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Fine Structure and Chemical Analysis of the Metathoracic Scent Gland of *Eurygaster maura* (Linnaeus, 1758) (Heteroptera: Scutelleridae)

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The morphology and ultrastructure of the metathoracic scent glands (MTG) of *Eurygaster maura* were studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Also, extracts of the volatile fraction of the MTG secretion from males and females were analyzed by capillary gas chromatography-mass spectrometry (GC-MS). In SEM investigations, MTG are composed of a reservoir and a pair of lateral glands connected to the reservoir by a duct. MTG are open in between the meso- and the metacoxae. These areas, called evaporation areas, are composed of mushroom-like elements. In TEM investigations, the reservoir walls contained two types of cells. Generally, a reservoir is lined by a single layer of epithelial cells, type I cells, which have numerous organelles. Type II cells are found only in a certain area of the reservoir wall. These cells have large secretory ducts lined by a cuticular intima layer. The lateral glands are lined by secretory cells and a secretory duct found in their cytoplasm. Nuclei of secretory cells are closed to the basal region of the cells and circular-shaped. In GC-MS investigations, the MTG exhibited a typical scutellerid composition. In general, (E)-2-hexanal, (E)-2-hexenyl acetate, n-tridecane, n-hexanoic acid, octadecanoic acid, and n-dodecane compounds were present, while diisooctyl acetate and 14-Beta-H-Pregna were detected only in the male extracts of *Eurygaster maura.*

Key words: Heteroptera, *Eurygaster maura*, scent glands, TEM, gas chromatography-mass spectrometry.

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Stink bugs, as their name suggests, 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 (ALDRICH 1988). Odorous compounds are produced by both adults and immature stages (STADDON 1979; PAVIS *et al.* 1994; HO *et al.* 2001) and numerous reports attest to their efficacy as defense against predation (KRALL *et al.* 1999; HO *et al.* 2001). They also may be used as alarm pheromones (KOU *et al.* 1989), demonstrated for similar types of compounds produced by bug species in other families (GUNAWARDENA & BANDUMATHIE 1993; LEAL *et al.* 1994).

A single or a pair of median or ventral scent glands is usually found in the metathoracic region of Hemiptera (CARAYON 1971). *Eurygaster maura* is a cosmopolitan species and lives on the Gramineae (LODOS 1986). These bugs are important pests of economic crops. They are known as the stink bugs because they usually retaliate by discharging volatile secretions from their MTG when they are disturbed or molested. In general, chemical analyses were done on most of the stink bugs. But there are few studies on the ultrastructure of the scent glands.

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

Material and Methods

Insect material

Adult *Eurygaster maura* were collected from various Gramineae in Ayas, 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 the laboratory. Bugs were maintained on fresh host-plants until dissection. The insects were dissected in insect saline (0.7% NaCl + 0.3% KCl) (SANTOS-MALLET & DE SOUZA 1990).

Scanning electron microscopy (SEM)

For scanning electron microscopy the thoracic region was dissected, the tergites 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 post fixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, dehydrated in graded ethanol, dried 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 1 h with 3 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 °C. MTG were washed with sodium phosphate buffer pH 7.2 for 2 h at 4 °C and postfixed in 1% OsO_4 in sodium phosphate buffer pH 7.2 for 1 h at 4 °C. Tissue samples were washed with the same buffer for 3 h at 4 °C, dehydrated in a graded ethanol series and embedded in 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 microscope (Jeol Ltd, Japan) at 80 kV.

Chemical analysis

The MTG were removed and immersed in ~100 μl analytical grade hexane distilled from calcium hydride (CaH_2) and stored at -20 °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 × 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 of authentic samples. The relative amount of each compound was determined from the area under GC peaks.

Results

Scanning electron microscope (SEM) results

In SEM investigations the MTG of *E. maura* posses a well developed reservoir and paired glands located in the upper-lateral region of the reservoir (Fig. 1). The reservoir is 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. 1).

