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Research paper

Spermatozoa morphology of the giant water bug *Belostoma anurum* (Herrich-Schäffer, 1848) (Heteroptera: Belostomatidae)

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ABSTRACT

Giant water bugs (Belostomatidae) have important functions in aquatic ecosystems with wide tropical and subtropical distribution. This study describes the spermatozoa morphology of the predator *B. anurum*. The spermatozoa have not polymorphism and they are scattered without bundles into the seminal vesicle. The mean length of the spermatozoa is 510 μm, including the 35 μm length of the nucleus, which is the longest germ cell, reported for Belostomatidae. The head of the spermatozoa is formed by the acrosome and the nucleus with strongly condensed chromatin. In the nucleus−flagellar transition occurs a C-shaped centriole adjunct, partially surrounding the posterior end of the nucleus and totally surrounding the anterior ends of the two mitochondrial derivatives. In the flagellum, the axoneme has a $9 + 9 + 2$ microtubular pattern, and it is linked to the two mitochondrial derivatives by protein bridges. The mitochondrial derivatives are symmetrical surrounding almost entirely the axoneme. The morphology of *B. anurum* spermatozoa differs from those found in other Pentatomomorpha by the acrosome that does not protrude along with the nucleus and by the single centriole adjunct.

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1. Introduction

Hemiptera has more than 100,000 described species with monophyly strongly supported by both morphological and molecular characters (Misof et al. 2014). However, phylogenetic relationships within the group are still discussed (Cryan & Urban, 2012).

The order is composed of three main clades: Sternorrhyncha, Auchenorrhyncha, and Heteropteroidea (Kristensen, 1975; Hennig, 1981), the last including Coleorrhyncha + Heteroptera (Kristensen, 1975). Heteroptera has seven infraorders: Enicocephalomorpha, Dipsocoromorpha, Gerromorpha, Nepomorpha, Leptopodomorpha, Pentatomomorpha and Cimicomorpha (Wheeler et al. 1993). In Nepomorpha, Belostomatidae has 11 genera and approximately 150 species (Estévez & Ribeiro, 2011; Moreira et al. 2011). Among the Belostomatidae, representatives of *Belostoma* are predators (Ohba et al. 2006; Ouyang et al. 2017; Ohba, 2019). These giant water bugs are natural enemies of insects of medical importance including the mosquitoes *Culex quinquefasciatus* Say, 1823 and *Aedes aegypti* (Linnaeus, 1762) (Valbon et al. 2019), vectors of filariasis, yellow fever, dengue fever, chikungunya and zika diseases.

The morphology of insect spermatozoa provides important characters for the systematic of some orders (see Jamieson et al., 1999; Birkhead et al. 2009; Dallai et al. 2016). The spermatozoa structure has been suggested to be conserved in Heteroptera, with some synapomorphies, such as the presence of two opposite bridges in the axoneme connecting the mitochondrial derivatives that have matrix rich in paracrystalline content, and the absence of accessory bodies throughout the flagellum (Dallai et al. 2016). However, the diversity of spermatozoa characters is important to understand the phylogenetic relationships in Heteroptera.

Intraspecific variations may occur in the spermatozoa morphology if insects (Hudgson, 1999) resulting in sperm polymorphism, which is characterized by different germ cell types, including their sizes and organelles (Jamieson et al. 1999).

This study describes the spermatozoa morphology of *Belostoma anurum* (Herrich-Schäeffer, 1848) (Nepomorpha: Belostomatidae),

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contributing to new data that can be useful in the future systematic studies of these important aquatic insects.

2. Material and methods

Adult males of *B. anurum* were obtained from a pond in the Federal University of Viçosa (20° 45′ S 42° 52′ W, 648 m asl), Viçosa, state of Minas Gerais, Brazil. Five insects were used for light microscopy and five for transmission electron microscopy.

