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Anatomical and histochemical descriptions of the male reproductive system of *Diachasmimorpha longicaudata* (Ashmed) (Hymenoptera: Braconidae)

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ABSTRACT

The parasitoid *Diachasmimorpha longicaudata* is an important control agent for several species of fruit flies. Research on the characteristics of the reproductive system and reproductive biology of this parasitoid can be valuable for studies in taxonomy and phylogeny of insects of the order Hymenoptera. In this study we analyzed the histology and histochemistry of the male reproductive system of *D. longicaudata*. In this species the male reproductive system consists of a pair of testes, two accessory glands, and an ejaculatory duct. Each testicle consists of only one follicle. The testicular follicles are filled with cysts in different stages of spermatogenesis. Histochemical analyses detected proteins and carbohydrates in the cytoplasm of secretory cells and in the lumen of accessory glands. The morphology of the male reproductive system of *D. longicaudata* differs in some respects from other species of Hymenoptera.

Introduction

Hymenoptera is one of the four largest insect orders with approximately 115.000 described species (Sharkey, 2007). This order has great ecological and economic importance, is represented by phytophagous insects, predators and parasitoids. Braconidae is one of the most numerous and important families within the order Hymenoptera, representing the second largest family, with about 19.000 described species (Yu, 2021). Braconids form one of the most studied groups of parasitoids, as they have a large number of species with potential to be used in biological pest control programs (Fernandez and Sharkey, 2006).

The parasitoid wasp *Diachasmimorpha longicaudata* (Ashmed) (Hymenoptera: Braconidae) is one of the most important species used in biological control of fruit flies worldwide (Montoya et al., 2000; González et al., 2007; López et al., 2009; Meirelles et al., 2016). This braconid parasitizes second and third-instar larvae of fruit flies of the family Tephritidae (Van Nieuwenhove and Ovruski, 2011). It was introduced into Brazil by Embrapa Mandioca e Fruticultura Tropical in 1994, aiming to control *Anastrepha* spp. and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Carvalho, 2005).

Diachasmimorpha longicaudata has excelled in augmentative-release programs for control of fruit flies of the genera *Bactrocera*, *Anastrepha* and *Ceratitis* (Carabajal-Paladino et al., 2010). Mass-rearing of this parasitoid in the laboratory is necessary to carry out augmentative releases. Therefore, knowledge of the characteristics of its reproductive system and reproductive biology can be valuable for the success of biological-control programs, as it would help to optimize production of this control agent in the laboratory.

The male reproductive system in Hymenoptera, in general, is formed by a pair of testicles, two seminal vesicles, a pair of deferent ducts, and a pair of accessory glands that connect to the deferent duct,

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forming the ejaculatory duct (Moreira et al., 2008; Araújo et al., 2010; Chapman, 2013). Within this general pattern, structural variations in the morphology of the hymenopteran reproductive system occur, and have served as a source of phylogenetic information (Dallacqua and Cruz-Landim, 2003; Tavares et al., 2003; Ferreira et al., 2004). These variations can occur in the number or size of the structures, in the absence of any of them, or in their position along the reproductive system (Moreira et al., 2008). In some cases, for example, the number of follicles per testicle may vary from one, which may occur in Braconidae, Chalcidoidea and Ichneumonidae (Huang et al., 2007; Fiorillo et al., 2008; Moreira et al., 2010; Uzbekov et al., 2017) to four, in most species of Apinae, Mellitinae and Megachilinae (Ferreira et al., 2004; Araújo et al., 2005). Other variations may also occur, such as the duration of sexual maturation, the number of spermatozoa per cyst, and the presence and number of accessory glands (Moreira et al., 2008).

Although there are studies on the morphology, anatomical, histology and ultrastructure characteristics of the male reproductive system of several species of Hymenoptera (Ferreira et al., 2004; Moreira et al., 2008, 2010; Araújo et al., 2010; Gomes et al., 2012; Moreira et al., 2012), few studies have been carried out on the morphology, histology and ultrastructure description of the male reproductive system of species of Braconidae (Maetô, 1987; Huang et al., 2007; Uzbekov et al., 2017).

No study has examined the morphology of the male reproductive system of *D. longicaudata*, which can contribute to knowledge of the reproductive biology and the success of this biological-control agent, and provide useful information for future studies of the taxonomy and phylogeny of Hymenoptera. The present study describes the anatomy and histology of the male reproductive system of the parasitoid *D. longicaudata*.

Materials and methods

Obtaining insects

Parasitoids and hosts used in this study were obtained from the Biological Control Laboratory of the State University of Montes Claros, Minas Gerais, Brazil, where these species are reared under controlled conditions (temperature 26 ± 2 °C, RH 65 ± 10% and photophase 12 h).

Larvae of *Ceratitis capitata* were used to maintain the colony of *D. longicaudata*. To the female parasitoids were offered hosts for 24 h, through an oviposition device that consisted of an embroidery ring 10 cm in diameter and 0.8 cm high, supporting a voile sandwich. Inside this sandwich were *C. capitata* larvae (third instar) and a small amount of artificial diet. After exposure to the parasitoids, the host larvae were transferred to plastic containers containing moist vermiculite and covered with voile to allow ventilation, until the parasitoids emerged. The emerged parasitoids were placed in acrylic cages (29.5 cm × 29.5 cm × 29.5 cm) and fed on artificial diet based on honey and water, following the method proposed by Carvalho et al. (1998).

