TECHNICAL NOTE

A comparison of volatiles emitted by adults of three triatomine species

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Introduction

Adult triatomine bugs (Hemiptera: Reduviidae: Triatominae), blood-sucking insect vectors of Chagas' disease, possess three types of exocrine glands that are absent in nymphs. The paired metasternal glands are located on the ventral metathorax, while the paired Brindley's glands are situated on the dorsolateral metathorax. In addition, glandular areas associated with the male genitalia were recently described (Weirauch, 2003). Nothing is known about the stimulus for metasternal or genitalia-associated gland secretion in triatomines, but disturbance of adult bugs induces the release of an odour, probably from Brindley's glands, that is repugnant to the human nose (Kälin & Barrett, 1975; Schofield, 1975).

It was reported that Brindley's gland secretion and the headspace over disturbed adult *Triatoma infestans* (Klug) consisted of a mixture of carboxylic acids that included isobutyric acid (Hack et al., 1980; Juárez & Brenner, 1981). More recently, Cruz López et al. (1995) analysed Brindley's gland secretion, and the headspace of disturbed adult *T. infestans* and found isobutyric acid, together with a mixture of aliphatic alcohols and esters, and aromatic compounds. No carboxylic acid other than isobutyric acid was detected by them, thus contradicting the findings of Hack et al. (1980) and Juárez & Brenner (1981). Analysis of Brindley's gland secretion of the triatomine *Rhodnius prolixus* (Stål) suggested the presence of isobutyric acid, other carboxylic acids, and three unidentified esters (Rojas et al., 2002).

While the secretion from Brindley's gland was suggested to represent an alarm signal (Kälin & Barrett, 1975; Schofield, 1975; Barrett, 1976; Ward, 1981), the existence of a sex/aggregation signal produced by adult triatomines during copulation has also been reported by different authors (Baldwin et al., 1971 for *R. prolixus*; Manrique & Lazzari, 1996 and Fontan et al., 2002 for *T. infestans*; for a review see Cruz López et al., 2001).

Given the variability of the results obtained for *T. infestans* by previous authors we asked if the blend of odours released by disturbed adult triatomines, supposedly originating in the Brindley's gland secretion, is characteristic of each species. The headspace vapour over disturbed adults of different triatomine species [*R. prolixus*, *T. infestans,* and *Dipetalogaster maxima* (Uhler)] that were reared and tested under similar conditions was analysed here. These tests were aided by the use of solid phase micro-extraction (SPME), a technique that does not require the use of solvents for the analysis of volatiles. In addition, efforts were made to identify any mating-associated odour for *R. prolixus*.

Materials and methods

Colonies of *R. prolixus, T. infestans,* and *D. maxima* were maintained in our laboratory as described in Taneja & Guerin (1997). Only adult triatomines were used in the experiments.

Odour collection from disturbed adult triatomines

Groups of eight 3–9-week-old (four males plus four females) *R. prolixus* or *T. infestans* starved for 1 week at 22 ± 1 °C were used. The bugs were introduced into an empty 300 ml Erlenmeyer flask with a Teflon septum and then disturbed by vigorously tapping and shaking the flask for 15 s. The flask was then placed in a climate chamber at 26 ± 1 °C during the bugs' virtual day, and 30 min later a polyacrylate SPME fibre (85 μm film thickness; Supelco, USA) was introduced. Gender differences in the compounds released by disturbed *R. prolixus* were assessed by testing a group of five males and a group of five females as described above. The odour present over a group of three male *D. maxima* (10-week-old

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males, starved for 1 week) was sampled using both the polyacrylate and polydimethylsiloxane (100 µm film thickness) SPME fibres. These animals were disturbed using an agitator at 3 Hz (amplitude of movements: 4 cm) for 15 min at room temperature. No compounds were detected from *R. prolixus* or *T. infestans* using this method.

For all three species, the sampling period by SPME was 50 min, and a control under the same conditions was made just before each test, using the same empty flask. Before use, fibres were conditioned for 2 h at 300 °C in a stream of N_2.

