Morphology and ultrastructure of salivary glands of male treehopper *Tricentrus brunneus* Funkhouser (Hemiptera: Membracoidea)

Haiying Zhong, Yalin Zhang & Cong Wei

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The salivary glands of male treehopper *Tricentrus brunneus* Funkhouser comprise a pair of acinous principal glands each with an anterior lobe and a posterior lobe; a pair of elbow-shaped accessory glands. Every accessory gland connects with the principal gland via a thin accessory salivary duct. The anterior lobe contains three types of acini (I, II and III), whereas the posterior lobe possesses only one type of acinus (IV). Two efferent salivary ducts fuse into a common salivary duct. Different acinis cells are filled with secretory granules that are distinct in size, number, shape and electron-density, indicating a maturation process before secreted. Infoldings in different acini suggest dilution of substances before being secreted. The presence of microvilli in acinus IV and the accessory gland duct possibly indicates the undergoing maturation of secretions. Electron-dense fine granules existed in the accessory gland cells, possibly related to virus transmission.

H. Zhong, Key Laboratory of Plant Protection Resources and Pest Management, Ministry of Education, Entomological Museum, Northwest A&F University, Yangling, Shaanxi 712100, China; and, State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China; E-mail: zhy8085@163.com
Y. Zhang & C. Wei, Key Laboratory of Plant Protection Resources and Pest Management, Ministry of Education, Entomological Museum, Northwest A&F University, Yangling, Shaanxi 712100, China

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1. Introduction

Treehoppers and leafhoppers (Membracoidea) are the most diverse group within Cicadomorpha (Shcherbakov 1996), with approximately 25,000 species described worldwide (Deitz & Dietrich 1993, McKamey 1998, Dietrich *et al.* 2001). Treehoppers are characterized by their extremely developed and peculiar-shaped pronotum which resembles thorns, parts of plants, or other objects in their habitat. They are also best known for their special behaviors, such as parental care, ant-mutualism, secreting brochosomes and anointing behavior, and acoustic communication (Gouranton & Maillet 1967, Day 1993, Wood 1993, Rakitov 1996, Cocroft *et al.* 2006, Morales & Beal 2006, Zhong *et al.* 2011).

The phytophagous treehoppers cause damage not only by sucking juice from the plants, but also by ovipositing into the tissue of the hosts (Butcher 1953, Yuan & Chou 2002, Wallace & Deitz 2007). The most serious damage caused by treehoppers is usually through the transmission of virus or bacteria during sap sucking (Simons & Coe 1958, Ammar & Nault 2002, Lashomb *et al.* 2002, Yuan & Chou 2002), which can cause pseudo-curly top of certain plants such as the ragweed (*Ambrosia* sp.), chickweed (*Stellaria media* (L.)), tomato (*Lycopersicum esculentum* (L.)), nightshade (*Solanum nigrum* L.), tobacco (*Nicotiana glutinosa* L.) and jimsonweed (*Datura stramonium* L.) (Simons & Coe 1958, Simons 1962, Tsai 1989, Briddon *et al.* 1996).

Although the transmission of pathogens is closely related to salivary glands of some insects (Grylls *et al.* 1947, Conti & Plumb 1977, Shikata 1979, Wijkamp *et al.* 1993, Ammar & Nault 2002, Ammar & Hogenhouta 2005, Froissart *et al.* 2010), the morphology and ultrastructure of the salivary glands of treehoppers have not been well/properly documented to date. Herein, we investigate the salivary glands of the treehopper *Tricentrus brunneus* Funkhouser, a widely distributed species mainly feeding on the paper mulberry (*Broussonetia papyrifera* (L.)), the black locust (*Robinia pseudoacacia* L.) and the thorny elaeagnus (*Elaeagnus pungens* Thunberg) in China (Yuan & Chou 2002), using both light microscopy and transmission electron microscopy. We are aiming to provide a detailed description of the salivary glands of *T. brunneus* at both the morphological and ultrastructural levels, and to supply information for future investigation about the pathogen transmission of treehoppers.

2. Materials and methods

2.1. Treehopper collection

As the salivary glands of the adult males and females exhibit great similarity, males were used for this study. Live *T. brunneus* (at least 50 individuals) were collected from the plant *B. papyrifera* in Yangling, Shaanxi Province, China in August 2010 and 2012. The individuals were anesthetized by chilling before they were dissected.

2.2. Light microscopy

The treated individuals were dissected in a phosphate buffered saline (PBS, 0.2 M, pH 7.2) under a Stereoscopic Zoom Microscope Motic SMZ168 (Xiamen, China). Their salivary glands were dissected out and photographed by a scientific digital micrography system equipped with an Auto-Montage Imaging system and a Qimaging Retiga 2000R digital camera (QImaging, Surrey, B.C., Canada).

2.3. Transmission electron microscopy

The salivary glands were fixed in 2.5% glutaraldehyde in PBS (0.1 M, pH 7.2) for 12 h at 4 °C. Before being post-fixed in 1% osmium tetroxide for 2 h, the samples were rinsed five times (5, 10, 15, 20, and 30 min, respectively) in PBS, then dehydrated in an ethanol series and 100% (v v) acetone. The materials were infiltrated by a mixture of epoxy resin (Epon 812) in acetone, and were embedded in pure Epon 812 for 24 h at 30 °C, then for 48 h at 60 °C. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined under a JEM-1230 TEM at 80 kV.

The terminology for the salivary gland structures mainly follows that of Tsai and Perrier (1996).

