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Research article

Behavioral and Toxicological Responses of *Rhodnius prolixus* (Hemiptera: Reduviidae) to the Insect Repellents DEET and IR3535

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Abstract

N,N-diethyl-3-methylbenzamide (DEET) is a broad-spectrum insect repellent used by millions of people since the 1950s. Ethyl 3-[acetyl(butyl)amino]propanoate (IR3535) is a repellent developed more recently that is still not used as extensively. This study compares the behavioral and toxicological effects of both substances in fifth-instar nymphs of the blood-sucking bug *Rhodnius prolixus* Stål (Hemiptera: Reduviidae), one of the main vectors of American trypanosomiasis (Chagas disease). Repellency was quantified using filter paper discs as experimental arenas. Half the discs were treated with solution of repellent in acetone, and the other half with acetone alone. The lowest observed effect level was identical for both substances, 74 μg/cm^2. Nymph age (between 1–3 h and 99 d from last ecdysis) had no influence on repellency. Topical application of 750 μg of DEET per nymph produced a mortality rate between 0% (24 h after application) and 40% (7 d later). The same dose of IR3535 produced no mortality during the same period of time. Simultaneous treatment with piperonylbutoxide (a mixed function microsomal oxidase inhibitor) synergized the lethal effect of DEET. Only DEET increased locomotor activity in nymphs exposed to a treated surface. Nymph antennectomy abolished DEET repellency but not its effect on locomotor activity. The concentrations of both these compounds required to produce either behavioral or toxicological effects are too high to have any practical applications in the control of *R. prolixus*.

Key words: *Rhodnius prolixus*, IR3535, Chagas disease vector, repellency, locomotor activity

An insect repellent has been defined as "something that causes insects to make oriented movements away from its source" (White and Moore 2015). *N,N*-diethyl-3-methylbenzamide (DEET) has been used as a commercial insect repellent for >60 yr and still remains the most widely used product for protecting people against hematophagous insects (Frances 2007). Its success is due to its high efficiency, broad spectrum of activity, and low toxicity to mammals (US Environmental Protection Agency [EPA] 2000). The EPA approves and recommends the use of DEET because it presents a low risk to human health (Antwi et al. 2008). However, it can occasionally cause skin reactions (itching, inflammation, and irritation) and other effects on the nervous system (convulsions, especially in children) (Osimitz et al. 2010). Other DEET disadvantages are its strong irritating effect on mucous membranes (reversible), capacity to dissolve rayon, plastics, and vinyl, oiliness, and unpleasant smell (Brown and Hebert 1997, Nentwig 2003, Osimitz et al. 2010).

Ethyl 3-[acetyl(butyl)amino]propanoate (IR3535) is an insect repellent developed in 1975 by the German company Merck. Compared with DEET, dermal or oral exposure to IR3535 is less toxic to mammals, causes less irritation to mucous membranes, and has more user-friendly physical properties (it does not dissolve plastics, is less oily and odorless) (World Health Organization [WHO] 2001a, 2006; Nentwig 2003). According to the WHO, it is "effective and safe for use in human beings" (WHO 2001b). In Argentina, the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (National Administration for Drugs, Food, and Medical Technology, ANMAT) authorized the sale of >20 repellent products containing IR3535 (ANMAT 2012). The main disadvantage of IR3535 is that it can be very irritating to the eyes. However, after 30 yr of use, no adverse effects have been reported (Puccetti 2007).

Chagas disease is an endemic disease that affects >10 million people in Latin America (Ministerio de Salud de la Nación 2014). It is an infectious disease produced by *Trypanosoma cruzi* Chagas, a protozoan transmitted by blood-sucking bugs of the Reduviidae family (Rassi et al. 2012). *Rhodnius prolixus* Stål is one of the main vectors of Chagas in Venezuela and Colombia (Rodrigues Coura 2015).

There are many scientific reports on DEET repellency in insects. On the contrary, studies on IR3535 are comparatively scarce, and

most have been carried out on mosquitoes that are vectors of human diseases. The objective of the present study was to compare the behavioral and toxicological responses of fifth-instar nymphs of *R. prolixus* to both repellents, DEET and IR3535.

Materials and Methods

Biological Material

Fifth-instar nymphs, 7–15 d old (except where otherwise indicated) and starved since last ecdysis, came from a colony reared at the Centro de Investigaciones de Plagas e Insecticidas (CIPEIN-UNIDEF-CONICET, Villa Martelli, Buenos Aires, Argentina). The colony is maintained at 26 ± 2°C and 60–90% relative humidity (RH), and fed on pigeon blood once a week.

