to Dufouriellini, and transferred four of the five tribes from Lyctocorinae to Anthocorinae. Finally, Schuh & Slater (1995), following the phylogenetic analysis of Schuh & Štys (1991), elevate the Lasiochilinae and Lyctocorinae to family rank (see Horton, 2008). In this article, we follow this classification. According to Tian et al. (2008), "further exploration of the cimicomorphan true bugs biology and evolution has been hindered by the lack of well-supported phylogenetic hypotheses".

It is common sense among researchers of the subject, as well as in several other studies, which have been demonstrated (e.g., Jamieson et al., 1999; Birkhead, 2009; Dallai & Afzelius, 1980), that the knowledge about the structural and ultrastructural characters of spermatids and, especially spermatozoa, can contribute significantly to the construction of phylogenetic hypotheses in insects. However, studies showing morphological features on cimicomorphan spermatozoa are still very scarce. Searching in the literature, we found only four works, which are: Dallai & Afzelius (1980); Dolder (1988); Báo & de Souza (1994); Baffa et al. (2017). What's more, they are all about Reduviidae, and only the work of Dallai & Afzelius (1980) shows a cross-section of the axoneme of the *Cimex lecticularius* sperm (Cimicidae). Therefore, in order to begin to expand the information about the morphology of this special type of cell in the cimicomorphan, we describe here the sperm morphology of *A. constrictus* and *B. pallescens* representing two Anthocoridae tribes, being therefore, the first work with morphology of spermatozoa in this family.

The Anthocoridae family comprises 500 to 600 species worldwide (Shapiro et al., 2010), and because they are all predators, they certainly play an important role in the control of other insects in natural systems, and some species have the potential to be used in biological control in various cultivated systems. The species Amphiareus constrictus Stal 1860 is found in the tropics of the Old and New World and are predators of small arthropods (Péricart, 1996). *Blaptostethus pallescens* Poppius 1909 is found abundantly in mango orchards and other cropping systems in Egypt (Sobhy et al., 2014) and prey eggs and larvae young lepidopteran (Tawfik and El-Husseini, 1971). Also, these two species were also found in tomato plants in Brazil where they are predators of many pest of this culture, including the moth *Tuta absoluta* Meyrick 1917 (Lepidoptera: Gelechiidae) (Pereira et al., 2014), one of the most important pests of the tomato.

Materials and methods

Adult males of *A. constrictus* and *B. pallescens*were obtained from colonies maintained at the Laboratório de Manejo Integrado de Pragas, Departamento de Entomologia, Universidade Federal de Viçosa (UFV). Three specimens each species were used for light microscopy, and five for transmission electron microscopy.

Light microscopy

Seminal vesicles were dissected and broken open on clean glass microscope slides, where spermatozoa were spread and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), for 15–20 min. The samples were then washed in running water and dried at room temperature. To measure the spermatozoa total length, some samples were stained with Giemsa for 15 min, then washed in running water and dried at room temperature. The analysis and photodocumentation of the spermatozoa were done in a photomicroscope (Olympus BX-60). To measure the length of the nuclei, some slides were stained with 4.6 diamino-2-phenylindole (DAPI) 0.2 μg/mL in PBS for 20 min, then washed in running water, dried at room temperature and mounted with 50% sucrose. These slides were examined on an epifluorescence microscope (Olympus, BX-60) equipped with BP360-370 nm filter. All the measurements were obtained with the software Image Pro-Plus, version 4.5 (Media Cybernetics Inc., MD, USA), and the average size was obsteined for 10 spermatozoa and 10 nuclei of each specimen, totaling 30 sperm and 30 nuclei.

Transmission electron microscopy (TEM)

The seminal vesicles of five adult males of each species were dissected in sodium cacodylate buffer 0.1 M, pH 7.2, and fix and transferred to 2.5% glutaraldehyde solution, picric acid 0.2%, sucrose 3% and $CaCl_2$ 5 mM in the same buffer. After 24 hours, the material was washed with the same buffer, post-fixed in 1% osmium tetroxide solution for 2 h, dehydrated in a gradea acetone series and embebed a in Epoxy resin (Epon 812). The ultrathin sections were obtained with automatic ultramicrotome Sorval MT2-B with diamond knife, collected on copper grids, and stained with 3% uranyl acetate and 2% lead citrate. All the samples were analyzed in a TEM Zeiss EM109, operating at 80 kV.