Odour collection from males and mating pairs of *Rhodnius prolixus*

The insects were 3 months old, and had been starved for 3 weeks at 22 ± 1 °C.

Virgin mating pairs. After moulting to the adult stage, 17 virgin females contained in a plastic container (500 ml, 7 cm diameter, 12 cm high), were carefully transferred to another similar clean container using a brush. A folded filter paper disc (125 mm diameter) manipulated with plastic gloves was then introduced into this container, which was sealed with netting. The animals were held in this plastic container for 2 h during which time they spontaneously moved to the filter paper, where they stayed. The same procedure was repeated using 17 males in another container. After the 2-h period, the filter paper disc with females and that with males were carefully introduced into a 1.2 l desiccator (11 cm wide mouth). The initial headspace over the animals was evacuated by passing room air at 250 ml min^{-1} for ca. 6 min before starting the odour collection. Forty min was allowed after closing the desiccator for mating pairs to form before starting the odour sampling. Nine couples copulated during the collection. A control experiment consisted of sampling the air from the glass container with only two filter paper discs just before the test with the triatomine couples.

Non-virgin males. Air from 34 non-virgin males was sampled using the same protocol as for the virgin mating pairs. Five copulation attempts between males were observed during odour collection. Females alone were not tested.

A closed-loop stripping apparatus (CLSA; Grob & Zürcher, 1976) with a 1.5 mg activated charcoal trap held in a 4 mm i.d. glass tube was used to collect the odours. An airflow of 200 ml min^{-1} passed through the desiccator on to the charcoal trap for 30 min. In the CLSA, volatiles breaking through the charcoal trap are restored into the collection loop via the pump. The trap was extracted by passing 16 µl of dichloromethane (DCM, Merck, analytical grade) through the charcoal bed 30 times. After this, 8 µl of extract was recovered, and 2.5 µl analysed by gas chromatography linked mass-spectrometry (GC-MS, see below). Before use, the trap was conditioned by washing it with DCM and heating it at 50 °C in a N_2 stream at 100 ml min^{-1}. These experiments were done at dusk at 23 ± 1 °C.

Gas chromatography coupled mass-spectrometry (GC-MS)

Samples collected on SPME fibres or on charcoal were analysed on a Hewlett Packard 5890 series II chromatograph linked to mass selective detector (MSD; 5971A, ionisation chamber temperature 180 °C; ionisation energy 70 eV, scanning for masses 19–300) using a fused-silica free fatty acid phase capillary column (FFAP, 30 m, 0.25 mm i.d., 0.25 µm film thickness; BGB Analytik, Switzerland). The samples collected on charcoal were injected on-column, and the samples collected on the SPME fibre were injected using the split/splitless injector at 240 °C to desorb volatiles from the fibre for 1 min in splitless mode. The carrier gas was He, maintained under constant flow (velocity 30 m s^{-1} at 40 °C) when injecting on-column, or under constant pressure (50 kPa) when injecting splitless, starting from 40 °C and temperature programmed at 8 °C min^{-1} to 180 °C and 5 °C min^{-1} to 220 °C and held for 10 min. When the headspace over a group of four male and four female disturbed *R. prolixus* or *T. infestans* was sampled, analyses were done using a DBWAX capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, USA). The identification of volatiles was based on the match of the mass spectrum of the natural products with that of a known product stored in the computer-based HP CHEMSTATION library of mass spectra linked to the mass selective detector. The mass spectrum and retention time of a natural product were then compared with those of the library-proposed synthetic equivalent injected under the same conditions.

