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Ultrastructural and Functional Aspects of the Spermatheca in the American Harlequin Bug, *Murgantia histrionica* (Hemiptera: Pentatomidae)

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Abstract

The spermatheca of *Murgantia histrionica* (Hahn) was investigated using fluorescence, scanning and transmission electron microscopy. The aim of the study was to elucidate the structure of this organ, pointing out differences between mated and unmated females. Results have shown an elaborated cuticular structure associated with muscular and glandular tissues. The spermatheca is joined with the common oviduct by the spermathecal duct, forming a thin saccular dilation through two consecutive invaginations. The distal part of the organ is formed by a series of two communicating cuticular chambers. The first cylindrical-shaped chamber, corresponding to the coiled region, is wrapped by longitudinal muscular fibers suspended between two cuticular flanges. The contractions of these fibers compress a deformable zone of the cylinder, pumping the sperm toward the spermathecal duct. Without contractions the cylinder results to be isolated from the proximal part of the spermatheca by means of a valve. The second chamber, corresponding to the spermatheca, is made of two parts: a truncated-conical sub chamber, with a constant cuticular thickness, bearing on itself the distal flange, where muscular fibers are attached. The second part is a bulb-like structure wrapped in a glandular epithelium. The secretory units are composed by two cells: a secretory cell and an associated duct cell. Every evacuating duct shows a little reservoir just after the terminal apparatus, and converge inside the distal bulb after a tortuous path. The functional implications of this structure in the reproductive biology of *M. histrionica* are discussed.

Introduction

Host location by insect parasitoids is a process mainly driven by three broad categories of cues, i.e. stimuli from the host microhabitat or from the plant (Potting *et al* 1999, Krugner *et al* 2008), stimuli indirectly associated with the presence of the host (i.e. host traces left on the substrate) (Colazza *et al* 1999, Conti *et al* 2003, Steiner *et al* 2007), and stimuli arising from the host itself (Godfray 1994, Meyhöfer & Casas 1999, Conti *et al* 2003, Laumann *et al* 2007, Segura *et al* 2007). For egg parasitoids, host location is often mediated by the perception of chemicals, either released as volatile (Colazza *et al* 1997, Fatouros *et al* 2005) or left as a trace on the substrate. In the association *Murgantia histrionica* Hahn - *Trissolcus brochymenae* Ashmead (Hymenoptera: Scelionidae), the parasitoid is able to discriminate between chemical traces (footprints) left by host adult males and females

(Conti *et al* 2003, Salerno *et al* 2009). Additionally, *T. brochymenae* females prefer the chemical traces left by mated females of *M. histrionica,* but that have not laid eggs (Salerno *et al* 2009). In this context, it has been hypothesized that the female parasitoid has evolved a strategy finely tuned with the host's physiological condition for host location.

In fact, during mating the male of *M. histrionica* transfers to the female sperm and other substances produced by glands associated with the male reproductive apparatus. These substances are often stored within specialized structures connected with the female reproductive organs. The spermatheca plays a key role in this view, allowing the maintenance of spermatozoa for long periods of time and an efficient use of them during fertilization (Parker 1970). Besides that, in many insects the spermatheca receives and stores secretion from male accessory glands transferred during mating. Unfortunately, most of the data available in literature dealing with the spermatheca are related to systematics, while the ultrastructure of this organ has been studied in only 11 species belonging to the following five orders: Dyctioptera (Gupta & Smith 1969), Orthoptera (Lay *et al* 1999), Diptera (Clements & Potter 1967, Filosi & Perotti 1975, Kokwaro *et al* 1981, Fritz & Turner 2002), Coleoptera (Happ & Happ 1970, Tombes & Roppel 1971, 1972, Villavaso 1975a,b), and Hymenoptera (Dallai 1975). The structure of this organ is very diversified among insects, varying from simple tube to complex organs (Matsuda 1976, Winterton *et al* 1999, Dallai *et al* 1996). In Heteropterans, the spermatheca has been studied in a few species (Huebner 1980, Gschwentner & Tadler 2000) after the pioneering study of Pendergrast (1957), without the aid of electron microscopy.

The aim of this work was to investigate the fine structure of the spermatheca in *M. histrionica*, using both light and electron microscopy techniques. The elucidation of the spermatheca structure will be of great value to clarify in future investigations the role of the male accessory glands secretions once it is transferred to *M. histrionica* female.

Material and Methods

Insects

Adults of *M. histrionica* were obtained from a laboratory colony maintained under controlled conditions (temperature 24 ± 2°C, 50-60% RH, 16:8 h photoperiod L:D).