Chemicals

DEET (97%) and piperonylbutoxide (90%) were bought from Sigma Aldrich (Buenos Aires, Argentina). IR3535 (99.6%) was a gift from Merck Argentina (Buenos Aires, Argentina). Analytical grade acetone (Merck, Darmstadt, Germany) was used as solvent.

Recording Equipment

A black and white closed-circuit video camera (VC 1910, Sanyo Electrical Co., Tokyo, Japan) and an image analyzer (Videomex V, Columbus Instruments, Columbus, OH) were used to evaluate repellency and locomotor activity. The video camera captures the image of the insects placed on a circular piece of treated filter paper. The image analyzer converts the analog signal input from the video camera into digital data with a resolution of 256 × 192 pixels and an acquisition and processing speed of 30 frames per second. On the screen, the video signal colors are inverted, i.e., white objects appear black and black ones, white. Therefore, the presence of insects on the filter paper is determined by visual contrast between the subjects (white) and the paper background (black), and is scored as the number of enlightened pixels. To quantify nymph movement, Videomex-V uses Multiple Zone Motion Monitor software that compares consecutive frames captured by the camera and records the number of pixels that change from "on" to "off" or vice versa. The sum of pixels that change during the experimental time is called motion (M). The software also calculates the average number of pixels that remain "on" during the experiment. This parameter is called area (A) and represents the average area occupied by the insects on the video image.

The experimental arena was illuminated with a cold light lamp (22 watts; Luxa, Shangai, China) located at the zenith. Temperature was maintained at 26 ± 2°C. Each set of data was imported and processed on a personal computer.

Evaluation of Repellency

The experimental arena was a single filter paper circle 70 mm in diameter (101 FAST, Hangzhou Xinxing Paper Industry and Co., Ltd., Fuyang, China). The circle was divided in half. One half was impregnated by a pipette with 0.35 ml of a solution of DEET or IR3535 in acetone and the other half with 0.35 ml of the solvent alone (acetone). After the solvent evaporated, the filter paper circle was placed on a horizontal surface and surrounded by a glass ring (2.5 cm high, 10 cm in diameter). A nymph was then placed in a plastic vial (5.5 cm high, 2.5 cm in diameter) which was then held a few millimeters above the experimental arena and gently inclined downward so that the nymph carefully slid down onto the center of the ring-enclosed arena.

Each nymph was randomly assigned to one of four treatments: solvent alone (control), 7.4, 74, and 740 μg/cm^2 of repellent. The image analyzer was programmed to record the motion parameters on each zone separately for 15 min (as a sum for the entire assay). Results were expressed using the following Distribution Coefficient (Moretti et al. 2013):

$$DC = (AT - At)/AT$$

where AT is the total area occupied by nymphs on the arena and At the area occupied by nymphs on the treated zone throughout the experiment. This coefficient varies between 0 and 1, where 0 represents the case in which the substance causes maximum attraction, and 1 represents maximum repellency. The value of 0.5 indicates an equal distribution of the insects between treated and untreated zones (random distribution).

Eight independent replicates were performed for each assay (different replicates were performed on different days, using different insects and different solutions).

In a separate experimental series, nymph antennas were dissected using dissection scissors (BioQuip, Compton, CA) under a magnifying glass (SMZ800, Nikon, Melville, NY). Both antennae were removed at their point of attachment to the head. Dissections were performed 24 h before assays. Each nymph was randomly assigned to one of two treatments: solvent alone (control) and 74 μg/cm^2 of repellent.

Evaluation of Toxicity

Groups of 10 nymphs were topically treated with solutions of DEET or IR3535 in acetone with or without piperonylbutoxide, using a microsyringe with dispenser (Hamilton, Reno, NE). Each nymph received 10 μl of DEET or IR3535 solution on the abdomen (750 μg/nymph). Each nymph treated with a solution containing piperonylbutoxide received 200 μg of this compound. A preliminary assay showed that this dose of piperonylbutoxide does not produce any symptoms of intoxication in the nymphs. Negative control groups (10 nymphs each) were treated with acetone alone (10 μl/nymph).