Results and discussion

The headspace over disturbed *R. prolixus* adults consisted of a blend of five carboxylic acids: acetic, propionic, butyric, 2-methylbutyric, and isobutyric acid (77–91% of the blend; Figure 1a). The identity of the compounds was confirmed by matching the mass spectra and retention times of the synthetic equivalents injected under the same conditions (indicated with an asterisk in Figure 1a). Two of the components of this blend (acetic and isobutyric acid) were also found in the Brindley's gland of *R. prolixus* and were described as triatomine attractants (Rojas et al., 2002). However, we did not find any ester, as reported in the secretion of the gland (Rojas et al., 2002). 2-Methylbutyric acid is reported here from triatomines for the first time. An olfactory receptor cell on the antenna of *T. infestans* nymphs, which is most sensitive to isobutyric acid, also responds to 2-methylbutyric acid (Guerenstein & Guerin, 2001). No

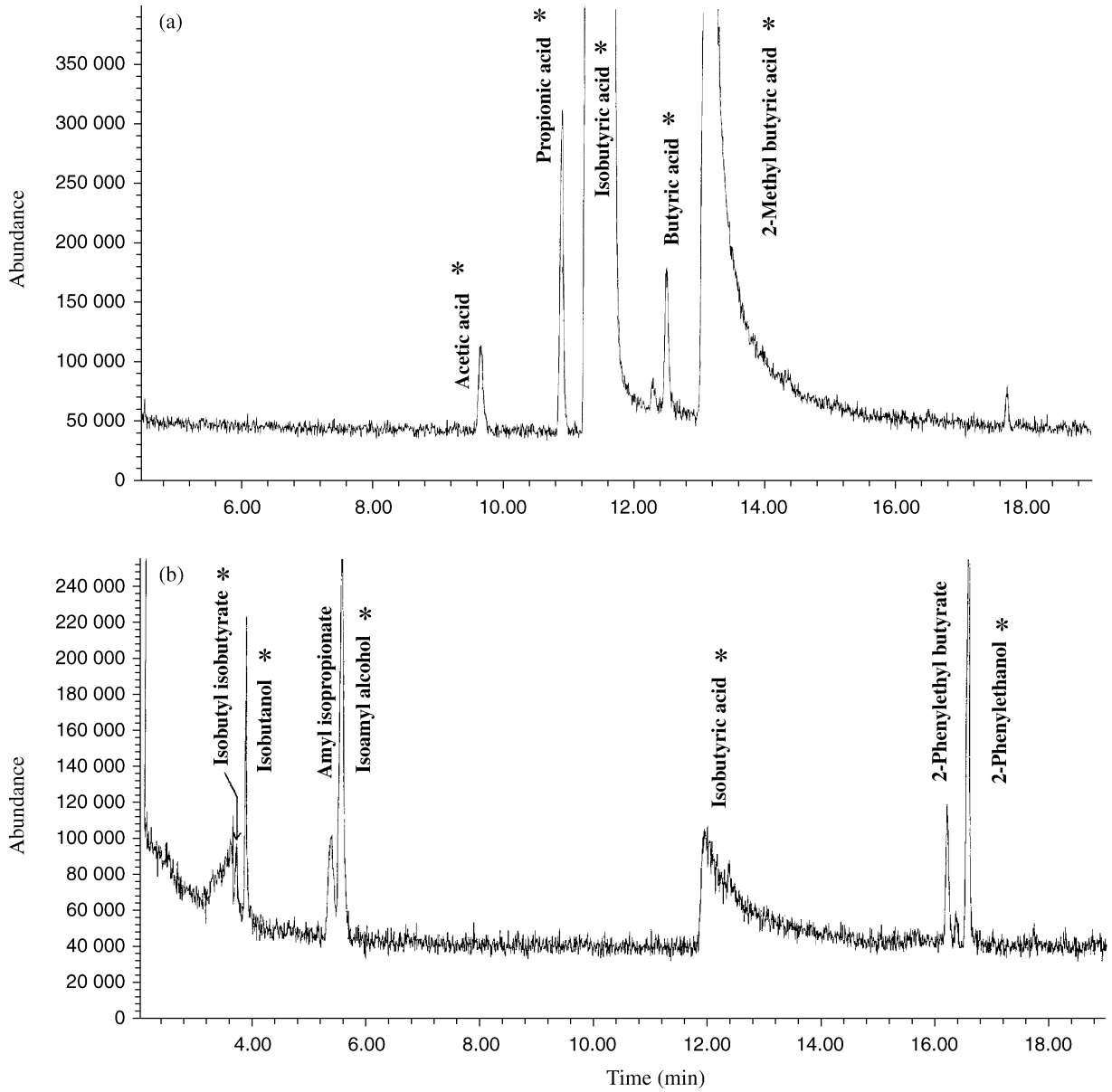

Figure 1 Total ion chromatogram of volatiles collected over eight (four male plus four female) disturbed adult *R. prolixus* (a), and *T. infestans* (b), sampled with a polyacrylate SPME fibre and analysed by GC-MS on a DB-WAX capillary column. Ordinate, total ion count detected by the MSD, and abscissa, retention times of the eluting compounds. Each 1 min interval is equivalent to an 8 °C rise in oven temperature, starting at 40 °C (isobutyric acid elutes at ca. 130 °C). The peaks that were identified represent >3 ng. Compounds whose identity was confirmed by machine mass spectra and retention times of synthetic equivalents are marked with an asterisk. Note that the ordinate scale in (b) is different from that in (a).

qualitative differences between the mixtures released by disturbed male or female *R. prolixus* were found (not shown), in agreement with the findings on Brindley's gland content in *R. prolixus* (Rojas et al., 2002), and as reported for *T. infestans* (Hack et al., 1980; Juárez & Brenner, 1981).

Seven compounds were tentatively identified by GC-MS in the headspace over disturbed *T. infestans* (isobutanol, isoamyl alcohol, amyl isopropionate, isobutyl isobutyrate, 2-phenylethanol, and 2-phenylethyl butyrate, as well as isobutyric acid; Figure 1b). Five of these, for which synthetic equivalents were available (isobutanol, isoamyl alcohol, isobutyric acid, isobutyl isobutyrate, and 2-phenylethanol), matched the retention times of the synthetic products injected under the same conditions (indicated

