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Volatile compounds emitted by *Triatoma dimidiata*, a vector of Chagas disease: chemical analysis and behavioural evaluation

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Abstract. In this study, we evaluated the responses of *Triatoma dimidiata* Latreille (Hemiptera: Reduviidae) to volatiles emitted by conspecific females, males, mating pairs and metasternal gland (MG) extracts with a Y-tube olfactometer. The volatile compounds released by mating pairs and MGs of *T. dimidiata* were identified using solid-phase microextraction and coupled gas chromatography-mass spectrometry (GC-MS). Females were not attracted to volatiles emitted by males or MG extracts; however, they preferred clean air to their own volatiles or those from mating pairs. Males were attracted to volatiles emitted by males, females, mating pairs, pairs in which the male had the MG orifices occluded or MG extracts of both sexes. However, males were not attracted to volatiles emitted by pairs in which the female had the MG orifices occluded. The chemical analyses showed that 14 and 15 compounds were detected in the headspace of mating pairs and MG, respectively. Most of the compounds identified from MG except for isobutyric acid were also detected in the headspace of mating pairs. Both females and males were attracted to octanal and 6-methyl-5-hepten-2-one, and males were attracted to 3,5-dimethyl-2-hexanol. Males but not females were attracted to a seven-compound blend, formulated from compounds identified in attractive MG extracts.

Key words. *Triatoma dimidiata*, Chagas disease, metasternal glands, sexual communication.

Introduction

Chagas disease represents an important public health problem for Latin America, where an estimated 10 million people are infected by the protozoan parasite *Trypanosoma cruzi* (Chagas), and more than 25 million are at risk of the disease (WHO, 2010). The parasite is transmitted to humans and other animals by bloodsucking bugs in the subfamily Triatominae, which includes 140 described species (Schofield & Galvão, 2009). The distribution of vectors and wild reservoirs of *T. cruzi* extends from the United States to Argentina and Chile (Coura & Albajar-Viñas, 2010). Of the 31 species or subspecies of triatomines in the North American region, most have been found to be infected with *T. cruzi*, so that the risk of infection in rural populations is estimated between 370 000 and 1 million individuals (Vidal *et al*., 2000; Guzmán, 2001; Ramsey *et al*., 2003). The most important vectors of Chagas disease are *Triatoma infestans* (Klug, 1834), *Triatoma dimidiata* Latreille *Triatoma brasiliensis* Neiva, *Rhodnius prolixus* Stål and *Panstrongylus megistus* Burmeister mainly because they are adapted to living in close contact with humans (Najarian, 1969; Cruz-Reyes & Camargo-Camargo, 2001). Efficient control of these vectors is the most effective method for preventing Chagas disease in Latin America. However, species which maintain sylvatic colonies, such as *T. dimidiata* and most North American species groups, are difficult to

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control because of the ongoing potential for re-colonization of treated houses (Enger *et al*., 2004; Barbu *et al*., 2010).

Chemical communication plays an important role in almost all aspects of the life cycles of insects. The chemical ecology of triatomine bugs has been studied for almost four decades, beginning with a pioneering study with *R. prolixus* (Baldwin *et al*., 1971). Currently, there is consensus that semiochemicals (e.g. pheromones and kairomones) play an important role in the aggregation, alarm, defence, host-finding and sexual behaviour of triatomines (for reviews see Cruz-López *et al*., 2001; Guerenstein & Lazzari, 2009). For example, mating pairs of *R. prolixus*, *T. infestans* and *T. brasiliensis* are known to release volatile compounds that are attractive and promote male aggregation (Baldwin *et al*., 1971; Manrique & Lazzari, 1995; Vitta *et al*., 2009). There is also growing evidence that the compounds involved in the sexual communication of these species are produced in the mestasternal glands (MGs) (Manrique *et al*., 2006; Pontes *et al*., 2008; Vitta *et al*., 2009; Pontes & Lorenzo, 2012). However, relatively few compounds have been identified from the Triatominae, and most studies have been conducted with only a few species, primarily *T. infestans*, *T. brasiliensis* and *R. prolixus*. There are two important reasons to conduct current research on the chemical ecology of other triatomine vector species. First, knowledge of the diversity of exocrine compounds produced by the Triatominae is important to understand how chemical communication has evolved within this subfamily, especially in light of their domestication processes. Second, from a practical point of view, the compounds produced by triatomine bugs during intra- and inter-specific interactions could be useful in the development of novel strategies to monitor and/or control domestic triatomine populations (Lazzari & Lorenzo, 2009).

Triatoma dimidiata is distributed from northern South America (Colombia, Venezuela, Ecuador and Peru), throughout all countries of Central America and into southern and both coastal areas of Mexico (Pacific and Gulf) (Ibarra-Cerdeña *et al*., 2009). It is the only species to naturally span North and South America, whereas three of the recognized genotype species of the *T. dimidiata* complex occur in Mexico (Lehmann *et al*., 2005; Bargues *et al*., 2008). The objectives of the present study were: (a) to evaluate the behavioural responses of *T. dimidiata* to volatiles emitted by conspecific females, males, mating pairs and MG extracts; (b) to identify the volatile compounds released by mating pairs and in MGs of *T. dimidiata*; and (c) to evaluate the behavioural responses of both sexes of this triatomine species to some of the compounds identified.

