SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Morphology of Male Reproductive System in Three Species of *Trypoxylon* (*Trypargilum*) Richards (Hymenoptera: Crabronidae)

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Morfologia do Sistema Reprodutor Masculino em Três Espécies de Trypoxylon (Trypargilum) Richards (Hymenoptera: Crabronidae)

ABSTRACT - Variations in the adult male reproductive system among different groups of Hymenoptera offer characteristics that help studies on behavior and phylogenetics. The objective of this study was to describe the adult male reproductive system of three *Trypoxylon* (*Trypargilum*) species. For that, tissues were disseced, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 and postfixed in 1% osmium tetroxide. The material was dehydratated and embedded for light and electron transmission microscopes. The species have similar reproductive systems, which are formed by a pair of testes, each one with three fusiforme follicles, from which emerges an efferent duct that later joins forming a deferent duct. The deferent duct opens into an ejaculatory duct. The first half of the deferent duct is enlarged and differentiated in a region specialized in sperm storage, the seminal vesicle. The accessory gland flows in the post-vesicular region of the deferent duct. The testes and vesicles are both covered with a conjunctive capsule. Sexually mature individuals have all spermatogenesis stages in their follicles. Sperms are released from testes in bundles which are disorganized inside seminal vesicles.

KEY WORDS: Histology, testes, spheciform wasp

RESUMO - Variações no sistema reprodutor entre os diferentes grupos de Hymenoptera oferecem caracteres que auxiliam nos estudos de comportamento e filogenia. O objetivo deste trabalho foi descrever o sistema reprodutor masculino de três espécies de *Trypoxylon* (*Trypargilum*). Para isso, os tecidos foram dissecados, fixados em glutaraldeído 2,5% em tampão cacodilato de sódio 0,1 M, pH 7,2 e pós-fixados em tetróxido de ósmio a 1%. O material foi desidratado e incluído para microscopias de luz e eletrônica de transmissão. As espécies possuem os sistemas reprodutores muito semelhantes, formados por um par de testículos, cada um com três folículos fusiformes, a partir dos quais emerge um ducto eferente que depois se juntam formando o ducto deferente. O ducto deferente termina no ducto ejaculatório. A primeira metade dos ductos deferentes é dilatada e diferenciada em uma região especializada no armazenamento de espermatozóides, a vesícula seminal. A glândula acessória desemboca na região pós-vesicular do ducto deferente. Testículos e vesículas seminais são envoltos por uma única cápsula conjuntiva. Indivíduos maduros sexualmente apresentam todos os estágios da espermatogênese em seus folículos. Os espermatozóides são liberados dos testículos em feixes, os quais estão desorganizados na vesícula seminal.

PALAVRAS-CHAVE: Histologia, testículo, vespa esfeciforme

The *Trypoxylon* Latreille genus has wide geographic distribution (Bohart & Menke 1976) and more than 660 species have been reported (Hanson & Menke 1995) in the *Trypoxylon* and *Trypargilum* Richards subgenera. The female *Trypoxylon* supply their nests with spiders; the nests are built completely with mud (Bohart & Menke 1976) or in pre-existing cavities, such as those made in wood by other insects.

The Hymenoptera reproductive system is usually formed by one pair of testes, two deferent ducts and one ejaculatory duct. There is a seminal vesicle in each deferent duct, where the spermatozoa are stored until copulation and in each duct opens an accessory gland. However, there are structural variations in this pattern among groups of Hymenoptera, which have been used as sources of phylogenetic information (Cruz-Landim & Cruz-Hofling 1969, Gotwald & Burdette

1981, Wheeler & Krutzsch 1992, Duvoisin *et al*. 1999, Baer & Schmid-Hempel 2000, Cruz-Landim 2001, Cruz-Landim & Dallacqua 2002, Baer 2003, Dallacqua & Cruz-Landim 2003, Tavares *et al*. 2003, Ferreira *et al*. 2004).

