J. Ent. Res. Soc., 11(2): 21-29, 2009 *ISSN:1302-0250*

Scanning Electron Microscopic and Chemical Study of the Metathoracic Scent Glands System of *Rhaphigaster nebulosa* (Poda, 1761) (Heteroptera: Pentatomidae)

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ABSTRACT

The metathoracic scent glands (MTGs) system of adult *Rhaphigaster nebulosa* (Heteroptera: Pentatomidae) was described by electron microscopy and secretion from them was analysed by a combination of gas chromatography and mass spectrometry (GC-MS). The MTGs is anatomically complex. It opens ventrally by paired openings located between the metathoracic furcae. It belongs to the paired MTG type and comprises on each side: median reservoir, lateral glands and evaporation surfaces. Median reservoir is sac-like and flanked by multitubular lateral glands. It is interconnected by a narrow bridge in the ventral mid-line. Metathoracic coxae are associated with crescent-like evaporation areas. Chemical analyses showed a typical pentatomid MTG composition. The glands of *R. nebulosa* males and females contain 9 same compounds: *n*-undecane, *n*-dodecane, *n*-tridecane, *n*-nonacosane, *(E)*-2-hexenal, *(E)*-2-octenal, *(E)*-2-decenal, *(E)*-2-hexenyl acetate, 2(5H)-furanone being the two major compounds n-tridecane and (E)-2-hexanal, which represent 90-95% of the secretion in the this species. The compounds *n*-tricosane, *(Z)*-cyclodecene, and *(E)*-2-decen-1-ol were detected only in females, while *n*-tetracosane, *n*-hexacosane, and 1-tridecene were detected only in males.

Keywords: Electron microscopy, gas chromatography-mass spectrometry, *Rhaphigaster nebulosa,* scent glands.

INTRODUCTION

In previous works (Nagnan *et al*., 1994; Zarbin *et al*., 2000; Durak & Kalender, 2007a,b,c) the authors made some observations on the anatomy and chemical structure of the MTGs of Heteroptera. Some species of the order Heteroptera have well developed metathoracic dorsal abdominal, ventral abdominal, sternal and Brindley's glands or secretory setae (Oliver *et al*., 1985; Aldrich, 1988a). A single or a pair of median or ventral scent glands is usually found in the metathoracic region of Heteroptera (Carayon, 1971). Their MTGs are composed of a reservoir and a pair of lateral glands (Waterhouse & Gilby, 1964; Santos-Mallet & De Souza, 1990; Nagnan *et al*., 1994; Durak, 2008).

Stink bugs produce various communication or defensive volatile chemicals in large, well-defined, and usually colored metathoracic glands. The most often found compounds are saturated linear hydrocarbons, short-chain unsaturated aldehydes, (E)-4-oxo-2-alkenals and unsaturated esters, such as n-undecane, n-tridecane, (E)-2-hexanal, (E)-2-octenal, (E)-4-oxo-2-hexenal, (E)-2-octenyl acetate (Aldrich 1988a; Durak & Kalender, 2007a,b,c; Moraes et al., 2008). These chemical are released when the insects are disturbed or molested. They may have a role as alarm pheromones (Kou *et al*. 1989), as has been demonstrated for similar types of compounds produced by bug species in other families (Gunawardena & Bandumathie, 1993; Leal *et al*., 1994). Stink bug defensive compounds have received considerable study, in part because they constitute such an obvious defense, and because they are produced in simple mixtures in comparatively large quantities, making them easy to analyze and identify (Ho & Millar, 2001).

As showy and often abundant insects, pentatomid have been studied more than other stink bugs (Heteroptera) (Aldrich, 1988a; Borges & Aldrich, 1992; Durak & Kalender, 2007c; Pareja *et al*., 2007; James *et al*., 1996). *Rhaphigaster nebulosa* (Poda, 1761) lives on Gramineae (Lodos, 1986). This bug is an important pest of economic crops. They are known as the stink bugs because they usually retaliate by discharging volatile secretions from their MTGs when they are disturbed or molested. In general, chemical analyses were done on most of the stink bugs especially Pentatomidae. But there are few studies on the structure of the scent glands.

The main purpose of the present work was to characterize morphology and chemical compouds of the metathoracic glands of *R. nebulosa* and to identify those compounds that elicit a physiologic approach compared to other species.

MATERIAL AND METHODS

Insect material

Adult *R. nebulosa* were collected from various Gramineae in Ayas, Ankara, Turkey, during June through September, 2007. Insects were reared and maintained at 22-24 ^{o}C and 70% RH 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 solution (0.7% NaCl + 0.3% KCl) (Santos-Mallet & De Souza, 1990).

