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Fine structure and chemical analysis of the metathoracic scent gland secretion in *Graphosoma lineatum* (Linnaeus, 1758) (Heteroptera, Pentatomidae)

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Abstract

Morphology and ultrastructure of metathoracic scent glands (MTGs) of *Graphosoma lineatum* (Heteroptera, Pentatomidae) were studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Extracts of the volatile fraction of the MTG secretion from males and females were subjected to initial analysis. One pair of the MTG is composed of a reservoir and a pair of lateral glands connected to the reservoir with a duct. MTGs are open in between the meso- and metacoxae, on evaporation areas with a mushroom-like structure. Reservoir walls embody two types of cells, type I and type II, respectively. Cells of type I have numerous organelles, while type II cells have only been found in a certain area of the reservoir wall. They have large secretory ducts lined by a cuticular intima layer. The lateral glands connected to reservoir have two further types of cells. Lateral glands are lined by type A secretory cells and secretory duct is found in their cytoplasm. Type B cells are poor in organelles and are smaller than type A cells. Coupled gas chromatography-mass spectrometry examinations revealed that both in males and females of *G. lineatum* MTGs 14 chemical compounds occur, among which only 10 are common. These secretions indicate information such as defensive and pheromonal activities, other functions could be attributed to the secretion of the MTG of *G. lineatum* by comparison with other closely related bug species. In the analyses of MTGs of females of *G. lineatum*, *n*-octadecanoic acid was observed at the most and *n*-undecane was determined at lowest level, while males of *G. lineatum* *n*-tridecane was determined at the highest level; and (*E*)-2-hexenyl acetate has been observed at the lowest. ***To cite this article: D. Durak, Y. Kalender, C. R. Biologies 332 (2009).***

Keywords: Electron microscopy; Gas chromatography-mass spectrometry; *Graphosoma lineatum*; Scent glands; Volatile compounds

1. Introduction

Stink bugs are characterized by the production of large quantities of strong-smelling and irritating defensive chemicals, which are released when the bugs are disturbed or molested [1]. Odorous compounds are pro-

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duced by both immature stages and adults [2–4] and numerous reports attest to their efficiency for their being effective guards against predation [4,5]. They may also act as alarm pheromones, 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-glands is usually found in the metathoracic region of Hemiptera [8–11]. The adult metapleural and the development of the larval dorso-abdominal scent glands are considered as an autapomorphy of the taxon Heteroptera within Insecta. MTGs in Pentatomomorpha are well-developed, open through paired orifices on metapleuron (metepisterum) and those reviewed published opinions on phylogeny of the scent glands [12]. Davidova-Vilimova et al. (2000) [13] illustrated the dorso-abdominal scent glands and occurrence of the metapleural scent gland evaporatoria in adults of Rhopalidae (Heteroptera), and systematic and phylogenetic implications were reviewed. Furthermore, the function of MTGs is not only defending but also intraspecific aggregation and mating behavior [14]. This intriguing mechanism has led to the isolation and identification of several defensive compounds released by a number of heteropteran species [1,15].

Graphosoma lineatum (L., 1758) is a cosmopolitan species and lives on the Umbellifers. This species is a representative of the subfamily Podopinae (Pentatomidae). They are known as a large quantity volatile releasing species. In general terms, chemical analyses were carried out on most stink bugs [1–3], but there is little research about the ultrastructure of MTG.

The main purpose of the present work was to characterize the morphology and fine structure using scanning and transmission electron microscopies) and chemical composition of the MTGs of female and male of *G. lineatum* and to identify the compounds that elicit a physiologic approach compared with other species.

2. Methods

2.1. Insects

Adult *G. lineatum* were collected from various wild umbellifers in Ayas, Ankara, Turkey, between June and September, 2005. Insects were reared and kept in plastic jars in the laboratory at 22–24 °C and 70% r.h. with a 12:12 h light–dark photoperiodic regime. Bugs were kept on fresh host-plants until dissection. The insects were dissected in insect saline (0.7% NaCl + 0.3% KCl) [16].

