Dislodgement effect of natural semiochemicals released by disturbed triatomines: a possible alternative monitoring tool

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ABSTRACT: The quick detection of domestic and peridomestic triatomines in their environments becomes difficult without the use of dislodgement substances that flush them out from their shelters. At present, tetramethrin 0.2% is being widely used in control programs. Although it is an efficient dislodging agent, its toxicity might affect the health of captured triatomines, of other insects and, to a lesser extent, of other animals, including humans. Here, we tested if semiochemicals released by disturbed adults of *Triatoma infestans* and/or *Rhodnius prolixus* can make larvae of the same species exit from their refuges. In a walking olfactometer we found that: 1) larvae of *T. infestans* were repelled by the odors released by disturbed adults of their own species and of *R. prolixus*, 2) larvae of *R. prolixus* did not change their behavior in the presence of odors released by adults of both species, and 3) activity levels were not modulated by these odors in any of both species. Besides, in pseudo-natural conditions we found an increased flushing-out activity of larvae of *T. infestans* when their shelters were sprayed with isobutyric acid or 3-pentanol, and of larvae of *R. prolixus* when sprayed with 3-methyl-1-butanol. We succeeded in this work to dislodge larvae of triatomines from artificial shelters using natural volatile compounds, allowing the capture of live bugs for further investigations (e.g., xenodiagnosis or genetic studies) and favoring ecological aspects (e.g., minimizing environmental insecticide-contamination and non-targeted mortality). *Journal of Vector Ecology* 38 (2): xxx-xxx. 2013.

Keyword Index: Triatomines, dislodgement, exocrine glands, detection, Chagas, behavior.

INTRODUCTION

Triatomine bugs (Hemiptera: Reduviidae) are haematophagous vectors of Chagas disease in Latin America (Lent and Wygodzinsky 1979, Zeledon and Rabinovich 1981). Most of the described species have nocturnal habits, displaying their activities mainly during the night, remaining aggregated inside their shelters during the day, usually in a quiescent state (akinesis) (Lazzari 1992, Lorenzo and Lazzari 1998). *Triatoma infestans* Klug 1834 and *Rhodnius prolixus* Stål 1859 develop large colonies in poorly maintained thatch and mud houses where cracks provide abundant shelters in close proximity to humans and their domestic animals. This fact makes them two of the most important epidemiological species, as they are mainly responsible for the transmission of the protozoan parasite *Trypanosoma cruzi,* the causative agent of Chagas disease, to humans: *T. infestans* in southern South America and *R. prolixus* in northern South America and Central America (Schofield 1994). Determining whether a house is infested with triatomines becomes very difficult to achieve, as field campaigns are performed during daylight hours, the moment in which these species are akinetic, hidden inside shelters. Detection of triatomine-infested houses (see Gurtler et al. 1995) is either made by the use of passive methods (Schofield 1978, Pinchin et al. 1981) in which different types of boxes are attached to the walls and secondary signals of the presence of triatomines (such as fecal marks, exuviae,

and eggs) are detected, or active methods (Gurtler et al. 1993, Garcia-Zapata et al. 1985), which involve the search for bugs by trained personnel who capture insects using a flushing-out agent. Passive methods do not need the application of toxic compounds inside dwellings but require longer periods of time for the detection of triatomines. Alternatively, the visual active detection and capture of flushed-out individuals after spraying with tetramethrin 0.2% is quite rapid but includes the application of pyrethroid-based insecticides in dwellings.

Tetramethrin is an efficient dislodging agent for triatomines, but its toxicity does not allow the capture of healthy triatomines for posterior studies (EPA 2010). Moreover, the chronic use of pyrethroids may cause an accumulation of the insecticide in the environment and might also generate genetic resistance. The use of natural dislodgement compounds that could be used instead of the pyrethroids present in tetramethrin would be of great help in control campaigns.