Light microscopy

In order to analyze and describe the morphology of the male reproductive system of *D. longicaudata*, 30 individuals 6 days old, were dissected in 0.1 M sodium phosphate buffer solution, pH 7.2, under a stereomicroscope (Leica EZ4 HD). Then, the reproductive systems were transferred to a glass container containing Zamboni fixative solution and kept in a refrigerator for 24 h (Stefanini et al., 1967). After this period, they were dehydrated in increasing ethanol concentrations (70%, 80%, 90% and 95%), for 15 min in each concentration. The dehydrated material was placed in plastic microtubes with a capacity of 1.5 ml, and the historesin solution (Leica) was added. The material in these microtubes was refrigerated for 24 h to allow infiltration of the resin. After this period, the material was transferred to a histomold to add historesin and the hardener in a 15: 1 ratio (historesin: hardener), and then transferred to an oven at 60 °C for 48 h, for complete polymerization. The dried material was glued to a small wooden block and sections 2 µm thick were cut with a self-advancing rotary ultamicrotome (Leica RM2255), using glass knives. The sections were transferred to histological slides and stained with toluidine blue for 15 min. The slides were assembled with coverslips, using Permount™ medium, to be analyzed and photographed.

The histochemical analyses were performed with the Xylidine Ponceau test (Vidal, 1970) to identify proteins, and the Periodic Acid Schiff-PAS test (O'Brien and McCully, 1981) to identify neutral polysaccharides. Photographic documentation for the anatomical and histochemical analyses was performed under a light microscope, model OPTICAM 400S, with an OPTICAM 100 camera coupled.

Results

The male reproductive system of *D. longicaudata* consists of a pair of testes connected to two deferent ducts, a pair of seminal vesicles, two accessory glands, and an ejaculatory duct (Figs. 1A and B).

Part of each deferent duct is differentiated into an enlarged region, which constitutes the seminal vesicle, where spermatozoa are stored until copulation. The deferent duct is connected in the median part of the accessory glands, and they later join together to form the ejaculatory duct, which is connected to the external genitalia, the aedeagus (Fig. 1B).

In this species, the testes have an ovoid shape and the two can be together, with the deferent ducts and their respective seminal vesicles covered by a single capsule of connective tissue (Fig. 2A). They can also be separated, that is, each testicle with the deferent duct and seminal vesicle is encapsulated separately, forming two units (Fig. 2B).

Each testis consists of only one follicle (Fig. 3A). Testicular follicles are filled with cysts at different stages of spermatogenesis and each cyst is surrounded by non-germ cells (Figs. 3D and E). In each cyst, all germ cells are at the same stage of spermatogenesis. It is possible to observe cysts with cells in the spermatocyte phase (Fig. 3B), spermatogonia (Fig. 3C), spermatids in bundles (Fig. 3D) and already mature spermatozoa (Fig. 3E).

In the follicles the germ cells are organized into cysts. A considerable variation was found in the number of spermatozoa per cyst, which ranged from 64 to 150 (Fig. 3E). After maturation, the spermatozoa, already free, leave the testicles and migrate to the seminal vesicles, where they are stored until they are transferred to the females (Figs. 4A and B). The fact that the spermatozoa already arrive free in the seminal vesicles and not in bundles, is evidenced by the presence of individualized spermatozoa inside the deferent ducts (Fig. 4C).

The epithelium of the deferent ducts, through which spermatozoa leave the testes, is formed by squamous cells (Fig. 4C). The seminal vesicles are tubular, have lumen completely filled with free spermatozoa, and their walls are composed of simple epithelial tissue with pavement cells (Figs. 4A and B).

The accessory glands are connected to the ejaculatory duct and are well developed. They are tubular and consist of pavement epithelial cells, and the lumen is filled with a granular secretion (Figs. 5A and D). The accessory gland walls have a large amount of secretory vesicles (Fig. 5C). The histochemical test (Xylidine Ponceau) showed the presence of proteins in the cytoplasm of the secretory cells, as well as in the secretion of the lumen of the accessory glands (Figs. 5A-C). The PAS test was positive for the secretion of the lumen and for the substances present in the apical region of the secretory cells (Figs. 5D and E).

