Effect of the combination of DEET and flupyradifurone on the tick Ixodes ricinus: Repellency bioassay and pharmacological characterization using microtransplantation of synganglion membranes

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Original article

Effect of the combination of DEET and flupyradifurone on the tick *Ixodes ricinus*: Repellency bioassay and pharmacological characterization using microtransplantation of synganglion membranes

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ABSTRACT

Ticks are vectors of many human and animal pathogens, and represent a major threat to public health. In recent years, an increase in tick-borne diseases has been observed, and new strategies are therefore needed in order to control tick numbers and reduce human tick bites. In the present study, we adapted the previous tick repellency bioassay based on the exploration behavior of the tick, using the ToxTrac software and video-tracking, to compare the repellent effect of two compounds on the tick *Ixodes ricinus*: N,N-diethyl-methyl-m-toluamide (DEET), and butenolide, flupyradifurone (FLU). We found that when applied alone, 10% DEET or FLU have no/or low repellency effect. But, the combination of both 10% DEET and FLU demonstrated a significant repellency effect against *I. ricinus*, similar to the repellency of 20% DEET. Using membrane microtransplantation, we evaluated the effect of DEET and FLU on native acetylcholine receptors expressed on the tick synganglion. We found that DEET has no effect on acetylcholine-evoked currents, but significantly reduced nicotine-induced current amplitudes. FLU induced an ionic current but was not able to reduce acetylcholine or nicotine evoked currents. The combination of both DEET and FLU strongly reduced nicotine-evoked currents. Finally, we demonstrated that our recording device for repellency, as well as the use of membrane microtransplantation, could be used as methods to study the mode of action of active compounds on ticks.

1. Introduction

Ticks are arthropods that are distributed worldwide, and can transmit numerous pathogens in the form of viruses, bacteria, protozoa, and nematodes, thus constituting a major parasite group and a health threat to both wild and domestic animals and humans (de la Fuente et al., 2008; Egyed et al., 2012; Jongejan and Uilenberg, 2004; Stuen et al., 2013). Over the past few decades, it has been demonstrated that climate change increases the risk of becoming infected with a tick-borne disease, in particular for people engaged in outdoor occupational or recreational activities (Eisen and Eisen, 2018; Ogden and Lindsay, 2016). In Europe, the castor bean tick, *Ixodes ricinus* can transmit pathogens leading to the development of tick-borne diseases such as Lyme borreliosis or tick-borne encephalitis (Barbour and Benach, 2019; Cardenas-de la Garza et al., 2019; Lindquist, 2008; Rizzoli et al., 2011). To avoid tick bites, repellents (natural or synthetic substances that causes ticks to either avoid or leave the host, stopping them from attaching, biting, or feeding) are used to prevent animal-vector contact (Pages et al., 2014). Thus, the term repellency which was classically used to describe the effects of a substance that causes a flying arthropod, such as mosquitoes, to make oriented movements away from its source, suggests for ticks, a range of behavioral responses (Halos et al., 2012). Recent studies using tick horizontal movement demonstrated that they can walk and explore a horizontal platform (Faraone et al., 2019; Herrmann and Gern, 2012; Kagemann and Clay, 2013). Ticks were placed in a petri dish containing concentric circles (outer untreated zone, treated ring, and inner untreated ring) drawn on a disk of filter paper. A volume of the selected treatment was uniformly applied on the paper ring, and an observer facing the petri dish recorded tick locations (Faraone et al., 2019). Consequently, repellency can be defined as the ability of a compound to

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cause ticks "avoiding" a particular location (for example, ticks that do not cross into the treated section) (Faraone et al., 2019; Hogenbom et al., 2021).

N,N-diethyl-methyl-m-toluamide (DEET) is considered the gold standard for evaluating the efficiency of new repellent compounds, and is the main compound present in repellents used on the skin against insect and tick bites (Bissinger and Roe, 2010; Cisak et al., 2012). DEET was therefore used to assess the repellency of several other compounds (Hogenbom et al., 2021; Lee et al., 2022). For example, the effects of using essential oil was compared to 20% DEET with the aim of demonstrating their effective tick repellent activity (Soutar et al., 2019). Following these studies, we hypothesized that mixing DEET with compounds acting on the cholinergic system, such as nicotinic acetylcholine receptors (nAChRs), could lead to an increase in the repellent effect of DEET and/or the mixture. Indeed, studies suggest that DEET alone or in combination with other compounds is able to act on the cholinergic pathways through muscarinic or nicotinic receptors (Abd-Ella et al., 2015; Abou-Donia et al., 2004; Legeay et al., 2016). For example, it can potentiate the effects of the carbamate propoxur via muscarinic receptor activation (Abd-Ella et al., 2015). Unfortunately, few compounds acting on nAChRs are used as tick repellents. The nAChR agonist, spinosad, was shown to be effective against resistant *Rhipicephalus microplus* ticks (Miller et al., 2011), and no tick resistance to this pesticide has been observed (Agwunobi et al., 2021). In addition, it was found that spinosad in rotation with amitraz was also effective for the control of the amitraz-resistant strain of the tick *R. microplus* (Jonsson et al., 2010). Consequently, we were interested in studying the repellent effect of the butanolide insecticide, flupyradifurone (FLU), in combination with DEET on the tick *I. ricinus*. FLU is a new pesticide developed in 2010 by Bayer CropScience, and commercialized under the Sivanto® formulation. It was demonstrated that FLU acts by activating the nAChRs present in the arthropod's central nervous system (Nauen et al., 2015), and is described as a pesticide with a low toxicity for non-target species (Nauen et al., 2015).

In this study, we developed an easy-to-use laboratory assays coupled to ToxTrac software video recording, and validated our method by evaluating FLU's repellent effect compared to DEET. This was carried out alongside the membrane microtransplantation method, which was recently validated for the tick *I. ricinus* (Le Mauff et al., 2020).

2. Materials and methods

2.1. Tick sample

Female adult *I. ricinus* were collected in the field, in the state forest of Chizé (Deux-Sèvres, Nouvelle Aquitaine, France). Female ticks were held at 24 h at 4°C in the dark with enough moisture to slow down their development and allow the ticks to survive for longer. They were placed

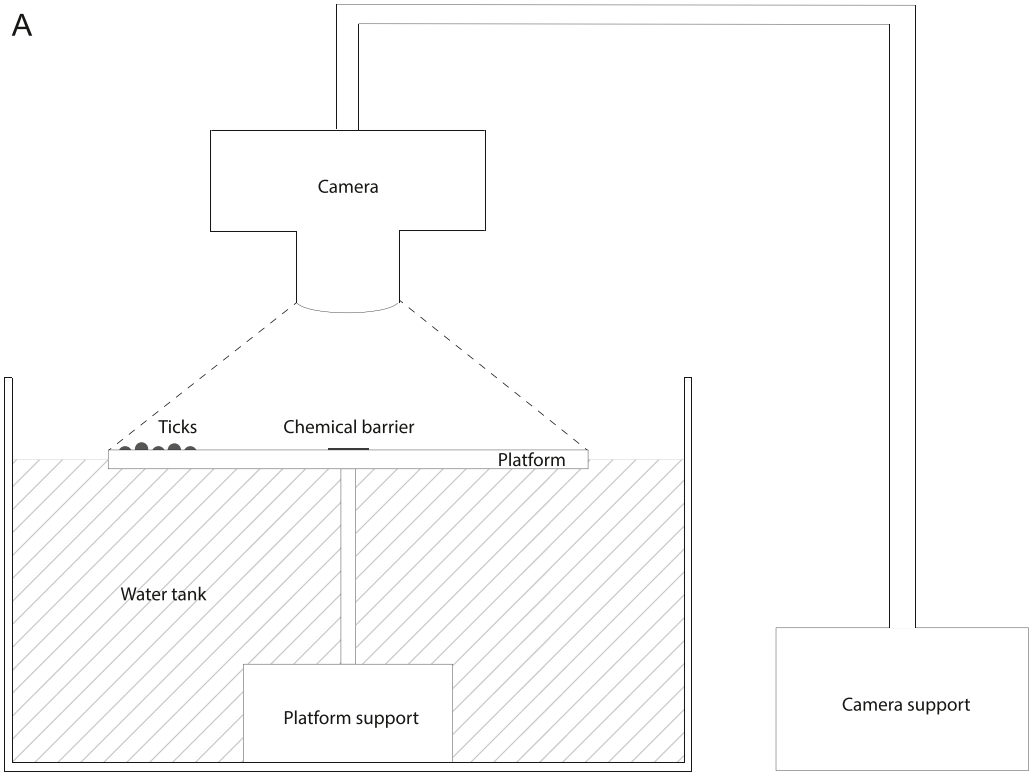

Fig. 1. Description of video-tracking set-up used in repellent behavioral assays. (A) In front view, the platform, which is surrounded by water, is fixed in the center to the top of the water tank. Half grey dots represent where ticks are released. The chemical barrier is put in the center of the platform and the camera is fixed above to record all tick movements across the platform. (B) Through the camera view, we can see the starting zone, where ticks (grey dots) are deposited at the edge of the platform (Zone 1), next the chemical barrier and then the last zone where ticks can go when they cross the barrier (Zone 2).

at room temperature 30 min before the behavioral experiment.