2.1. Light microscopy

The insects were anesthetized at - 20 °C for 5 min and dissected in the presence of 125 mM NaCl to remove the male reproductive tract. Then, the spermatozoa were extracted from the seminal vesicles in the presence of 0.1 M sodium phosphate buffer, pH 7.2 (PBS), spread onto histological slides and fixed with 4% para-formaldehyde for 15–20 min at room temperature. The samples were washed in distilled water and air-dried. To measure the total length of the spermatozoa, five samples of one seminal vesicle were stained with Giemsa for 15 min, washed in water, air-dried and analyzed with an Olympus BX-60 photomicroscope. To measure the nucleus length, five samples of the other seminal vesicle were stained with 0.2 μg/mL of 4,6-diamino-2-phenylindole (DAPI) in PBS, for 20 min, then washed in water and mounted with 50% su-crose. These samples were examined using an Olympus BX-60 epifluorescence microscope, equipped with a BP 360–370 nm filter. All measurements were obtained using the computer program Image Pro-Plus. In total, 30 spermatozoa and 30 nuclei randomized from the five males analyzed were measured.

2.2. Transmission electron microscopy

After dissection of the male reproductive tract in 0.1 M sodium cacodylate buffer, pH 7.2, the seminal vesicles were transferred to 2.5% glutaraldehyde with 0.2% picric acid solution in the same buffer, for 24 h at 4 °C. The pieces were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded acetone series (50%, 70%, 80%, 90%, and 99%) - for 10 min each and embedded in Epon resin. The ultrathin sections (70–90 nm) were obtained using a Sorval MT2-B ultramicrotome with a diamond knife, collected on copper grids, were stained with 2% aqueous uranyl acetate in and lead citrate (Reynolds, 1963) and analyzed with a transmission electron microscope, Zeiss EM 109.

3. Results

B. anurum has one spermatozoa morphotype, which are stored without sperm aggregation in the seminal vesicle. These germ cells measured 514.29 ± 9.35 (502.73–533.31) μm in length, including 35 ± 1.07 (34.88–38.55) μm of the nucleus (Fig. 1A and B).

The head of the spermatozoa is formed by a small acrosome that has a subacrosomal space and the nucleus. The acrosome has ca. 4.5 μm in length being 1.8 μm in the head apex and 1.5 μm in the region closely to the nucleus where they overlap (Fig. 1C–E). In the anterior region, the nucleus is beak-like flute shaped (Fig. 1D and E), followed by a median cylindrical region (Fig. 1F–G), and a sharpened posterior region (Fig. 1H). The nucleus is filled by condensed chromatin (Fig. 1I).

In the nucleus–flagellum transition region, there was a C-shaped centriole adjunct. In the anterior part, the centriole adjunct partially envelops the posterior end of the nucleus (Fig. 1F and G), whereas in the posterior part it covers the anterior ends of the two mitochondrial derivatives (Fig. 1H).

The flagellum has an axoneme and two mitochondrial derivatives (Fig. 1J). The axoneme has a 9 + 9 + 2 microtubular pattern, with nine outer (accessory), nine peripheral and two central pairs of microtubules. The terminal portion of the flagellum is sharpened with disorganization of the microtubular pattern.

The mitochondrial derivatives are symmetrical and, in cross-section, almost completely surround the axoneme (Fig. 1J). The axoneme and the mitochondrial derivatives are linked by electron-dense inter-microtubular bridges (Fig. 1J).