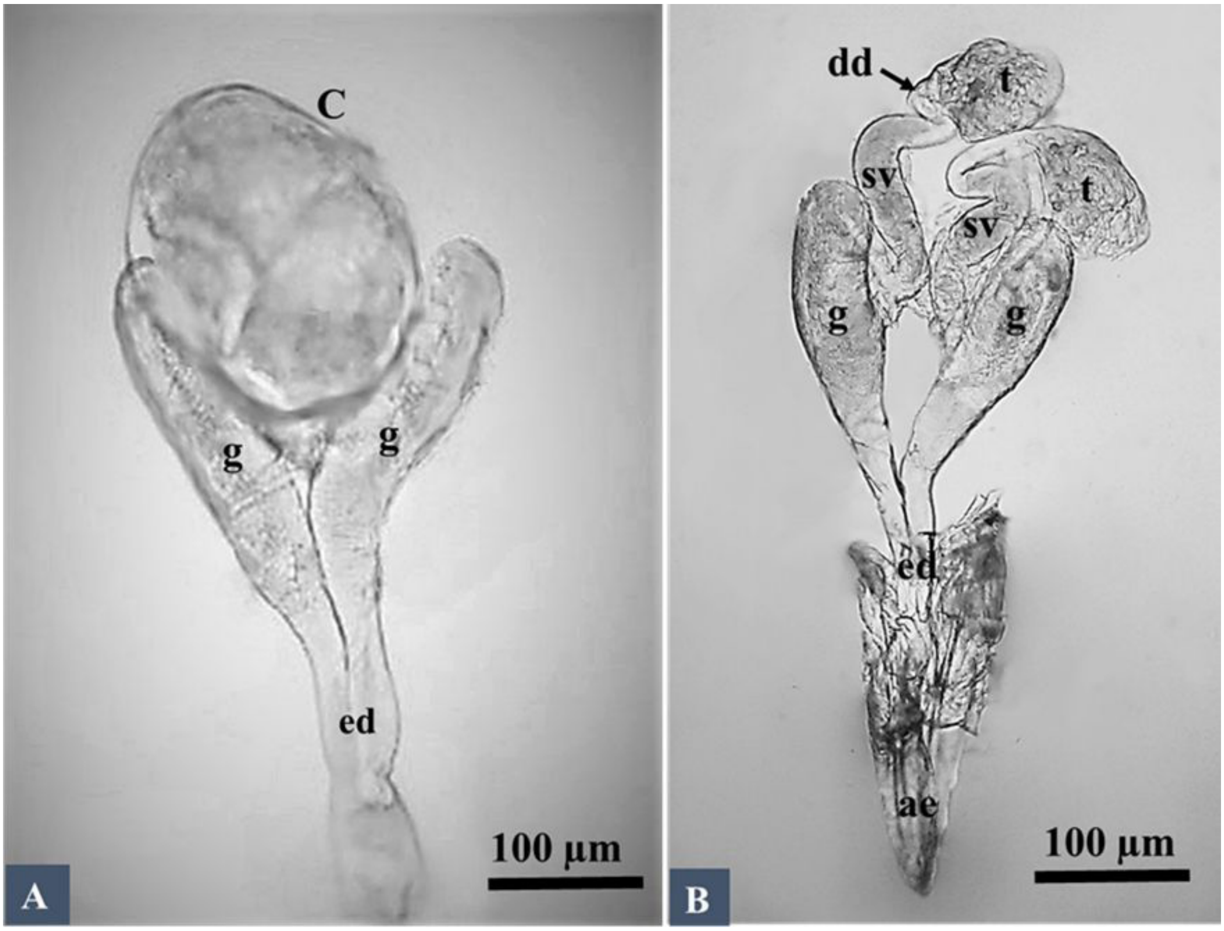

Figure 1 Photomicrograph of the male reproductive system of *Diachasmimorpha longicaudata*. **A.** The testes and seminal vesicles are surrounded by the capsule (c), accessory gland (g), ejaculatory duct (ed), **B.** testicles after capsule rupture. Testis (t), deferent duct (dd), seminal vesicle (sv), accessory glands (g), ejaculatory duct (ed) and aedeagus (ae).

