

Compounds released by disturbed adults of the haematophagous bug *Triatoma infestans* (Hemiptera: Reduviidae): behavioural effects of single compounds and binary mixtures

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Abstract. Adults of the Chagas disease vector *Triatoma infestans* Klug (Hemiptera: Reduviidae: Triatominae), possess paired exocrine glands: the metasternal and Brindley's glands. Both glands are discharged by disturbed adults, releasing an alarm pheromone that elicits an escape response of larvae. The present study analyzes the individual (or combined) effects of some of the volatiles of the whole pheromone blend released under disturbance, searching for active compounds and for possible interactions (e.g. synergism, additive effects) between them. Using an experimental arena, different doses of components emitted by disturbed adults are tested against larvae. Larvae show escape responses to some of the acids, as well as to one alcohol, but no response to ketones. This is observed with certain doses of compounds from Brindley's glands (isobutyric, butyric and acetic acid; 2-methyl-1-butanol), although the compounds tested in the present study that are produced by metasternal glands are shown to evoke random responses, suggesting that mainly Brindley's glands are involved in the alarm context. Two combinations of two individually repellent compounds (2-methyl-1-butanol with acetic or isobutyric acid) evoke escape responses, although other combinations make the individual effect disappear. A different mixture of two individually repellent compounds (butyric and isobutyric acid) evokes attraction, although these are also host odours. The potential use of the active compounds released by disturbed adults to monitor triatomine populations is discussed.

Key words. Alarm pheromone, Brindley's glands, Chagas disease, Triatominae, volatiles.

Introduction

Triatomines are haematophagous insects capable of transmitting the flagellate parasite *Trypanosoma cruzi*, which is the causative agent of Chagas disease in humans. Among the more than 130 species of triatomine bugs, *Triatoma infestans* Klug is the main vector responsible for the transmission of this

trypanosomiasis in the Southern Cone of South America. This disease affects 6–7 million people, with a further 75–90 million being at risk of potential transmission in Latin America (Coura & Dias, 2009). To date, no vaccine exists against *T. cruzi*, and vector control comprises the main method for reducing Chagas disease.

As for most insects, chemical communication plays a significant role in the biology of triatomines. Most species, including *T. infestans*, use chemical cues and signals to detect their hosts and to communicate with conspecifics. This species utilizes the chemical cues released by their hosts to locate them in space and time. Moreover, pheromones are released to evoke an assembling behaviour, to locate mates and to alert conspecifics about a potential danger, amongst other behaviours (Ward, 1981;

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Manrique & Lazzari, 1995; Manrique *et al.*, 2006; Crespo & Manrique, 2007; Cocchiararo-Bastías *et al.*, 2011).

Adults of most triatomines possess paired exocrine glands in the thorax (metasternal glands) and in the abdomen (Brindley's glands), which are absent in larvae (Schofield & Upton, 1978). In *T. infestans*, the secretion from the metasternal glands contains several compounds, some of which are highly volatile aliphatic ketones, acetals and short-chain alcohols: 3-pentanone (the major component), 2-butanone, 3-pentanol, 3-hexanol, 2-methyl-1-butanol (Manrique *et al.*, 2006) and (4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane and related compounds (Unelius *et al.*, 2010; Bohman *et al.*, 2011). The metasternal gland volatiles are suggested to mediate sexual communication in several triatomine species (Manrique *et al.*, 2006; Crespo & Manrique, 2007; Pontes *et al.*, 2008; Vitta *et al.*, 2009; Zacharias *et al.*, 2010; May-Concha *et al.*, 2012; Pontes *et al.*, 2014).