with an asterisk in Figure 1b). The blend of compounds collected in this study was similar to that reported by Cruz López et al. (1995) but different to that found by Juárez & Brenner (1981). The latter authors detected a mixture of C_2–C_5 acids (acetic, propionic, isobutyric, and isovaleric acid) in the headspace over disturbed adult *T. infestans* – a blend more akin to that found in this study from disturbed *R. prolixus*. Both blends reported from *T. infestans* have a counterpart in the profiles of compounds found in Brindley's gland by different authors (Hack et al., 1980; Cruz López et al., 1995).

Isobutyric acid was the only product common to the headspaces from disturbed *R. prolixus* and *T. infestans* reared and examined under the same conditions. This may indicate that the odour blend released by triatomines after mechanical disturbance is species-specific. However, isobutyric acid is not a universal secretory product among disturbed adult triatomines. GC-MS analysis of the headspace of three disturbed adult male *D. maxima* suggested the presence of 3-methyl-2-hexanone in the samples obtained with both polyacrylate and polydimethylsiloxane SPME fibres. Single-ion analysis using the MSD at the m/z = 73 typical of $[CH_3CHCO_2H]^+$ on these chromatograms detected no isobutyric acid. 3-Methyl-2-hexanone was also found by Rossiter & Staddon (1983) in the metasternal glands of *D. maxima.* It therefore seems likely that, at least in this species, the secretion from this gland functions as a defensive or alarm signal.

The only compound detected in headspace samples over both males alone and mating pairs of undisturbed *R. prolixus* was isobutyric acid. Ríos-Candelaria (1999) also found this compound from mating pairs of *R. prolixus.* In addition, isobutyric acid, together with other compounds, was also found in the headspace from mating *T. infestans* (Fontan et al., 2002). It seems unlikely that the release of this acid in our experiments was due to an inadvertent disturbance of the bugs. Firstly, as mentioned above (see Materials and methods), the disturbance of adult *R. prolixus* had to be relatively strong for detection of compounds in the headspace of the disturbed bugs. Secondly, in an additional series of odour collections (not shown) where the loop of the CLSA was opened, the vibrations generated by the air-pump (the only apparent source of a possible disturbance to the insects during the sampling period) were avoided by placing it in a neighbouring room. In these samples, isobutyric acid was still collected in one out of three experimental groups of 20 insects each, at a level close to the detection threshold of the MSD.