Immediately after the treatment, nymphs were placed in an incubator FOC 225E provided with a thermoregulation system (Velp Scientifica, Usmate, Italy) programmed at 26 ± 2°C (RH was 60–90%). The number of affected nymphs was recorded every 24 h. To quantify the toxicological effect, a circle of filter paper 15 cm in diameter (101 FAST, Hangzhou Xinxing Paper Industry and Co., Ltd., Fuyang, China) was placed within a plastic container (32 cm long, 25 cm wide, and 8 cm high; Colombraro, Buenos Aires, Argentina). A nymph was then placed in the center of the paper circle using a soft pair of tweezers and observed for 1 min. In all cases, control nymphs rapidly abandoned the paper circle (in <5 s) following an approximately straight line toward the side of the plastic container. Nymphs treated with the repellent that did not abandon the paper circle after 1 min and that manifested visible symptoms of intoxication (difficulty to walk or to remain standing up, no movement after being softly touched with soft tweezers) were considered affected. Three independent replicates were made for each assay. Results were expressed as a percentage of affected nymphs.

Quantification of Locomotor Activity

The experimental design was similar to the one used for measuring repellency but with the following modifications: 1) the circles of filter paper were not divided into zones; therefore, only one zone was used which consisted in the entire paper circle, 2) the image analyzer was programmed to record the motion parameters in just one zone,

3) the circles of filter paper were impregnated using a pipette with 0.7 ml of a solution of DEET or IR3535 in acetone. Once the solvent evaporated, a nymph was placed in the center of the experimental arena as previously described. Each nymph was randomly assigned to one of five treatments: solvent alone (control), 7.4, 74, 740, and 3,700 μg/cm^2 of repellent.

Due to changes in the positions of the nymphs, the number of total "on" pixels varies during an experiment. To standardize the data for the size of the nymphs, the results were expressed in terms of the quotient "Locomotor Activity = *M*/*A*" (see the section "Recording equipment" for the definition of these parameters) (Alzogaray et al. 1997). As both, *M* and *A*, are expressed in units of pixels, the quotient *M*/*A* has no unit.

In a separate experimental series, nymph antennas were dissected as previously described, and each insect was randomly assigned to one of two treatments: solvent alone (control) and 3,700 μg/cm^2 of repellent. Only DEET was used in these assays, because IR3535 failed to hyperactivate nymphs.

Statistical Analysis.

Data from repellency and locomotor activity bioassays were analyzed using one-way ANOVA. When *P*-values were <0.05, Tukey's post hoc comparisons were used to detect significant differences between pairs of treatments. In the second experimental series (repellency vs. nymph age), data failed to meet the ANOVA's assumption of normality; therefore, they were analyzed using the Kruskal–Wallis test. A *t*-test was used to analyze the percentage of affected nymphs in toxicity experiments and the results of the assays using nymphs with and without antennas. Percentages of affected nymphs were arcsin square root transformed prior to analysis.

Results

The first experimental series was carried out to determine the repellent effect of DEET and IR3535 on fifth-instar nymphs of *R. prolixus* (Fig. 1). Both substances were significantly repellent (DEET: $F = 37.1$; df = 3, 28; $P < 0.001$; IR3535: $F = 32.37$; df = 3, 28; $P < 0.001$), and in both cases, the lowest observed effect level was 74 μg/cm^2.

The objective of the second experimental series was to evaluate the repellent effect in nymphs of different ages between 1–3 h and 99 d exposed to 74 μg/cm^2 of DEET or IR3535 (Fig. 2). The effect of both substances was similar in all the groups, and the values of DC were not significantly different (DEET: $H = 4.52$, df = 3, $P = 0.21$; IR3535: $H = 5.18$, df = 3, $P = 0.159$).

The third experimental series assessed the toxicity of DEET and IR3535 when applied topically (Fig. 3). The mean percentage of nymphs affected by a dose of 750 μg of DEET per nymph varied between 0% (24 h after application) and 40% (7 d later). No mortality was observed in the controls throughout the entire test. When nymphs were treated simultaneously with DEET and piperonylbutoxide, the mortality rate was significantly higher after 7 d (82.5%) than in