The Brindley's glands of *T. infestans* contain isobutyric acid (the major component), butyric acid, acetic acid, propionic acid, 2-butanone, 2-methyl-1-butanol, 2-methyl-1-propanol and other compounds, such as esters, in lesser amounts (Manrique *et al.*, 2006). These glands are likely to be associated with alarm and defence functions (Schofield, 1979; Ward, 1981; Cruz-López *et al.*, 1995; Rojas *et al.*, 2002; González Audino *et al.*, 2006; Manrique *et al.*, 2006; May-Concha *et al.*, 2015). In particular, the existence of an alarm pheromone released by disturbed *T. infestans* adults that elicits an escape response of conspecific larvae is suggested (Manrique *et al.*, 2006). Minoli *et al.* (2013a) report that the escape response to the alarm pheromone can be widely modulated by associative and non-associative conditioning protocols. Furthermore, isobutyric acid alone is involved in the modulation of the behaviour of this species, being attractive or repellent in a dose-dependent manner (Ward, 1981; Guerenstein & Guerin, 2001). Interestingly, it is suggested that both pairs of glands (i.e. metasternal and Brindley's glands) discharge their contents during mechanical disturbance (Manrique *et al.*, 2006). Apart from isobutyric acid, it is still unclear whether other compounds secreted by Brindley's or the metasternal glands of *T. infestans* are relevant in both alarm and defensive contexts.

Although the escape response of larvae of *T. infestans* evoked by the blend of volatiles emitted by disturbed adults is already demonstrated, it is not yet known which of the individual compounds (or combinations of compounds) are able to trigger this response. Based on the proportion present in the secretion released by disturbed *T. infestans* adults (Manrique *et al.*, 2006), the present study aims to identify which of the most abundantly emitted compounds of the whole blend exhibit an active effect in the escape response of larvae of the same species. Specifically, the present study analyzes the behavioural responses of larvae evoked by different doses of some of the compounds from the Brindley's and metasternal glands. In addition, in an attempt to clarify how combinations of compounds can affect the behaviour of individuals and to understand any possible interactions (e.g. synergic), the behavioural responses of larvae exposed to mixtures of compounds that individually evoke an escape response are evaluated. Finally, the potential use of active compounds released by adults of *T. infestans* as dislodgement

agents in natural environments is discussed as a means of driving them from refuges and improving the monitoring and/or control of domestic triatomine populations.

Materials and methods

The *T. infestans* used in the present study were obtained from a colony established at the Servicio Nacional de Chagas (Punilla, Córdoba, Argentina) and reared in the insectary under an LD 12:12 h photoperiod at $28 \pm 1^\circ\text{C}$ and 30–60% relative humidity. The colony was established for more than 20 years and is frequently reinforced with wild-caught insects. Animals were handled according to the biosafety rules from the Servicio de Higiene y Seguridad of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. Behavioural experiments were performed using unfed fourth-instar larvae that were 10–25 days post-moulting (test insects) and unfed 1–3-day-old adults (as stimulus source). Adults under these conditions have their scent glands full and will release their contents after mechanical disturbance (Palottini *et al.*, 2014). Insects were used once and then discarded.

Measurement of the escape response of larvae of T. infestans exposed to synthetic compounds from the blend released by disturbed adults

The responses of larvae to some of the synthetic compounds from the total blend of volatiles released by disturbed adults were evaluated using an experimental arena based on Minoli *et al.* (2013b). Briefly, the arena consisted of a rectangular acrylic container (15 × 10 × 4 cm) with 32 equidistant holes (diameter 2 mm) in its base. Below this, a lower chamber (15 × 10 × 1 cm), divided into two equal sections by an odour-impermeable transversal plate, was attached. Each section of the lower chamber communicated to a detachable stimulus flask (10 mL) containing the odour source.

For each assay, the base of acrylic arena was covered with a new filter paper, with holes matching those on its base to avoid any chemical contamination left by the previous larvae. Then, the stimulus flasks containing the volatile compound(s) were attached to each section of the lower chamber and one larva was placed on the filter paper, in the middle of the arena, and left covered with a flask for 1 min of context familiarization and also to allow diffusion of odours on the assumption that a chemical gradient was generated. Next, the flask was gently removed, the arena was covered with a transparent lid and the behaviour of the released larva was recorded for 4 min using a video camera connected to a digital recorder.

