Fine Structure and Chemical Analysis of the Metathoracic Scent Glands *Graphosoma semipunctatum* (Fabricius, 1775) (Heteroptera, Pentatomidae)

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Abstract

Morphology and ultrastructure of the metathoracic scent glands (MTG) of *Graphosoma semipunctatum* (Heteroptera: Pentatomidae) were studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Extracts of the volatile fraction of the MTG secretions from male and female were analyzed by capillary gas chromatography-mass spectrometry (GC-MS). In SEM investigations, MTG are composed of reservoir and a pair of glands located at the lateral site. Lateral glands connect to the reservoir with a duct. MTG open between the meso- and the metacoxae. These areas which have mushroom-like structures are called evaporation area. In TEM investigations, reservoir walls contain two types of cells. Generally, reservoir is lined by a single layer epithelial cells. These cells which have numerous organelles are named Type I cells. Type II cells are found only in a certain area of reservoir wall. These cells contain big secretory ducts lined by cuticular intima layer. Lateral glands which have two types of cells connected to the reservoir. Lateral glands which have secretory duct in their cytoplasm are lined by type I secretory cells. Type II cells have poor organelles. They are smaller than type I cells. In GC-MS investigations, MTG of each male and female of *G. semipunctatum* are very different in chemical coumpounds. In the MTG of females of *G. semipunctatum,* n-tridecane was determined maximum 44,97% and 3-Hexadecene was determined minimum 0,02% while in the males of *G. semipunctatum* n-tridecane was determined maximum 45,49% and 2-Dipentyl acetate was determined minimum 0,01%.

Key words: *Graphosoma semipunctatum*, scent glands, electron microscopy, gas chromatography-mass spectrometry

INTRODUCTION

Stink bugs, as their names suggest, are characterized by the production of large quantities of strong-smelling and irritating defensive chemical which are released when the bugs are disturbed or molested [1]. Odorous compounds are produced by both adults and immatures [2-4] and numerous reports attest to their efficacy as effective defenses against predation [4, 5]. They also may have a role as alarm pheromones [5], as has been demonstrated for similar types of compounds produced by bug species in other families [6, 7]. A single or a pair of median or ventral scent gland is usually found in the metathoracic region of Hemiptera [8].

G. semipunctatum is cosmopolitan species and live on the Umbellifers. This species is known as a large quantities released volaties species. In general, chemical analyses were done on most of stink bugs. But there are few studying about ultrastructure of scent glands.

In this paper, the MTG were studied by scanning and transmission electron microscopy and volatiles were analyzed by GC-MS.

MATERIALS AND METHODS

Insect material

Adults of *G. semipunctatum* (Fabricius, 1775) were collected from various wild umbellifers in Ayaş, Ankara, Turkey, during June through September, 2005. Insects were reared and maintained at 22-24 ^{o}C and 70% r.h. with a 12:12 light-dark photoperiodic regime in plastic jars in laboratory. Bugs were maintained on fresh host-plant until dissection. The insects were dissected in insect's saline (0.7% NaCl + 0.3% KCl) [9].

Scanning electron microscopy (SEM)

For scanning electron microscopy the thoracic region was dissected, the tergites were removed and the MTG (reservoir and glands) were fixed for 3 h with 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). After washing in the same buffer the MTG were fixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, dehydrated in graded ethanol, and drayed using 1,1,1,3,3,3-hexamethyldisilazane (HMDS), and coated with gold. The observations were made in a Jeol JSM 5600 scanning electron microscope.

Transmission electron microscopy (TEM)

For transmission electron microscopy the MTG (reservoir and glands) were dissected and fixed for 3 h with 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 ^{o}C. MTG were washed with sodium phosphate buffer pH 7.2 for 2 h at 4 ^{o}C and postfixed in 1% OsO_4 in sodium phosphate buffer pH 7.2 for 1 h at 4 ^{o}C. Tissue samples were washed with the same buffer for 1 h at 4 ^{o}C, and dehydrated in graded ethanol series and embedded in Araldite. Thin sections were cut with Leica EM UC6 (Leica Co., Austria) ultramicrotome. These sections were viewed and photographed on a Jeol 100 CX II transmission electron microscope (Jeol Ltd, Japan) at 80 kV.