Light microscopy

Twelve adult females were used for the observations. Insects were anaesthetized using CO_2 and kept at -18°C until death. Then, individuals were dissected in 2% NaCl physiological saline, removing the spermatheca from the abdomen. The spermatheca soft tissues were digested using a KOH solution (1% w/v) and mounted on a glass slide with a glycerin drop. For the observation, a LEICA® microscope DMLB was used, exploiting the natural fluorescence of the cuticular parts of the spermatheca. Pictures were obtained using a LEICA® ICCA camera, and images were acquired with the software IM 1000 LEICA®. Digital pictures were mounted using "The Gimp 2.4.6" software.

Scanning electron microscopy (SEM)

Twenty-four adult females, twelve mated and twelve unmated, were used for the observations. Insects were anaesthetized using CO_2 and kept at -18°C until death. Then, individuals were dissected in 2% NaCl physiological saline, removing the spermatheca from the abdomen. For each typology (virgin and mated) three different groups were used: 1) no digestion; 2) specimens digestion in a potassium hydroxide solution (1%) 15 min at 65°C; or 3) 5 min at 65°C. Then, specimens were washed in saline and dehydrated in a series of graded ethanol. After dehydration, the specimens were treated with hexamethyldisilazane (HMDS) (Sigma®), allowed to dry under a hood and gold-sputtered using a "Balzers Union® SCD 040" unit. On each aluminum stub, five specimens were mounted. The observations were carried out using a scanning electron microscope Philips® XL 30.

Transmission electron microscopy (TEM)

Twenty adult females, ten mated and ten unmated, were anaesthetized with CO_2 and immediately immersed in a solution of 5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer with 5% sucrose (pH 7.2-7.3). The spermathecae were extracted and left at 4°C for 2h. After rinsing overnight in cacodylate buffer 0.1 M, the specimens were post fixed in 1% osmium tetroxide for 1h, and rinsed in the same buffer (two times, 15 min each). Dehydration in a graded ethanol series was followed by embedding in Epon-Araldite with propylene oxide as bridging solvent. Thin sections (90-120 nm) were taken with a diamond knife Drukker® on a LKB® "Nova" ultramicrotome, and mounted on formvar coated 50 mesh grids.

The sections were investigated with a Philips® EM 208, after staining with uranyl acetate 1.8 % (15 min, room temperature) and lead citrate (0.88 g sodium citrate and 0.66 g lead nitrate in 25 ml distilled water) (5 min, room temperature). Digital pictures (1376 x 1032 pixels, 8b, uncompressed grayscale Tiff files) were obtained using a high resolution digital camera MegaViewIII (SIS®) connected to the TEM.

Results

External morphology

In *M. histrionica*, the spermatheca complex originates as an invagination of the ectoderm. After dissection, the spermatheca complex is enveloped by numerous tracheoles, fat body and connective tissues, that forced us to digest soft tissues for a correct description of the structures. The spermatheca complex consists of three main regions (Fig 1a): a distal region, composed by the spermatheca surrounded by glandular tissues, a medial region, composed by the coiled region delimited by two cuticular flanges, and a proximal region made by the spermathecal duct connected with the common oviduct.

Distal region

In preparations after the complete removal of the soft tissues, the coiled region showed a sub-cylindrical structure (250 µm x 100 µm) (Fig 1b), directly connected with the spermathecal duct (Fig 1c) and characterized by the presence of two laminar expansions (cuticular flanges) (Fig 1b). The distal flange (Ø ≈ 255 µm) is larger than the proximal one (Ø ≈ 175 µm), both having the edge folded toward the centre of the cylinder in a way that one is facing the other (Fig 1b). The distal part of the coiled region looks like a deformable ring; at this level the cuticle shows numerous longitudinal folds (Fig 1d). The spermatheca appears to be wrapped by a dense net of tubular elements (Ø ≈ 0.5 µm): the evacuating ducts of the secretory units that constitute the spermathecal gland (Fig 2a). Each duct ends penetrating the bulb's cuticle through cuticular pores (Fig 2b), and is characterized distally by two elliptical dilations: the end apparatus and a small, spherical reservoir (Ø ≈ 5 µm), where the secretion is temporarily stored (Fig 2c). In partially digested specimens, it is possible to distinguish the single secretory units, similar to spherical bodies (Fig 2d). In mated females, the lumen of the bulb appears full of spermatozoa and the gland epithelium is thicker than in virgin females, in which the bulb is empty (Fig 3a-b). The coiled region lumen (180 µm x 85 µm) is isolated