2.2. Electron microscopy

For electron microscopy, the MTGs (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 °C. MTGs were washed with sodium phosphate buffer for 1 h at 4 °C and postfixed in 1% OsO_4 in sodium phosphate buffer (pH = 7.2) for 1 h at 4 °C.

For the scanning electron microscopy, the MTGs were dehydrated in graded ethanol, dried by the use 1.1.1.3.3.3-hexamethyldisilazane (HMDS), and coated with gold after being washed in the same buffer. The observations were made with a Jeol JSM 5600 scanning electron microscope.

For transmission electron microscopy tissue samples were washed with the same buffer for 1 h at 4 °C, and dehydrated in graded ethanol series and embedded in epoxy resin Araldite. Thin sections were cut with a Leica EM UC6 (Leica Co., Austria) ultramicrotome. The sections were viewed and photographed on a Jeol 100 CX II transmission electron microscopy (Jeol Ltd, Japan) at 80 kV.

2.3. Terminology [13]

Ostiole: External opening of the glands;
Evaporatorium: A part of external surface of metapleura, connected with the ostiole, and covered with a specific, complex, mushroom-like structure;
Peritreme: Ostiolar groove, running from the ostiole, mostly laterally;
Reservoir: Term used for the membranous structure, mostly sac-shaped, beneath the ostiole;
Lateral glands: Term used for glands on the lateral side of the reservoir.

2.4. Chemical analysis

The MTGs (reservoir and glands) were removed and immersed in ∼100 µL analytical grade *n*-hexane which was distilled from calcium hydride (CaH_2) and stored at −20 °C. Extracts were analyzed (∼2 µL of the extract) by GC-MS which is splitless, with an Agilent 6890 series fitted with a HP-5 MS column (30 m × 0.25 mm I.D. × 0.25 µm film) and interfaced to an Agilent 5973 mass selective detector (electron impact ionization, 106 eV). The GC was programmed at 50 °C/2 min then 5 °C/min to 250 °C, with injector and transfer 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

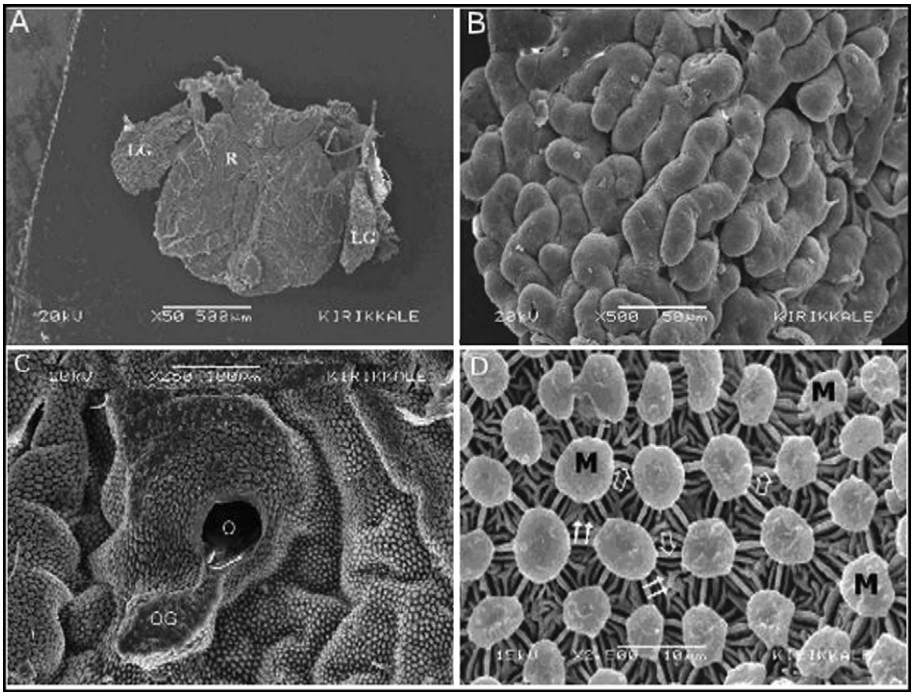

Fig. 1. (A) Reservoir and lateral glands of metathoracic scent glands, (B) Lateral glands of metathoracic scent glands, (C) Ostiole and evaporative area of metathoracic scent glands of *G. lineatum*, (D) Structure of evaporative area of metathoracic scent glands of *G. lineatum*. **R**: reservoir, **LG**: lateral gland, **O**: ostiole, **OG**: ostiolar groove, **M**: mushroom-like structure, ⇨: ridge, ⇉: trabecule.