The amount of isobutyric acid from undisturbed males and mating *R. prolixus* pairs was, on average, more than 100-fold smaller than that from disturbed adult *R. prolixus.* This difference was not due to the different odour sampling method. Thus, it can be suggested that the amount of acid per insect released by undisturbed males and mating pairs was considerably lower than that from disturbed insects. This may suggest that the bugs continuously release low quantities of the acid. The release mechanism of the secretion from Brindley's gland has already been described (Barrett et al., 1979). However, it is not known if the gland is hermetic in the 'closed' state, and if not, this could lead to a slow and constant release of the secretion.

Many of the compounds (including isobutyric acid) detected in this study from the headspace of adult triatomines are known constituents of vertebrate odour (Wellington et al., 1979; Albone, 1984; Jemiolo et al., 1994). Isobutyric acid has been considered the 'alarm pheromone' of triatomines (Kälin & Barrett, 1975; Barrett, 1976) due to its prevalence in the headspace vapour over adults of several triatomine species when physically disturbed, and because of its dispersal effect on bugs. That isobutyric acid might induce different behavioural effects as a function of dose was first suggested by Schofield (1975), who demonstrated that high levels of this compound repelled *T. infestans* (alarm signal role) in an olfactometer in still air, whereas lower levels attracted these bugs. Data from experiments on a servosphere suggested that isobutyric acid is attractive at levels that are near the electrophysiological threshold of its olfactory receptor cell (ca. 0.1 p.p.b.), but this attraction disappears at levels 3 log steps higher (Guerenstein & Guerin, 2001).

A few undisturbed adults could release low levels of acid that may cause the animals to aggregate in a refuge, just as similar concentrations of the product may play a role in attracting hungry bugs to vertebrates (Guerenstein & Guerin, 2001). Although the existence of an aggregation pheromone in 'triatomine odour' has not been clarified, it was suggested that during copulation, adult *T. infestans* form aggregations using odour cues for orientation (Manrique & Lazzari, 1996). In addition, undisturbed adult *T. infestans* were reported to attract other adults of the same species even before copulation (Fontan et al., 2002). In ticks, isobutyric acid is the predominant compound of the aggregation-attachment pheromone of *Amblyomma hebraeum* (Koch) (Apps et al., 1988).

A parsimonious use of isobutyric acid (and maybe some of the other compounds secreted by the exocrine glands) by triatomines can be proposed as this compound could be used as an alarm signal, a host attractant, and as an intraspecific aggregation signal, depending on the concentration and physiological state of the perceiving bug. Products that function as alarm pheromones for a species when present in relatively high concentrations, and that promote aggregation when present at lower levels, are known for other insects (Blum, 1996).

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References

Albone E (1984) Mammalian Semiochemistry, 1st edn. John Wiley & Sons Ltd, Chichester, UK and New York.

Apps PJ, Viljoen HW & Pretorius V (1988) Aggregation pheromones of the bont tick *Amblyomma hebraeum*: identification of candidates for bioassay. Onderstepoort Journal of Veterinary Research 55: 135–137.

Baldwin WF, Knight AG & Lynn KR (1971) A sex pheromone in the insect *Rhodnius prolixus* (Hemiptera: Reduviidae). Canadian Entomologist 103: 18–22.

Barrett TV (1976) Parasites and predators of Triatominae. Scientific publications. Pan-American Health Organisation 318: 24–30.

Barrett FM, Millen BF & Lai-Fook J (1979) The Brindley's glands of *Rhodnius prolixus*. I. Structure of the mature gland. Canadian Journal of Zoology 57: 1109–1119.

Blum MS (1996) Semiochemical parsimony in the Arthropoda. Annual Review of Entomology 41: 353–374.

Cruz López L, Morgan ED & Ondarza RN (1995) Brindley's gland exocrine products of *Triatoma infestans*. Medical and Veterinary Entomology 9: 403–406.