Based on previous literature describing the composition of the blend released by disturbed *T. infestans* adults (Manrique *et al.*, 2006), the escape responses of larvae exposed to isobutyric acid (Fluka, Germany; 98% purity), butyric acid (Fisher Scientific Co., Pittsburgh, Pennsylvania; 98% purity), propionic acid (Merck, Germany; 98% purity), acetic acid (Anedra,

Argentina; 98% purity), 3-pentanone (Sigma, Germany; 98% purity), 2-butanone (Sigma; 99% purity), 2-methyl-1-butanol (Sigma; 99% purity) and 2-methyl-1-propanol (Sigma; 98% purity) were tested. This choice was taken considering the proportion of individual compounds present in the secretion released by disturbed adults (Manrique *et al.*, 2006). However, it should be acknowledged that this might lead to exclusion of other relevant compounds that may be responsible, in part, for the escape responses evoked. Different doses (0.1, 10 or 1000 µg) of each compound were prepared in 50 µL of dichloromethane (99% purity; Merck). The stimulus solution (50 µL containing the different compounds) was loaded onto a small strip of filter paper (0.5 cm × 2.5 cm), which was inserted into one stimulus flask, whereas, in the other flask, a filter paper was loaded with 50 µL of dichloromethane alone (control). The solvents were allowed to evaporate for 30 s and the stimulus flasks were then attached to the lower chamber. In this way, stimuli entered by diffusion into each of the two parts of the subchamber independently, and continued to diffuse up to the arena through the holes, where one larva per assay was released.

In addition, the behavioural responses of larvae to the natural alarm pheromone released by disturbed adults were evaluated. To assess the natural blend for the tests, one undisturbed adult was carefully inserted into the stimulus flask and, immediately before attaching the flask to the chamber, disturbance was achieved by grabbing its legs with forceps for 30 s inside the flask. Previous observations revealed that this practice is sufficient for a complete release of the contents of the exocrine glands (Palottini *et al.*, 2014). The other stimulus flask held an undisturbed adult, which remained undisturbed during the assay.

The assignment of the odour sources to each flask was reversed after every replicate to eliminate directional bias. The experiments were conducted during the initial hours of the scotophase (i.e. 1–5 h after lights were turned off) to match the maximal activity period observed for these insects (Lazzari, 1992). Assays were performed under conditions of total darkness to match the phase of the photoperiod in which animals were tested (i.e. scotophase), as well as to exclude the possible use of visual cues by larvae. The temperature of the experimental room was set to 25 ± 1 °C before the beginning of each assay. The relative humidity range in the room where the assays were completed was $50 \pm 10\%$.

Measurement of the escape response of larvae of T. infestans exposed to mixtures of synthetic compounds from the blend released by disturbed adults

The behavioural responses of larvae to binary mixtures generated artificially with the volatiles tested individually as described above were analyzed in a similar way. Using the same experimental device, protocols and conditions described above, different mixtures were prepared and deployed. For each binary mixture, paper strips loaded with the corresponding combined compounds were added to one stimulus flask. In an attempt to determine the interactions between compounds from the blend released by disturbed adults, binary mixtures were prepared combining the repellent dose (10 µg) of each of the four active

compounds (Fig. 1) together with 0.1, 10 and 1000 µg of the other three active volatiles. The assignment of the odour source to each flask was reversed after every replicate to eliminate directional bias.

Behavioural recordings and statistical analysis

For each individual assay, the time that larvae spent in each side of the arena was recorded and a preference index (PI) ranging from -1 to 1 was calculated as $PI = (t - 120)/120$, where t is the time in seconds spent on the side of the arena containing the stimulus (i.e. synthetic compound/s or disturbed adult). Therefore, PIs near -1 , 0 or 1 indicate repellence, no effect or attraction to the stimulus, respectively. The orientation responses in the behavioural bioassays (PI) were assessed by means of one-sample t -tests (H_0 : $PI = 0$). Differences between responses evoked by the synthetic volatiles were assessed by means of one-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparisons. Twenty to forty replicates were completed for each treatment. In addition, all treatments were performed simultaneously up to completion of the stipulated replicates, aiming to avoid temporal and biological bias.