Ferreira *et al*. (2004) in a study on 51 bee species from six families (according to the Michener classification, 1965) divided the male reproductive systems into four types according to the number of testicular follicles and the portion of the deferent duct covered by the scrotal membrane. In addition to this study, there are only two other studies on the male reproductive system of Apoidea, one by Dallacqua & Cruz Landim (2003) and another by Araújo *et al*. (2005) that both report bees. The present study describes the male reproductive system of three species of *Trypoxylon* (*Trypargilum*) and is the first study on the subject in spheciform wasp.

Materials and Methods

Adult male *Trypoxylon lactitarse* (Saussure), *T. rogenhofrei* (Kohl) and *T. aurifrons* (Shuckard) were obtained from trap-nests placed in the Central Apiary of the Federal University of Viçosa, Viçosa, MG and on the farm Bela Vista, Conceição do Castelo, ES, Brazil. The trap-nests were made of bamboo were 10 to 18 cm long and 6 to 12 mm in diameter. Those occupied by *Trypargilum* were taken to the laboratory where they were kept until the emergence of the individuals.

Light microscopy. For the histological analysis, reproductive systems from male adults were fixed for 12h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 and post fixed in 1% osmium tetroxide. They were then dehydrated in increasing alcohol concentration and embedded in Historesin® (GMA, Leica). Semithin sections were stained with 1% sodium toluidine borate and amounted in Entelan® (Merck). The analysis and photographic records were made in an Olympus CX-31 microscope. For the anatomical analyses, shortly after fixing, some reproductive systems were photographed under an Olympus CX-31 microscope and an Olympus SZ40 stereoscopic microscope.

Transmission electron microscopy. Seminal vesicles were fixed for 3h in a solution of 2.5% glutaraldehyde, in 0.1 M cacodylate buffer, pH 7.2, 0.2% picric acid, 3% sucrose and 5 mM $CaCl_2$. They were post fixed in 1% osmium tetroxide in the same buffer and then dehydrated in acetone and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with the Zeiss LEO 906 transmission electron microscope.

Results

The reproductive systems of these three *Trypargilum* species are similar (Fig. 1A). They have two testicles, each one with three fusiform follicles or testicular tubules (Figs. 1B, C). An efferent duct emerges from each follicle (Figs. 1B, C); three efferent ducts join forming the deferent duct (Fig. 1B). The anterior half of the deferent duct is dilated and differentiated in a seminal vesicle, where the spermatozoa are stored until copulation (Figs. 1B, 2D). The seminal vesicle is tubular and presents a fold in the middle region that divides it into two regions, which are parallel to each other and with the testicle (Figs. 1B, D). The seminal vesicles and the testicles are covered by a single conjunctive tissue capsule (Figs. 1A, D). In the deferent duct, shortly after the seminal vesicles region, the accessory gland opens (Figs. 1A, B) and, later, the deferent duct opens into the ejaculatory duct (Fig. 1B).

