



Rhodnius prolixus smells repellents: Behavioural evidence and test of present and potential compounds inducing repellency in Chagas disease vectors



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ABSTRACT

Insect repellents are known since many decades ago and constitute a major tool for personal protection against the biting of mosquitoes. Despite their wide use, the understanding of why and how repellents repel is relatively recent. In particular, the question about to what extent insects other than mosquitoes are repulsed by repellents remains open. We developed a series of bioassays aimed to test the performance of well established as well as potential repellent molecules on the Chagas disease vector *Rhodnius prolixus*. Besides testing their ability to prevent biting, we tested the way in which they act, i.e., by obstructing the detection of attractive odours or by themselves. By using three different experimental protocols (host-biting, open-loop orientation to odours and heat-triggered proboscis extension response) we show that DEET repels bugs both in the presence and in the absence of host-associated odours but only at the highest quantities tested. Piperidine was effective with or without a host and icaridine only repelled in the absence of a living host. Three other molecules recently proposed as potential repellents due to their affinity to the *Ir40a*⁺ receptor (which is also activated by DEET) did not evoke significant repellency. Our work provides novel experimental tools and sheds light on the mechanism behind repellency in haematophagous bugs.

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1. Introduction

The terms “repellent” and “repellency” have been defined in different ways since the first classification of behavioural responses of insects to chemicals by Dethier (1947) and Dethier et al. (1960). One of the most acknowledged definitions was that of Barton-Browne (1977) who stated that a repellent is “a chemical that acting in the vapour phase prevents an insect from reaching a target to which it would otherwise be attracted”. Currently, the definitions take into account the behavioural responses of insects to these compounds. The most recent and comprehensive handbook on repellents (Debboun et al., 2015) defines a repellent

simply as “something that causes insects to make oriented movements away from its source” (White and Moore, 2015). The expression “oriented movements” seems to indicate a displacement based on directional information, and not only activity modulation. Different derivations of this term as spatial repellent, excitorepellent and others, designate substances that induce a particular response, not only orientated ones, but also modifications in the insect's activity or in its action radius. Repellency, in its turn, designates the end result of the action of the repellent including behavioural reactions but not a reaction itself (Obermayr, 2015).

A major difficulty to apprehend the biological meaning of repellents and understand consequently their actual effect on insects is the reduced information that we have about their action mechanisms. Hence, repellents are defined primarily by the practical consequences of their use, rather than by their biological action. Furthermore we can say that they are defined by their action on mosquitoes more than on any other insect.

The gold standard and largely most used repellent is NN-diethyl-3-methylbenzamide, usually abbreviated as DEET in the literature. After it was discovered by the USDA ARS and developed by the U.S. Army in 1946, DEET was introduced for use by the general public in 1957, becoming its use worldwide since the

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1960s (Katz et al., 2008). In spite of its long history and efficacy for personal protection against disease vectors, its action mechanism remained obscure until recent years.

The way DEET interacts with the sensory system of insects started to be unravelled just in recent years and has been the matter of an interesting controversy that pushed forward the research on these compounds. A first proposal on the mode of action of DEET was made by McIver (1981), who suggested that this compound might interact with the lipidic portions of the cell membranes in mosquitoes' olfactory neurons, preventing the insect from sensing host odours. However, the first experimental evidence of its action mechanism was only provided many years later by Ditzen et al. (2008), who suggested that DEET blocks the reception of attractive host-odours, such as octenol. Later on, Syed and Leal (2008) showed that mosquitoes actually smell DEET, which stimulates receptors sensitive to plant odours such as linalool. These authors, as well as Pickett et al. (2008), attributed the previous finding to an experimental artefact related to the way in which chemicals were presented. These findings served as a basis for the work of Bohbot and Dickens (2010), who shed additional light on the problem by studying the molecular basis of the action of different repulsive compounds. They concluded that “repellents can act as olfactory agonists or antagonists thus modulating OR [odorant receptor] activity bringing concordance to conflicting models.”

Recently Kain et al. (2013) presented evidence supporting that DEET activates the molecular receptor *IR40a*⁺ in *Drosophila* and mosquitoes. If confirmed in other insects, this could represent a real breakthrough, with important consequences for the development of repellents for two main reasons. On the one hand, this receptor is highly conserved across insect species and it would represent a common target for repellency. On the other hand, this receptor would also be activated by other chemical molecules, some of them less toxic than the DEET (Corbel et al., 2009), or even not toxic at all (Kain et al., 2013). Very recently, however, this hypothesis has been seriously challenged by the discovering that, in *Culex quinquefasciatus*, it is not the ionotropic receptor *IR40a*⁺, but the olfactory receptor *CquiOR136* the responsible for the action of DEET and other repellents in these mosquitoes. This finding reinforces the idea of a possible link between natural products with long insect–plant evolutionary history and synthetic repellents (Xu et al., 2014; Leal, 2014). All these results have changed (or should change) the way in which we deal with repellents, for two main reasons. First, mechanisms and molecular targets seem to vary according to the species (e.g., Kain et al., 2013; Leal, 2014), making it necessary to avoid generalizations. Second, the knowledge of the molecular targets opens the possibility of rational design of novel repellent molecules (Kain et al., 2013).

