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# Ultrastructure of the Salivary Glands of the Stink Bug Predator *Podisus distinctus*

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**Abstract:** *Podisus distinctus* (Hemiptera: Pentatomidae) is a zoophytophagous insect with significant potential for use as a biological control agent in agriculture and forestry because their nymphs and adults actively prey on diverse insect species. The saliva of this insect possesses active substances that cause paralysis and death of the prey. As the first step in identifying compounds of *P. distinctus* saliva, this study describes the ultrastructure of the salivary glands of this predator. The salivary system of *P. distinctus* possesses a pair of main salivary glands with a short anterior lobe, a long posterior lobe, and a pair of tubular accessory glands. The main salivary gland of *P. distinctus* has no associated muscles, suggesting that the saliva-release mechanism occurs with the help of certain thorax muscles. The main salivary gland epithelium has a single layer of cells (varying from cubical to columnar) with cytoplasm rich in rough endoplasmic reticulum, spherical granules of different sizes, a nucleus with a predominance of decondensed chromatin, and nucleolus. The apical cell region has a few short microvilli and the basal region has plasma membrane infoldings. The epithelium of the accessory salivary glands possesses a single-layered epithelium of cubic cells delimiting a narrow lumen. The apical cell region has a high density of microvilli and pleomorphic mitochondria, whereas the central cell region is rich in rough endoplasmic reticulum with a well-developed nucleus and decondensed chromatin. The basal cell region is characterized by the presence of several basal plasma membrane infoldings associated with mitochondria and numerous openings to the hemocoel forming large channels. The ultrastructural characteristics suggest that the main salivary glands and accessory salivary glands play a vital role in protein synthesis for saliva production and that the accessory glands are involved in transport of materials of the hemolymph.

**Key words:** saliva, extra-oral digestion, salivary gland, secretory cells, zoophytophagy

## Introduction

Biological control is an important strategy for the management of agricultural and forest pests and includes the process of conservation and release of natural enemies in the concerned region. The interaction of predatory natural enemies of insect pests occurs in natural and artificial habitats, as recognized in biological control studies (Zanuncio et al., 1994; Eubanks & Denno, 1999; De Clercq, 2002; Symondson et al., 2002).

Predators play an important role in the dynamics of insect communities due to the variation in food or prey availability that affects survival, dispersion, and population dynamics of these organisms (Cohen, 1990; Coll & Gershon, 2002). Predatory insects, including representatives of Coleoptera, Diptera, Hemiptera, and Hymenoptera, feed on specific or general prey (Cohen, 1990; Memmott et al., 2000; Richter, 2000). These predators can be classified as opportunistic, obligates, or facultative (Coll & Gershon, 2002). The opportunistic predators include phytozoophagous insects (which are herbivores and may feed on prey) and zoophytophagous (which are carnivores and may feed on plants) (Memmott et al., 2000; Coll & Gershon, 2002).

Pentatomidae (Hemiptera: Heteroptera) includes zoophytophagous stink bug species that are the main representatives of the genus *Podisus* (Zanuncio et al., 2008; Jesus et al., 2014) used in biological control of insect pests (Torres & Boyd, 2009; Torres et al., 2010).

*Podisus distinctus* (Stål) is a zoophytophagous insect used in biological control in agriculture and forestry against defoliating caterpillars (Lepidoptera) of *Eucalyptus* species (Matos Neto et al., 2004; Zanuncio et al., 2011, 2013). The importance of *P. distinctus* in biological control has stimulated several studies on its development, morphology, reproduction, predator–prey interactions, and feeding strategies (Lacerda et al., 2004; Matos Neto et al., 2004; Santos et al., 2004; Pires et al., 2009; Sá et al., 2013; Zanuncio et al., 2013).

Some predatory insects have a feeding process involving extra-oral digestion, which begins with saliva injection into the prey body before ingestion. In this case, the saliva may have digestive enzymes that liquefy prey tissues and facilitate the absorption of nutrients (Miles, 1972; Terra & Ferreira, 1994; Cohen, 1995). The salivary glands of predatory Hemiptera produce saliva with a mixture of different

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compounds including water, lipids, carbohydrates, and enzymes that play an important role in extra-oral digestion (Miles & Slowiak, 1976; Terra & Ferreira, 1994; Zeng & Cohen, 2000; Oliveira et al., 2006). In this context, *P. distinctus* inserts the mouth stylet into the prey body and regurgitates saliva, causing rapid paralysis and death of the prey (Cohen, 1998; Lemos et al., 2003; Oliveira et al., 2006). However, *Podisus nigrispinus* Dallas has only collagenase as the digestive enzyme in its saliva (Fialho et al., 2012), suggesting that saliva can be primarily used to cause paralysis and death of the prey rather than extra-oral digestion (Martínez et al., 2014).

