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A CO_2-Free Synthetic Host–Odor Mixture That Attracts and Captures Triatomines: Effect of Emitted Odorant Ratios

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Abstract

Triatomines, vectors of Chagas Disease, are hematophagous insects. Efforts have been made to develop synthetic attractants based on vertebrate odor—to lure them into traps. However, because those lures are not practical or have low capture efficiency, they are not in use in control programs. Therefore, more work is needed to reach a practical and efficient odor lure. Recently, a three-component, CO_2-free, synthetic blend of vertebrate odor (consisting of ammonia, L-(+)-lactic acid, and hexanoic acid), known as Sweetscent (Biogents AG, Regensburg, Germany), was shown to attract and capture triatomines in the laboratory. In this study, using a trap olfactometer and an odor blend with constituents similar to those of Sweetscent (delivered from low-density polyethylene sachets) we found that the odorant ratios of the mixtures have a strong effect in the capture of triatomines. The blend with the most efficient combination of odorant ratios evoked ca. 81% capture in two relevant triatomine species. In the case of the most effective odor mixtures, we measured the odor mass emission for the three components of the mixture and therefore were able to estimate the odorant ratios emitted that were responsible for such a high capture performance. Thus, in those mixtures, pentanoic acid was the main component (ca. 65 %) followed by ammonia (ca. 28%) and, L(+)-lactic acid (ca. 7 %). Our results are encouraging as efficient, practical, and cheap odor baits to trap triatomines in the field would be within reach. The odor-delivery system used should be improved to increase stability of odor emission.

Key words: Chagas disease, hematophagous insect, attractant, odor, bait.

The triatomines (*Hemiptera*: *Reduviidae*) are insects that feed on the blood of vertebrates. They are vectors of the protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease. *Rhodnius prolixus* Stal and *Triatoma infestans* Klug are two of the epidemiologically most important species. According to WHO (2015), vector control is the most effective method to prevent Chagas disease.

Due to their epidemiological importance, efforts have been made to develop a number of devices to attract triatomines to actively detect and capture them. Those devices contain a bait which consists of a live host (e.g., Noireau et al. 2002), another natural source of odor (e.g., Guerenstein et al. 1995), a CO_2-containing synthetic host–odor blend plus an artificial source of heat (Ryelandt et al. 2011), or an aldehyde (Rojas de Arias et al. 2012). However, because those devices are not practical or have low capture efficiency, they are not in use in control programs. Recently, a CO_2-free commercial mosquito attractant consisting of a synthetic mixture of human skin odors showed to attract and capture triatomines efficiently, demonstrating that a relatively cheap, simple, practical, and efficient odor bait is a reachable aim (Guidobaldi and Guerenstein 2013).

The effectiveness of odor-baited devices for sampling, surveillance, and control of harmful insects is strongly influenced by the odor-delivery system used (Cork 2004, Torr et al. 1997). Low-density polyethylene (LDPE) sachets have proved to be useful as odor-delivery systems because odors are released at predictable rates over relatively prolonged periods of time (Mukabana et al. 2012, Torr et al. 1997). The ratios at which the components of an odor mixture are delivered are very important to determine their behavioral effect (e.g., attraction, Christensen and Hildebrand 1997, Mukabana et al. 2012). The odor delivering system determines not only the dynamics of emission of individual odorants but also the ratio of emitted blend constituents and consequently the attraction efficiency of the odor mixture (Mukabana et al. 2012). In order to standardize an LDPE odor-delivery sachet system, it is necessary to take into account the following variables: sachet size, thickness of the LDPE sheet, volume

of the odor solution (or pure odor), solvent used (if any), and concentration of the solutions. Using a three-component CO_2-free synthetic host–odor mixture and LDPE sachets as odor-delivery system, we report here the effect of changing the volumes of the odor solutions on the emitted odorant ratios and its result on the attraction and capture of triatomines. Moreover, we estimate the odorant ratios emitted by the mixtures that evoke very high capture of triatomines.