Results

In *A. constrictus* and *B. pallescens*, we observe only one spermatozoa morphotype. The spermatozoa are slender and long, which measured 220 ± 3.82 (216.16-225.47) µm in *A. constrictus* (Fig. 1A) and 185 ± 12.59 (144.75-189.33) µm in *B. pallescens* (Fig. 1D). The nuclei of these cells exhibited distinct lengths between the two species: they measured 36 ± 7.31 (28.80-42.07) µm (Fig. 1B) and 17 ± 7.46 (12.57-36.25) µm (Fig. 1E), respectively. In addition to these differences in measurements, the spermatozoa of both species were easily distinguished when they were stained with DAPI. In *A. constrictus*, the nucleus was slightly tapered, about 0.30 µm in diameter at the anterior tip, and 0.50 µm near the base. Furthermore, in this region it exhibited a thin extension of about 0.25 µm in diameter × 6 µm in length (Fig. 1C). Already in *B. pallescens*, the nucleus is thicker (~ 0.85 µm) in the median region, and sharpens at both ends. In addition, its posterior region exhibited lower fluorescence by almost half (~ 8 µm) of its length, and the extremity of this region (~ 2 µm) sharpens rapidly and is bevelled (Fig. 1F).

The ultrastructure showed that in both species, the spermatozoa head region is formed with acrosome and nucleus, and the flagellar region has an axoneme, two mitochondrial derivatives (MDs), and in the transition between these two regions, a well-developed centriolar adjunct, especially in *B. pallescens*, although it has shorter spermatozoa (Figs. 2A-I, 3A-I). In *A. constrictus*, the acrosomal complex measured about 2.2 µm in length (Fig. 2A). In the cross-section, it is circular (Fig. 2B), accompanying the shape of the nucleus (Fig. 2C).It is formed by a cuneiform acrosome with a small subacrosomal region of lower electron-density, and an extra-acrosomal coating measuring about 30 nm thick (Fig. 2A, B). The nucleus contained compact chromatin, but with electron lucent areas in the central region (Fig. 2C-D). Also, mainly in the central region, a large amount of small particles (~ 15 nm) with electron density higler than that of chromatin and electron-lucent contour were observed (Fig. 2C-D). The centriole adjunct is quite developed (~ 6 µm in length, Fig. 1C), surroundiyng that posterior extension of the nucleus, and it shows an indent along its ventral surface (Fig. 2E-H). Its posterior region circles, in addition to the nuclear tip, the anterior tips of the two MDs, at different levels (Fig. 2G, H), and then the axoneme tip that is positioned in line with the nucleus.

The flagellum is formed by one axonema and two MDs (Fig. 2I). The axoneme has the 9 + 9 + 2 microtubule pattern, with much intertubular material. The MDs begin at different heights, next to the nuclear base, surrounded by the centriole adjunct (Fig. 2G, H). In cross-section of the flagellum, they exhibit an irregular contour, and are rather asymmetrical, the smallest MD being about 22% larger than the axonema area (~ 0.045 µm^2), while the largest is about twice that area. Each MD displays two paracrystalline materials: one small situated on the ventral side, and the other occupying almost the entire area of the derivative, and with two regions of different electron densities (Fig. 2I). Also, externally involving the two paracrystallines, the mitochondrial cristae region was also observed (Fig. 2I, detail). Notewortly the flagellar components are very compact, making it difficult to see the bridges connecting the axoneme to each MD (Fig. 2I). Moreover, the whole spermatozoon is covered by aelectron-dense and thick glycocalyx (Fig. 2B-I).

In *B. pallescens*, it was possible to observe that the acrosome fits over the nuclear tip asymmetrically and shows a narrow crevice along its median region (Fig. 3A). In cross-section, it is oval (Fig. 1A detail), following the nuclear shape (Fig. 1C, D), which has the chromatin uniformly compact (Fig. 3A-G) with few smal particles (Fig. 3C). Notewortly that grossty the posterior nuclear half (~ 6 µm) is narrower laterally (Fig. 3B), acquiring crescent moon shape in transverse direction (Fig. 3D), where the anterior projection of the centriole adjunct fits (Fig. 3B). Finally, the posterior nuclear portion

(~ 2 µm) narrows uniformly, assumes the cylindrical shape, and becomes fully surrounded by the centriolar adjunct (Fig. 3B, E-G).