Chemical analysis

The MTG (reservoir and glands) were removed and immersed in ~100 µL analytical grade hexane distilled from calcium hydride (CaH_2) and stored at -20 ^{o}C. Extracts were analyzed (~2 µL of the extract) by splitless coupled gas chromatography-mass spectrometry (GC-MS) with a Agilent 6890 series fitted with a HP-5 MS column (30m x 0.25 mm I.D. x 0.25 µm film) and interfaced to an Agilent 5973 mass selective dedector (electron impact ionization,

106 eV). The GC was programmed at 50 °C/ 2 min then 5 °C/min to 250 °C, with injector and transfered line temperatures of 250 and 280 °C, respectively, with helium carrier gas. Compounds were tentatively identified by GC-MS, and identifications were confirmed by comparison of the retention times and mass spectra with those of authentic samples. The relative amount of each compound was determined from the area under GC peaks.

RESULTS

***Scanning electron microscope (SEM) findings of* MTG**

MTG of *G. semipunctatum* have a well developed reservoir and paired glands which are located in the lateral of this reservoir (Figure 1). Reservoir is bag-shaped. There are irregular projections and intrusions on reservoir surface. Reservoir is connected to the glands by a canal in the apical surface (Figure 1).

MTG open from two ostioles to the outside. The ostioles located between 2nd and 3rd coxae show globular shape. Ostiolar groove extends downwards from ostiole (Figure 2). While this structure is much narrow in basale, its anterior demonstrates to a wider structure. After MTG secretion exists from ostiole, it spreads out the evaporation surface by ostiolar groove. There are mushroom-like structures on the evaporation surface. So, ostiole, ostiolar groove and their surroundings are called evaporation area (Figure 3). Mushroom-like structures which are polygonal and slightly concave in the center have irregular projections. They are linked to each other by ridges. (Figure 3). Furthermore they are connected to each other tightly by numerous trabecules found under the ridges (Figure 3).

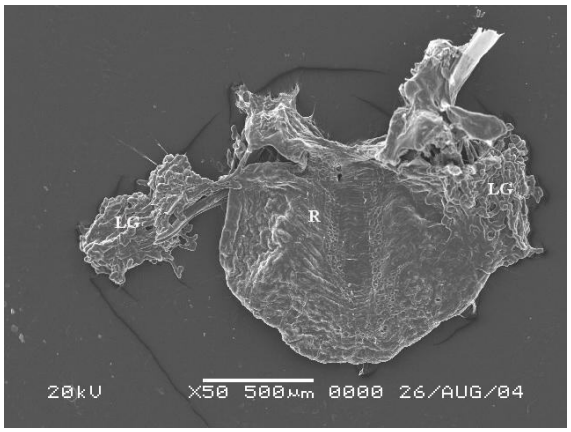

Figure 1. Scanning electron micrograph metathoracic scent glands of *G. semipunctatum* LG: lateral gland, R: reservoir

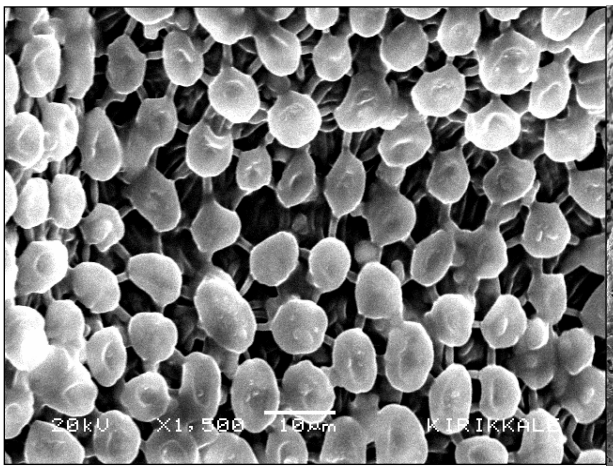

Figure 2. Evaporation areas in *G. Semipunctatum*

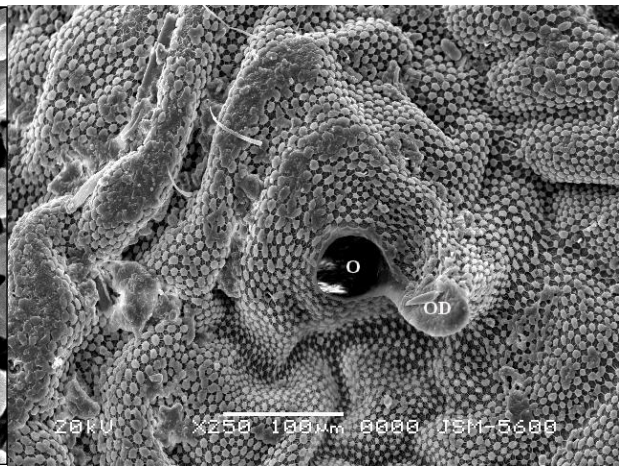

Figure 3. Ostiole and evaporation areas in *G.semipunctatum*, O: ostiole, OD: ostiolar duct