Materials and methods

Insect rearing

The insects used in this study were obtained from a colony established at the Centro Regional de Investigación en Salud Pública, Instituto Nacional de Salud Pública (CRISP-INSP), Tapachula, Chiapas. The bugs were originally collected from homes in the community of El Manacal (92°09′38″N, 14°54′59″W) in Tapachula, Chiapas, Mexico. The bugs used in this study correspond to *T. dimidiata* genotype 3 (Pacific Coast, Lehmann *et al*., 2005). Prior to our study, adults were reared for two generations in the insectary. The insects were maintained at 27 ± 1 °C, 70 ± 5% RH, with a photoperiod of LD 12 : 12 h. New Zealand White (NZW) rabbits were used as a bloodmeal source for all insects; the bugs used in the experiments were fed 8 days before sampling their volatiles. Soon after emergence, adults were separated by sex and held in single-sex cohorts in plastic containers to ensure that adult insects used in the experiments were virgins. Experiments were conducted using male and female adults between 15 and 30 days old. Each insect was sampled only once.

Behavioural responses of T. dimidiata *to volatiles emitted by females, males and mating pairs*

The behavioural responses of both sexes of *T. dimidiata* to volatiles emitted by live bugs were evaluated using a Y-tube olfactometer consisting of a Y-shaped glass tube (2.5 cm diameter; length of the common tube and two side arms = 12 cm) and two sample chambers (15 cm high × 4.5 cm diameter). Activated charcoal-filtered air was pushed into each sample chamber at 500 mL/min. The airflow was regulated by flowmeters (Gilmont Instruments, Barnant Co., Barrigton, IL, U.S.A.) and the air was humidified by passing it through a water jar before introducing it into the olfactometer. One sample chamber held the test material (i.e. two females, two males or a mating pair) and the other was used as a control (air). The assignment of odour source to each sample chamber was reversed after every replicate to eliminate directional bias. One female or male *T. dimidiata* was gently introduced into the base of the Y-olfactometer and given 5 min to walk to the end of one of the olfactometer arms. A choice was recorded when a bug reached the end of the arm and remained there for at least 30 s. If the bug had not reached the end of one of the two arms after 5 min, the test was stopped and the bug was considered a non-responder. After each set of trials, the olfactometer was washed with detergent, rinsed with distilled water and acetone, and dried at 120 °C for 30 min. All bioassays were conducted between 18.00 and 22.00 hours at 27 ± 1 °C and 55 ± 5% RH. Illumination was provided by a 60-w red bulb mounted 120 cm above the olfactometer, giving a light intensity of 20 lux.

The following treatments were offered as odour baits to male or female *T. dimidiata*: (a) two females, (b) two males, (c) one female and one male, (d) one female whose MG orifices were occluded and one male, and (e) one female and one male whose MG orifices were occluded. The orifices of the MGs were occluded with a water-based correction fluid (Aqua fluid, Pelikan Mexico, S. A. de C. V., Puebla, Mexico). Preliminary observations indicated that this material did not affect the bug's mating behaviour. For each treatment, we conducted 30 replicates on several days using different odour sources.

Behavioural responses of T. dimidiata *to MG extracts*

The responses of both sexes of *T. dimidiata* to MG extracts were evaluated in the Y-tube olfactometer described above.

For preparing the extracts, bugs were placed in a freezer at −20 °C for 10 min to avoid discharge of the gland contents during manipulation. The glands of females and males were dissected separately under water using a binocular microscope. Ten MGs were placed into a 2-mL glass vial containing 1 mL of dichloromethane. Typically, the extracts were prepared in the morning and evaluated in the evening. Pilot studies showed that glands dissected during the day or the night have similar profiles of volatile compounds. The conditions and bioassay technique used in this experiment were similar to those described above. One sample chamber held the test material (1 μL of the extract loaded on a small strip of filter paper) and the other was used as a control (1 μL of dichloromethane applied on filter paper). The solvent was allowed to evaporate and the filter papers were placed into the sample chambers. The assignment of odour source to each sample chamber was reversed after every replicate to eliminate directional bias. For each treatment, we conducted 30 replicates over several days using different odour sources.

Volatile sampling and chemical analysis

The volatiles emitted by mating pairs and by MGs were sampled with solid phase microextraction devices (SPME) fitted with fibres coated with 65-μm polydimethylsiloxane-divinylbenzene (PDMS-DVB; Supelco, Bellefonte, PA, U.S.A.). All samples were maintained under the same temperature and relative humidity conditions (25 ± 2 °C and 65 ± 10% RH). In all samplings, a control was performed before each test under the same conditions, using an empty flask. After the sampling period, the fibre was withdrawn and inserted into the injector of a gas chromatography-mass spectrometry (GC-MS). The samples were desorbed for 1 min in the GC injector for analysis.

GC-MS analyses were performed with a GC Varian model CP-3800 coupled with a Varian Saturn 2200 mass spectrometer (Varian, Palo Alto, CA, U.S.A.). Insect volatiles were analysed using a polar CP-Wax 57CB capillary column (25 m × 0.32 mm i.d., film 0.20 μm; Varian). The column was programmed from 40 °C for 1 min, then 10 °C/min to 75 °C for 0 min, then 15 °C/min to 200 °C, and hold for 15 min. The splitless mode was used for the injector with the inlet temperature set to 250 °C. Helium was used as a carrier gas at 1.0 mL/min. Ionization was by electron impact at 70 eV. Compounds were tentatively identified by matching the mass spectra of GC peaks with those in the MS library (NIST 2002). The identities of these compounds were confirmed by comparing their retention times and mass spectra with those of authentic standards. Compounds were quantified using GC peak areas, and were then expressed as a percentage of the summed peak areas from all targeted peaks.