The salivary glands of predatory Pentatomidae have been morphologically and histologically studied in a limited number of species (Baptist, 1941; Oliveira et al., 2006; Martínez et al., 2014). Although these studies have provided significant data on the morphology of the salivary glands of some species, more detailed information corresponding to other species is necessary. The objective of this study was to describe the morphology of salivary glands in the stink bug predator *P. distinctus* in order to provide additional data for the comprehension of predator–prey interaction and ecological relationships of this predator in biological control programs.

## Material and Methods

### Insects

Adults of *P. distinctus* were obtained from a mass-rearing facility in the Laboratório de Controle Biológico of the Instituto de Biologia Aplicada à Agricultura e Pecuária, Federal University of Vicosa, Minas Gerais, Brazil and maintained at 25 ± 2°C, 75 ± 5% relative humidity, and 12-h photophase conditions. These insects were fed *ad libitum* on *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) pupae and *Eucalyptus grandis* (W. Hill ex. Maiden) leaves (Zanuncio et al., 1994; Lemos et al., 2005).

### Scanning Electron Microscopy

Ten adults of *P. distinctus* were cryoanesthetized at −4°C for 5 min, and the salivary glands were dissected out in the saline solution for insects (0.1 M NaCl, 0.1 M $KH_2PO_4$, 0.1 M $Na_2HPO_4$) and transferred to Zamboni's fixative solution (Stefanini et al., 1967) for 12 h at 5°C. Then, the samples were dehydrated in a graded ethanol series (70, 80, 90, and 98%), transferred to hexamethyldisilazane for 5 min, air dried, coated with 20-nm-thick layer of gold, and observed with a LEO VP1430 scanning electron microscope (Carl Zeiss, Jena, Germany).

### Light Microscopy

Ten adults of *P. distinctus* were cryoanesthetized at −4°C, the salivary glands were dissected in saline solution for insects, and transferred to Zamboni's fixative solution for 24 h at 5°C. Then, the samples were dehydrated in a graded ethanol series (70, 80, 90, and 95%) and embedded in historesin JB4 (Electron Microscopy Sciences, Fort Washington, PA, USA). Sections (3 μm thick) were stained with hematoxylin and eosin and analyzed by light microscopy.

### Transmission Electron Microscopy

In total, 20 salivary glands of *P. distinctus* were dissected and transferred to 2.5% glutaraldehyde prepared in sodium cacodylate buffer (0.2 M; pH 7.2) containing 0.2 M sucrose for 4 h at room temperature. Then, the main salivary gland was divided into the anterior and posterior lobes, and the accessory gland was isolated. The pieces were postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, followed by washing in buffer and dehydration in a graded ethanol series (70, 80, 90, and 99%). The samples were embedded in LR White Resin (Electron Microscopy Sciences, Fort Washington, PA, USA), and ultrathin sections (50–90 nm) obtained with a glass knife in a Sorvall MT2-BMT2-B ultramicrotome (Sorvall Instruments, Wilmington, DE, USA). Sections were stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963) and examined with a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany).

## Results

### Anatomy

The salivary system of *P. distinctus* showed a pair of main salivary glands and a pair of tubular accessory salivary glands, extending from the prothorax to the metathorax, which were white and translucent in the physiological solution for insects.

The main salivary glands were bilobed, with the anterior lobe smaller than the elongated posterior one (Fig. 1a). The anterior lobe was semioval in shape with a short projection into the insect head and enlarged toward the posterior lobe (Figs. 1a, 1b). The posterior lobe was located in the prothorax, which was more enlarged at the junction of the anterior lobe and sharper at the posterior end (Figs. 1a, 1b). In the hilum region, between the anterior and posterior lobes, a narrow salivary duct was (Fig. 1c) inside the head and connected with the salivary duct of the other main salivary gland to form a single salivary duct opening in the mouthpart stylet.

Accessory salivary glands were tubular and narrower than the main salivary glands (Fig. 1b), opening by a narrow glandular duct in the hilum of the main salivary gland (Fig. 1c). In the portion near the hilum, the duct accessory salivary gland presented regular U-shape folds (Fig. 1c).

### Main Salivary Glands

The main salivary gland epithelium was composed of a single layer of cells varying from cubic to columnar in shape with a well-developed spherical nucleus rich in decondensed chromatin and nucleolus (Figs. 2a, 2b). The cytoplasm was homogeneous with differently sized vacuoles and some granules. The luminal content was homogeneous and acidophilus in both the lobes (Figs. 2a, 2b). Externally, the main salivary glands were coated with a thin basal membrane (Figs. 2a, 2b).

