Volatile compounds secreted by Brindley's glands of adult *Triatoma infestans*: identification and biological activity of previously unidentified compounds

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ABSTRACT: Volatile emissions of adult male *Triatoma infestans* were collected on non-polar SPME fibers and analyzed by gas chromatography linked to a mass spectrometer. A complex mixture of 16 short-chain esters and acids were identified. The composition of short-chain aliphatic acids (ethanoic to nonanoic acids) was similar to previously reported results. The most abundant aliphatic acid was 2-methylpropanoic acid, constituting 18% of the total volatile content. Also abundant were the esters 2- and 3-methylbutyl 2-methylpropanoate, which constituted 30% and 22%, respectively, of the total volatile content. A similar pattern of compounds was observed in the volatiles secreted by dissected male Brindley's glands; however, in this case, 2- and 3-methylbutan-1-ol were detected which were not found in live insect volatile emissions. Large variability in volatile composition was also observed among the glands excised from different insects. Electroantennographic (EAG) evaluation of the components of Brindley's gland showed significant responses for 2- and 3-methylbutyl 2-methylpropanoate compared to controls. The mixture of volatiles secreted by excised Brindley's glands and the isolated 2- and 3-methylbutyl 2-methylpropanoate had repellent effects on both male and female *T. infestans*, possibly associated with a defensive strategy.
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Keyword Index: Triatoma infestans, Brindley's gland, repellency, EAG.

INTRODUCTION

The behavior of *Triatoma infestans* (Klug) has been studied extensively for many years (Schofield 1979). Many behaviors, such as aggregation, sexual communication, host location, and alarm responses, appear to be mediated by semiochemicals. Feces have been identified as the source of pheromones mediating aggregation in both nymphs and adults (Lorenzo Figueiras et al. 1994, Lorenzo and Lazzari 1996). Our laboratory has recently reported that 4-methyl and 2,4-dimethylquinazolines, present in feces, are *T. infestans* attractants (Alzogaray et al. 2006). A footprint assembling factor (substances present in the feces or cuticle that elicit aggregation in the tested insects), which acts in an intra-, as well as interspecific fashion, has been reported by Lorenzo Figueiras and Lazzari (1998). Electrophysiological studies have shown that copulating pairs release a pheromonal compound that attracts male *T. infestans* (De Brito Sánchez et al. 1995). Nonetheless, there is still scarce information on the actual chemicals that mediate these behavioral responses. In a previous study, we reported that volatiles emitted by male and female *T. infestans* collected on Porapak-Q were attractive to both sexes (Fontán et al. 2002). Several aldehydes were identified in extracts prepared from the Porapak-Q entrainments, which were then found to be attractive in an arena bioassay: hexanal and benzaldehyde proved to attract females and nonanal attracted males. However, the pattern of release of these compounds was not consistent with the previously reported copulation pheromone (De Brito Sánchez et al. 1995).

Short-chained acids have been identified in secretions from disturbed adults (Juárez and Brenner 1981) and from extracts of Brindley's glands from males and females (Hack et al. 1980). Ward (1981) reported that short-chain acids, mainly 2-methylpropanoic acid, elicited an alarm response in adult *T. infestans*. Cruz Lopez et al. (1995) identified isobutyric acid as the main component in Brindley's gland together with isobutyl, isoamyl, and amyl alcohols, 2-phenylethanol and other carboxylic acids and esters. In addition, isobutyric acid, isobutyl, isoamyl, and amyl alcohols, and esters, were also found in volatile emissions of adult *T. infestans,* and the authors hypothetically related them to a mechanism of defense. However, it has also been shown that acids released by Brindley's gland elicit a dose-dependent behavioral pattern, with low doses inducing attractive effects on immature stadia instead of an alarm response (Guerenstein and Guerin 2001).