Volatiles released by mating pairs of T. dimidiata

Preliminary studies showed that this species mated more often during the scotophase rather than the photophase. Consequently, the volatiles released by mating pairs of *T. dimidiata* were sampled during the scotophase (18:00–22:00 hours). Typically, a male and a female were gently introduced into a 50-mL borosilicate glass Erlenmeyer flask. The mouth of the flask was then covered with aluminum foil and sealed with masking tape. Copulation typically occurred almost immediately (<10 s) as soon as both sexes were in contact, and lasted on average 13.8 min (range 12–16 min). At the initiation of copulation, an SPME fibre was exposed for 15 min to the headspace through a pin-size hole in the top of the aluminum foil, after which the fibre was withdrawn and the trapped volatiles were analysed by GC-MS. Ten replicates were performed.

Volatiles produced by MGs

Bugs were frozen and the MGs were dissected as described above. The glands were placed into a 2-mL glass conical vial and the mouth of the vial was covered with aluminum foil and sealed with masking tape. Glands were crushed using a thin wire which was introduced into the vial through a pin-size hole in the aluminum foil and then an SPME fibre was exposed to the headspace for 60 min, then the trapped volatiles were immediately analysed by GC-MS. Ten replicates per treatment were performed.

Chemicals

Most of the volatile compounds found in the headspace of mating pairs or exocrine glands were obtained from commercial sources (Sigma/Aldrich, Toluca, Mexico). The compounds were 97–99% pure according to the manufacturer. The compounds 3-methyl-2-hexanone and 3-methyl-2-pentanone were donated by Dr William F. Wood, Chemistry Department, Humboldt State University, Arcata, CA, U.S.A. 3-Methyl-2-hexanol was prepared from 3-methyl-2-hexanone by reduction with sodium borohydride in methanol. 1-Octen-3-one was prepared by sodium hypochlorite oxidation of 1-octen-3-ol. The last two compounds were prepared according to protocols from Pavia *et al*. (1990).

Synthesis of 3,5-dimethyl-2-hexanone

A solution of diisopropylamine (1.54 mL, 11 mmol) in 20 mL of dry tetrahydrofuran (THF) was cooled to −78 °C and buthyllithium (2.8 M in hexanes, 3.57 mL, 10 mmol) was added dropwise. The mixture was stirred for 1 h and then 5-methylhexan-2-one (1.14 g, 10 mmol; TCI America, Portland, OR, U.S.A.) was added dropwise. The mixture was allowed to warm to room temperature, then cooled to −78 °C again and methyl iodide (1.56 ml, 25 mmol) was added dropwise. The mixture was slowly warmed to room temperature overnight, then quenched with 0.5 M aqueous KH_2PO_4 and extracted with pentane. The extract was backwashed with brine, dried over anhydrous Na_2SO_4

and concentrated under reduced pressure at room temperature, yielding, in order of GC elution, a mixture containing unreacted 5-methylhexan-2-one (23%), the desired 3,5-dimethylhexan-2-one (20%), 6-methylheptan-3-one (40%) and products from the addition of two methyl groups (9%). EI mass spectrum of 3,5-dimethyl-2-hexanone (m/z, abundance): 128 (M^+, trace), 85 (11), 72 (60), 69 (5), 57 (13), 43 (100) and 41 (15).

Synthesis of 3,5-dimethyl-2-hexanol

Approximately half of the crude mixture of methylated products generated above, dissolved in 5 mL ether, was added dropwise to a slurry of $LiAlH_4$ (0.19 g, 5 mmol) in 10 mL ether at 0 °C. The mixture was warmed to room temperature and stirred for 1 h, then cooled to 0 °C again and quenched by cautious addition of water (0.15 mL), 20% NaOH (0.7 mL) and water (0.2 mL). The resulting mixture was diluted with pentane and stirred for 15 min to allow the salts to coalesce, then filtered through a plug of Celite. The resulting clear solution was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure at room temperature, yielding, in order of GC elution, a mixture containing 5-methyl-2-hexanol (∼20%), the desired 3,5-dimethyl-2-hexanol as a mixture of diastereoisomers (∼20%), and 6-methylheptan-3-ol (∼40%), and higher molecular weight products. EI mass spectrum of 3,5-dimethyl-2-hexanol (m/z, abundance): 115 (M^+- 15, 2), 97 (8), 85 (20), 84 (31), 71 (9), 69 (18), 57 (18), 55 (10), 45 (100), 43 (55) and 41 (22).