The follicles are covered by a layer of conjunctive tissue and entirely filled with cysts, which consist of germinative cells covered by somatic cells (Figs. 1C, E). Even in the sexually mature individuals, the follicles are completely filled by cysts at different phases of spermatogenesis. In each cyst, the spermatogenetic process is synchronized with all of the germinative cells in the same differentiation phase. During this process, the cysts migrate from the more anterior region of the follicle to the region close to the efferent duct. Thus, the follicles have differentiated regions along their length, with the youngest cells situated at the tip (Fig. 1C). Each cyst has up to 32 cells in the spermatid phase, a number that is maintained in the spermatozoa phase (Figs. 1E, 2F). At the end of the spermatogenesis a substance is secreted that assumes the shape of a hood, in which the anterior portion of the sperm heads are embedded (Figs. 2E, F). This substance keeps the spermatozoa together in bundles and thus they are transferred to the seminal vesicles (Fig. 2A) where this bundles will become disorganized (Figs. 2D, E). All the ducts present single epithelium, with spherical and basal cellular nuclei. A thin basal membrane separates the epithelial cells from a tunic formed by longitudinal and transversal bundles of muscular fibers (Figs. 2A-E, 3A-C, 4D, F, H). The efferent duct and the pre-vesicle region of the deferent duct have epithelium with cubic cells with cytoplasmatic inclusions, heavily stained with toluidin blue in the tip domain (Figs. 2A-C). The seminal vesicles have histological differences between the anterior and posterior regions of the fold. The anterior region has a homogeneous secretion and the muscular tunic is thin (Figs. 3A, D), while in the posterior region the secretion is dense and heterogeneous and the muscular tunic is thick (Figs. 3C, E). The epithelium is formed by prismatic cells with spherical and basal nucleus. Above the nucleus, there are myelin figures and secretion vesicles with varied contents. At the tip, a large quantity of mitochondria is observed and the plasmatic membrane has microvilli (Figs. 3A, B). After the seminal vesicle, the deferent duct becomes narrow, almost obliterated, and projects into the region of the accessory gland insertion.

The accessory glands are oval (Fig. 4A) with epithelium with prismatic cells with small nucleus located in the basal third, and many secretion granules throughout the cytoplasm (Figs. 4B, C, E).

The deferent duct, after the glandular opening, has cubic cells with fairly evident microvilli (Figs. 4C, F). The deferent duct opens into the ejaculatory duct (Fig. 4G), whose epithelium is completely covered by a thin cuticle (Fig. 4 H).