Previous studies have shown that metasternal and Brindley's glands, present only in adults of triatomines (Brindley 1930, Schofield and Upton 1978, Staddon 1983), release different blends of volatile compounds in a context-dependent manner. Although the metasternal glands are the main source of sexual communication signals of these insects (Manrique et al. 2006, Crespo and Manrique 2007, Pontes et al. 2008, Vitta et al. 2009, Zacharias et al. 2010, Manrique and Lorenzo 2012), it has been suggested that the

secretions of Brindley's glands are also involved in triatomine sexual communication (Fontan et al. 2002, Rojas et al. 2002, Guerenstein and Guerin 2004). However, the occlusion of the secretion openings of metasternal glands affects mating success and male aggregation around mating pairs described in *T. infestans* and *R. prolixus*, while the occlusion of Brindley's glands does not (Crespo and Manrique 2007, Pontes et al. 2008, Pontes and Lorenzo 2012, Manrique and Lorenzo 2012). The Brindley's glands volatiles appear to act mostly as an alarm pheromone (AP), as it has already been shown that the blend released by disturbed *T. infestans* adults repels individuals of the same species in a walking olfactometer (Manrique et al. 2006). Moreover, a defensive function was also suggested for this blend (Ward 1981, Manrique et al. 2006, Audino et al. 2007). Chemical analyses were carried out by several authors identifying the content and/or the volatiles released by each type of gland in a sexual and/or in an alarm context (Schofield 1979, Hack et al. 1980, Juarez and Brenner 1981, Cruz-López et al. 1995, Rojas et al. 2002, Guerenstein and Guerin 2004, Manrique et al. 2006, Pontes et al. 2008, Unelius et al. 2010, Bohman et al. 2011). Nevertheless, not much is known about the repellent effect of individual substances released by adults during a disturbance event on the behavior of conspecifics. Only the behavioral response to isobutyric acid, the main compound of Brindley's glands, has been studied in triatomines (Kälin and Barrett 1975, Pinchin et al. 1981, Ward, 1981).

The main objective of this study was to find alternative natural compounds that could evoke a flushing-out activity in larvae of triatomines, to avoid or decrease the systematic use of tetramethrin 0.2% as a dislodgement agent in natural environments. We tested here, in two different conditions, the effect of volatiles released by disturbed adults of *T. infestans* and/or *R. prolixus* on the escape behavior of larvae of the same species. Repellence or attraction and activation of larvae as a response to odors released by the metasternal and Brindley's glands of disturbed adults were quantified in individual assays in the laboratory. Flushing-out activity caused by single compounds produced by these glands was studied under simulated natural conditions.

MATERIALS AND METHODS

Insects

Insects of both species (*T. infestans* and *R. prolixus*) were reared in the laboratory at 28±2° C and 60±20% relative humidity, under a 12:12h L/D illumination regime. All instars were fed once a week on live hens. For the assays, 5-15 days old non-fed 4th instar larvae of *T. infestans* and 5th instar larvae of *R. prolixus* were separated weekly. Different instars of both species were used as they have approximately the same size. Insects were used only once and then discarded.

Experiment 1: Activity and repellence quantification in a spatial olfactometer

Changes in the activity levels and repellence effect caused by odors released by disturbed adults of *T. infestans* or *R. prolixus* were tested on 4th instar larvae of *T. infestans*

and 5th instar larvae of *R. prolixus* using a dual-choice walking olfactometer without air current (Figure 1). Briefly, a rectangular acrylic arena (15x10x4cm) with holes on the floor, attached to a sub-chamber divided in two equal parts by an odor-impermeable transversal plate, communicated to independent lower openings into which removable flasks (10ml) containing the stimuli were attached, was used. Either an undisturbed or a disturbed adult of *T. infestans* or *R. prolixus* was inserted in each flask. In this way, stimuli (the volatiles emitted by disturbed or undisturbed adults) entered by diffusion into each of the two parts of the sub-chamber and continued to diffuse up to the arena through the small holes, where one experimental insect per assay was released. Filter paper with holes matching the floor holes served as a substrate and avoided possible chemical contamination between assays, as it was changed every time new bugs were released. The temperature of the experimental room was set to 25±1º C before the beginning of each assay with an electric fan heater, which was turned off before the start of the experiment. Relative humidity varied between 30% and
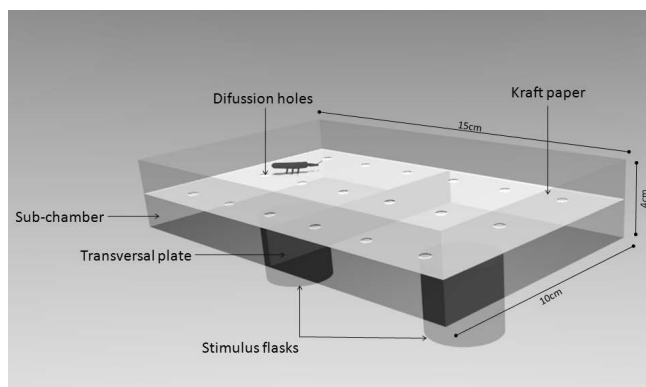