Behavioural responses of T. dimidiata *to synthetic compounds*

The responses of both sexes of *T. dimidiata* to the synthetic compounds were evaluated using the Y-tube olfactometer described above. One sample chamber held the test material (dichloromethane solutions of individual compounds or blends loaded onto a small strip of filter paper) and the other was used as a control (1 μL of dichloromethane applied on filter paper). The solvent was allowed to evaporate and the filter papers were placed into the sample chambers. The assignment of odour source to each sample chamber was reversed after every replicate to eliminate directional bias. The individual compounds identified in the headspace of mating pairs were dissolved in dichloromethane, and evaluated at the dose of 10 μg, because preliminary studies showed that insects did not respond to a lower (1 μg) or higher dose (100 μg). In addition, we tested a seven-component blend formulated from some of the compounds identified in attractive MG extracts. The blend was evaluated at the dose of 10 μg. The composition of the seven-compound blend was: 3-methyl-2-hexanone, 3, 5-dimethyl-2-hexanone, octanal, 1-octen-3-one, 3-methyl-2-hexanol, 3, 5-dimethyl-2-hexanol and 1-octen-3-ol (ratio: 72.2 : 5.2 : 1.7 : 9.2 : 4.2 : 6.1 : 1.3). Ratios (shown in parenthesis) correspond to the natural ratios of compounds present in female MG extracts. For each treatment, we conducted 30 replicates on several days using different odour sources.

Statistical analyses

The data were analysed with the MINITAB Statistical Software, version 15 (Minitab Inc., PA, U.S.A.). We used a multivariate analysis of variance (MANOVA) to determine whether there was a significant difference in the relative abundance of compounds found in the MGs between females and males. The olfactometric responses of *T. dimidiata* were analysed using the log-likelihood ratio test (G-test) for goodness of fit with Williams' correction for sample size (Sokal & Rohlf, 1995).

Results

Behavioural responses of T. dimidiata *to volatiles emitted by females, males and mating pairs*

Females did not show a preference for volatiles emitted by live males ($G = 0.13$, d.f. $= 1$, $P > 0.05$), or pairs where males had MG orifices occluded ($G = 2.09$, d.f. $= 1$, $P > 0.05$), but females significantly preferred clean air to volatiles emitted by females ($G = 8.39$, d.f. $= 1$, $P < 0.01$), mating pairs ($G = 8.39$, d.f. $= 1$, $P < 0.01$) and pairs where females had MG orifices occluded ($G = 6.43$, d.f. $= 1$, $P < 0.01$) (Fig. 1A). In contrast, males were attracted to volatiles emitted by males ($G = 6.43$, d.f. $= 1$, $P < 0.01$), females ($G = 4.72$, d.f. $= 1$, $P < 0.05$), mating pairs ($G = 13.11$, d.f. $= 1$, $P < 0.001$) or pairs where males had MG orifices occluded ($G = 10.62$, d.f. $= 1$, $P < 0.001$), but they were not attracted to volatiles emitted by pairs where females had MG orifices occluded ($G = 0.13$, d.f. $= 1$, $P > 0.05$) (Fig. 1B).

Behavioural responses of T. dimidiata *to MG extracts*

Females were not attracted to male MG extracts as compared with clean air ($G = 0.13$, d.f. $= 1$, $P > 0.05$), but they showed a preference for clean air over female MG extracts ($G = 6.43$, d.f. $= 1$, $P < 0.01$) (Fig. 2A). In contrast, males were more attracted to female ($G = 15.86$, d.f. $= 1$, $P < 0.001$) and male ($G = 8.39$, d.f. $= 1$, $P < 0.01$) MG extracts than to clean air (Fig. 2B).

Volatiles released by mating pairs of T. dimidiata

The SPME and GC-MS analysis showed that several compounds were found in the headspace of mating pairs (Table 1), and from these 14 compounds were consistently detected, the major component was isobutyric acid (approx. 53% of the total peak area), followed by 3-methyl-2-hexanone, decanal, 6-methyl-5-hepten-2-one and nonanal. The compounds 2-methyl-3-buten-2-ol and 3,5-dimethyl-2-hexanone were found only in traces (Table 1).

Volatiles produced by MGs

The SPME and GC-MS analyses showed that the secretion of MGs of *T. dimidiata* contained 3-methyl-2-hexanone as the

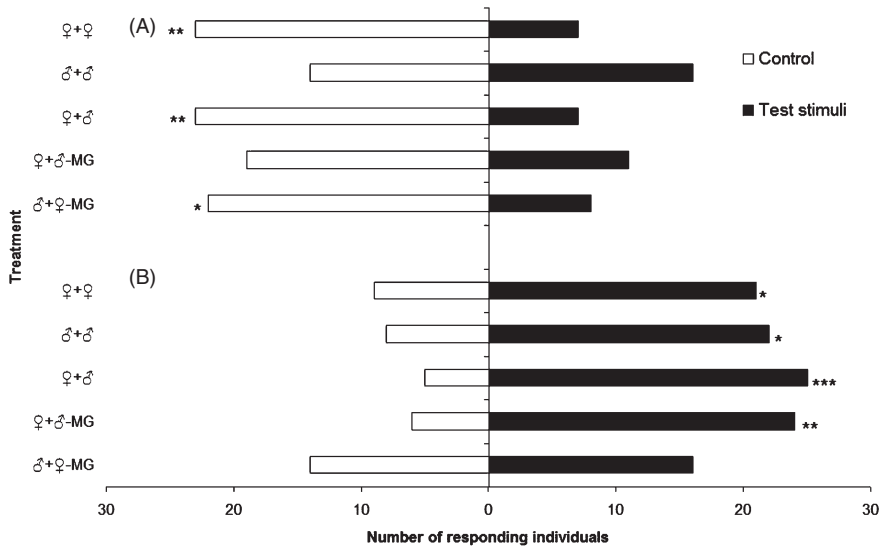

Fig. 1. Responses of *Triatoma dimidiata* females (A) and males (B) to volatile compounds emitted by conspecific females (♀ + ♀), males (♂ + ♂), mating pairs (♀ + ♂), one female and one male whose metasternal gland (MG) orifices were occluded (♀ + ♂-MG) and one female whose MG orifices were occluded and one male (♂ + ♀-MG). Pure air was used as a control. Significant differences between paired bars (G test) indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