The actual value of repellents for personal protection against the biting of disease vectors other than mosquitoes remains unclear, provided that the efficacy of repellents has not been fully validated and tests rendered contradictory results. For instance, whereas Usinger (1966) and recently Wang et al. (2013) reported repellency of bedbugs by DEET, this compound is still reported as ineffective in these insects by different organisms and agencies (e.g., Ohio Department of Health, 2011).

Concerning triatomine vectors of Chagas disease, different aspects of their response to DEET have been analysed in detail, as for example the spatial distribution and olfactory orientation of *Triatoma infestans* (Alzogaray et al., 2000), the effect of nitric oxide on repellency induced by DEET in *Rhodnius prolixus* (Sfara et al., 2008) and the loss of sensitivity to DEET following pre-exposition in the same species (Sfara et al., 2011). However, some other relevant aspects, such as the exact way in which it modulates the behaviour of bugs or its relative efficacy as compared to other compounds, remain unknown.

Taking together all these results, some relevant questions arise. First, if mosquitoes only detect DEET by the stimulation of receptors devoted to the detection of specific plant-odours, what should we expect for its action on insects lacking any evident association with plants? Second, do all haematophagous actually smell repellents or do these compounds block the reception of host-odours? And finally, are *IR40a*⁺ stimulating compounds other than DEET able to induce repellency in insects other than mosquitoes?

In this work we provide some answers to these questions by presenting the results of a series of experiences conducted using different bioassays and contexts to assess the performance and mode of action of DEET, icaridine and other molecules (methyl-, ethyl- and butyl-anthranilate) which could potentially act as repellents on *R. prolixus*.

2. Material and methods

2.1. Insect rearing

Bugs were reared in the laboratory at 25 ± 2 °C under a 12L:12D illumination and $65 \pm 5\%$ RH regimes. Fourth-instar nymphs of *R. prolixus* were fed on heparinized sheep blood using an artificial feeder and used for experiments about two weeks after their moult to the fifth instar. All experiments were conducted in dim light (0.012 to 0.040 ± 0.001 $\mu\text{W}/\text{cm}^2$), and under 25 ± 2 °C and $65 \pm 5\%$ RH regimes.

2.2. Orientation and PER towards a living host in the presence of repellent

The orientation of the bugs towards a living host and their biting attempts were quantified in the presence of repellents. An exposure device was made using a polystyrene tube (Fig. 1, 10×1.7 cm, 14 cm³), which allows measuring activity, approach to the host and proboscis extension response (PER). This, avoiding the host to have any contact with the chemicals or to get bitten by the experimental insects.

Insects were individually placed inside the tube in the refuge zone (Fig. 1) and were allowed 5 min of familiarisation, after which the Whatman filter paper was introduced in the tube and the refuge gate was open. During the following 5 min three variables were registered with the aid of an event recording software: activity, time spent in each zone and number of PER. An insect was considered to be active either when it was walking or trying to bite.

Insects were randomly assigned to one of 14 treatments: no stimulus aside from that of the host odours (control group), distilled water, ethanol, piperidine (Hexahydropyridine C₅H₁₁N, 99% Reagent Plus®, Sigma–Aldrich) 1%, 10%, 50% and 90% in distilled water, icaridine (C₁₂H₂₃NO₃, 25%, as the commercial repellent Moustidose®, Laboratoires Gilbert) and DEET 10%, 50% and 90% in ethanol (NN-Diethyl-meta-toluamide C₁₂H₁₇NO, 97% Sigma–Aldrich). For each treatment 10 individuals were tested using 10 μl of the compounds loaded on the filter paper and host stimuli (experimenter's arm). Additionally, DEET 90% and ethanol were tested in a volume of 50 μl , in order to evaluate further effects depending on quantity of substance applied, and a further negative control was performed by placing a paper soaked with 10 μl ethanol, but without host stimuli.

2.3. Orientation in the presence of repellent

The orientation of insects when presented with airstreams loaded or not with repellent was tested using a locomotion compensator, under open-loop condition for distance and closed-loop for direction. The device was set after Barrozo and Lazzari (2004)