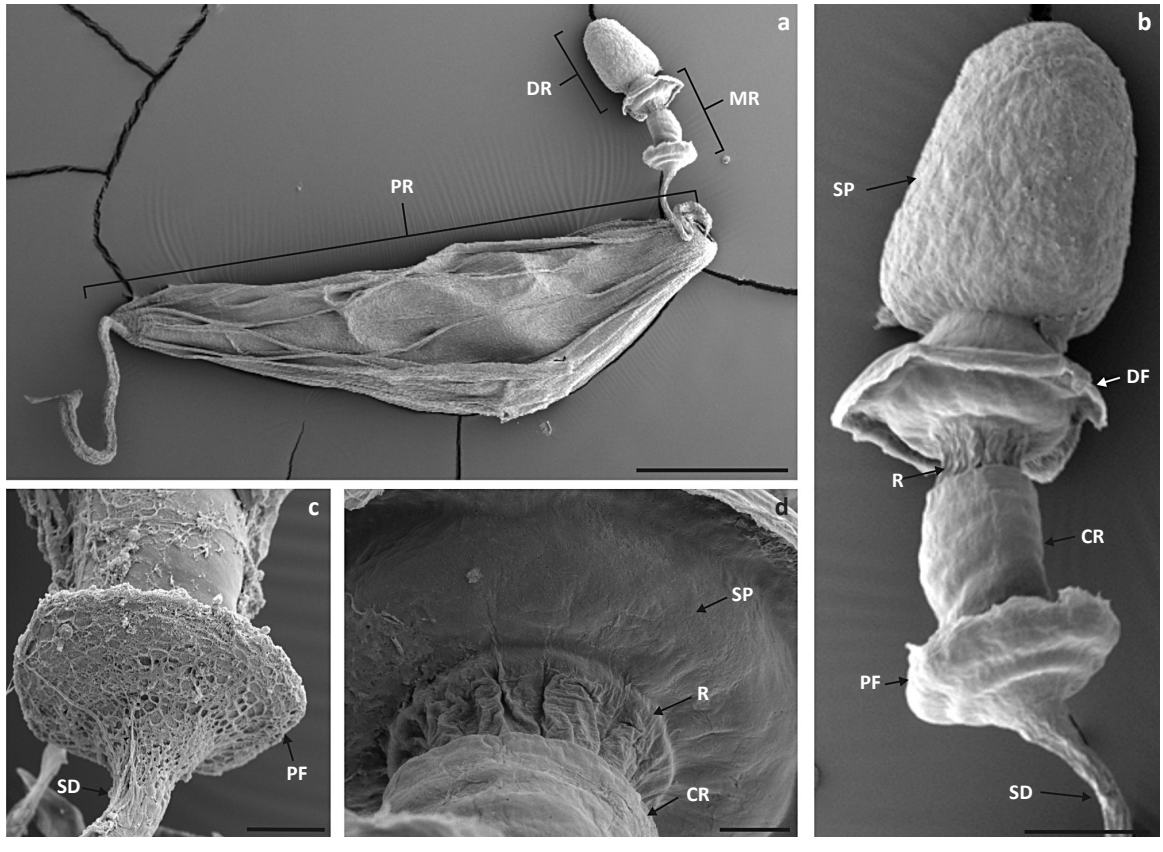

Fig 1 SEM images. a) General view of the spermathecal complex composed by the proximal region (PR), the medial region (MR) and the distal region (DR); b) Distal and medial region of the spermathecal complex with details of the spermathecal duct (SD), the proximal flange (PF), the coiled region (CR), the deformable ring (R), the distal flange (DF) and the spermatheca (SP); c) Detail of the connection between the spermathecal duct (SD) and the proximal flange (PF); d) Particular of the region just below the distal flange in which the deformable ring (R) is visible under the spermatheca (SP). Scale bar a: 500 µm b: 100 µm c: 50 µm d: 20 µm.

