# A Novel *Ex Vivo* Bioassay Suggests DEET is an Effective Repellent of *Rhipicephalus Sanguineus*[1]

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**ABSTRACT** Ticks are vectors that pose a threat to public health. N,N-diethyl-3-methylbenzamide (DEET) is commonly applied as a repellent to prevent attachment of ticks to humans and animals. Typical commercially available repellents contain between 5–100% DEET. Lower concentrations of DEET may be necessary to minimize potential health risks associated with DEET. To characterize the repellency of low concentrations of DEET, we performed an *in vitro* vertical bioassay, and developed a novel *ex vivo* vertical bioassay using porcine skin for use with the adult brown dog tick, *Rhipicephalus sanguineus* (Latreille) (Acari: Ixodidae). DEET applied at concentrations of 0.19% *in vitro* and 12.5% *ex vivo* immediately after application, and at 0.38% *in vitro* and 40% *ex vivo* at 4 h after application, repelled over 90% of ticks. In both *in vitro* and *ex vivo* assessments, and at both 0 and 4 h post application, the repellency against female ticks was similar to that against male ticks. This study demonstrates that concentrations of DEET lower than those in commercial repellents may provide sufficient repellency when potential tick exposure occurs shortly after application. Additionally, the development of a porcine *ex vivo* bioassay provides an alternative assessment tool for future repellency studies.

**KEY WORDS** Parasitiformis, Ixodidae, repellency bioassay, DEET, porcine

Ticks are vectors of human and animal pathogens (Parola & Raoult 2001, Shaw et al. 2001), such as *Rickettsia rickettsii* (da Rocha-Lima) (Rickettsiales: Rickettsiaceae) and *Borrelia burgdorferi* (Johnson et al. emend. Baranton et al.) (Spirochaetales: Spirochaetaceae), the etiologic agents of Rocky Mountain Spotted Fever and Lyme disease, respectively (Burgdorfer 1975, Dantas-Torres 2008). Ticks require a blood meal to develop (Oliver 1989). Ticks have evolved several behaviors to ease host finding and attachment, including long grass questing behaviors, or staying near or in their host's dwelling for nidicolous species (Parola & Raoult 2001). In addition to public health concerns, ticks are responsible for livestock diseases and economic losses. For example, hard tick infestation of meat and dairy animals is linked to severe anemia and immunosuppression, and damages skin, reducing hide quality (Rajput et al. 2006). In Texas, outbreaks of cattle fever ticks (*Rhipicephalus annulatis* (Say) and *Rhipicephalus microplus* (Canestrini);

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both Acari: Ixodidae) have led to estimated loss and control efforts costs greater than US$100 million per outbreak (Anderson et al. 2010)

*Rhipicephalus sanguineus* (Latreille) (Acari: Ixodidae), commonly known as the brown dog tick, is an endophilic tick with a wide geographical distribution. It primarily attaches to dogs and occasionally humans (Gray et al. 2013). It is a known vector of the causative agents of Rocky Mountain Spotted Fever, Mediterranean Spotted Fever, Israeli Spotted Fever, Tularemia, several canine diseases (Goodman 2005), and ehrlichiosis (Bowman et al. 2004). The public health threat posed by *R. sanguineus* may increase if average global temperatures increase by as little as 2–3°C, mainly through increased distribution and seasonal activity of this species (Dantas-Torres 2010; Beugnet et al. 2011).