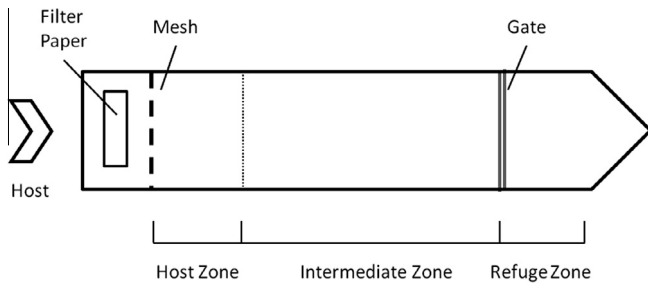


Fig. 1. Exposure device to test orientation and PER of *Rhodnius prolixus* in the presence of repellent towards a living host. Horizontal polystyrene tube (10×1.7 cm 14 cm³) open in its end but separated from the environment by means of a mesh placed 1 cm away from the extreme. The tube was divided into three zones: a proximal zone (up to 2 cm from the mesh Host Zone) and intermediate zone and a distal Refuge Zone which was determined by placing a mobile gate 2 cm from the closed distal extreme. Odours were presented inside the tube by placing a rolled filter paper (1×3 cm) soaked with the compound of interest in the space between the extreme and the mesh. Host stimuli were provided by holding the open end of the tube against the arm of the experimenter. In this way bugs could get close enough to the host without actually biting and the repellents could be interposed between the host and the bugs.

and consisted of a Styrofoam ball (2.5 g 9.7 cm diameter) suspended by an air current, which movement is tracked by means of an optic sensor placed underneath. Insects were dorsally attached to a rotating steel rod with double-sided adhesive tape and allowed to walk freely on the ball in all directions.

Two horizontal air streams, one containing clean air and the other containing the compound of interest, were presented from exact opposite directions (flow rate: 3.76 cm³ s⁻¹) at a distance of 3 cm from the experimental individual. Before reaching the insect, airstreams passed through glass bottles in which 10 μ l of the compounds tested were sowed on filter paper (4×0.8 cm) 2 min before assays were performed.

Insects were randomly assigned to one of 5 treatments: clean air, volatiles produced by an aerobic culture of baker's yeast (2.5 g yeast *Saccharomyces cerevisiae*, 2.5 g sugar and 5 ml water), which are known to act as an attractant to triatomine bugs (Guerestein et al., 1995; Lorenzo et al., 1999), piperidine, DEET and DEET + yeast odours.

Before each trial bugs were familiarised with the experimental device for 2 min, after which they were subjected to the opposed airstreams during 3 min. The orientation behaviour of the bugs on the locomotion compensator was registered as x - and y -coordinates every 200 ms. The position of the loaded and the clean air-currents was randomly changed from one individual to the next one.

2.4. PER in the presence of repellent

In *R. prolixus* the PER triggered by heat constitutes a robust and easily observable appetitive behaviour (Fresquet and Lazzari, 2011; Vinauger et al., 2013). We tested the responsiveness of bugs confronted to a warm object in the presence of different repellents. The rationale behind this procedure is to test the effect of the repellents *per se*, in the absence of host's odours. In this way, the interference in the detection of attractive odours and the direct reception of repellents could be distinguished, shedding some light on the repellent's action mechanism.

Insects were randomly assigned to one of seven treatments: no stimulus (positive control group), ethanol (solvent control), icaridine (25% as the commercial repellent Moustidose®), DEET, methyl anthranilate (C₈H₉NO₂, natural 99%, Sigma–Aldrich), ethyl anthranilate (C₉H₁₁NO₂, $\geq 99\%$ Sigma–Aldrich) and butyl anthranilate (C₁₁H₁₅NO₂, Analytical standard, Sigma–Aldrich). All these

substances except the icaridine were diluted to 50% in ethanol. Although findings on the previous experiments suggested that neither DEET 50% nor icaridine 25% would have an effect on the PER rate in the proximity of the host (see Section 3), we chose to test these concentrations in this experiment considering that the absence of host odour cues might lower the response threshold of the bugs to the drugs.

An experimental device was set after Fresquet and Lazzari (2011) in which insects were presented with an adjustable thermal source. Bugs were dorsally attached to a steel rod with double-sided adhesive tape and were allowed to secure a Styrofoam ball in order to provide tarsal contact. The temperature of a water-cooled Peltier element (4×4 cm QuickCool Germany) was defined by an accurate controller (Peltron GmbH Peltier-Technik Germany), which allowed a quick and precise variation of the temperature at the Peltier surface. Insects were placed at a distance in relation to the Peltier element so that they could nearly contact its surface by extending the proboscis.

Before each trial bugs were familiarised with the device for 1 min and the temperature of the Peltier element was fixed at 25 °C. Each trial consisted of five consecutive stimulation cycles in which the bugs were exposed to the Peltier element at 35 °C for 10s followed by 50s at 25 °C. Second to fourth stimulation cycles (repellent test) were done exposing the bugs to the potential repellents. Exposure to the compounds was achieved by sticking a filter paper (6×6 mm) embedded with 5 μ l of the tested substance to the Peltier element by means of double-sided adhesive tape. For the fifth cycle the stimulus was removed. For the control group only a filter paper was presented. PER events were recorded when the proboscis of an insect was observed to be fully extended. Within the repellent test, the proportion of bugs that elicited 3 PERs, as well as the proportion of bugs that elicited PER in the first cycle with the substance, were calculated for the each experimental series.