Transmission electron microscope (TEM) findings of MTG

Reservoir of MTG is lined by a single layer of columnar epithelial cells. These cells are called Type I cells (Figure 4). They are surrounded by a thin basal lamina and by a cuticular intima layer in the apical surface. Cuticular intima layer does not show flattened structure, it makes projections and intrusions. Nuclei of type I cells found in the center or near of the basal. There are numerous spherical crystals in their cytoplasm (Figure 4).

There are secretory cells which contain ducts. These cells found in the certain area of reservoir wall as a group take place among Type I cells (Figure 5). They are named as Type II cells. Secretory ducts of these cells are lined by cuticular intima layer. While nuclei of Type II cells are found in the basal region, secretory ducts of type II cells located in apical region. It is supposed that these ducts transport special secretion to the reservoir (Figure 5).

Lateral glands cells arranged around a lumen which star-like shaped (Figure 6). Its surrounding is lined by epicuticular intima layer. Around a lumen 3-5 cells are found. These cells are surrounded by a common basal lamina. These are secretory cells and named Type I secretory cells. Their nuclei are adjacent to the basal and

circular-shaped. There are different size secretory material in their cytoplasm (Figure 7). They have rich ribosomes, endoplasmic reticulums and mitochondria. There are secretory canalicule lined radial around a central lumen in their cytoplasm (Figure 8). These cells transport secretion to the lumen by these canalicule and from here secretion is transferred to the reservoir by a duct.

There are flattened cells among Type I secretory cells around the lumen of lateral glands. These cells are called Type II cells. Cytoplasms of these cells have poor organelles (Figure 6). We believe that these cells secrete cuticular intima layer.

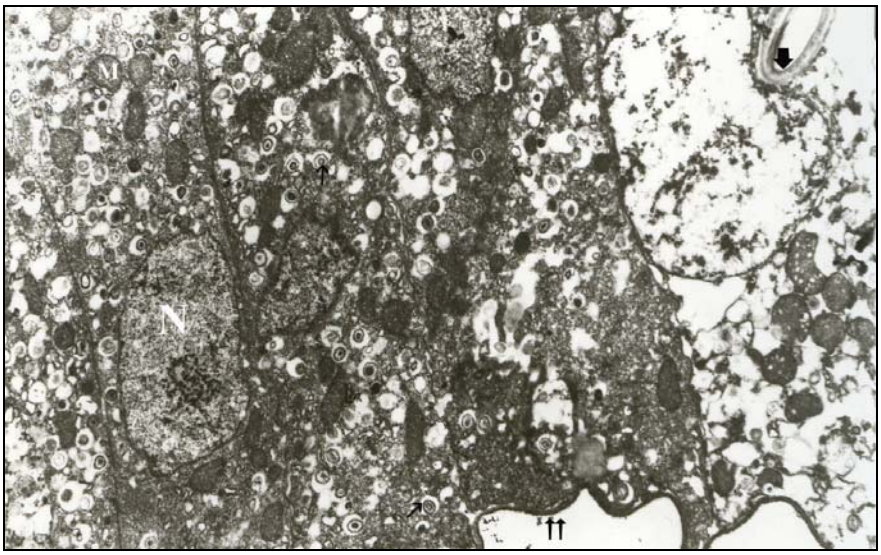

Figure 4. Single layer cylindirical epithelial cells in the reservoir of *G. semipunctatum*
M: mitochondria, N: nucleus, ➔: intima, →: spherical crystals, ⇉: basal membrane, X8750

