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Adaptation of the repellency response to DEET in *Rhodnius prolixus*

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ABSTRACT

For many years it has been accepted that DEET interferes with the detection of odours from the host instead of having a repellent effect. However, recent work showed that DEET acts as an odorant molecule and elicits a behavioural response in the absence of other stimuli. Therefore, DEET must promote some phenomenon connected with the stimuli–sensory system interaction, such as a sensory adaptation, where the sensory system regulates its sensitivity to different stimuli intensities during continuous or repetitive exposure. In this work, we studied different aspects of the insect–DEET interaction through behavioural observations. Previous exposure of fifth instar *Rhodnius prolixus* nymphs to DEET decreased the behavioural response to this repellent. We observed a decrease in repellence after different times of continuous stimulation with DEET in a time-dependent manner. The response to DEET was recovered 10 min after exposure, when insects were continuously stimulated during 5 or 10 min; maximum repellence was recovered 20 min after exposure when insects were stimulated for 20 min. DEET produced a repellent effect when nymphs were exposed only to its vapours. These results suggest that exposure to DEET produces adaptation in *R. prolixus* nymphs, and that the behavioural response elicited by DEET occurs via olfaction when no other stimuli are present.

1. Introduction

An insect repellent has been defined as a chemical that in insects produces oriented movements away from its source (Dethier et al., 1960). It is currently accepted that these compounds act as repellents in the vapour phase (Barton Browne, 1977). *N*,*N*-diethyl-3-methylbenzamide (DEET) is an insect repellent used worldwide. The repellent properties of DEET were discovered in 1946. Ten years later, it arrived on the market and become a successful product due to its effectiveness, persistence and low human toxicity (Frances, 2007). The effect of DEET has been proved in several insect species, including the haematophagous bugs *Triatoma infestans* (Alzogaray et al., 2000; Sfara et al., 2006) and *Rhodnius prolixus* (Sfara et al., 2008).

Although DEET has been commercially available since 1956, little is known about its mode of action. It has been widely accepted that it interferes with the detection of odours emanated from the host rather than having a repellent effect in the absence of other odour molecule stimuli (Ditzen et al., 2008; Dogan et al., 1999; McIver, 1981). However, electrophysiological and biochemical evidence shows that DEET acts as an odorant molecule (Alzogaray et al., 2000; Davis and Rebert, 1972; Syed and Leal, 2008).

As an odour molecule, DEET should promote some phenomenon associated with the stimulus–receptor interaction, such as sensory adaptation, a property of all sensory cells (Dolzer et al., 2003). In this phenomenon, the sensory system regulates its sensitivity to different stimulus intensities (Zufall and Leinders-Zufall, 2000), preventing saturation of the cellular transduction machinery and allowing the retention of high sensitivity during continuous or repetitive exposure to stimuli. Three types of sensory adaptations that differ in the time the effect lasts, the time of recovery and pharmacological properties have been identified in the olfactory receptor neurons of vertebrates and insects (Zufall and Leinders-Zufall, 2000). The duration and intensity of the stimulus determine the type of adaptation elicited. Adaptation is associated with a shift in the stimulus–response curve to higher concentrations (Borroni and Atema, 1988).

R. prolixus is a haematophagous bug distributed in the north of South America and some countries of Central America. As other haematophagous insects, it has conspicuous host-seeking behaviours associated with the detection of chemical cues, mainly olfactory, emanated by the host (Lehane, 1991). Repellents also elicit an easily measurable behavioural response in these insects (Sfara et al., 2008, 2009). Hence, we studied different aspects of insect–DEET interaction via behavioural observations. In this work we described a phenomenon of adaptation in *R. prolixus* nymphs exposed to DEET and the repellent effect of this substance in the absence of other stimuli.

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2. Materials and methods

2.1. Chemicals

N,*N*-diethyl-3-methylbenzamide (DEET) was from Aldrich (Milwaukee, WI, USA). Acetone was from Merck (Darmstadt, Germany).

2.2. Biological material

Experiments were performed using fifth instar *R. prolixus* nymphs from a colony reared in our laboratory. Insects were starved for 7–30 days after moulting and kept in an environmental chamber at 28 °C under a 12:12 L:D photoperiod. All experiments were performed at the beginning of the scotophase.