Recently, Rojas et al. (2002) performed a chemical, electrophysiological, and behavioral study of the volatile compounds of Brindley's gland in *Rhodnius prolixus*. Six compounds were found: acetic, isobutyric, and caproic acids, and three preliminary identified compounds: propionate,

butyrate, and valerate esters. The results of this study indicated that isobutyric and caproic acids were attractive to male and female *R. prolixus* in a dose-dependent manner.

Most studies of Brindley's gland suggest that its function is probably related to the secretion of compounds with alarm pheromone activity (Ward 1981, Cruz López et al. 1995). Alarm behaviors can include movements towards (Blum 1977) or away (Jutsum and Gordon 1989) from the pheromonal source. In most species, the usual response to alarm pheromones of nearby individuals is to move away from the emitter, a classical repellent effect (Baker 1985).

In the present study, we have identified the volatiles secreted by adult *T. infestans* males and their excised Brindley's glands, using non-polar solid phase micro-extraction (SPME) fibers. An assay of the repellency of the gland's volatile components was performed to further understand the function of Brindley's gland and demonstrate whether secretion from Brindley's gland releases an alarm response. The electroantennographic (EAG) response to the main components, as well as the response produced by the main ester components, was also determined.

MATERIALS AND METHODS

Biological material

Insects were collected in infested houses from Acambuco, Salta, Argentina. They were reared at 28±1° C, 50% HR, and a photoperiod of 12:12 (L:D) h and were fed on pigeons weekly.

Gland extraction

Three groups of three males were separated from the colony and placed in large insect rearing containers filled with folded paper. Seven days later, the insects were anaesthetized with a very low flow rate of CO_2. Once the insects dropped from the papers, usually after 5 min exposure, they were removed and Brindley's glands were dissected as follows: lateral incisions were made running from the 2nd abdominal tergite up to the thorax. A median incision was made along the abdomen thus joining the lateral ones, the dorsal cuticle was then removed revealing the gland which was then excised and placed in an individual conical flask (0.5 ml). The glands were immediately stored at -20° C for not more than 48 h before analysis.

Synthetic chemicals

Chemicals were obtained from commercial sources or synthesized by standard synthetic methods (Furniss et al. 1989). 2-Methylpropyl propanoate; 2-methylpropyl 2-methylpropanoate; 2-methylbutyl propanoate; 3-methylbutyl propanoate; 2-methylbutyl 2-methylpropanoate; 3-methylbutyl 2-methylpropanoate; 2-methylbutyl butanoate; 2-methylbutyl 2-methylbutanoate; 3-methybutyl 2-methylbutanoate; 2-methylbutyl 3-methylbutanoate; 3-methylbutyl 3-methylbutanoate; phenylethyl 2-methylpropanoate; phenylethyl propanoate; phenylethyl butanoate; phenylethyl 2-methylbutanoate, and phenylethyl 3-methylbutanoate were all synthesized. Esters were obtained either by acid catalysis with *p*-toluenesulphonic acid and Dean-Stark removal of water, or by forming their respective acyl chloride with thionyl chloride and then adding the corresponding alcohol. In order to purify the esters, they were washed with a base, subjected to flash chromatography on silica gel and eluted with 10% diethyl ether in hexane. When quantities permitted, the esters were purified by Kugelrohr distillation. Synthesized esters were characterized by mass spectrometry. All chemicals used for bioassays were determined as >98% chemically and isomerically pure by capillary GC analysis.

3-methylbutyl acetate (99%); 3-methyl butan-1-ol (>99%); 2-methyl butan-1-ol (>99%); propanoic acid (>99.5%); 2-methyl propanoic acid (isobutyric acid) (99%); butanoic acid (>99%); 2-methylbutanoic acid (98%); and 3-methyl butanoic acid (isovaleric acid) (99%) were purchased from Aldrich (St. Louis, MO), and 3-methylbutyl butanoate (99%) was purchased from Fisher Ltd.