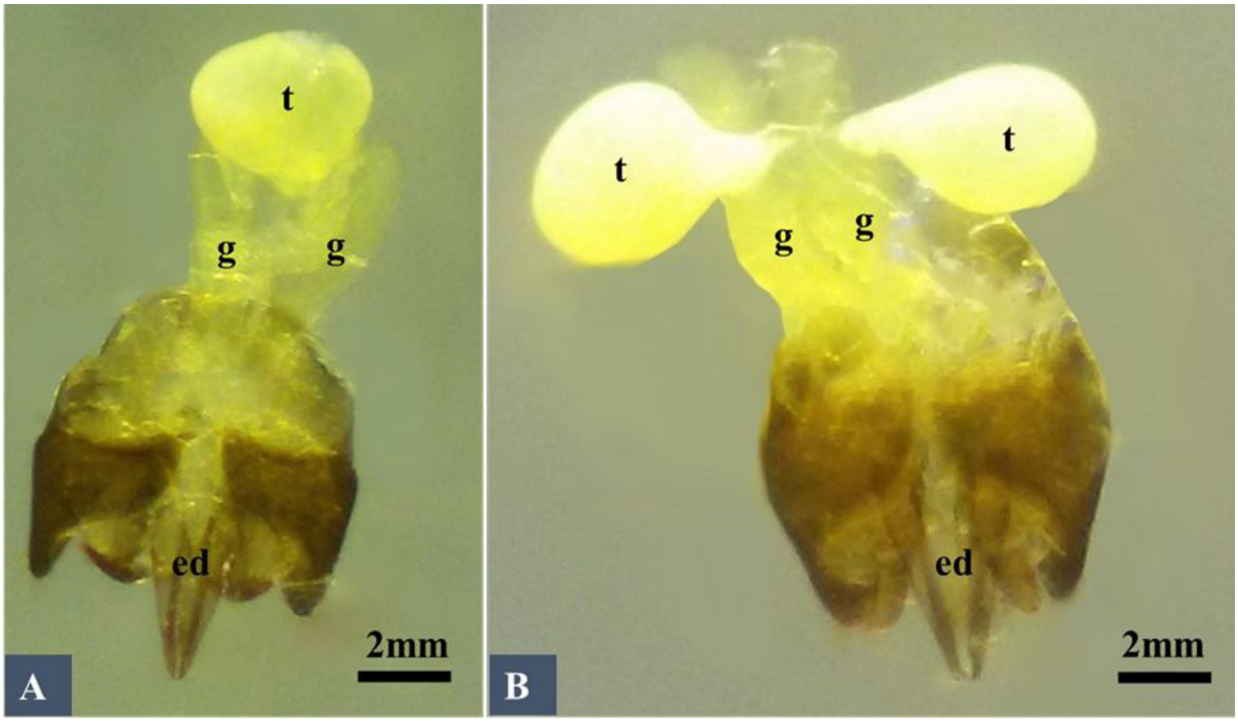

Figure 2 The general view of the reproductive system of *Diachasmimorpha longicaudata*. **A**. The two testicles, are surrounded by a single capsule of connective tissue; **B**. Each testicle, seminal vesicle and deferent duct are encapsulated separately. Testicle (t), accessory glands (g), ejaculatory duct (ed).