Results

Behavioural responses of T. infestans larvae exposed to individual synthetic compounds from the blend released by disturbed adults

The escape response of larvae of *T. infestans* was elicited by four of the eight tested components from the blend released by disturbed adults when presented individually at particular doses (Fig. 1). Insects were significantly repelled by 10 µg of isobutyric acid ($PI = -0.09 \pm 0.04$, $t_{39} = -2.04$, $P = 0.04$) (Fig. 1A), 10 µg of butyric acid ($PI = -0.35 \pm 0.08$, $t_{39} = -4.15$, $P = 0.0001$) (Fig. 1B), 10 µg of acetic acid ($PI = -0.27 \pm 0.08$, $t_{39} = -3.03$, $P = 0.004$) (Fig. 1D) and 10 µg of 2-methyl-1-butanol ($PI = -0.21 \pm 0.08$, $t_{39} = -2.44$, $P = 0.02$) (Fig. 1G). Conversely, larvae remained unaware of the presence of the remaining of the doses of these volatiles that were tested, and of the presence of all doses of each of the other compounds tested (i.e. propionic acid, 3-pentanone, 2-butanone and 2-methyl-1-propanol) (t -test, $P > 0.05$ for all cases). The control series of assays (i.e. dichloromethane versus dichloromethane) that were carried out in parallel for each treatment yielded random distributions in all cases (data not shown, t -test, $P > 0.05$ for all cases).

Furthermore, as expected (Manrique *et al.*, 2006), larvae showed an escape response (Fig. 1, vertical line) in response to odours released by disturbed adults ($PI = -0.18 \pm 0.07$, $t_{39} = -2.58$, $P = 0.013$). This escape response of the larvae exposed to the natural alarm pheromone did not differ significantly from that evoked by larvae exposed to 10 µg of isobutyric acid, 10 µg of butyric acid, 10 µg of acetic acid or 10 µg of 2-methyl-1-butanol (one-way ANOVA, $P > 0.05$).