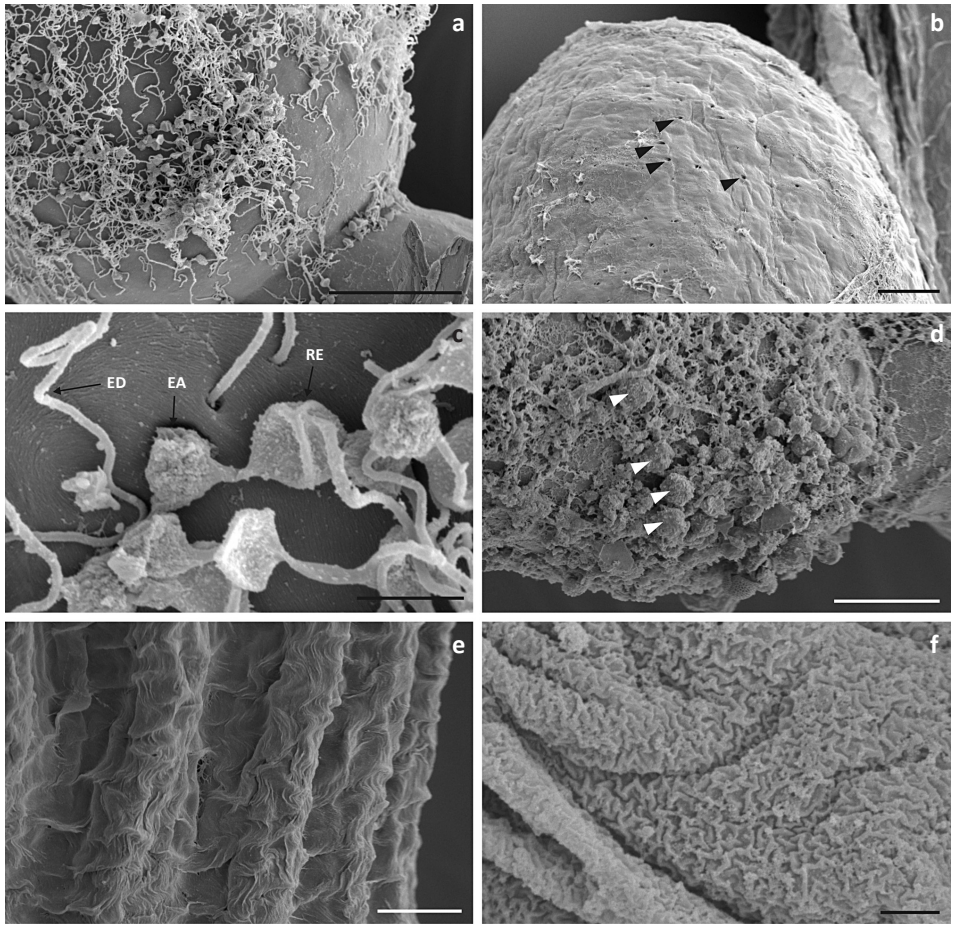

Fig 2 SEM images. a) General view of the spermatheca surface after treatment with KOH, showing the net of the evacuating ducts of the glandular units; b) Surface of the spermatheca after the complete removal of the spermathecal gland: the pores through which the evacuating ducts penetrate the cuticular wall are visible (arrowheads); c) Detail of the evacuating ducts (ED) in which the end apparatus (EA) and the reservoirs (RE) are shown; d) Spermathecal surface after the partial digestion of the cellular components, the remains of the secretory cells are visible (arrowheads); e) External surface of the saccular dilation; f) Internal surface of the saccular dilation. Scale bar a,d: 50 µm b,e: 20 µm c: 5 µm f: 2 µm.