The ostioles located between the 2^{nd} and 3^{rd} coxae open to the outside. The MTG open through paired ostioles. In *E. maura* ostioles have a globular shape. A groove-like structure extends downwards from the ostiole (Fig. 2). While this structure is long and wide, its ostiole is circular. This structure is named the ostiolar groove or peritreme. After secretion through the ostiole, the contents of the MTG spread out from this region via the ostiolar groove. Therefore the ostiole, the ostiolar groove and their surroundings are termed the evaporation area. Mushroom-like structures exist

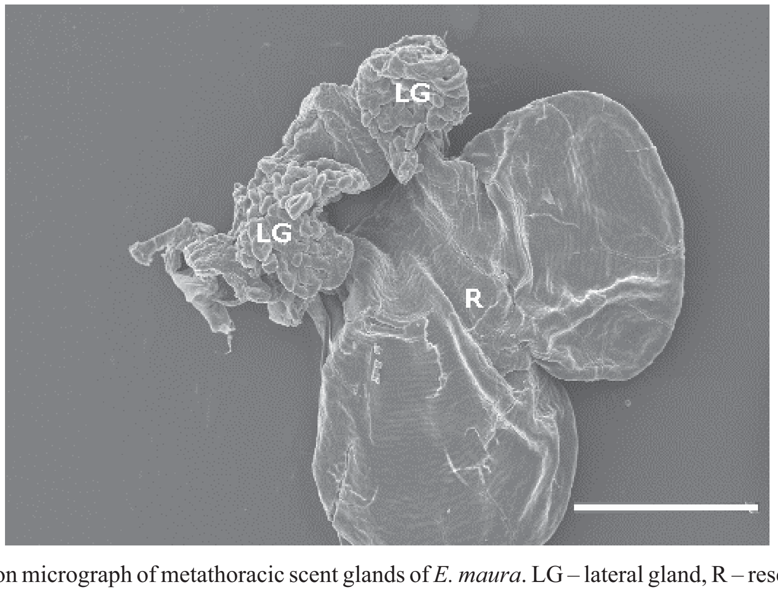

Fig. 1. Scanning electron micrograph of metathoracic scent glands of *E. maura*. LG – lateral gland, R – reservoir. Bar=500 μm.

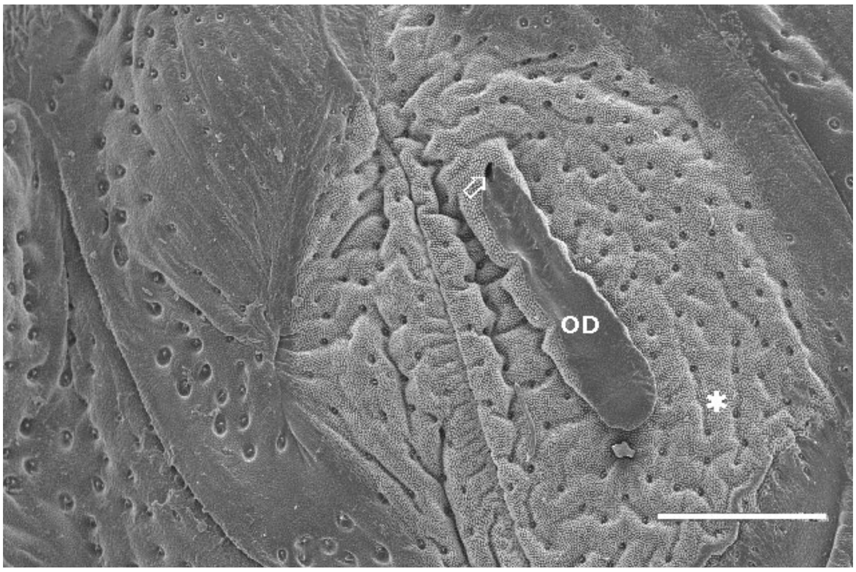

Fig. 2. Ostiole (arrow), ostiolar duct (OD) and evaporation areas (✱) in *E. maura*. Bar=500 μm.

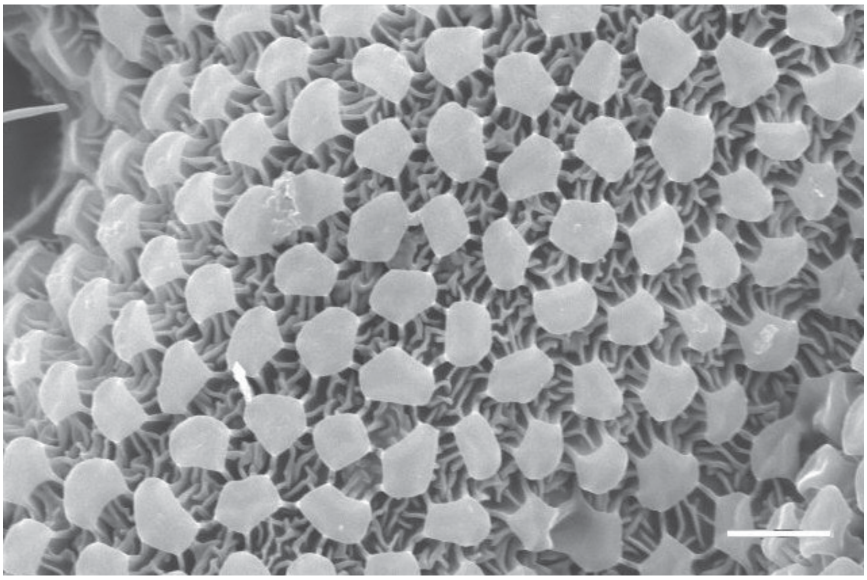

Fig. 3. Evaporation areas in *E. maura*. Bar=10 μm.