Materials and Methods

Insects

Third-instar larvae of *R. prolixus* and *T. infestans* were used. The insects were obtained from our colony, originally from insects provided by the Servicio Nacional de Chagas (Córdoba, Argentina). The colony was fed once every 2 wk on hens, and maintained in the laboratory at 27 ± 1°C under a 12:12 (L:D) h illumination regime. For experiments, insects were fed as second-instar larvae and, upon moulting, were starved for 45–60 d for *R. prolixus,* and 30–40 d for *T. infestans* (values chosen according to data obtained by Guerenstein and Núñez 1994 on feeding motivation and resistance to starvation).

Olfactometer Tests

Experimental Design. To study the attraction evoked by different odorants and test the ability of those odorants to evoke capture-related behavior, insects were tested individually using a dual-choice olfactometer (Guerenstein et al. 1995, Guidobaldi and Guerenstein 2013). This device consists on a polystyrene rectangular arena (11.5-cm length, 7-cm thickness, and 1-cm height) with a glass lid and three holes that are each connected to a plastic tube from below (Fig. 1). The three tubes have cloth mesh at the bottom. On one side of the arena, one of the tubes (the release-tube) contains the insect at the beginning of the experiment. Insects could reach the surface of the arena by climbing onto a piece of cardboard inside the tube. On the opposite side of the arena, two capture-tubes (emanating control and test odor) are placed. The insects can let themselves drop into the capture-tubes (triatomines let themselves drop when sensing host–odor from below; Guerenstein et al. 1995) and cannot escape from them (unpublished results) as the piece of cardboard within does not reach the surface of the arena. The olfactometer had a paper septum (3.5-cm length, 1-cm thickness, and 0.5-cm height) on the arena, between the capture-tubes, from the wall opposite the release-tube up to the center of the arena (Fig. 1).

Below each, the test and control capture-tubes, a 1-liter open glass bottle containing the odorant source or its control was placed. The position of the test and control bottles was switched in successive trials. Air was pulled from the bottom of the release-tube at 6 ml/min and, therefore, an airstream from the odorant source and its control constantly reached the release tube via the capture-tubes and the arena (Guidobaldi and Guerenstein 2013). Tests were carried out at 27 ± 1°C, in darkness, from 17:00 h to 8:00 h of the following day. Experiments started at the beginning of the insects scotophase as in Guidobaldi and Guerenstein (2013). Before the experiment began, an insect was individually placed in the release-tube. Insects were allowed 5 min to habituate to the experimental situation and during this time the exit of the release-tube was closed. An experiment started when the exit was opened so that the insect was free to leave the release-tube. In the following morning, the number of insects in the capture-tubes was recorded.

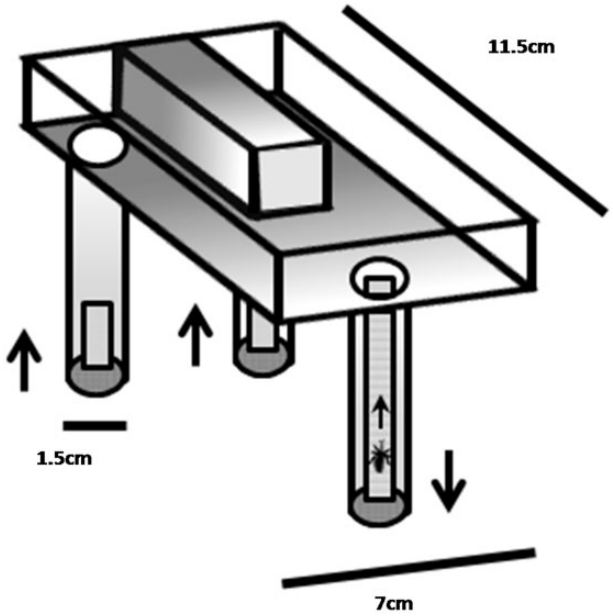

Fig. 1. Dual-choice trap olfatometer consisting on a polystyrene rectangular arena with a glass lid and three holes that are each connected to a plastic tube from below: one release-tube, and two capture-tubes. Thick arrows indicate the direction of the airstream. Thin arrow indicates the trajectory of the bug to exit the release-tube. Modified from Guidobaldi and Guerenstein (2013).