Cruz López L, Malo EA, Rojas JC & Morgan ED (2001) Chemical ecology of triatomine bugs: vectors of Chagas disease. Medical and Veterinary Entomology 15: 351–357.

Fontan A, Audino PG, Martinez A, Alzogaray RA, Zerba EN, Camps F & Cork A (2002) Attractant volatiles released by female and male *Triatoma infestans* (Hemiptera: Reduviidae), a vector of Chagas disease: Chemical analysis and behavioral bioassay. Journal of Medical Entomology 39: 191–197.

Grob K & Zürcher F (1976) Stripping of trace organic substances from water, equipment and procedure. Journal of Chromatography 117: 285–294.

Guerenstein PG & Guerin PM (2001) Olfactory and behavioural responses of the blood-sucking bug *Triatoma infestans* to odours of vertebrate hosts. Journal of Experimental Biology 204: 585–597.

Hack WH, Ricciardi AI, Oscherov I & de Olivetti B (1980) Composition of the secretion of Brindley's gland in Triatominae. Medicina (Buenos Aires) 40: 178–180.

Jemiolo B, Gubernick DJ, Yoder MC & Novotny M (1994) Chemical characterization of urinary volatile compounds of *Peromyscus californicus*, a monogamous biparental rodent. Journal of Chemical Ecology 20: 2489–2500.

Juárez P & Brenner RR (1981) Bioquímica del ciclo evolutivo del *Triatoma infestans* (vinchuca). V. Emision de ácidos grasos volátiles. Acta Physiologica Latino-Americana 31: 113–117.

Kälin M & Barrett FM (1975) Observations on the anatomy, histology, release-site, and function of Brindley's glands in the blood-sucking bug, *Rhodnius prolixus*. Annals of the Entomological Society of America 68: 126–134.

Manrique G & Lazzari CR (1996) Existence of a sex pheromone in *Triatoma infestans* (Hemiptera: Reduviidae): I.Behavioural evidence. Memorias do Instituto Oswaldo Cruz (Rio de Janeiro) 90: 645–648.

Ríos-Candelaria E (1999) Análisis químico y conductual de los compuestos volátiles emitidos por *Rhodnius prolixus*. Hemiptera: Reduviidae), vector de la enfermedad de Chagas. PhD Thesis, Universidad Autónoma de Chiapas, Mexico.

Rojas JC, Ríos-Candelaria E, Cruz-López L, Santiesteban A, Bond-Compean JG, Brindis Y & Malo EA (2002) A reinvestigation of Brindley's gland exocrine compounds of *Rhodnius prolixus* (Hemiptera: Reduviidae). Journal of Medical Entomology 39: 256–265.

Rossiter M & Staddon BW (1983) 3-Methyl-2-hexanone from the triatomine bug *Dipetalogaster maximus* (Uhler) (Heteroptera; Reduviidae). Experientia 39: 380–381.

Schofield CJ (1975) The behavioural biology of Triatominae (Heteroptera, Reduviidae) with special reference to intraspecific communication mechanisms. PhD Thesis, London University, UK.

Taneja J & Guerin PM (1997) Ammonia attracts the haematophagous bug *Triatoma infestans:* behavioural and neurophysiological data on nymphs. Journal of Comparative Physiology A 181: 21–34.

Ward JP (1981) A comparison of the behavioural responses of the haematophagus bug, *Triatoma infestans*, to synthetic homologues of two naturally occurring chemicals (n- and iso-butyric acid). Physiological Entomology 6: 325–329.

Weirauch C (2003) Glandular areas associated with the male genitalia in *Triatoma rubrofasciata* (Triatominae, Reduviidae, Hemiptera) and other Reduviidae. Memorias do Instituto Oswaldo Cruz (Rio de Janeiro) 98: 773–776.

Wellington JL, Byrne KJ, Preti G, Beauchamp GK & Smith AB (1979) Perineal scent gland of wild and domestic guinea pigs. A comparative chemical and behavioural study. Journal of Chemical Ecology 5: 737–751.