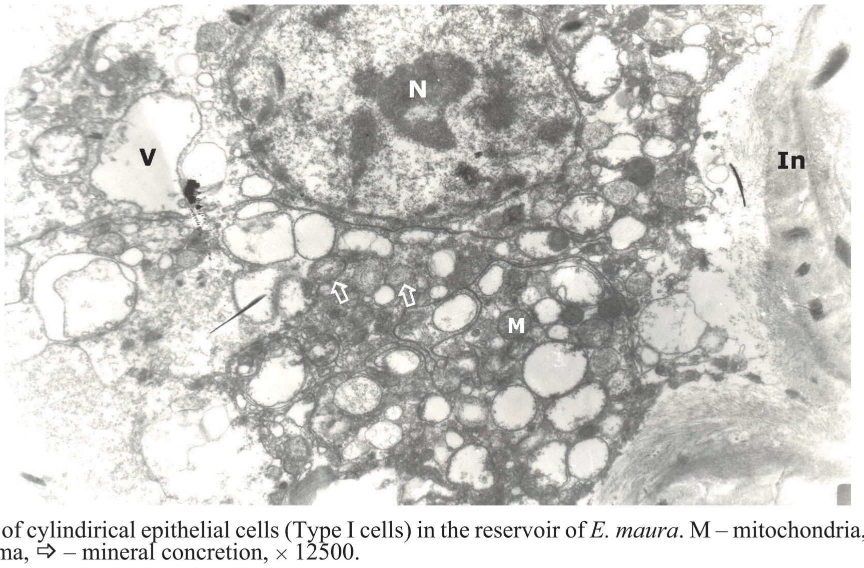

Fig. 4. Single layer of cylindirical epithelial cells (Type I cells) in the reservoir of *E. maura*. M – mitochondria, N – nucleus, V – vacuole, In – intima, ⇨ – mineral concretion, $\times$ 12500.

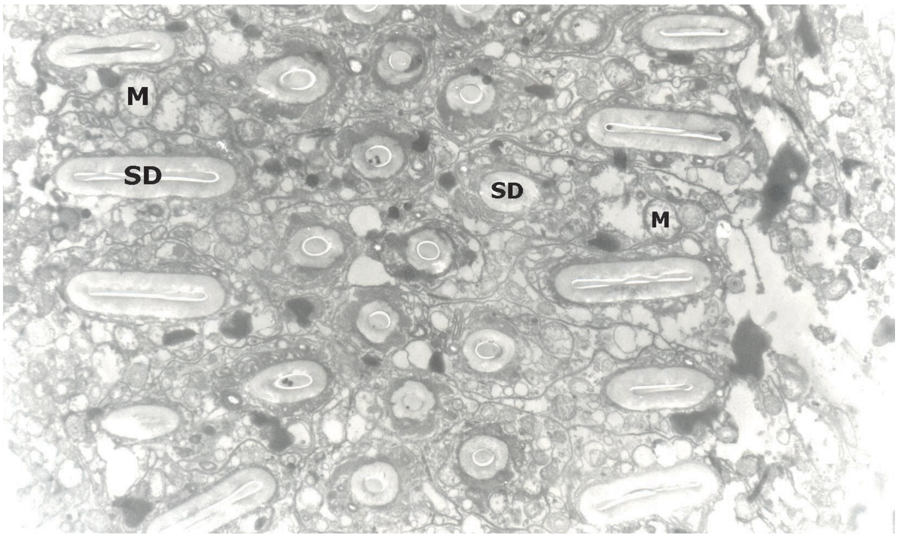

Fig. 5. Type II cell and seceretory ducts (SD) in the reservoir of *E. maura*. M – mitochondria, × 8750.