2.2. Repellency bioassay

2.2.1. Experimental set-up

The tick repellency bioassay was based on the exploration behavior of the tick *I. ricinus*, inspired by the work done by Nchu et al. (2012) on *Hyalomma rufipes* ticks, with the following modifications. A polystyrene platform (20×5 cm) was fixed in a plastic container (L = 36.5 cm, W = 26.5 cm, H = 14 cm) and was completely surrounded by water (Fig. 1A). The platform was completely covered with a protective paper containing a waterproof side (Sodipro, Echirolles, France). The filter paper (Whatman No. 1, 1 cm x 5 cm, Sodipro, Echirolles, France) was treated with 7.5 µl of the tested compound or solvent (absolute ethanol), placed from one side to the other in order to form a chemical barrier in the center of the filter paper. After, it was left to dry for 20 min. The filter paper was fixed to the center of the platform using double-sided tape (Fig. 1B). Next, a group of five field collected ticks was put on one side of the platform (zone 1, Fig. 1B). The ticks' movements were recorded for 30 min using a SX430 IS camera (Canon, Montevrain, France) fixed above the device. For each group of ticks, a first round of recording was performed with the solvent, corresponding to the control condition. The second round of recording was done by adding either DEET or FLU, alone or in combination, and at different concentrations (10% and 20%). For each compound and concentration, between 6 and 10 groups of ticks were used (each group corresponding to five ticks).

2.2.2. ToxTrac software

Tick mobility was recorded and analyzed using ToxTrac software (Rodriguez et al., 2017; Rodriguez et al., 2018). For video analysis, three zones were defined on the platform: the area where the tick started the experiment (zone 1); the filter paper in the center of the platform (chemical barrier, zone 3); and the final zone (zone 2), which ticks could access if they crossed the chemical barrier (Fig. 1B). Tick detection was calibrated on ToxTrac software using size (15–300 pixels) and contrast (55–80 intensity level). After the recording, the software provided data to characterize the ticks' movement on the platform. Thus, the following parameters were automatically recorded: the rate of tick presence inside zone 2 which is the rate of non-repelled ticks relative to the control, the exploration rate (over the whole platform) which is the number of explored areas/total number of areas (the platform is divided into regular 50 mm squares, without any overlaps, and the presence of ticks was recorded for each square), the mobility rate which represents the total distance covered by tick in platform/mean distance covered by the control group under the same experimental condition, and the velocity rate which is the ratio of the instantaneous speed of tick relative to the mean speed of control group under the same experimental condition. The ToxTrac software could also indicate recording bias, such as the frozen rate (proportion of time during which ticks are immobile), and the invisibility rate (proportion of time during which the software is not able to detect the tick: frame where no tick detection was made during the analysis of the video relative to control group). We performed 10 trials of each experimental condition (10% DEET and 20%, 10% FLU and 20%, combinations of DEET and FLU) on untreated and treated platforms, using the ToxTrac software and video tracking recordings.

2.3. Membrane microtransplantation experiments

2.3.1. Synganglion membrane preparation

Membrane preparation using field collected female adult tick synganglia was performed as described previously (Le Mauff et al., 2020). A pool of 150 synganglia was mechanically dissociated in 500 µl of extraction buffer (50 mM Tris/HCl, 1 mM EGTA, 3 mM EDTA, supplemented with protease inhibitors) using a pellet Argos mixer (World Precision Instruments, Hertfordshire, UK). All experiments were conducted at 4°C. The solution was first centrifuged 1 min at 1,500 rpm. The supernatant was collected in a new tube with 5 ml of extraction buffer, then homogenized and centrifuged at 30,000 rpm (154,224 g) for 70 min. The pellet containing the synganglion membranes was further concentrated using a sucrose gradient (7 ml of 20% sucrose and 5 ml of 50% sucrose, respectively), and centrifuged at 40,000 rpm (274,174 g), for 2 h 45 min. The membrane fraction located between the 20% and 50% sucrose phases was supplemented with 20 ml of extraction buffer, then centrifuged at 40,000 rpm (274,174 g), for 50 min. The pellet was resuspended in 5 ml of glycine buffer, centrifuged 45 min at 40,000 rpm (274,174 g). Finally, the pellet was collected in 100 µl of glycine buffer and stored at -80°C.

2.3.2. Xenopus oocytes preparation

Oocytes from *Xenopus laevis* (African clawed frog) were provided by the Center for Biological Resources (CRB) (University of Rennes, Rennes, France). The CRB Xenope is a French national platform dedicated to *X. laevis* breeding for experimental research. Oocytes were defolliculated according to Cartereau et al. (2018), and incubated in a standard oocyte saline (SOS) solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.5) supplemented with antibiotics (50 mg/ml gentamycin, 100 U/ml penicillin, 100 mg/ml streptomycin and 2.5 mM pyruvate). Defolliculated oocytes were microinjected with 50.6 nl of the tick membrane solution using a nanoliter injector (Nanoliter 2010, World Precision Instruments) and were incubated in the SOS solution at 18°C for 24 h before being subjected to electrophysiological measurements.

2.3.3. Electrophysiological recording

The two-electrode voltage clamp technique was used to record neuronal receptor responses from *I. ricinus* synganglion membranes micro-transplanted into *Xenopus* oocytes. Electrodes were prepared with a glass puller (model P-97, Sutter Instrument, Novato, California, USA) and filled with a 3 M KCl solution. Their resistance was controlled to be between 0.1 and 3 MΩ. After incubation, oocytes were placed in a recording chamber at room temperature (20 - 22°C), with a continuous flow of SOS supplemented with atropine. Atropine inhibits muscarinic acetylcholine receptor responses in order to be able to selectively record the nAChR responses. A Digidata-1322A (Axon Instruments, San Jose, California, USA) A/D converter was used to make the recordings, which were analyzed with pCLAMP 10 (Molecular Devices, Union City, CA, USA). The oocyte membrane potential was held at -60 mV and perfusion for each molecule tested was performed for 20 s with a flow rate of 5 ml/min. The pre-treatment of DEET or FLU was applied for 15 min before the application of the agonist. Three pre-treatment conditions were tested, each at 5 nM: 1) DEET alone, 2) FLU alone and 3) combination of DEET and FLU. After the application of each molecule, oocytes were washed with a continuous flow of SOS with atropine for 5 min.

2.4. Chemicals

Acetylcholine (ACh), nicotine (Nic), atropine, PNU-120956, DEET and flupyradifurone (FLU) were purchased from Sigma-Aldrich (St Quentin, France). They were dissolved in DMSO (dimethylsulfoxyde), with a final concentration of 0.01% DMSO for the electrophysiological recordings. DEET and FLU were dissolved in absolute ethanol for the repellent bioassay.

2.5. Statistical analysis

Two types of statistical analyses were performed. For the behavioral study, differences between the control (ticks on untreated platform) and tested conditions (ticks on a treated platform) were analyzed using a One tailed Wilcoxon paired test. Data were shown as means ± SEM of tick groups. All recorded parameters (zone 2 inside, exploration, immobility, frozen, velocity and invisibility rates) were normalized using the mean ± SEM of data under control condition using the same

experimental conditions. Data were analyzed using Prism 7 (GraphPad Software, La Jolla, CA, USA). For the electrophysiological results, currents were normalized using the mean current amplitudes under the same control conditions (I/Imean). Data were shown as means ± SEM of the current amplitudes. Differences between current amplitudes in control conditions and currents recorded under application of DEET, FLU or the combination of both DEET and FLU were analyzed using a one-way Anova and One tailed Wilcoxon paired test. The concentration giving half of the maximum response (EC_{50}) values for ACh, nicotine and FLU were determined using nonlinear regression on normalized data (1 mM ACh or nicotine as maximal response) using Prism 7 (GraphPad Software, La Jolla, CA, USA). The dose–response curves were derived from the fitted curve following the equation: $Y = Imin + (Imax - Imin)/(1 + 10(\log(EC_{50}–X)H)$ where Y is the normalized response, Imax and Imin are the maximum and minimum responses, H is the Hill coefficient and X is the logarithm of the compound concentration. For behavioral and electrophysiological experiments, "n" represents the number of ticks and recorded oocytes, respectively.