2.3. Evaluation of repellency

The device used to quantify the repellent effect of DEET is showed in Fig. 1. A circular piece of Whatman No. 1 filter paper (Whatman International Ltd., Miadstone, UK) (diameter: 11 cm) was cut into halves (Zone I and Zone II). Zone I was treated with 0.35 ml of acetone alone, and Zone II was treated with 0.35 ml of DEET dissolved in acetone (100 mg/ml). After acetone evaporation, both filter paper halves were fitted together and located on the test arena floor. A glass ring (high: 4.5 cm; diameter: 9 cm) was used to prevent the insect from leaving the filter paper. A fifth instar nymph was gently released in the centre of the filter paper and recorded with a closed-circuit digital camera (Sony, Tokyo, Japan) connected to a colour monitor (Sony, Tokyo, Japan). The image of the nymph was monitored visually and the time spent in Zone II was recorded during 300 s using a chronometer.

Results were expressed as Repellency Coefficients [RC = (Total Experimental Time − Time in Zone II)/Total Experimental Time]. RC values vary between 0 (maximum attraction) and 1 (maximum repellency). RC = 0.5 indicates that the insect spent the same time in both zones (random distribution of the insects). As controls, one insect was located in an arena where both halves were treated with acetone alone. Ten independent replicates were performed for each bioassay.

2.4. Adaptation to DEET

A circular piece of Whatman No. 1 filter paper (Whatman International Ltd., Miadstone, UK) (diameter: 9 cm) was treated with 0.5 ml of a DEET solution in acetone (200 mg/ml). After solvent evaporation, the filter paper was placed on the bottom of a circular plastic container (high: 4 cm; diameter: 9 cm) with a lid. The container was closed for 5 min to stabilize the system. A fifth instar nymph was placed into the container through a little opening, and was allowed to walk on the DEET-treated filter paper during the exposure time. Then, the repellent effect of DEET was evaluated for each individual at 0, 10, 20 and 30 min after exposure. Insects were kept in an open plastic container with a clean filter paper on its floor (DEET free atmosphere) for 5 min between measurements. Four exposure times were evaluated (1, 5, 10 or 20 min), and ten nymphs were used per exposure time. As controls, a nymph was placed on a filter paper treated with 0.5 ml of acetone alone. Ten independent replicates were performed for each bioassay.

2.5. Repellency: smell or contact?

The device showed in Fig 2A was used to evaluate the repellency produced by DEET vapours. A circular filter paper (diameter: 11 cm) was divided in half. One half was treated with 0.35 ml of acetone alone and the other half with 0.35 ml of a DEET solution in acetone (100 or 500 mg/ml). Both halves were fitted together and placed in the bottom of a plastic lid. The lid was covered with a piece of gauze, and a circular plastic container (diameter: 9 cm) was placed on the lid, keeping the gauze in place 1 mm above the treated filter paper. This prevented the nymph from coming into direct contact with the source of DEET. A fifth instar nymph was gently placed into the container through a little opening and repellency was evaluated as described above.

In another similar experiment both halves were treated with DEET (100 mg/ml); one half was placed in the bottom of the lid, while the other half was placed on the gauze (Fig 2B). This way, the nymph was exposed to DEET vapours and was able to contact the repellent while walking on the filter paper but was only exposed to DEET vapours when walking on the gauze. Repellency was evaluated as described above.

2.6. Shift of the stimulus–response curve

In order to study the shift of the stimulus–response curve of DEET after exposure to the repellent, the following experiments were performed. Repellency of three concentrations of DEET (100, 200 and 340 mg/ml) was determined in two groups of insects: one group was previously exposed to a surface treated with

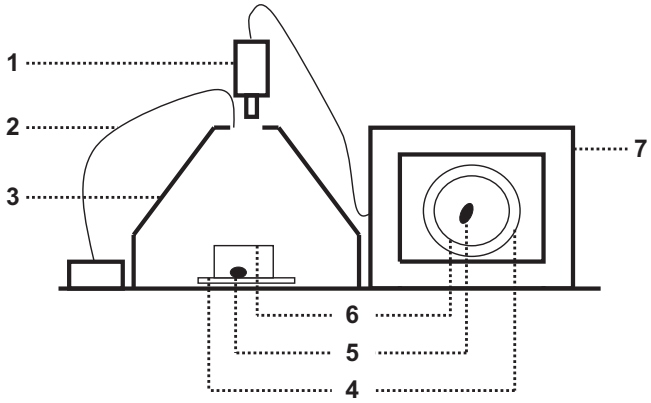

Fig. 1. Experimental arena used to determine the spatial distribution of the insects. (1) video camera; (2) optic fibre of light source; (3) dark chamber; (4) filter paper; (5) insect; (6) glass ring; (7) monitor.