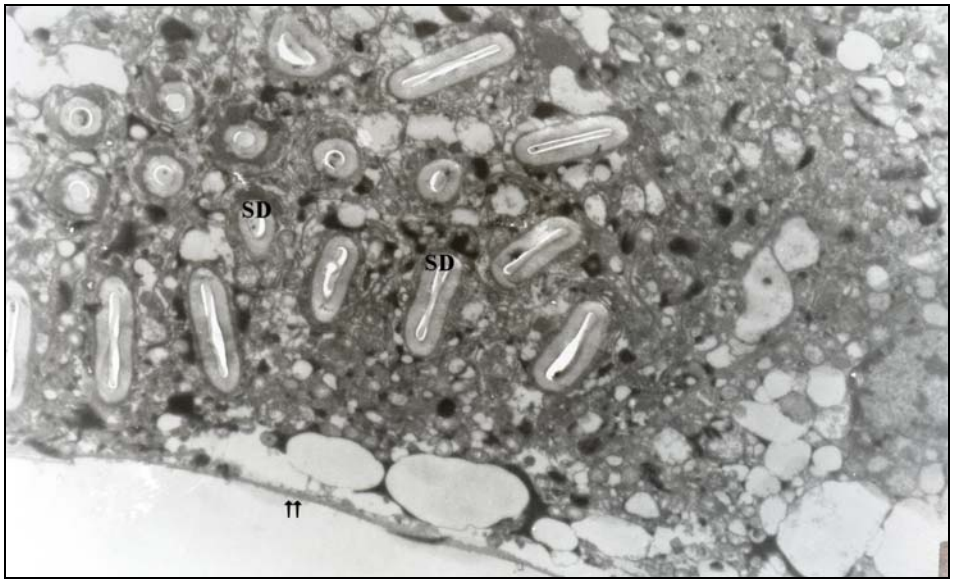

Figure 5. Type II cell and secretory ducts in the reservoir wall of *G. semipunctatum,*
SD: secretory duct, ⇉: basal membrane, X75000

Figure 6. General appearance of lateral gland connected to the reservoir of *G. semipunctatum,* BL: basal lamina L: lumen, M: mitochondria, N: nucleus, T: Trakea, T I: type I cell, T II: type II cell, X8750

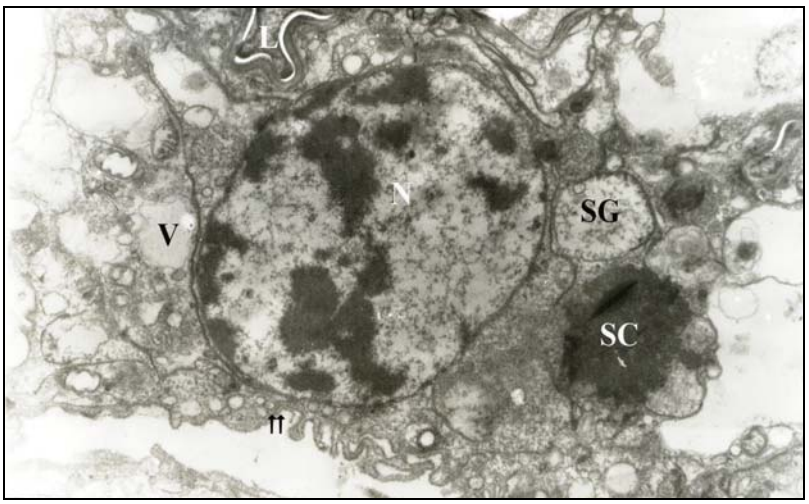

Figure 7. Type I cells of lateral glands of *G. semipunctatum*, L: lumen, N: nucleus, SG: secretion granule, SC: secretory canalicule, ⇉: basal lamina, X12500