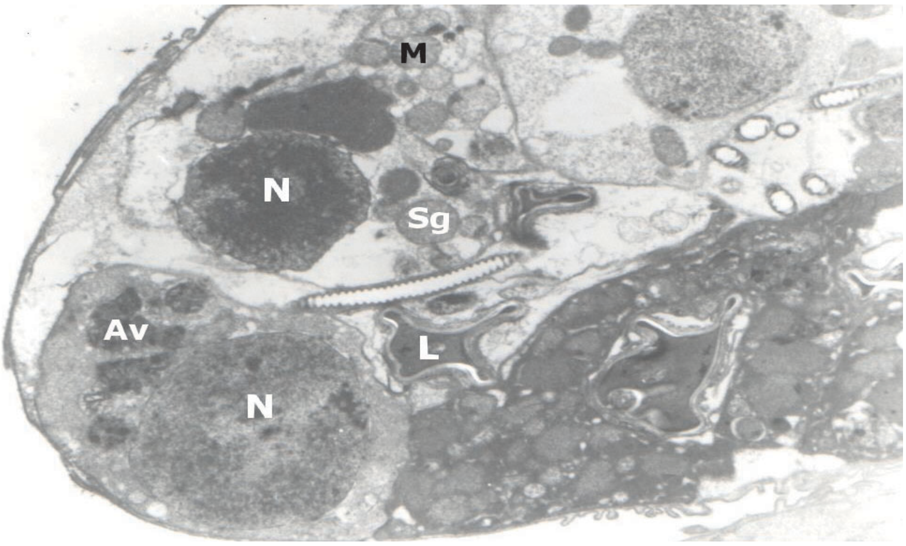

Fig. 6. General appearance of secretory cells of lateral gland of *E. maura*. L – lumen, M – mitochondria, N – nucleus, Sg – secretion granule, Av – autophagic vacuole, × 8750.

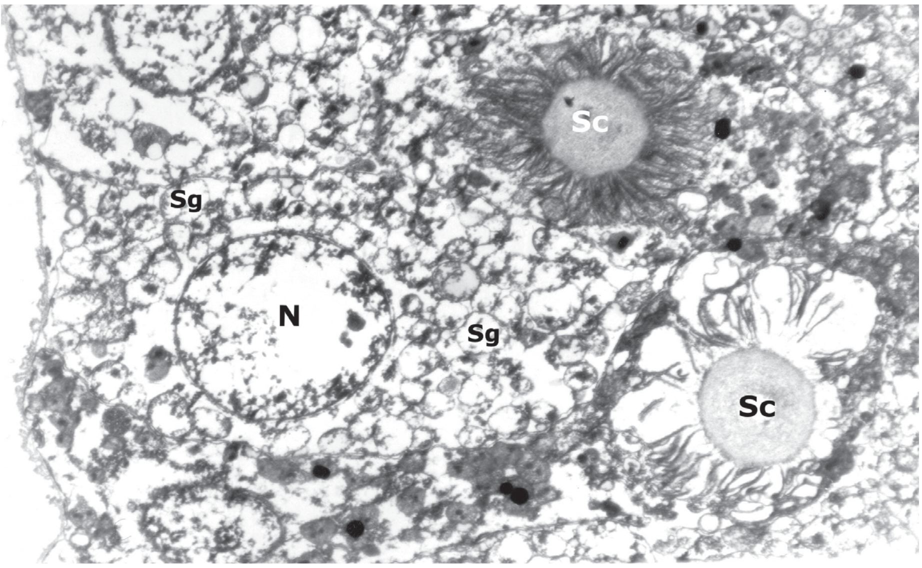

Fig. 7. Secretory ducts of secretory cell of lateral gland of *E. maura*. N – nucleus, Sg – seceretion granule, Sc – secretory canalicule, × 7500.

on the surface of the evaporation area (Fig. 3). These globular structures are slightly concave in the center and have irregular projections. The mushroom-like structures are linked to each other by ridges and numerous trabecules found under the ridges (Fig. 3).

Transmission electron microscope (TEM) results

The reservoir of MTG is lined by a single layer of columnar epithelial cells. These cells are called Type I cells (Fig. 4) and are surrounded by a thin basal lamina and a cuticular intima layer on the apical surface. The cuticular intima layer does not have a flattened structure, it possesses projections and intrusions. Nuclei of reservoir cells are generally found in the center or near the basal region of the cells. There are numerous mitochondria in the cytoplasm. Additionally, abundant mineral concretions and small vacuoles were observed in the cytoplasm (Fig. 4).