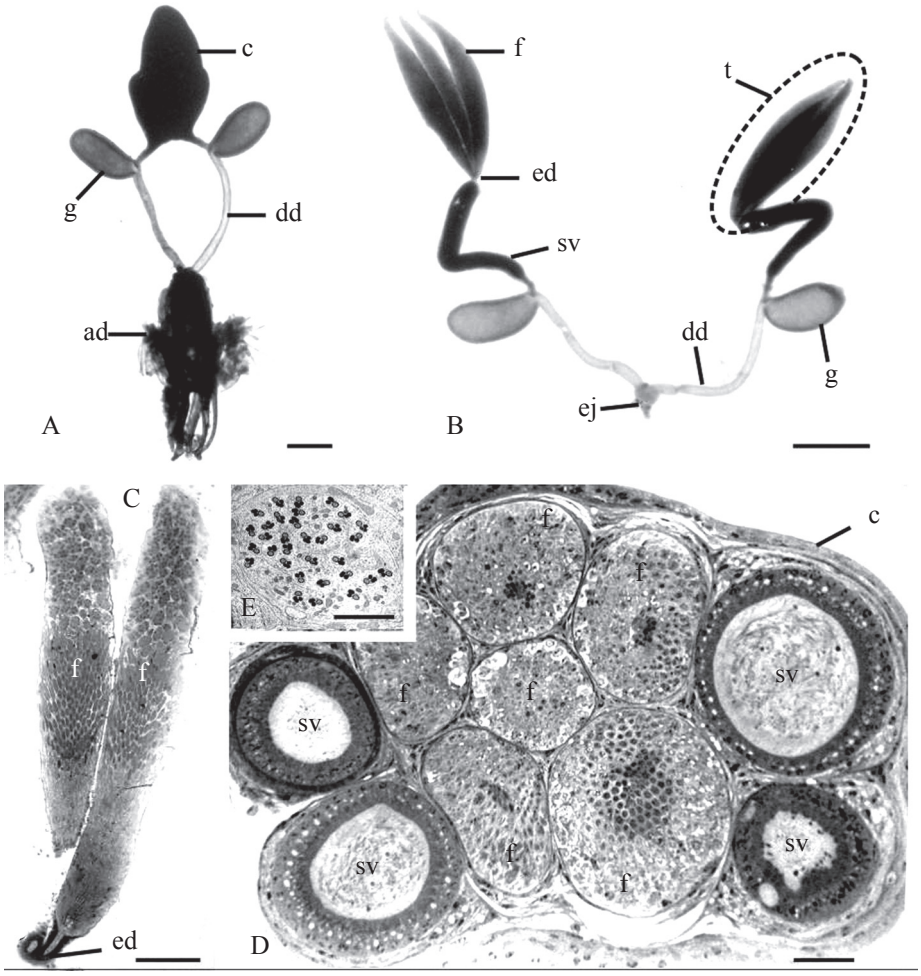

Fig. 1. Anatomy and histology of the reproductive system of male *Trypoxylon* (*Trypargilum*). A - The testicles and seminal vesicles are wrapped in a capsule (c) the accessory glands (g), the deferent duct (dd) and the aedeagus (ad). B - The capsule is broken, showing the testes (t) and its three follicles (f), the efferent duct (ed) seminal vesicles (sv) and the ejaculatory duct (ej). C - Longitudinal section of a follicle (f) where the different maturity zones of the spermatozoa and their release into the efferent duct (ed) are observed. D - Cross section of the encapsulated region, showing the organization of the seminal vesicles (sv) and the testicular follicle (f). E - Cross section, in the flagellum region, of a cyst with 32 spermatozoa. Accessory gland = g; capsule = c; deferent duct = dd; aedeagus = ad; follicle = f; efferent duct = ed; seminal vesicles = sv; testes = t; ejaculatory duct = ej. Bars: A and B = 0.4 mm; C = 0.2 mm; D = 50 µm; E = 2 µm.

Discussion

The reproductive system of the species studied here is similar to that of Type I reported for bees of the Colletidae, Andrenidae, Halictidae and Megachilidae families (Ferreira *et al*. 2004). It is particularly similar to that of the Colletidae because of the presence of three follicles in the testicle, a single capsule covering the testicles and seminal vesicles, whose diameter is similar to those of the deferent duct. Furthermore, in both cases the post-vesicles deferent duct is short and opens in the first part of the accessory gland. The similarity observed among the bees and in these three wasp species coincide with the morphological structural and molecular data that indicated the Crabronidae as a brother group of the bees (Lomholdt 1982, Melo 1999, Michener 2000). Further, Colletidae is considered a more basal group of bees (Michener 2000) what is reinforced by the similarity between the Crabronidae reproductive system and that of these bees.

The number of spermatozoa per bundle may present interspecific variations that have been used for phylogenetic analyses (Cruz-Landim 2001, Schiff *et al*. 2001). In the species studied here, up to 32 spermatozoa were found per bundle. However, we observed up to 128 spermatozoa per bundle in *Sceliphron laetum* Smith (Sphecidae *stricto sensu*). In most of the corbiculada bees, the bundles have up to 64 spermatozoa, except in Meliponi where up to 128 spermatozoa are observed in the bundle (Cruz-Landim 2001). According to Virkki (1970, 1973), more derived insects tend to have fewer spermatozoa per bundle than the more basal insects. This coincides with that observed for *Trypoxylon* and *Sceliphron*, because the Sphecidae are considered more basal than the Crabronidae (Lomholdt 1982, Melo 1999).