To discourage tick attachment, chemical repellents are typically applied to skin and clothing (Carroll et al. 2004). Most modern arthropod repellents use varying concentrations of N,N-diethyl-3-methylbenzamide (DEET) as their active ingredient. DEET concentrations in commercial products typically range from 5–100% and do not specify how long they are effective, especially for tick repellency. The Environmental Protection Agency (EPA) states that no harmful effect is found in repellents containing DEET (US EPA 2016). Further, most studies on the safety of repellents containing DEET revealed no specific harm to adult, children, pregnant women, or lactating women (Koren et al. 2003, Menon & Brown 2005). However, health concerns regarding the application of DEET have been raised in recent years (US EPA 2016), and six deaths have been associated with DEET exposure (Katz et al. 2008). The American Association of Pediatrics has recommended concentrations no higher than 10–30% for children of any age (Katz et al. 2008). In Canada, DEET-containing repellents are not recommended for children under 6 months of age, and only those containing less than 10% DEET for children younger than 12 years (Anonymous 2015). Therefore, there remains public health interest to limit exposure of high concentrations of DEET both in regard to human use and environmental exposure.

Previous studies conducted on tick repellency use one or a combination of vertical bioassays, such as the repellent treated clothing assay, the filter paper bioassay, the fingertip bioassay, the human leg bioassay, or the moving-object bioassay to evaluate repellency (Schreck et al. 1995, Büchel et al. 2015, Solberg et al. 1995, Dautel et al. 1999, Carroll et al. 2004, Dautel et al. 2013, Kröber et al. 2013). This study evaluated the effectiveness of varying concentrations of DEET using an *in vitro* vertical filter paper bioassay, as well as a novel *ex vivo* porcine skin bioassay. The porcine skin *ex vivo* bioassay was designed to emulate physiological prey cues, such as skin texture, and was tested for its suitability as a repellency assay. Porcine skin has been used in chemical absorption studies as an analog to human skin (Baynes et al. 2002), but not as a bioassay for tick repellency. The current study also attempted to identify the minimal concentration at which DEET would effectively repel *R. sanguineus* when exposed immediately after application or 4 h after application. Development of the *ex vivo* bioassay will allow researchers to study alternative repellents using the *ex vivo* model. Additionally, our findings provide new information as to the minimal concentration of DEET that may be effective as a repellent to adult *R. sanguineus*, which does not coincide with contemporarily available commercial repellents.

## Materials and Methods

**Tick acquisition and culture conditions.** A total of 400 *R. sanguineus* were purchased from the Tick Rearing Facility (TRF) at Oklahoma State University (Stillwater, OK) and maintained within a growth chamber in clean 4-L desiccator jars at 16°C and 12:12 (L:D) h photoperiod. 500 mL aqueous solution of 1.7 M potassium sulfate (Sigma Aldrich, St. Louis, MO) was added to the base of each desiccator jar as a mold inhibiting agent and to maintain relative humidity near 100%. The ticks were segregated by gender and held in small, 5 mL lid-perforated vials within these jars, in groups of ten, for one week prior to use.

Due to limited resources available for the study, ticks were reused in some instances. For the 0- and 4-h *in vitro* vertical assay (described below), 100 DEET-naïve ticks (for each exposure period) were reused a maximum of 12 times each with a minimum of 24 h between exposures to minimize chemical tolerance. For the 0- and 4-h *ex vivo* assay (described below), 200 DEET naïve ticks (for both exposure periods) were reused a maximum of three times each with a minimum of 7 d between exposures to minimize chemical tolerance. Although reuse of ticks has been described in previous studies (Pretorius et al. 2003, Jensenius et al. 2005) and efforts to avoid chemical tolerance were taken in this study, we cannot preclude unknown knock-on effects of tick reuse. Therefore, we present the data as preliminary (Schreck et al. 1995).