of commercial standards. The molecular structures of compounds were determined by a comparison of the recorded mass spectra with the reference spectra of the NIST and WILEY library and coinjections of the extracts and synthetic compounds on the 2 columns. The relative proportions of the compounds in the extracts were obtained by integration of the GC-peak areas.

(E)-2-decenal, n-dodecane, n-undecane, (Z)-cyclodecene, n-tetracosane, n-hexacosane, n-octacosane (Fluka Chemical Co), (E)-2-hexenal, n-tridecane, n-heptadecane, cyclohexane standards and n-hexane (Merck Chemical Co.) were used.

3. Results

3.1. Studies on MTGs by scanning electron microscopy

The MTGs of *G. lineatum* possess a well-developed reservoir and paired glands which are located in the lateral of this reservoir (Fig. 1A), the reservoir being bag-shaped. There are irregular projections and intrusions on its surface. The reservoir is connected to the lateral glands by a canal in the apical surface (Fig. 1B).

The ostioles located between 2nd and 3rd coxae open to the outside. The MTGs open through paired ostioles. In *G. lineatum*, ostioles are observed in globular shapes. A groove-like structure extends downwards from the ostiole (Fig. 1C). While this structure is quite narrow at the basale, its anterior demonstrates larger structure. This structure is called as ostiolar peritreme or groove. Due to this peritreme and their surroundings are called as evaporation area. There are mushroom-like structures on the evaporation surface (Fig. 1D), which are slightly concave in the center, polygonal and have irregular projections. Mushroom-like structures are connected to each other by ridges. Generally six ridges extracted from each mushroom-like structure and these are connected to other mushroom-like structures (Fig. 1D). Moreover, mushroom-like structures are tightly connected to each other by numerous trabecules found under the ridges (Fig. 1D). These structures are almost the same on the whole evaporatorium. Trabecules are the main and thick bridges between mushroom-like structures, whereas ridges are thin and small.

3.2. Studies on MTGs by transmission electron microscopy

The reservoir of MTGs is lined by a single layer of columnar epithelial cells (Fig. 2E). These cells are