Figure 1. Spatial dual-choice walking olfactometer. The activity and odor preference of larvae of *T. infestans* or *R. prolixus* were analyzed when undisturbed and/or disturbed adults of *T. infestans* were inserted in the stimulus flasks. Activity levels were assessed by counting the number of virtual lines crossed by larvae during the experimental time (4 min). Odor preference was quantified by calculating the proportion of time spent on each side of the arena.

60%. No considerable changes of temperature or humidity were registered in the room during the experimental time. Experiments were performed during the first hours of the scotophase of the insects (one to five h after lights were turned off), as to match their maximal activity period.

In each individual assay, one adult of *T. infestans* or *R.prolixus* was inserted in each of the two stimulus flasks. One of the adults was left undisturbed and the other was disturbed as to promote the AP release. The disturbance was achieved by grabbing its legs with forceps for 30 s. Previous observations revealed that this practice is enough for a complete release of the content of the exocrine glands (Palottini, personal observation). Once disturbed (or not), the flask was attached to the experimental arena and one larva (*T. infestans* or *R.*

prolixus) was placed in the middle of the upper arena and left covered with an inverted flask over it. After 1 min of context familiarization and odor diffusion, the larva was carefully released and its behavior was registered for 4 min by means of a video camera connected to a digital recorder. Control series were performed leaving undisturbed adults in both stimulus flasks. The position of the stimulus was alternated between assays.

For each assay, the number of lines (four vertical and two horizontal lines were drawn over the transparent lid of the arena) crossed by each larva over 4 min was counted and registered as a measure of its activity level. Differences in activity levels between species (*T. infestans* and *R. prolixus*) and treatments (presence or absence of odors released by disturbed adults) were compared by means of a one-way ANOVA. The time spent on each side of the arena (a line was drawn vertically in the middle of the arena) was registered as a measure of its spatial preference (in relation to the position of the odors emitted by disturbed adults). A preference index (PI) ranging from -1 to 1 was calculated as $PI=(120-T)/120$, where T is the time in seconds spent at the side of the arena where the stimulus was added. In control series, T is the time in seconds spent on one side of the arena chosen randomly. PIs near -1, 0, or 1 indicate repellence, random distribution, or attraction to the added stimulus, respectively. Deviations from a random distribution (i.e., PI=0) of the larvae over the arena were assessed for each species and treatment by means of One Sample T-Tests. A total of 40 replicates was achieved for each treatment.

Experiment 2: Flushing-out activity from artificial shelters

The flushing-out activity of 4th instar larvae of *T. infestans* and 5th instar larvae of *R. prolixus* caused by individual odors present in the AP released by disturbed *T. infestans* and/or *R. prolixus* adults was assessed in pseudo-natural conditions. Two bricks (6x26x10 cm) were placed side by side in an experimental glass arena (40x30x20 cm) with filter paper as
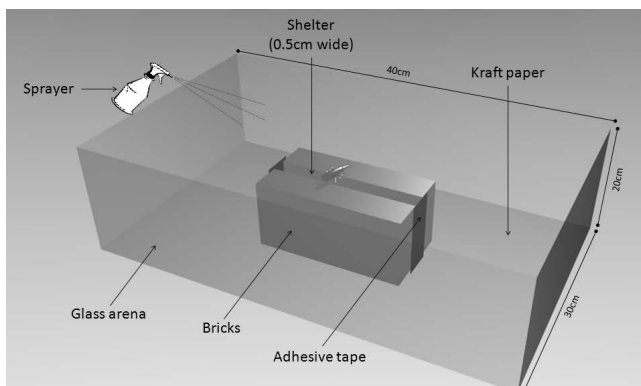

Figure 2. Artificial shelters and spraying method. Different compounds released by disturbed adults of *T. infestans* and/ or *R. prolixus* were sprayed over shelters bearing a single larva of *T. infestans* or *R. prolixus* inside. The exit of larvae was visually registered during the experimental time (2 min) and the number of assays in which larvae were dislodged was quantified.

substrate (Figure 2). The space between the bricks (ca. 0.5 cm wide) served as shelter for the insects. The lateral openings were permanently occluded with adhesive tape, while a mobile silicone stick (0.7 cm diameter) was used to close the upper opening (the only exit for insects during assays). The temperature inside the crack was set to 27±2º C before the beginning of each assay with an electric fan heater, which was turned off before the beginning of the experiment. Relative humidity varied between 30% and 70%. No considerable changes of temperature were registered inside the artificial shelter during the experimental time.