4. Discussion

The absence of spermatozoa polymorphism in *B. anurum* has been also reported in other Heteroptera (Araújo et al. 2011; Dias et al. 2016; Novais et al. 2017; Santos & Lino-Neto, 2018; Munhoz et al., 2020), except in some representatives of Pentatomidae (Bowen, 1922; Schrader & Leuchtenberger, 1950; Araújo et al. 2011), Largidae (Araújo et al. 2012), and in the Auchenorrhynca Cicadidae (Kubo-Irie et al. 2003; Chawanji et al. 2005, 2006).
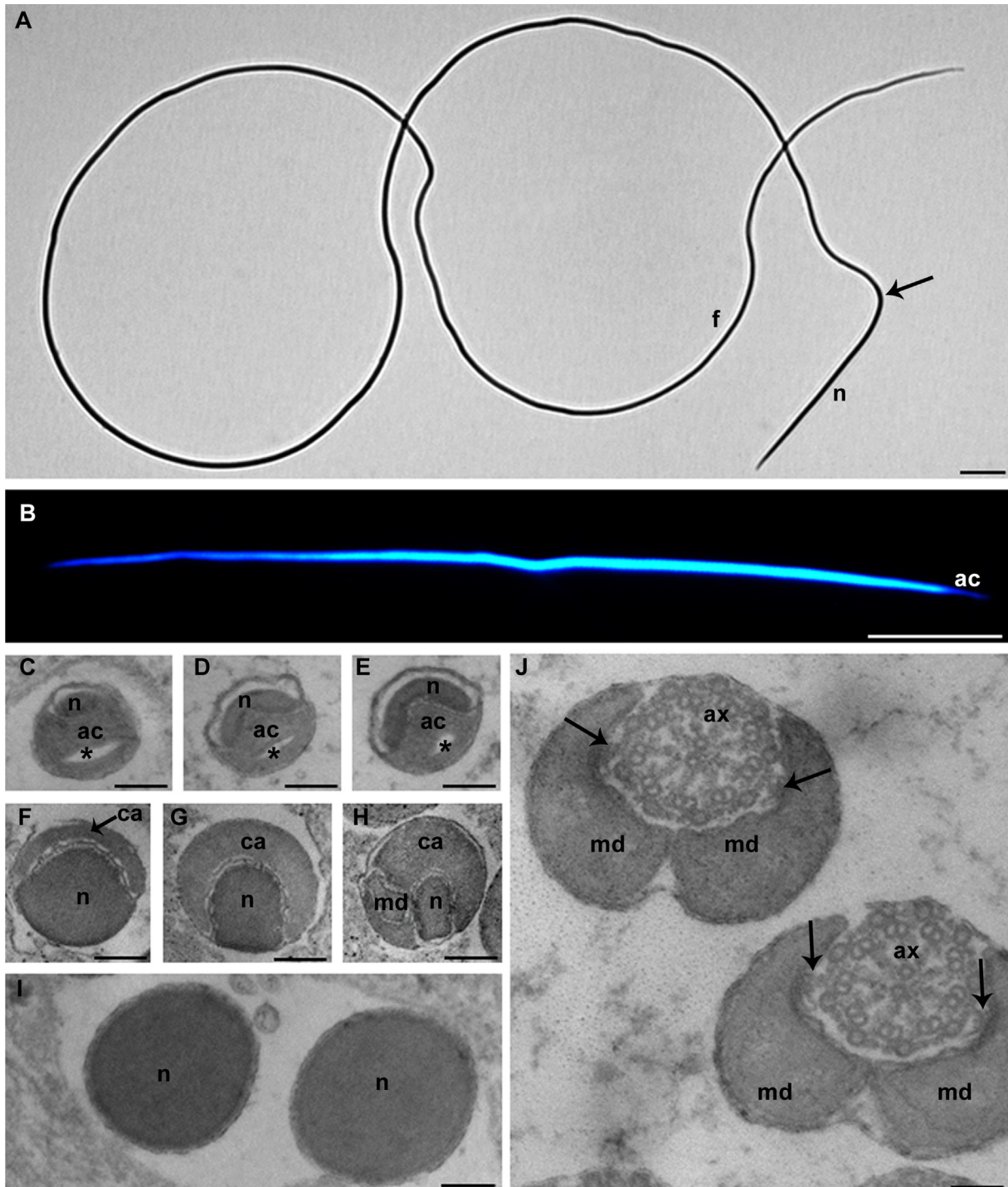
Individualized spermatozoa without bundles in the seminal vesicle of *B. anurum*, suggest spermatozoa bundles are broken when they reach the seminal vesicle, such as reported for other Nepomorpha (Lee & Lee, 1992). However, in Auchenorrhyncha these germ cells occur in bundles in the seminal vesicle (Folliot & Maillet, 1970; Cruz-Landim & Kitajima, 1972; Kubo-Irie et al. 2003; Chawanji et al. 2005, 2006; Araújo et al. 2010; Zhang & Dai, 2012).