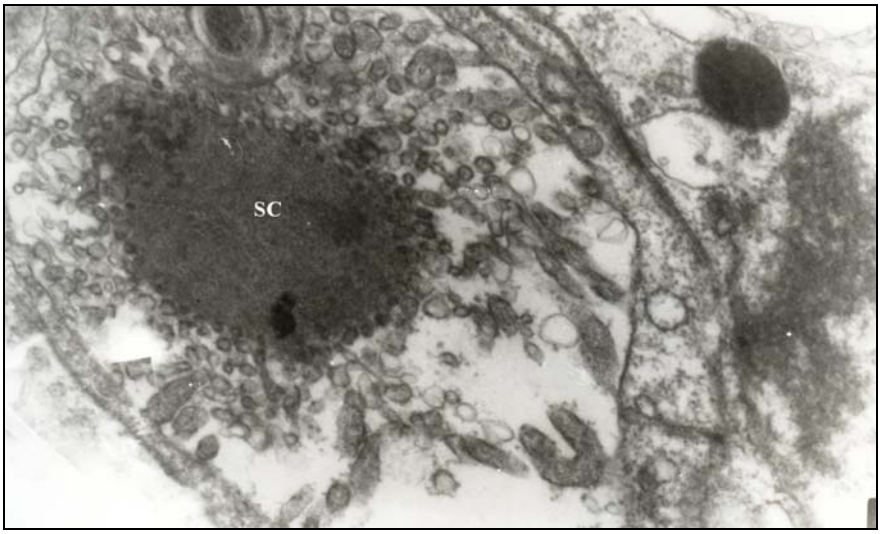

Figure 8. Secretory ducts of lateral glands cells of *G. semipunctatum*, SC: Secretory canalicule, X37500

Gas chromotography-mass spectrometry (GC-MS) findings of MTG

Analyses of MTG of *G. semipunctatum* were done on individually for male and female. Twenty types of chemical substances were determined in female while 23 types of chemical substances were determined in male (Table I). But

quantitative and qualitative compositions of these substances differ in both sexes. In the females of *G. semipunctatum* 4 types alcanes (n-Dodecane, n-Tridecane, n-Undecane, Heptadecane), 2 types alcenes (Cyclodecene, 3-Hexadecene), 4 types acetates [Methyl acetate, Ethyl acetate, Dioctyl acetate, L-Histidinemethylesterdihydrochloride], 5 types aldehydes [(E)-2-hexenal, (E)-2-Decenal, (E)-2-Octenal, 2-Undecenal, 2,6-Nonadienal], 2 types acids (n-Hexadecanoic acid, Octadecanoic acid), 1 type alcohol (2-Cyclohexen-1-ol), 1 type lactone [2(5H)-Furanone], 1 type urethan (n-Methyl carbamate) were found. In the analyses of MTG of females of *G. semipunctatum* n-tridecane was determined maximum 44,97% and 3-Hexadecene was determined minimum 0,02% (Figure 9 and Table 1).

In the males of *G. semipunctatum* 8 types alcanes (n-Dodecane, n-Tridecane, n-Undecane, Heptadecane, Heptacosane, Octacosane, Docosane, Tetracosane), 2 types alcenes (Cyclodecene, 3-Hexadecene), 3 types acetates [(E)-2-hexenyl acetate, 2-Dipentyl acetate, 6-(phenylsulfonyl)-diacetate], 4 types aldehydes [(E)-2-hexenal, (E)-2- Octenal, 2-Undecanal, 2,6-Nonadienal], 2 types acids (n-Hexadecanoic acid, Octadecanoic acid), 1 type steroid (14-Beta-H-Pregna), 1 type lactone [2(5H)-Furanone], 1 type ether (cetyl vinyl ether), 1 type alcohol (2-Cyclohexen-1-ol) were found. In the analyses of MTG of males of *G. semipunctatum* n-tridecane was determined maximum 45,49% and 2-Dipentyl acetate was determined minumum 0,01% (Figure 10 and Table 1).

Table 1. Persentages of compounds in metathoracic scent secretion of female and male of *G. semipunctatum.*

Group	Chemical Compounds	*G. semipunctatum*	
		FEMALE %	MALE %
ALCANES	n-Dodecane	2,44	3,43
	n-Tridecane	44,97	45,49
	n-Undecane	0,19	0,56
	Heptadecane	0,15	0,26
	Heptacosane	n.d	0,45
	Octacosane	n.d	0,02
	Tetracosane	n.d	0,09
	Docosane	n.d	0,11
ALCENES	3-Hexadecene	0,02	0,07
	Cyclodecene	2,01	5,02
ALDEHYDES	(E)-2-hexenal	0,36	0,36
	(E)-2-Octenal	0,61	11,79
	2-Undecenal	0,08	0,06
	2,6-Nonadienal	0,15	0,12
	(E)-2-Decenal	25,37	n.d
ACETATES	2-Dipentyl acetate	n.d	0,01
	Dioctyl acetate	0,27	n.d
	6-(phenylsulfonyl)-diacetate	n.d	0,1
	L-Histidinemethylesterdihydrochloride	0,03	n.d
	(E)-2-hexenyl acetate	n.d	0,10
	Methyl acetate	0,06	n.d
	Ethyl acetate	0,04	n.d
ACİDS	n-Hexadecanoic acid	0,12	0,63
	Octadecanoic acid	1,17	5,12
ETHER	Cetyl vinyl ether	n.d	0,24
ALCOHOL	2-Cyclohexen-1-ol	11,42	14,89
STEROİD	14-Beta-H-Pregna	n.d	0,15
LACTONE	2(5H)-Furanone	8,21	2,12
URETHAN	n-Methyl carbamate	0,16	n.d