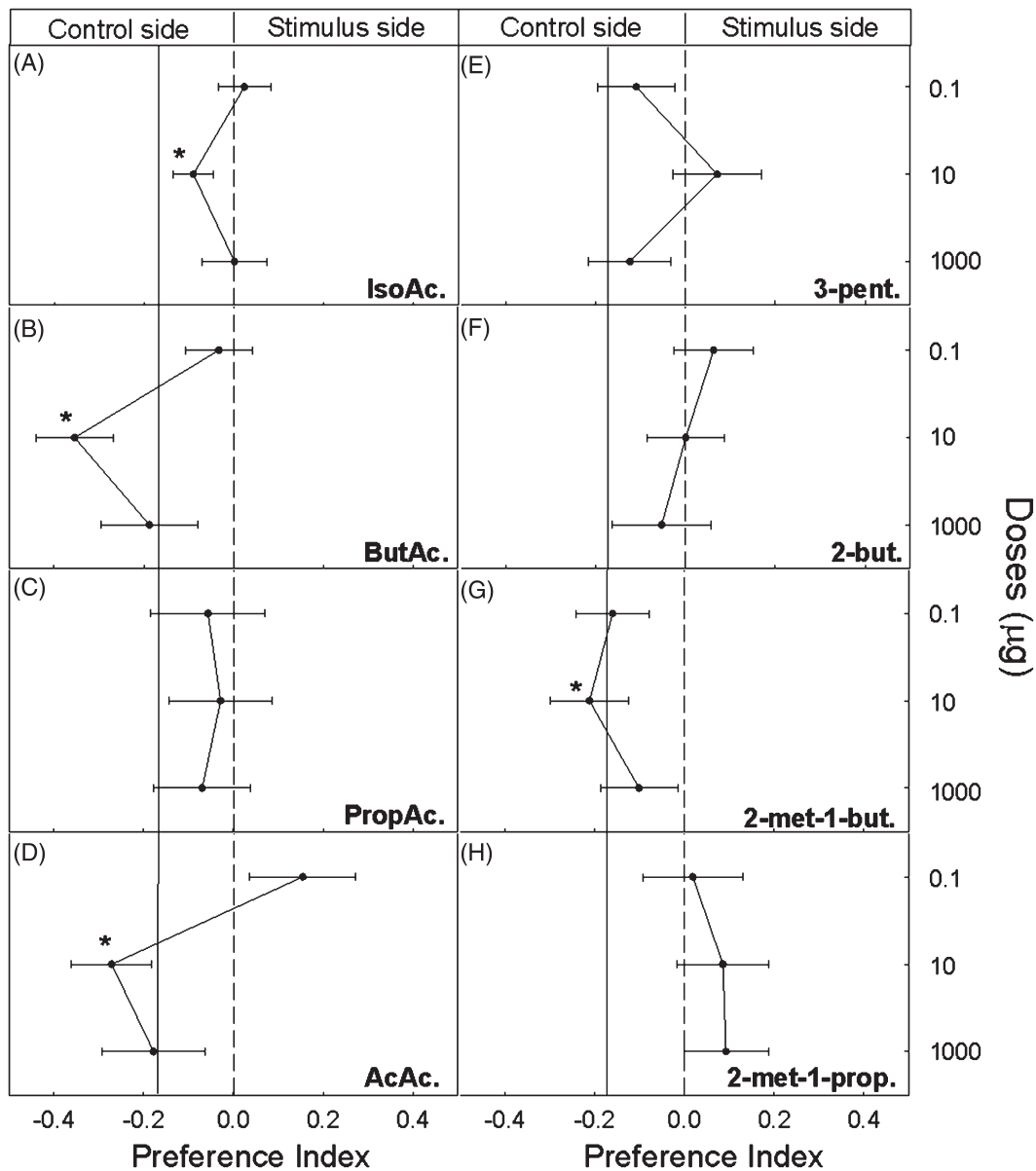


Fig. 1. Behavioural response (represented as preference index; PI) of larvae of *Triatoma infestans* exposed in an experimental arena to different doses of synthetic components of the volatile blend released by disturbed adults. (A) IsoAc, isobutyric acid. (B) ButAc, butyric acid. (C) PropAc, propionic acid. (D) AcAc, acetic acid. (E) 3-pent, 3-pentanone. (F) 2-but, 2-butanone. (G) 2-met-1-but, 2-methyl-1-butanol. (H) 2-met-1-prop, 2-methyl-1-propanol. The vertical line represents the PI value corresponding to the escape response evoked by the natural alarm pheromone. Error bars represent the SEM ($n = 40$ replicates for each treatment); an asterisk indicates a significant preference toward the control side (PI different from zero, t -test, $P < 0.05$).

Behavioural responses of T. infestans larvae exposed to mixtures of synthetic compounds from the blend released by disturbed adults

Larvae exhibited an escape response when exposed to $10 \mu\text{g}$ of isobutyric acid alone ($\text{PI} = -0.09 \pm 0.04$, $t_{39} = -2.04$, $P = 0.04$) (Fig. 2A) and when combined with $0.1 \mu\text{g}$ of 2-methyl-1-butanol ($\text{PI} = -0.19 \pm 0.05$, $t_{19} = -3.45$, $P = 0.002$) (Fig. 2A). Conversely, no systematic orientation/escape responses were observed when other doses (of 2-methyl-1-butanol) and other

compounds were added to the isobutyric acid ($P > 0.05$ for all cases).

Similarly, larvae avoided the side of the arena containing $10 \mu\text{g}$ of 2-methyl-1-butanol alone ($\text{PI} = -0.21 \pm 0.08$, $t_{39} = -2.44$, $P = 0.02$) (Fig. 2B) and when $0.1 \mu\text{g}$ of acetic acid was added ($\text{PI} = -0.29 \pm 0.12$, $t_{19} = -2.44$, $P = 0.02$) (Fig. 2B). When other compounds and doses were added to 2-methyl-1-butanol, no orientation response was registered ($P > 0.05$ for all cases).