Our experimental design consisted of a battery of 12 olfactometers, so that 12 insects could be simultaneously tested in a single night. This allowed us to simultaneously test all stimuli and controls included in the experiments. After each experimental night, olfactometers were disassembled, all material was thoroughly washed and the cardboards pieces were discarded.

Stimuli. We tested a series of three-component CO_2-free synthetic host–odor blends, which contained ammonium hydroxide (dissolved in water, 29 ± 1%, Cicarelli, San Lorenzo, Santa Fe, Argentina), L-(+)-lactic acid (>98%, Sigma, St. Louis, Mo., USA), and pentanoic acid (>99%, Aldrich, Milwaukee, Wis., USA). The solvent was distilled water in all cases. The three compounds used are the same or similar to those included in Sweetscent (ammonia, lactic, and hexanoic acid), which was tested in Guidobaldi and Guerenstein (2013).

In a preliminary experimental series, odorants at different ratios were delivered from filter paper strips (5 by 1 cm, one for each compound). In positive-control experiments for this experimental series a mouse (BALB-C strain) was placed below the test capture tube whereas in negative-control experiments a filter paper strip with water (solvent) was placed in each capture tube. Experiments using filter paper as odorant dispensers showed the critical importance of odorant ratios to capture *R. prolixus*.

Taking into account the results from those preliminary experiments, odor blends using the same three odorants were tested (Table 1) in LDPE (20-μm thickness, Jumboplast S.R.L., Santa Fe, Argentina) sachets, aiming at increasing the duration, and stability of the odorant emission of the bait. For this, diluted solutions of the three compounds were prepared to reach the values shown in Table 1. Each odorant was presented in an individual LDPE sachet of a fixed size depending on the compound held: 3 by 5 cm for lactic and pentanoic acid, and 6 by 5 cm for ammonium hydroxide. Thus, for lactic acid and ammonium hydroxide sachet size, thickness of

Table 1. Synthetic blends tested, dispensed from individual LDPE sachets

Blend ID	Constituent	Volume (µl)	Concentration (µg/µl)	Total mass (mg)
LDPD1	L-(+)-lactic acid	200	50	10
	Pentanoic acid	400	46.95	18.78
	Ammonium hydroxide	2000	58	116
LDPD2	L-(+)-lactic acid	400	50	20
	Pentanoic acid	400	46.95	18.78
	Ammonium hydroxide	500	58	29
LDPD3	L-(+)-lactic acid	400	50	20
	Pentanoic acid	400	46.95	18.78
	Ammonium hydroxide	4000	58	232
LDPD4	L-(+)-lactic acid	200	50	10
	Pentanoic acid	400	46.95	18.78
	Ammonium hydroxide	1000	58	58
LDPD5	L-(+)-lactic acid	200	50	10
	Pentanoic acid	400	46.95	18.78
	Ammonium hydroxide	500	58	29

the LDPE sheet, solvent, and concentration of the solution were kept constant whereas the volume of the odor solution varied. In the case of pentanoic acid all variables (including volume of solution) were constant (see Table 1). Odorant emission data as well as behavioral data suggest that different volumes of solution result in different odorant emissions (see "Results" section).

The sheets were carefully thermally sealed. Positive and negative control experiments were as described above except that in negative controls the solvent (water) was inside a LDPE sachet.

Estimation of Odorant Emission

The mass of each of the three odorants emitted from each of the three LDPE sachets of two of the blends was determined at 25 ± 1°C. L-(+)-lactic and pentanoic acid were analyzed by combining gravimetric measures with gas chromatography analysis. Ammonium hydroxide was analyzed by combining gravimetric measures with colorimetric determination using Nessler tinction.