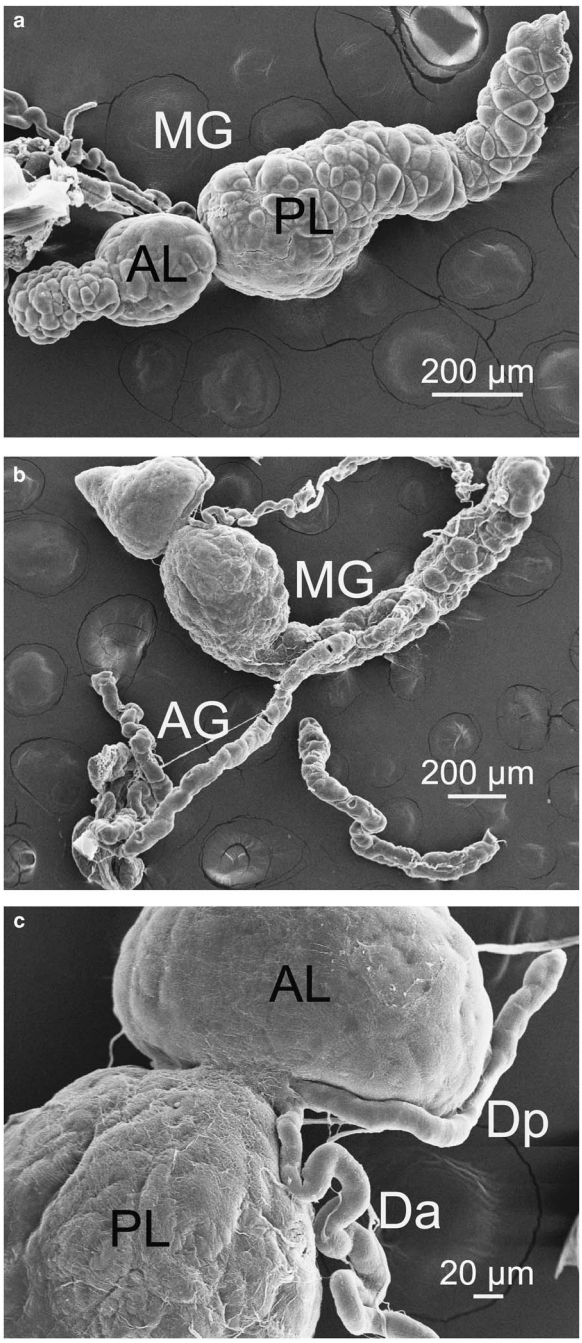


**Figure 1.** Scanning electron micrographs of salivary glands of *Podisus distinctus*. **a:** General view showing the main salivary gland (MG) with anterior lobe (AL) and posterior lobe (PL). **b:** MG and accessory gland (AG). **c:** Detail of hilum between AL and PL with ducts of accessory gland (Da) and main gland (Dp).

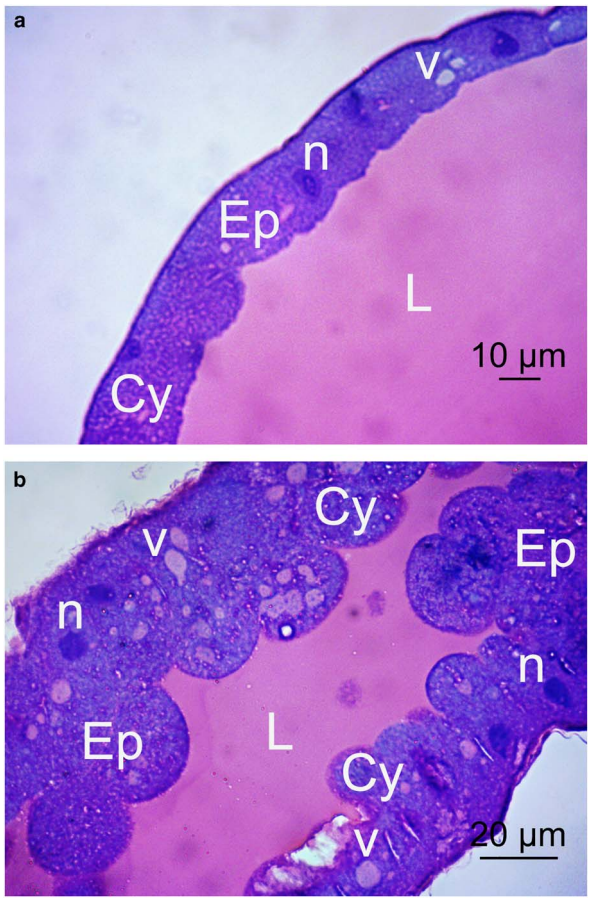


**Figure 2.** Histological sections of main salivary glands of *Podisus distinctus*. **a:** Anterior lobe of main gland showing epithelium (Ep) with well-developed nucleus (n), vacuoles (v), and basophilic cytoplasm (Cy) and acidophill gland content in the lumen (L). **b:** Posterior lobe of main gland showing epithelium (Ep) with some cells containing two nuclei (n), vacuoles (v) and basophilic cytoplasm (Cy).