3. Results

3.1. Morphology of the salivary glands

The salivary glands of *Tricentrus brunneus* are paired structures located centrally inside the head and thorax. Each salivary organ consists of a principal gland and an accessory gland. The principal gland is acinous and contains an anterior lobe and a posterior lobe, with the former possessing three types of acini (I, II, III) but the latter having only one type of acinus (IV). The acini of types I, II and III are all one in number; while the rosette-shaped acini of type IV are six in number and are closely arranged concentrically. Two thin efferent salivary ducts fuse into a thick common salivary duct that opens into the salivary syringe. The ar-

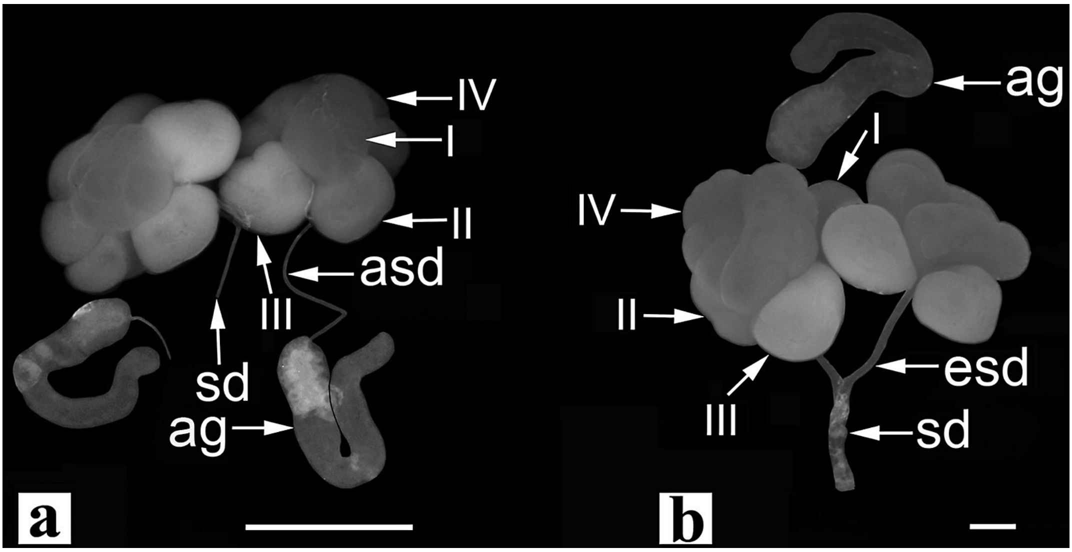

Fig. 1. Light micrographs of salivary glands of male *Tricentrus brunneus*. – a. Anterior view. – b. Lateral view. Abbreviations: ag, elbow-shaped accessory gland; esd, efferent salivary duct; asd, accessory salivary duct; sd, common salivary duct; I–III, three types of acini contained in anterior lobes of principal gland; IV, the fourth type of acinus of posterior lobe in principal gland. Scale bars: 0.5 mm in a, 0.1 mm in b.

rangement of the acini of the salivary gland is shown in Fig. 1. The accessory gland is elbow-shaped and joins the principal gland via a thin and long accessory salivary duct.

3.2. Ultrastructure of the salivary glands

3.2.1. Principal gland

Different types of secretory cells are identified in the principal gland (acini I, II, III, IV), based on their cytoplasmic characteristics. Both the semithin section and ultrathin section of the tissue (acini I, II, III) show that their cells are composed of abundant secretory granules throughout, and are devoid of other elements such as microvilli. The acini I and II possess one type of cell. The efferent salivary duct, common duct, and accessory salivary duct exhibit a great similarity in the cell characteristics (for details, see below).

3.2.1.1. Acinus I

The basal plasma membrane of the secretory cells invaginates into developed infoldings associated with mitochondria (Fig. 2a). Scattered elements of rough endoplasmic reticulum and numerous secretory granules are observed in the cytoplasm. The secretory granules are different in number, size, shape and electron-density; of these, some are electron-dense in the center and electron-lucent at the periphery. Some secretory vesicles of irregular shape are evident around the secretory granules; inside, they appear to contain many fine granular materials.

3.2.1.2. Acinus II

Secretory cells are characterized by poorly-developed basal infoldings, abundant secretory granules and sparsely scattered secretory vesicles filled with granular material (Figs 2b, 3a, b). The secretory vesicles are large and the secretory granules are small, but both irregular in shape (Fig. 2c). Three types of secretory granules are observed in the cells, which vary in number, size, shape and electron-density: electron-dense granules without a black border; electron-dense granules with a black border; and granules with electron-dense center and electron-lucent periphery (Figs 2d, 3a). Small oval mitochondria scattered around the secretory vesicles are also found in the cytoplasm (Fig. 3b).