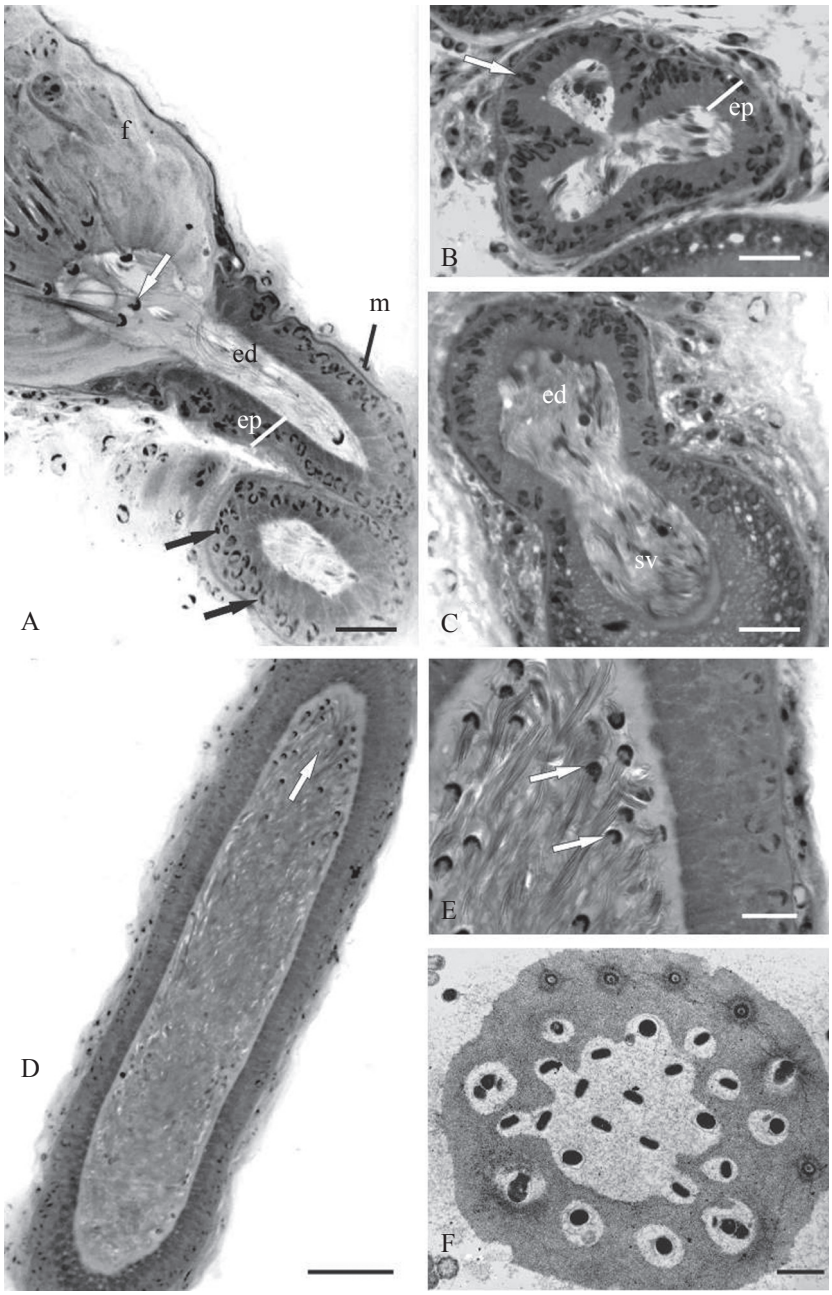

Fig. 2. A - Longitudinal section of the posterior region of a follicle (f) with its respective efferent duct (ed) and transversal section of a efferent duct (ed) where the epithelium (ep) is observed with its basal nuclei (black arrows), wrapped in a muscular layer (m) the white arrow shows a spermatozoa bundle migrating to the efferent duct. B - Cross section of the junction region of the three efferent ducts, forming a deferent duct, observe the base nuclei (arrow) in the epithelium (ep). C - Longitudinal section in the transition region between the initial region of the deferent duct (dd) and the seminal vesicles (sv) observe the difference between the epitheliums. D - Longitudinal section in the seminal vesicle, showing the anterior region with several spermatozoa bundles (arrow) and the posterior region where free spermatozoa predominate. E - Detail of the previous photography, note the sickle shaped material region of the spermatozoa bundles (arrow). F - Cross section of a spermatozoa bundle, where the spermatozoa are observed cut in the head region, covered on the outside by a nuclear protein substance. Epithelium = ep; efferent duct = ed; muscular layer = m; follicle = f; seminal vesicles = sv. Bars: A-C = 25 μm; D = 50 μm; E = 10 μm; F = 0.5 μm.