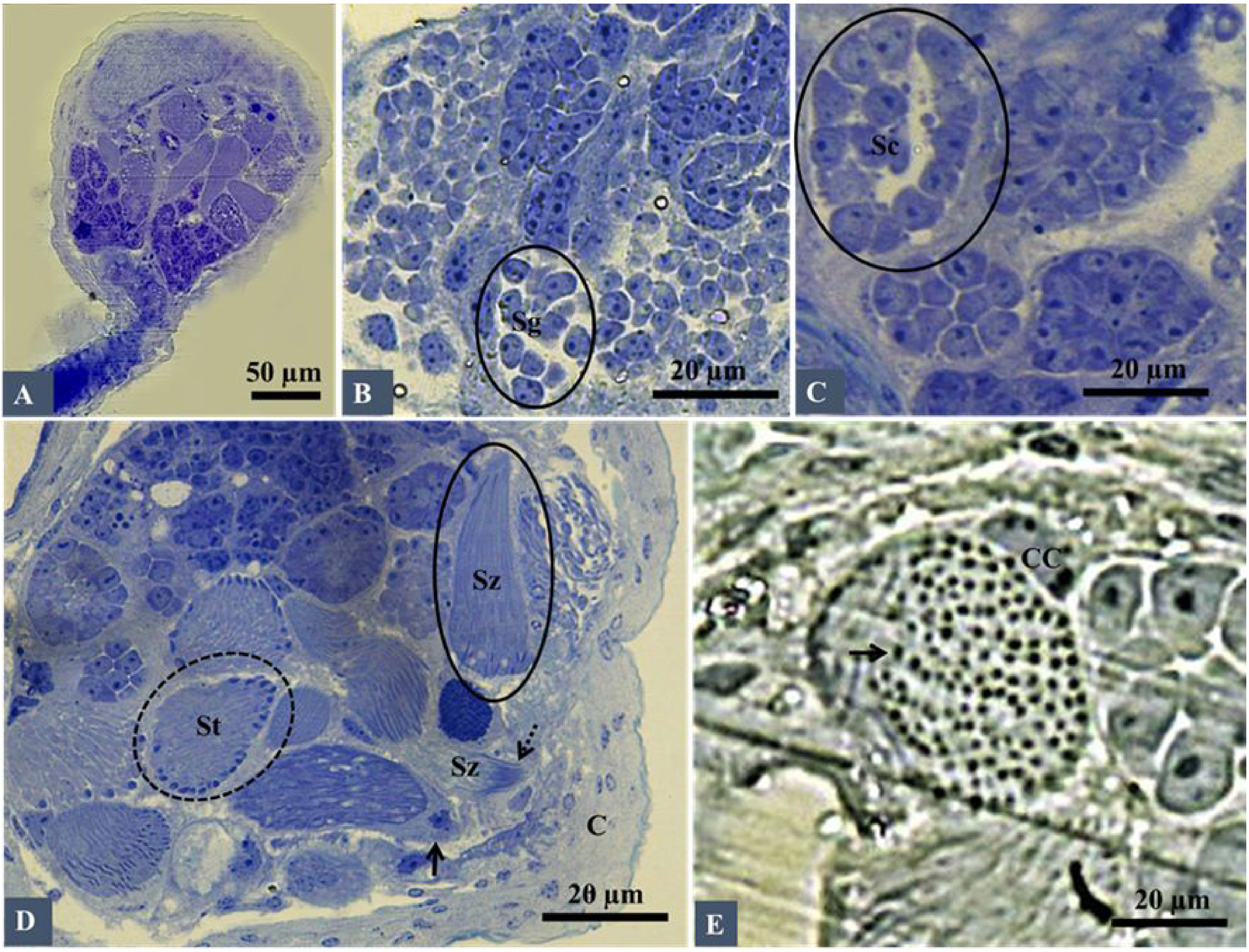

Figure 3 Histology of the male reproductive system of *Diachasmimorpha longicaudata*. **A**. Longitudinal section of the testicle, showing the presence of only one follicle. **B.** Germarium region with spermatogonia from the testicular follicle (circle). Spermatogonia (Sg). **C.** Cysts with spermatocytes in the testicular follicle growth zone (circle). Spermatocytes (Sc). **D.** Testicular follicle with cysts at different stages of spermatogenesis: Cystic cell (arrow continues), spermatids (dashed circle), spermatozoa in bundles with elongated shape (continuous circle), spermatozoa bundles (dotted arrow), conjunctiva capsule (C). Spermatids (St); Spermatozoa (Sz). E. Cyst containing mature spermatozoa (arrow), cystic cell nucleus (CC).

Discussion

The general morphology of the male reproductive system of the parasitoid *D. longicaudata* is similar to other species of Braconidae (Huang et al., 2007; Uzbekov et al., 2017), characterized by the presence of a pair of testicles, two deferent ducts, and two seminal vesicles, followed by two well-developed accessory glands and an ejaculatory duct.

Here, we observed that *D. longicaudata* can present the two testicles together, covered by a single capsule of connective tissue, or they can be encapsulated separately. This characteristic has not yet been reported in the literature for any species of parasitoid Braconidae or in the order Hymenoptera. In *Cotesia vestalis* (Haliday) (Hymenoptera, Braconidae) and *Cotesia congregata* Say (Hymenoptera, Braconidae) each testicle and the seminal vesicles are involved separately by the conjunctive capsule (Huang et al., 2007; Uzbekov et al., 2017). Ferreira et al. (2004) reported variations in the morphology of the male reproductive system among several species of bees, even being from the same family.

The morphology of the male reproductive system of Hymenoptera differs considerably among species. These differences may be related to the presence, size or shape of the structures, as well as their position along the reproductive tract (Ferreira et al., 2004; Araújo et al., 2005; Bushrow et al., 2006; Moreira et al., 2008; Chapman, 2013).

The number of follicles per testicle may vary among the groups of Hymenoptera. In *D. longicautada*, only one follicle was observed in each testicle. Other studies of parasitic wasps have also reported only one follicle per testicle (Huang et al., 2007; Fiorillo et al., 2008; Moreira et al., 2010; Paoli et al., 2013; Uzbekov et al., 2017), which indicates that it is a common morphological characteristic among parasitoids. On the other hand, in bees the number of follicles can vary considerably. The presence of three follicles per testicle has been reported in bee species from the subfamilies Colletinae, Andreninae and Halictinae (Ferreira et al., 2004; Araújo et al., 2005), and in Crabronidae wasps (Moreira et al., 2008). In most species of the subfamilies Apinae, Mellitinae and Megachilinae, four follicles were observed (Ferreira et al., 2004; Araújo et al., 2005); exceptions were observed in *Hypanthidium faveolatum* (Alfken), with 28 follicles (Gracielle et al., 2009), and *Apis melífera* (Linnaeus) (Apidae), with about 250 follicles in each testicle (Chapman, 2013). Thus, within the order Hymenoptera, differences