## Anterior Lobe of the Main Salivary Gland

In the anterior lobe of the main salivary glands, the apical surface of the secretory cells had a few short microvilli (~2.0 $\mu$m long) (Fig. 3a). The apical and median cytoplasm was almost entirely filled with rough endoplasmic reticulum cisterns (Figs. 3a, 3b) with some forming stacks and concentric arrays (Figs. 3b, 3c). The cytoplasm of these cells showed several electron-lucent vesicles and some membranous content similar to that of myelin figures (Figs. 3b, 3d). In addition to the rough endoplasmic reticulum and vesicles, some mitochondria were found in the apical and perinuclear cytoplasm (Figs. 3b–3d). The adjacent secretory cells showed a narrow intercellular space (Fig. 3c) with a short septate junction at the apex (Fig. 3d). The median nucleus showed decondensed chromatin with some heterochromatin and one or two large nucleoli (Fig. 3e) with evident amorphous and fibrillar regions (Fig. 3f). In the basal region of the secretory cells of the main salivary glands, the cytoplasm was slightly electron-dense with several electron-lucent vesicles (Fig. 3g). The basal plasma membrane had a few short infoldings (Fig. 3h). Secretions from the anterior lobe of the main salivary gland were strongly electron dense and generally reaches the microvilli tip of the secretory cells (Figs. 3i, 3j). Some of the secretory cells showed highly vacuolated cytoplasm with some autophagic vacuoles (Fig. 3k) containing cytoplasmic and mitochondrial debris (Fig. 3l).