The MDs begin inside the centriole adjunct, on the side of the posterior cylindrical portion of the nucleus (Fig. 3B, F and G). As in *A. constrictus*, in longitudinal sections, the MDs show peripheral mitochondrial cristae with a parallel arrangement at a regular distance of ~38 nm (Fig. 3I), and in cross-section they are asymmetrical (Fig. 3B, G and H). However, in *B. pallescens*, they have a regular contour, and the smaller one has the crescent moon shape, while the larger one the drop shape. This latter exhibits, at the tapered end (ventral and near to axoneme), a projection with sickle shape that is electron-denser and attaches to the axoneme (Fig. 3B, H and detail). The axonema also exhibits the 9 + 9 + 2 microtubular pattern.

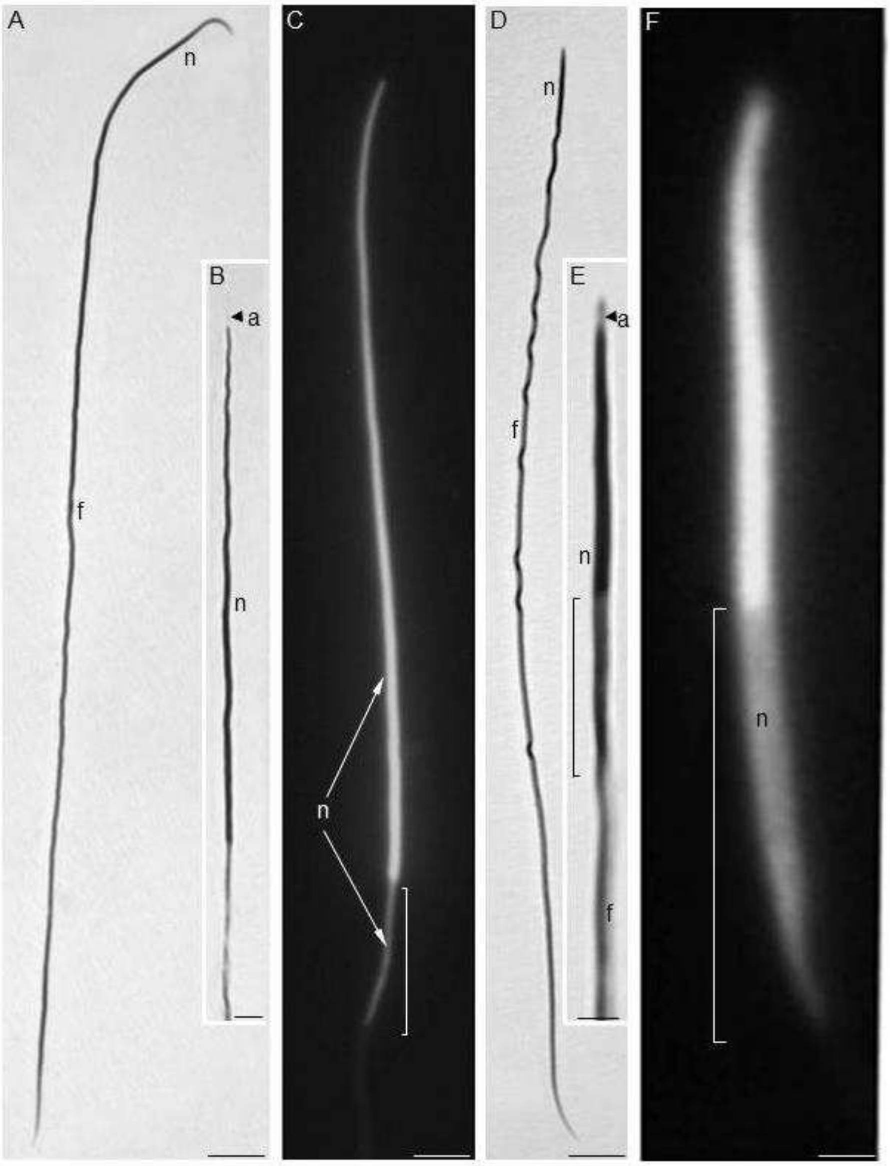

Fig. 1. Spermatozoa under light microscopy of *Amphiareus constrictus* (A-C) and *Blaptostethus pallescens* (D-E), stained with Giemsa (A, B, D and E) and DAPI (C and F). Note that almost the posterior half of the nucleus (n) is weakly stained in *B. pallescens* sperm, whereas in *A. constrictus* only the thin end of the nucleus is less stained. f = flagellum; a = acrosome. Scale bars: A, D = 10 µm; B, C, E, F = 2 µm.