The continuous spermatozoa production in these three species, shown by the presence of cysts in difference phases of spermatogenesis in their testicles, is in line with the observation that these individuals mate throughout the adult phase, which lasts for about two months in *Trypoxylon lactitarse* (Buschini 2007). This behavior was observed in other *Trypargilum* such as *T. monteverdeae* (Brockmann 1992), *T. rogenhoferi* (Garcia & Adis 1995) and *T. vagulum*

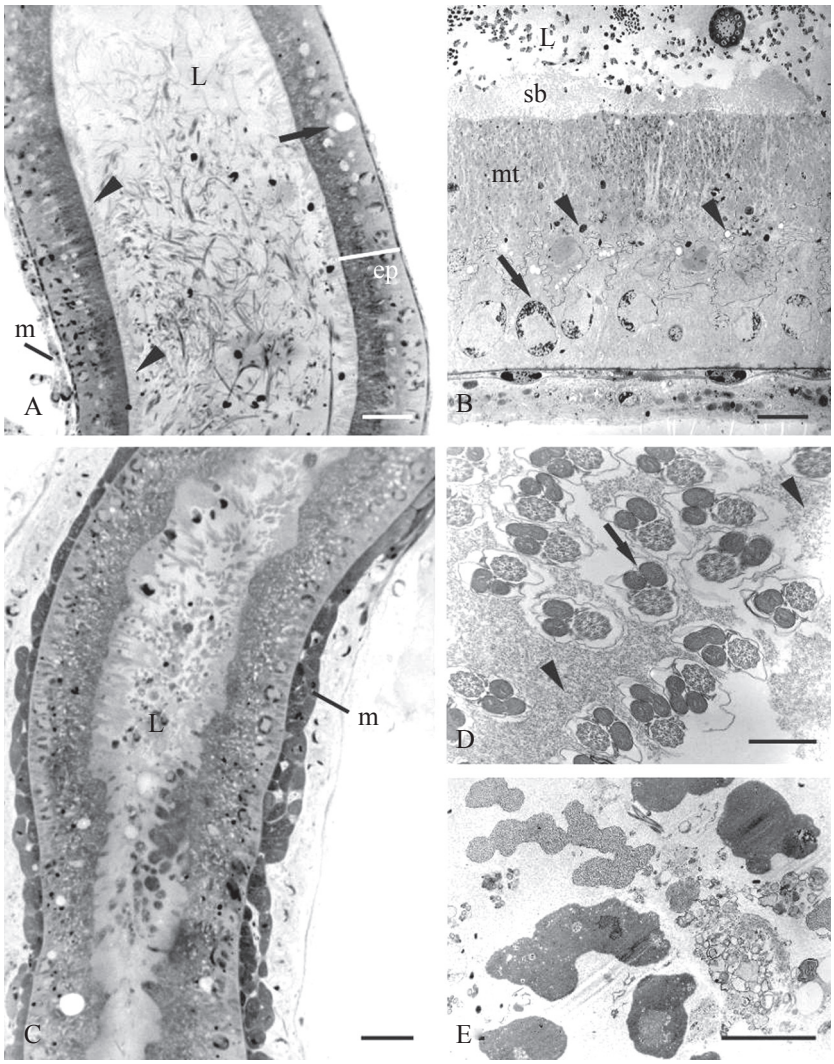

Fig. 3. Light (A and B) and transmission electron (B, D and E) micrographs of seminal vesicles. A - Longitudinal section of the anterior region of the seminal vesicle. In this region the muscular layer (m) is inconspicuous, the epithelium (ep) has several vesicle structures (arrow) and evident striated border (arrowheads). B - The seminal vesicle wall showing the spherical and basal nuclei (arrow), vesicle structures in the middle region (arrowheads) and the mitochondria (mt) and striated border (sb) in the tip region. C - Longitudinal section of the posterior region of the seminal vesicle. In this region the muscular layer (m) is thick. D - Seminal vesicle lumen in the anterior region, showing a homogeneous secretion (arrowheads) covering the spermatozoa (arrow). E - Seminal vesicle lumen in the posterior region showing dense and heterogeneous secretion. Lumen = L; muscular layer = m; epithelium = ep; striated border = b; mitochondria = mt. Bars: A and C = 25 μm; B e E = 5 μm; D = 0.5 μm.