Collection of the volatiles released by adults

Two *T. infestans* males were introduced into Erlenmeyer flasks (250 ml). Three independent assays were done. The insects were handled with great care to minimize any disturbances. A SPME fiber (PDMS 100 µm) was held in the air space for 30 min at 20° C. The volatiles collected on the fiber were then analyzed by GC-MS.

Collection of the volatiles released from excised Brindley's glands

Individual excised glands (n=9) were placed in glass vials and sealed with a rubber septum. The glands were punctured with a needle to induce volatile release immediately before introducing the SPME fiber into the vial. Headspace volatiles were collected on the SPME fibers during 30 min at 25° C, and then analyzed by GC-MS. Highest and lowest concentrations of the identified compounds obtained in different insects were reported.

Identification of volatile components

Extracts were analyzed by GC-MS using a Shimadzu QP 5050A instrument in the electron impact (70 eV) mode. Samples were analyzed on polar (30 m x 0.32 µm film thickness, CP Wax 52CB Chrompack, The Netherlands) and non-polar (30 m x 0.25 µm film thickness, DB1, J & W Scientific, CA) GC columns. Volatiles from the SPME fibers were desorbed in the injector port for 1 min at 250° C. The GC columns were maintained at 50° C for 2 min and then temperature was increased by 6° C/min up to 220° C and then held for 20 min. When a higher degree of resolution was needed, the columns were initially maintained at 40° C for 20 min, then temperature was increased by 10° C/min up to 220° C and held there for 10 min. Helium was used as the carrier gas, at a head pressure of 14 kPa. Compounds identified in the samples were confirmed by comparing the GC retention times and MS data with authentic compounds.

Electroantennograms (EAG)

Insects were immobilized on their dorsal surface in a notch in a Plasticine® block and restrained with a strip of polystyrene that was held in place with pins. The exposed antennae were held firmly on the surface of the Plasticine® block using U-shaped copper wires. Glass microelectrodes were made from borosilicate glass tubing (2 mm OD, 1.16 mm ID, Clark Electromedical Instruments, Reading, UK) using a micro-electrode puller (PUL-I, WPI) and filled with ringer solution (Roelofs and Comeau 1971). The micro-electrodes were held with micromanipulators (Leica, WILD Heerbrugg, Switzerland) and connected to a high input impedance ($10^{12}\Omega$) AC/DC micro-amplifier (Syntech, Hilversum, The Netherlands) using Ag/AgCl junctions. The recording electrode was inserted into the distal end of an antennal flagellum and the reference electrode into the basal scape of the same antenna. Amplified EAG responses were digitized using an IDAC-2 board and displayed and processed on a PC using EAG software (Syntech, Hilversum, The Netherlands).

Pasteur pipettes containing test samples on filter paper strips (0.04 µM) were positioned 1 cm above the mid-point of the EAG preparation. Test samples were exposed to the EAG preparation by blowing a 3 s pulse of nitrogen (500 ml/min) through the pipette and over the preparation. A minimum interval of 60 s was allowed between each exposure. Each sample was tested at least twice for each EAG preparation, and controls (1 µl, dichloromethane) were run before and after each sample. Each series of samples was repeated in random order with three different male *T. infestans* EAG preparations. Mean EAG responses were divided by the mean control response taken before and after the analysis. Octanal and nonanal were used as positive control compounds (Fontán et al. 2002). The doses of the compounds used for these studies were measured in µM to compensate for differences in the molecular weight of the test chemicals (Burguiere et al. 2001).

Bioassays

The effects of Brindley's gland extracts and 2-methylpropanoate esters on the behavior of adult *T. infestans* were evaluated using the method described by Lorenzo Figueiras et al. (1994). A circular arena (28 cm in diameter) was used. It was lined on the bottom with filter paper divided in three areas. Groups of nine insects were placed in the center of the arena using an inverted bowl. After 10 min, an intact, recently dissected Brindley's gland was placed on a cover glass in one of the sections. The gland was crushed using a glass rod. Then, the inverted bowl was pulled up by a nylon thread attached outside the device. This way, the insects were released without being disturbed. Thirty min later the number of insects in each area was counted. Male and female insects were tested in separate experiments. The rationale of this experimental design was to offer sources of chemicals and to record the aggregation or attraction induced in the insects.