The rationale behind this protocol was that the first stimulation without any chemical allowed testing the responsiveness of bugs to heat and only insects extending the proboscis during the first thermal stimulation were considered. The final (fifth) exposition to only heat allowed evaluating possible after effects.

2.5. Data analysis and statistics

Data corresponding to the three variables measured in experiments performed on living host (i.e., activity, permanence near the host and number of PER) were tested for normality and homoscedasticity. In order to determine the possible effect of solvents used, the responses of the bugs on the three variables were analysed using Student t -test (ethanol 10 μ l + host vs. host alone; ethanol 50 μ l + host vs. ethanol 10 μ l + host; ethanol 10 μ l + host vs. ethanol 10 μ l alone). To determine the effect of icaridine, a Student t -test was performed for each variable, comparing against its correspondent solvent control (distilled water in host proximity). Effects of DEET and piperidine on the bugs' behaviour and the dose dependency for each drug were tested by means of One-Way ANOVAs and post hoc Tukey contrasts, for each drug and variable of interest separately. For DEET, the groups under analysis were: Ethanol (10 μ l) + Host (solvent control), DEET 10%, DEET 50%, DEET 90% (10 μ l), and DEET 90% (50 μ l). For piperidine, the groups under analysis were: Distilled water + Host (solvent control), piperidine 1%, piperidine 10%, piperidine 50% and piperidine 90%.

Orientation on the locomotion compensator data was analysed using circular statistics. Trajectories were reconstructed and the sequence of x - and y -coordinates were used to obtain the mean angular trajectory of each individual during the stimulation time (see Barrozo and Lazzari, 2004 for details). All the individual angles