For the chromatographic analysis, (A) LDPE sachets containing the odorant solutions were weighted after different periods of time, after which a 100-µl sample of its content was taken. (B) This sample was diluted 1/10 in absolute ethanol. From this dilution, 1 µl was manually injected in a gas chromatograph (Agilent 7820 A), using a split/splitless injector, an FID detector, and a capillary column DB-WAX (30-m long, 0.25-mm i.d., Agilent technologies). Chromatographic settings were injector at splitless mode, carrier flow constant at 1 ml/min, injector temperature: 250°C, 11.567 psi; FID temperature: 300°C, flux: $H_2 = 30$ ml/min, Air = 400 ml/min; oven: initial column temp.: 40°C, temp. rising rate: 10°C/min to 75°C, temp. then rising rate: 15°C/min to 200°C constant. Difference between signal integration at different times is related to the concentration of injected sample.

For colorimetric determination with Nessler tinction, the initial steps are the same for part (A) chromatographic analysis. From that point, the odorant solution sample taken from the LDPE sachet was diluted (to ensure working into the technique range) and the protocol suggested by the manufacturer (Hach, Loveland, CO, USA) was followed. Absorbance was measured at 425 nm using a UV–VIS spectrophotometer (Metrolab 330).

Ten repetitions were made for all determinations. Using gravimetric measure plus chromatographic or colorimetric determination per each emission time period allowed the estimation of the odorant mass emitted per period.

Data Analysis

By quantifying the number of insects in the capture-tubes, the percentage total capture (%TC) was assessed. The %TC was defined as the total number of insects in the capture-tubes (test + control) over the total of insects tested. This parameter was statistically analyzed using contingency tables (Sokal and Rohlf 2009). Thus, we compared the odorant-induced %TCs with those of the basal %TCs that were obtained in the negative control experiments. We also compared %TC between treatments. We also defined the percentage of oriented capture (%OC) as the percentage of insects captured in the test tube respect to the total captured. This was analyzed with one-tail binomial test (Zar 1999).

RESULTS

Olfactometer Tests

In preliminary tests with three-component synthetic blends dispensed from filter paper strips the best mixture (2 mg L-(+)-lactic acid, 1.9-mg pentanoic acid, and 4.8-mg ammonium hydroxide) evoked 31.3% TC ($N = 32$), capturing significantly more insects than the negative control (7.5 %TC, $N = 50$, $P < 0.05$) but less than a mouse (68.0 %TC, $N = 50$, $P < 0.0001$).

Tests with Three-Component Synthetic Blends Dispensed from LDPE Sachets. The percentage of *R. prolixus* caught in the capture-tubes (%TC) in negative control experiments was 17.8% ($N = 90$) whereas a mouse evoked 74.2 %TC ($N = 89$, Fig. 2). The %TC evoked by the different blends tested in *R. prolixus* is shown in Fig. 2 and the statistical analysis is summarized in Table 2. Overall, significant differences were found between treatments—stimuli—($\chi^2_{(6,346)} = 74.7$, $P < 0.0001$). The mixtures LDPE 4 and LDPE 5 evoked the highest %TC, not different from that evoked by a mouse. The %OC ranged from 92 to 100% and were significant in all cases ($P < 0.05$).

The blends LDPE 3, 4, and 5 were also tested in *T. infestans*. Mouse was again used as positive control evoking 92.6 %TC ($N = 54$) whereas the negative control evoked 21.7 %TC ($N = 60$). The %TC evoked by the different blends tested in *T. infestans* is shown in Fig. 3 and the statistical analysis is summarized in Table 3. Overall, significant differences were found between treatments—stimuli—($\chi^2_{(4,221)} = 72.5$, $P < 0.0001$). As in the case of *R. prolixus*, the mixtures LDPE 4 and LDPE 5 evoked the highest %TC, not