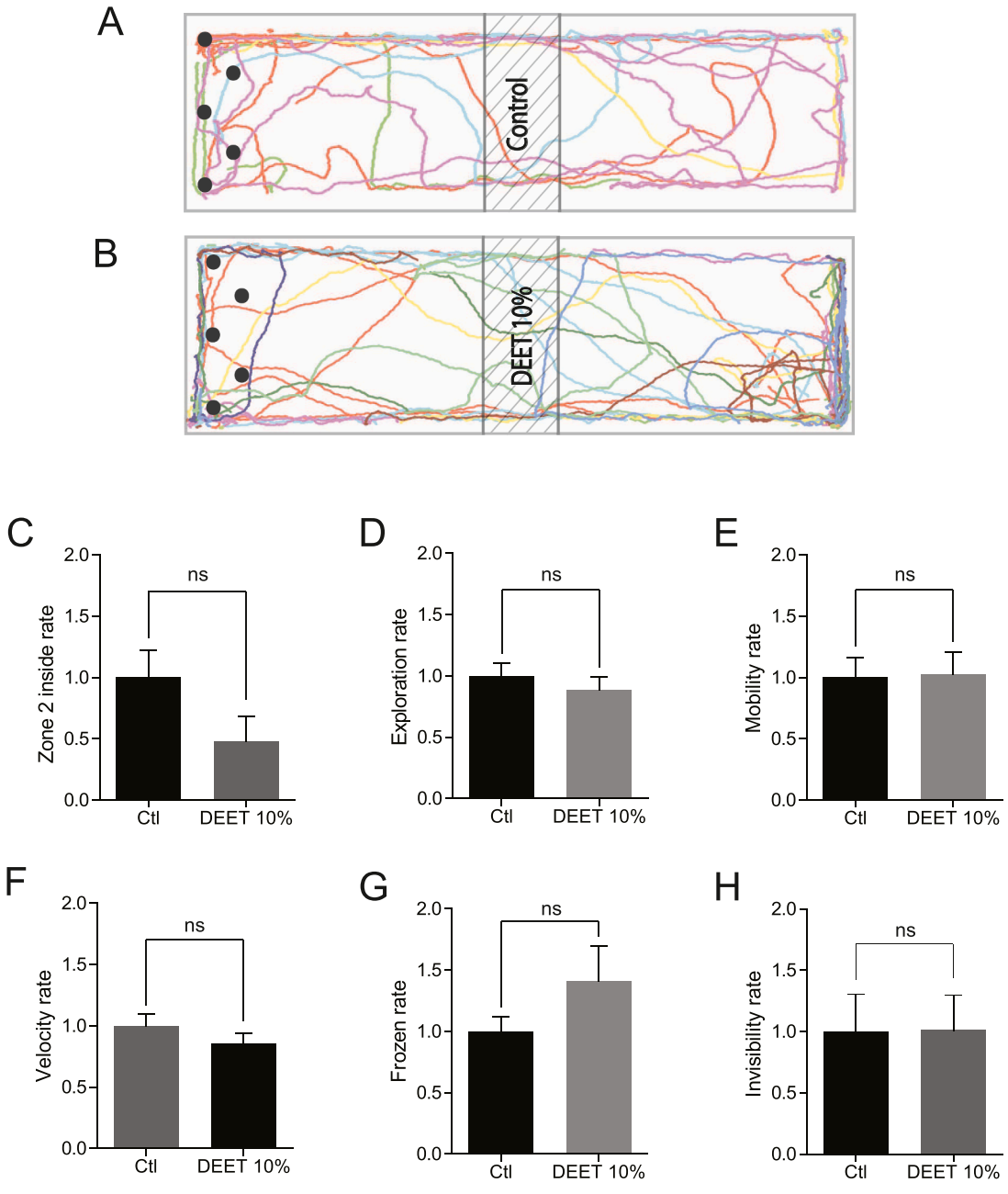

Fig. 2. Repellent effect of 10% DEET on adult female *Ixodes ricinus*. (A and B) Captures representing tick movement tracking during the video recording with (A) the control compound and (B) 10% DEET. Tick individual trajectories are labelled using different colors. (C–F) Histograms representing the different parameters which were recorded by the ToxTrac software. In particular, the zone 2 inside rate, exploration rate, mobility rate, velocity rate, frozen rate and invisibility rate. Note that for Figs. 3–6, we recorded the same parameters. Control conditions (Ctl) are represented by black bars and 10% DEET by grey bars. Data are normalized by the mean of the control values, $n = 50$. Histograms represent mean ± S.E.M, $\alpha = 0.05$; significant differences are marked with *, ns: not significant.

3. Results

3.1. Repellent effect of DEET and flupyradifurone tested alone and in combination on adult female I. ricinus

First, we evaluated the repellent effect of two different concentrations of DEET (10% and 20%), with the aim of validating the platform assay, in particular the ability of ticks to cross the chemical barrier. With 10% DEET, using the video-tracking associated to ToxTrac software video-tracking, we found that ticks were able to cross the chemical barrier as found with ticks for an untreated platform (Fig. 2A and B). This finding was confirmed when we analyzed the recorded parameters. Indeed, statistical analysis using parameters from ToxTrac software demonstrated that despite an apparent decrease, no significant differences were found for the parameter zone 2 inside rate ($n = 50$ ticks, $p = 0.0654$, Fig. 2C) which recorded the number of ticks in zone 2. Similarly, the exploration rate (number of explored areas compared to control conditions), mobility rate (distance covered by tick compared to control conditions) and the velocity rate (average speed relative to control conditions) of ticks did not present a significant difference ($n = 50$ ticks, $p > 0.05$, Fig. 2D–F). These results demonstrated that tick repellent behavior is not modified in the presence of 10% DEET. Also, the frozen rate (time that tick stayed in frozen state relative to control conditions) and the invisibility rate (no tick detection during the recording relative to control conditions) were not different between the control and 10% DEET, indicating the absence of experimental bias (Fig. 2G and H, $n = 50$

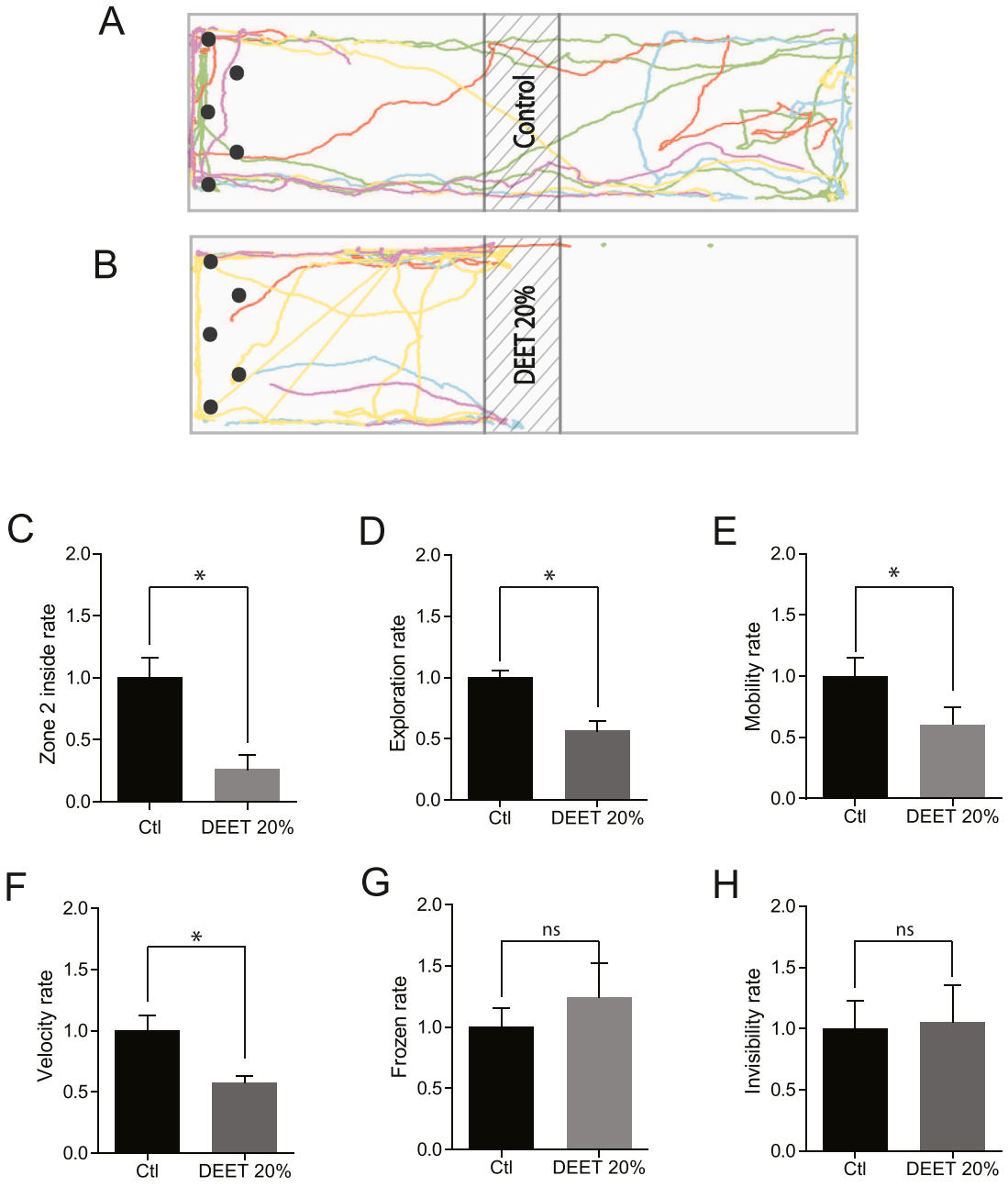

Fig. 3. Repellent effect of 20% DEET on adult female *Ixodes ricinus*. (A and B) Captures represented tick movements tracking during the video recording with (A) control and (B) 20% DEET application at the chemical barrier. Tick individual trajectories are labelled using different colors. (C–F) Histograms representing the difference between the control (black bars) and DEET (grey bars) conditions for the different parameters defined by the ToxTrac software. Data are normalized by the mean of the control values, $n = 50$. Histograms represent mean ± S.E.M, $\alpha = 0.05$; significant differences are marked with *, ns: not significant.

ticks, $p > 0.05$). With 20% DEET, we first observed that the number of ticks crossing the chemical barrier was significantly reduced, in contrast to the ticks under control condition (Fig. 3A and B). This was confirmed by the finding that the parameter, the zone 2 inside rate showed a significant decrease compared to ticks under control condition (Fig. 3C, $n = 50$ ticks, $p < 0.05$), and the exploration rate of ticks inside zone 2 decreased by 50% (Fig. 3D, $n = 50$ ticks, $p < 0.05$). The mobility and velocity rates were also significantly different in the presence of 20% DEET (Fig. 3E and G, $n = 50$ ticks, $p < 0.05$). The frozen and invisibility rates were not modified (Fig. 3F and H, $n = 50$ ticks, $p > 0.05$). These results confirmed the repellent effect of 20% DEET on the tick *I. ricinus*, as found in several studies (Buchel et al., 2015; Carroll et al., 2004).