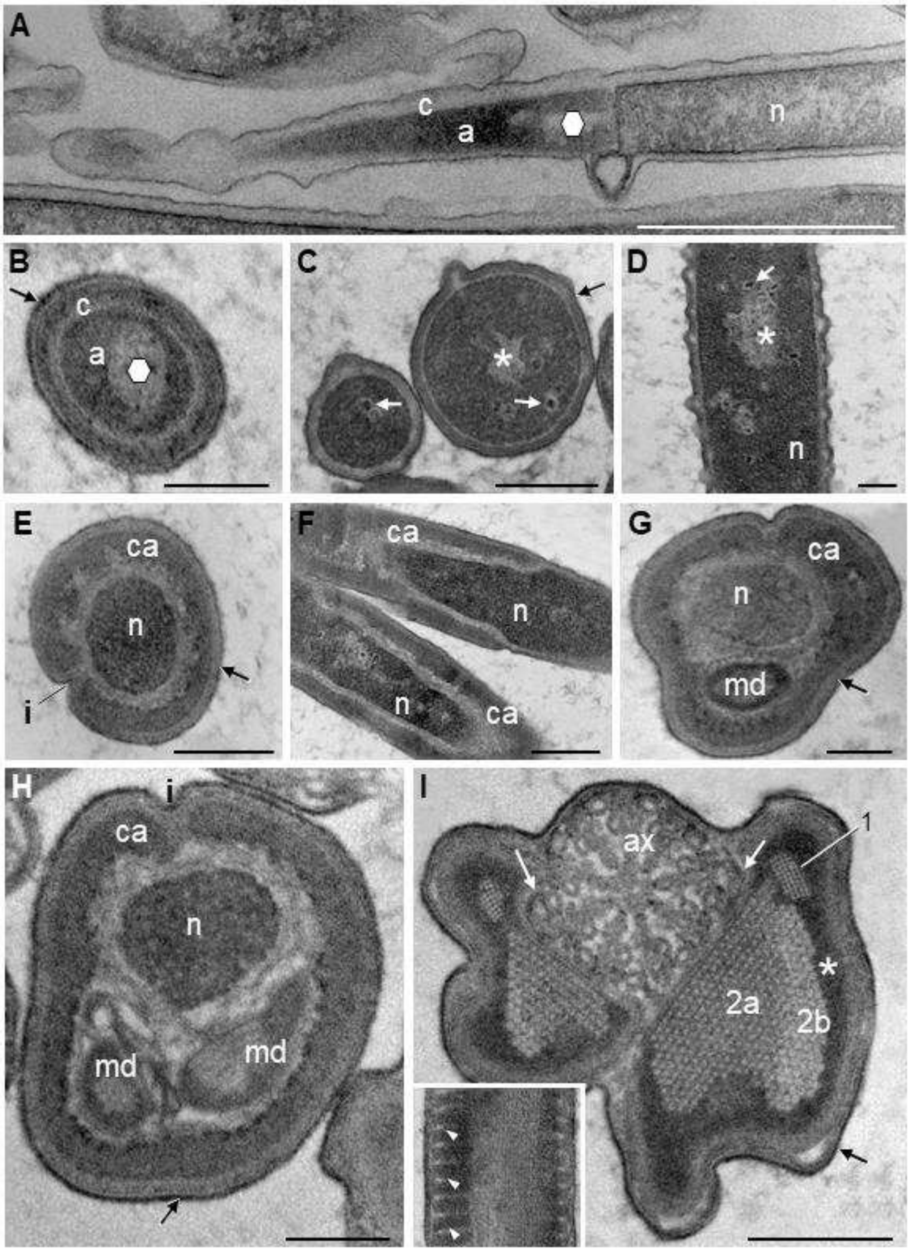

Fig. 2. Transmission electronic micrographs of *Amphiareus constrictus* sperm in longitudinal (A, D, F) and cross (B, C, E, G-I) sections. (**A, B**) Acrosomal region showing the acrosome (a) with a small subacrosomal region of lower electron-density (white hexagon), and the extra-acrosomal coating (c). n = nucleus. (**C, D**) Nuclear region showing areas of lower density(asterisks), and the virus-like particles (white arrows). (**E-H**) Nucleus-flagellum transition region, note the centriole adjunct (ca) surrounding completely the nuclear end, and then the anterior tips of the two mitochondrial derivatives (MDs). i = the indent on the ventral region of centriole adjunct. (**I**) Flagellar region showing the axoneme (ax), and the two asymmetric MDs. Note the two paracrystalline materials in each MD (1 and 2),and the mitochondrial cristae region indicated by the asterisk and arrow heads in detail. White arrows =the bridges connecting the axoneme to MDs. The black arrows in all the figures indicate the dense layer surrounding the sperm. Scale bars: A = 1 µm; B, D, G, H = 0.1 µm; C, E, I = 0.2 µm; F = 0.3 µm.