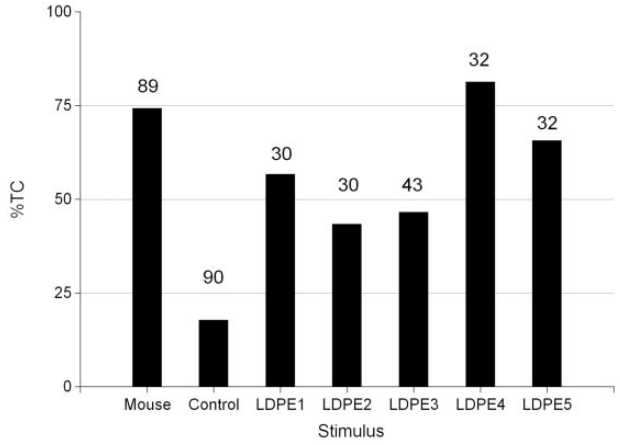

Fig. 2. %TC evoked in *R. prolixus* by synthetic blends assayed in trap olfactometers using LDPE sachets as odor dispensers. Mouse is the positive control and the different synthetic host–odor blends are named with consecutive numbers. Numbers above bars indicate N for each stimulus tested.

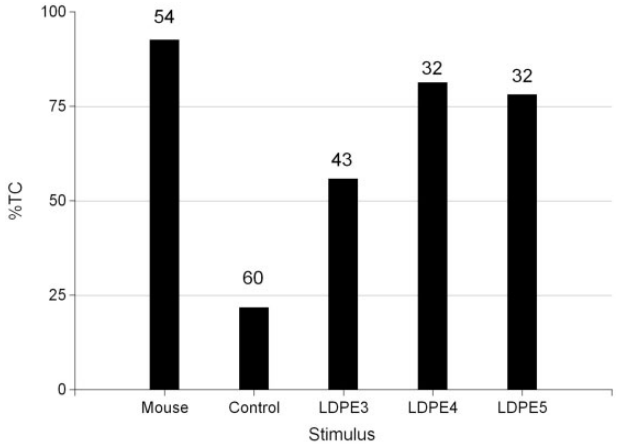

Fig. 3. %TC evoked in *T. infestans,* by synthetic blends assayed in trap olfactometers, using LDPE sachets as odor dispensers. Mouse is the positive control and the different synthetic host–odor blends are named with consecutive numbers. Numbers above bars indicate N for each stimulus tested.

Table 2. Statistical analyses of %TCs for *R. prolixus*. *P* values of pairwise comparisons indicating statistically significant differences are shown in bold.

%TC comparison	χ^2 test
Mouse–Control	$\chi^2_{(1,179)} = 57.30$; $\mathbf{P < 0.0001}$
LDPE1–Control	$\chi^2_{(1,120)} = 17.07$; $\mathbf{P < 0.0001}$
LDPE2–Control	$\chi^2_{(1,120)} = 8.02$; $P = 0.0046$
LDPE3–Control	$\chi^2_{(1,133)} = 12.17$; $\mathbf{P = 0.0005}$
LDPE4–Control	$\chi^2_{(1,122)} = 42.13$; $\mathbf{P < 0.0001}$
LDPE5–Control	$\chi^2_{(1,122)} = 25.58$; $\mathbf{P < 0.0001}$
LDPE1–Mouse	$\chi^2_{(1,119)} = 3.25$; $P = 0.0713$
LDPE2–Mouse	$\chi^2_{(1,119)} = 9.55$; $\mathbf{P = 0.0020}$
LDPE3–Mouse	$\chi^2_{(1,132)} = 9.76$; $\mathbf{P = 0.0018}$
LDPE4–Mouse	$\chi^2_{(1,121)} = 0.65$; $P = 0.42$
LDPE5–Mouse	$\chi^2_{(1,121)} = 0.85$; $P = 0.36$

According to Bonferroni correction, $P < 0.0045$ for significance in pairwise comparisons.

Table 3. Statistical analyses of %TCs for *T. infestans*. *P* values of pairwise comparisons indicating statistically significant differences are shown in bold.