Thus, in a second set of experiments, we evaluated the repellent effect of FLU on ticks *I. ricinus*. No significant difference was observed on the capacity of ticks to cross the chemical barrier compared to control conditions when using a dilution of 10% FLU (Fig. 4A and B). In some recordings, we found that ticks did not present trajectories along the strip edges with the zone. In correlation with this result, no significant differences were found for the zone 2 inside rate, the exploration rate, the mobility rate, or the velocity rate, when comparing the control and 10% FLU conditions (Fig. 4C, D, E and F, $n = 30$ ticks, $p > 0.05$). As with previous results, we also found that frozen rate and invisibility rate were not different between the control and 10% FLU (Fig. 4G and H, $n = 30$ ticks, $p > 0.05$). Using the same conditions, when FLU was tested at

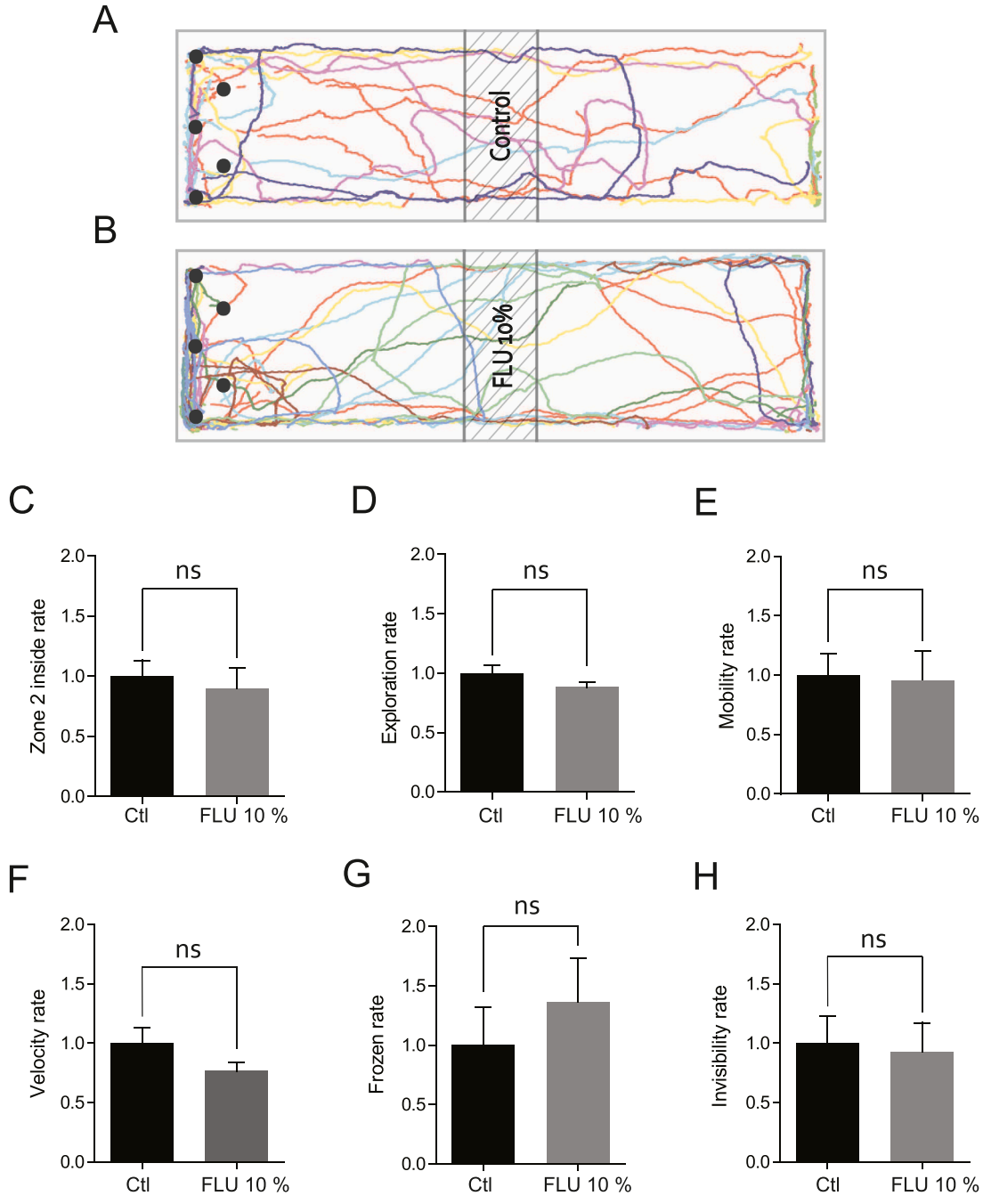

Fig. 4. Repellent effect of 10% flupyradifurone on adult female *Ixodes ricinus*. (A and B) Captures represent tick movement tracking during the video recording with (A) control and (B) 10% FLU application to the chemical barrier. Tick individual trajectories are labelled using different colors. (C–F) Histograms representing the difference between the control (black bars) and FLU (grey bars) conditions for the different parameters defined by the ToxTrac software. Data are normalized by the mean of the control values, $n = 30$. Histograms represent mean ± S.E.M, $\alpha = 0.05$; significant differences are marked with *, ns: not significant.

higher concentration (20%) no significant difference was observed compared to the control condition (Fig. 5). The platform was extensively explored by ticks both in the control and with 20% FLU (Fig. 5A and B). None of the different parameters measured showed any difference compared to the control: zone 2 inside rate, exploration rate, mobility rate, and velocity rate (Fig. 5C, D, E and F, $n = 30$ ticks, $p > 0.05$). Frozen and invisibility rates were also similar in all experimental conditions (Fig. 5G and H, $n = 30$ ticks, $p > 0.05$). These results demonstrated that in contrast to 20% DEET, FLU alone is unable to have a repellent effect on the tick *I. ricinus*. Consequently, we evaluated the repellent effect of 10% DEET and FLU in combination. Firstly, we found that a combination of 10% DEET and 10% FLU induced a repellent effect, with a reduction of the number of ticks unable to cross the chemical barrier containing the combination, in contrast to the control conditions (Fig. 6A and B, $n = 30$ ticks, $p < 0.05$). The repellent effect of the combination of DEET and FLU was confirmed by an 88% decrease in the zone 2 inside rate (Fig. 6C, $n = 30$ ticks, $p < 0.05$) and a 30% decrease in the exploration rate (Fig. 6D, $n = 30$ ticks, $p < 0.05$). The mobility rate and velocity rate were also lower in the presence of the mixture (Fig. 6E and F, $n = 30$ ticks, $p < 0.05$). Interestingly, the frozen rate and invisibility rate were similar in the control and the combination of DEET and FLU (Fig. 6G and H, $n = 30$ ticks, $p > 0.05$), confirming that the bioassay protocol was valid. Following these results, we proposed that the combination of 10% DEET and FLU has a similar effect to 20% DEET. These results also demonstrated the synergistic effect on tick behavior caused by the combination of DEET and FLU at low doses (10%).

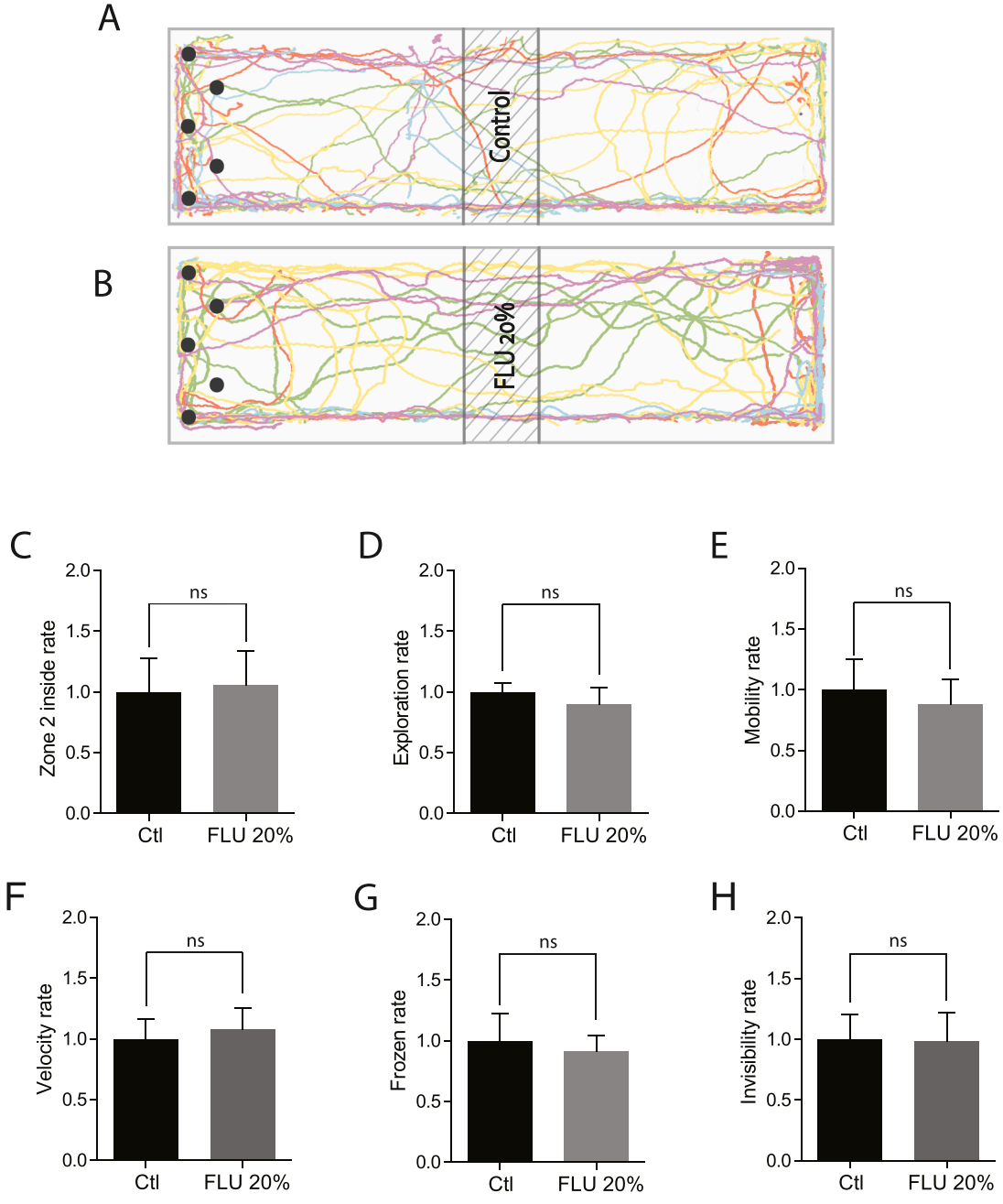

Fig. 5. Repellent effect of 20% flupyradifurone on adult female *Ixodes ricinus*. A and B represent tick movement tracking during the video recording with (A) control) and (B) 20% FLU application at the chemical barrier. Tick individual trajectories are labelled by different colors. (C–F) Histograms representing the difference between the control (black bars) and FLU (grey bars) conditions for the different parameters defined by the ToxTrac software. Data are normalized by the mean of the control values. $n = 30$. Histograms represent mean ± S.E.M, $\alpha = 0.05$; significant differences are marked with *, ns: not significant.