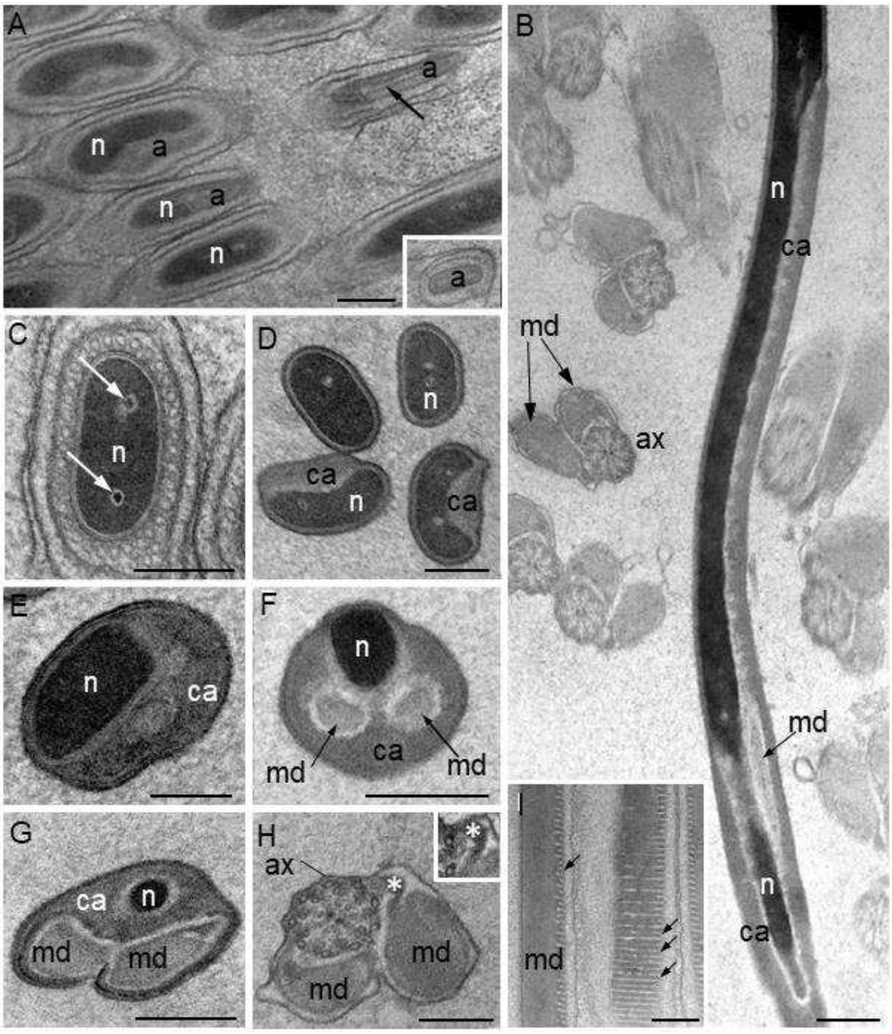

Fig. 3. Transmission electronic micrographs of *Blaptostethus pallescens* spermatozoa in cross (A, C-H) and longitudinal (B, I) sections. (**A**) The acrosome (a) fitted over the nuclear tip (n) asymmetrically. Note the narrow crevice along its median region (arrow), and its oval shape (detail). (**B, D-G**) Nucleus-flagellum transition region, note (in B, D) the anterior projection of the centriole adjunct (ca) side by side with the nucleus (n), and then the ca envelops totally the thin nuclear end, and the anterior tips of the MDs (E-G). (**C**) Nuclear region showing the virus-like particles (arrows), and that in this species the nucleus is oval in transverse view. (**H**) Flagellar region showing that in this species the MDs are also asymmetric, however they exhibit fairly regular contour, and a sickle-shaped projection (asterisks) on the larger MD associated with the axoneme (ax). (**I**) MDs showing the mitochondrial cristae region (arrows). Scale bars: A, B = 0.3 µm; C-H = 0.2 µm.