Scanning electron microscopy (SEM)

For scanning electron microscopy the thoracic region was dissected, 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 MTGs were post fixed with 1% osmium tetroxide in 0.1M sodium phosphate buffer, dehydrated in graded ethanol, dried using 1,1,1,3,3,3- hexamethyldisilazane, and coated with gold. The observations were made in a Jeol JSM 6060 scanning electron microscope.

Chemical analysis

The MTGs (15 males and females) were removed and immersed in ~100 µL analytical grade *n*-hexane which was 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 an 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 detector (electron impact ionization, 70 eV). The GC was programmed at 50 ^{o}C/ 2 min then 5 ^{o}C/ min to 250 ^{o}C, with injector and transfer line temperatures of 250 and 280 ^{o}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 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-octenal (Aldrich Chemical Co.) *(E)*-2-decenal, *n*-dodecane, *n*-undecane, *(Z)*-cyclodecene, *n*-tetracosane, *n*-hexacosane (Fluka Chemical Co), *(E)*-2-hexenal, *n*-tridecane, 2(5H)-furanone standards, and hexane (Merck Chemical Co.) were used.

RESULTS

Scanning electron microscopy (SEM) results

In SEM investigations the MTGs of *R. nebulosa* posses a well developed reservoir and paired glands located in the upper-lateral region of the reservoir (Fig. 1A). The reservoir being bag-shaped. There are irregular projections and intrusions on its surface. The glands is multitubular (Fig. 1B). The ostioles located between the 2nd and 3rd coxae open to the outside. The MTGs open through paired ostioles. In *R. nebulosa* ostioles have a globular shape. A groove-like structure extends downwards from the ostiole (Fig. 1C). While this structure is long and wide, its ostiole is circular. This structure is named the ostiolar groove or peritreme. Mushroom-like structures exist on the surface of the evaporation area. Generally six ridges extracted from each mushroom-like structure and these are connected to other mushroom-like structures. Moreover mushroom-like structures are tightly connected to each other by numerous trabecules found under the ridges (Fig. 1D). These globular structures are slightly concave in the center and have irregular projections.

Gas chromotography-mass spectrometry (GC-MS)

Analyses of MTG of *R. nebulosa* were carried out separately for both sexes. 12 chemical substances were determined both in females and males (Table 1). Quantitative and qualitative compositions of same substances differ in both sexes. In the females of *R. nebulosa* the following substances were found: 5 alkanes (*n*-undecane, *n*-dodecane, *n*-tridecane, *n*-tricosane, *n*-nonacosane), 1 cycloalkene [*(Z)*-cyclodecene)], 3 unsaturated aldehydes [*(E)*-2-hexenal, *(E)*-2-octenal, *(E)*-2-decenal], 1 unsaturated ester [*(E)*-2-hexenyl acetate], 1 unsaturated alcohol [(E)-2-decen-1-ol] and 1 lactone [2(5H)-Furanone]. In the analyses of MTGs of females of *R. nebulosa* *n*-tridecane (55.44%) was determined in the largest amount and *(E)*-2-decen-1-ol (0.03%) was determined in the smallest quantity (Fig. 2 and Table 1). In the males of *R. nebulosa* the following substances were found: 6 alkanes (*n*-undecane, *n*-dodecane, *n*-tridecane, *n*-tetracosane, *n*-nonacosane, *n*-hexacosane), 1 alkene (1-tridecene), 3 unsaturated aldehydes [*(E)*-2-hexenal, *(E)*-2-octenal, *(E)*-2-decenal], 1 unsaturated ester [*(E)*-2-hexenyl acetate], 1 lactone [2(5H)-Furanone]. In the chemical analyses of MTGs of males of *R. nebulosa* *n*-tridecane (66.51%) was determined in the largest amount and *n*-undecane (0.17%) was determined in the smallest quantity (Fig. 3 and Table 1).

GROUP	Chemical Compounds	*R. nebulosa*	
		Female %	Male %
ALKANES	*n*-undecane	0,13	0,17
	n-dodecane	0,93	1,14
	n-tridecane	55,44	66,51
	n-tricosane	0,11	n.d
	n-tetracosane	n.d	0,38
	n-nonacosane	0,34	0,30
	n-hexacosane	n.d	0,34
ALCENES	*(Z)*-cyclodecene	0,61	n.d
	1-tridecene	n.d	0,25
ALDEHYDES	*(E)*-2-hexenal	37,37	24,38
	(E)-2-octenal	0,63	1,79
	(E)-2-decenal	0,61	1,21
ESTERS	*(E)*-2-hexenyl acetate	0,33	0,21
ALCOHOL	*(E)*-2-decen-1-ol	0,03	n.d
LACTONE	2(5H)-Furanone	2,40	3,04

Table I. Percentages of compounds in metathoracic scent secretion of female and male of *R. nebulosa*, n.d= not detected