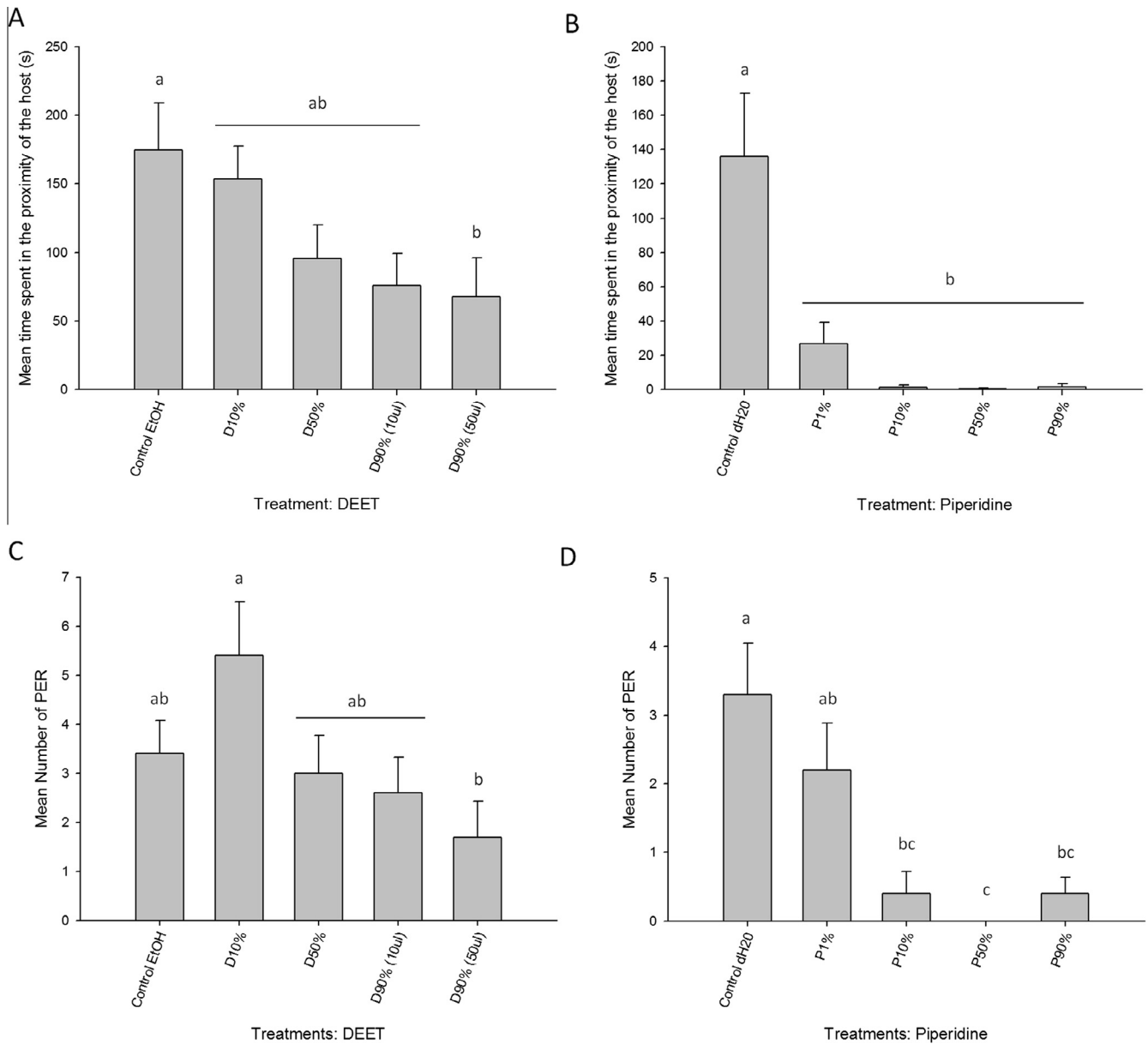


Fig. 2. Time spent in the proximity of a living host and number of PER elicited by *Rhodnius prolixus* individuals when exposed to repellents. Time spent near the host when exposed to DEET (A) or Piperidine (B) in different concentrations; and number of PER elicited when exposed to DEET (C) or Piperidine (D) in different concentrations. D: DEET, P: piperidine, EtOH: ethanol, dH₂O: distilled water. Letters a, b, c indicate distinct groups resulting from significant differences in post hoc Tukey comparisons after one-way ANOVA for each variable and drug tested.

of the insects belonging to each experimental group were analysed using three successive tests. First, Rayleigh Test was used to determine if the individual orientation angles were uniformly distributed within each treatment. In the cases in which the distribution was uniform, Rao spacing-test was used in order to assess whether the data were indeed uniformly arranged or if they were distributed following a predominant axis, but in opposite directions. In the cases showing no uniformity or axial distribution of trajectories, V-test was used to determine if the distribution of angles followed a particular expected direction. For computations, angular data were normalised to make 0° correspond to the direction of the air current loaded with the stimulus or stimuli and set 180° as the expected direction when a repellent was presented.

Results from PER experiments were analysed performing independent Chi-square tests between the proportions of bugs that elicit 3 consecutive PER for each treatment against the controls:

icaridine was compared against the positive control and the other compounds against ethanol.

3. Results

3.1. Inhibition of host-biting

The activity, the time spent in host-proximate zone and the number of PER elicited were analysed in insects exposed to different repellents.

The activity of the insects in the presence of the host did not differ among treatments, bugs showing activity during, on average, 68% of the experimental time (Mean activity time = $20.3.77 \pm 32.15$ s). On the other hand, when exposed to ethanol but deprived of the host stimuli, activity was significantly lower ($t_{18} = 2.2573$, $p = 0.0183$) than when exposed to ethanol 10 µl + host stimulus.