The same experiment was conducted using 10 µl of 2- or 3-methylbutyl 2-methylpropanoate instead of the intact Brindley's gland because they were believed to be behaviorally active compounds. Control experiments were also carried out by placing three clean cover glasses in each area. All the experiments were performed at room temperature (about 25° C) and repeated six times. Results were analyzed using the G-test (Sokal and Rohlf 1981).

RESULTS

Volatiles emitted by adult male *Triatoma infestans*

The volatile compounds emitted by male *T. infestans*, collected on SPME fibers, and identified by GC-MS analysis, are summarized in Table 1. The emitted volatiles are composed of a complex mixture of related esters, alcohols, and acids. The most abundant compounds identified in these secretions were the esters 2-methylbutyl 2-methylpropanoate and 3-methylbutyl 2-methylpropanoate, and 2-methylpropanoic acid.

Volatiles identified in Brindley's glands of male *Triatoma infestans*

The most abundant volatile compounds collected from excised Brindley's glands of male *T. infestans* (Table 1) were the same compounds identified in volatiles secreted by intact insects, suggesting that the compounds were stored and possibly even synthesized in the glands before being released. Furthermore, large variability between insects was observed for the composition of each component, as can be seen from the range of highest to lowest concentrations (Table 1). These results indicate that volatile emission of adult *T. infestans* probably originated in Brindley's gland.

The esters identified in the volatiles from intact insects were similar to the profile of esters from excised glands. Once again, the main esters were 2-methylbutyl 2-methylpropanoate and 3-methylbutyl 2-methylpropanoate. However, other acids and alcohols such as propanoic acid, 2-methyl propanoic acid, butanoic acid, 2- and 3-methylbutanoic acids, and 2- and 3-methyl butan-1-ol were also present in the glands of male insects. These compounds are possible precursors of the esters, which again suggest that the esters could be synthesized in the gland itself. Interestingly, the amount of 2- and 3-methylbutan-1-ol was inversely proportional to the quantity of esters in the same extracts, indicating that they may have been used for the synthesis of the corresponding esters.

A more complex pattern of esters was found in the gland and in volatile emissions. According to GC-MS data, these compounds could be esters derived from linear or branched C_5-acids and alcohols. However, esters derived from phenylethyl alcohol were more abundant in volatiles released from intact males than in the excised gland. These quantitative differences between volatiles released from intact insects and excised glands could be related to a differential volatility of these groups of compounds in particular blends.

Electroantennograms

EAG responses induced on male *T. infestans* by most of

Table 1. Percentage composition of male *T. infestans* volatiles emitted by intact insects or collected from dissected Brindley's glands, using non-polar SPME fibers and determined by GC-MS.