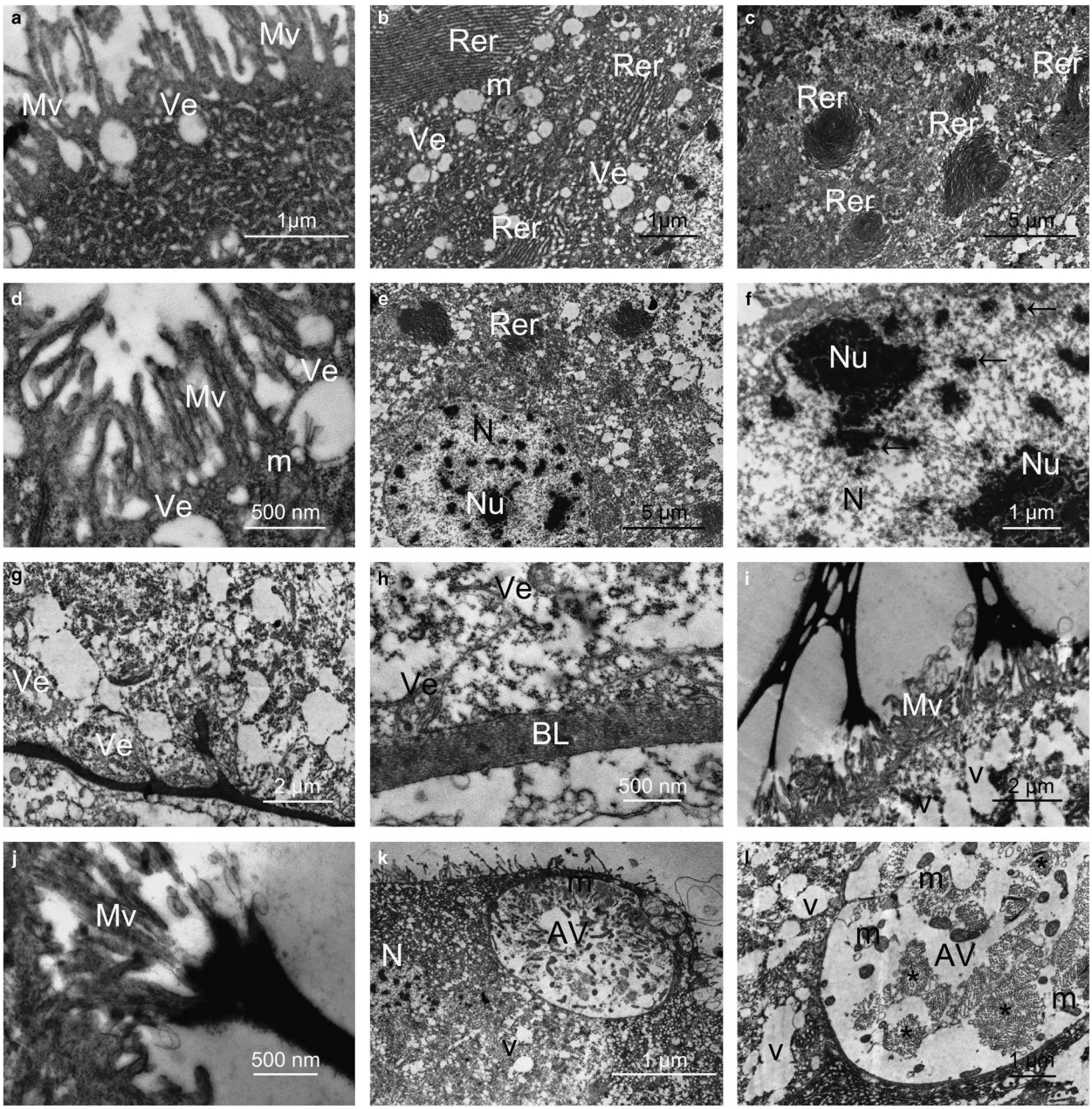


**Figure 3.** Transmission electron micrographs of secretory cells of anterior lobe of the main salivary gland of *Podisus distinctus*. **a:** Apical cell region showing short microvilli (Mv) and vesicles (Ve). **b:** Apical cytoplasm showing cisternae of rough endoplasmic reticulum (Rer) and electron-lucent vesicles (Ve). **c:** Perinuclear cytoplasm with rough endoplasmic reticulum (Rer) cisternae with concentric array. **d:** Apical cell region showing enlarged electron-lucent vesicles (Ve) closely associated with microvilli (Mv). **e:** Median cell region showing nucleus (N) with decondensed chromatin, nucleolus (Nu). **f:** Detail of the nucleus (N) with two well-developed nucleoli (Nu) and condensed chromathin (arrows). **g:** Basal cell region with electron-lucent vesicles (Ve). **h:** Basal cell region showing absence of plasma membrane infoldings and an electron-dense basal lamina (BL). **i:** Apical cell surface showing contact of electron-dense gland content with microvilli (Mv). **j:** Detail of the contact of gland content with cell microvilli (Mv). **k:** Secetory cell with enlarged autophagic vacuoles (AV). **l:** Detail of autophagic vacuole with mitochondria (m) and cytoplasm debris (stars). v, vacuoles.

## Posterior Lobe of the Main Salivary Gland

In the posterior lobe of the main salivary gland, the surface of the secretory cells had thin and short microvilli (~4.0 $\mu$m long) (Fig. 4a). The apical and median cytoplasm regions were electron dense due to the presence of large amounts of rough endoplasmic reticulum (Fig. 4a) and vesicles with homogeneous, membranous, or electron-lucent content (Fig. 4b). The nuclei of these cells were well developed with a predominance of decondensed chromatin and multiple nucleoli (Fig. 4c). The basal cytoplasm of the