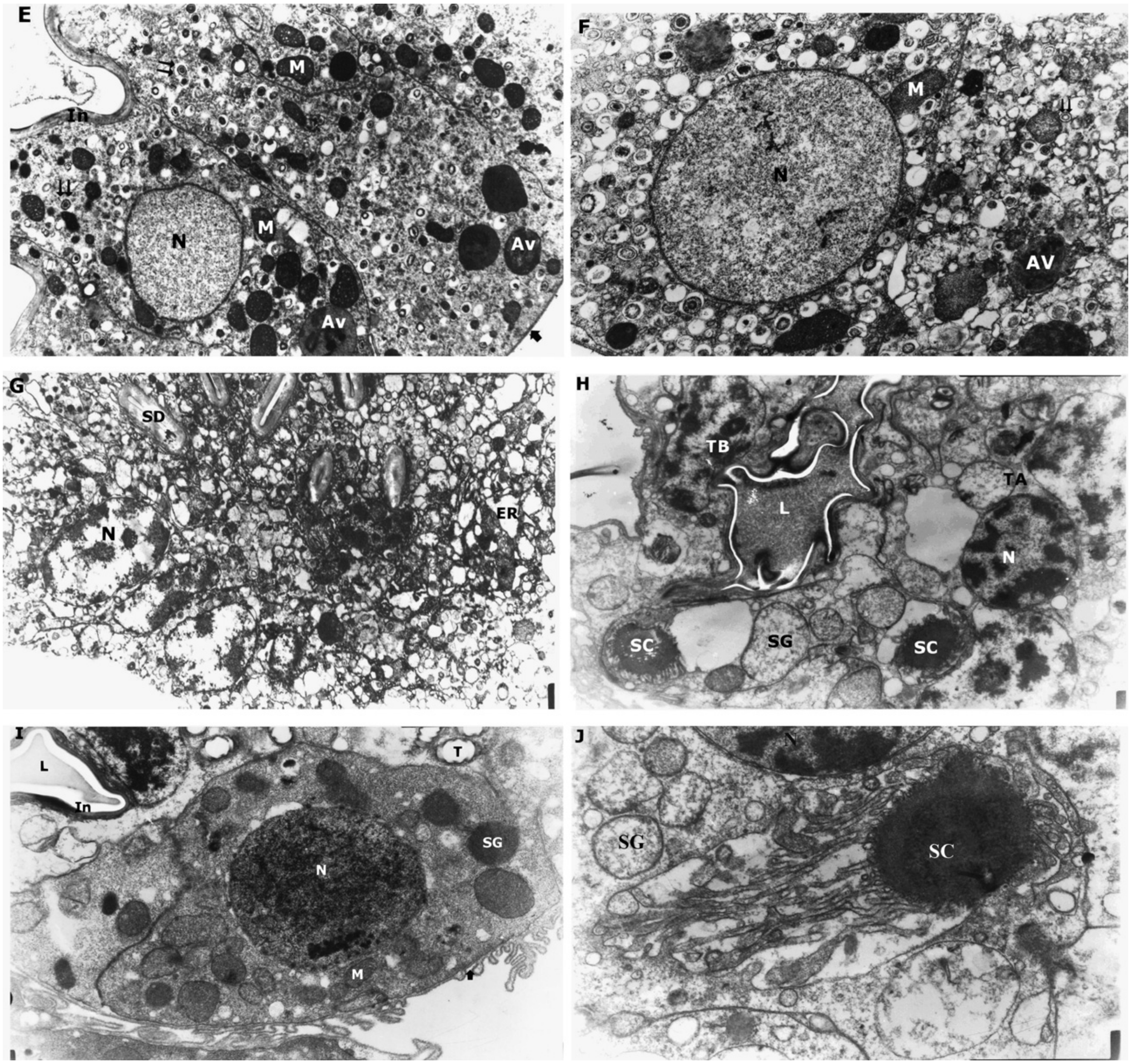

Fig. 2. (E, F) Type I cells in reservoir of MTG X8750, X12000, (G) Type II cell and secretory ducts in reservoir of MTG X8750, (H) One lateral gland of MTG X11000, (I) Type A secretory cell in lateral gland of MTG X16000, (J) Secretory duct of Type A secretory cells in lateral gland of MTG of *G. lineatum* X22500. **Av**: autophagic vacuol, **M**: mitochondria, **N**: nucleus, **In**: intima, ⇉: mineral concretion, ➡: basal membrane, **SD**: secretory duct, **ER**: endoplasmic reticulum, **TA**: type A cell, **TB**: type B cell, **SC**: secretory canalicule **L**: lumen, **SG**: secretion granule, **T**: trakea.

called as Type I cells (Fig. 2F). They are surrounded by a thin basal lamina and cuticular intima layer at the apical surface. The cuticular intima layer does not have flattened structure, it makes projections and intrusions. Nuclei of reservoir cells are generally found in the center or near of the basal. There are many mitochondria in the cytoplasm. Besides these, large autophagic vacuoles, numerous mineral concretions and small vacuoles are observed in the cytoplasm (Fig. 2E, F).

Secretory cells which embody ducts in the reservoir wall of MTGs were also detected. These cells are found in a certain area of reservoir wall as a group belonging to Type I cells. These secretory cells found in this section are called Type II cells (Fig. 2G). Secretory ducts of these cells are lined by cuticular intima layer. While nuclei of Type II cells are found in the basal part, their secretory ducts are located in the apical section. These ducts are close to the apical surface and located in the apical-basal direction. The endoplasmic reticulum is well-developed in these cells. Moreover, mineral concretions also exist in the cytoplasm. It is supposed that these ducts transport special secretory material of epithelial cells to the reservoir (Fig. 2G).