	Emitted (%)	Gland (%)	Ki polar	Ki non polar
2+3-Methylbutyl acetate	-	+	1045	n.d.
Ethylbutanoate	0.8	-	1054	n.d.
Ethyl 3-methylbutanoate	5.8	-	1065	n.d.
2-Methylpropyl propanoate	-	+	1071	850
2-Methylpropan-1-ol	-	+	1081	n.d.
2-Methylpropyl 2-methylpropanoate	3.9	6	1089	904
2-Methylbutyl propanoate	-	+	1173	954
3-Methylbutyl propanoate	-	+	1176	952
2-Methylpropyl-3-methylbutanoate	-	+	1181	993
3-Methylbutyl 2-Me propanoate	22.1	12-21*	1183	998
2-Methylbutyl 2-Me propanoate	29.6	36-60*	1185	1002
2- & 3-Methylbutan-1-ol	-	1-31*	1206	716/718
3-Methylbutyl butanoate	-	+	1283	1042
2-Methylbutyl butanoate	-	+	1292	1044
3-Methybutyl 2-methylbutanoate	-	+	1301	1086
2-Methybutyl 2-methylbutanoate	-	3	1305	1096
2-Methylbutyl 3-methylbutanoate	-	1-3*	1334	1094
3-Methylbutyl 3-methylbutanoate	-	3	1336	1091
Propanoic acid	0.6	+	1564	n.d.
2-Methylpropanoic acid	18.2	15-35*	1570	n.d.
Butanoic acid	-	+	1625	n.d.
2- & 3-Methylbutanoic acid	1.5	+	1668	n.d.
4-methyl pentanoic acid	2.6	-	1774	n.d.
Hexanoic acid	8.4	-	1842	n.d.
2-Phenylethyl 2-methylpropanoate	-	+	1875	1371
2-Phenylethyl propanoate	-	+	1877	1325
2-Phenylethyl alcohol	0.1	1-3*	1906	1084
Heptanoic acid	1.3	-	1949	n.d.
Octanoic acid	1.8	-	2054	n.d.
Nonanoic acid	2.0	-	2163	n.d.
2-Phenylethyl butanoate	0.9	+	1959	1431
2-Phenylethyl 2-methylbutanoate	-	+	1964	1466
2-Phenylethyl 3-methylbutanoate	-	+	1983	1468

+ Traces.

* Highest and lowest concentrations obtained in five determinations with different Brindley's glands.

the main components of Brindley's gland at a dose of 0.04 µmoles were not significantly different from those induced by solvent controls (Table 2). However, 3-methylbutyl-2-methypropanoate and 2-methylbutyl-2-methypropanoate generated responses that were significantly higher than those of the control (P<0.005) (Table 3). This increased response was also observed with the aldehydes used as positive controls (Fontán et al. 2002).

Bioassays

Both male and female *T. infestans* avoided the arena area containing a crushed male Brindley's gland (Figure 1). The distributions of the insects were significantly different from random (G-test, $P < 0.05$). Similar results were obtained when the main volatile compounds, 2-methylbutyl or 3-methylbutyl 2-methylpropanoate, were placed in the arena instead of the intact gland (Figure 2), where once again the distribution of the insects was significantly non-random (G-test, P<0.05).

DISCUSSION

When adult *T. infestans* are manipulated, they release a characteristic odor that has been identified as a blend of short-chained acids: 2-methylpropanoic, 3-methylbutanoic, ethanoic, and propanoic acids (Hack et al. 1980, Juárez and Brenner 1981). In a previous study, we collected the volatiles released by adult *T. infestans* using Porapak-Q (Fontán et al. 2002), confirming the presence of these short-chained acids. We also found a complex blend of short and long-chain fatty acids, aldehydes, and alcohols. In the present study, the volatiles released by male *T. infestans* and by their Brindley´s glands were collected on SPME fibers. The profile of the chemical blends obtained was similar to the one previously reported (Cruz López et al. 1995). We found significant quantities of short-chain esters and additionally, as has not been reported before, we could resolve 2- methylbutyl 2-methylpropanoate from 3-methylbutyl 2-methylpropanoate (isoamyl isobutyrate). The concentrations of these two esters were 30% and 22% respectively, in volatiles emitted by males and from 12-20% and 36-60% respectively, in volatiles from the glands. Aliphatic acids still represented a significant proportion of the blend, with concentrations ranging between 40% to 45% of total volatiles. Trace amounts of compounds not previously reported have been found: acetates, 2- and 3-methyl butyl propionates and butyrates and 2-methyl propyl and phenethyl alcohols. The presence of acetates could be explained by taking into account that acetic acid was the major compound found in volatiles from *T. infestans* by Juarez and Brenner (1981). Furthermore, great variability among insects was observed for the composition of each component, as can be seen from the range of highest to lowest concentration.