**In vitro vertical paper assay.** The bioassay was performed as previously described (Carroll et al. 2004) but with the following modifications. A large filter paper (9 × 4 cm) was divided into three zones 0–3 cm ("lower zone"), 3–8 cm ("repellency zone"), and 8–9 cm ("upper zone") from the bottom. A 97% DEET solution (Sigma-Aldrich, St. Louis, MO) was diluted to 50% to 0.05% with 100% ethanol (Fisher Scientific, Waltham, MA). Concentrations of DEET (μl of DEET/cm$^2$) were applied as follows: 50% (3.75 μl/cm$^2$), 25% (1.88 μl/cm$^2$), 12.5% (0.938 μl/cm$^2$), 6.25% (0.469 μl/cm$^2$), 3.125% (0.234 μl/cm$^2$), 1.56% (0.117 μl/cm$^2$), 0.76% (0.057 μl/cm$^2$), 0.38% (0.0285 μl/cm$^2$), 0.19% (0.0143 μl/cm$^2$), 0.08% (0.006 μl/cm$^{2)}$), and 0.04% (0.003 μl/cm$^2$). Ethanolic DEET solutions and ethanol controls were tested in a random order. For both 0- and 4-h post application bioassays, 150 μl of a prepared repellent concentration was applied drop-wise to the 'repellency zone' and allowed to dry for 5–10 min before use. The DEET-applied filter paper was aged for 4 h at room temperature on a plastic surface without exposure to light. Thirty male and 30 female ticks (60 ticks total) were tested for each DEET concentration in the 0- and 4-h bioassays. Since no difference in repellency was observed between males and females, the data collected for each DEET concentration and ethanol controls were grouped for statistical analysis.

Following placement on the lower zone of the filter paper, the location of each tick was monitored over a 10-min period. The ticks were considered repelled if they remained in the lower zone after 10 min, or dropped from the filter paper when approaching or crossing into the repellency zone. The ticks were considered not repelled if they crossed into the repellency zone, or traversed into the upper zone.

**Porcine skin preparation.** Porcine skin was purchased as a freshly cut "rib belly" from a Westchester County (New York State) supermarket. The cut was from the ventral side of the pig and was relatively flat. Prior to each trial, the rib belly was trimmed of fat, cut into 12 × 12 cm squares, and washed with unscented hand

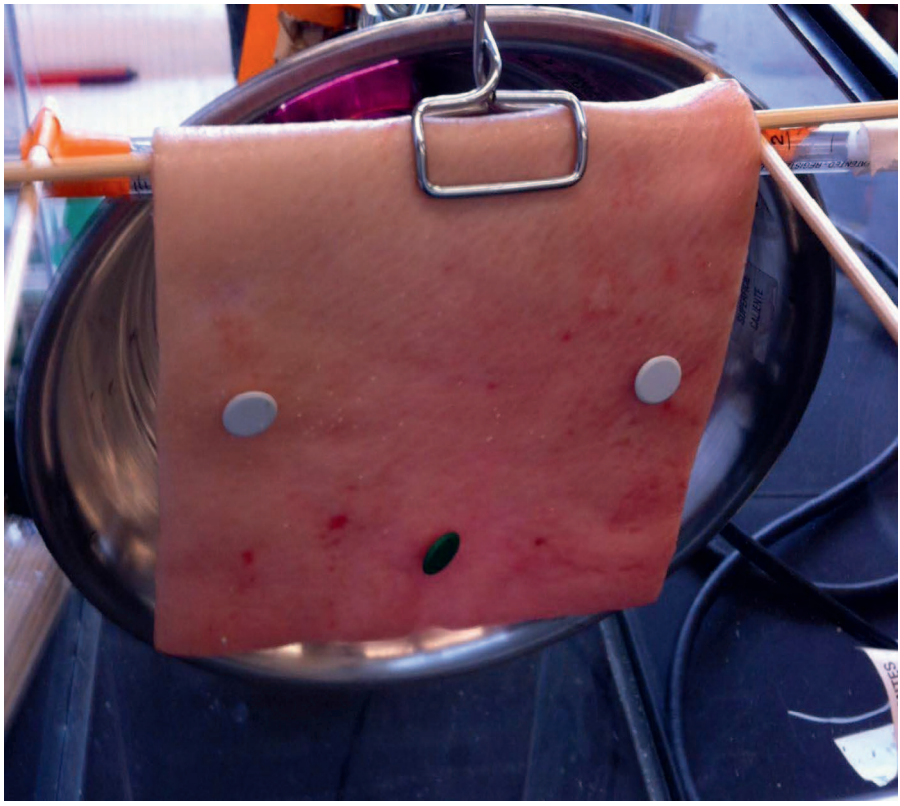

**Fig. 1.** Set up of the porcine skin *ex vivo* bioassay. The white pushpins denoted the boundary between untreated and treated skin. The green pushpin was the center of the untreated skin, where ticks were placed at the start of the experiment. When testing the effect of repellent 4 h after application of DEET, the white pushpins also served to adjoin the two pieces of skin (treated and untreated) together. The lamp was placed behind to maintain the skin at about 22°C.