DISCUSSION

The scent gland system of Heteroptera constitutes a principal autopomorphy of the suborder. Exocrine glands are connected to the lateral glands by a canal in the apical surface, and usually secrete volatile odoriferous substances, principally as means of chemical defence against predation. It is best developed in Pentatomidae where, in the adult, the glands open externally by paired ostioles on the metapleura (Carver, 1990). There are two types of MTGs in Hemiptera, the diastomian and the omphalian types (Carayon, 1971). In *R. nebulosa*, 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 *R. nebulosa*. The ostiolar grooves are long while the ostiole structures are circular. After secretion through the ostiole, the contents of the MTGs spread out from this region via the ostiolar groove. Therefore the ostiole, the ostiolar groove and their surroundings are termed the evaporation area.

In the evaporation areas, polygonal mushroom-like structures are connected to each other via many trabecules. No differences in the MTGs of males and females were observed. The contents of the MTGs are secreted through the ostiole (Carayon, 1971). The structure of the ostiole shows differences between species (Davidova-Vilimova *et al*., 2000; Eger & Baranowski, 2002). In the study of Kamaluddin & Ahmad (1988), five new stink bug species belonging to the subfamily Phyllocephalinae of the family Pentatomidae were described with special reference to MTGs ostioles placed in systematic keys and diagnostic features. *R. nebulosa* contains a metathoracic scent gland ostiole with ostiolar groove and surface of evaporation with a mushroom-like structure. These structures can be used in identification keys for *R. nebulosa* and compared with other species of the same genus in future studies.

The chemical compositions of Heteroptera exocrine secretions have been extensively studied, especially in Pentatomidae and Coreidae (Blum, 1981; Aldrich, 1988a, Marques *et al*., 2007, Moraes *et al*., 2008). Chemical analysis of the MTGs in both sexes of *R. nebulosa* revealed many compounds in every individual. There is a far greater basic similarity within the families of Pentatomidae bugs (Waterhouse *et al*., 1961; Nagnan *et al*., 1994; Ho & Millar, 2001). It is not know how the various secretions components interact. Some of these compounds are thought to be sex pheromones. In this regard, our observations reveal existence of numerous components that are characteristic of male or female. As suggested by Aller & Caldwell (1979), aggregation pheromones may serve as sex pheromones by promoting the encounters between the males and females. Release of sex pheromones by females and males has been demonstrated in various heteropteran families (Aldrich *et al*., 1986; Aldrich, 1988b). Regarding the qualitative variations of composition of MTGs secretions, an exclusive aggregative function for these glands is questionable and needs further investigation.

Chemical analysis showed that the aldehydes and hydrocarbons found in the MTGs in a number of Heteroptera have a dual function. These compounds exerted different effects according to either high or low viscosity (Farine *et al*., 1992). The aldehydes and esters are strongly scented and are strong irritants, providing both a warning signal and a strong defense. The function of the hydrocarbons is less clear, but they may serve as solvents and as controlled-release substrates for the more volatile aldehydes (Remold, 1962; Gunawardena & Herath, 1991). In this present chemical data, 3 aldehydes, 6 hydrocarbones (5 saturated hydrocarbones, 1 alkene) and 1 ester were identified in the females, 3 aldehydes, 7 hydrocarbones (6 saturated hydrocarbones, 1 alkenes) and 1 ester were identified in males *R. nebulosa*. These components may serve strong defense, irritants, and a warning signal in *R. nebulosa*.

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 this species, various concentrations of *n*-tridecane cause the same reactions (Farine *et al*., 1993). *(E)*-2-hexenal and *n*-tridecane were identified in males and females of *R. nebulosa.*

(E)-2-decenal is a compound that is one of the most toxic chemicals within the defensive secretion. It is not tolerated by many insects in high concentrations (Gilby & Waterhouse, 1965). *(E)*-2-decenal was detected in females and males of *R. nebulosa.*

The alkanes *n*-dodecane, *n*-tridecane and *n*-undecane compounds were identified as toxic, irritant or repellent (Borges *et al*., 1999, 2007). 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 *R. nebulosa.*

Special features of *R. nebulosa* can be pointed out in this paper. The first one concerns the structure of the lateral multitubular glands and reservoir. Adults produce large quantities of strong-smelling and irritating defensive chemicals in large, well-defined, and usually colored metathoracic glands. The second one concerns the chemicals of MTGs. The aldehydes and esters identified are strongly scented and strong irritants, providing both an easily detected warning signal and an effective defense. The function of the hydrocarbons is less clear, but they may serve as solvents and as controlled-release substrates for the more volatile aldehydes. The composition and structure of the MTGs have been identified and quantified in many Heteroptera, the biological function of each compound in the scent secretion still needs further study. So, MTGs compounds of *R. nebulosa* reported in Table 1 are probably the major contributors to the odours of the Pentatomidae investigated.