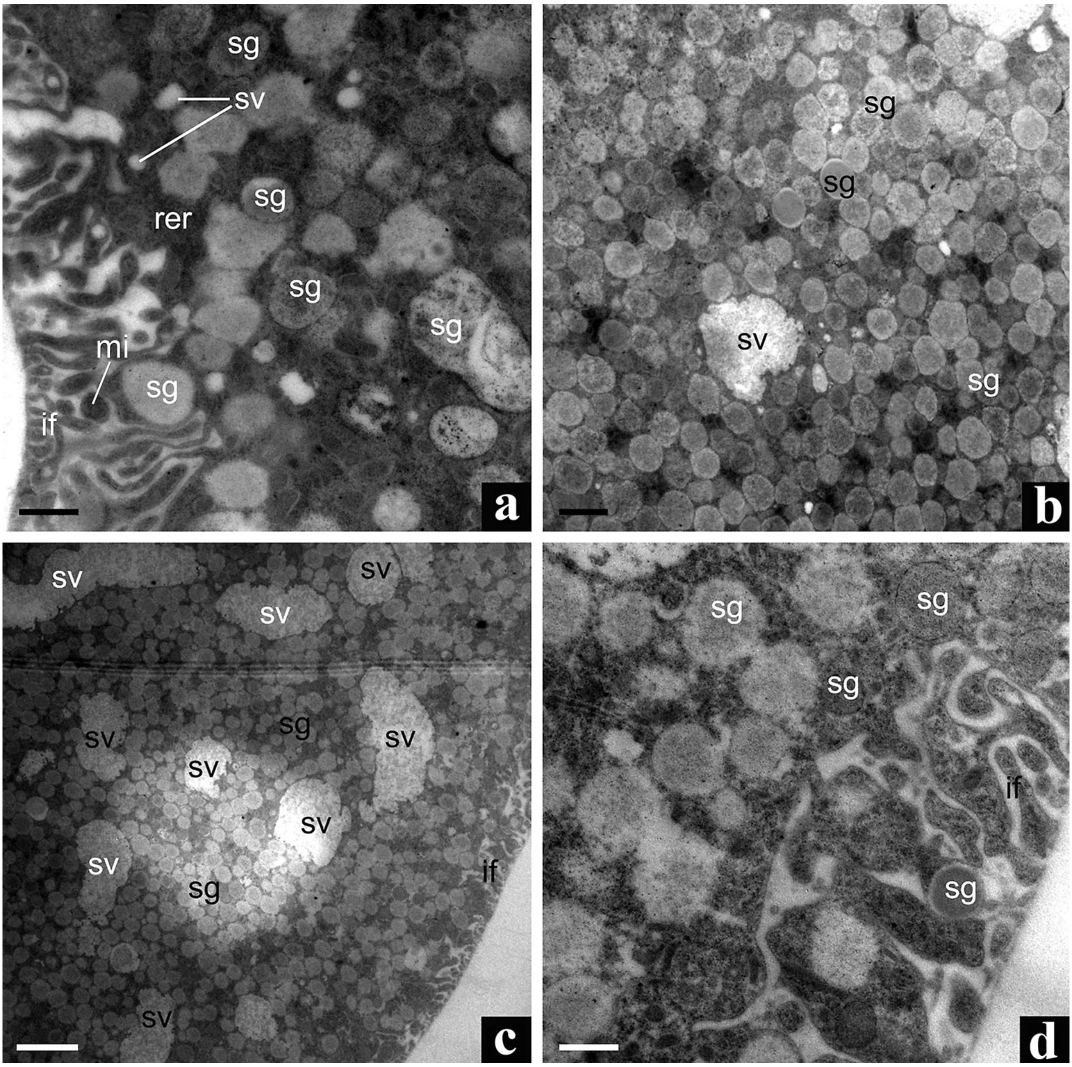

Fig. 2. Electron micrographs of acini I and II in anterior lobe of acinous principal salivary glands of male *Tricentrus brunneus*. – a. Peripheral part of a cell of acinus I with basal plasma membrane invaginating into infoldings. Mitochondria and rough endoplasmic reticulum scatter among secretory granules and secretory vesicles. – b. Cytoplasm of a cell of acinus II with abundant secretory granules filling cytoplasm. Secretory vesicles among secretory granules. – c. Peripheral portion of cells in acinus II with basal infoldings, secretory granules and secretory vesicles. – d. Peripheral portion of a cell in acinus II. Cytoplasm contains secretory granules. Abbreviations: if, infoldings; mi, mitochondria; rer, rough endoplasmic reticulum; sg, secretory granules; sv, secretory vesicles. Scale bars: 1.0 μm in a and d, 2.0 μm in b, 5.0 μm in c.

3.2.1.3. Acinus III

The cells are remarkably featured by large areas of rough endoplasmic reticulum and numerous electron-dense secretory granules. The secretory granules are rounded and vary in size. Some oval secretory vesicles are scattered among the secretory granules.

The secretory vesicles in the acini III cells are regular in shape and very small in size. Infoldings of the basal plasma membrane are poorly-developed (Fig. 3c, d).