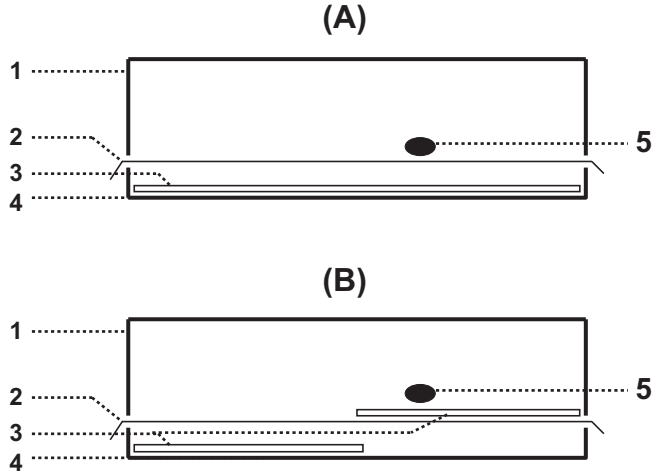

Fig. 2. Experimental device used to determine repellency caused by DEET via only vapours (A) and both contact or vapours (B). (1) plastic container; (2) gauze; (3) filter paper; (4) lid; (5) insect.

200 mg/ml of DEET during 10 min and the other one was not exposed to the repellent.

2.7. Statistical analysis

RC values were statistically compared using Kruskall–Wallis test, followed by Dunn's non-parametric post-hoc comparisons test when required. RC > 0.5 indicates repellency.

3. Results

Previous exposure of *R. prolixus* nymphs to DEET produced a time-dependent decrease in the subsequent behavioral response when insects were tested for repellency to the same substance (Fig 3). The RC value for nymphs previously exposed to DEET during 1 min was not significantly different from repellency controls (insects non-exposed to DEET) ($p > 0.05$). However, RC values were significantly lower than the repellency controls when nymphs were previously exposed to DEET during 5, 10 or 20 min ($p < 0.05$).

RC values of nymphs previously exposed to DEET and then maintained in a DEET free atmosphere are shown in Fig 4. All RC values from insects exposed 1 min to DEET were not significantly different from repellency controls ($p > 0.05$) (Fig 4A). When insects were exposed 5 or 10 min to DEET, recovery of the repellency occurred 10 min after exposure ($p < 0.05$) (Fig 4B and Fig 4C). When insects were exposed to DEET for 20 min, they recovered the repellency 20 min after exposure ($p < 0.05$) (Fig 4D).

Fig. 5 shows the repellency tested with three concentrations of DEET in insects exposed and non-exposed to 200 mg/ml of the same repellent. No decrease in the repellency was observed when insects were tested with the same or a higher concentration of DEET than the concentration used for the exposure ($p > 0.05$).

Fig. 6 shows the RC values for nymphs exposed to two concentrations of DEET vapours in an experiment where the repellent was out of reach from the nymphs. Both concentrations tested produced a significant repellent effect ($p < 0.05$). However, the RC value for the 100 mg/ml was lower than when the same concentration was tested in an experiment in which the nymphs were in contact with the repellent (see Control 1 in Fig 3).

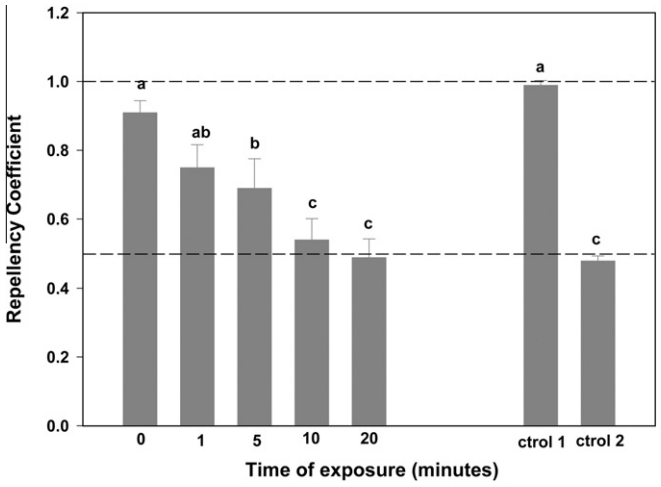

Fig. 3. Repellency Coefficients (RC) calculated for insects previously exposed to DEET for different times. Dotted lines indicate random distribution (RC = 0.5) and maximum repellency (RC = 1). Results are compared with two controls: Ctrol 1 = repellency tested in insects no previously exposed to DEET; Ctrol 2 = spatial distribution of insects exposed to an arena treated only with acetone. Repellency was tested with a 100 mg/ml solution of DEET. Different letters indicate significant differences ($p < 0.05$, Kruskall–Wallis test and Dunn's test for post-hoc comparisons).