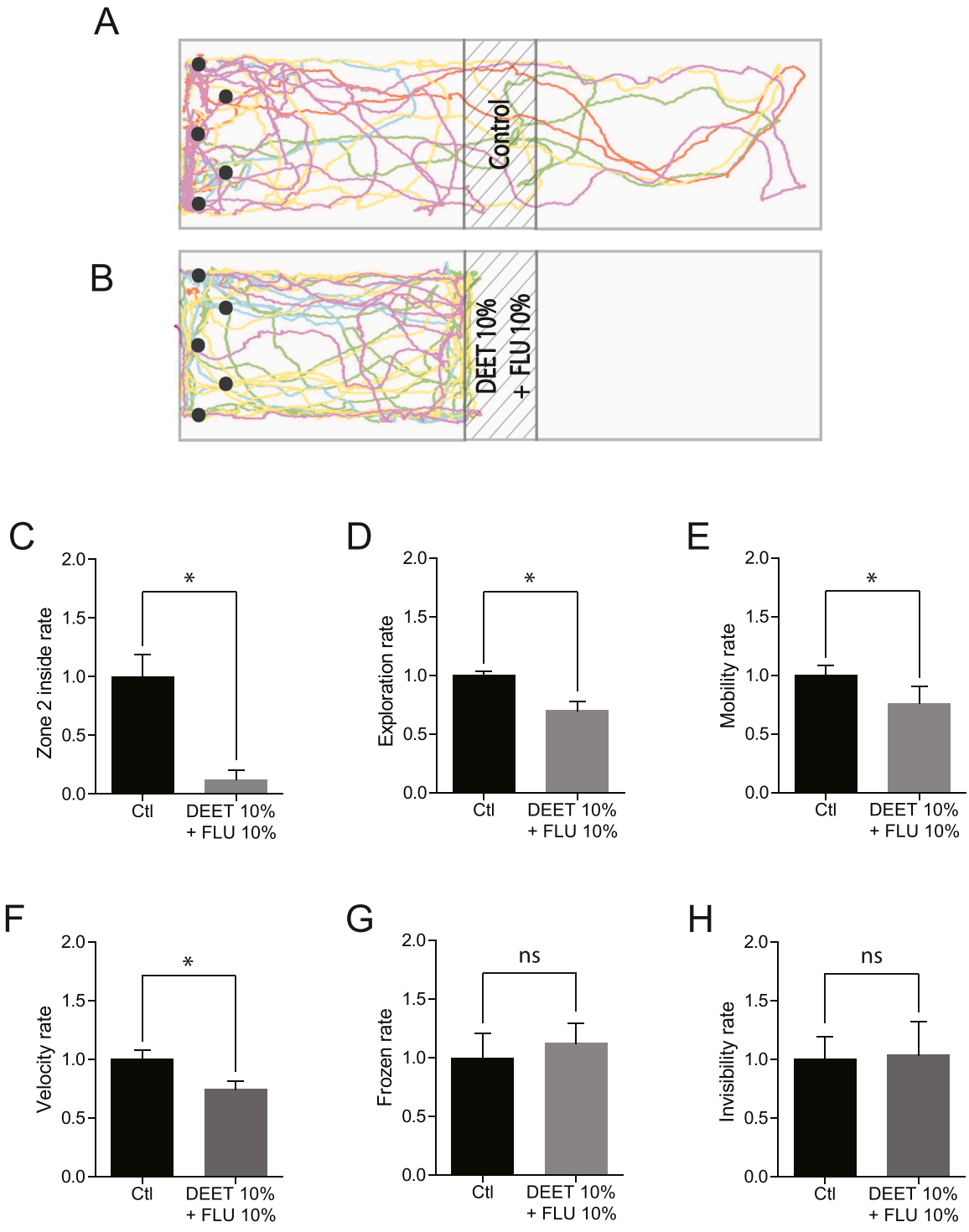

Fig. 6. Repellent effect of the combination of 10% DEET and 10% FLU on adult female *Ixodes ricinus*. A and B represent tick movement tracking during the video recording with (A) control and (B) 10% DEET /10% FLU application at the chemical barrier. Tick individual trajectories are labelled using different colors. (C–F) Histograms represent the difference between the control (black bars) and 10% DEET/ 10% FLU (grey bars) conditions for the different parameters defined by the ToxTrac software. Data are normalized by the mean of the control values. $n = 30$. Histograms represent mean ± S.E.M, $\alpha = 0.05$; significant differences are marked with *, ns: not significant.

3.2. Mode of action of DEET and flupyradifurone on native neuronal nicotinic receptors expressed on synganglion from adult female I. ricinus

As indicated in previous studies, DEET can activate or act on the arthropod cholinergic system (Koloski et al., 2019), and FLU is known to act as an agonist on nAChRs (Nauen et al., 2015). We recently used membrane microtransplantation to demonstrate that the membranes of tick synganglion in *Xenopus* oocytes express nAChRs (Le Mauff et al., 2020). We used this method to study the effect of DEET and FLU on ACh and nicotine-evoked currents. As demonstrated in Fig. 7, application of either 1 mM acetylcholine, or nicotine induced ionic currents with a dose-dependent effect confirmed the presence of functional nAChRs