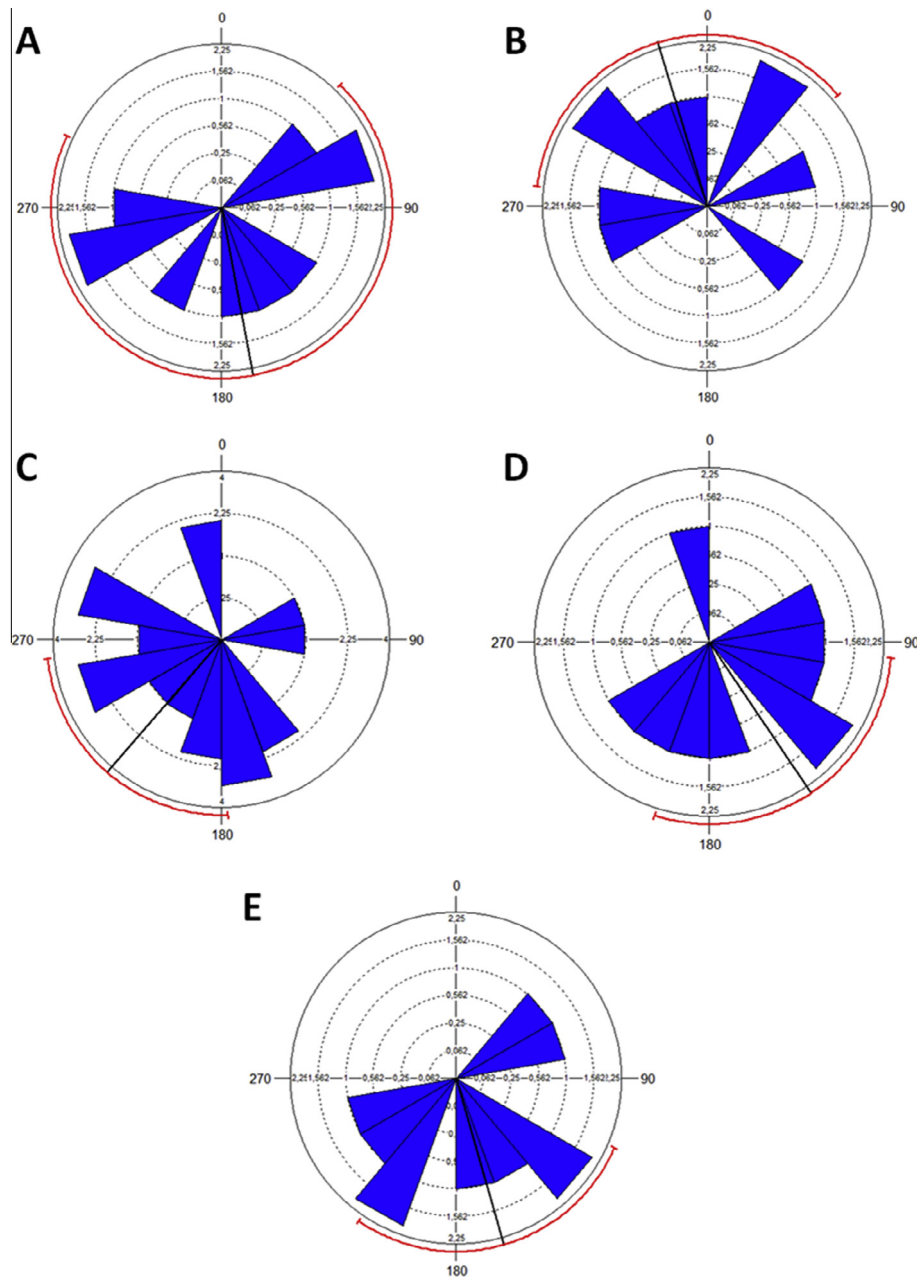


Fig. 3. Orientation behaviour of *Rhodnius prolixus* in a locomotion compensator when exposed to airstreams containing repellents. (A) Clean Air; (B) Yeast Odours; (C) DEET; (D) DEET + Yeast Odours; (E) Piperidine. Full radii and arches represent mean orientation angles and their errors.

The time spent in the proximity of the host (Zone 3 in Fig. 1) was significantly lower when bugs were exposed to ethanol, but deprived from host stimuli ($t_{18} = 2.2346$, $p = 0.0192$). Analysis of the effect of DEET on this variable showed significant differences ($F_{(4,45)} = 3.42$, $p = 0.0159$, Fig. 2A). This differences were evinced only in the comparison between the highest concentration of DEET (90% 50 μ l) and the control group (Tukey HSD $p = 0.0411$), although DEET 90% also showed a tendency towards diminishing the time spent near the host (Tukey HSD $p = 0.0697$). However, no differences in the time spent near the host were found among different concentrations of DEET. When exposed to piperidine, the time spent near the host was shorter than that of the control for all concentrations tested ($F_{(4,45)} = 12.66$, $p = 0$, Fig. 2B, Tukey HSD 1% vs Control: $p = 0.0002$, Tukey HSD 10%, 50%, and 90% vs Control: $p = 0$). No differences were found among the different

concentrations of piperidine tested. Icaridine (25%) did not show any effect on the time spent near the host ($t_{18} = 1.3486$, $p = \text{n.s.}$).

The number of PER performed by the insects, as expected, was significantly lower in the absence of the host than in its presence ($t_{18} = 4.6212$, $p = 0.0001$). Exposure to DEET affected the number of PER elicited ($F_{(4,45)} = 3.15$, $p = 0.0231$, Fig. 2C), but this was only evinced at the higher concentration and dose when compared to 10% DEET concentration, being the variable significantly lower (DEET 90%, 50 μ l, Tukey HSD $p = 0.0124$). Insects exposed to piperidine showed a lower number of PER ($F_{(4,45)} = 9.46$, $p = 0$, Fig. 2D), particularly at the three higher concentrations tested, (Tukey HSD 10% vs Control: $p = 0.0006$, 50% vs Control: $p = 0.0001$, 90% vs Control: $p = 0.0006$). The lowest concentration (1%) did not differ from the control, but did differ from the 50% treatment (Tukey HSD 1% vs 50%: $p = 0.0132$) and marginally from the 10% and 90%

treatments (Tukey HSD: $p = 0.0623$ in both cases). Exposure to icaridine (25%) did not affect the number of PER elicited ($t_{18} = 0.113$ n.s.).

3.2. Olfactory orientation

The orientation on a locomotion compensator was evaluated in the presence of repellents. While no effect of clean airstream was found (Rayleigh: 0; Rao: $U_{10} = 0.50 > p > 0.10$, Fig. 3A) when offered an airstream containing yeast-odours the bugs exhibited orientation towards it (V -test = 0.0380, Fig. 3B), which is in accordance with previous findings (Guerenstein et al., 1995; Lorenzo et al., 1999; Vinauger et al., 2011a,b). Conversely, when confronted with an airstream containing DEET the insects avoided the stimulus, significantly orientating in the opposite direction (V -test = 0.029, Fig. 3C). Furthermore, the addition of yeast-odours to the DEET did not counter its repellency, i.e., bugs kept avoiding the stimulus (V -test = 0.032, Fig. 3D). Piperidine was also found to act as a repellent, as in its presence bugs significantly orientated in the opposite direction (V -test = 0.014, Fig. 3E).

3.3. PER modulation by classical and presumed repellents

The proboscis extension response was tested in insects confronted with a source of heat in the presence of repellents and other substances that could potentially act as such.