Experiments were performed during the first hours of the photophase of the insects, in the presence of diurnal natural illumination, as to test the dislodgement capacity of the compounds in moments in which active search by sanitary agents would be performed. In each assay, one bug was released inside the shelter via the upper opening of the device. Once inside, the opening was covered with the silicone stick to prevent the bug from escaping before the beginning of the test. After 1 min of familiarization with the context, the silicone stick was removed and one of the tested substances was sprayed over the upper side of the bricks using a commercially available sprayer. The more representative compounds released by disturbed adults of *T. infestans* and/ or *R. prolixus* were tested. Isobutyric acid (Fluka, Germany, 98% purity) was the first choice as it has been described as the main volatile released by disturbed adults of both species, and its effect on the escape behavior of triatomines has already been shown for *T. infestans* (Manrique et al. 2006). Acetic acid (Anedra, Argentina, 98% purity), isobutyl alcohol (Sigma, Germany, 98% purity), 3-methyl-1-butanol (Sigma, Germany, 98% purity), 3-pentanone (Sigma, Germany, 98% purity), 2-methyl-1-butanol (Sigma, Germany, 99% purity), and 3-pentanol (Fluka, Switzerland, 99.5% purity) were also tested. Isobutyric acid and acetic acid are released by disturbed adults of both *T. infestans* and *R. prolixus* (Juarez and Brenner 1981, Cruz-López et al. 1995, Guerenstein and Guerin 2004, Manrique et al. 2006). Isobutyl alcohol, 3-methyl-1-butanol, 3-pentanone, and 2-methyl-1-butanol are released only by disturbed adults of *T. infestans* (Manrique et al. 2006, Cruz-López et al. 1995, Guerenstein and Guerin 2004). Although 3-pentanol was not detected in the blends released by disturbed adults of either species, it is present in metasternal glands of *T. infestans* (Manrique et al. 2006). Four concentrations of each substance diluted in distilled water were applied: 0.1%, 0.5%, 2%, or 10%. We decided to test low concentrations of all compounds as higher doses might be deleterious for the insects and probably noxious for other animals. In addition, the range of concentrations selected includes the amount of isobutyric acid present in the adults Brindley's glands (Palottini, personal observation), although we do not know the amount of the other compounds present in these glands. Three puffs (0.8±0.1 ml each) were delivered per assay at an angle of 65° with a vertical line and a distance of 25 cm from the opening. Control series consisted of either no spraying, three puffs of distilled water (negative control), or three puffs of Tetramethrin 0.2% (positive control). Only one concentration of one compound was tested for each

larva and then discarded. After spraying, the occurrence of a flushing-out within the following 2 min was registered. An insect was counted as flushed-out if its whole body was visible outside the crevice. A total of 24 replicates was carried out for each substance and concentration. We then calculated the proportion of the assays in which larvae walked out from the shelters for each treatment and compared it with a control series (distilled water and Tetramethrin 0.2%) by means of Chi-Square Tests of homogeneity.

After each assay, substrate (filter paper) and bricks were replaced by new ones and each experimental arena was gently cleaned with distilled water and ethanol (96%). The experimental area was then ventilated for 10 min with an electric fan. The mortality of the bugs was monitored during and up to 1 week after the assays, not finding differences across all treatments, except for tetramethrin 0.2%, in which a high mortality was observed (data not shown).

RESULTS

Experiment 1: Activation and repellence caused by the AP of adults

The activity levels of larvae of both species were modulated differently by odors released by disturbed adults of the same or the other species (Figure 3; one-way ANOVA, p=0.00001). Tuckey post-hoc comparisons showed that although larvae of *T. infestans* were more active than larvae of *R. prolixus*, the presence of the blend released by disturbed adults of *T. infestans* and *R. prolixus* did not change their activity levels ('undisturbed' vs 'disturbed'; p<0.05 in all cases). Additionally, activity levels of larvae of *T. infestans* seemed to decrease in the presence of disturbed or undisturbed adults of *R. prolixus*.