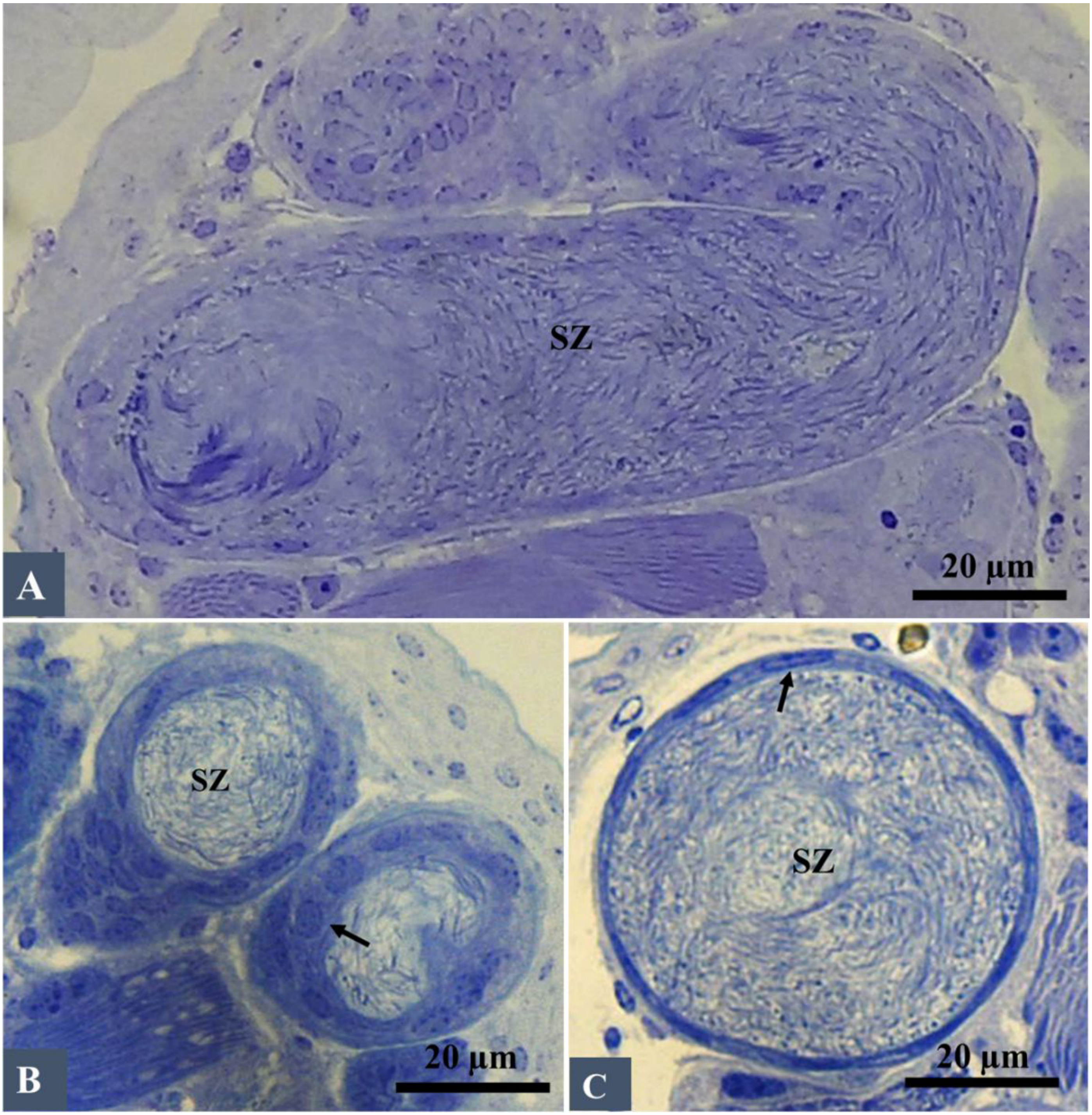

Figure 4 Histology of the seminal vesicle and the deferent duct of *Diachasmimorpha longicaudata*. **A**. Longitudinal section of the seminal tubular vesicle filled with free spermatozoa (sz), **B.** Cross section of the seminal vesicle, showing cubic epithelial cells (arrow). **C**. Deferent duct containing free spermatozoa (sz) and pavement epithelial cells (arrow).

in the number of follicles in each testicle is a characteristic that can separate families (Ferreira et al., 2004).

The presence of cysts at different stages of spermatogenesis in the testicles of *D. longicaudata* indicates that spermatozoa production in this species is continuous, as spermatozoa production was observed both in newly emerged males (one day old) and at six days old. Carbajal-Paladino et al. (2017) observed cysts at different stages of spermatogenesis in *D. longicaudata* aged 11 days. Continuous spermatozoa production generally occurs in species that have a long adult life and copulate throughout this phase (Buschini, 2007; Moreira et al., 2008). However, males that copulate for a short period produce spermatozoa only once, with the onset of spermatogenesis in the pupal phase, ending before or shortly after adult emergence (Boomsma et al., 2005; Lima et al., 2006). For example in several species of Hymenoptera, mainly social ones, males produce spermatozoa only once and then the testicles begin to degenerate immediately after the spermatozoa migrate to the seminal vesicles (Cruz-Landim and Dallacqua, 2002; Araújo et al., 2005; Lima et al., 2006). Species such as these can copulate many