There are secretory cells which contain ducts in the reservoir wall of the MTG of *E. maura.* These cells are found in a certain area of the reservoir wall and group among Type I cells (Fig. 5). The secretory cells found in this region are designated as Type II cells. Secretory ducts of these cells are lined by a cuticular intima layer. While nuclei of Type II cells are found in the basal region, secretory ducts of type II cells are located in the apical region. These ducts are closed at the apical surface and located in the apical-basal direction. It is assumed that these ducts transport the secretion of the epithelial cells to the reservoir (Fig. 5).

Lateral glands examined by TEM show that the cells are arranged around a lumen which is star-like in shape and narrow (Fig. 6). It is lined by an epicuticular intima layer. Three to five cells are found around a single lumen. Cells are surrounded by a common basal lamina. These are the secretory cells and their nuclei are located close to the basal region of the cells and circular-shaped. The secretory material was of different size and the number of mitochondria in the cytoplasm was variable (Fig. 6). The secretory cells have an intracellular canalicule around a central lumen in their cytoplasm (Fig. 7). The canalicule with the membrane foldings is directed towards the cytoplasm and transports the secretion to the lumen by the canalicule; afterwards the secretion is transfered to the central lumen of the lateral glands and further to the reservoir through ducts.

Gas chromotography-mass spectrometre (GC-MS) results

Analyses of MTG of *E. maura* were done separately for both sexes. While 16 different chemical substances were determined in the female, 20 different chemical substances were determined in the male (Table 1). Quantitative and qualitative compositions of these substances differ in both sexes.

Table 1

Percentages of compounds in metathoracic scent secretions of females and males of *E. maura*

Groups	Chemical Compounds	*E. maura*	
		Female %	Male%
Alcanes	n-Undecane	n.d	0.11
	n-Dodecane	0.3	0.34
	Pentadecane	n.d	0.46
	Hexadecane	0.33	0.28
	Nonadecane	1.12	3.82
	n-Tridecane	22.94	34
	Octadecane	1.68	0.43
	Tricosane	1.72	0.36
	Tetracosane	41.51	3.82
	Hexacosane	2.25	2.91
	Octacosane	5.52	26.58
Alcenes	1-Nonadecene	0.45	1.43
	Cyclodecene	1.29	n.d
Aldehyde	(E)-2-Hexanal	0.27	2.63
Acetates	(E)-2-Hexenyl acetate	0.39	0.19
	Diisooctyl acetate	n.d	1.00
Acids	n-Hexanoic acid	4.92	1.29
	n-Hexadecanoic acid	1.89	1.51
	Ocadecanoic acid	8.96	4.21
Alcohol	3,7,11-(3-metyl-trideuterio)-trimetyl-(4,4,5,5-tetradeuterio)dodeca-1,6,10-trien-3-ol	n.d	3.76
Steroid	14-BETA-H-Pregna	n.d	1.32

n.d.= not detected

In the females of *E. maura* the following substances were found: 9 types of alcanes (n-Dodecane, Hexadecane, Nonadecane, n-Tridecane, Octadecane, Tricosane, Tetracosane, Hexacosane, Octacosane), 2 types of alcenes (1-Nonadecene, Cyclodecene), 1 type of acetate [(E)-2-hexenyl acetate], 1 type of aldehyde [(E)-2-hexenal], and 3 types of acids (n-Hexenoic acid, n-Hexadecanoic acid, Octadecanoic acid). In the analyses of MTG of females of *E. maura* tetracosane (41.51%) was determined in the largest amount and (E)-2-hexenal (0.27%) was determined in the smallest quantity (Fig. 8 & Table 1).

In the males of *E. maura* the following substances were found: 11 types of alcanes (n-Undecane, n-Dodecane, Pentadecane, Hexadecane, Nonadecane, n-Tridecane, Octadecane, Hexacosane, Octacosane, Tricosane, Tetracosane), 1 type of alcene (1-Nonadecene), 2 types of acetates [(E)-2-hexenyl acetate, Diisooctyl acetate], 1 type of aldehyde [(E)-2-hexenal], 3 types of acids (n-Hexadecanoic acid, n-Hexanoic acid, Octadecanoic acid), 1 type of steroid (14-Beta-H-Pregna), 1 type of alcohol [3,7,11-(3-methyl-trideuterio)-trimethyl-(4,4,5,5-tetradeuterio)dodeca-1,6,10-trien-3-ol]. In the analy-