3.2.1.4. Acinus IV

Cells of the acinus IV undergoing different pro-

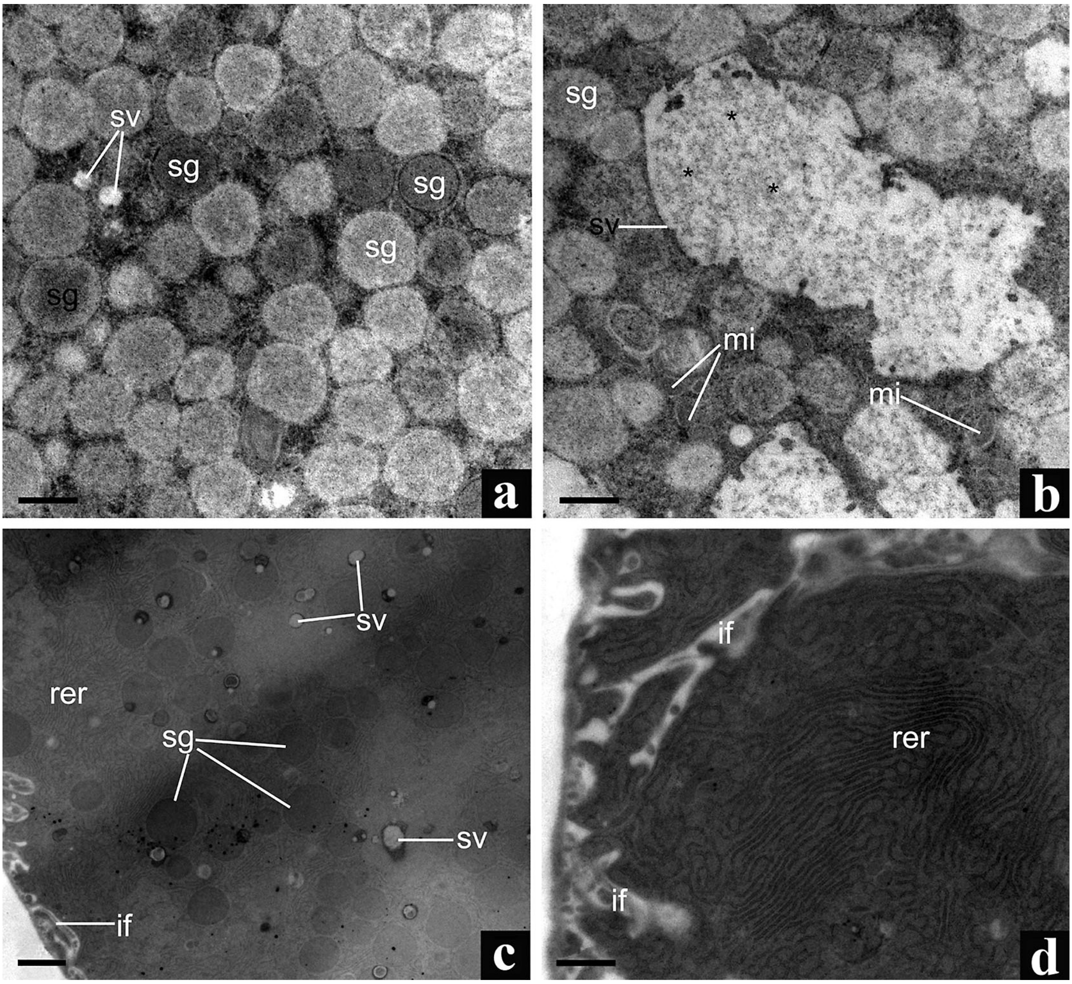

Fig. 3. Electron micrographs of acini II and III in anterior lobe of acinous principal salivary glands of male *Tricentrus brunneus*. – a. Cytoplasm of acinus II contains numerous secretory granules, with some granules having a black border. – b. Cytoplasm of acinus II contains mitochondria and large secretory vesicles with fine granular material (asterisks). – c. Peripheral portion of a cell of acinus III with many secretory granules, extensive rough endoplasmic reticulum and very shallow, poorly-developed basal infoldings. – d. Peripheral part of cell of acinus III with large area of rough endoplasmic reticulum and basal infoldings. Abbreviations: if, infoldings; mi, mitochondria; rer, rough endoplasmic reticulum; sg, secretory granules; sv, secretory vesicles. Scale bars: 1.0 µm in a, b and d, 2.0 µm in c.

cesses of the physiological development are recognized, with one characterized by peculiar secretory granules and wide basal infoldings associated with mitochondria. In the first process of the physiological development, secretory granules are prominent, around which there are some irregular-shaped secretory vesicles (Fig. 4a). These secretory granules vary in size and electron-density, and possess a black border (Fig. 4b).

In the second process of the physiological development, the basal plasma membrane invaginates into wide infoldings containing elongated mitochondria (Figs 4c, d). In the cytoplasm, three types of secretory granules with different number, size and electron-density are observed: electron-dense granules with an apparent black border; granules with an electron-dense center and an electron-lucent periphery; and large electron-lucent granules (Fig. 4d). Some small and electron-dense granules appear to fuse into larger electron-lucent granules (Fig. 5a).