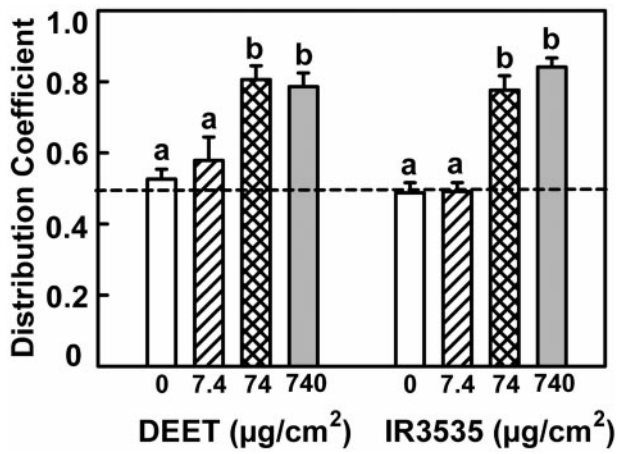

Fig. 1. Repellency produced by DEET and IR3535 on fifth-instar nymphs of *R. prolixus*. Distribution Coefficient = (*AT* − *At*)/*AT*, where *AT* is the total area occupied by nymphs on the arena, and *At* is the area occupied by nymphs on the treated zone, both during the experimental time. Each bar represents the mean of eight independent replicates. Vertical lines are SE. Inside each group, bars marked with the same letter are not significantly different ($P > 0.05$). The dashed line indicates 0.5 DC value (random distribution of nymphs).

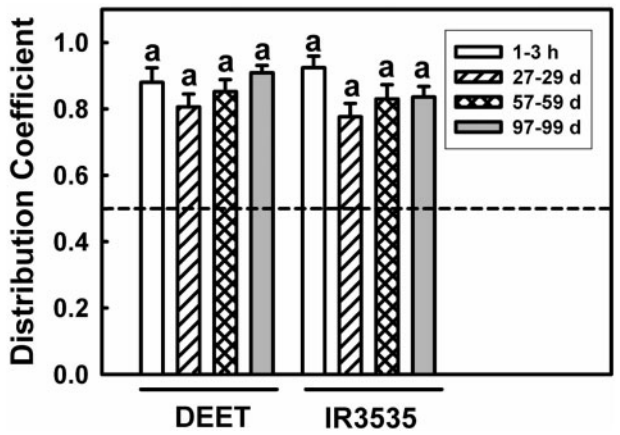

Fig. 2. Repellency produced by DEET and IR3535 on fifth-instar nymphs of *R. prolixus* of different ages. Both DEET and IR3535 were applied at 74 μg/cm^2. DC, Distribution Coefficient = (*AT* − *At*)/*AT*, where *AT* is the total area occupied by nymphs on the arena, and *At* is the area occupied by nymphs on the treated zone, both during the experimental time. Each bar represents the mean of eight independent replicates. Vertical lines are SE. Inside each group, bars marked with the same letter are not significantly different (DEET, $P > 0.05$; IR3535, $P > 0.05$). The dashed line indicates 0.5 DC value (random distribution of nymphs).

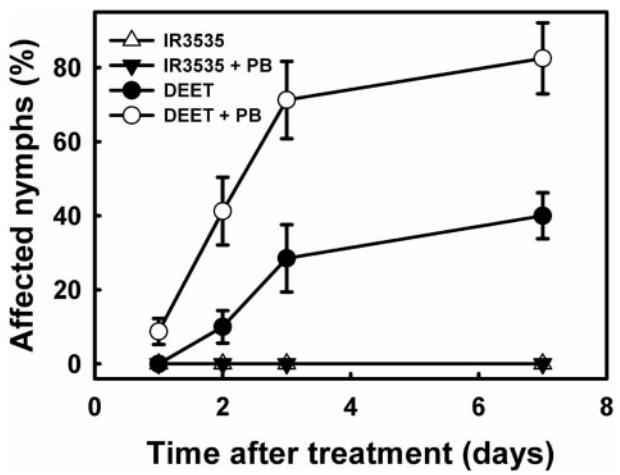

Fig. 3. Toxicity of DEET and IR3535 on fifth-instar nymphs of *R. prolixus*. PB, piperonylbutoxide. Each compound was applied at a dose of 750 μg/nymph. Each symbol represents the mean of three independent replicates. Vertical lines are SE.

nymphs treated only with DEET (40%; $T = 34.5$, $P = 0.009$). A dose of 750 µg of IR3535, with or without piperonylbutoxide, did not produce symptoms of poisoning.

In the fourth experimental series, nymphs exposed to a surface completely treated with DEET elicited a significant increase in locomotor activity that was concentration dependent ($F = 9.70$; df = 4, 35; $P < 0.001$; Fig. 4). The same concentrations of IR3535 produced no significant changes in locomotor activity ($F = 1.73$; df = 4, 32; $P = 0.17$).