The proportions of insects that elicited 3 consecutive PERs while being exposed to icaridine or DEET were significantly lower than the corresponding proportion in the controls ($\chi^2 = 29.2701$, $p = 0$; $\chi^2 = 9.727$, $p = 0.001816$ respectively, Fig. 4). On the other hand, when exposed to methyl-, ethyl- or butyl anthranilate, although the proportions of bugs that performed 3 PER were slightly lower than the control, neither of these differences was statistically significant ($\chi^2 = 1.3329$, n.s. for methyl- and butyl-; $\chi^2 = 0.3903$, n.s. for ethyl anthranilate).

4. Discussion

4.1. Repellents repel but...

From the several putative repellents tested in this work, some revealed as effective against *R. prolixus* but others were not able to inhibit the approaching and the biting attempts of bugs. DEET, the “gold standard” among repellents, was effective on bugs, in agreement with previous reports demonstrating the repellent effect of DEET on *R. prolixus* (Sfara et al., 2008) and the related species *T. infestans* (Alzogaray et al., 2000). Nevertheless, in our tests, this effect was only relevant at concentrations that are much higher (i.e., more than 90%) than those found in most commercial formulations. Conversely, piperidine revealed effective at relatively low concentrations (i.e., 10% is enough to modify the time the insect spends in proximity of the host and the biting attempt rate), but the toxicity of this substance, which is also an insecticide, limits its utilisation (Debboun et al., 2015). Icaridine, in its turn, inhibited the PER to a warm object, but was ineffective in the presence of a living host.

Hence, it seems that, despite both being haematophagous insects, repellents are not as effective against *R. prolixus* as they are against mosquitoes. The question arises then about the mechanisms behind the action of these substances on bugs.

4.2. Bugs smell repellents

Concerning the mode of action of repellents, two competing hypothesis have been proposed (Ditzen et al., 2008; Syed and

Leal, 2008; Pickett et al., 2008; Bohbot and Dickens, 2010). The first hypothesis establishes that the mode of action of repellents is through interaction with the host-associated volatiles, preventing the olfactory neurons of the bugs from sensing those cues. Alternatively, the second hypothesis establishes that repellents act by binding to specific receptors, and that its action is routed through central signal integration processes in the insect's brain. Distinguishing between these two possible alternatives has been in the centre of an important and scientifically fructiferous controversy.

In order to elucidate the mode of action of repellents in *R. prolixus*, we analysed the orientation behaviour of bugs on a locomotion compensator, when insects were presented with air currents loaded or not with chemical attractants and/or repellents. Our results showed that both repellent compounds tested, i.e., DEET and piperidine, induced avoidance in bugs when presented alone. As even in the absence of host-related stimuli the repellents were perceived by the insects' sensory system and a behavioural response was evinced, we conclude that the repellents must be acting through specific receptors, rather than interacting with the perception of host odours.

4.3. Repellents inhibit heat-triggered PER

For *R. prolixus*, as well as for other triatomine bugs, heat constitutes a major stimulus associated to feeding behaviour (Flores and Lazzari, 1996; Lazzari, 2009), being indeed the only stimulus that is both necessary and sufficient for triggering the PER that precedes biting. Besides, bugs possess the highest thermal sensitivity known at present in animals (Lazzari, 2009). Thus, it appeared relevant testing to what extent repellents are able to prevent biting: only impeding the approach, or also inhibiting the PER triggered by heat. An additional interest to test this is that PER is a highly stereotyped response of bugs that can be easily evoked and quantified (Vinauger et al., 2013), and that may constitute a reliable bioassay of repellents in these insects.

In the proximity of a host, two of the tested repellents, i.e., DEET and piperidine, were able to significantly reduce the rate of PER triggered by heat (Table 1). This result could be due to two possible reasons already exposed: chemicals could be interacting negatively on the perception of host's odours, or they could be perceived by themselves, hence preventing the bugs biting. Given the previous

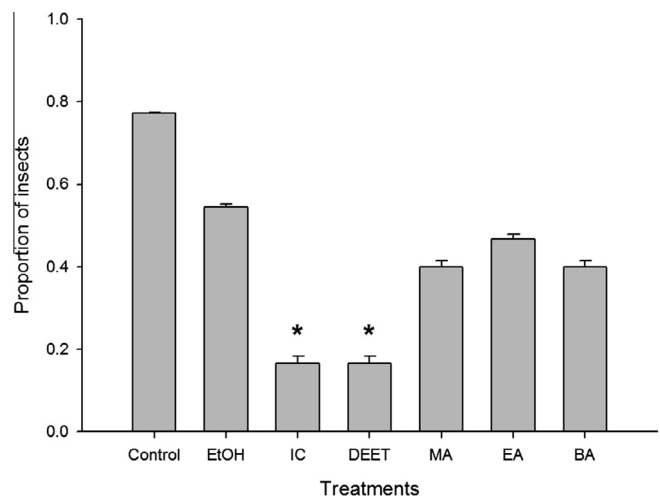


Fig. 4. Proboscis extension reflex (PER) of *Rhodnius prolixus* towards a heat source when exposed to repellents. Positive control (no compound added) solvent control (Ethanol EtOH) Icaridine (25% as Moustidose® IC) NN-Diethyl-meta-toluamide (DEET 50%) Methyl anthranilate (50% MA) Ethyl anthranilate (50% EA) and Butyl anthranilate (50% BA). *Indicates significant differences.