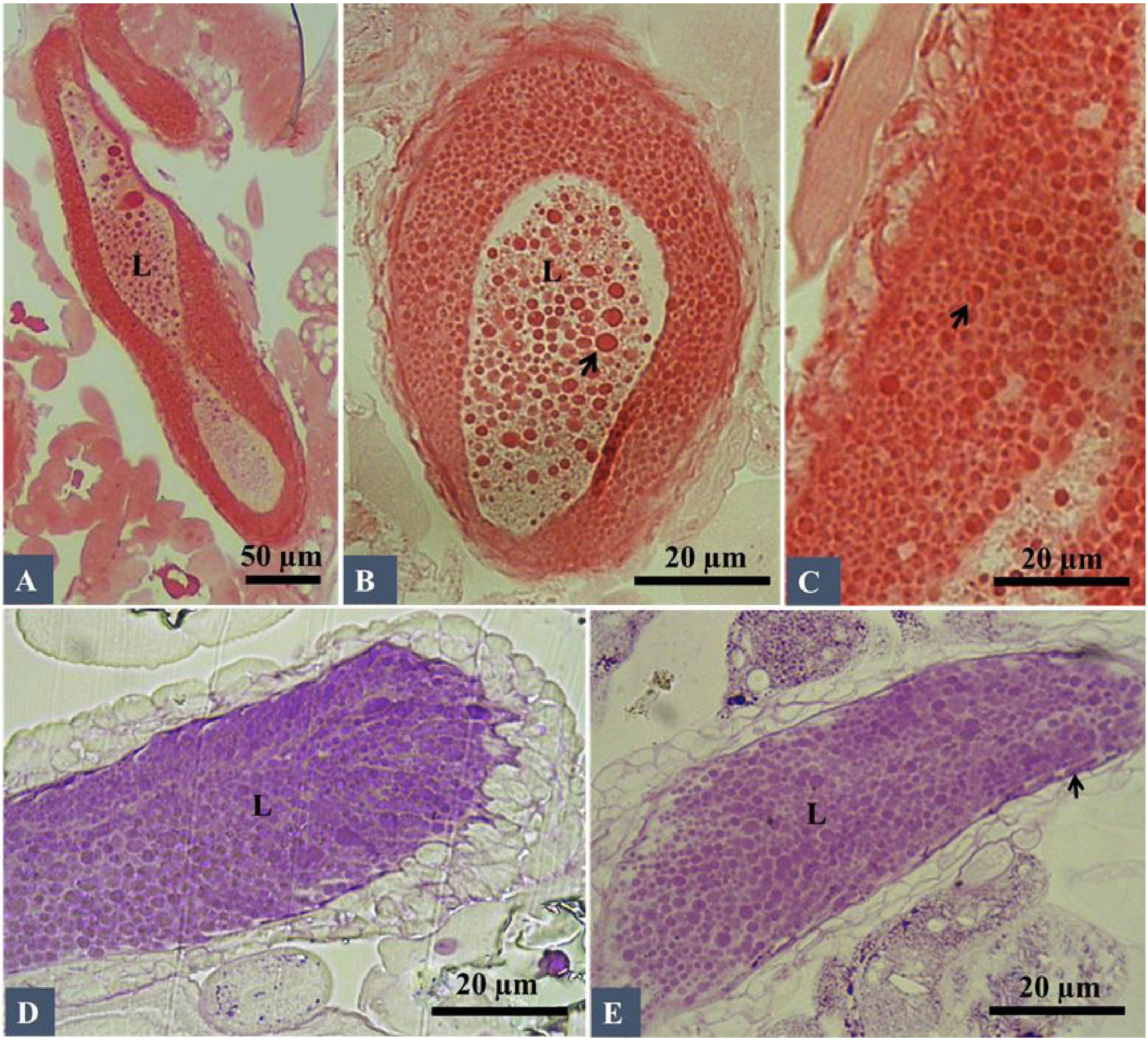

Figure 5 Histochemistry of the accessory glands of *Diachasmimorpha longicaudata*. **A.** Longitudinal section and **B.** cross section of the accessory gland stained with Xylidine Ponceau. Lumen (L), protein secretions (arrow). **C.** Accessory gland wall containing secretory vesicles (arrow). **D** and **E.** PAS. positive for secretions and substances present in the apical region of secretory cells, and pavement epithelial cells (arrow).

times, however, within short intervals, as they do not replenish the spermatozoa stock in the seminal vesicles (Baer, 2003).

Spermatogenesis occurs in the testicles, in compartments called cysts. In the present study, we observed cysts with cells in different stages of differentiation, that is, cysts with spermatogonia, spermatocytes and spermatids, with a more-elongated shape due to loss of cytoplasm, nuclear compaction and flagellar formation, which indicates maturation for the formation of spermatozoa. Other research reports a similar sequence of events in the male reproductive system of other insects orders, such as Coleoptera and Heteroptera (Ozyurt et al., 2015; Candan et al., 2018; Koçakoğlu et al., 2019, 2021). According to Chapman (2013), as the cysts are produced at the apex of the follicle, they displace those that developed previously, so that a series of developmental stages is present in each follicle, with the initial stages in the germ and the oldest in the proximal part of the follicle adjacent to the deferent duct.

The variation in the number of spermatozoa per cyst found in *D. longicaudata* (64 to 150) has also been observed in other hymenopterans. In the parasitic wasp *Arge pagana* (Panzer) (Symphyta), 240 to 350 spermatozoa were found per cyst (Lino-Neto et al., 2008). In the egg parasitoid *Gryon pennsylvanicum* (Ashmead) (Platygastridae) about 64 spermatozoa were found per cyst (Paoli et al., 2013). The presence of 64 spermatozoa per cyst has also been reported in euglossine bees (Zama et al., 2005) and *Xylocopa frontalis* (Olivier) (Fiorillo et al., 2010). According to Gomes et al. (2012), the plesiomorphic number of spermatozoa per cyst in Hymenoptera is 64, as also found in *Trypoxylon* (*Trypargilum*) Richards (Moreira et al., 2008) and *Microstigmus arlei* (Richards) (Zama et al., 2007). However, in some members of Vespidae up to 128 spermatozoa were observed (Brito et al., 2005), as in *Polistes versicolor versicolor* (Olivier) (Araújo et al., 2010), and in Apidae, tribe Meliponini (Lino-Neto et al., 2008). The number of spermatozoa per cyst is determined by the number of cell divisions and is constant for each species; however, it can vary from species to species. Therefore, this number has been used as supplementary information in the systematics of Hymenoptera (Zama et al., 2007; Lino-Neto et al., 2008).