soap and rinsed with tap water. During the testing, a heat lamp was positioned as necessary behind the skin to maintain the skin at approximately 22°C (Figure 1).

**DEET application to porcine skin.** Each ethanolic DEET solution was prepared as described above. Ethanolic DEET solutions and ethanol controls were tested in a random order. For each replicate, 200 μL of the prepared ethanolic DEET solution, or the ethanol control solution was applied drop-wise evenly across the upper half (6 × 12 cm) of the pig belly and spread with an unused straight razor. Ethanolic DEET solutions and ethanol controls were tested in a random order. Concentrations of DEET (μl of DEET/cm$^2$) were used as follows: 50% (1.39 μl/cm$^2$), 40% (1.11 μl/cm$^2$), 25% (0.69 μl/cm$^2$), 12.5% (0.34 μl/cm$^2$), 10% (0.28 μl/cm$^2$), and 6.25% (0.17 μl/cm$^2$). The skin was suspended vertically using 30 cm long bamboo skewers over a clean and empty 37.85-L fish tank. The untreated (bottom) half of the porcine skin hung downward.

**Tick attachment to porcine skin.** For each replicate, one tick was placed onto the center of the lower untreated area of porcine skin using forceps and monitored continuously. After the tick became active, observations were continued for 3 min. Observation time was decreased from 10 min in the *in vitro* bioassay to 3 min in the *ex vivo* bioassay to prevent desiccation of porcine skin, and to follow previously established observation protocols (Büchel et al. 2015). During that time, ticks that did not move onto the DEET-treated skin area or dropped off the skin within 3 s after crossing into the treated area were considered "repelled", while ticks that moved onto the treated upper skin and remained more than 3 s were considered "not repelled". A preliminary range finding test was completed using 5 male and 5 female ticks (10 ticks per bioassay) at the following DEET concentrations: 50.0%, 25.0%, 12.5%, 6.25%, and 3.125%. The range finding test noted repellency was lost in the *ex vivo* bioassay between 12.5% and 6.25% concentrations of DEET. Therefore, subsequent replicates were completed and focused on the following DEET concentrations: 25.0%, 12.5%, 10.0%, and 6.25%. For these tests, 30–50 ticks (with equal representation of male and female ticks) were used for these four selected concentrations. Treatment with 100% ethanol only as a control was conducted for each concentration with 5 male and 5 female ticks (50 ticks total).

Bioassays assessing the effectiveness of DEET at 4 h after the application of DEET to the substrate were conducted using the same method as described above, with the following differences. Prior to the application of DEET solution, the porcine skin was cut into two 6 $\times$ 12 cm pieces, one of which was subsequently treated. During the 4 h between application and bioassay, each piece of skin was kept in separate, sealed plastic bags to prevent excessive drying of the skin and to prevent diffusion of repellent to the untreated skin. The two pieces of skin were joined immediately prior to the bioassays. The untreated piece of skin was positioned to allow a 0.5 cm overlap of the treated skin along the 12-cm edge, and pushpins were inserted through both pieces of skin in this overlapping area to achieve attachment. DEET solutions with concentrations of 25.0%, 12.5% 10.0% and 6.25% were initially tested, followed by concentrations of 50.0% and 40.0% in order to characterize the loss of repellency in concentrations below 50% DEET. 30 to 50 ticks (with equal representation of male and female ticks) were performed for each concentration. 10 male and 10 female ticks were used for the ethanol control (20 ticks total).