(Coville *et al*. 2000). The *Trypargilum* males, in addition to copulating throughout the adult phase, also copulate several times in a short period of time. This behavior has also been observed in other Hymenoptera, in which the seminal vesicles have a valve or constriction that can regulate the quantity of spermatozoa in the copulation (Baer & Boomsma 2003, Damiens & Boivin 2005). Thus, it can be supposed that, in this species of *Trypargilum*, the fold that divides the seminal vesicle into two regions has the function of regulating the quantity of spermatozoa eliminated during copulation. Furthermore, the thicker muscular tunic in the posterior region of the seminal vesicles in the *Trypargilum* species also contributes to the assumption that only the spermatozoa in this region are transferred to the female in the copulation.

The tip portion of the seminal vesicles epithelium has many mitochondria, that has also been observed in other Aculeata such as *Apis mellifera* Lepeletier (Cruz-Landim & Cruz-Hofling 1969), *Camponotus* spp. (Wheeler & Krutzsch 1992), *Melipona bicolor bicolor* Lepeletier (Dallacqua & Cruz Landim 2003) and *Scaptotrigona xanthotricha* Moure (Araújo *et al*. 2005). It probably indicates high metabolic activity in these cells, involving mainly the regulation of the lumen pH (Wheeler & Krutzsch 1992).

As in most insects, the ejaculatory duct in these species is single, median and presents a cuticle, showing its ectodermic origin. Bushrow *et al*. (2006) reported the presence of two