When we analyzed changes in the spatial preference over the arena, the statistical analyses (one sample T-tests) showed that larvae of both species exhibited a random distribution over the experimental arena when both stimulus-adults were kept undisturbed (control series; Figure 4; p>0.05 in all cases). Larvae of *T. infestans* avoided the side of the arena where an adult of the same species (p=0.02) or of *R. prolixus* (p=0.007) was mechanically disturbed. Conversely, larvae of *R. prolixus* remained unaware of the presence of the blends released by adults of both species (p>0.05 in both cases).

Experiment 2: Flushing-out activity caused by single compounds of the AP

Larvae of both species exhibited a null spontaneous flushing-out activity when no spraying was delivered, an intermediate frequency when distilled water was added, and the highest proportions of bugs walking out the refuges when tetramethrin 0.2% was sprayed (Figures 5 and 6).

For *T. infestans*, significantly more bugs came out from the refuges when tetramethrin 0.2% was sprayed than when distilled water was poured (50% and 4.17%, respectively, p=0.0003). Similarly, in *R. prolixus*, tetramethrin 0.2% flushed out significantly more larvae than distilled water (62.5% and 12.5%, respectively, p<0.0003).

When the different concentrations of the tested substances were delivered, larvae of *T. infestans* and *R.*

prolixus behaved dissimilarly. For *T. infestans* (Figure 5), only 0.5% of isobutyric acid (p=0.04) and 2% of 3-pentanol (p=0.04) provoked a flushing-out activity significantly higher than the distilled water. A similar but not significant trend was observed when 10% of 3-pentanone (p=0.07), 0.5% of acetic acid (p=0.08), 2% of 2-methyl-1-butanol (p=0.08), or 10% of 3-pentanol was sprayed (p=0.08). The rest of the concentrations/compounds elicited responses similar to those elicited by the distilled water (all cases p>0.1).

On the other hand, the larvae of *R. prolixus* (Figure 6) exhibited a significant increase of flushing-out activity when 0.1% (p=0.04) or 0.5% (p=0.0001) of 3-methyl-1-butanol were sprayed as compared to distilled water. Particularly, 0.5% of 3-methyl-1-butanol was the only compound/concentration that elicited a behavior similar in intensity as tetramethrin 0.2% (p=0.89). Also, 2% of isobutyric acid seemed to flush-out larvae of this species, although not significantly (p=0.08) (Figure 6). The rest of the compounds did not differ significantly from distilled water (all cases p>0.1)

DISCUSSION

Triatomines are vectors of Chagas disease in Latin America. Vector control is actually the only feasible way to reduce Chagas disease transmission, as to date no vaccine exists against the protozoan *T. cruzi*, the causative agent of this disease. *T. infestans* and *R. prolixus* are among the main vectors of this disease in South America and Central America, respectively (Schofield 1994). Due to economic constraints and to the ecological impact of applying insecticides, sanitary control strategies include the spraying of pyrethroids or the appliance of knock-out strategies only in infested houses. The quick identification of infested dwellings is thus relevant for an adequate strategy functioning. Manual detection by sanitary agents is almost exclusively done during daylight hours, when triatomines are in an akinetic state inside their shelters. Tetramethrin 0.2% is commonly used to make these insects flush-out from their shelters. Sometimes the capture of healthy insects is necessary for the concretion of posterior analyses like, for example, xenodiagnosis or genetic studies. However, the toxicity of tetramethrin (EPA 2010) does not allow most of these post-capture studies. The application of the different compounds and concentrations did not change the larval survival for at least one week (data not shown). Contrarily, when tetramethrin 0.2% was sprayed, an almost immediate deleterious effect was observed over the major part of the assayed insects. Similarly, a relatively high mortality of bugs was obtained by other authors (Pinchin et al. 1981) who were able to flush-out *Panstrongylus megistus* using pyrethroids but did not succeed in dislodging bugs with isobutyric acid. To our knowledge, this is the first report in which triatomines are obliged to walk-out from their shelters using natural odors released by their conspecifics. Although we are aware that the amounts of insecticide actually sprayed during control activities are enormous, the reduction or even the avoidance of the application of tetramethrin 0.2% for the identification of infested dwellings should not be negligible.