For acetic acid presented individually, only a dose of $10 \mu\text{g}$ of was found to be repellent for larvae ($\text{PI} = -0.27 \pm 0.08$,

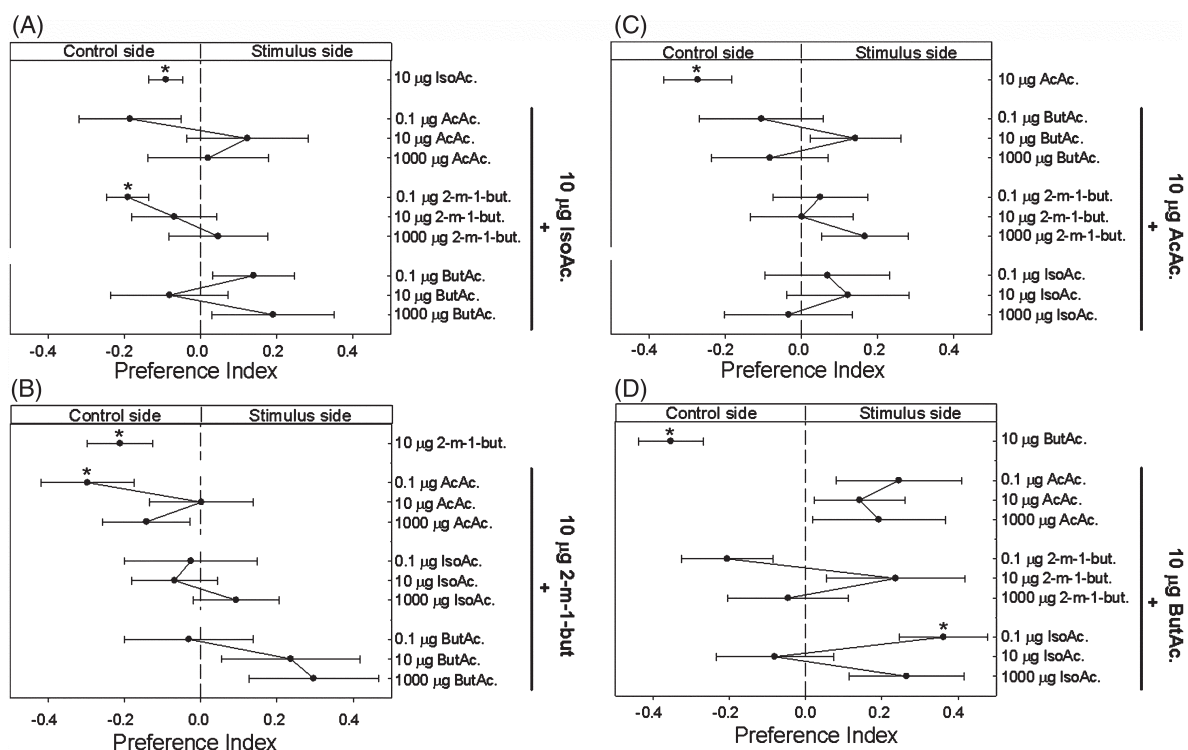


Fig. 2. Behavioural response (represented as preference index; PI) of larvae of *Triatoma infestans* exposed in an experimental arena to binary mixtures of synthetic components of the volatile blend released by disturbed adults. (A) 10 μg of isobutyric acid + 0.1–1000 μg of acetic acid, 2-methyl-1-butanol or butyric acid. (B) 10 μg of 2-methyl-1-butanol + 0.1–1000 μg of acetic acid, isobutyric acid or butyric acid. (C) 10 μg of acetic acid + 0.1–1000 μg of butyric acid, 2-methyl-1-butanol or isobutyric acid. (D) 10 μg of butyric acid + 0.1–1000 μg of acetic acid, 2-methyl-1-butanol or isobutyric acid. IsoAc, isobutyric acid; AcAc, acetic acid; ButAc, butyric acid; 2-met-1-but, 2-methyl-1-butanol. Error bars represent the SEM ($n = 20$ replicates for each treatment); an asterisk indicates a significant difference from a random distribution (PI = 0, t -test, $P < 0.05$).