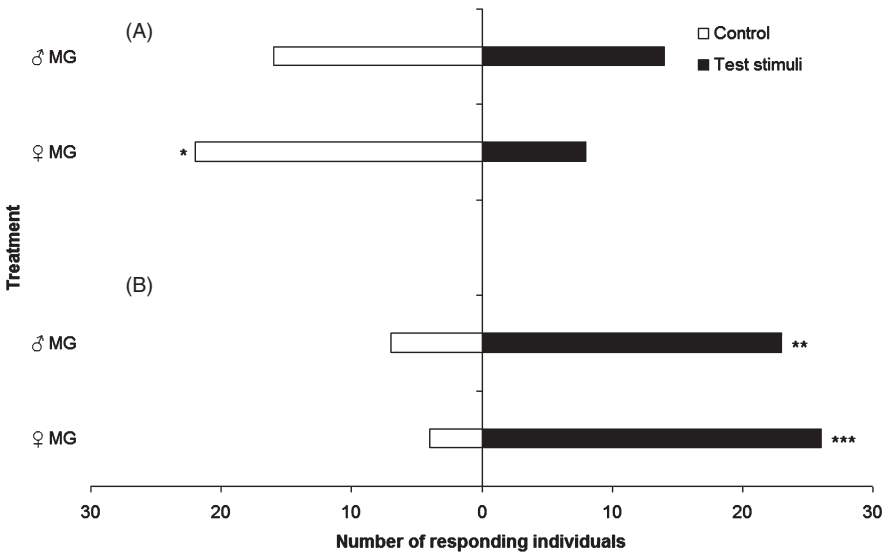

Fig. 2. Responses of *Triatoma dimidiata* females (A) and males (B) to extracts of conspecific metasternal glands (MG) vs. pure air (control). Significant differences between paired bars (G test) indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

major component (approx. 60% of the total peak area) and another 14 minor compounds were also consistently found (Table 2). There was no difference in the relative amounts of the compounds released by the MGs of females and males ($F = 2.29$; d.f. = 11, 4; $P > 0.05$).

Behavioural responses of T. dimidiata *to synthetic compounds*

Females were more attracted to octanal (G = 6.43, d.f. = 1, $P < 0.01$) and 6-methyl-5-hepten-2-one (G = 6.43, d.f. = 1, $P < 0.01$) than to clean air. In contrast, females showed preference for clean air over nonanal (G = 8.39, d.f. = 1, $P < 0.01$), decanal (G = 6.43, d.f. = 1, $P < 0.01$) and 1-octen-3-ol (G = 4.72, d.f. = 1, $P < 0.05$). The responses of females to isobutyric acid (G = 0, d.f. = 1, $P > 0.05$), 1-octen-3-one (G = 0.53, d.f. = 1, $P > 0.05$), 3-methyl-2-hexanone (G = 1.18, d.f. = 1, $P > 0.05$), 3-methyl-2-pentanone (G = 0, d.f. = 1, $P > 0.05$), 3,5-dimethyl-2-hexanone (G = 0.13, d.f. = 1, $P > 0.05$), 3,5-dimethyl-2-hexanol (G = 0, d.f. = 1, $P > 0.05$), 2-methyl-3-buten-2-ol (G = 0.13, d.f. = 1, $P > 0.05$) and 3-methyl-2-hexanol (G = 0, d.f. = 1, $P > 0.05$)

Table 1. Relative amount (%) of volatile compounds identified in the headspace of mating pairs of *Triatoma dimidiata.*

No	Compound	Mean ± SE
1	3-Methyl-2-pentanone	2.20 ± 1.48 (7)*
2	2-Methyl-3-buten-2-ol	t
3	3-Methyl-2-hexanone	8.89 ± 4.74 (5)
4	3,5-Dimethyl-2-hexanone	t
5	Octanal	4.24 ± 1.88 (10)
6	1-Octen-3-one	2.31 ± 1.32 (9)
7	3-Methyl-2-hexanol	0.35 ± 0.14 (8)
8	3-Methyl-2-hexanol isomer†	0.08 ± 0.02 (10)
9	6-Methyl-5-hepten-2-one	6.43 ± 2.81 (10)
10	3,5-Dimethyl-2-hexanol	0.23 ± 0.14 (9)
11	Nonanal	6.37 ± 3.62 (10)
12	1-Octen-3-ol	t
13	Decanal	8.34 ± 3.71 (10)
14	Isobutyric acid	52.45 ± 13.57 (4)

*Numbers between parentheses indicate the detection frequency for each compound (n = 10 samples).
†Compound indicated with a † was not identified by comparison of pure standards.
t, Traces, traces < 0.1% abundance.

were similar to clean air (Fig. 3). Females preferred the clean air ($G = 22.16$, d.f. = 1, $P < 0.001$) to the seven-component blend (Fig. 3).