Fig. 7 shows the RC values evaluated in a device where nymphs were exposed to DEET via both vapours and contact. The distribution of the insects was random. No significant differences were observed compared to the controls ($p > 0.05$).

4. Discussion

Although the repellent properties of DEET have been demonstrated in a number of insect species and it is the active principle of most commercially available repellents, certain aspects of its mode of action remain controversial. There are two main hypotheses regarding mode of action of DEET. The most widely accepted one proposes that DEET interferes with the detection of odours emanated by the host (Ditzen et al., 2008; Dogan et al., 1999; McIver, 1981). In this context, insects are not directly repelled by DEET, but are not oriented towards an attractant odour cue. The second hypothesis proposes that DEET is a chemical stimulus that generates an avoiding behaviour (i.e. repellency) in the insects when detected. Electrophysiological recordings from *Aedes aegypti* and *Culex quinquefasciatus* antennae in response to DEET support this hypothesis (Davis and Rebert, 1972; Syed and Leal, 2008).

Our results agree with the role of DEET as an odorant molecule. Using simple experimental designs we demonstrated that DEET is an effective repellent of *R. prolixus*. Our experimental designs allowed us to evaluate the properties of the repellent in the absence of other stimuli, as it is applied on an inert surface (filter paper). Therefore, every behavioural change observed can only be attributed to the effect of DEET.

Corbel et al. (2009) demonstrated that DEET is not only a behaviour-modifying chemical but also inhibits insect acetylcholinesterase. Symptoms of intoxication with anti-cholinesterasic compounds are well described and may be easily identified by simple observation of the intoxicated insect (Heath, 1961). Insects exposed to DEET in this study showed no signs of intoxication due to acetylcholinesterase inhibition. Moreover, the repellency observed when test concentrations of DEET were higher than the exposure concentration excludes any association of the studied phenomenon with toxic effects.

Alzogaray et al. (2000) observed a dose-dependent repellency in *T. infestans* using different experimental designs. They observed that the dose that elicited repellency in a test arena where insects were exposed only to DEET vapours was 100-fold higher than the dose that produced this effect in the arena where insects had direct contact with a DEET treated surface. In the present paper we found a dose-dependent repellency in the same range of doses used by Alzogaray et al. (2000). When *R. prolixus* nymphs were exposed to DEET vapours, the dose that elicited repellency was 5-fold higher than the repellent dose when insects had contact with the treated surface.

There are many reports of reduced responsiveness in insects after being exposed to stimuli (Boyle and Cobb, 2005; Cobb and Domain, 2000; Devaud et al., 2001; Kuenen and Baker, 1981). Several mechanisms have been proposed to explain this lack of responsiveness, either at the central (habituation) or at the receptor level (sensory adaptation). As both mechanisms are expressed at the behavioural level as a decrease in responsiveness either with constant or pulsed stimulation, the observation of the behavioural response makes it difficult to determine whether it is an adaptation or a habituation phenomenon. However, there are some features associated with sensory adaptation that allow discarding this phenomenon and confirming habituation in learning experiments. In sensory adaptation, stronger stimuli produce a higher decrease in the response; the opposite is expected in habituation, where response decrements to higher stimulus intensities take longer (Asztalos et al., 2007; Groves et al., 1969; Kuenen and Baker,