Lateral glands of MTG are recorded as cells which are formed around a lumen bearing a star-like shape. It is surrounded by epicuticular intima layer. Around a lumen, 3–5 cells exist (Fig. 2H). These cells are surrounded by common basal lamina. These are secretory cells and named Type A secretory cells. Their nuclei are adjacent to the basal of cell and they are circular-shaped. It has been recorded that there are secretory materials of different size in the cytoplasm (Fig. 2I). They are rich in ribosomes, endoplasmic reticulum as well as mitochondria. The perinuclear space of the nucleus membrane is filled with secretory material in these cells (Fig. 2I). There are secretory canaliculi lined radial around a central lumen in the cytoplasm of Type A secretory cells (Fig. 2J). Type A secretory cells transport material to the lumen via this canaliculi and secretion is transported to the central lumen of glands by a duct and from there, it is transferred to the reservoir by a duct.

There are flattened cells among Type A secretory cells around the lumen of lateral glands of MTG. These cells are called Type B cells. The cytoplasm of these cells contains only a few organelles (Fig. 2H). We consider that these cells secrete cuticular intima layer.

3.3. *Analysis of MTGs by gas cromotography-mass spectrometry (GC-MS)*

In the analyses of MTGs of *G. lineatum*, 14 different chemical substances were determined both in females and males (Table 1). However, quantitative and qualitative compositions of these substances differ between the sexes.

The volatile of MTG secretions of the female is composed of *n*-alkanes 23.97% (cyclohexane, *n*-undecane, *n*-dodecane, *n*-tridecane, *n*-heptadecane), alcene 12.86% [(*Z*)-cyclodecene], aldehydes 0.82% [(*E*)-2-hexenal, (*E*)-2-decenal], acetates 4.22% [(*E*)-2-hexenyl acetate, *Z*-17-nonadecen-1-ol acetate], acids 52.59% (*n*-hexadecanoic acid, *n*-octadecanoic acid, 1-phenanthrenecarboxylic acid) and steroid 0.96% (14-β-H-Pregna). In the MTG chemical analyses of *G. lineatum* females, *n*-octadecanoic acid was determined as the most frequent (31.15%) and *n*-undecane was determined as the least (0.02%) (Fig. 3K and Table 1).

The volatile of MTG secretions of the male is composed of *n*-alkanes 55.17% (cyclohexane, *n*-dodecane, *n*-tridecane, *n*-heptadecane, *n*-hexacosane, *n*-octacosane, *n*-tetracosane), alcene 16.51% [(*Z*)-cyclodecene], aldehydes 1.71% [(*E*)-2-hexenal, (*E*)-2-decenal], acetates 2.14% [(*E*)-2-hexenyl acetate, benzeneoctadecanoic acid 3,4-dimethoxy-methyl ester], acids 21.73% (*n*-hexadecanoic acid, *n*-octadecanoic acid). In the MTG analyses of males *n*-tridecane was determined as having the highest frequency (23.89%) and (*E*)-2-hexenyl acetate was determined as the lowest (0.8%) (Fig. 3L and Table 1).

Table 1
Percentages of compounds in metathoracic scent secretion of females and males of *Graphosoma lineatum*.

Groups	Chemical compounds	*G. lineatum*	
		female %	male %
Alcanes	cyclohexane	0.60	0.60
	n-undecane	0.02	n.d.
	n-dodecane	0.14	0.48
	n-tridecane	22.75	23.89
	n-heptadecane	0.46	2.96
	n-hexacosane	n.d.	4.47
	n-octacosane	n.d.	17.38
	n-tetracosane	n.d.	5.39
Alcenes	(*Z*)-cyclodecene	12.86	16.51
Aldehydes	(*E*)-2-hexenal	0.36	0.36
	(*E*)-2-decenal	0.46	1.35
Acetates	(*E*)-2-hexenyl acetate	0.23	0.28
	benzeneoctanoic acid 3,4-dimethoxy-methyl ester	n.d.	1.86
	Z-17-nonadecen-1-ol acetate	3.99	n.d.
Steroid	14-β-H-Pregna	0.96	n.d.
Acids	*n*-hexadecanoic acid	0.49	4.35
	n-octadecanoic acid	21.15	17.38
	1-phenanthrenecarboxylic acid	30.95	n.d.

n.d. = not detected.