**Statistical analysis.** First, male and female tick data were analyzed to determine if differences in repellency existed between male and female ticks for the various DEET solutions and bioassays by Sidak's multiple comparisons test at $\alpha = 0.05$. Since no significant trends were found between genders, individual data points of ticks studied for repellency at a given DEET solution and time (0 or 4 h) were grouped by bioassay (where ticks were considered either 'repelled' or 'not repelled'). Grouped data were analyzed to determine if they were parametric or non-parametric by D'Agostino-Peason Ominbus-K2 normality test. As data for each condition were found to be non-parametric, Kruskall-Wallis test with post-hoc Dunn's multiple comparisons test were completed to determine significance differences at $\alpha = 0.05$. Statistical analyses and other statistics presented (average repelled, SEM, and figures) were performed using GraphPad Prism version 6.07 for Windows (GraphPad Prism 2015, Miller 2003). Chi-square values were calculated using the GraphPad QuickCalcs (GraphPad 2017).

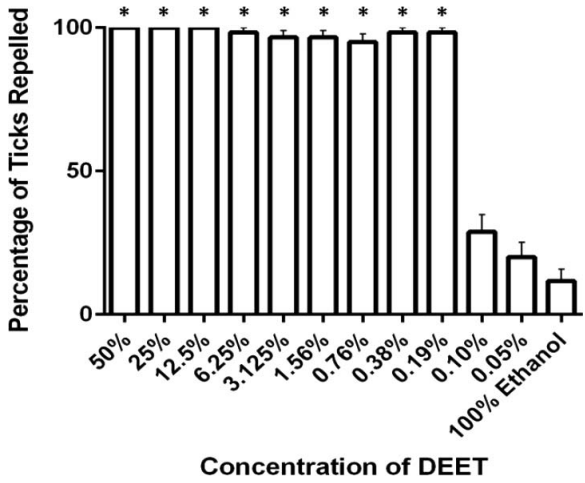


**Fig. 2.** Average numbers (means ± SEM) of ticks repelled immediately after application of varying concentrations of DEET in *in vitro* vertical filter paper bioassays. 60 ticks were studied per DEET solution, and 60 ticks were studied at the 100% ethanol control. Means topped with '*' were significantly different according to Dunn's multiple comparison test at $\alpha = 0.05$.

## Results

**In vitro vertical filter paper bioassay.** DEET was a highly effective repellent for *R. sanguineus* at very low concentrations in the *in vitro* bioassays. DEET applied at concentrations of 0.19% to 50% repelled 95–100% of ticks when the ticks were exposed immediately after application, and the ticks were more significantly repelled than those in the ethanol control group ($X^2 = 39.135$ df $= 12$, $P < 0.0001$) (Figure 2). Tick repellency decreased to 28.8% and 20% when DEET concentrations were lowered to 0.1% and 0.05%, respectively. Repellency at 0.1% and 0.05% were not significantly different than control. The ethanol control repelled 11.6% of ticks. No significant difference in repellency was observed between male and female ticks ($X^2 = 1.2378$, df $= 1$, $P = 0.2659$) (Figure 3A).

At 4 h after the application, 0.38% and 0.76% DEET were effective at repelling 100% and 96.7% of ticks, respectively; the repellency was significantly greater than that of the ethanol control ($X^2 = 21.1085$ df $= 3$, $P < 0.0001$) (Figure 4). Only 55% of ticks were repelled at 0.19% DEET, which was significantly less repellent when compared to the higher concentrations. No significant difference in repellency was noted between male and female ticks ($X^2 = 1.8092$ df $= 1$, $P = 0.1786$) (Figure 3B).