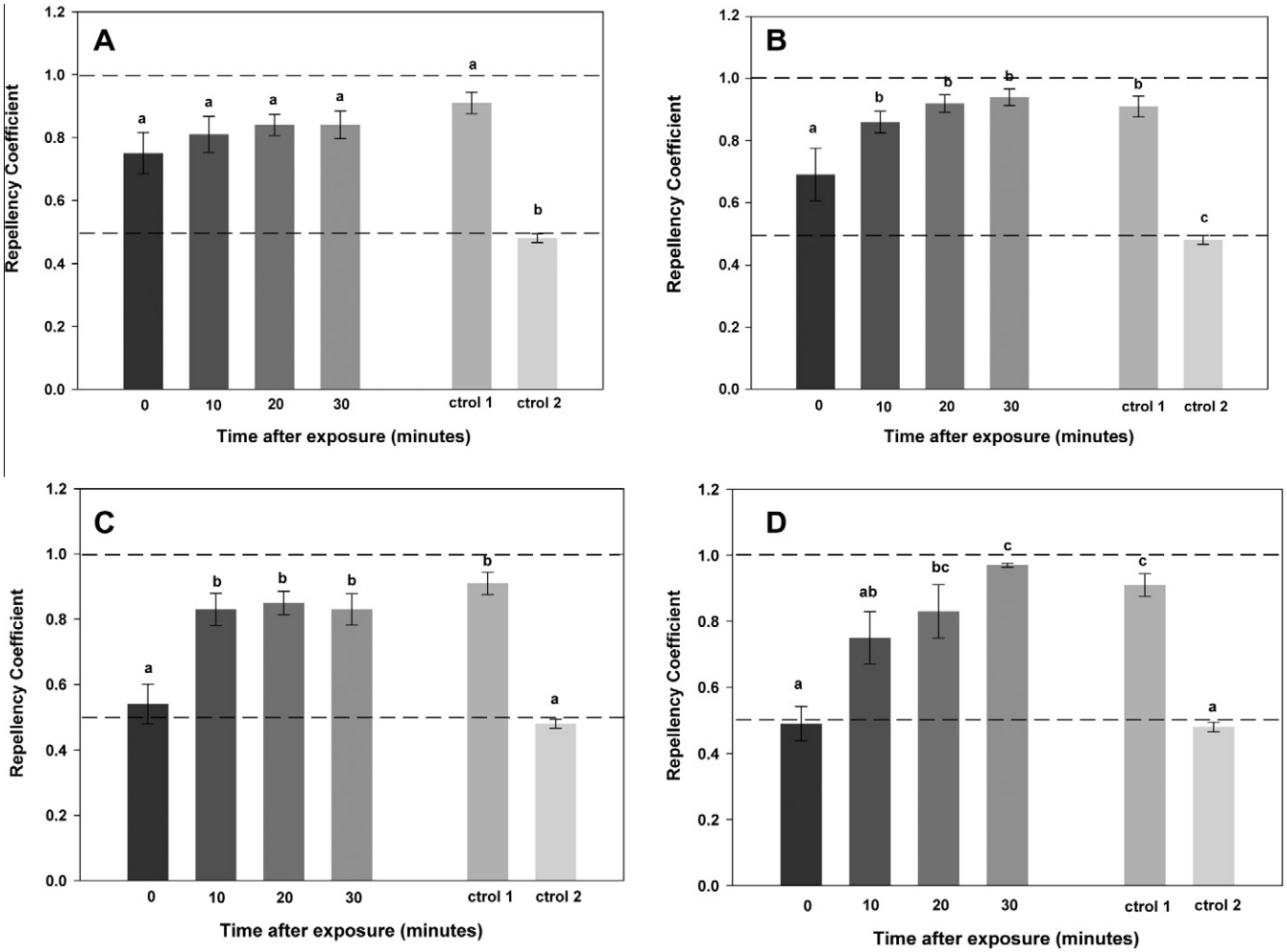

Fig. 4. Repellency Coefficients (RC) calculated for insects previously exposed to DEET and then kept for different times in an atmosphere free of the repellent. The times of previous exposure to DEET were 1 (A); 5 (B); 10 (C); or 20 (D) min. Dotted lines indicate random distribution (RC = 0.5) and maximum repellency (RC = 1). Results are compared with two controls: Ctrol 1 = repellency tested in insects no previously exposed to DEET; Ctrol 2 = spatial distribution of insects exposed to an arena treated only with acetone. Different letters indicate significant differences ($p < 0.05$, Kruskall–Wallis test and Dunńs test for post hoc comparisons).