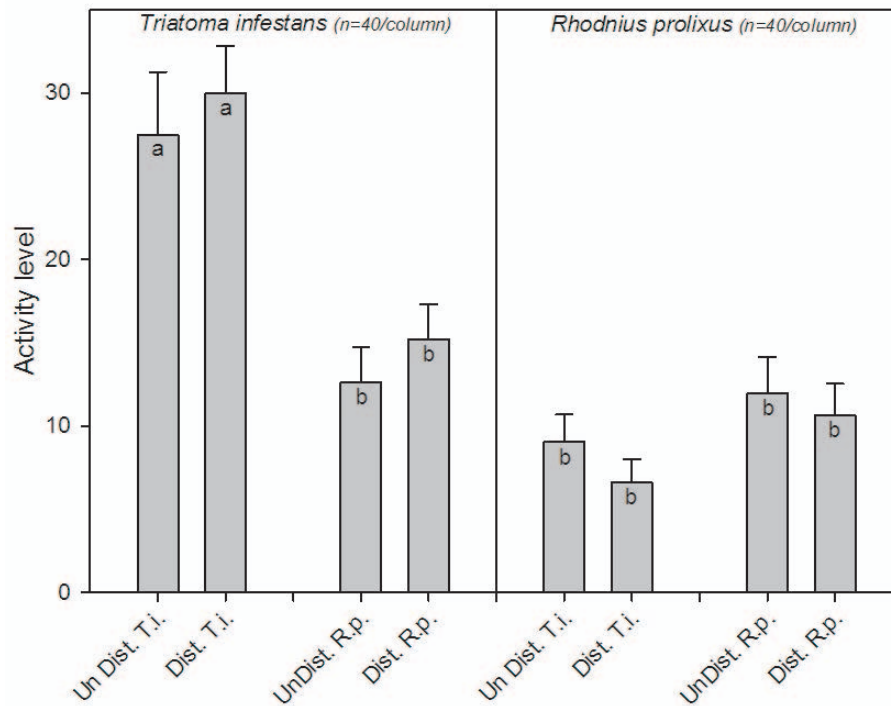Flushing-out activity, defined in this work as the behavior

Figure 3. Activity levels of larvae of *T. infestans* and *R. prolixus* confronted by volatiles released by disturbed adults. Larvae of *T. infestans* were more active than those of *R. prolixus*. However, no changes in activity in any of both species were observed when odors released by disturbed adults were added to the stimulus flask. A total of 40 replicates was achieved for each treatment. UnDist: undisturbed adults. Dist: disturbed adults. *T.i.*: *Triatoma infestans*. *R.p.*: *Rhodnius prolixus*. Different letters denote significant differences (One-Way ANOVA).
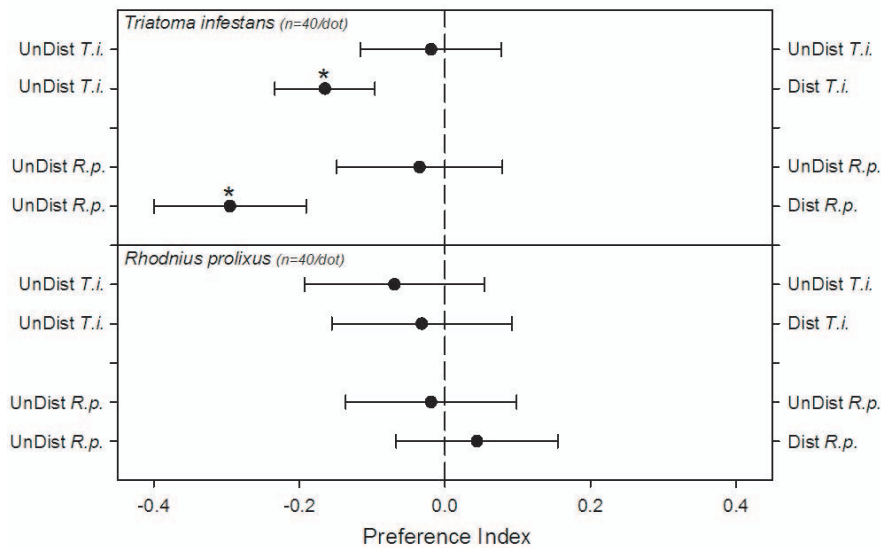