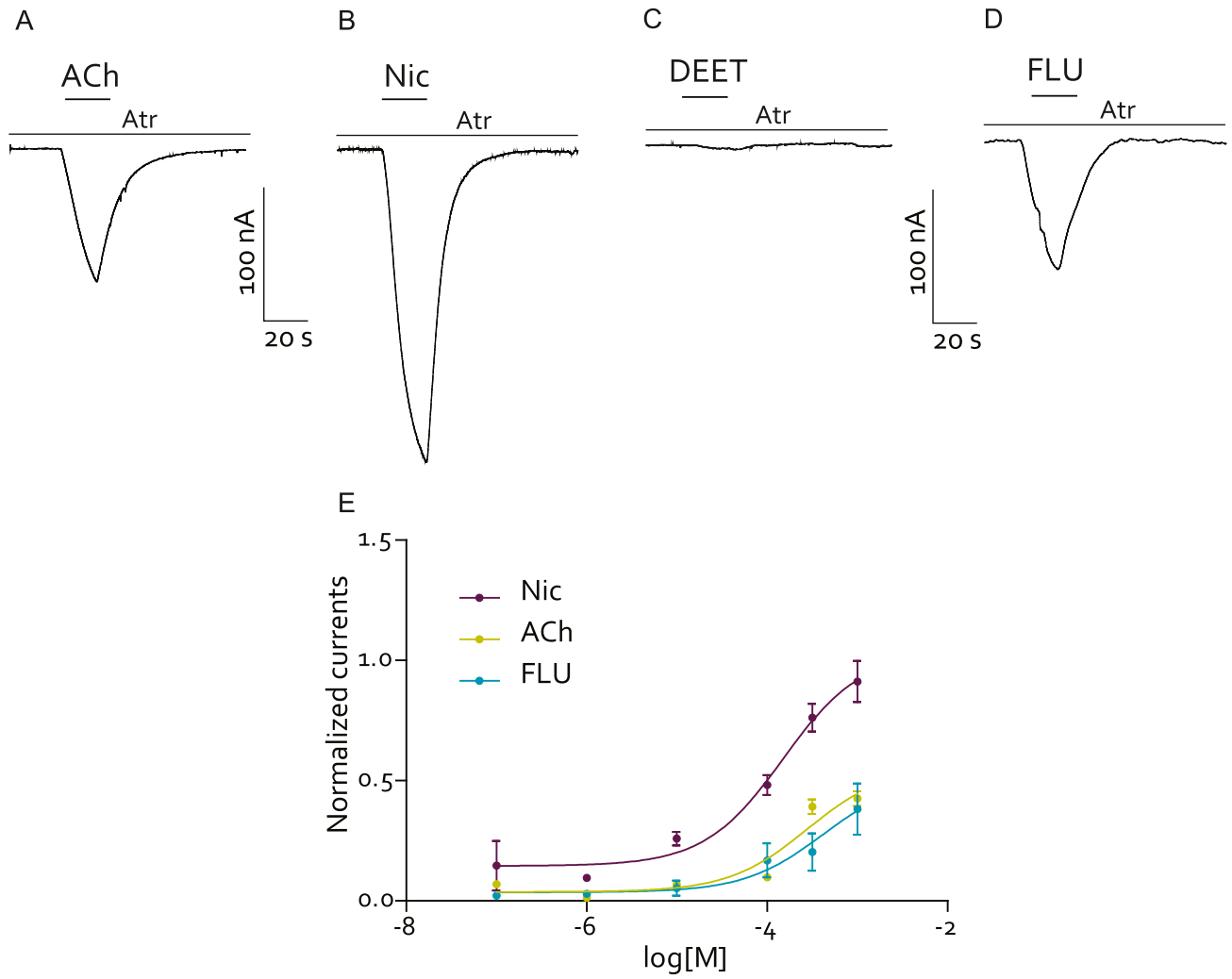

Fig. 7. Comparison of agonist action of acetylcholine (ACh), nicotine (Nic), DEET and flupyradifurone (FLU) on adult female *Ixodes ricinus* native nAChRs. Currents were recorded on oocytes microtransplanted with purified synganglion membranes. Example of 1 mM acetylcholine (ACh)- (A), nicotine (Nic) (B)-evoked currents. Bath application of 1 mM DEET did not induce an inward current (C), compared to 1 mM flupyradifurone (FLU) (D). For all illustrated currents, horizontal bar represents 20 s application of each compound. (E) Dose-response curves. Each point represents mean of $n = 15$ independent recordings. All experiments were performed in the presence of 0.5 μM atropine and 1 μM PNU-120596. Currents are normalized to the Imax of 1 mM ACh.

(Fig. 7). However, 1 mM DEET was not able to elicit an inward current whereas 1 mM FLU induced current of -78.8 ± 25 nA. This suggests that DEET did not have an agonist effect on tick nAChRs, which is in accordance with previous studies (Abd-Ella et al., 2015). Indeed, the dose-response curves showed an EC_{50} of 276 μM for acetylcholine, 149 μM for nicotine, and 521 μM for FLU, respectively (Fig. 7). To better evaluate the effect of both DEET and FLU on nAChRs, we studied their modulatory effect on ACh and nicotine-evoked current amplitudes. We found that pre-treatment of cells with 5 nM DEET (Fig. 8A, $n = 9$ oocytes, $p > 0.05$) or 5 nM FLU (Fig. 8B, $n = 6$ oocytes, $p > 0.05$) has no effect on ACh-induced current amplitudes. A similar lack of effect was also found with the combination of both 5 nM DEET and FLU (Fig. 8C, $n = 8$ oocytes, $p > 0.05$), demonstrating that the combination of both DEET and FLU has no additional effect. On the contrary, pre-treatment with 5 nM DEET significantly inhibited the nicotine-induced response by 45% (Fig. 8D, $n = 7$ oocytes, $p < 0.05$), whereas 5 nM FLU had no effect on nicotine-evoked currents (Fig. 8E, $n = 8$ oocytes, $p > 0.05$). A combination of both 5 nM DEET and FLU showed a significant inhibition of the nicotine-evoked responses by 46% (Fig. 8F, $n = 7$ oocytes, $p < 0.05$), similar to the decrease induced by DEET alone.

4. Discussion

4.1. Validation of the bioassay method on adult female I. ricinus

In the present work, we validated a new *in vitro* bioassay to study the repellent effect of the combination of DEET and FLU on adult female *I. ricinus*. The device developed here allowed the study of the explorative tick behavior on a horizontal platform to be exploited using ToxTrac software analysis (Rodriguez et al., 2017; Rodriguez et al., 2018), which made it possible to optimize the exploitation of the data by using automatic recording (Adenubi et al., 2018). Several parameters were selected in the tick behavioral assays and those described in the present manuscript are the most representative of the trajectories, immobility, and exploration of the ticks. Moreover, the parameter illustrating the time spent where the individuals were not visible to the camera allowed to demonstrate that this phenomenon is not a bias as no significant difference was observed between the control and tested groups. The study of tick avoidance behavior is widely used for the screening of new repellent compounds (Nchu et al., 2012, 2016) and several *in vitro* bioassays have been developed (Adenubi et al., 2018). *In vitro* experiments were more commonly used and were regularly performed on nymphs or adult ticks in small devices, such as petri dishes (Carroll et al.,

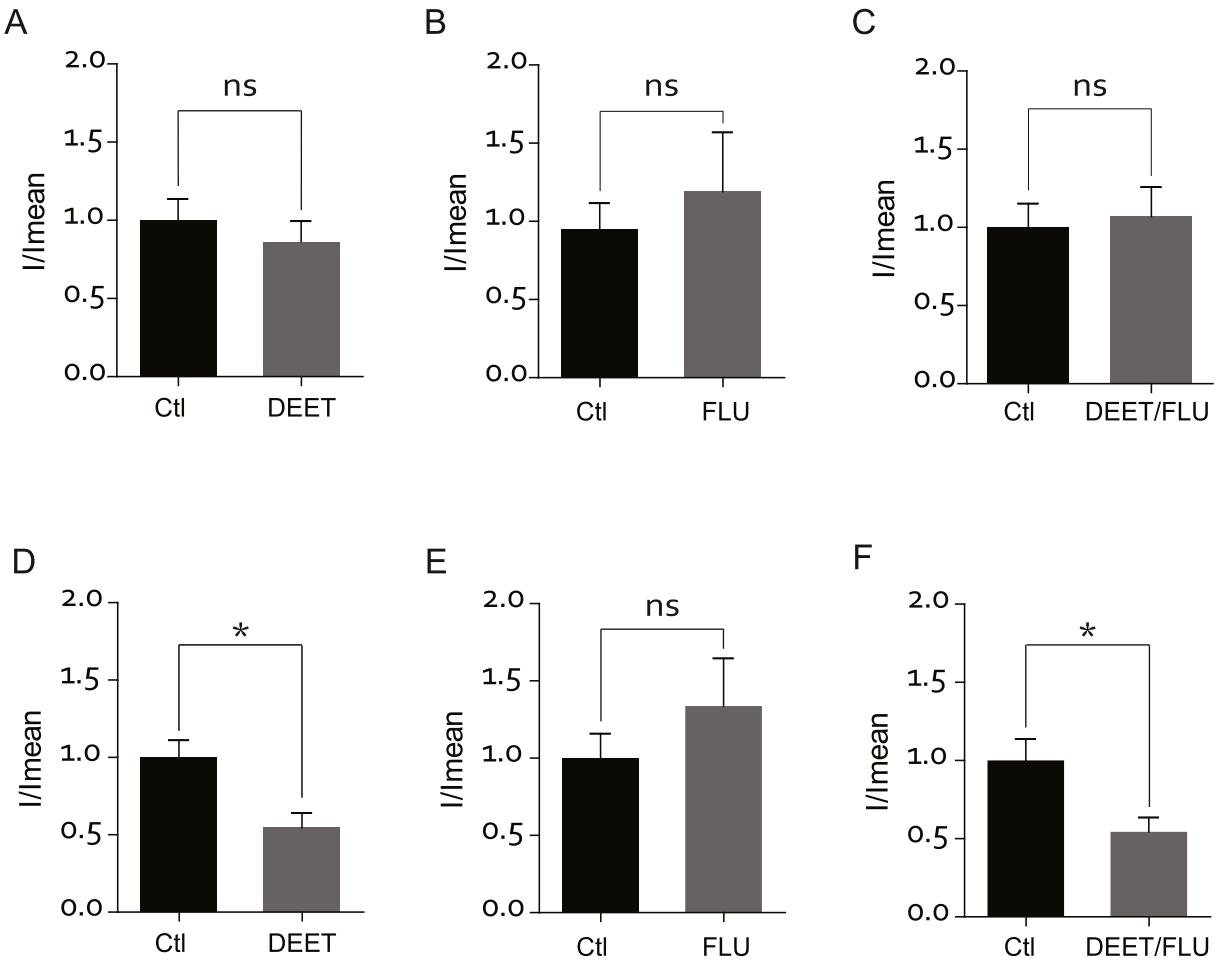

Fig. 8. Acetylcholine- and nicotine-evoked currents recorded after pretreatment of the synganglia with 5 nM DEET, 5 nM flupyradifurone (FLU) and the combination of both DEET and FLU. For each histogram, black bars, control condition (Ctl), represent acetylcholine (A–C) and nicotine (D–F) mean ± S.E.M of current amplitudes. Grey bars represent 5 min pretreatment with DEET, FLU or their combination. 5 min pretreatment with 5 nM DEET (Fig. A, $n = 9$, $p > 0{,}05$), 5 nM FLU (Fig. B, $n = 6$, $p > 0{,}05$), and the combination of both 5 nM DEET and FLU have no effect on acetylcholine-induced current amplitudes. Interestingly, pretreatment with 5 nM DEET induces a significant decrease in nicotine-induced current amplitudes (Fig. D, $n = 9$, $p < 0{,}05$). No significant difference is found after 5 min pretreatment with 5 nM FLU (Fig. E, $n = 8$, $p > 0{,}05$), but the combination of both 5 nM DEET and FLU induces a significant decrease of nicotine current amplitudes (Fig. F, $n = 8$, $p < 0{,}05$). * Significant difference. The perfusion time is 20 s.