n.d: not dedected

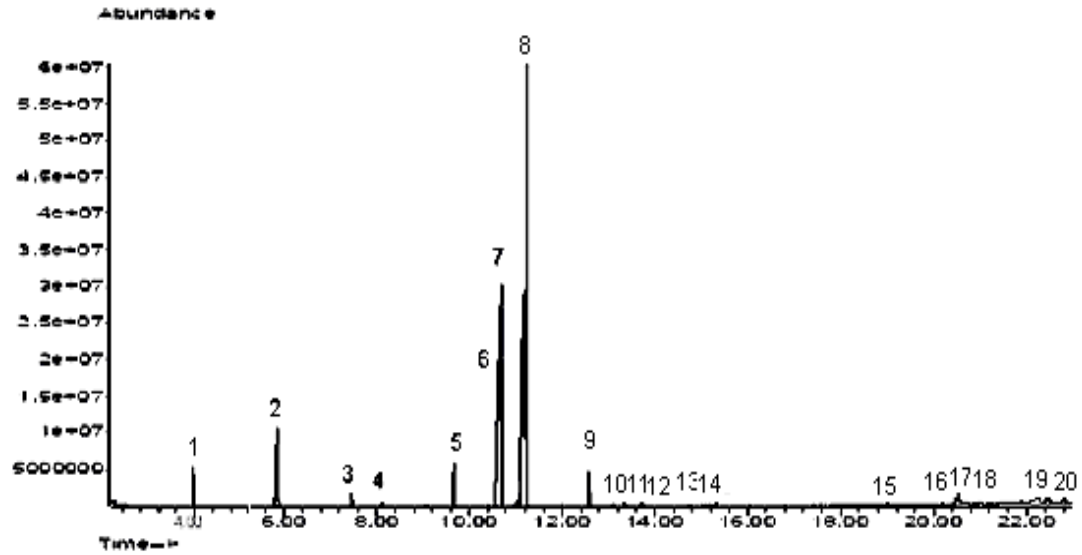

Figure 9. Gas chromatogram of an excract of the metathoracic scent gland secretion of *G. semipunctatum* female, 1-(E)-2-hexenal, 2-2(5H)-Furanon, 3-(E)-2-Octenal, 4- n-Undecane, 5- n-Dodecane, 6-2-Cyclohexen-1-ol, 7-(E)-2-Decenal, 8- n-Tridecane, 9- Cyclodecene, 10-2-Undecenal, 11-3-Hexadecene, 12-2,6-Nonadienal, 13- Ethyl acetate, 14- Methyl acetate, 15-n-Hexadecanoic acid, 16- L-Histidinemethylesterdihydrochloride, 17- n-Methyl carbamate, 18- Octadecanoic acid, 19-Heptadecane, 20- Dioctyl acetate, RT: Retention time in minutes.