**Ex vivo porcine skin bioassay.** Immediately after application to the substrate, DEET applied at concentrations of 12.5% and 25% was effective at repelling 88% and 95% of ticks, respectively ($X^2 = 23.5134$, df $= 4$, P $< 0.0001$) (Figure 5). A 10% DEET concentration repelled 60% of ticks, which was significantly less repellent compared to the higher concentrations. Repellency of DEET applied at 6.25% was not significantly different to the ethanol control group. There was no

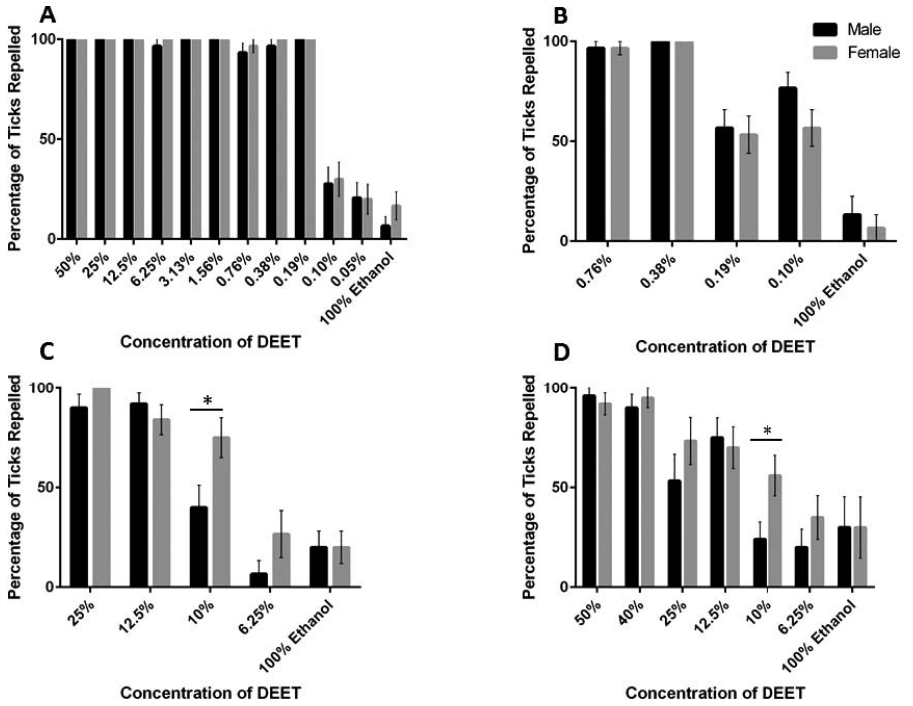


**Fig. 3.** Average numbers (means ± SEM) of male (black bars) and female ticks (grey bars) repelled by varying concentrations of DEET in A) *in vitro* bioassay immediately after application; B) *in vitro* bioassay 4 h after application; C) *ex vivo* bioassay immediately after application; and D) *ex vivo* bioassay 4 h after application. Means topped with '*' were significantly different according to Sidak's multiple comparisons test at $\alpha = 0.05$.

significant difference in repellency against male and female ticks immediately after application of DEET solutions to pig skin, with the exception of 10% ($X^2 = 4.7382$, df = 1, $P = 0.0295$) (Figure 3C).

At 4 h after application, 50% and 40% DEET solutions were able to repel 94% and 92.5% of ticks, respectively, significantly more than lower concentrations and ethanol control ($X^2 = 27.8569$, df = 6, $P < 0.0001$) (Figure 6). DEET solution at 6.25% repelled 27.5% of ticks, and was not significantly different in repellency to the ethanol control. There was no significant difference in repellency between male and female ticks at 4 h after application of DEET solutions to pig skin, with the exception of 10% ($X^2 = 2.9666$, df = 1, $P = 0.0850$) (Figure 3D).