2004; Ferreira et al., 2017). The main disadvantage of these experiments is that large amounts of repellents can quickly saturate the air within a petri dish (Adenubi et al., 2018). Another type of device, used to study the climbing behavior of ticks, is made of a platform with a rod in its center allowing the ticks to ascend and be in contact with filter papers soaked with substances to be tested. This technique has been used with several species of adult ticks, including *Rhipicephalus pulchellus* (Zorloni et al., 2010), and *Hyalomma rufipes* (Nchu et al., 2012, 2016), or with larvae, as in the case of *I. ricinus* (Tabari et al., 2017). We propose that our method could be used to study the repellent effect of new active compounds. Indeed, the ability of a tick to move on a horizontal platform has been demonstrated in previous studies and this behavior can be used to study the repellent effect of active compounds (Faraone et al., 2019; Herrmann and Gern, 2012; Kagemann and Clay, 2013). During the experiment, and as found in other studies, we found that ticks explore the platform without a specific stimulus. Despite that identifying the stimuli that could induce horizontal movements observed in ticks with an ambush strategy is beyond the scope of this study, we argue that at least two different types of behaviors could involve horizontal movements. Firstly, tick may explore horizontally their environment horizontally in order to look for a suitable support on which they could climb (and once found, exhibit vertical movements). Secondly, ticks exhibit movement on a "flat surface" (without trying to climb) once they are on the host, to look for a suitable site for blood feeding.

4.2. *Effect of flupyradifurone, DEET and their combination on tick avoidance behavior*

In the first set of experiments, we demonstrated that FLU, which belongs to the butenolide family, did not have a repellent effect on *I. ricinus,* in contrast to when DEET was applied. Although 10% DEET did not alter the explorative behavior, 20% DEET had a significant repellent effect, consistent with previous studies demonstrating that products containing 30% DEET or less provided an adequate protection (Buchel et al., 2015; Diaz, 2016; Katz et al., 2008; Soutar et al., 2019). Moreover, when using a combination of 10% DEET and FLU, we obtained a tick repellent effect which was higher than those observed for each compound alone at this concentration, demonstrating that the mixture was more efficient in terms of tick avoidance behavior. In addition, the repellent effect observed with the mixture is superior even to that obtained with 20% DEET, suggesting that it could be used as an alternative for tick bite prevention. Nevertheless, the adverse effects of FLU on human skin, at the tested concentrations, is unknown and should be considered before using it in combination. Indeed, when used alone, FLU has no repellent effect at any tested concentration and could therefore be acting as a potentiator of the DEET repellent effect. Our hypothesis is

confirmed by a recent study on the repellent effect of a combination of the pyrethroid, transfluthrin, and DEET on the mosquito, *Aedes aegypti* (da Silva Mesquita et al., 2020). The current study did not focus on either the molecular mechanisms or anatomical structures involved in DEET and FLU repellency. Indeed, ticks have specific and complex modes of parasitism. They can detect several stimuli emitted by the host through their sensorial organs (Sonenshine et al., 2002). A previous study using the thermotaxis assay on *Amblyomma americanum* and *Dermacentor variabilis* ticks demonstrated that DEET eliminated thermotaxis without affecting olfaction-stimulated host-seeking behavior (Carr and Salgado, 2019). The DEET effect was associated to the Haller's organ, as ticks were introduced in arena with a closed air circulation system and a control of warm (Carr and Salgado, 2019). Our experimental conditions using the open platform, Toxtrac software and video tracking did not fit with their experimental conditions, but we confirmed the repellent effect of DEET on tick behavior. Another study showed that the activation of ion channels by transfluthrin could have a positive synergistic effect on the repulsive activity of DEET (Andreazza et al., 2021). Similarly, a synergistic effect was recently identified between two components of pyrethrum, which can each activate a specific type of olfactory neuron (Liu et al., 2021). Thus, we investigated the agonist and modulatory action of DEET and FLU on ACh and nicotine-evoked currents. We demonstrated that DEET had no agonist effect on native nAChRs expressed on tick synganglia, but that pre-treatment with a low concentration of DEET inhibited nicotine-induced currents. FLU showed an agonistic effect but no modulatory effect on nicotine-induced currents. In our study, we cannot conclude that FLU directly activates tick neuronal nAChRs, because other acetylcholine receptors could be involved in the pharmacological properties of FLU despite that the sensitivity of native nAChRs to FLU was demonstrated using binding assays on a fly's (*Musca domestica*) head membrane preparations, as well as electrophysiological measurements on neurons isolated from *Spodoptera frugiperda* (Nauen et al., 2015). Moreover, it is conceivable that complex mechanisms occur, leading to the effect of DEET or the combination of DEET and FLU. It was recently demonstrated that DEET targets octopaminergic synapses to induce neuroexcitation and toxicity in insects (Swale et al., 2014). Thus, it is possible that the DEET effect occurred through the activation of different synapses other than cholinergic system.

5. Conclusion

The present study combined an original behavioral assay with the recent improved technique of tick membrane microtransplantation. This integrative approach has been revealed to be particularly relevant for the evaluation of the repellent effect of both DEET and FLU, due to its correlation with the mode of action of each compound at molecular level. In this work, we determined that FLU alone does not have a repellent effect on ticks, even though it could have an agonist action on native nAChRs. Interestingly, a combination of FLU and DEET proved to have a repellent effect, and the molecular mechanisms of this synergy remains to be elucidated. Nevertheless, the use of this combination could be a promising strategy for studying drug discovery in the aim of controlling tick-resistant populations.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.102079.

References

Abd-Ella, A., Stankiewicz, M., Mikulska, K., Nowak, W., Pennetier, C., Goulu, M., Fruchart-Gaillard, C., Licznar, P., Apaire-Marchais, V., List, O., Corbel, V., Servent, D., Lapied, B., 2015. The repellent DEET potentiates carbamate effects via insect muscarinic receptor interactions: an alternative strategy to control insect vector-borne diseases. PLoS One 10 (5), e0126406. https://doi.org/10.1371/journal.pone.0126406.

Abou-Donia, M.B., Dechkovskaia, A.M., Goldstein, L.B., Abdel-Rahman, A., Bullman, S. L., Khan, W.A., 2004. Co-exposure to pyridostigmine bromide, DEET, and/or permethrin causes sensorimotor deficit and alterations in brain acetylcholinesterase activity. Pharmacol. Biochem. Behav. 77 (2), 253–262. https://doi.org/10.1016/j.pbb.2003.10.018.

Adenubi, O.T., McGaw, L.J., Eloff, J.N., Naidoo, V., 2018. *In vitro* bioassays used in evaluating plant extracts for tick repellent and acaricidal properties: a critical review. Vet. Parasitol. 254, 160–171.

Agwunobi, D.O., Yu, Z., Liu, J., 2021. A retrospective review on ixodid tick resistance against synthetic acaricides: implications and perspectives for future resistance prevention and mitigation. Pestic. Biochem. Phys., 104776

Andreazza, F., Valbon, W., Wang, Q., Liu, F., Xu, P., Bandason, E., Chen, M., Wu, S., Smith, L., Scott, J., Jiang, Y., Jiang, D., Zhang, A., Oliveira, E., Dong, K., 2021. Sodium channel activation underlies transfluthrin repellency in Aedes aegypti. PLoS Negl. Trop. Dis. 15, e0009546 https://doi.org/10.1371/journal.pntd.0009546.

Barbour, A.G., Benach, J.L., 2019. Discovery of the Lyme disease agent. MBio 10 (5). https://doi.org/10.1128/mBio.02166-19.

Bissinger, B.W., Roe, R.M., 2010. Tick repellents: past, present, and future. Pestic. Biochem. Phys. 96 (2), 63–79.

Buchel, K., Bendin, J., Gharbi, A., Rahlenbeck, S., Dautel, H., 2015. Repellent efficacy of DEET, Icaridin, and EBAAP against Ixodes ricinus and Ixodes scapularis nymphs (Acari, Ixodidae). Ticks Tick Borne Dis. 6 (4), 494–498. https://doi.org/10.1016/j.ttbdis.2015.03.019.

Cardenas-de la Garza, J.A., De la Cruz-Valadez, E., Ocampo-Candiani, J., Welsh, O., 2019. Clinical spectrum of Lyme disease. Eur. J. Clin. Microbiol. Infect. Dis. 38 (2), 201–208. https://doi.org/10.1007/s10096-018-3417-1.

Carr, A.L., Salgado, V.L., 2019. Ticks home in on body heat: a new understanding of Haller's organ and repellent action. PLoS One 14 (8), e0221659. https://doi.org/10.1371/journal.pone.0221659.

Carroll, J.F., Solberg, V.B., Klun, J.A., Kramer, M., Debboun, M., 2004. Comparative activity of deet and AI3-37220 repellents against the ticks ixodes scapularis and amblyomma americanum (acari: ixodidae) in laboratory bioassays. J. Med. Entomol. 41 (2), 249–254. https://doi.org/10.1603/0022-2585-41.2.249.

Cartereau, A., Martin, C., Thany, S.H., 2018. Neonicotinoid insecticides differently modulate acetycholine-induced currents on mammalian alpha7 nicotinic acetylcholine receptors. Br. J. Pharmacol. 175 (11), 1987–1998. https://doi.org/10.1111/bph.14018.

Cisak, E., Wojcik-Fatla, A., Zajac, V., Dutkiewicz, J., 2012. Repellents and acaricides as personal protection measures in the prevention of tick-borne diseases. Ann. Agric. Environ. Med. 19 (4), 625–630.

da Silva Mesquita, R., Kyrylchuk, A., Grafova, I., Kliukovskyi, D., Bezdudnyy, A., Rozhenko, A., Tadei, W.P., Leskelä, M., Grafov, A., 2020. Synthesis, molecular docking studies, and larvicidal activity evaluation of new fluorinated neonicotinoids against Anopheles darlingi larvae. PLoS One 15 (2), e0227811.

de la Fuente, J., Kocan, K.M., Almazan, C., Blouin, E.F., 2008. Targeting the tick-pathogen interface for novel control strategies. Front. Biosci. 13, 6947–6956. https://doi.org/10.2741/3201.