A fifth experimental series was carried out to determine the effect on locomotor activity and repellency in nymphs with and without antennae (Fig. 5). Locomotor hyperactivity by DEET was significantly observed in both nymphs with antennae and nymphs that had been antennectomized (with antennae: $t = -6.42$, df = 14, $P < 0.001$; antennectomized: $t = -2.15$, df = 21, $P = 0.043$; Fig. 5A). IR3535 was not tested here because it failed to hyperactivate the nymphs in the fourth experimental series.

Concentrations of DEET and IR3535 that produced significant repellency in nymphs with antennae had no repellent effect on antennectomized nymphs (DEET, with antennae, $F = -4.51$, df = 14, $P < 0.001$; DEET, antennectomized, $F = -1.52$, df = 15, $P = 0.15$; IR3535, with antennae, $t = -4.46$, df = 14, $P < 0.001$; IR3535, antennectomized, $t = 1.02$, df = 14, $P = 0.32$; Fig. 5B).

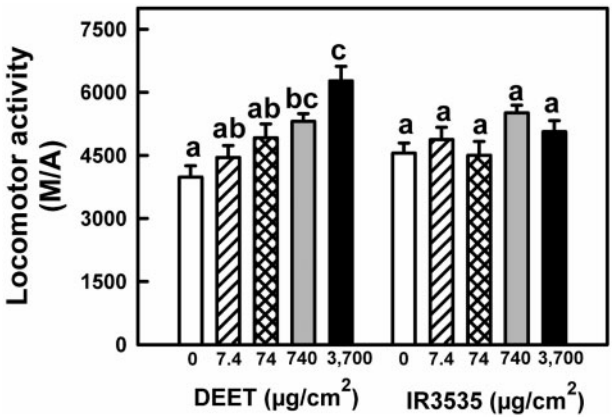

Fig. 4. Locomotor activity of fifth-instar nymphs of *R. prolixus* exposed to DEET or IR3535. *M*, movement (expressed in pixels); *A*, area occupied by the nymph (expressed in pixels). Each bar represents the mean of eight independent replicates. Vertical lines are SE. Inside each group, bars marked with the same letter are not significantly different ($P > 0.05$).

Discussion

Repellency

There are many studies on DEET repellency in insects, but very few on IR3535. Most studies of the latter were carried out on mosquitoes that are vectors of human diseases. The general conclusion is that in both laboratory and field conditions, IR3535 is as efficient as DEET in repelling *Aedes* and *Culex* mosquitoes, but less efficient against *Anopheles* (Costantini et al. 2000, 2004; Thavara et al. 2001; Barnard et al. 2002; Fradin and Day 2002; Barnard and Xue 2004; Cilek et al. 2004; N'Guessan et al. 2006). IR3535 also proved to be an effective repellent against ticks, sandflies, stable flies, horseflies, wasps, and bees (Puccetti 2007). In this study, the repellent effect of IR3535 on fifth-instar nymphs of *R. prolixus* was similar to that of DEET (Fig. 1) and both substances had the same lowest observed effect level of 74 µg/cm^2.

Two hypotheses have been proposed to explain the way in which repellents interact with the nervous system of insects: 1) blocking the perception of host odors, and 2) stimulating specific receptors (insects actually smell repellents). Although the first hypothesis has not been discarded, in the last few years there have been more evidences supporting the second (Pickett et al. 2008, Syed and Leal 2008). The results we present here agree with other studies showing that the triatomines *Triatoma infestans* Klug and *R. prolixus* respond to DEET in the absence of host odors (Alzogaray et al. 2000, Sfara et al. 2008, Zermoglio et al. 2015).

DEET and IR3535 are detected by the insects' olfactory sense, via the interaction between repellent molecules and odorant receptors located on olfactory sensory neurons (Bohbot and Dickens 2010, Kain et al. 2013). As both these substances are synthetic, their perception in insects is not the product of an evolutionary process as occurs with naturally produced smells. The receptors that interact with DEET and IR3535 must have appeared at some point of the insect's evolution as a consequence of exposure to other smells.