In *B. anurum* the spermatozoa measure 510 μm in length, longer than those in the Belostomatidae *Muljarus japonicus* (Vuillefroy) with 220 μm (Lee & Lee, 1992) and *Diplonychus esakii* Miyamoto & Lee, 1966 with 260 μm (Lee & Lee, 1988). However, the spermatozoa nucleus length (ca. 35 μm) of *B. anurum* is similar to those from *M. japonicus* (Lee & Lee, 1992). Considering that the nucleus has DNA and, generally, in the same species the quantity of DNA is constant (Barcellos et al. 2015), the similar size of the spermatozoa nuclei in *B. anurum* and *M. japonicus* indicates a possible closely phylogenetic relationships between them and a distance between both and *D. esakii* that has the longer nucleus (ca. 65 μm) (Lee & Lee, 1988).
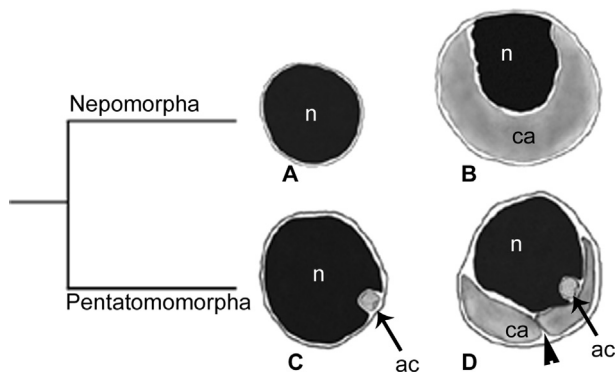
In *B. anurum* and in other Nepomorpha Belostomatidae (Lee & Lee, 1992; Lee & Lee, 1988), the acrosome does not prolong along the entire nucleus length such as reported to occurs in the Penta-tomomorpha Largidae (Araújo et al. 2012) and Plataspididae (Dias et al. 2016), suggesting that this is a more derived characteristic because Pentatomomorpha is more derived than Nepomorpha (Schu et al. 2009).

In the nucleus–flagellum transition of the spermatozoa of some insects, an electron-dense structure, the centriole adjunct, usually connect the flagellar elements with the head of the spermatozoa. In *B. anurum*, the centriole adjunct is a single not bifurcated C-shaped structure likely found in the Belostomatidae *M. japonicus* and *D. esakii* and Nepidae *Laccotrephes japonensis* Scott, 1874 and *Ranatra chinensis* Mayr, 1865 (Lee & Lee, 1992; Lee & Lee Lee, 1987). On the other hand, in the Pentatomomorpha the centriole adjunct has a cleft, resulting in a bifurcated structure (Cossolin, 2015; Araújo et al. 2012; Dias et al. 2016).

In addition to conserved structures in the spermatozoa of Heteroptera, including the presence of two opposite bridges in the axoneme connecting the mitochondrial derivatives, absence of accessory bodies in the flagellum, and presence of paracrystalline material in the matrix of the mitochondrial derivatives, our results show some important features of *B. anurum* occurring in Nepo-morpha. The acrosome not prolonged laterally along the entire length of the nucleus and the non-bifurcated centriole adjunct in the spermatozoa of *B. anurum* are characters that distinguish

Fig. 1. A). Contrast of phase microscopy from *Belostoma anurum* spermatozoa showing the nucleus (n) and flagellum (f). The arrow indicates the nucleus/flagellum transition. B) Nucleus stained with DAPI and acrosome (ac). C-J) Transmission electron micrographs of *B. anurum* spermatozoa in cross sections. C-E Transition acrosome-flagellum from the apex to the base, showing nucleus (n), acrosome (ac) and subacrosomal space (asterisks). F–I) Details of C-shaped centriole adjunct (ca), nucleus (n) with condensed chromatin and the beginning of one mitochondrial derivative (md). J) Cross section of flagella showing the axoneme (ax) with 9 + 9 + 2 microtubule pattern, two mitochondrial derivatives (md) and bridges (arrows) connecting the axoneme to the mitochondrial derivatives. Scale bars: A-B = 5 μm; C-J = 1 μm.

Fig. 2. Schematic reconstructions. Cross section (A, C) of the nucleus region (n) and nucleus—flagellum transition (B, D) showing the absence of the acrosome (ac) prolonging laterally the entire length of the nucleus in Nepomorpha such as occurs in Pentatomomorpha. In Nepomorpha, the centriole adjunct (ca) is a single structure and in Pentatomomorpha it is bifurcated by a cleft (arrowhead).

Nepomorpha from Pentatomomorpha, in which the acrosome prolongs along the nucleus and the centriole adjunct has a cleft resulting in a bifurcated structure (see Fig. 2 for comparison). The total spermatozoa length in *B. anurum* is longer than the other species of Belostomatidae. Overall, this study provided new data contributing to understanding the morphology and systematics of giant water bugs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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