Males were more attracted to octanal ($G = 8.39$, d.f. = 1, $P < 0.01$), 6-methyl-5-hepten-2-one ($G = 6.43$, d.f. = 1, $P < 0.01$) and 3,5-dimethyl-2-hexanol ($G = 8.39$, d.f. = 1, $P < 0.01$) than to clean air. In contrast, males preferred clean air to nonanal ($G = 10.61$, d.f. = 1, $P < 0.001$) and decanal ($G = 6.42$, d.f. = 1, $P < 0.01$). The male responses to isobutyric acid ($G = 1.18$, d.f. = 1, $P > 0.05$), 1-octen-3-one ($G = 0.13$, d.f. = 1, $P > 0.05$), 3-methyl-2-hexanone ($G = 1.18$, d.f. = 1, $P > 0.05$), 1-octen-3-ol ($G = 2.10$, d.f. = 1, $P < 0.05$), 3-methyl-2-pentanone ($G = 0.53$, d.f. = 1, $P > 0.05$), 3,5-dimethyl-2-hexanone ($G = 0.53$, d.f. = 1, $P > 0.05$), 2-methyl-3-buten-2-ol ($G = 0$, d.f. = 1, $P > 0.05$) and 3-methyl-2-hexanol ($G = 0.13$, d.f. = 1, $P > 0.05$) were similar to that elicited by clean air (Fig. 4). Males were significantly attracted to the seven-compound blend ($G = 4.72$, d.f. = 1, $P < 0.05$) as compared with clean air (Fig. 4).

Discussion

The present results indicate that *T. dimidiata* release volatile compounds that affect the behaviour of conspecifics. We found that males were attracted to volatiles released by conspecific females, males and mating pairs, whereas the female's behaviour was negatively affected by their own volatiles and those from mating pairs. The MGs appeared to be the source of at least some of these compounds, because occlusion of the MGs resulted in loss of activity. In spite of production of the same MG compounds by both sexes, only males were attracted to them, and females released attractive compounds during mating. When the MGs of *T. dimidiata* females were occluded, the proportion of mating pairs decreased (I. J. May-Concha *et al*., unpublished data, 2010) and males were not attracted to mating pairs. Similarly, *T. brasiliensis* males were attracted to volatiles emitted by conspecific males or females, but attraction ceased when the MGs of females were occluded (Vitta *et al*., 2009). As in the present study, the occlusion of MGs of *T. brasiliensis* males did not affect male attraction (Vitta *et al*., 2009). In *R. prolixus*, the occlusion of the MG orifices of female, male or both female and male resulted in a decrease in copulation frequency (Pontes *et al*., 2008). Another study with this species showed that males did not aggregate near mating couples when the female had the MG orifices occluded, but they did aggregate near mating pairs with males that had occluded MGs (Pontes & Lorenzo, 2012).

The fact that both *T. dimidiata* female and male MGs produce the same compounds, as also occurs in *T. infestans*

Table 2. Relative amount (%, mean ± SE) of volatile compounds in the headspace of metasternal glands of female and male *Triatoma dimidiata*.

No	Compound	♀	♂
1	3-Methyl-2-pentanone	1.67 ± 0.53 (9/10)*	1.26 ± 0.19 (10/10)
2	2-Methyl-3-buten-2-ol	5.21 ± 2.12 (10/10)	6.77 ± 3.35 (10/10)
3	3-Methyl-2-hexanone	59.95 ± 10.14 (10/10)	64.42 ± 8.91 (10/10)
4	3,5-Dimethyl-2-hexanone	0.94 ± 0.33 (10/10)	1.61 ± 0.33 (10/10)
5	3,5-Dimethyl-2-hexanone isomer	0.16 ± 0.14 (3/10)	0.09 ± 0.02 (6/10)
6	Octanal	3.37 ± 1.12 (10/10)	2.47 ± 1.15 (10/10)
7	1-Octen-3-one	1.77 ± 1.10 (5/10)	14.26 ± 7.41 (10/10)
8	3-Methyl-2-hexanol	1.93 ± 0.93 (10/10)	0.62 ± 0.21 (10/10)
9	3-Methyl-2-hexanol isomer†	0.48 ± 0.18 (9/10)	0.40 ± 0.15 (8/10)
10	6-Methyl-5-hepten-2-one	t	0.47 ± 0.18 (10/10)
11	3,5-Dimethyl-2-hexanol	0.67 ± 0.40 (8/10)	0.85 ± 0.22 (9/10)
12	3,5-Dimethyl-2-hexanol isomer†	0.54 ± 0.20 (6/10)	0.46 ± 0.32 (5/10)
13	Nonanal	t	0.84 ± 0.31 (10/10)
14	1-Octen-3-ol	0.99 ± 0.43 (6/10)	2.21 ± 0.99 (9/10)
15	Decanal	t	t

*Numbers between parentheses indicate the detection frequency for each compound (n = 10 replicates per sex, two glands/sample were used).
†Compounds indicated with a † were not identified by comparison of pure standards.
t, t races, traces <0.1% abundance.