Insect response to olfactory stimuli is influenced by their age and physiological state (den Otter et al. 1991, Qiu et al. 2013). In fifth-instar *R. prolixus* nymphs, the behavioral response to carbon

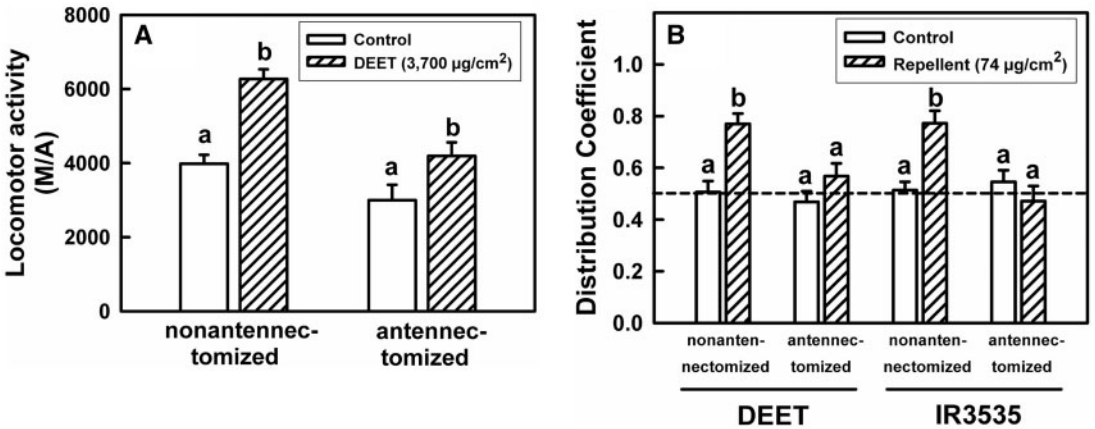

Fig. 5. Repellency and locomotor activity evaluated on fifth-instar nymphs of *R. prolixus* with or without antennas, exposed to DEET or IR3535. (A) Effect on locomotor activity produced by DEET. *M*, movement (expressed in pixels); *A*, area occupied by the nymph (expressed in pixels). Each bar represents the mean of eight independent replicates. Vertical lines are SE. Inside each group, bars marked with the same letter are not significantly different ($P > 0.05$). (B) Repellency produced DEET and IR3535. Each bar represents the mean of eight independent replicates. Vertical lines are SE. Inside each group, bars marked with the same letter are not significantly different ($P > 0.05$).

dioxide (a long-distance cue associated with host localization) is developed in an all-or-none way and clearly depends on the time since the last ecdysis (Bodin et al. 2009). During the first week following ecdysis, there is no response, but after this period the nymphs are strongly attracted to carbon dioxide. This delay could be due to the presence of a mechanism that allows the insects locate its host only after achieving the appropriate physiological state for feeding. This type of modulation would not be physiologically justified for an olfactory stimulus unrelated to host localization.

The repellency elicited by DEET and IR3535 was not influenced by the age or nutritional condition of *R. prolixus* fifth-instar nymphs (Fig. 2). The response of the nymphs was similar between 1–3 h after ecdysis to 99 d later under fasting conditions (the younger group of nymphs used for this experiment had a pale-pink coloring, indicating that their cuticle was still not fully sclerotized).

Unlike the response to carbon dioxide, perception and behavioral response to artificial repellents seem to be permanent in this species. It would be interesting to determine whether these insects elicit the same response when exposed to other natural or synthetic odors that are foreign to their environment.

Toxicity

The insecticide activity of DEET and IR3535 has received little attention. Topical applications of DEET showed moderate toxicity at low microgram doses in *Ae. aegypti* (L.), *An. gambiae* Giles, and the common housefly (Swale et al. 2014), and was between 2 and 7.6 times more toxic than IR3535 in four species of mosquitoes (Pridgeon et al. 2009). No symptoms of intoxication were observed in nymphs or adults of *T. rubida* Uhler exposed to filter papers treated with low concentrations of DEET (1–10%) (Terriquez et al. 2013).

A topical application of 750 µg of DEET per nymph produced an effect in *R. prolixus* nymphs that varied between 0 and 40% at 24 h and 7 d, respectively (Fig. 3). The same concentration of IR3535 produced no toxic effects within the same period of time. DEET toxicity can be classified as extremely low for this species, considering the fact that the lethal dose for 50% of treated insects (LD_{50}) of some cyanopyrethroid insecticides range between 40 and 250 ng/nymph for this same *R. prolixus* instar (de Oliveira Filho 1999).

DEET affects the insect nervous system, but its mode of action is still not clear. In vitro, it showed to be a poor inhibitor of acetylcholinesterase activity in flies and mosquitoes (Corbel et al. 2009). Neurophysiological recordings suggest that octopaminergic synapses could be its target (Swale et al. 2014).