%TC comparison	χ^2 test
Mouse-Control	$\chi^2_{(1,114)} = 57.83$; $\mathbf{P < 0.0001}$
LDPE3 -Control	$\chi^2_{(1,104)} = 11.97$; $\mathbf{P = 0.0005}$
LDPE4 -Control	$\chi^2_{(1,92)} = 30.34$; $\mathbf{P < 0.0001}$
LDPE5 -Control	$\chi^2_{(1,92)} = 27.44$; $\mathbf{P < 0.0001}$
LDPE3 -Mouse	$\chi^2_{(1,97)} = 17.9$; $\mathbf{P < 0.0001}$
LDPE4 -Mouse	$\chi^2_{(1,86)} = 2.52$; $P = 0.113$
LDPE5 -Mouse	$\chi^2_{(1,86)} = 3.77$; $P = 0.052$

According to Bonferroni correction, $P < 0.007$ for significance in pairwise comparisons.

different from that evoked by a mouse. The %OC ranged from 82 to 85% and were significant in all cases ($P < 0.05$).

Estimation of Odorant Emission

The odorant emission during different periods of time from LDPE sachets containing different solutions is shown in Table 4. The measurements correspond to the two blends that evoked the highest %TC (LDPE 4 and 5). During the first 2 h pentanoic acid was the main constituent of the emitted blends, followed by ammonia, and lactic acid. However, the proportions changed after that period of time.

Discussion

Because the most effective way to control Chagas disease is through the control of its vector (WHO 2015), a practical, cheap, and sensitive tool for early detection of low-density populations of triatomines is needed (Tarleton et al. 2007). A highly attractive baited field trap could represent a powerful tool for a cost-effective and efficient control strategy.

It has been established that triatomines are attracted by the natural, complex, odor blend emanating from their hosts (e.g., Ortiz and Molina 2010) and by single synthetic host odorants, including CO_2 (e.g., Barrozo and Lazzari 2004, Guerenstein and Guerin 2001, Otálora-Luna et al. 2004). It has also been shown that a synthetic host–odor blend designed for mosquitoes (Sweetscent), that consists of ammonia, hexanoic acid, and L(+) lactic acid, evokes attraction and trapping in *R. prolixus* and *T. infestans* when tested in dual choice trap-olfactometers (Guidobaldi and Guerenstein 2013). Here, we used a blend with constituents similar to those of the mixture tested in Guidobaldi and Guerenstein (2013). One of our aims was to study the effect of the odorant-constituents ratios on the attraction and capture of triatomines. Moreover, we asked if the attraction and trapping evoked by that mixture is significantly higher using a certain combination of odorant–source ratios. We also aimed at knowing if the LDPE sachets could represent useful odorant delivery devices for the use of this blend in triatomines. Finally, we aimed at estimating the odorant emission and emitted ratios that evoked the highest attraction and trapping.

We suggest that, for the odor-mixture tested, LDPE sachets are more efficient odorant-delivery systems for capturing triatomines than filter papers as the maximum capture obtained with the papers (after testing 14 combinations of odorant ratios; data not shown) was 31.3% TC comparing to 81.3%TC obtained with an LDPE sachets. This would be the result of the more stable odorant delivery by the sachets. Regarding the capture efficiency of the blend tested,

Table 4. Odor mass emission from synthetic blends delivered from LDPE sachets

Emission period (h)	Constituent	LDPE4 sachet		LDPE5 sachet	
		Emitted mass (μg)	% of total mass	Emitted mass (μg)	% of total mass
0–2	L-(+)-lactic acid	710 ± 8	7.03	710 ± 8	6.79
	Pentanoic acid	6640 ± 177	65.74	6640 ± 177	63.54
	Ammonia	2750 ± 32	27.23	3100 ± 22	29.67
2–4	L-(+)-lactic acid	590 ± 18	14.64	590 ± 18	15.49
	Pentanoic acid	1450 ± 200	35.98	1450 ± 200	38.06
	Ammonia	1990 ± 44	49.38	1770 ± 37	46.46
4–6	L-(+)-lactic acid	260 ± 56	8.15	260 ± 56	8.36
	Pentanoic acid	1800 ± 190	56.43	1800 ± 190	57.88
	Ammonia	1130 ± 96	35.42	1050 ± 44	33.76