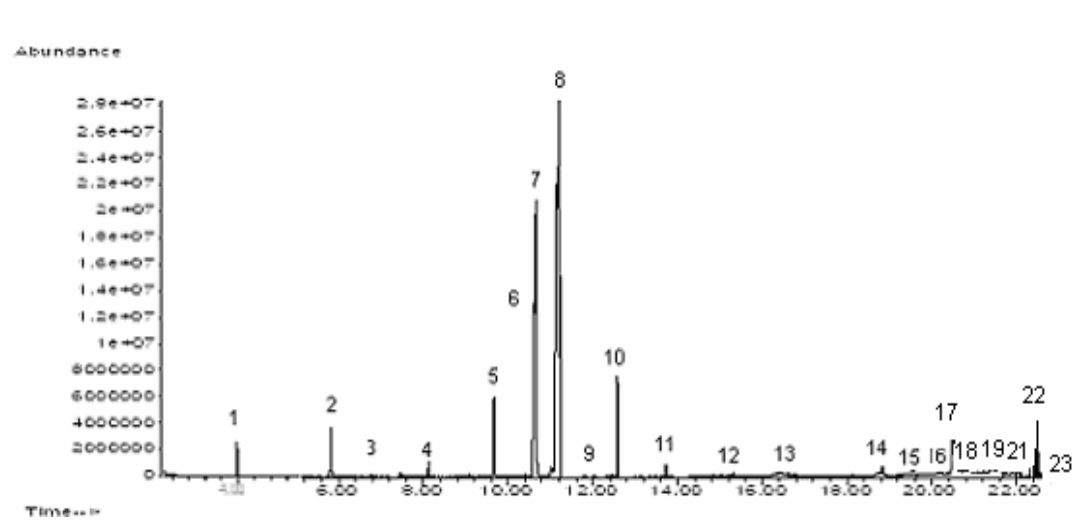

Figure 10. Gas chromatogram of an excract of the metathoracic scent gland secretion of *G. semipunctatum* male. 1-(E)-2-hexenal, 2-2(5H)-Furanone, 3-(E)-2-hexenyl acetate, 4- n-Undecane, 5- n-Dodecane, 6-(E)-2-Octenal, 7-2-Cyclohexen-1-ol, 8- n-Tridecane, 9-2-Undecenal, 10-Cyclodecene, 11-2,6-Nonadienal, 12-3-Hexadecene, 13-14-Beta-H-Pregna, 14- n-Hexadecanoic acid, 15- Tetracosane, 16- Heptacosane, 17- Octadecanoic acid, 18-Heptadecane, 19-Docosane, 20-Octacosane, 21- Cetyl vinyl ether, 22-6-(phenylsulfonyl)-diacetate, 23-2- Dipentyl acetate, RT: Retention time in minutes.

DISCUSSION

As a result of some researches three types of scent glands have been observed in insects until today. They are metathoracic scent gland, Brindley's glands and ventral glands. Most of Hemipteres consist of MTG. Metathoracic scent glands and Brindley's glands found in the insect's metathoracic region [10]. Kalin & Barret [11] described the Brindley's glands dorsally located extending into the 2nd abdominal segment. Glands of Brindley are found only Reduviids [9]. MTG are found in the 3rd thorax region in *G. semipunctatum*.

There are two types of MTG which are distomien type and omphalien type in Hemipteres. In *G. semipunctatum*, MTG belong to the diastomien type. Diastomien type scent glands open externally by paired ostioles on to the outside. There is only one ostiole between 2nd and 3rd coxa in the *G. semipunctatum.*

Kamaluddin & Ahmad [12] described five new stink bugs belonging to subfamily Phyllocephalinae of the family Pentatomidae with their special features. Their metathoracic scent glands ostioles also categorized in systematic keys and diagnostic features. *G. semipunctatum* contains ostiole, ostiolar duct and mushroom-like structures. These structures may be used for systematic keys and diagnostic features of *G. semipunctatum.* With the following studies, these special feature can be compared with other species of same genus. In evaporation areas mushroom-like structures may show

difference between species of same family. As suggested by Carayon [8], mushroom-like structures and ostiolar grooves are different in the evaporation areas of species belong to *Xylocoris* genus. In this study, ostiolar groove is wide while ostioles structures are circular in the *G. semipunctatum*. In the evaporation areas mushroom like structures which are polygonal and connected to each other with the trabecules were recorded in high number. It is believed that many of ridges found in the evaporation areas reason to staying a long time of volatile in this region. Also, mushroom-like structures function as a physical barrier for dispersal of discharged secretion.

Type I and type II cells were described by TEM in the reservoir's wall in *G. semipunctatum*. Type I cells are epithelial. These basic cells secrete intima layer. Type II cells embedded completely in the small region of reservoir's wall transport secretion to the reservoir via their ducts. These structures are similar to *Lincus* species (Heteroptera: Pentatomidae) were studied beforehand by Nagnan et al. [13]. We think that type II cells may give some scent compounds to the secretion. An important special feature of these cells is that they contain numerous spherical crystals. Spherical crystals are formed in the other bugs' tissue too. These structures which are usually seen in the digestion and excretion system are also called mineralized granules or mineral concretions [14-17]. Grodowitz et al. [15] say that sferocrystals are composed of numerous concentric thin layers as evidenced by TEM and SEM of fractured granules. Demonstrated by X-ray diffraction, spherical crystals are composed of water, P, Ca, Mg, K, Fe, Cu, Al, Zn and carbohydrates [15,16]. Sferocrystals are known that they arrange ion metabolism and the larval-pupal stage. We think that sferocrystals play an important role on developing reservoir and also manufacture scent in *G. semipunctatum*.