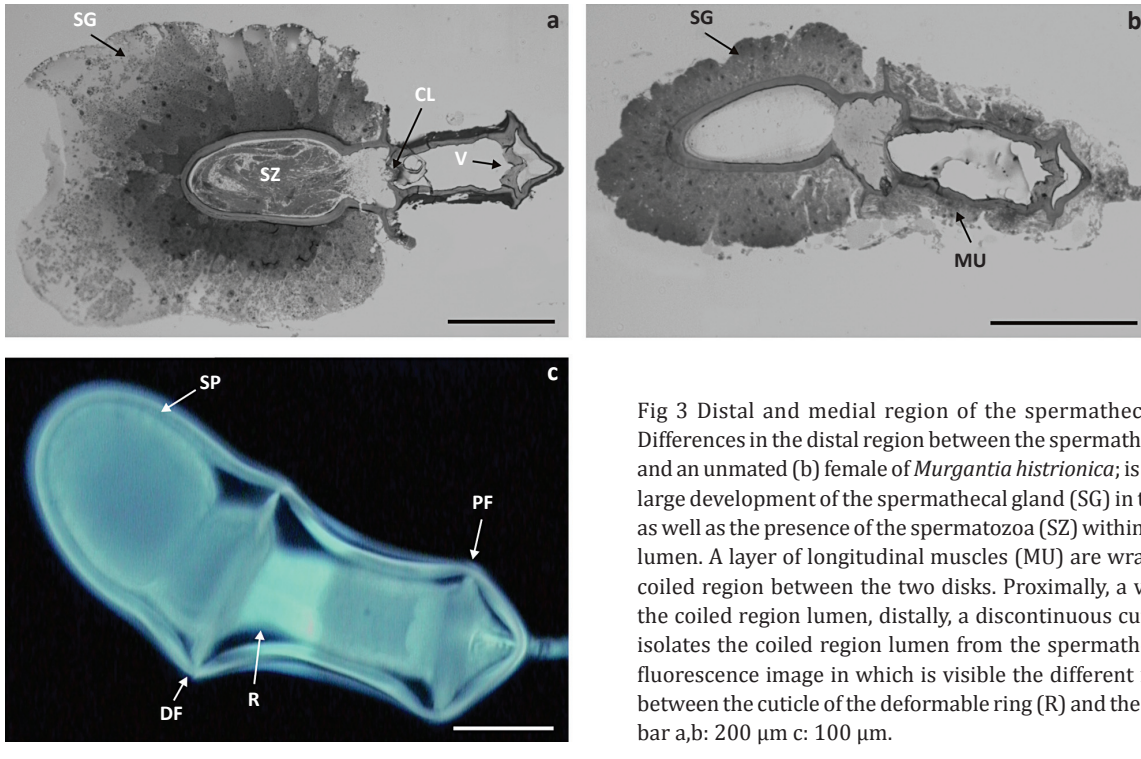

Fig 3 Distal and medial region of the spermathecal complex. a-b) Differences in the distal region between the spermatheca of a mated (a) and an unmated (b) female of *Murgantia histrionica*; is clearly visible the large development of the spermathecal gland (SG) in the mated female, as well as the presence of the spermatozoa (SZ) within the spermatheca lumen. A layer of longitudinal muscles (MU) are wrapped around the coiled region between the two disks. Proximally, a valve (V) delimits the coiled region lumen, distally, a discontinuous cuticular layer (CL) isolates the coiled region lumen from the spermatheca lumen; c) UV fluorescence image in which is visible the different intensity of color between the cuticle of the deformable ring (R) and the other parts. Scale bar a,b: 200 µm c: 100 µm.

proximally from the spermathecal duct by a layer of endocuticle, operating like a valve. This valve is formed by two parts, oriented toward the duct (Fig 3a). The cuticle making the valve is thicker (≈ 24 µm) than the one of the cylinder walls (≈ 10 µm). Distally, the coiled region lumen is separated from the spermatheca lumen by a discontinuous cuticular structure (thickness 5-10 µm), having numerous projections oriented toward the spermatheca (Fig 3a). The valve, the discontinuous cuticular layer and the distal part of the coiled region walls show a different organization in the chitinous layers of the cuticle; at this level the non-sclerotized endocuticle is abundant, while the rigid exocuticle is scant. In epifluorescence observations, the difference in the cuticular structure is clearly visible (Fig 3c). The coiled region is surrounded by longitudinal muscular fibers (Fig 3b), suspended between the two cuticular rings and connected with their internal sides through tonofibrillae.

The spermatheca is formed by a unique chamber having a structure that define two regions, a proximal (75 µm x125 µm), and a bulb-shaped (175 µm x 105 µm) distal one (Fig 2b, 3a-b). The cuticular walls of the proximal region present a constant thickness (6.5 µm); in the distal bulb the cuticle is crossed by numerous canals that allow the evacuating ducts to flow the secretion into the lumen. The spermathecal gland completely wraps the spermatheca, and is formed by several glandular units, each one composed of a secretory cell and a duct cell. The glandular epithelium has a maximum thickness of about 320 µm, and is bounded by a basal lamina with wide invaginations (Fig 4a). The secretory cells are positioned on the external part of the epithelium, in contact with the basal lamina. They show a large nucleus, a cytoplasm with a remarkable presence of rough endoplasmic reticulum, tracheolae and a well developed end apparatus. The spherical end apparatus is surrounded by numerous electron-lucent vesicles and presents a receiving canal with abundant microvilli (Fig 4b). A short cuticular duct (1.9 µm) rises from the end apparatus and widens in a rounded reservoir (Ø ≈ 2.5 µm), then the duct develops with a tortuous course until the bulb, crossing the cuticular layers (Fig 4c-d). The duct cell shows a reduced cytoplasm and a small nucleus (Fig 4e). The evacuating