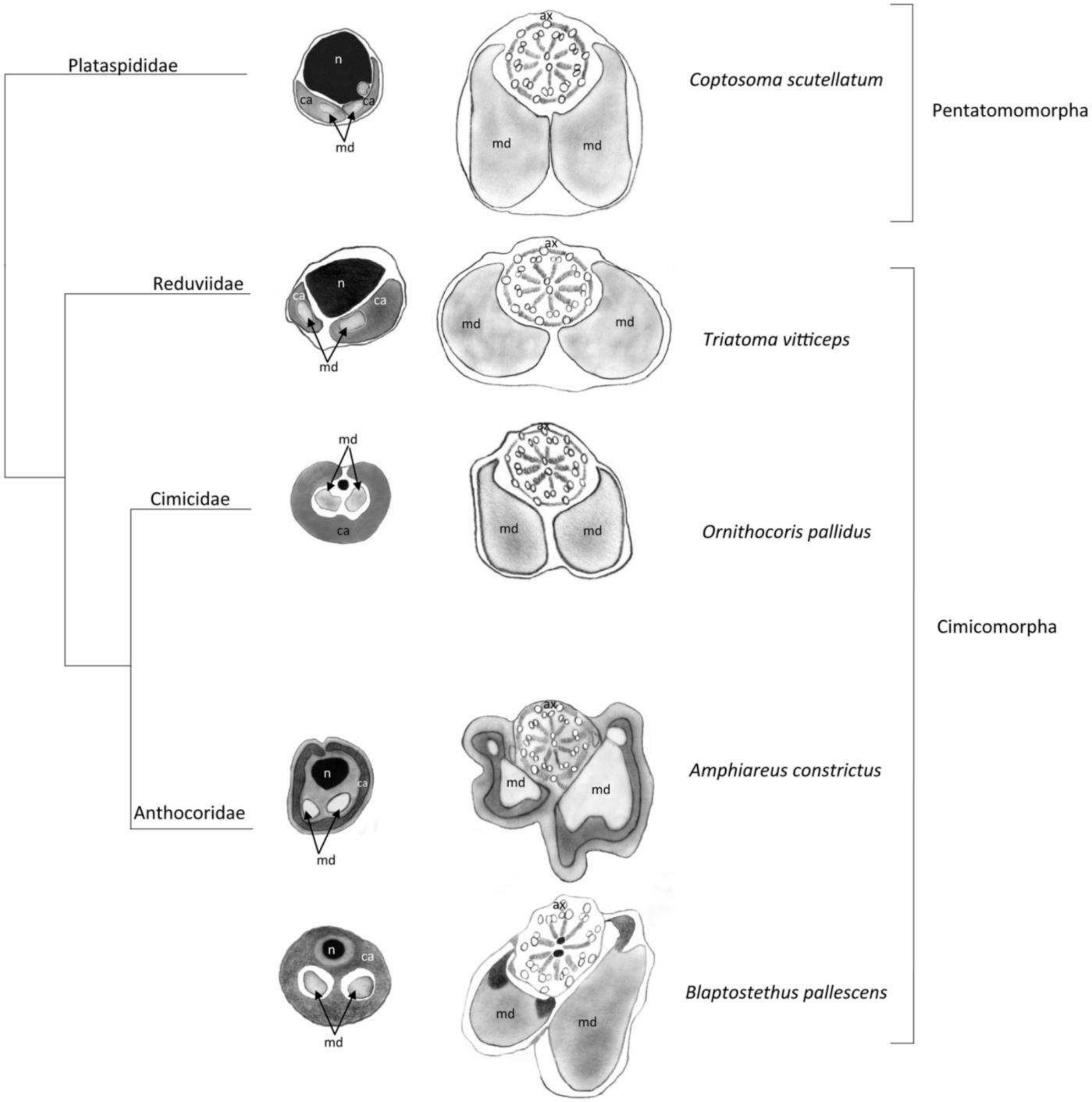

Fig. 4. Schematic reconstruction of the nucleus-flagellum transition region and the flagellar region in cross-sections. Note that the centriole adjunct is bifurcated in Reduviidae (Cimicomorpha), as in Pentatomorpha (e.g., the Plataspididae *Coptosoma scutellatum*), whereas in cimicid and anthocorids it is unique and completely surrounds the nuclear tip. Still the MDs are asymmetric in the anthocorids, and symmetrical in the spermatozoa of the other species shown here. From: Dias et al., 2016 (*C. scutellatum*); Maia, 2009, unpublished data (*T. vitticeps*), and Novais, 2017, unpublished data (*O. pallidus*).

Discussion

The observation of only one spermatozoa morphotype, as in these two anthocorid species, has been recorded in other heteropteran species (Dias et al., 2016; Novais et al., 2017). However, in this suborder, the production of more than one spermatozoa morphotype (polymorphism) by the same individual has been observed in Auchenorrhyncha (Cicadomorpha: Chawanji et al., 2005, 2006; Araújo et al., 2010), Pentatomomorpha (Bowen, 1920; Schrader &Leuchtenberger, 1950; Araújo et al., 2011; Araújo et al., 2012; Mercati & Dallai, 2016), and Cimicomorpha. In the latter, in which are the species studied here, the spermatozoadimorphism was observed in Reduviidae (Baffa et al., 2017), and it is interesting to note that this family was considered sister-group of the other cimicomorphan(Schuh & Štys, 1991). This leads us to wonder whether there is a direct relation between the spermatozoa polymorphism and the position of this family closer to the basal node of the cimicomorphan cladogram.