Figure 4. Odor preference of larvae of *T. infestans* and *R. prolixus* confronted by volatiles released by disturbed adults.. Larvae of *T. infestans* were repelled by the odors emitted by disturbed adult *T. infestans* and *R. prolixus*. Contrarily, larvae of *R. prolixus* did not modify their spatial distribution with the presence of odors released by adult *T. infestans* and *R. prolixus*. UnDist: undisturbed adults. Dist: disturbed adults. *T.i.*: *Triatoma infestans*. *R.p.*: *Rhodnius prolixus*. A total of 40 replicates was achieved for each treatment. Asterisks denote significant differences (One Sample T-Test).
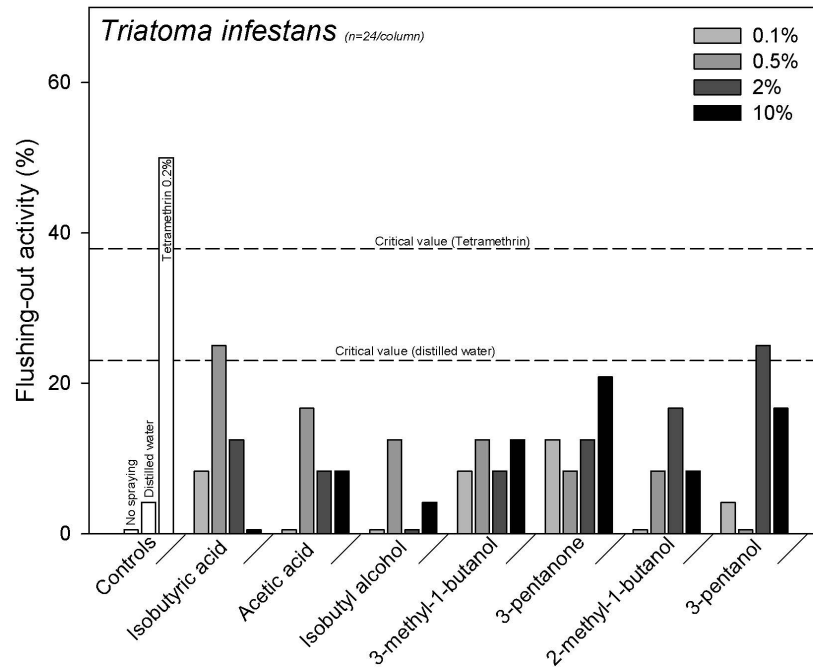
Figure 5. Flushing-out activity of larvae of *T. infestans* sprayed with volatiles from the AP. Both 0.5% of isobutyric acid (Chi-Square test, p=0.04) and 2% of 3-pentanol (p=0.04) provoked higher flushing-out activities than distilled water. No concentration/compound dislodged as much larvae as tetramethrin 0.2% (p>0.05 in all cases). Lower and upper dashed lines show critical values for statistical comparison (Chi-Square test) with distilled water or tetramethrin 0.2%, respectively. A total of 24 replicates was achieved for each treatment.
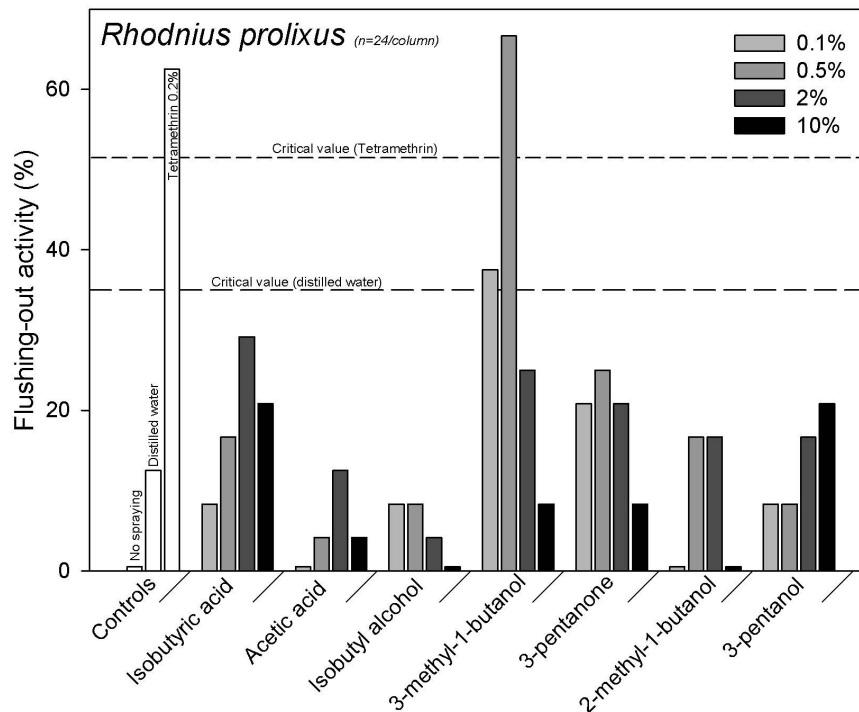