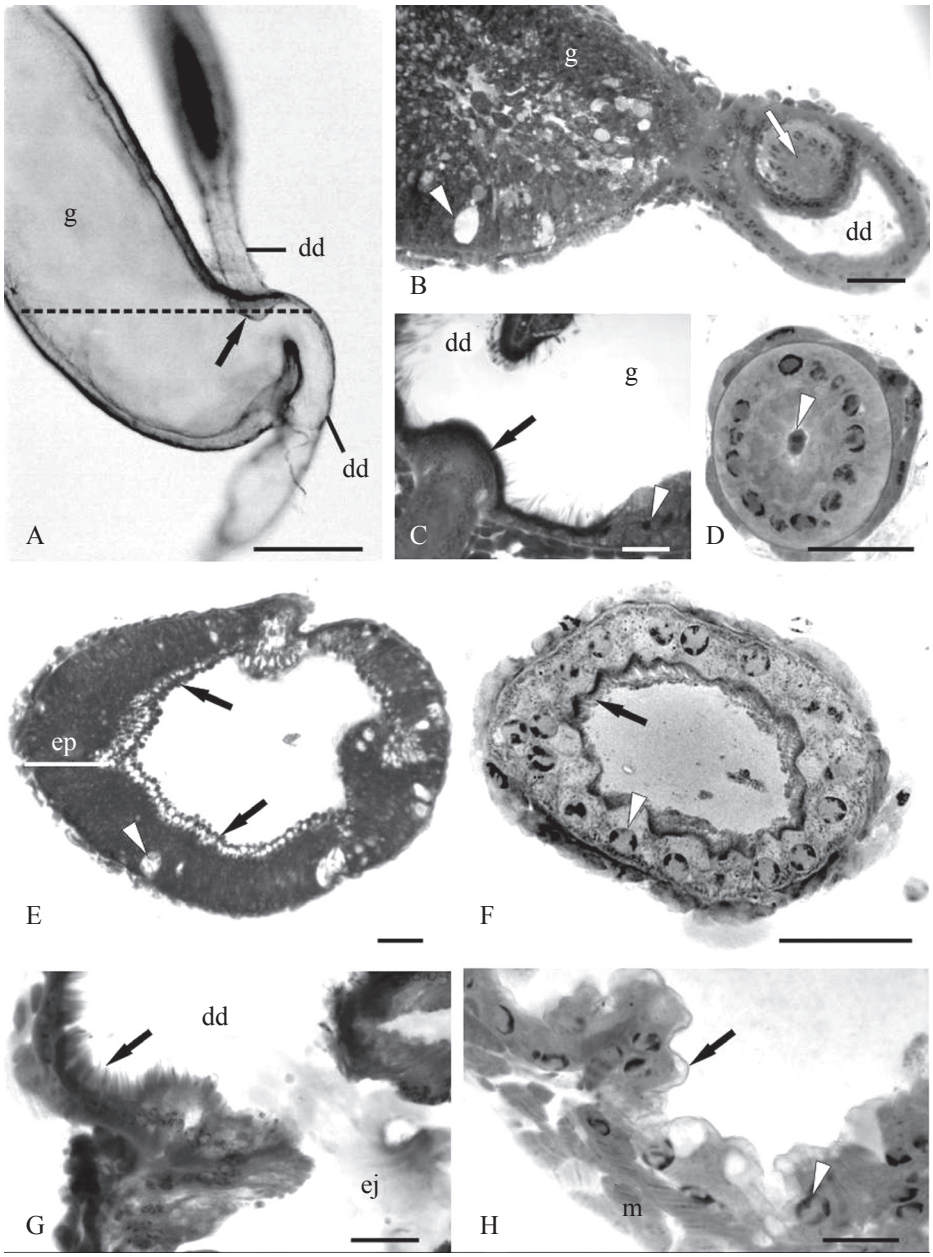

Fig. 4. A - Light micrograph of part of the accessory gland (g) showing its insertion in the deferent duct (dd) and the papilla formed in this region (arrow). B - Transversal section in the traced region of the anterior figure, showing the papilla formed (arrow) and the presence of secretion vesicle in glandular epithelium (arrowheads). C - Longitudinal section in the insertion region of the accessory gland (g) in the deferent duct (dd) where the papilla is again observed (arrow) and the glandular epithelium cells with basal nucleus (arrowhead). D - Transversal section of the post-vesicle deferent duct showing the reduced lumen (arrowhead). E - Cross section of the accessory gland showing epithelial cells (ep) with several secretion vesicles (arrowhead) and apocrine secretion release (arrow). F - Cross section of deferent duct in a region after the insertion of the accessory gland showing epithelial cells with basal nucleus (arrowhead) and the striated border (arrow). G - Longitudinal section of the transition between the deferent duct (dd) and the ejaculatory duct (ej). Note the difference between the epithelium of these regions, with the presence of striated border on the deferent duct. H - Cross section of the ejaculatory duct showing the cuticle at the tip of the epithelium (arrow), the spherical cellular nuclei (arrowhead) and the muscular layer (m). Accessory gland = g; deferent duct = dd; epithelium = ep; ejaculatory duct = ej; muscular layer = m. Bars: A = 0.2 mm; B-E = 50 μm; G and H = 15 μm.

ejaculatory ducts in *Ancistrocerus antilope* Panzer (Vespidae) that began at the base of the anterior accessory glands and joined later forming the common ejaculatory duct later. However, as these authors did not examine the wall of these ducts, we cannot know whether the two ducts that leave the accessory glands have cuticle and, therefore, whether they

are ejaculatory ducts or the posterior region of the deferent duct as we have reported for the three *Trypargilum* species.

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