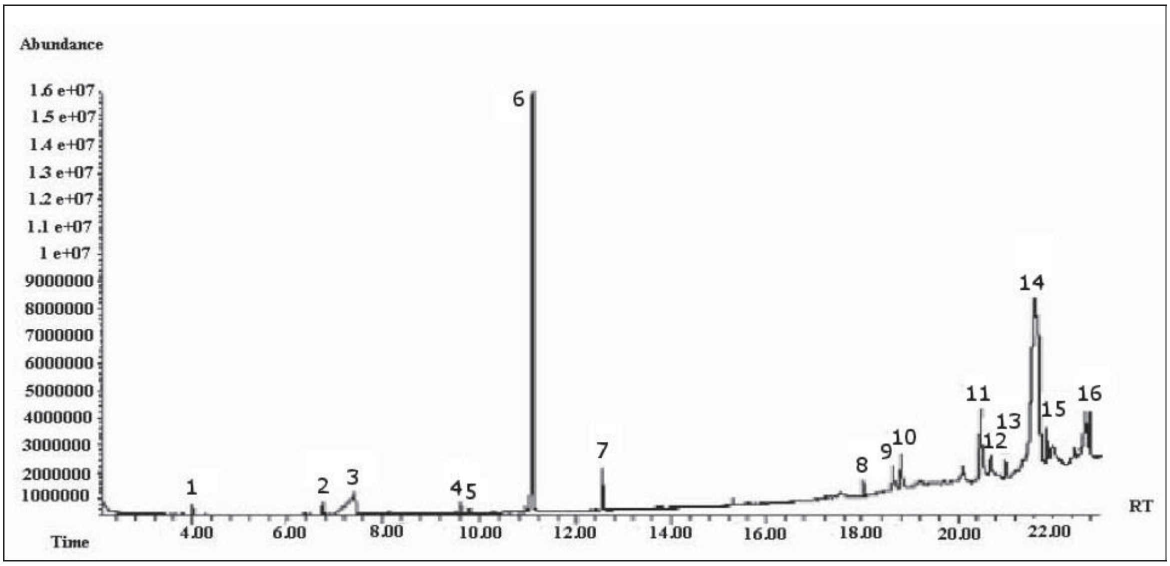

Fig. 8. Gas chromatogram of an excract of the metathoracic scent gland secretion of *E. maura* female, 1 – (E)-2-Hexanal, 2 – (E)-2-Hexenyl acetate, 3 – n-Hexanoic acid, 4 – n-Dodecane, 5 – Hexadecane, 6 – n-Tridecane, 7 – Cyclodecene, 8 – Octadecane, 9 – 1-Nonadecen, 10 – n-Hexadecanoic acid, 11 – Hexacosan, 12 – Octacosane, 13 – Tricosane, 14 – n-Tetracosane, 15 – Octadecanoic acid, 16 – Nonadecane, RT – Retention time in minutes.