## Discussion

Traditional studies comparing tick repellency to chemical repellents have utilized the *in vitro* filter paper bioassay, the human fingertip bioassay, and the

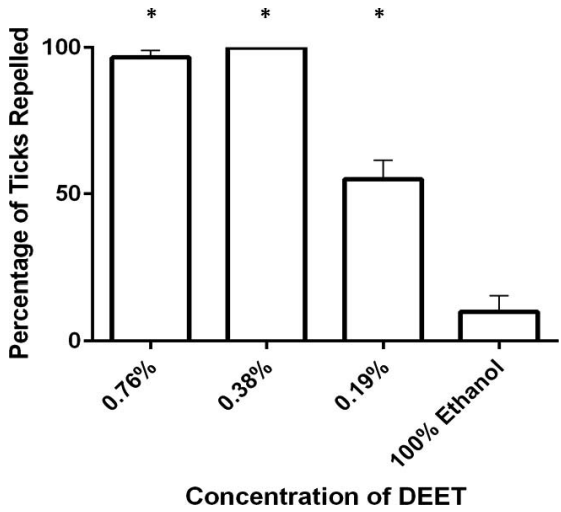


**Fig. 4.** Average numbers (means ± SEM) of ticks repelled 4 h after application of varying concentrations of DEET in *in vitro* vertical filter paper bioassays. 60 ticks were studied per DEET solution, and 30 ticks were studied for the 100% ethanol control. Means topped with '*' were significantly different according to Dunn's multiple comparison test at $\alpha = 0.05$.

simulated–moving-bioassay (Dautel 2004). The filter paper bioassay offers an inexpensive, quick and convenient method to examine the tick response to the tested repellent. Though economical, the vertical assay lacks certain motivational cues inherent to tick motivation, such as but not limited to residual $CO_2$ trails, skin and hair texture cues, and vibration and movement, all of which favorably impact tick attachment (Balashov 1968, Steullet & Guerin 1992, Dautel et al. 1999). Techniques such as the moving bioassay and fingertip bioassay overcome these barriers, but exacerbate other issues, such as recruitment of volunteers and potential chemical exposure, as well as a potentially cost-prohibitive associated setup, especially for research at Primarily Undergraduate Institutions (PUIs) and other small institutions. In this study, we developed a simple and inexpensive alternative to these bioassays based on an *ex vivo* porcine skin repellency bioassay to simulate tick motivation cues. Pig skin offers a more accurate simulation of mammalian physiological conditions, such as those associated with human skin, including cues related to skin texture, the presence of hair follicles, host odor, and vertical climbing cues (Balashov 1968). In addition to putatively increasing tick motivation, the porcine skin model offers several advantages over the traditional fingertip bioassay. It eliminates the requirement of human volunteers for tick repellency studies, reduces volunteer chemical exposure, and removes the necessity for institutional review board approval; making this an applicable model to PUIs and other institutions.

Admittedly, there are drawbacks associated with the *ex vivo* porcine skin bioassay. While porcine skin provides mammalian physiological cues to ticks, it does not

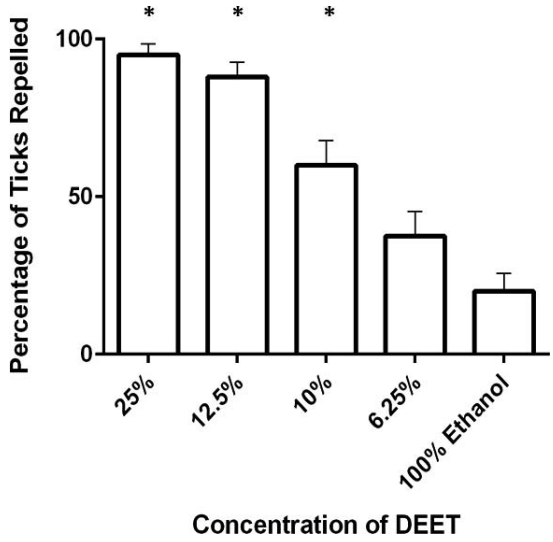


**Fig. 5.** Average numbers (means ± SEM) of ticks repelled immediately after application of varying concentrations of DEET in *ex vivo* porcine skin bioassays. 30 to 50 ticks were studied for each concentration, and 50 ticks were tested in the ethanol control. Means topped with '*' were significantly different according to Dunn's multiple comparison test at $\alpha = 0.05$.