When the spermatozoa are formed, the cysts rupture and the spermatozoa travel to the deferent ducts and then to the seminal vesicles, where they are stored until they are transferred to the females during copulation. We observed that in *D. longicaudata*, the spermatozoa leave the testicles individually and remain free in the seminal vesicles. This was evidenced by the presence of free spermatozoa in the deferent ducts. This characteristic has also observed in other species of Hymenoptera (Lino-Neto et al., 1999; Fiorillo et al., 2008; Moreira et al., 2010), including braconids (Uzbekov et al., 2017), all belonging to the group of parasitic wasps. It is believed that this characteristic may be common in parasitic wasps, as in these insects there is still no report of the occurrence of sperm bundles, either in the seminal vesicles or in the deferent ducts (Schiff et al., 2001). According to Lino-Neto et al. (2008), it is likely that the organization of sperm in bundles in seminal vesicles is an ancestral characteristic common to Hymenoptera, and its absence in parasitic wasps would be a more recent apomorphy. In most hymenopterans, the spermatozoa are released from the testicles in bundles. Aculeata release their sperm from the testicles in bundles and they separate only in the seminal vesicles (Cruz-Landim, 2001; Moreira et al., 2004; Zama et al., 2005). In Symphyta, the spermatozoa leave the testicles and remain in bundles in the seminal vesicle (Quicke et al., 1992; Schiff et al., 2001; Lino-Neto et al., 2008). According to Lino Neto et al. (2008), this difference may indicate that the seminal vesicle of members of Aculeata differs from that of Symphyta in its enzyme content and/or the pH of fluids in the lumen.

In Hymenoptera, the seminal vesicles comprise an enlarged and differentiated region of the deferent ducts, usually the median region as observed in *D. longicaudata*. Most, but not all hymenopterans possess seminal vesicles (Moreira et al., 2010). In addition to storing spermatozoa, seminal vesicles can also have other functions, for example nourishing spermatozoa (Gillott, 2005; Chapman, 2013), especially in species that lack accessory glands. This is not the case for *D. longicautada*, which has well-developed accessory glands, formed by cells that contain secretory vesicles throughout their cytoplasm. As with the other organs of the reproductive system, the accessory glands have wide morphological diversity and may be tubular, spherical or oval. In some species, such as meliponine bees (Apidae), accessory glands are absent (Ferreira et al., 2004). Each accessory gland consists of a single layer of epithelial cells, which vary in structure depending on the stage of development and the nature of the secretion produced (Chapman, 2013).

The histochemical test Xylidine Ponceau and PAS of the accessory glands of *D. longicautada* indicated the presence of proteins and carbohydrates in the secretion of the lumen and in the secretory vesicles of cells, respectively. Proteins and carbohydrates have also been found in the accessory glands of other insect species, as the main components of glandular secretion (Marchini et al., 2003; Fernandez and Cruz-Landim, 2004; Cruz-Landim and Dallacqua, 2005; Gillott, 2005; Braswell et al., 2006).

The accessory glands become functional in the early adult life of the insect, and the secretions they produce are transferred to the females along with the spermatozoa during copulation (Gillott, 2005; Chapman, 2013). These secretions have several functions, including induction and acceleration of oviposition, increased fertility, decreased female receptivity or attractiveness, maintenance and activation of spermatozoa, and success in sperm competition (Gillott, 2005; Huang et al., 2007; Avila et al., 2011; King et al., 2011). These secretions may also be related to the male reproductive strategy, contributing to the mechanism that guarantees copulation fidelity and keeps the sperm viable in the female's spermateca for a longer period (Araújo et al., 2010).

It is clear that the male reproductive system of *D. longicaudata* possesses morphological diversity capable of differentiating it from other species of Hymenoptera. An example is the organization of the testicles in the testicular capsule, in which the two may be enclosed by a single conjunctive capsule, or by separate capsules, forming two structures. The morphological characteristics of the male reproductive system of *D. longicaudata* for example the continuous production of spermatozoa, provide information about the reproductive behavior of this species. The diversity of information provided by the morphological characteristics of the reproductive system of this important biological control agent can be used in future studies of behavior, taxonomy and phylogeny of insects of the Hymenoptera group.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contribution statement

MDCS: Investigation; Methodology; Data curation; Formal analysis; Writing - original draft; Writing - review & editing. MPG: Conceptualization; Formal analysis; Writing - review & editing. EAS: Investigation; Methodology; Writing - review & editing. WOB: Investigation; Methodology. TAG: Writing - review & editing. CGC: Investigation; Methodology. CDA: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Data curation; Supervision; Writing - original draft; Writing - review & editing.

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