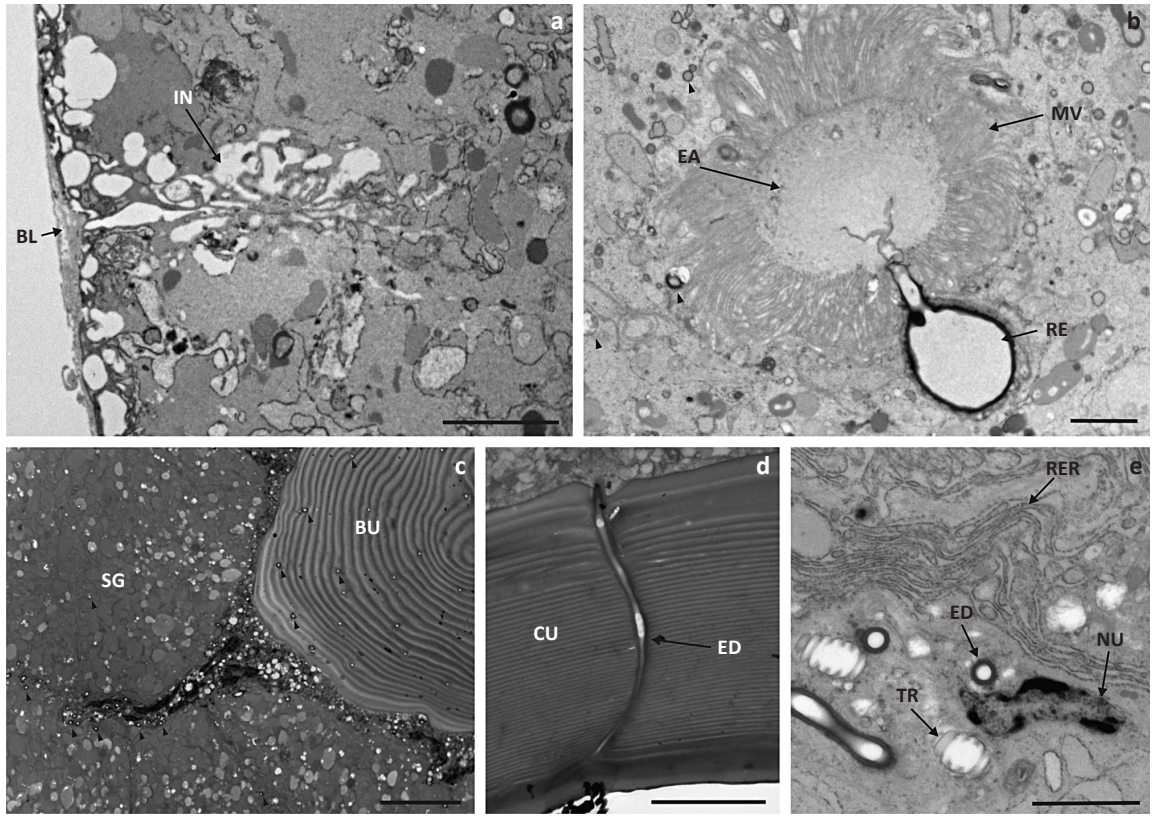

Fig 4 TEM micrographs a) Cross section of the spermathecal gland, a basal lamina (BL) with invaginations (IN) wraps the epithelium; b) Cross section of a secretory cell with the end apparatus (EA) surrounded by numerous microvilli (MV) and connected with the reservoir (RE); c) Cross section at the bulb (BU) tip level, many evacuating ducts (arrowheads) are visible, both in the bulb's cuticle and in the spermathecal gland (SG); d) Longitudinal section of an evacuating duct (ED) crossing the cuticular layer (CU) of the bulb; e) Cross section of a duct cell showing the nucleus (NU) and a tracheole (TR). The abundant rough endoplasmic reticulum (RER) belongs to the adjacent secretory cell. Scale bar a,d: 5 µm b,e: 2 µm c: 10 µm.

ducts, from the various glandular units, are organized to form bundles orthogonal to the bulb surface.

Proximal region

In the central part, the spermathecal duct (Ø ≈ 25 µm) gives rise to a deformable saccular dilation, extended to surround the duct itself (Fig 5a). On both sides (external and internal), the surface of the saccular dilation show abundant folds (Fig 2e-f). Internally, through consecutive invaginations, two cuticular channels are present (Fig 5b). The outer cuticular channel is the widest (Ø ≈ 110 µm) and contains the inner channel (Ø ≈ 20 µm) (Fig 5c). Cross sections show differences between the cuticular thickness of the saccular dilation (≈ 4 µm), the outer channel (≈ 18 µm) and the inner channel (≈ 11 µm). An electrondense material fills completely the lumen between the saccular dilation and the outer channel (Fig 5c).

Discussion

The spermatheca is an organ playing a key role in insect reproduction, allowing the temporary storage of spermatozoa and making them available when needed, ensuring at the same time an efficient use of spermatozoa during the fertilization process (Parker 1970). In *M. histrionica*, the spermatheca consists of a complex structure composed by a long duct that opens at the level of the common oviduct. The investigations have not displayed the presence of muscles associated to the duct surface, like in other insects (Ilango 2005). The saccular dilation is probably delegated to the storage of the material transferred by the male during mating. This structure is formed by a thin cuticle and a single layer of epithelial cells. Salerno *et al* (2009) reported that the saccular expansion appears full of material and with a significantly greater volume in mated than in unmated females of *M. histrionica*. It may be possible to correlate this fact with the ability of the egg parasitoid *Trissolcus brochymenae* to discriminate between the traces left on the substrate by males and females and, among females, between mated and unmated individuals. The preference of *T. brochymenae* for mated females of *M. histrionica* has been correlated with the sperm and other substances transferred during mating. This fact has been observed also in protracted mating in other species belonging to the same family (Wang & Millar 1997, Ho & Millar 2001).