In the third process of the physiological de-

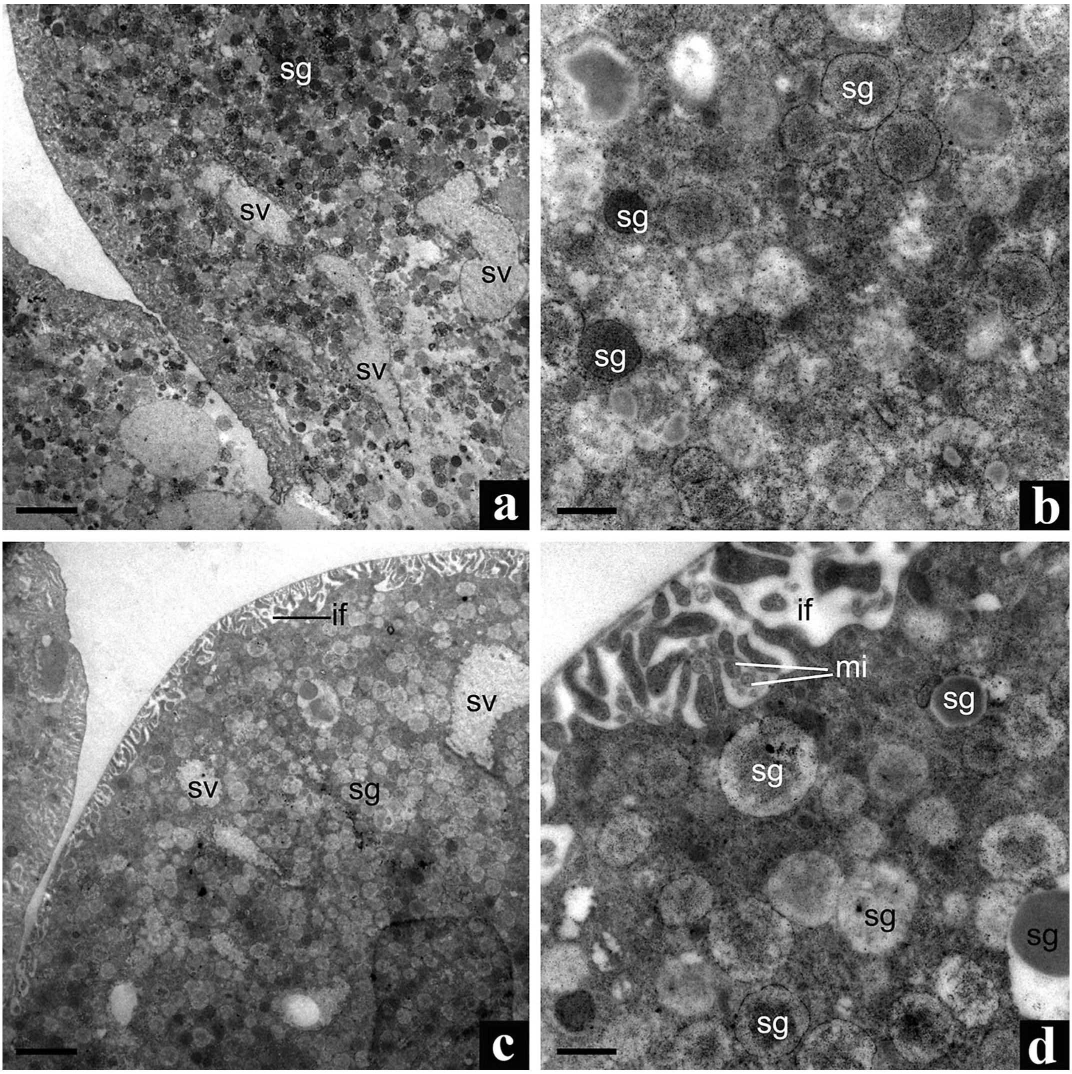

Fig. 4. Electron micrographs of acinus IV in posterior lobe of acinous principal salivary glands of male *Tricentrus brunneus*. – a. Peripheral part of a cell in the first process of the physiological development. Cytoplasm contains abundant secretory granules and secretory vesicles. – b. Cytoplasm of a cell undergoing the first process of the physiological development possesses border-black secretory granules of different electron-density. – c. Peripheral part of a cell in the second process of the physiological development, showing numerous secretory granules, interspersed with secretory vesicles. Basal infoldings are wide. – d. Peripheral portion of a cell in the second process of the physiological development possesses basal plasma membrane invaginating into wide infoldings associated with mitochondria. Many secretory granules of different size and electron-density exist near infoldings. Abbreviations: if, infoldings; mi, mitochondria; sg, secretory granules; sv, secretory vesicles. Scale bars: 5.0 µm in a and d, 1.0 µm in b, 5.0 µm in c.

velopment, the basal plasma membrane invaginates deeply and forms infoldings that are regular (Fig. 5b). Such cell shows electron-lucent secretory granules filled with abundant fine granular materials (Fig. 5a, d). Mitochondria and intracellular canaliculi coated with short microvilli facing the lumen are found among the secretory granules (Fig. 5b). A large amount of fine granular materials fill the lumen of the intracellular canaliculi (Fig. 5b). Inside the lumen of some intracellular canaliculi, a cloud of myelin figure is also observed (Fig. 5d). Large nuclei with evident