The esters identified in the SPME samples represent permutations of the short-chain acids and alcohols previously identified by other techniques. The detection of trace esters not previously reported could be explained because of the greater sensitivity of the SPME methods compared to Porapak-Q collection methods (recovery threshold for 2-methylpropyl 2-methylpropanoate was 6.1 ng for Porapak-Q and 0.08 ng for SPME, Martínez and González Audino, unpublished data), and because very volatile compounds like acetates or propionates may be lost in a Porapak-Q collection and are not lost with SPME. Also, some mixtures of isomeric esters were not resolved in previous work because only a polar column was used. We used both a polar and a non-polar column to reach more resolution in some zones of the GC trace. The chemical composition and relative proportions of the volatile compounds collected from the insects with SPME fibers was similar to those released from crushed Brindley's glands, suggesting that the volatiles are stored and probably even synthesized in the organ.

In the EAG study of the main volatile components of Brindley's gland, a dose of 0.04 µM 2-methylbutyl and 3-methylbutyl 2-methylpropanoate induced a significant

Table 2. Mean EAG responses of male T. infestans elicited by the main components of Brindley's gland (except isobutyrate esters).

Substance	Mean EAG*	SE
Solvent**	1[a]	
2 -methylbutanol	1.019[a]	0.077
3-methylbutanol	1.076[a]	0.032
2-methylpropanoic	1.094[a]	0.073
2-phenylethyl alcohol	0. 973[a]	0.078
Octanal***	1.346[b]	0.037
Nonanal***	1.168[b]	0.032

*Mean EAG (n = 3) compared to the solvent control.
**Mean absolute EAG elicited by the solvent, - 0.30 mV.
***Reference compounds (Fontan et al. 2002). Values with the same letter are not significantly different (ANOVA).

Table 3. Mean EAG responses of male *T. infestans* elicited by 2-methylpropanoates (isobutyrates) found in Brindley's gland.

Substance	Mean EAG*	SE
Solvent**	1[a]	
2-methyl propyl-2-methyl propanoate	1.044[a]	0.02
3-methylbutyl-2-methypropanoate	1.189[b]	0.058
2-methylbutyl-2-methypropanoate	1.329[b]	0.116
2-Phenylethyl 2-methyl propanoate	0.83[a]	0.031
Octanal***	1.346[b]	0.037
Nonanal***	1.168[b]	0.032

*Mean EAG response (n = 3) compared to the solvent control. **Mean absolute EAG response elicited by the solvent, -0.30 mV; ***Reference compounds (Fontán et al, 2002). Values followed by the same letter are not significantly different (ANOVA).

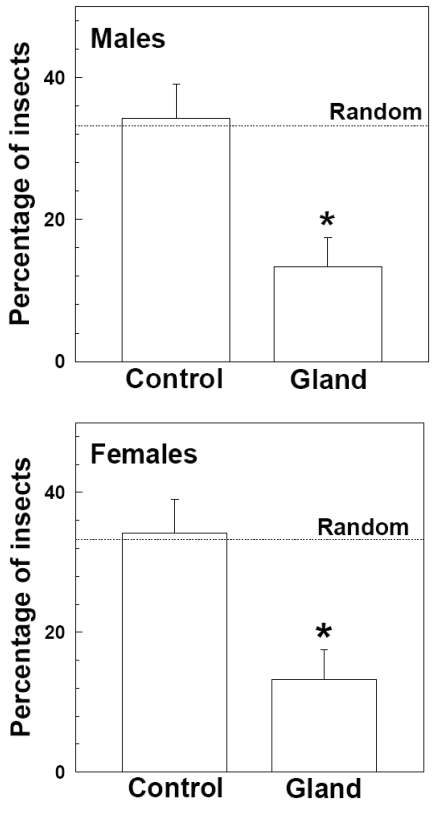

Figure 1. Behavioral response of male (top, N = 108) and female (bottom, N = 108) *T. infestans* adults to volatile compounds emitted by intact Brindley's glands from conspecific males. Bars represent the percentage of insects in the arena area where the gland was placed. Vertical lines represent SE. *Significantly different from a random distribution (dashed line) (G-test, $P < 0.05$).