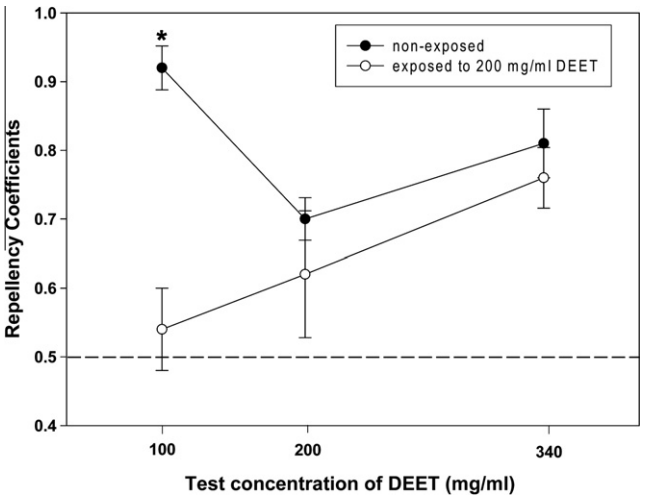

Fig. 5. Repellency Coefficients (RC) for different concentrations of DEET evaluated in insects previously exposed (white circles) or non-exposed (black circles) to DEET. Asterisks indicate significant differences ($p < 0.05$; Mann–Whitney test for two samples).

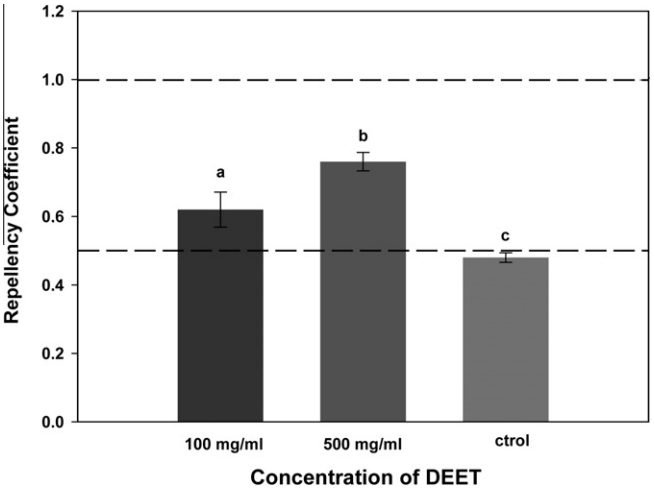

Fig. 6. Repellency Coefficients (RC) calculated for insects exposed to DEET vapours. Control groups were exposed to acetone alone. Dotted lines indicate random distribution (RC = 0.5) and maximum repellency (RC = 1). Different letters indicate significant differences ($p < 0.05$; Kruskall–Wallis test and Dunńs test for post hoc comparisons).

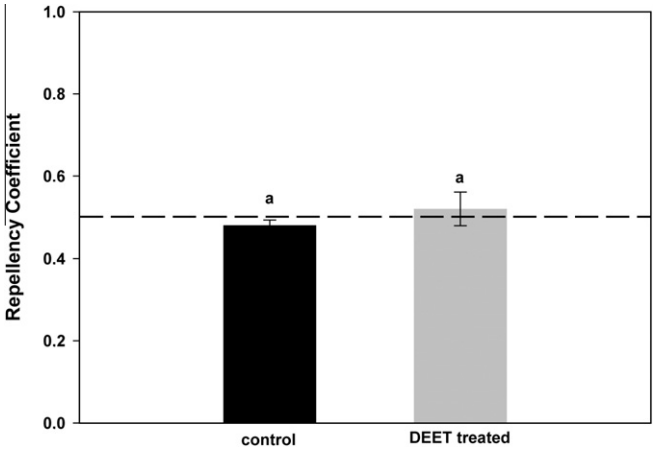

Fig. 7. Repellency Coefficients (RC) calculated for insects exposed to DEET via both vapours and contact. Control insects were exposed to filter papers treated with acetone alone. Dotted line indicates random distribution (RC = 0.5). Equal letters indicate no significant differences ($p > 0.05$; Mann–Whitney test for two samples).

1981). In addition, the recovery of the response is usually faster in sensory adaptation, while habituation shows more temporal stability (Devaud et al., 2001; Kuenen and Baker, 1981). The results presented here are coincident with the statements regarding sensory adaptation, so we consider that the decrease in repellency we observed corresponds to an adaptation phenomenon. Analyzing the dynamics of repellency extinction, we observed a gradual decrease in repellency according to the previous time of exposure to the repellent. Thus, longer exposure times caused greater decreases in repellency, as expected for adaptation. As increasing exposure times to the same concentration of DEET are equivalent to increasing doses, the adaptation is a dose-dependent effect rather than an all-or-none phenomenon.