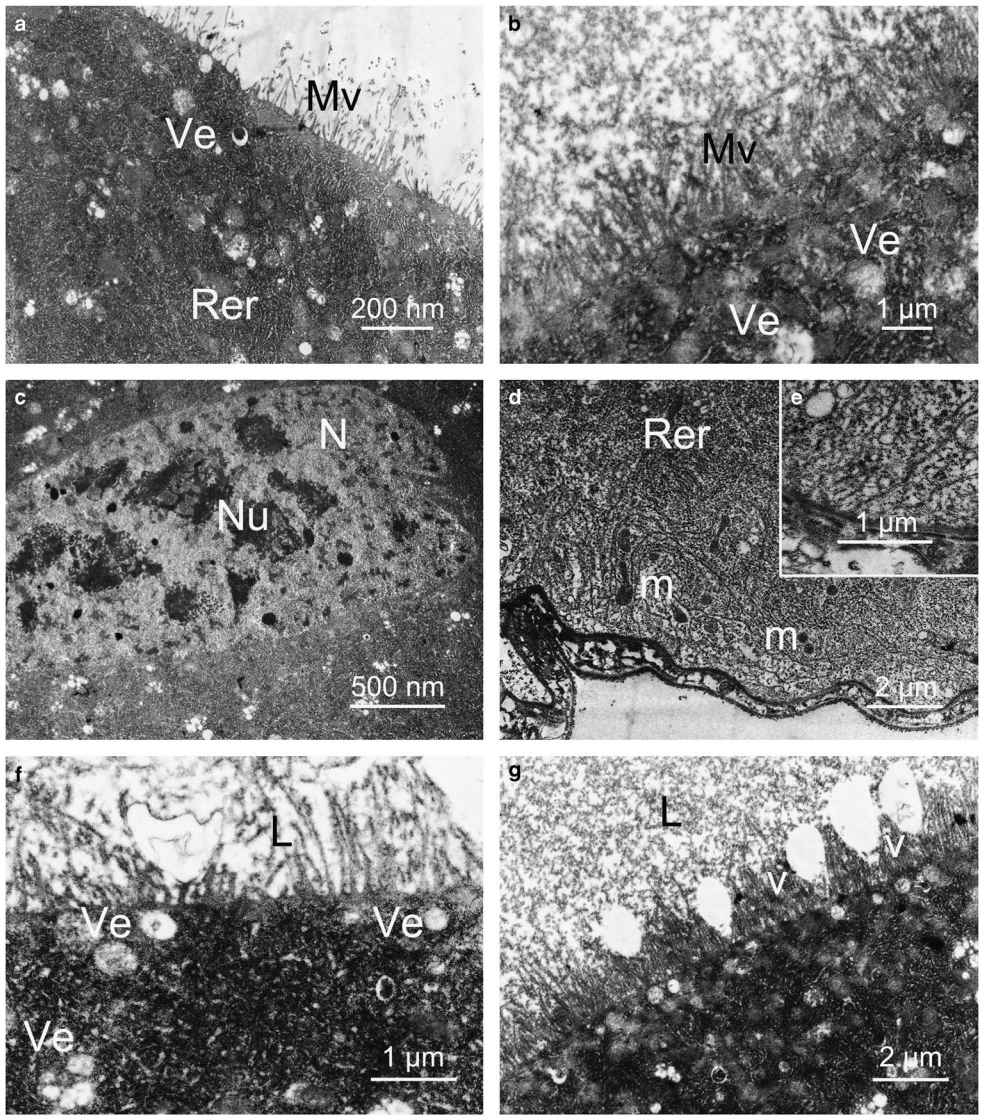


**Figure 4.** Transmission electron micrographs of secretory cells of the posterior lobe of the main salivary gland of *Podisus distinctus*. **a:** Apical cell region showing thin and short microvilli (Mv), vesicles (Ve), and rough endoplasmic reticulum (Rer). **b:** Apical cytoplasm rich in vesicles (Ve). **c:** Nucleus (N) with decondensed chromatin and many nucleoli (Nu). **d:** Basal cell region showing rough endoplasmic reticulum (Rer) and mitochondria (m). **e:** Detail of the rough endoplasmic reticulum cisterns in the basal cell region. **f:** Apical cytoplasm with large vesicles (Ve). **g:** Apical cell surface showing large vacuoles (V) among the microvilli (Mv) and flocculent content in the lumen (L).

secretory cells of the posterior lobe of the main salivary gland had some cisterns of rough endoplasmic reticulum and mitochondria (Fig. 4d), whereas the basal plasma membrane had some short infoldings (Fig. 4e). The secretion stored in the lumen of the posterior lobe was flocculent. It is possible that the secretions could be released from the cytoplasmic vesicles, which were pinched-off into the gland lumen, where they fused to form large vacuoles (Figs. 4f, 4g).

## Accessory Salivary Gland

The epithelium of the accessory salivary gland was formed by a single-layered epithelium of cubic cells lining a narrow lumen (Fig. 5). The apical surface of the secretory cells of the accessory salivary gland had a high density of microvilli (~6.0 $\mu$m long) (Fig. 6a). The apical cytoplasm showed some pleomorphic mitochondria (Fig. 6b), lipid droplets, and rough endoplasmic reticulum cisterns (Fig. 6c). The large nucleus showed a predominance of decondensed chromatin and clumps of heterochromatin (Fig. 6a). The basal cell region was characterized by the presence of several plasma membrane infoldings (Fig. 6a) that were associated with the mitochondria and extended to the middle one-third of the cell (Fig. 6a) with numerous openings to the hemocoel forming large channels (Fig. 6d), some of which were swollen toward the apex cell, but never reaching the lumen of the gland (Fig. 6e).

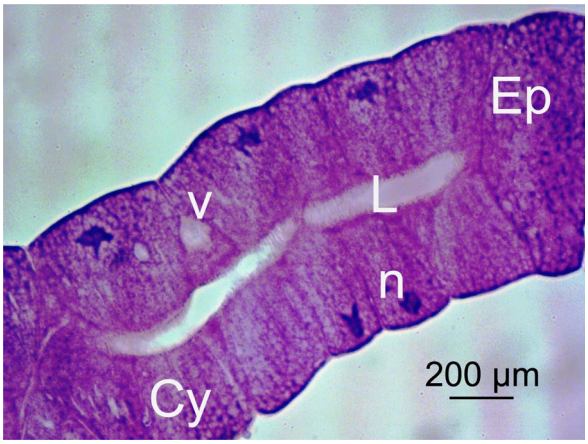


**Figure 5.** Histological section of accessory salivary gland of *Podisus distinctus* showing cubic epithelium (Ep) with small nucleus (n) and cytoplasm (Cy) with vacuoles (v). L, lumen.