4. Discussion

There are two types of MTGs in Hemipteres, namely diastomian type and omphalian type. In *G. lineatum*, the MTGs belong to the diastomian type with scent glands always open to the outside with two ostioles. There is an ostiole between the 2nd and 3rd coxae in *G. lineatum*.

Structure of ostiole varies between species [13,20–23]. In a study of Kamaluddin and Ahmad (1988) [21] it was found that the structure of MTG ostioles has also been located in systematic and phylogenetic implications while five new stink bug species of the Phyllocephalinae subfamily belonging to the Pentatomidae family were described with a special reference. *G. lineatum* contains MTGs ostiole with peritreme elongate and evaporation surface with mushroom-like structure. These structures

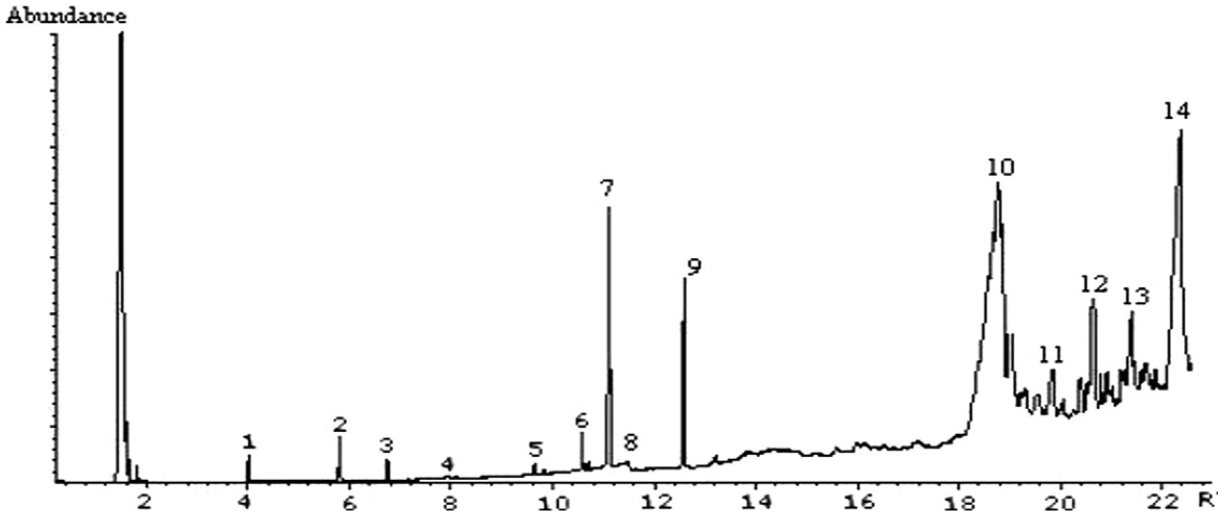

Fig. 3K. Gas chromatogram of extract of the metathoracic scent gland secretion of *G. lineatum* female. 1: (*E*)-2-hexenal, 2: cyclohexane, 3: (*E*)-2-hexenyl acetate, 4: *n*-undecane, 5: *n*-dodecane, 6: (*E*)-2-decenal, 7: *n*-tridecane, 8: *n*-hexadecanoic acid, 9: (*Z*)-cyclodecene, 10: *n*-octadecanoic acid, 11: *n*-heptadecane, 12: *Z*-17-nonadecen-1-ol acetate, 13:14-β-H-Pregna, 14: 1-phenanthrenecarboxylic acid, RT: Retention time in minutes.