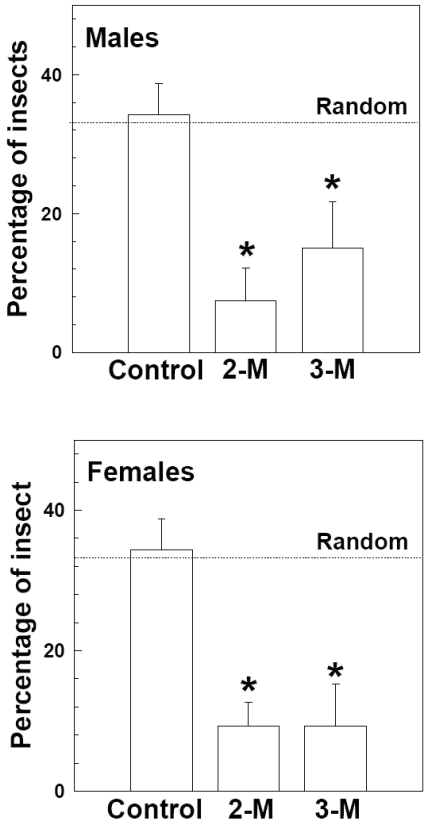

Figure 2. Behavioral response of male (top, N = 162) and female (bottom, N = 162) *T. infestans* adults to 2-methylbutyl (2-M) and 3-methylbutyl 2-methylpropanoate (3-M). Bars represent the percentage of insects in the arena area where each compound was placed. Vertical lines represent SE. *Significantly different from a random distribution (dashed line) (G-test, $P < 0.05$).

electrophysiological response, which could probably be related to a possible biological role. The bioassay experiments indicated that both the intact Brindley's gland, and the two isolated esters, have repellent effects on male and female *T. infestans*. These results indicate that the blend of volatile compounds emitted by the gland possibly acts as an alarm pheromone. In particular, 2-methylbutyl and 3-methylbutyl 2-methylpropanoate could be partially responsible for inducing this behavioral response in conspecifics. There are previous reports on repellent activities of alarm pheromones. The Heteroptera are characterized by well-developed scent glands, the contents of which provide an effective chemical defense against predation. Typical defensive compounds include short-chain alcohols, aldehydes, and esters, (*E*)-2-alkenals, 4-oxo-(*E*)-2-alkenals, alkanes, monoterpenes, and aromatic alcohols and aldehydes. Males of coredi bug, *Leptoglosus australis* F., produce 2-octenyl propionate (Aldrich 1988). Isovalerate esters have been identified from secretions of male predaceous pentatomids (Asopinae) (Aldrich 1988). Propionate, valerate, and butyrate esters have been described in *R. prolixus* (Rojas et al. 2002) and in other species of Hemiptera (Aldrich, 1988, McBrien and Millar 1999).

For female and male *Lygus lineolaris*, blends released by disturbed insects differed quantitatively from blends released by calm insects, with amounts of compounds increasing 75–350 times in samples from disturbed insects. In static air bioassays, both females and males were repelled by natural volatiles collected from females, indicating that these volatiles may serve an alarm or epideictic function, as well as a possible role as defensive allomones (Wardle et al. 2003). In his review, Aldrich (1988) described pheromone analysis of Heteroptera as a semiochemical quagmire because of the difficulty of herding bugs into suitable apparatus without eliciting a chemical discharge.

In conclusion, the results of the present study suggest that the Brindley's gland is not only a reservoir but also a place where compounds with pheromonal activity, particularly 2-ethylpropanoate esters, are synthesized.

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