Table 1

Summary of the bioassays and the repellents tested in this work.

Assay		Repellent					
		DEET	Piperidine	Icaridine	MA	EA	BA
No living host	Orientation	Repellency	Repellency	–	–	–	–
	PER	Repellency	–	Repellency	No effect	No effect	No effect
With living host	Activity	No effect	No effect	No effect	–	–	–
	Attraction	Repellency in high concentrations	Repellency	No effect	–	–	–
	PER	Repellency in high concentrations	Repellency	No effect	–	–	–

results (on the locomotion compensator), there is evidence to believe the second mechanism to have stronger support. A way to further test this hypothesis was to subject the insects to the repellents in the presence of cues from the host that are of a different nature (heat-triggered) and that are hence necessarily routed through different receptors. In this experiment, we found that three repellents, DEET, piperidine and icaridine were able to significantly reduce the insect's PER. Together with our previous findings, these results constitute strong evidence supporting the hypothesis that repellents have an effect by themselves, and that their mode of action is not regulated by interactions with other molecules' (host-related odours) receptors. It is possible that the integration of signals from different sources, mediated by distinct receptors, could be determining the behaviour of the insects, and that they evaluate the benefits and risks involved given the strength of the signals. This could explain that, while icaridine had no effect on the bugs' behaviour in the proximity of the host, it did show a reduced response in its absence. Further research is needed to elucidate the neuronal pathways of such interactions. In addition, these experiments helped validate the induction of PER as a simple, powerful and reliable bioassay for testing repellency in bugs.

4.4. The repellent performance of well-known and novel potential molecules

In this study we performed several experiments in which we tested different concentrations of distinct repellents. Our results show that, for both DEET and piperidine, there exists a dose-dependent effect on the repellency performance of the compounds. This effect was strongly evinced when testing DEET, which only seemed to be effective at the highest concentration tested, which is much lower than that used in commercial products. This suggests that DEET, although broadly used against other insects in safety-approved concentrations, would probably require higher quantities to provide protection against *R. prolixus*. In the case of piperidine, we observed that only the lowest concentration had a different performance, particularly in preventing the PER, hence suggesting that small quantities of this repellent could be enough to deter the bug from its host. However, as previously discussed, the toxicity of this compound renders it less adequate for personal protection usage (Debboun et al., 2015).

Finding novel, safer and more effective repellent molecules constitutes a major challenge in medical entomology. Much attention is nowadays directed towards finding substances that could block or activate olfactory pathways, using as experimental models *Drosophila* and mosquitoes. Among them, those targeting the *IR40a*⁺ receptor have been presented as promising solutions (Kain et al., 2013; Afify et al., 2014). This receptor, which is highly conserved across insect species, would play a key role on the effect that DEET and a series of anthranilate compounds have been proposed as repellents acting on vinegar flies and on mosquitoes (Kain et al., 2013).

Three of the substances previously shown to interact with *IR40a*⁺ receptor (methyl-, ethyl- and butyl-anthranilate), were

tested in this study using the PER triggered by heat as experimental bioassay. None of the compounds evinced repellent properties in *R. prolixus*. Given that the insects do respond to DEET, which for flies and mosquitoes is sensed through *IR40a*⁺ receptor, but do not respond to the other compounds that act through the same receptor, our results suggest that the *IR40a*⁺ receptor is not involved in repellency in this species, in spite of it being highly conserved across insect taxa.

5. Concluding remarks

In this study we shed light on the repellency of several compounds against *R. prolixus*, a main vector of Chagas disease. Importantly, we provide several easy-to-perform bioassays which can be used to test repellents and their action mode in triatomine bugs. We could determine that bugs are able to smell repellents independently of the presence of host-related stimuli, which means the compounds' action mode involves a specific receptor which binds the repellent's molecules. However, we could not discard the possibility that the repellents could be acting through two distinct pathways, both being sensed by themselves and blocking the host-associated volatiles perception. To further discern if these two mechanisms coexist, molecular and physiological studies are needed. Although our results suggest that *IR40a*⁺ receptor would not be the main target of repellents in triatomines, the molecular pathways underlying their perception also deserves further research. Finally, an important question remains on the original function that repellents' receptor would play on normal bugs' physiology and behaviour. It has been suggested that repellents receptors in mosquitoes could be originally related to their close association with plants, and that receptors for plants' volatiles might be also used to sense repellents. For haematophagous bugs, such association would not be relevant, and hence the origin and primary function of the receptor still need to be elucidated.

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