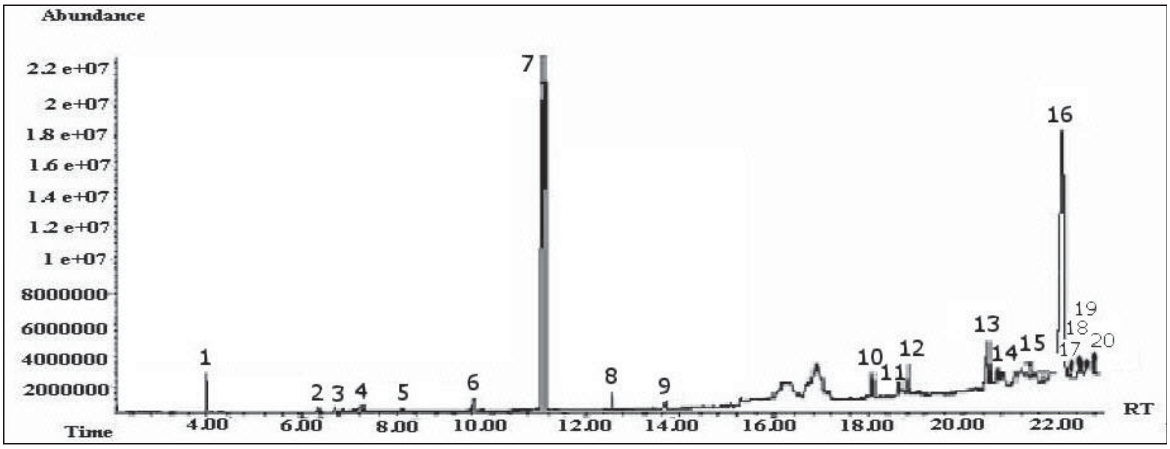

Fig. 9. Gas chromatogram of an excract of the metathoracic scent gland secretion of *E. maura* male, 1 – (E)-2-Hexanal, 2 – (E)-2-Hexenyl acetate, 3 – n-Hexanoic acid, 4 – n-Undecane, 5 – n-Dodecane, 6 – Hexadecane, 7 – n-Tridecane, 8 – Pentadecane, 9 – 1-Nonadecene, 10 – Nonadecane, 11 – 14-BETA-H-Pregna, 12 – n-Hexadecanoic acid, 13 – Octadecanoic acid, 14 – Octadecane, 15 – Hexacosane, 16 – Octacosane, 17 – Tricosane, 18 – [3,7,11-(3-metyl-trideuterio)-trimetyl-(4,4,5,5-tetradeuterio)dodeca-1,6,10-trien-3-ol], 19 – Diisooctyl acetate, 20 – Tetracosane, RT – Retention time in minutes.

ses of MTG of males of *E. maura* n-tridecane (34%) was determined in the largest amount and n-undecane (0.11%) was determined in the smallest quantity (Fig. 9 and Table 1).

Discussion

Three different categories of scent glands, i.e the MTG, Brindley's glands and ventral glands, are found in insects. The MTG are present in most Hemiptera. The MTG and Brindley's glands are situated in the insect's metathoracic region (SCHOFIELD & UPTON 1978). KALIN & BARRET (1975) described Brindley's glands as dorsally located and extending into the 2nd abdominal segment. Brindley's glands are found only in Reduviids (SANTOS-MALLET & DE SOUZA 1990). The MTG are usually found in the 3rd thorax region in *E. maura*.

The shape, size and aspect of the glands vary according to the feeding status (SANTOS-MALLET & DE SOUZA 1990). However, KALIN and BARRET (1975) did not observe any difference between insects unfed for 30-40 days and those fed for 1 or 2 days before examination. After the dissection of *E. maura* (15 males and 15 females), the position of MTG becomes stable when they are observed for examination. However, various differences were observed in the reservoir depending on the nutritional state of the insects. In well-fed insects the glands reached the second abdominal segment, whereas in starved insects they were only in the thoracic region.

There are two types of MTG in Hemiptera, the diastomien and the omphalien types. In *E. maura*, the MTG belong to the diastomien type with scent glands always open to the outside with two ostioles. There is an ostiole between the 2nd and 3rd coxa in *E. maura.* The ostiolar grooves are long while the ostiole structures are circular. In the evaporation areas, polygonal mushroom-like structures are connected to each other via many trabecules. No differences in the MTG of males and females were observed. The contents of the MTG are secreted through the ostiole. The structure of the ostiole shows differences between species (DAVIDOVA-VILIMOVA *et al.* 2000). In the study of KAMALUDDIN and AHMAD (1988), five new stink bug species belonging to the subfamily Phyllocephalinae of the family Pentatomidae were described with special reference to MTG ostioles placed in systematic keys and diagnostic features. *E. maura* contains a metathoracic scent gland ostiole with ostiolar groove and surface of evaporation with a mushroom-like structure. These structures can be used in systematic keys for *E. maura* and compared with other species of the same genus in future studies.

Type I and type II cells were described in the wall of the reservoir in *E. maura* by TEM. The type I cell is an epithelial cell. These are basal cells and are composed of an intima layer, which not only protects the cells but also blocks the shriveling of the reservoir. There are type II cells in the small region of the reservoir. The cell which is embedded completely in the wall of the reservoir transports the secretion to the reservoir via their ducts. This structure is similar in *Lincus* species (Heteroptera: Pentatomidae), which were studied beforehand by NAGNAN *et al.* (1994). The authors suggest that type II cells may produce secretory scent compounds because they possess canals, particularly in the apical region of these cells. An important feature of these cells is that they contain numerous spherical crystals. Spherical crystals are formed in other insect tissues also (WESSING *et al.* 1992; GRODOWITZ *et al.* 1987; KRUEGER *et al.* 1987; KALENDER *et al.* 2001). These structures are usually seen in the digestion and excretion systems and are also called mineralized granules or mineral concretions. According to GRODOWITZ *et al.* (1987), spherocrystals are composed of numerous concentric thin layers as evidenced by TEM and SEM of fractured granules. Demonstrated by X-ray diffraction, spherical crystals composed of water, P, Ca, Mg, K, Fe, Cu, Al, Zn and carbohydrates were also detected (GRODOWITZ *et al.* 1987; KRUEGER *et al.* 1987). Spherocrystals are known to influence ion metabolism and play a role in the larval-pupal stage. The authors think that spherocrystals play an important role in developing the reservoir and also in the production of odourous substances in *E. maura*.