As might be expected, *A. constrictus* and *B. pallescens* spermatozoa are formed by the same structural components found in the spermatozoa of the other Heteroptera, which are: an acrosome and a nucleus in the head region, an axoneme in the flagellar region with a 9 + 9 + 2 microtubular pattern, two mitochondrial derivatives, and in the nucleus-flagellum transition region, a centriole adjunct. In addition, in these anthocorid spermatozoa, flagellar structures called accessory bodies were not observed, but they exhibited a bridge linking each MD to the axoneme (in the double nº. 1 and 5, Dallai & Afzelius, 1980), and at least two paracrystalline materials in each MD. Thus, demonstrating the wide distribution of these three characteristics in the heteropteran sperm, and therefore, supporting the synapomorphic condition for the suborder (Dallai & Afzelius, 1980; Dallai et al., 2016). On the other hand, the relative size and shape of each structure, as well as the arrangement between them, make the spermatozoa of these two species unique among those observed in the other Hemiptera. Interestingly, these same characteristics easily differentiate *A. constrictus* from *B. pallescens*, and likewise, Dufouriellini from Blasptostethini.

As for the sperm-head region, these two species can be distinguished from each other by the extracrosomal layer in *A. constrictus*, and the cleft in the acrosome of *B. pallescens*, and also by the shape of the acrosome and nucleus (in cross-section, circular, and oval, respectively). Moreover, we did not find the first two acrosome features described for any other Heteroptera sperm, and for Cimicomorpha, this is the first description of acrosome. In relation to the nucleus, the lower intensity of staining observed under light microscopy, both in the final portion of the nucleus in A. constrictus and in the posterior nuclear half in B. pallescens, is due to the presence of the centriole adjuvant in this region, as later verified with the transmission electron microscopy images.