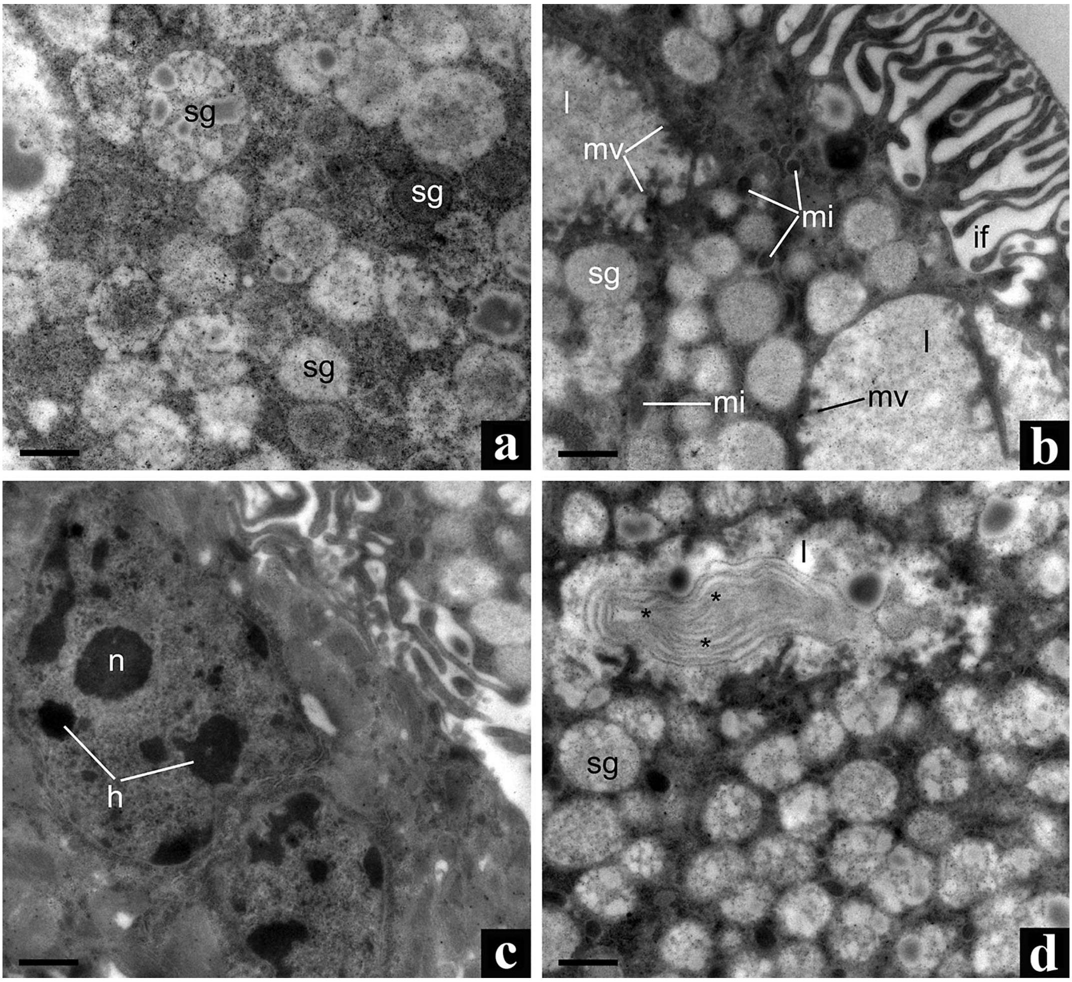

Fig. 5. Electron micrographs of acini IV in posterior lobe of acinous principal salivary glands of male *Tricentrus brunneus*. – a. Cytoplasm of cells in the second process of the physiological development contains abundant secretory granules varying in electron-density. – b. Peripheral part of cells in the third process of the physiological development, showing abundant electron-lucent secretory granules and well-developed basal infoldings. Intercellular channels is lined with short and sparse microvilli. Cytoplasm also contains many mitochondria. – c. Cytoplasm of cells in the third process of the physiological development, showing large nucleus with clumps of heterochromatin (h). – d. Cytoplasm of cells in the third process of the physiological development, showing fine granular materials contained in numerous electron-lucent secretory granules. Lumen of intercellular channels possessing myelin figure (asterisks). Abbreviations: if, infoldings; mi, mitochondria; rer, rough endoplasmic reticulum; sg, secretory granules; sv, secretory vesicles; l, lumen; mv, microvilli; n, nucleus. Scale bars: 1.0 μm in a–d.

nucleoli and small clumps of heterochromatin are also present in the cytoplasm (Fig. 5c).

In the fourth process of the physiological development, infoldings of the basal plasma membrane are poorly-developed, and have a very shallow profile (Fig. 6a). This cell is featured by abundant electron-dense secretory granules and a large area of rough endoplasmic reticulum (Fig. 6b c). The secretory granules vary in shape and size, with some smaller granules appearing to fuse into larger ones. Very small secretory vesicles also exist near the rough endoplasmic reticulum that extends to the basal infoldings (Fig. 6d).

3.2.2. Accessory gland

In the cells of the accessory gland and the accessory salivary duct, the basal infoldings are not ob-