## Discussion

The salivary system of *P. distinctus* is composed of a pair of main salivary glands and a pair of accessory salivary glands with a similar anatomy to that described for other Asopinae such as *Brontocoris tabidus* Signoret (Azevedo et al., 2007), *P. nigrispinus* Dallas (Martínez et al., 2014), and *Supputius cincticeps* Stål (De Castro et al., 2013), suggesting an anatomical pattern similar within the Pentatomidae predators. However, anatomical variations of the salivary glands have been reported for other Hemiptera such as *Belostoma lutarium* Stål (Belostomatidae) (Swart & Felgenhauer, 2003), *Cimex hemipterus* Fabricius (Cimicidae) (Serrão et al., 2008), *Karenia caelatata* Distant (Cicadidae) (Zhong et al., 2013), *Mahanarva posticata* Stål (Cercopidae) (Roma et al., 2003), and *Triatoma infestans* Klug (Reduviidae) (Reis et al., 2003). The morphological diversity of salivary glands may be attributed to the difference in the feeding habits of Hemiptera, which are zoophagous, phytophagous, zoophytophagous, phytozoophagous, or hematophagous (Miles, 1972; Terra & Ferreira, 1994; Cohen, 1995; Zeng & Cohen, 2000). In this sense, the midgut of predatory Heteroptera has three morphological and physiological differentiations (Guedes et al., 2007; Fialho et al., 2009, 2013), whereas phytophagous ones have four midgut regions (Silva et al., 1995; Pires et al., 2007; Uceli et al., 2011). Salivary gland anatomy of *P. distinctus* is more closely related with that of Asopinae species (Azevedo et al., 2007; De Castro et al., 2013; Martínez et al., 2014), suggesting that the phylogenetic position is an important factor in morphology of the salivary glands, as also reported for the midgut of insects (Terra & Ferreira, 1994; Serrão & Cruz-Landim, 1995, 2000; Fialho et al., 2012).

Morphology of the salivary system of *P. distinctus* indicate the responsibility of three regions for saliva production: anterior and posterior lobes of the main and accessory salivary glands, as demonstrated by the occurrence of glandular epithelium composed of cylindrical or cuboidal cells in the three gland regions (Del Bene et al., 1991; Ghanim et al., 2001; Reis et al., 2003; Sais et al., 2003; Azevedo et al., 2007; Serrão et al., 2008; Martínez et al., 2014).

The secretory cells of the anterior and posterior lobes of the main salivary glands of *P. distinctus* possess a cytoplasm rich in rough endoplasmic reticulum and granules, indicating protein synthesis. Protein secretion by the main salivary gland has been reported in other hemipterans (Miles, 1972; Cohen, 1998; Swart & Felgenhauer, 2003; Nunes & Camargo-Mathias, 2006; Azevedo et al., 2007; Serrão et al., 2008; Fialho et al., 2012; Zhong et al., 2013), indicating that the main function of the salivary glands was conserved in these insects regardless of their feeding habits. However, the presence of thin and short basal plasma membrane infoldings associated with few mitochondria in the secretory cells of the anterior and posterior lobes of the main salivary glands of *P. distinctus* suggests low activity of substance transport from the hemolymph.

The accessory salivary glands of *P. distinctus* are tubular, with a narrow lumen and a duct opening in the hilum between the two lobes of the main salivary gland, similar to that described for the predatory Hemiptera *B. tabidus* (Azevedo et al., 2007), *P. nigrispinus* (Martínez et al., 2014), and *S. cincticeps* (De Castro et al., 2013), which suggests that secretions produced in the accessory salivary gland are transported to the lumen of the main salivary gland. The linking via a duct between the main and accessory salivary glands has been reported in other Hemiptera (Terra & Ferreira, 1994; Cohen, 1995; Swart & Felgenhauer, 2003; Zhong et al., 2013), suggesting that the composition of saliva produced in both the glands may vary due to continuous mixing.

The epithelial cell ultrastructure of accessory salivary glands of *P. distinctus* suggests its role in the transport of substances from the hemolymph as well as in proteins synthesis. The presence of several basal plasma membrane infoldings indicate transport of substances from the hemolymph without storage of substances in its narrow lumen, which may be constitutively released into the lumen of the main salivary glands. Our results indicate that the accessory salivary gland of *P. distinctus* is responsible for the water present in the saliva (Miles & Slowiak, 1976), but the occurrence of protein synthesis due to the presence of well-developed rough endoplasmic reticulum cannot be ruled out, as it is the organelle responsible for production of membranes and released proteins. In this sense, Martínez et al. (2014) reported the presence of proteins and lipids in the accessory salivary gland of *P. nigrispinus*.

The salivary gland complex of predatory bugs is important in the production of enzymes and other substances useful in extra-oral digestion (Swart & Felgenhauer, 2003; Azevedo et al., 2007; Martínez et al., 2014) as in prey digestion before ingestion (Cohen, 1990, 1995, 1998; Mohaghegh et al., 2001; Eubanks et al., 2003; Fialho et al., 2012), whereas secretory cells of the accessory salivary gland produce other substances such as carbohydrates, lipids, and proteins, as well as transport of water, which contribute