The low number of studies on the toxicity of repellents in addition to the methodological and biological differences between *R. prolixus* and the aforementioned mosquitoes does not allow making specific comparisons. In general, it is evident that DEET tends to be more toxic than IR3535 in both these groups of insects. To explain the cause of this differential toxicity, it is first necessary to find differences between the toxicokinetics and toxicodynamics of these repellents in the insects, on which there is little or no information.

DEET and IR3535 metabolism has been studied in mammals, but there are hardly any studies carried out on insects. In humans and rats, DEET undergoes oxidation and dealkylation through mixed function microsomal oxidase (MFMO) activity (Selim et al. 1995, Constantino and Iley 1999, Sudakin and Trevathan 2003). IR3535 is rapidly biotransformed by ester cleavage to IR3535-free acid in humans, rats, and rabbits, probably via the activity of esterases (van Dijk 1996, Broschard et al. 2013).

Simultaneous application of DEET and piperonylbutoxide doubled the mean percentage of affected nymphs (Fig. 3). This suggests that MFMO are involved in the biotransformation of DEET in *R. prolixus*. Piperonylbutoxide also increased the toxicity of DEET in a strain of *Ae. aegypti* (Bonnet et al. 2009). However, it did not modify the toxicity of this repellent in *An. gambiae*, *Musca domestica*, and a strain of *Ae. aegypti* different to the one used in the aforementioned study. Further investigation is necessary to determine whether these results are due to interspecific differences in the metabolic pathways of DEET.

No symptoms of intoxication were observed in nymphs treated with IR3535 with or without piperonylbutoxide. This might indicate that MFMO are not involved in the metabolism of this repellent in *R. prolixus*. However, this result could also be merely the consequence of the very low toxicity of IR3535 in this insect.

Effect on Locomotor Activity

Hyperactivity is the first sign of intoxication that is easily observed in insects exposed to pyrethroids and other insecticides (Alzogaray and Zerba 2001, Moretti et al. 2013). Compounds that produce hyperactivity in triatomines make them abandon their shelters where they usually remain most of the day (Pinchin et al. 1980). This phenomenon is known as "flushing-out." WHO recommends the use of pyrethroids for controlling triatomines because their flushing-out activity makes the insects leave their shelters and become exposed to the surfaces treated with the insecticide (WHO 2002). Flushing-out is also used for determining whether a home is infested with triatomines (Gürtler et al. 1993).

Tetramethrin is a first-generation pyrethroid with very low toxicity to triatomines (Casabé et al. 1988). Its effect generating hyperactivity in these insects is low compared with other pyrethroids (Alzogaray et al. 1997). At present, the effectivity of tetramethrin as a flushing-out agent is limited by the appearance of pyrethroid-resistant triatomine populations in Argentina (Vassena et al. 2000, Picollo et al. 2005) that are resistant to both knockdown and hyperactivity (Sfara et al. 2006). Therefore, it is increasingly necessary to identify flushing-out agents to replace tetramethrin.

DEET generated hyperactivity in *R. prolixus* nymphs in a concentration-dependent way (Fig. 4). The absence of hyperactivity in nymphs exposed to IR3535 is coherent with its aforementioned low toxicity. Hyperactivity by DEET was equally elicited in nymphs with and without antennae, but repellency only occurred in nymphs with intact antennae (Fig. 5). These results suggest that the effect of DEET on locomotor activity is a separate phenomenon to the mechanism of sensory perception involved in the repellent effect. They also indicate that the sensory organs that detect DEET and IR3535 appear to be mainly located on the antennae.

In conclusion, this is the first study evaluating the behavioral and toxicological responses of a triatomine vector of Chagas disease exposed to the synthetic insect repellent IR3535. It is also the first time that DEET toxicity has been assessed in a species of the genus *Rhodnius*. The results show that both repellents elicit a behavioral response in the lack of a host-related stimuli, independently of nymph age and nutritional state. The presence of insect antennas was essential for the behavioral response to occur. The toxicity of both repellents was very weak, and MFMO seem to be involved in the metabolism of DEET. The concentrations of these compounds required to produce either behavioral or toxicological effects are too high to have any practical applications in the control of *R. prolixus*.

Further investigation is necessary to find out whether these repellents have greater effect on other Chagas disease vectors.

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