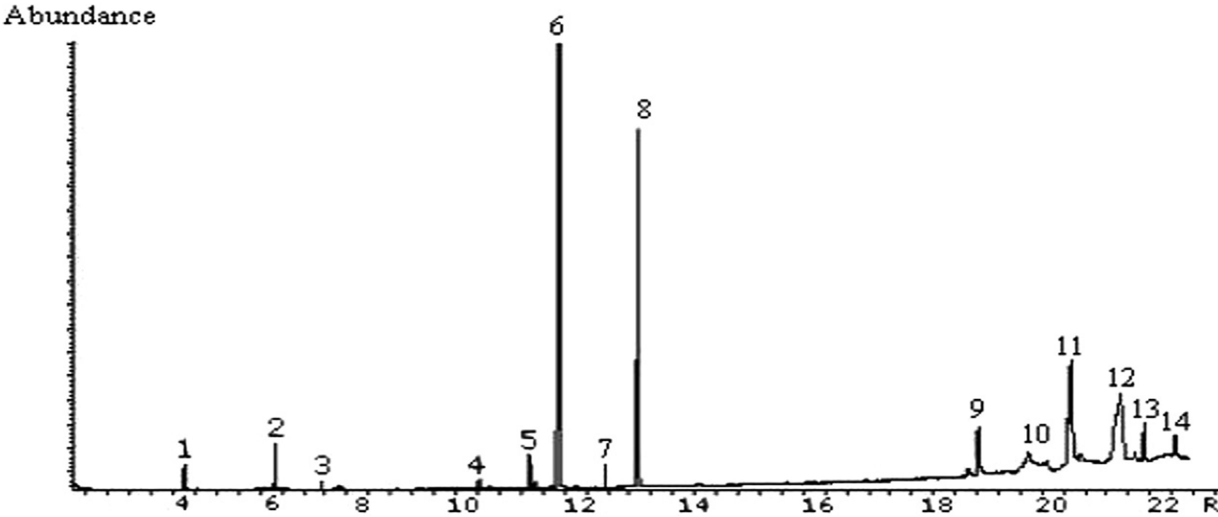

Fig. 3L. Gas chromatogram of extract of the metathoracic scent gland secretion of *G. lineatum* male. 1: (*E*)-2-hexenal, 2: cyclohexane, 3: (*E*)-2-hexenyl acetate, 4: *n*-dodecane, 5: (*E*)-2-decenal, 6: *n*-tridecane, 7: *n*-hexadecanoic acid, 8: (*Z*)-cyclodecene, 9: *n*-octadecanoic acid, 10: *n*-heptadecane, 11: *n*-tetracosane, 12: *n*-hexacosane, 13: *n*-octacosane, 14: benzeneoctanoic acid 3,4-dimethoxy-methyl ester, RT: Retention time in minutes.

can be used in systematic and phylogenetic implications of *G. lineatum*. In the next research, these special features can be compared with other species of the same genus. In evaporation areas, mushroom-like structure may show difference between the species of the same family [8]. Carver (1990) [22] examined the mushroom-like structure of the ventral thoracic scent gland of *Poecilometis longicornis* (Hemiptera: Pentatomidae) in detail and put forth its special feature. In this present research, it has been found that peritremes are wide, whereas ostioles are circular in the *G. lineatum*. The evaporation areas which are polygonal and connected to each other with trabecules are found in large numbers. It has been believed that many of ridges found in the evaporation areas have caused to long time of volatility in this section. Also, mushroom-like structure of the integument served as a physical barrier to dispersal of discharged secretion [22].

Schofield and Upton (1978) [17] showed that in *Panstrongylus megistus* the gland is composed of two types of cells: the secretory cells and the epithelial cells. Later, Barret et al. (1979) [24] also confirmed the earlier data in *Rhodnius prolixus* and reported the existence of another type of glandular unit named type A, which is composed of the same elements of type B but differs in the structural aspect, like the shape of the secretory apparatus. Type I cells and type II cells were described in the reservoir wall in *G. lineatum* by TEM. Type I cells secrete intima layer. Type II cells transport their secretion to a reservoir via their ducts. This structure similarity in *Lincus* species (Heteroptera: Pentatomidae) has been examined before by Nagnan et al. (1994) [19]. We consider that type II cells may have added some scent compounds to the secretion product. Canals have been observed especially in the apical section of these cells that are located in this area.