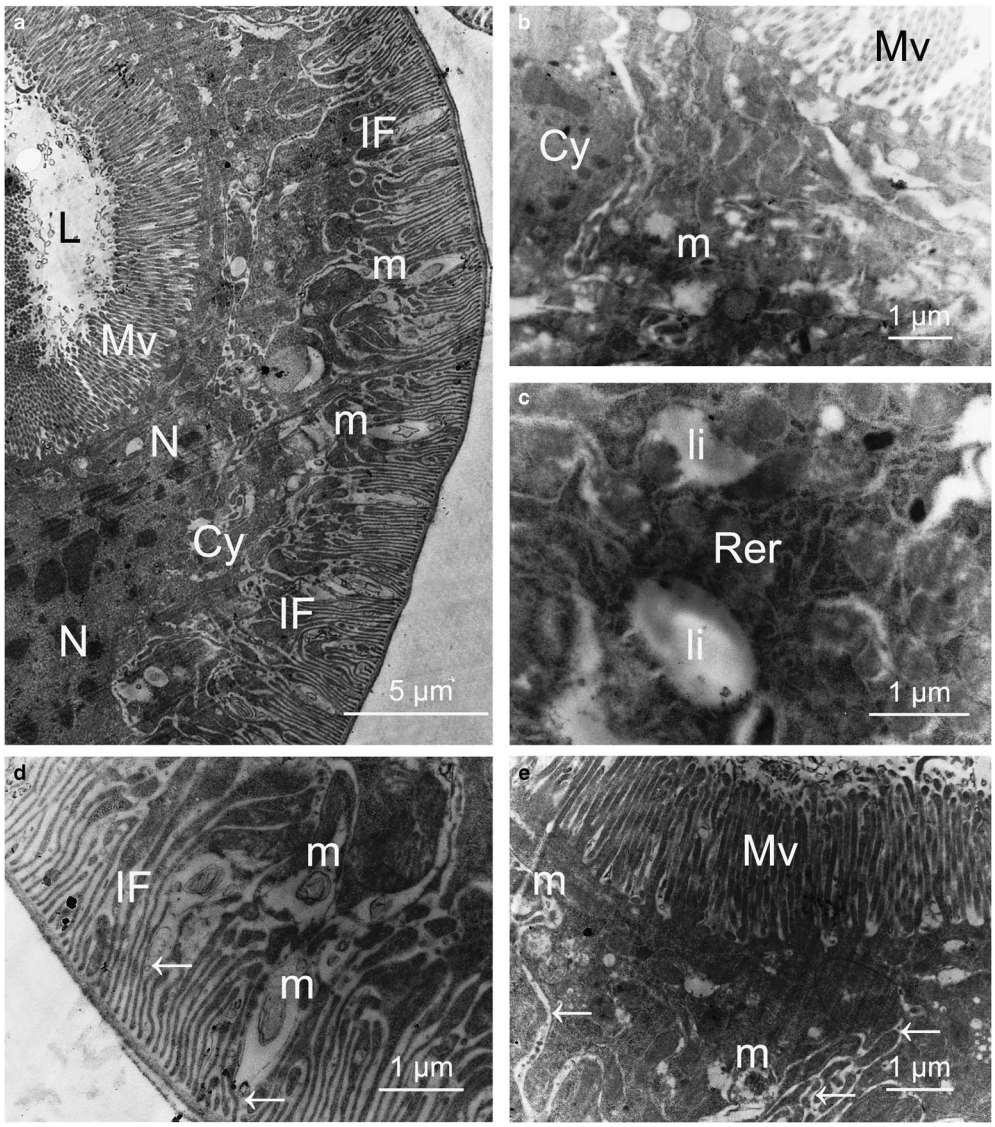


**Figure 6.** Transmission electron micrographs of secretory cells of accessory salivary gland of *Podisus distinctus*. **a:** General view of secretory cell showing the apical cell region with many microvilli (Mv), basal cell region with numerous plasma membrane infoldings (IF), and well-developed nucleus (N). **b:** Apical cell region with numerous mitochondria (m). **c:** Apical cell region with lipid droplets (li) and rough endoplasmic reticulum (Rer). **d:** Detail of the basal cell region showing plasma membrane IF forming enlarged extracellular spaces with many openings (arrows) to the hemocoel and mitochondria (m). **e:** Detail of the apical cell region with presence of enlarged extracellular space (arrows) formed by basal plasma membrane infoldings. L, lumen; Cy, cytoplasm.

to the final composition of saliva (Miles & Slowiak, 1976; Terra & Ferreira, 1994; Cohen, 1998; Torres et al., 2010; Martínez et al., 2014).

## Conclusions

The present study describes the morphology of the salivary complex of the predatory stink bug, *P. distinctus* that has a pair of bilobed main salivary glands and a pair of tubular accessory salivary glands. The salivary complex of *P. distinctus* has a high production and diversity of substances for saliva composition, which contributes to the ability of this predator to feed on a large amount of prey. Our study of the salivary complex of *P. distinctus* histologically and ultrastructurally provided new insights to the function of secretory cells in the salivary gland and the production and diversity of substances in the saliva used for extra-oral digestion and/or prey paralysis and death. Overall, this study contributes toward the comprehension of the ecological relationships in predator–prey interactions. Future studies should involve detection of toxins or enzymes in the saliva that could be sensitive to insecticidal proteins in transgenic plants on which their prey feeds. This is an important subject matter for biological control strategies.

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