Diaz, J.H., 2016. Chemical and plant-based insect repellents: efficacy, safety, and toxicity. Wilderness Environ. Med. 27 (1), 153–163. https://doi.org/10.1016/j.wem.2015.11.007.

Egyed, L., Élő, P., Sréter-Lancz, Z., Széll, Z., Balogh, Z., Sréter, T., 2012. Seasonal activity and tick-borne pathogen infection rates of Ixodes ricinus ticks in Hungary. Ticks Tick Borne Dis. 3 (2), 90–94. https://doi.org/10.1016/j.ttbdis.2012.01.002.

Eisen, R.J., Eisen, L., 2018. The blacklegged tick, Ixodes scapularis: an increasing public health concern. Trends Parasitol. 34 (4), 295–309. https://doi.org/10.1016/j.pt.2017.12.006.

Faraone, N., MacPherson, S., Hillier, N.K., 2019. Behavioral responses of Ixodes scapularis tick to natural products: development of novel repellents. Exp. Appl. Acarol. 79 (2), 195–207. https://doi.org/10.1007/s10493-019-00421-0.

Ferreira, L.L., de Oliveira Filho, J.G., Mascarin, G.M., de León, A.A.P., Borges, L.M.F., 2017. *In vitro* repellency of DEET and β-citronellol against the ticks Rhipicephalus sanguineus sensu lato and Amblyomma sculptum. Vet. Parasitol. 239, 42–45.

Halos, L., Baneth, G., Beugnet, F., Bowman, A.S., Chomel, B., Farkas, R., Franc, M., Guillot, J., Inokuma, H., Kaufman, R., Jongejan, F., Joachim, A., Otranto, D., Pfister, K., Pollmeier, M., Sainz, A., Wall, R., 2012. Defining the concept of 'tick repellency' in veterinary medicine. Parasitology 139 (4), 419–423. https://doi.org/10.1017/S0031182011002228.

Herrmann, C., Gern, L., 2012. Do the level of energy reserves, hydratation status and Borrelia infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks ? Parasitology 139 (3), 330–337. https://doi.org/10.1017/S0031182011002095.

Hogenbom, J., Istanbouli, M., Faraone, N., 2021. Novel beta-cyclodextrin and catnip essential oil inclusion complex and its tick repellent properties. Molecules 26 (23). https://doi.org/10.3390/molecules26237391.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129, S3–14. Suppl.

Jonsson, N., Miller, R., Kemp, D., Knowles, A., Ardila, A., Verrall, R., Rothwell, J., 2010. Rotation of treatments between spinosad and amitraz for the control of Rhipicephalus (Boophilus) microplus populations with amitraz resistance. Vet. Parasitol. 169 (1-2), 157–164.

Kagemann, J., Clay, K., 2013. Effects of infection by Arsenophonus and Rickettsia bacteria on the locomotive ability of the ticks Amblyomma americanum, Dermacentor variabilis, and Ixodes scapularis. J. Med. Entomol. 50 (1), 155–162. https://doi.org/10.1603/me12086.

Katz, T.M., Miller, J.H., Hebert, A.A., 2008. Insect repellents: historical perspectives and new developments. J. Am. Acad. Dermatol. 58 (5), 865–871. https://doi.org/10.1016/j.jaad.2007.10.005.

Koloski, C.W., LeMoine, C.M., Klonowski, A.R., Smith, C.M., Cassone, B.J., 2019. Molecular evidence for the inhibition of cytochrome p450s and cholinesterases in ticks by the repellent DEET. Ticks Tick Borne Dis. 10 (3), 515–522.

Le Mauff, A., Chouikh, H., Cartereau, A., Charvet, C.L., Neveu, C., Rispe, C., Plantard, O., Taillebois, E., Thany, S.H., 2020. Nicotinic acetylcholine receptors in the synganglion of the tick Ixodes ricinus: functional characterization using membrane microtransplantation. Int. J. Parasitol. Drugs Drug Resist. 14, 144–151. https://doi.org/10.1016/j.ijpddr.2020.10.005.

Lee, X., Wong, C., Coats, J., Paskewitz, S., 2022. Field evaluations of three botanically inspired repellents against the blacklegged tick, Ixodes scapularis (Acari: Ixodidae). J. Med. Entomol. 59 (5), 1694–1699. https://doi.org/10.1093/jme/tjac111.

Legeay, S., Clere, N., Hilairet, G., Do, Q.T., Bernard, P., Quignard, J.F., Apaire-Marchais, V., Lapied, B., Faure, S., 2016. The insect repellent N,N-diethyl-m-toluamide (DEET) induces angiogenesis via allosteric modulation of the M3 muscarinic receptor in endothelial cells. Sci. Rep. 6, 28546. https://doi.org/10.1038/srep28546.

Lindquist, L., 2008. Tick-borne encephalitis (TBE) in childhood. Acta Paediatr. 97 (5), 532–534. https://doi.org/10.1111/j.1651-2227.2008.00761.x.

Liu, F., Wang, Q., Xu, P., Andreazza, F., Valbon, W.R., Bandason, E., Chen, M., Yan, R., Feng, B., Smith, L.B., 2021. A dual-target molecular mechanism of pyrethrum repellency against mosquitoes. Nat. Commun. 12 (1), 1–9.

Miller, R.J., White, W.H., Davey, R.B., George, J.E., Perez de Leon, A., 2011. Efficacy of spinosad against acaricide-resistant and -susceptible Rhipicephalus (Boophilus) microplus and acaricide-susceptible Amblyomma americanum and Dermacentor variabilis. J. Med. Entomol. 48 (2), 358–365. https://doi.org/10.1603/me08222.

Nauen, R., Jeschke, P., Velten, R., Beck, M.E., Ebbinghaus-Kintscher, U., Thielert, W., Wolfel, K., Haas, M., Kunz, K., Raupach, G., 2015. Flupyradifurone: a brief profile of a new butenolide insecticide. Pest Manag. Sci. 71 (6), 850–862. https://doi.org/10.1002/ps.3932.

Nchu, F., Magano, S.R., Eloff, J.N., 2012. *In vitro* anti-tick properties of the essential oil of Tagetes minuta L. (Asteraceae) on Hyalomma rufipes (Acari: Ixodidae). Onderstepoort J. Vet. Res. 79 (1), E1–E5. https://doi.org/10.4102/ojvr.v79i1.358.

Nchu, F., Magano, S.R., Eloff, J.N., 2016. Repellent activities of dichloromethane extract of Allium sativum (garlic) (Liliaceae) against Hyalomma rufipes (Acari). J. S. Afr. Vet. Assoc. 87 (1), e1–e5. https://doi.org/10.4102/jsava.v87i1.1356.

Ogden, N.H., Lindsay, L.R., 2016. Effects of climate and climate change on vectors and vector-borne diseases: ticks are different. Trends Parasitol. 32 (8), 646–656. https://doi.org/10.1016/j.pt.2016.04.015.

Pages, F., Dautel, H., Duvallet, G., Kahl, O., de Gentile, L., Boulanger, N., 2014. Tick repellents for human use: prevention of tick bites and tick-borne diseases. Vector Borne Zoonotic Dis. 14 (2), 85–93. https://doi.org/10.1089/vbz.2013.1410.

Rizzoli, A., Hauffe, H.C., Carpi, G., Vourc'h, G., Neteler, M., Rosa, R., 2011. Lyme borreliosis in Europe. Eurosurveillance 16 (27), 19906.

Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, M., 2017. ToxId: an efficient algorithm to solve occlusions when tracking multiple animals. Sci. Rep. 7 (1), 14774. https://doi.org/10.1038/s41598-017-15104-2.

Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, P.L., Andersson, M., 2018. ToxTrac: a fast and robust software for tracking organisms. Methods Ecol. Evol. 9 (3), 460–464. https://doi.org/10.1111/2041-210X.12874.

Sonenshine, D.E., Ceraul, S.M., Hynes, W.E., Macaluso, K.R., Azad, A.F., 2002. Expression of defensin-like peptides in tick hemolymph and midgut in response to challenge with Borrelia burgdorferi, Escherichia coli and Bacillus subtilis. Exp. Appl. Acarol. 28 (1-4), 127–134. https://doi.org/10.1023/a:1025354326877.

Soutar, O., Cohen, F., Wall, R., 2019. Essential oils as tick repellents on clothing. Exp. Appl. Acarol. 79 (2), 209–219. https://doi.org/10.1007/s10493-019-00422-z.

Stuen, S., Granquist, E., Silaghi, C., 2013. Anaplasma phagocytophilum—a widespread multi-host pathogen with highly adaptive strategies. Front. Cell. Infect. Microbiol. 3 (31) https://doi.org/10.3389/fcimb.2013.00031.

Swale, D.R., Sun, B., Tong, F., Bloomquist, J.R., 2014. Neurotoxicity and mode of action of N, N-diethyl-meta-toluamide (DEET). PLoS One 9 (8), e103713. https://doi.org/10.1371/journal.pone.0103713.

Tabari, M.A., Youssefi, M.R., Maggi, F., Benelli, G., 2017. Toxic and repellent activity of selected monoterpenoids (thymol, carvacrol and linalool) against the castor bean tick, Ixodes ricinus (Acari: Ixodidae). Vet. Parasitol. 245, 86–91. https://doi.org/10.1016/j.vetpar.2017.08.012.

Zorloni, A., Penzhorn, B.L., Eloff, J.N., 2010. Extracts of Calpurnia aurea leaves from southern Ethiopia attract and immobilise or kill ticks. Vet. Parasitol. 168 (1-2), 160–164. https://doi.org/10.1016/j.vetpar.2009.10.026.