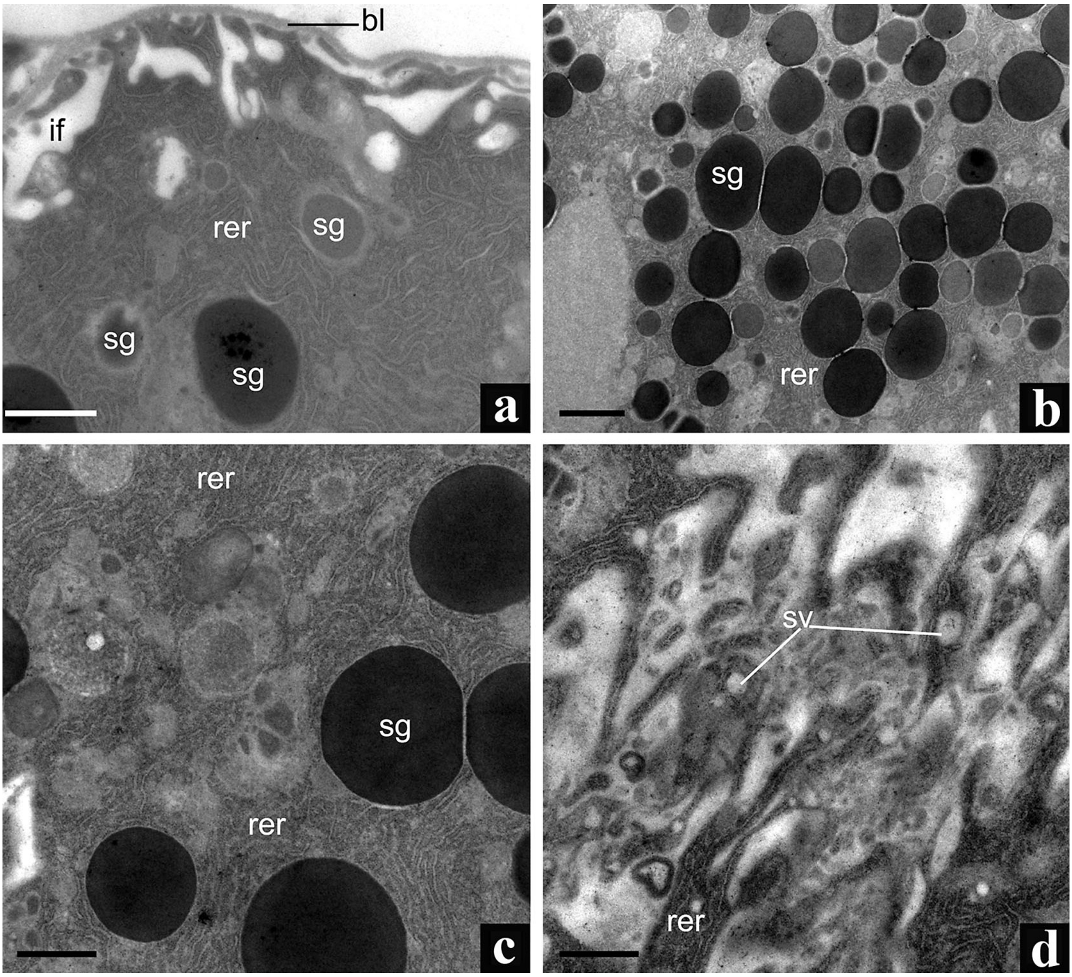

Fig. 6. Electron micrographs of acini IV in posterior lobe of acinous principal salivary glands of male *Tricentrus brunneus*. – a. Peripheral part of cells in the fourth process of the physiological development contains basal infoldings, secretory granules and extensive rough endoplasmic reticulum. – b. Cytoplasm undergoing the fourth process of the physiological development shows abundant secretory granules of different electron-density and size. Extensive rough endoplasmic reticulum exists among secretory granules. – c. Cytoplasm of cells in the fourth process of the physiological development has electron-dense secretory granules and rough endoplasmic reticulum. – d. Rough endoplasmic reticulum of cells in the fourth process of the physiological development extends into basal plasma membrane invaginations. Some small secretory vesicles exist adjacent to rough endoplasmic reticulum. Abbreviations: if, infoldings; rer, rough endoplasmic reticulum; sg, secretory granules; sv, secretory vesicles; bl, basal lamina. Scale bars: 1.0 μm in a, c and d, 2.0 μm in b.

served, but other organelles exhibit some differences.

Cells of the accessory gland contain secretory vesicles, oval nuclei, and electron-dense fine granules (less than 0.3 μm in diameter) (Fig. 7a). Scattered elements of rough endoplasmic reticulum are observed among the granules (Fig. 7b). Microtubules and myelin figure are also visible in the cytoplasm (Fig. 7c). Cells of the accessory salivary duct are characterized by a thick basal lamina and developed microvilli at the apical border. In the cytoplasm, abundant oval mitochondria, spherical electron-dense granules, and large nuclei with nucleoli and clumps of heterochromatin are observed (Fig. 7d).