In some other reports for Blattodea (Mullinus & Keil 1980), Orthoptera (Gwynne 1988), Lepidoptera (Bogges & Gilbert 1979) and Coleoptera (Boucher & Huignard 1987), the role played by the secretion of the glands associated with male genitalia has been investigated. In *Locusta migratoria* L. (Orthoptera: Acrididae), substances produced by male accessory glands are transferred toward the spermatheca during mating, like the LOM-AG Myotropin II factor that breaks out the oviduct

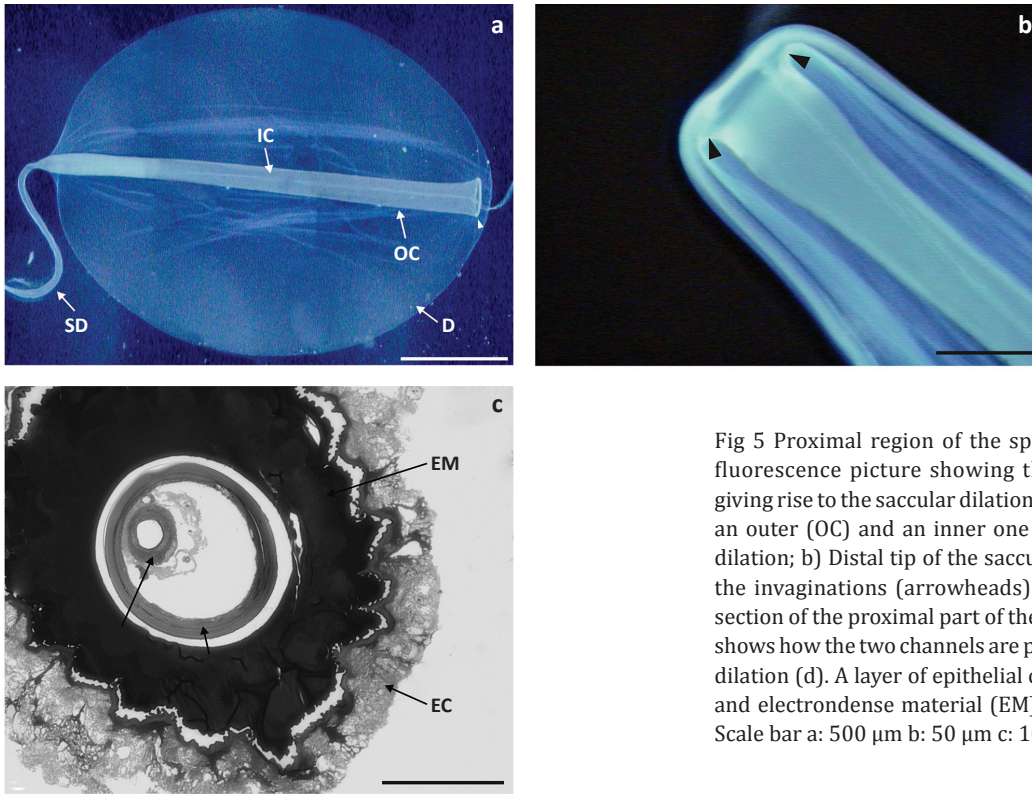

Fig 5 Proximal region of the spermathecal complex. a) UV fluorescence picture showing the spermathecal duct (SD) giving rise to the saccular dilation (D). Two cuticular channels, an outer (OC) and an inner one (IC), are present inside the dilation; b) Distal tip of the saccular expansion with detail of the invaginations (arrowheads); c) Light microscope cross section of the proximal part of the spermathecal complex that shows how the two channels are positioned inside the saccular dilation (d). A layer of epithelial cells (EC) bound the dilation and electrondense material (EM) completely fills the lumen. Scale bar a: 500 µm b: 50 µm c: 100 µm.

contractions (Paemen *et al* 1991), or the stimulating factor discovered by Lange & Loughton (1985). Zahn *et al* (2008) hypothesized that the male of *M. histrionica* can discriminate between mated and unmated females, perceiving variations of the cuticular components induced by the mating. The same strategy could be adopted by the parasitoid. The role played by the male accessory glands secretion is however not yet clear.