Figure 6. Flushing-out activity of larvae of *R. prolixus* sprayed with volatiles from the AP. Only 0.1% and 0.5% of 3-methyl-1-butanol (Chi-Square test, p=0.04 and p=0.0001, respectively) elicited higher flushing-out activities than distilled water. Also, 0.5% of 3-methyl-1-butanol achieved a dislodgement effect similar as tetramethrin 0.2% (p=0.89). Lower and upper dashed lines show critical values for statistical comparison (Chi-Square test) with distilled water or tetramethrin 0.2%, respectively. A total of 24 replicates was achieved for each treatment.

of exiting the shelters, is not only dependent on the locomotion activity levels of bugs, but also on the directionality of the displacement. The more active they become, the higher the probability of exiting the shelter, even if there is no directionality of the movement. Moreover, if triatomines try to escape from the source of the AP, the higher the probability to get out from the shelter would be. In the spatial preference box, we were able to analyze the two behaviors of activation and orientation separately, for larvae stimulated with blends emitted by disturbed *T. infestans* or *R. prolixus* adults. We found in both species that basal activity levels were not increased by the presence of the AP of *T. infestans* or by odors from disturbed adults of *R. prolixus*, but that larvae of *T. infestans* actively escaped from the odor source, suggesting that the alarm pheromone might be more relevant for the life of a domestic species (as *T. infestans*) than for a sylvatic one (as *R. prolixus*). These results suggest that some volatiles or mixtures of individual odors present in the AP of adults might be good candidates to act as natural dislodgement agents for some triatomine species. It is worth noting that Manrique et al. (2006) found an increased locomotor activity promoted by odors released by disturbed *T. infestans* adults, while in this work no differences in activity levels were found for any species. However, a different experimental setup was used in this work.

Compounds tested along this work are part of the chemical communication used by triatomines. In such cases, the concentration and even the ratio among the compounds are relevant factors as to specifically transfer a message. That is probably why we obtained responses at certain concentrations and not at others. However, we cannot strictly discard the occurrence of a sensory adaptation at different central nervous system levels.

Previous studies showed that 3-methyl-1-butanol is present in Brindley's glands and in the blend released by disturbed adults of *T. infestans* (Manrique et al. 2006) and not in *R. prolixus* (Rojas et al. 2002, Guerenstein and Guerin 2004). Strikingly, we found that the higher flushing-out activity of *R. prolixus* larvae was evoked by this compound. Unexpectedly, although isobutyric acid is the main component of the blend released by disturbed adults of both species, higher flushing-out activities (as compared to distilled water) were not observed for *R. prolixus*. The results presented here do not allow us to speculate about possible mechanisms and/or reasons involved in the modulation of the flushing-out response of triatomines.

This work allows us to speculate about the importance of finding a dislodging agent to use as an alternative to tetramethrin 0.2% but lacking its deleterious effect over bugs and, what is probably more important, with low impact over the environment. However, our results still prevail as a first step, as other series of compounds and/or concentrations or mixtures could be tested in the future. We show here that natural non-insecticidal substances can be used to facilitate the detection of triatomines in their natural environments. In this way, if further investigation is performed to analyze other substances in various concentrations and combinations, a very useful and much safer tool could be developed to be used in field campaigns for monitoring and controlling the Chagas disease vectors.

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