The reservoir of the MTG consists of paired colorless lateral glands in both side and the lateral glands empty through ducts into it. The task of the Type A secretory cells of the lateral glands is to produce secretory material. These cells have intracellular canaliculi in their cytoplasm. Scent is transported lumen of lateral glands by intracellular canaliculi. In previous study, these structures were described as intracellular canaliculi and endoplasmic reticulum [16,19]. We argue that this structure is an intracellular canalicule. Rather it may be named as endoplasmic reticulum, since it may be intracellular transport system. We believe that the cell is stimulated and the secretion is introduced into the canalicule and passes trough the collecting duct up to the glandular lumen where it is stored for future liberation.

Chemical analysis of the MTG in both sexes of *G. lineatum* revealed many compounds in each individual. Due to their general organization and the composition of the scent products, volatile compounds of the MTGs and difference between females and males of *G. lineatum* have been unknown up to now [25,26]. There is a far greater basic similarity within the families of Pentatomidae bugs [4,19,27]. However, some distinctiveness of various secretions may play a sexual or alarm pheromone role. Since, *n*-undecane, *Z*-17-nonadecen-1-ol acetate, 14-β-H-pregna and 1-phenanthrenecarboxylic acid were found to be specific to only females, however, *n*-hexacosane, *n*-octacosane, *n*-tetracosane and benzeneoctadecanoic acid 3,4-dimethoxy-methyl ester were found to be specific to males. Some of these compounds are thought to be possibly sex pheromone. 14-β-H-Pregna, which is a kind of steroid, was also found in the gland. This compound, which is secreted by the MTG, may give rise to not only defensive chemicals but also to sex pheromone and, may also facilitate reproduction within the species.

Chemical analysis put forth that the aldehydes and hydrocarbons found in the MTG, and a number of the species of Heteroptera have a dual function. That means that this compound indicated different effects according to their either heavy viscosity or light viscosity [28]. An identified dual role for (*E*)-2-hexenal was found for *Nezara viridula* and some species of Pentatomidae. This compound becomes attractive at low concentration, and repellent at high concentration. In the pentatomid *N. viridula*, various concentrations of *n*-tridecane lead the same reactions [29]. Similar results were observed through the use of high or low concentrations of aldehydes, keto-aldehydes and saturated hydrocarbons in *Dyoderus fasciatus* and *D. cingulatus* [30]. (*E*)-2-hexenal was identified male and female of *G. lineatum* and we consider that these compounds may be effective in a dual role in *G. lineatum*.

n-undecane, *n*-dodecane, *n*-tridecane compounds were identified as toxic, irritant or repellent [31]. They are released by stink bugs as a reaction to disturbance. These facts indicate that they are responsible for chemical defenses. Thus, these compounds may also have a similar function in *G. lineatum* species.

As suggested by Waterhouse and Gilby (1964) [18], the various paraffin compounds such as *n*-hexacosane, *n*-tricosane, *n*-docosane have been indicated to help penetration through the cuticule of insect enemies and, their acting as ‘odour fixatives’ for the more volatile scent constituents by delaying evaporation. These components do not exist in the MTGs of all bug species. It would be interesting to know the relative efficiencies of scents with and without paraffin in deterring the natural enemies. These compounds were identified in *G. lineatum* species, which may be blocking the evaporation of scent compounds very quickly after they are released. Thus, these compounds may also serve as odour fixatives for *G. lineatum* species.

Special features of *G. lineatum* can be pointed out in this paper. The first one concerns to the structure of the lateral multitubular glands and reservoir. The defensive secretions of insects always included compounds in small or trace amounts. These components may represent only a few per cent or less of the total secretion. MTG contents are primarily for defense and have role as sex pheromones. In addition to these hypothetical defensive and pheromonal activities, other functions could be attributed to the secretion of the MTG of *G. lineatum* by comparing with other close bug species. In addition, since they are usually not identified, their phylogenetic significance remains ignored. As the identification of other compounds in other species continues to be implemented, more data will become available for the systematic and phylogenetic analyses [29]. With our present study we have aimed to provide contributions to systematic and phylogenetic research. The composition and structure of the MTG has been identified and quantified in many Heteroptera, the biological function of each compound in the scent secretion still needs further study. So, MTG compounds of *G. lineatum* reported in Table 1 are probably the major contributors to the odours of the Pentatomidae investigated.

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