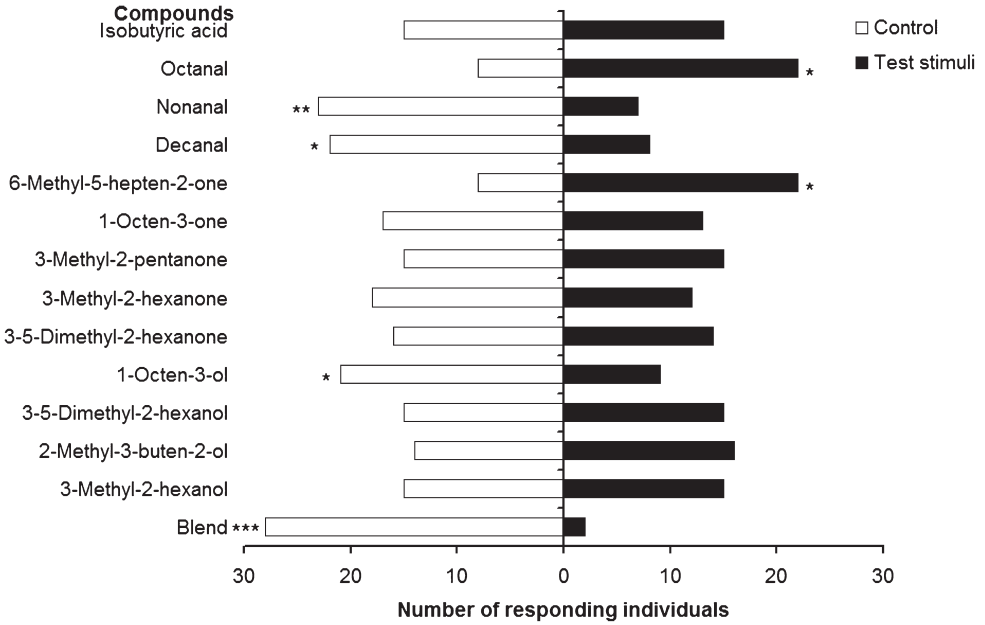

Fig. 3. Responses of *Triatoma dimidiata* females to single synthetic compounds or a seven-component blend vs. pure air (control). Significant differences between paired bars (G test) indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

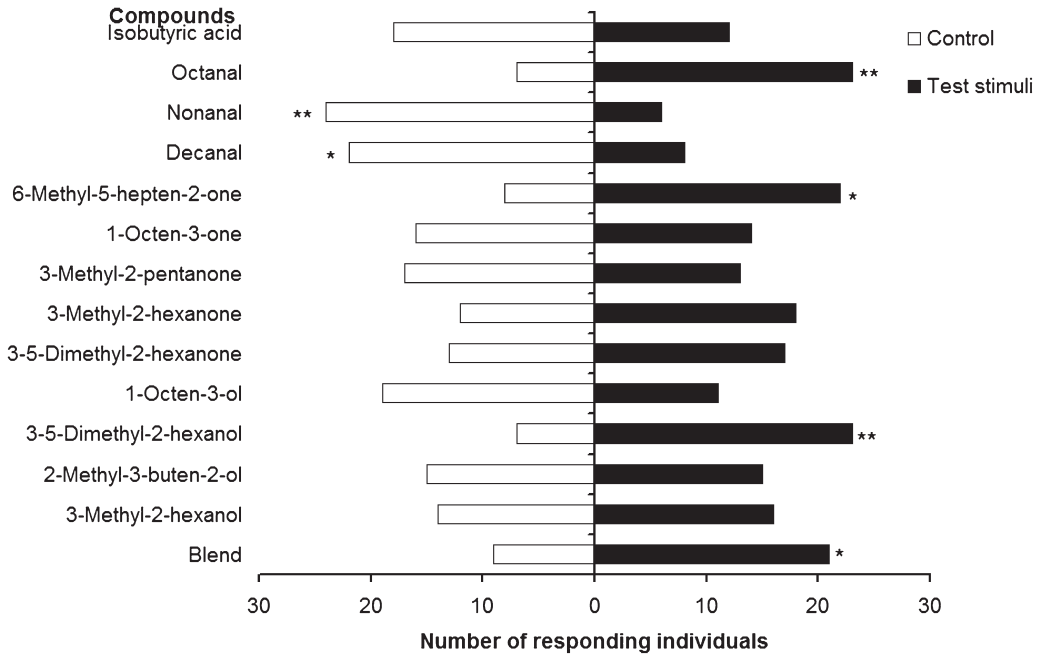

Fig. 4. Responses of *Triatoma dimidiata* males to single synthetic compounds or a seven-component blend vs. pure air (control). Significant differences between paired bars (G test) indicated by $*P < 0.05$, $**P < 0.01$.

(Manrique *et al*., 2006), suggests that the primary role of these molecules may be for other reasons rather than sexual communication. For example, initially these compounds may have functioned as a defensive substance and then evolved into sex pheromones, as suggested for other insects (Leal, 1997; Ruther *et al*., 2001). Future studies need to analyse the possible role of the MG volatiles of this triatomine bug as defensive substances. Functional shifts of defensive compounds occur in several species of insects where the compounds have been co-opted to function as aggregation, sex, alarm and trail pheromones (Steiger *et al*., 2011). Several compounds used as sex pheromones by beetles of the subfamily Melolonthinae have antimicrobial activity against bacteria and fungi, which indicates that these compounds may have taken on

a secondary function as sex pheromones (Leal, 1997). The primary function of the secretion of the pygidial gland of beetles of the subtribe Stizopina is to deter predators, but in addition, the secretion also functions as an aggregation pheromone (Geiselhardt *et al*., 2009).