We also report here that the behavioural response to the repellent is recovered in a few minutes when nymphs previously exposed to DEET are kept in a repellent-free atmosphere before repellency is assessed. This is consistent with the fact that adaptation quickly reverts, in contrast with central nervous system events that generally have temporal stability (Devaud et al., 2001). On the other hand, a complete loss of the repellent effect was observed in nymphs previously exposed during 10 or 20 min to DEET. Although the decrease was similar in both cases, there was a difference in the time required for reverse adaptation. The dynamics of the behavioural response to DEET recovery does not seem dependent on the amount of response lost, but on the time of previous exposure to the repellent.

Sensory adaptation produces a shift of the stimulus–response curve to higher concentrations, maintaining the sensitivity of the olfactory system (Randall et al., 1997; Zufall and Leinders-Zufall, 2000). The three concentrations of DEET used in this study showed high repellency in insects that had not been previously exposed. After exposure to 200 mg/ml, a decrease in the repellency proportional to the test concentration was observed. There was a clear displacement in the repellency curve due to exposure to the repellent. We interpret these results as evidence of sensory adaptation, since this kind of shift of the stimulus–response curve is expected in adaptation.

Insects respond less to previously experienced chemical stimuli. However previous experience does not always cause a decrease in the sensitivity of neurons and the behavioural associated response. It has been recently reported that previous experience with female sex-pheromones increases the sensitivity of neurons in the antennal lobe and its associated behavioural response in males of *Spodoptera littoralis* (Anderson et al., 2007). In these experiments insects were exposed to physiological concentrations of pheromone. Work reporting decreases in response after exposure to pheromones was performed using pheromone concentrations higher than those emitted by females (Kuenen and Baker, 1981). There may be an association between the concentration used for the pre-exposition phase and the effect observed in the responsiveness of the neurons and the behavioural response. In our work we previously exposed insects to DEET concentrations that are higher than the repellent concentrations reported by Alzogaray et al. (2000) for *T. infestans*.

As we believe that DEET interacts with the insect as a sensory input, moreover as a chemical stimulus, we wanted to determine if olfactory or contact chemoreceptors, or both, are involved in the reception of this repellent. Insects were effectively repelled whether they entered into contact with DEET or if they were exposed to DEET vapours. However, the same concentration of DEET produced a lower repellent effect when insects were exposed only to its vapours. It was evident that when insects were separated from the DEET-treated surface by a piece of gauze, more concentration was needed to elicit the same effect than when they were in direct contact with the repellent. These results suggest that olfaction is involved in the detection of DEET.

There is some evidence in the literature showing that olfactory receptors are necessary to detect DEET. In a previous work, we found that the treatment of *R. prolixus* antennae with a nitric oxide donor produced a decrease in DEET repellency (Sfara et al., 2008). These results are in agreement with results from other authors who showed that similar insect antennae treatments in different species affected the olfactory function, such as pheromone detection (Redkozubov, 2000). Moreover, electrophysiological activity of DEET at the olfactory receptor level was measured in mosquitoes and fruit fly antennae (Davis and Rebert, 1972; Syed and Leal, 2008; Syed et al., 2011).

Alzogaray et al. (2000) demonstrated that DEET repellency was detected by the sensory system of the haematophagous bug *T. infestans*. These authors showed that treatment with the sulphidryl reagent *N*-ethyl maleimide, that blocks chemoreception, produced a significant decrease in repellency. However these results were not enough to determine whether the repellent is detected by contact or olfactory (or both) chemoreceptors, since the sulphidryl reagents block either contact or olfactory chemoreception (Koyama and Kurihara, 1971; Singer et al., 1975; Villet, 1974). We asked if contact chemoreceptors present in *Rhodnius* antennae could also participate in repellent detection when insects are in contact with the DEET-treated surface. We showed lack of repellency in a particular experimental arena where insects were exposed to a DEET saturated atmosphere and only contacted the repellent in one half of the arena. In this device insects could not differentiate the half treated with DEET by means of olfaction. As insect spatial distribution was random, we speculate that contact chemoreceptors are not involved in the detection of the repellent DEET.

In summary, this work confirms the repellent effect of DEET in *R. prolixus* nymphs that are not exposed to other stimuli. We also describe for the first time an adaptation phenomenon to DEET after continuous exposure to this repellent. These results provide behavioural evidence regarding DEET as a chemical stimulus, presumably olfactory, with a repellent effect in the absence of other stimuli.

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