There are two types of cells in lateral glands. Type I secretory cells are responsible for producing secretion. There are intracellular canalicule in cytoplasm of these cells. Scent produced in Type I secretory cells is transported lumen of lateral glands by intracellular canalicule. Secretion is carried to reservoir by a duct. In previous studies, these structures have been described as intracellular canalicule and endoplasmic reticulum [9,13]. We thought that this structure is intracellular canalicule. It may also be named as endoplasmic reticulum which is transportation system of intracellular.

Chemical analyses of MTG of species of Phyrrhocoridae were divided into 11 chemical groups: aldehydes, saturated hydrocarbons, acetates, alcohols, terpenes, lactones, ketones, esters, alcenes, acids and miscellaneous compounds [18]. 10 types of chemical groups were detected as alcanes, alcenes, aldehydes, acetates, steroid, acids, ether, alcohol, lactone, urethan in chemical analysis of MTG of *G. semipunctatum* Steroid coumpound has never been reported previously in chemical analysis of MTG of Heteroptera. 14-Beta-H-Pregna is a kind of steroid. It has been found in only female bugs. This compound may be defensive. It also facilitates reproduction between same species.

The metathoracic scent reservoir receives secretions both the lateral glands and secretic cells of reservoir which differentiate from its epithelium. Their compounds are characterized as acetate [19]. In this study, some acetates were identified both male and female of *G. semipunctatum*.

The chemical analyses showed that the aldehydes and hydrocarbons are found in the scent glands a number of the species of Heteroptera. These compounds indicated different effect according to high viscosity or light viscosity [20]. Alcanes and alcenes are hydrocarbons. In this chemical data, 5 aldehydes and 6 hydrocarbones (4 alcanes, 2 alcenes) were identified in female, 4 aldehydes and 10 hydrocarbones (8 alcanes, 2 alcenes) were identified in male of *G. semipunctatum*. (E)-2-hexenal which has a dual role was found in *Nezara viridula* and some species of Pentatomidae. This compound becomes attractive at low concentration or repellent at high concentration. In the pentatomid *N. viridula*, various concentrations of n-tridecane cause the same reactions [18]. (E)-2-hexenal was identified male and female of *G. semipunctatum.* We think that it may provide a dual role in *G. semipunctatum*.

(E)-2-hexenyl acetate is found in only male of *Lethocerus indicus* (Le Peletier & Serville) (Heteroptera: Belostomatidae) [21]. It stated that (E)-2-hexenyl acetate is found in only female of *Lygocoris communis* (Heteroptera: Miridae). It works as a sex pheromone [22]. This identified (E)-2-hexenyl acetate may be sex pheromone for *G. semipunctatum* male.

N-undecane, n-dodecane, n-tridecane compounds were identified as toxic, irritant and repellent [23]. They are secreted by stink bugs for disturbance. These facts show that they are responsible for chemical defense. They may be in same function for *G. semipunctatum.*

As suggested by Waterhouse & Gilby [24], the various paraffins compounds such as hexacosane, tetracosane, docosane have provided to aid penetration of the cuticule of insect enemies and, by delaying evaporation, to act as 'odour fixatives' for the more volatile scent constituents. These components are not found in every MTG of bug. It would be interesting to know the relative efficiencies of scents with and without paraffins in deterring natural enemies. These compounds were identified in *G. semipunctatum* male. These compounds may block evaporation scent compounds very quickly after they release. These compounds may also be an odour fixatives for *G. semipunctatum* male.

(E)-2-decenal which presents in defensive MTG of *Nezara viridula* (L.) (Heteroptera: Pentatomidae) was identified as kairomone compound. Preliminary laboratory studies showed that *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae) which is parasitoid for this species eggs is attracted to an area containing adult *N. viridula's* eggs. *T. basalis* is a parasitoid for 14 genus of Pentatomidae eggs and Scutelleridae eggs [25]. (E)-2-decenal is one of the most toxic chemicals within the defensive secretion. It is not tolareted by many insects at high concentrations [26]. It was found in female of *G. semipunctatum.* It may play role as a kairomone for *G. semipunctatum.* Suggested that it is effective for defensive condition because of more toxic.

n-Methyl carbamate detected in *G. semipunctatum* female is a kind of carbamate insecticide. This compound may have contaminated from environmental. So, we didn't evaluate it as a new group.

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