Undoubtedly the fact that *T. dimidiata* females released volatiles during mating to attract males provides an advantage for polyandry. Melgar *et al*. (2007) suggested that polyandry exists as a mating system for *T. dimidiata*, and that the female volatiles that attract males could be related to this mating system, as also suggested for other triatomine bugs (Crespo & Manrique, 2007; Pontes & Lorenzo, 2012). However, the biological significance of the attraction of males to volatiles from other males is difficult to explain. In the laboratory it is common to observe homosexual interactions between *T. dimidiata* males (i.e. male-male mounting and attempts of copulation), but the occurrence of these interactions in nature is unknown. Males of the dung fly, *Hydromyza livens* (Fabricius), might reduce the reproductive success of competitors, and thereby increase their own, through same-sex interactions (Preston-Mafham, 2006). Immature males of *Drosophila* spp. learn more successful courtship and mating skills through same-sex activity with conspecifics (McRobert & Tompkins, 1988). Further studies in the Triatominae may clarify the behavioural advantage of same sex activity.

Volatiles from the headspace of *T. dimidiata* mating pairs contained numerous compounds, including alcohols, ketones, aldehydes and one carboxylic acid. The major component was isobutyric acid, followed by 3-methyl-2-hexanone. Most of the compounds found in the headspace of mating pairs were also detected in MGs of this triatomine species, except isobutyric acid, which is produced in the Brindley's gland (May-Concha, 2010). The compound 3,5-dimethyl-2-hexanone and an isomer of 3,5-dimethyl-2-hexanol appeared in trace amounts in the MG extracts, but they were not detected in the headspace of mating pairs. It is possible that these compounds were not seen from the insect extracts because of the minor amounts present. Almost half of the compounds identified in the effluvia of mating pairs of *T. dimidiata* are reported for the first time herein, for any species of Triatominae, including 3-methyl-2-pentanone, 3,5-dimethyl-2-hexanol, 3,5-dimethyl-2-hexanone, 6-methyl-5-hepten-2-one, 1-octen-3-one, 1-octen-3-ol and decanal. Other compounds identified in *T. dimidiata* have been reported as triatomine MG volatiles, including 3-methyl-2-hexanone in *Dipetalogaster maximus* (Uhler) (Rossiter & Staddon, 1983), 3-methyl-2-hexanol in *T. brasiliensis* (Vitta *et al*., 2009) and 2-methyl-3-buten-2-ol in *R. prolixus* (Pontes *et al*., 2008). Octanal and nonanal have been found in volatiles emitted by *T. infestans* in copula (Fontán *et al*., 2002).

Isobutyric acid was only detected in four samples of the headspace of copulating pairs, but it is also detected in extracts from agitated bugs (May-Concha, 2010). Its presence in the effluvia of only a few pairs may have been the result of an alarm rather than attractive response. The behavioural evaluation of isobutyric acid showed that this compound did not influence the attraction of *T. dimidiata* males or females. There have been conflicting reports regarding the emission of isobutyric acid from *R. prolixus*; whereas Guerestein & Guerin (2004) reported this compound from volatiles of copulating pairs, Pontes *et al*. (2008) were unable to detect it. A similar situation was reported for *T. infestans* (Fontán *et al*., 2002; Manrique *et al*., 2006).

Ketones and alcohols have been proposed as mediators of sexual communication in *T. infestans*, *R. prolixus* and *T. brasilensis* (Manrique *et al*., 2006; Pontes *et al*., 2008; Vitta *et al*., 2009). However, no study has evaluated the behavioural activity of these compounds. In the present study, we found that *T. dimidiata* males, but not females, were attracted to 3,5-dimethyl-2-hexanol and the seven-compound blend (three ketones and three alcohols), supporting their role in the sexual behaviour of *T. dimidiata*. Possible functions of 3-methyl-2-hexanone in *D. maxima* include intra- or inter-specific (e.g. in interactions with formicid predator) excitation of an alarm behaviour (Rossiter & Staddon, 1983). Interestingly, *T. dimidiata* females were not attracted to volatiles from live insect or to MG extracts, but they were attracted to two compounds (octanal and 6-methyl-5-hepten-2-one) present in the extracts, when these compounds were evaluated individually. Several compounds, including octanal and 6-methyl-5-hepten-2-one, detected in the exocrine glands of triatomines have also been found in the odour of birds and mammals (Meijerink *et al*., 2000; Guerenstein & Guerin, 2001, 2004; Syed & Leal, 2009; Rajchard, 2010), common hosts of triatomines. Thus, the attraction of *T. dimidiata* females and males, to these compounds could be interpreted as responses to host cues. Several aldehydes have been identified in the effluvia of mating pairs of *T. infestans*, and their behavioural evaluation showed that hexanal and heptanal were attractive to females, and nonanal was attractive to males (Fontán *et al*., 2002).

In summary, this study shows that males are attracted to conspecific females, males and mating pairs. The sources of the attractive compounds were the MGs of males and females; however, odours from female MGs play the primary role during sexual communication. Forty per cent of the compounds identified in *T. dimidiata* have not been previously identified in other triatomine species. Behavioural evaluation showed that males were attracted to 3,5-dimethyl-2-hexanol and a seven-compound blend formulated from compounds identified in an attractive female MG extract. Future studies should determine how many of the seven components are necessary to elicit a behavioural response from *T. dimidiata* males. The results obtained herein represent a significant advance in the search for attractants for *T. dimidiata* that could be used to develop novel strategies for surveillance or the control of this important Chagas disease vector.

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