$t_{39} = -3.03$, $P = 0.004$) (Fig. 2C). The addition of all other compounds, at all of the tested doses, to 10 μg of acetic acid resulted in a lack of oriented responses ($P > 0.05$ for all cases) (Fig. 2C).

By contrast to all the above results, although larvae were repelled by 10 μg of butyric acid (PI = -0.35 ± 0.08 , $t_{39} = -4.15$, $P = 0.0001$) (Fig. 2D), the addition of 0.1 μg of isobutyric acid to this quantity of butyric acid switched the repellence to attraction (PI = 0.36 ± 0.11 , $t_{19} = 3.09$, $P = 0.005$) (Fig. 2D). The combination of butyric acid with all other compounds and doses resulted in a lack of response ($P > 0.05$ for all cases).

Discussion

The present study analyzes the individual contributions of the more abundant components found in the blend of volatile compounds discharged from adults of *T. infestans* as a disturbance response. Different doses of these components are tested behaviourally against conspecific larvae, with consideration being given to the relative abundance of different volatiles in the blend (Manrique *et al.*, 2006). Larvae show an escape response when exposed to some of the acids (but not to all), as well as to one alcohol, but no response to any ketones. Escape responses are observed in response to the presence of compounds that

derive from Brindley's glands (i.e. isobutyric acid, butyric acid, acetic acid, 2-methyl-1-butanol). By contrast, none of the compounds that are produced by the metasternal glands evoke a behavioural response in larvae. These results suggest that the metasternal glands are not associated with an alarm function, supporting evidence for these glands being involved in sexual communication, whereas Brindley's glands are previously reported to be implicated in alarm and/or defence contexts in several triatomines (Crespo & Manrique, 2007; Pontes *et al.*, 2008; Vitta *et al.*, 2009; Zacharias *et al.*, 2010; May-Concha *et al.*, 2012; Pontes *et al.*, 2014). An exception is the species *Dipetalogater maxima* that lacks Brindley's glands and instead produces 3-methyl-2-hexanone, a volatile with possible alarm or defensive functions that is detected when insects of this species are disturbed (Guerenstein & Guerin, 2004), from the metasternal glands (Rossiter & Staddon, 1983).

When mixtures of the *T. infestans* volatiles are tested, distinct interaction scenarios are displayed. First, there is no single instance in which a combination of two active and repellent compounds results in an increase in the escape response compared with that obtained with the volatiles evaluated individually. In many cases, when two individually repellent volatiles are presented together, the individual effect of each disappears entirely. Additionally, in one situation, the larvae showed the opposite response (i.e. attraction) when exposed to a mixture of two

originally repellent compounds (10 µg of butyric acid + 0.1 µg of isobutyric acid). In triatomines, the lack of knowledge of the neuronal network involved in the alarm context does not allow any definitive explanation of why, in some instances, two doses of repellent compounds can turn into a neutral response when presented together. It is not clear whether triatomines are able to discriminate between short-chain fatty acids (Barrozo & Lazzari, 2004). If this is indeed the case, the larvae would perceive the mixture as only one compound but with an increased dose, which may be responsible for the absence of a response to many of the binary mixtures in the present study. It is evident that further investigations are required to test this hypothesis. The perception of a mixture of odours is an extremely complex process in insects, and needs to be studied not only at the peripheral, but also at higher levels of the central nervous system, such as the antennal lobes or mushroom bodies. Nevertheless, the present study is the first report to describe, under controlled experimental conditions, the behaviour of larvae of *T. infestans* in response to individual components of the volatile blend (apart from isobutyric acid) released by disturbed adults. In turn, it is the first study to analyze the effect of binary mixtures of some of these compounds at behaviourally relevant doses. Further studies are necessary to test other compounds (or combinations) of the blend released under disturbance, with the aim of identifying other active compounds of the alarm pheromone of *T. infestans*.