The presence of small electron-dense particles, observed inside the nucleus of these two species, was recorded by Báo et al. (1996) in *Bemisia tabaci* (Sternorryncha: Aleyrodidae), who described them as virus-like particles. This same type of particles was also observed in Auchenorryncha by Araújo et al. (2010), therefore, the transfer of viral particles through the sperm nucleus was already observed in three of the four suborders of Hemiptera. Mercati & Dallai (2016) observed the presence of virus in the membrane of one of the two types of spermatozoa produced by Heteroptera *Raphigaster nebulosa*, and the interesting this particular type of spermatozoa is the one that carries out fertilisation. The transmission of viral particles via spermatozoa has also been reporteedin Diptera (Schrankel & Schwalm, 1975) and Coleoptera (Kitajima et al., 1985).

The centriole adjunct completely surrounding the nuclear posterior end, as in *A. constrictus* and *B. pallescens* spermatozoa. Until then it was observed only in Cimicidae sperm (Fig. 4), indicating that this family and Anthocoridae are closely , as has been suggested in all phylogenetic analyses of Cimicomorpha (e.g. Tian et al., 2008).On the other hand, in Reduviidae, unlike in the two families mentioned above, the centriole adjunct is bifurcated and parallel to the nuclear base (Maia, 2009, unpublished data; Baffa et al., 2017). Notewortly these same characteristics of the centriole adjunct have been observed in Pentatomomorpha sperm (e.g. Dias et al., 2016), thus indicating thesister-group condition of Reduvidae of the other Cimicomorpha, as proposed by Schuh & Štys (1991), and differing from the phylogenetic hypothesis of Kerzhner (1981). Furthermore, the centriole adjunct distinguishes *A. constrictus* from *B. pallescens* since only *A. constrictus* spermatozoa exhibited the indent along the ventral face, while *B. pallescens* spermatozoa showed a long anterior projection disposed laterally to the nucleus. This projection makes the nucleus of these spermatozoa easily recognizable even under light microscopy, thus making it possible to group those species that have this characteristic.

Analysing diligently, it is possible to observe that the asymmetry of MDs is the only characteristic that, in fact, allows to group these two species. Since in Cimicidae, a family considered phylogenetically closer to Anthocoridae, the MDs are symmetrical, as they are also in Reduviidae and in the Pentatomomorpha (see Fig. 4 and Dallai & Afzelius, 1980). However, the shape and contour of the MDs are clearly different between the two species: irregular in *A. constrictus*, and regular in *B. pallescens*. According to Gottardo et al. (2016), asymmetric MDs are a apomorphy condition, so it is possible to assume that Anthocoridae, and possibly Lasiochilidae and Lyctocoridae, because of the taxonomic proximity between them, are the families most derived in Cimicomorpha.

In conclusion, in the spermatozoa of these two species, the absence of accessory bodies, and the presence of more than one paracrystalline material in each MD, and the bridges connecting the MDs to the axonema, are features shared with the other Heteroptera; the centriole adjunct completely surrounds the nuclear-tapered posterior end is shared with the Cimicidae (also Cimicomorpha) and, finally, the asymmetry of the MDs links these two species. However, their spermatozoa exhibit at least five traits that differentiate one species from the other: acrosome and nucleus format, extra-acrosomal layer in *A. constrictus*, cleft in *B. pallescens* acrosome, indent in the *A. constrictus* centriole adjunct, MDs contour and sperm compaction. This number of differences is not commonly observed among closely species, and this leads us to suggest that the sperm morphology may provide insights on the relationships among families, tribes and genera of minute pirate bugs.

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3. Conclusão

Nos espermatozoides de *A. constrictus* e *B. pallescens,* a ausência de corpos acessórios e a presença de mais de um material paracristalino em cada derivado mitocondrial e as pontes que conectam os derivados mitocondriais ao axonema são características compartilhadas com os outros Heteroptera;

O adjunto do centríolo que envolve completamente a extremidade posterior cônica nuclear é compartilhado com Cimicidae (também Cimicomorpha) e;

A assimetria dos derivados mitocondriais agrupa essas duas espécies, no entanto, seus espermatozoides exibem pelo menos cinco traços que diferenciam uma espécie da outra, como: formato do acrossomo e núcleo, camada extra-acrossomal em *A. constrictus*, fenda no acrossomo de *B. pallescens,* endentação no adjunto centríolo de *A. constrictus*, contorno dos derivados mitocondriais e compactação do espermatozoide. Esse número de diferenças não é comumente observado entre espécies próximas, o que nos leva a sugerir que a morfologia do espermatozoide pode esclarecer as relações entre famílias, tribos e gêneros de Anthocoridae.