it should be mentioned that in preliminary tests nonanal (an odorant detected by triatomines, Guerenstein and Guerin 2001) at different doses (0.17, 1.66 and 4.15 source doses, in distilled water) delivered from filter paper as described in this study did not capture a statistically significant number of *R. prolixus* nymphs in our experimental device ($P > 0.05$ comparing with the negative control). The maximum capture evoked by nonanal was 30 %TC ($N = 10$). Although this %TC is similar to the maximum value found for the blend when using filter paper as dispenser (the lower number of repetitions and higher negative-control values in the nonanal experiments would be responsible for the lack of statistical significance), it should be noted that nonanal evoked just 33.3 %OC at the most, whereas the blend evoked 70 %OC. This suggests that nonanal increases the locomotor activity of the insects (as suggested by Guerenstein and Guerin 2001), which results in some captures in both the test and control capture tubes, whereas it's attractive efficiency is rather low. This contrasts with the higher %OC found for the blend which would indicate that that capture is due to the higher attractive efficiency of the stimulus. This suggests that the best blends tested here are very promising odor baits for a field trap, as even nonanal *per se* was able to attract triatomines in the field (Rojas de Arias et al. 2012). It should be mentioned that preliminary tests suggested that, surprisingly, addition of nonanal to one of the attractive synthetic odor blends resulted in a decreased capture, and this effect was dose-dependent (not shown).

We found that the odorant ratios of the mixture tested has a strong effect in attraction and capture as the range of %TC for *T. infestans* is from 55.8 to 81.3 and for *R. prolixus* from 43.3 to 81.3. Moreover, the fact that the most efficient of the combinations of odorant ratios used resulted in ca. 81 %TC for both species suggests very high capture performance, which is not different from that evoked by a mouse.

As we wanted to estimate the odorant mass emission and odorant ratios responsible for such a high capture performance, we measured odorant emission for the three components of the two mixtures that performed best. For those two blends, little difference in the constituents proportion was observed between them, and that is consistent with the similar capture values obtained. However, the ratios changed with time. In another experiment carried out in our laboratory (unpublished results), we studied the temporal dynamics of host seeking behavior of *R. prolixus* and *T. infestans* in our dual-choice trap olfactometers, when they were confronted with host–odor stimuli. That study suggests that in our experimental device triatomines are attracted and get trapped mostly during the first hour of the assay. Therefore, the captures reported here might have resulted from within 1–2 h of the trial beginning and thus, the odorant ratios that evoke high attraction and trapping would be those occurring during the first 2 h of assay. In the most efficient blends, those ratios are as follows: pentanoic acid is the main component (ca. 65%) followed by ammonia (ca. 28%) and lactic acid (ca 7%; Table 4). This is consistent with a recent study in mosquitoes using the highly attractive lure Sweetscent (Owino et al. 2014) where it was found that hexanoic acid is the main emitted component (73%) of such lure. Thus, we propose that three-component blends using the odorants and proportions mentioned above are very promising candidates to be tested as lures for a triatomine field trap.

Our results are encouraging as efficient, operatively convenient, cheap, and environmentally friendly odor baits could be within reach. The fact that the odor mixture used is CO_2-free is advantageous because, even when CO_2 is an efficient attractant, it is not practical for use in the field. The use of LDPE sachets as odorant delivery system represents a practical tool to develop attractive lures securing reproducibility. However, the efficiency of the delivery system used here should be improved to prolong the period of efficient trapping of triatomines.

It has been suggested that continuous surveillance followed by selective intervention is necessary even in regions where triatomine control has already been successful (Gürtler et al. 2007, Schofield et al. 2006). An odor baited trap that efficiently captures triatomines could become an important tool for the monitoring and sustainable control of triatomines.

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