The reservoir of the MTG consists of paired, colorless lateral glands. The lateral glands empty the secretion through ducts into the reservoir. The star-like canal of the lateral glands is located in their central part. Secretory cells of the lateral glands are responsible for producing the secretion. These secretory cells have their own reservoir and intracellular canalicule. The odorous secretion is synthesized by these cells and discharged by canalicule into the reservoir. In the previous study, these structures were described as intracellular canalicule and endoplasmic reticulum (SANTOS-MALLET & DE SOUZA 1990; NAGNAN *et al.* 1994). We think that this structure is an intracellular canalicule. It may also be classified as endoplasmic reticulum because this structure functions as the intracellular transport system.

Chemical analysis of the MTG in both sexes of *E. maura* revealed many compounds in every individual. N-undecane, pentadecane, diisooctyl acetate, 3,7,11-(3-methyl-tridöterio)-trimethyl-(4,4,5,5 tetradöterio) dodeka-1,6,10-trien-3-ol and 14-Beta-H-pregna are specific only for the male, while cyclodecene is specific for the female. Some of these compounds are thought to be sex pheromones. The authors suggest that diisooctyl acetate may be a sex pheromone because it is found only in male bugs. 14-Beta-H-Pregna is a kind of steroid and was also found only in male bugs. This compound, secreted by the MTG, is not only a defense chemical but also a sex pheromone and facilitates reproduction in conspecifics.

The compounds identified in the MTG of Phyrrhocoridae species were divided into 11 chemical groups: aldehydes, saturated hydrocarbons, acetates, alcohols, terpenes, lactones, ketones, esters, alcenes, acids and miscellaneous compounds (FARINE *et al.* 1993). A steroid compound has never been previously detected in the chemical analysis of the MTG in Heteroptera. Therefore this steroid constitutes the 12th new group in Heteroptera.

Chemical analysis showed that the aldehydes and hydrocarbons found in the MTG in a number of Heteroptera have a dual function. These compounds exerted different effects according to either high viscosity or low viscosity (FARINE *et al.* 1992). In this study, 1 aldehyde and 11 hydrocarbons (9 saturated hydrocarbons, 2 alcenes) were identified in the female and 1 aldehyde and 12 hydrocarbons (11 saturated hydrocarbons, 1 alcene) were identified in male *E. maura*. A dual role of (E)-2-hexenal was found for *Nezara viridula* and some species of Pentatomidae: this compound becomes an attractant at low concentrations and a repellent at high concentrations. In the pentatomid *N. viridula*, various concentrations of n-tridecane cause the same reactions (FARINE *et al*., 1993). (E)-2-hexenal was identified in males and females of *E. maura* and the authors suggest that (E)-2--hexenal may have a dual role in *E. maura*.

(E)-2-hexenyl acetate was found only in the male of *Lethocerus indicus* (Heteroptera: Belostomatidae) and it may have a sexual function as an attractant or may produce an alarm effect (PATENDEN & STADDON, 1970). (E)-2-hexenyl acetate was detected only in the female of *Lygocoris communis* (Heteroptera: Miridae) and it acts as a sex pheromone. In this study, (E)-2-hexenyl acetate has also been identified in males and females of this species and may be a sex pheromone in *E. maura*.

Nonadecane, n-dodecane and n-tridecane compounds were identified as toxic, irritant or repellent (ZARBIN *et al.* 2000). They are released by stink bugs in response to disturbance, showing that they are responsible for chemical defenses and may also have the same function in *E. maura.*

The various paraffin compounds such as hexacosane, tricosane and octacosane have been shown to aid penetration of the cuticule of insect enemies (REMOLD 1963) and, by delaying evaporation, to act as 'odour fixatives' for the more volatile constituents. These compounds also were identified in *Graphosoma semipunctatum* males (Heteroptera: Pentatomidae) (DURAK & KALENDER 2007). These components were not found in the MTG of every bug. It would be interesting to know the relative efficiencies of scents with and without paraffins in deterring natural enemies (WATERHOUSE & GILBY 1964). These compounds were identified in *E. maura* species and may quickly block the evaporation of scent compounds after their release. These compounds may also be odour fixatives for *E. maura* species.

ALDRICH *et al.* (1978) stated that esters are produced in the lateral glands, while aldehydes are produced in the reservoir. Aldehydes come into existence through alcohol oxydation and enzymatic hydrolysis of esters in the reservoir (ALDRICH *et al.* 1978; STADDON & DAROOGHEH 1981). *E. maura*'s lateral glands and reservoir contain a number of esters and aldehydes which appeared during extraction.

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