ACKNOWLEDGEMENTS

We thank Ayse Öğütcü, Meltem Uzunhisarcıklı and Özlem Özsaraç for their help in this study.

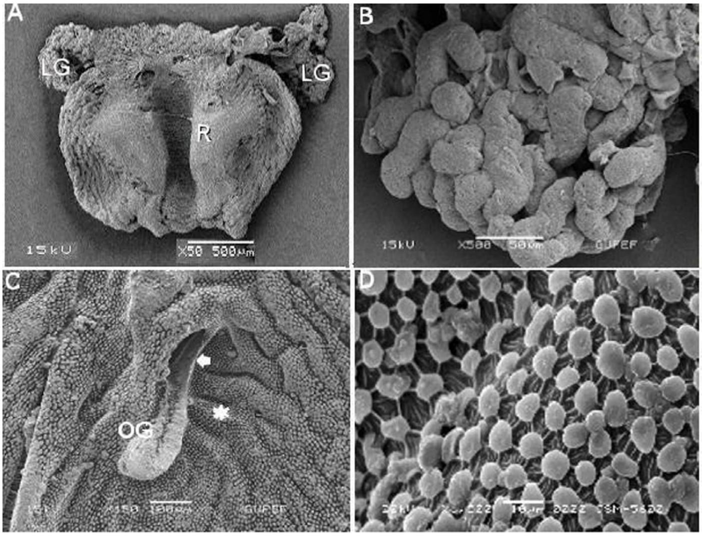

Fig. 1a-d. 1a. Scanning electron micrograph metathoracic scent glands and structures associated with *R. nebulosa*. LG: Lateral gland, R: Reservoir. 1b. Multitubular lateral glands of metathoracic scent glands. 1c. Ostiole (⇨) and OG: Ostiolar groove, evaporation area (✶). 1d. Evaporation area.

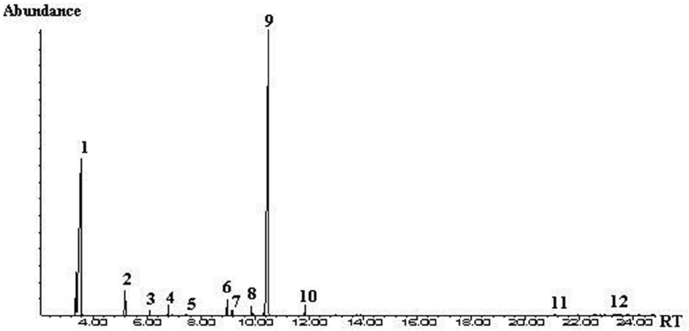

Fig. 2. Gas chromatogram of excract of the metathoracic scent gland secretion of *R. nebulosa* females, 1-*(E)*-2-hexenal, 2-2(5H)-furanone, 3-*(E)*-2-hexenyl acetate, 4-*(E)*-2-octenal, 5-*n*-undecane, 6-*n*-dodecane, 7-*(E)*-2-decen-1-ol, 8-*(E)*-2-decenal, 9-*n*-tridecane, 10-*(Z)*-cyclodecene, 11-*n*-tricosane, 12-*n*-nonacosane, RT: Retention time in minutes.

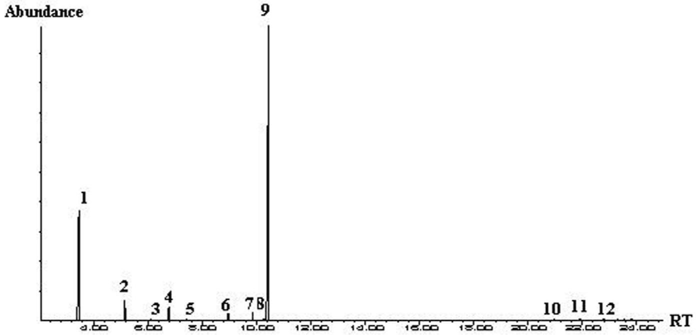

Fig. 3. Gas chromatogram of excract of the metathoracic scent gland secretion of *R. nebulosa* males, 1-*(E)*-2-hexenal, 2-2(5H)-furanone, 3-*(E)*-2-hexenyl acetate, 4-*(E)*-2-octenal, 5-*n*-undecane, 6-*n*-dodecane, 7-*(E)*-2-decenal, 8-1-tridecene, 9-*n*-tridecane, 10-*n*-tetracosane, 11-*n*-nonacosane, 12-*n*-hexacosane, RT: Retention time in minutes.

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Received: December 03, 2008 Accepted: March 28, 2009