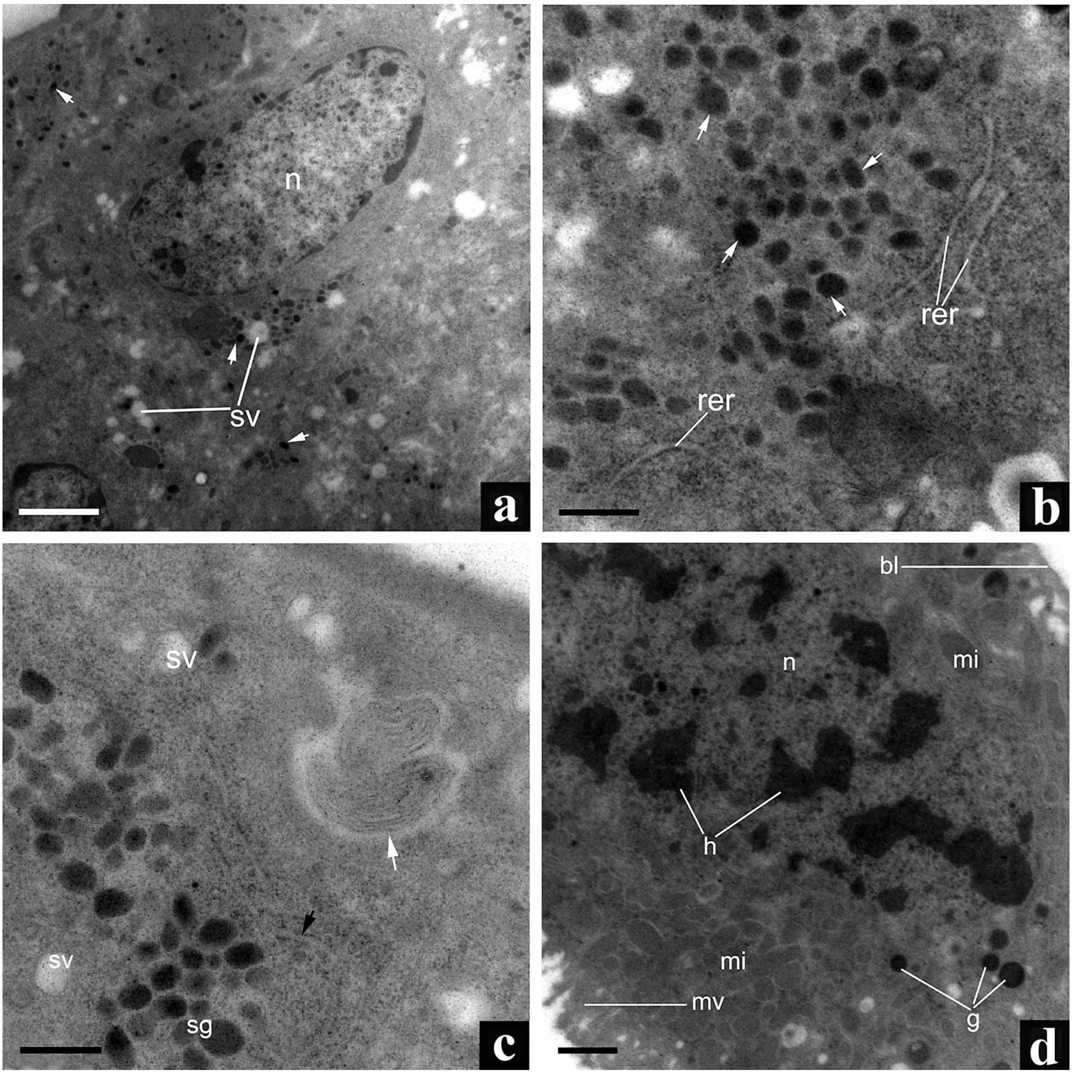

Fig. 7. Electron micrographs of cells of accessory gland and accessory salivary duct. – a. Peripheral part of a cell of accessory gland with ovoid nuclei, small electron-lucent secretory vesicles and fine electron-dense granules (white arrows). – b. Fine electron-dense granules (white arrows) and rough endoplasmic reticulum in accessory gland cells. – c. Microtubules (black arrows) and myelin figure (white arrow) in accessory gland cells. – d. Several mitochondria, electron-dense granules and large nuclei with clumps of heterochromatin in cytoplasm of accessory salivary duct. Apical border of cells is densely packed with microvilli. Basal lamina is thick. Abbreviations: mi, mitochondria; rer, rough endoplasmic reticulum; sv, secretory vesicles; sg, secretory granules; mv, microvilli; n, nucleus; bl, basal lamina; g, electron-dense granules; h, clumps of heterochromatin. Scale bars: 2.0 µm in a, 0.5 µm in b and c, 1.0 µm in d.

4. Discussion

Morphologically, the salivary glands of *T. brunneus* are similar to those found in some leafhoppers (Dobroscky 1931, Berlin & Hibbs 1963, Gil-Fernandez & Black 1965, Nasu 1965, Sogawa 1965, Tsai & Perrier 1996, Wayadande *et al.* 1997). However, each type of acinus of the anterior lobe in *T. brunneus* is only one in number, which is much less than that described in Cicadellidae. The differences among the salivary glands might be a characteristic of different families within Membracoidea.

Basal infoldings and apical microvilli greatly increase the effective surface area of cells, and effectively enhance the exchange and transport of substances (O'Donnell & Maddrell 1983). The infoldings can increase the area for transport of water from the hemolymph to the cells, thus changing the concentration of saliva (Serrão & Cruz-Landim 1996, 2000, Abdalla & Cruz-Landim 2005, Serrão *et al.* 2008). The existence of infoldings in cells of different acini of *T. brunneus* suggests that the dense secretions are diluted to facilitate saliva secretion into the efferent salivary duct. Microvilli play a vital role in the reabsorption of water, electrolytes, and other components of initial secretion (Nunes & Camargo-Mathias 2006). The presence of microvilli in acinus IV and the accessory gland duct of the salivary glands of *T. brunneus* probably indicate that the secretions in these organs are undergoing maturation before being discharged.

The presence of large areas of rough endoplasmic reticulum in the cells of the principal gland of *T. brunneus* indicates proteinaceous granules may be produced there. This is consistent with the suggestion that the principal gland plays an important part in the synthesis of protein (Baptist 1941, Miles 1960, 1972, Reis *et al.* 2003, Swart & Felgenhauer 2003, Swart *et al.* 2006, Azevedo *et al.* 2007). However, existence of scattered elements of rough endoplasmic reticulum and lack of basal infoldings in the cells of the accessory gland of *T. brunneus* might indicate the secretions produced by the accessory gland are watery and possess a few enzymes, as has been suggested by Ponsen (1972).

Secretory granules of different size, morphology and electron-densities are observed in the same type of cells and/or different type of cells of the principal gland in *T. brunneus*, suggesting that substances of different nature might be synthesized, or that the synthesized materials experience a maturation process, as has been indicated for the salivary glands of *Peregrinus maidis* (Ashmead) by Ammar (1986) and *Mahanarva fimbriolata* (Stål) by Nunes & Camargo-Mathias (2006). Electron-dense fine granules being different from the above mentioned secretory granules are found in the cells of the accessory gland of *T. brunneus*. Virus-like granules and viruses such as luteoviruses and the pea enation mosaic enamovirus (PEMV) closely associated with the accessory gland (especially with the anterior portion of the gland) have been observed in the cells of the accessory gland of aphids, such as the *Myzus persicae* (Sulzer) (Gildow *et al.* 1997, Gildow 1999). Therefore, the fine granules present in the cells of the accessory gland of *T. brunneus* are probably related to virus transmission. However, their nature and function need to be investigated further.

In conclusion, the ultrastructural characters indicate the salivary glands of *T. brunneus* have the following functions: (1) the principal gland is able to produce concentrated or stocked protein-rich secretions, which have to be diluted before discharge into the efferent salivary duct; (2) cells of the accessory gland may be closely related to the transmission of virus or bacteria; and (3) secretory cells synthesize and secrete the saliva containing enzymes and other substances.

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