Our investigations revealed a high degree of complexity in the organization of the spemathecal complex in *M. histrionica*, in accordance to what has already been described in other true bugs, particularly lygaeids (Gschwentner & Tadler 2000, Chiang 2010). However, we were unable to find in *M. histrionica* a structure that can be related to the "medial tube" described by Chiang (2010) in *Leptoglossus occidentalis* (Heidemann) (Hemiptera: Coreidae). In fact, the saccular dilation of the spermatecal duct originates as a double folding of the spermatecal duct itself, and no other cuticular ducts have been observed serving as possible sperm transport systems. It is possible that Lygaeidae and Pentatomidae evolved different strategies in terms of sperm transport systems, as reflected by the difference in the spermathecal complex organization. The functional significance of such complicated structures could be interpreted in terms of cryptic female choice, i.e. occurring after insemination, as already proposed by Chiang (2010).

Differently from other species, the spermatheca in *M. histrionica* has a single glandular structure localized at the distal part, completely wrapping the spermatheca. Contrarily to what was observed in other heteropteran species, i.e. *Rodnius prolixus* (Stål) (Huebner 1980) and *Lygaeus simulans* (Deckert) (Gschwentner & Tadler 2000), where the glandular epithelium is formed by class 1 secretory cells (Noirot & Quennedey 1974, Quennedey 1998), the secretory units in *M. histrionica* belong to class 3. Each secretory unit is composed of a secretory cell and a duct cell responsible for the production of the cuticular evacuating duct (Fig 6). In this simple scheme, the secretion is produced by the secretory cell and is released through the cuticular evacuating duct. The transfer of the secretion from one cell to the other takes place at the level of the end apparatus, a specialized semi-permeable cuticular structure interfacing the apical part of the secretory cell with the basal region of the duct cell. The ultrastructural features of secretory cells (i.e. the presence of large basal infoldings, abundant stacks of rough endoplasmic reticulum, mitochondria and secretory vesicles within the cytoplasm) are related to the production and release of chemical substances into the lumen of the spermatheca. These secretions should play an important role in maintaining the spermatozoa alive, as well as in their mobility when insemination occurs.

The spermatheca shows variations regarding the structure of the cuticular intima, in particular if we consider the structure of the distal part. The spematheca is made of thick cuticle typically arranged in layers. At this level, the evacuating ducts of the spemathecal glands penetrate the thick cuticular layer in order to ooze the secretion within the bulb lumen. The ring below the distal flange shows a thick layer of endocuticle and a thin exocuticle layer. This can be related with a possible deformability of this part of the coiled region, probably as a consequence of the tightening of the muscles connected with the two cuticular rings. Muscular contractions cause an increase of the internal pressure in the coiled region and a consequent exit of the sperm. In this view, the discontinuous cuticular layer between the spermatheca and the coiled region could have an important role in the modulation and regulation of the sperm flow, avoiding the sudden escape of an excessive number of gametes. The cuticular valve positioned in the proximal part of the coiled region is also composed by endocuticle, therefore it can be deformed due to changes in the pressure within the cylinder. Since the valve opens toward the spermathecal duct, its function could be to prevent the backflow of the sperm toward the spermatheca when the muscles are relaxed. However, a possible role in the modulation of the sperm acquisition during mating with the male cannot be ruled out.

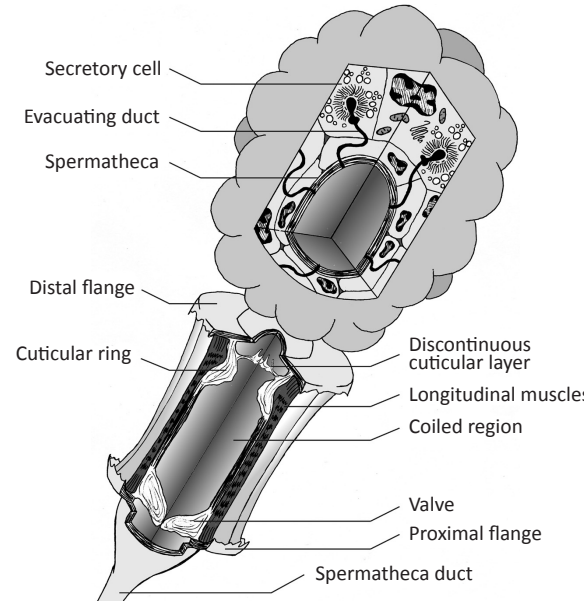

Fig 6 Schematic drawing of the distal region of the spermatheca in *Murgantia histrionica* (not in scale).

In conclusion, the spermatheca in *M. histrionica* shows some peculiar ultrastructural features that make possible for the female to finely regulate and tune the spermatozoa use, as well as to possibly store molecules transferred by the male during copulation, the role and chemistry of which still remains unknown. A follow up study will be carried out on *M. histrionica* male accessory glands in

order to fully understand the fine interactions between individuals during reproduction.

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