With regard to the volatile compounds that are evaluated in the present study, there are reports describing their role or potential roles in different insect species. Although the present study finds no responses that differ from a random distribution when ketones are tested, 2-butanone forms part of the alarm pheromone released by the bed bug *Cimex lectularius*, another haematophagous hemipteran (Levinson *et al.*, 1974), albeit of a different family. Also, 3-pentanone is one of the compounds released by the cockroach *Periplaneta americana* as a component of its defence allomone (Brossut, 1983). At present, it cannot be discounted that compounds showing no alarm effect in the current experiments may act as defensive allomones, although, in *T. infestans*, the possible defensive role of odours emitted under disturbance has not been tested previously. In addition, both of the ketones (3-pentanone and 2-butanone) are components of the volatiles emitted by the metasternal glands of *T. infestans*, with 3-pentanone being the major component detected in the headspace over copulating pairs (Manrique *et al.*, 2006). The secretion from these glands promotes the copulation and aggregation behaviour of males around mating couples of this species (Crespo & Manrique, 2007), although there is currently no clear understanding of which of the compounds may be responsible for such activity. Two alcohols are tested in the present study, yet larvae only respond to 2-methyl-1-butanol. According to the literature, this compound is not associated with communication between insects but, instead, is described as part of the defensive secretion released by two coleopteran species (Dettner & Reissenweber, 1991). Fatty acids are reported as typical components of the defensive secretions of many insects, probably as a result of their acid nature that can be irritant or distasteful for predators. Normally, the acetic, butyric, isobutyric and 2-methyl butyric acids are present together in alarm pheromones or defence allomones,

mainly in a kinetic chemical equilibrium, because some of them are subproducts of the others (Attygalle *et al.*, 2004).

The short-chain fatty acids, including isobutyric acid, butyric acid and acetic acid, are commonly present in odours, such as in the breath (Cork & Park, 1996) of vertebrates and the axilla of humans (Zeng *et al.*, 1991), which are hosts of triatomine bugs. Although isobutyric acid can evoke an escape response when it is released from the Brindley's glands of disturbed adults, this compound can also assume a different function and act as a cue for the location of the food source related to host odours (Ward, 1981; Guerenstein & Guerin, 2001). This may also occur with butyric and acetic acids because they are present in host odours (Barrozo & Lazzari, 2004) and in the aggregation pheromones present in triatomine faeces (acetic acid) (Mota *et al.*, 2014), and they also evoke an escape response in the larvae of *T. infestans*, as observed in the present study.

The development of precise and reliable methods to achieve early detection of triatomines in natural environments is necessary for the effective prevention of Chagas disease. Vector control is now considered to be the main way of reducing the transmission of Chagas' disease as a result of the lack of a vaccine and sufficiently effective therapeutic drugs (Coura & Dias, 2009). At present, the detection of domiciliary infestations in control programmes is carried out by a manual search for triatomines and/or of particular signs of settling and infestation, such as faeces, eggs and exuviae. In situations where infestation rates are low, the use of chemical dislodging agents such as tetramethrin 0.2% is introduced to generate the flushing out of insects from their refuges, rendering them exposed. From a practical point of view, and in a wider context, the use of chemical mixtures based on pheromones or host odours is proposed to comprise an effective tool with low cost. Furthermore, these compounds are usually environmentally benign and can be very effective for the detection and control of many pest insects (El-Sayed *et al.*, 2009). Particularly for the kissing-bugs, the chemical compounds produced during intra- and interspecific interactions might be useful for the development of new strategies aiming to monitor and/or control populations of domestic triatomines. The results obtained in the present study demonstrate that several volatile components of the suggested alarm pheromone can generate an escape response in *T. infestans* larvae, even when presented individually. Further field studies will be necessary to determine whether deploying these compounds in the natural environments of *T. infestans*, or even of related species, can exert a specific dislodging effect on the larvae from their shelters. Accordingly, Minoli *et al.* (2013b) report that 3-methyl-1-butanol can be as effective as tetramethrin (0.2%; i.e. a pesticide commonly used as dislodging agent) in making larvae of triatomines exit from artificial shelters under pseudo-natural conditions.

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