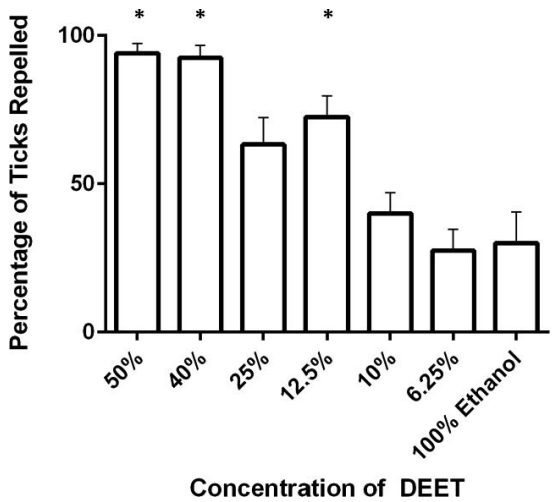


**Fig. 6.** Average numbers (means ± SEM) of ticks repelled 4 h after application of varying concentrations of DEET in *ex vivo* porcine skin bioassays. 30 to 50 ticks were studied for each concentration, and 20 ticks were tested in the ethanol control. Means topped with '*' were significantly different according to Dunn's multiple comparison test at $\alpha = 0.05$.

perfectly model the physiological and environmental conditions of human skin, which may provide additional attachment motivation to the tick. One cue that could not be precisely mimicked by the *ex vivo* porcine skin bioassay is the temperature of human skin. Our initial trials determined that the porcine skin bioassay works well at or slightly above room temperature (i.e., 22°C), which prevents the skin from drying (Meade, personal observation). While this is not physiological mammalian internal temperature, this temperature, which was higher compared to the laboratory (approximately 20°C), approximates a change in temperature when in contact with human skin, which is typically below physiological body temperature (31–34°C) (Kopp & Haraldson 1983, Andal et al. 2011). An additional concern is the treatment of the thick subdermal adipose layer found in pork belly skin. This thick layer of tissue must be cleaned and removed thoroughly prior to use of the porcine skin. Therefore, care must be taken during cleaning and fat removal to avoid using perturbing chemicals, such as strong solvents or perfumed soaps, that may impact tick repellency. As such, control data was useful to ensure that chemicals used in washing the skin were satisfactorily removed prior to experimentation and did not impact the observed results.

DEET was found to be more highly repellent to ticks at very low concentrations (e.g., 0.19% or 0.0143 $\mu$l/ cm$^2$) in *in vitro* filter paper bioassay when compared to a 12.5% (0.34 $\mu$l/ cm$^2$) DEET solution necessary to repel ticks in *ex vivo* porcine skin bioassay. While not exactly comparable, the increased concentration of DEET required for the *ex vivo* assay may be due to the different absorption characteristics of porcine skin when compared to filter paper; the porcine skin may more effectively absorb DEET similar to previously studied solvents (Shang et al. 2014). This may lead to a lower concentration of DEET being present on the surface of the skin and thus available as a repellent to ticks. Alternatively, ticks could be less deterred by higher concentrations of DEET when in the presence of porcine skin due to increased motivation from the aforementioned physiological cues. Similar results demonstrated 4 h after application of DEET solutions to pig skin may also be explained by the above mentioned rationales. Although the porcine skin bioassay may require modifications to increase efficacy for future studies, it provides an inexpensive, safe, and alternate method to test for tick repellency.

In addition to a different repellency pattern, there was also a higher fluctuation amongst replicates in the porcine skin bioassay (Figures 5 and 6) than in the filter paper bioassay (Figures 2 and 4). This fluctuation may be attributed to modest variations in porcine skin samples along with the above mentioned tick motivation cues, such as a slight change in light pattern or vibration during replicates, both of which simulate animal movement, which may have influenced tick climbing behavior (Balashov 1968).

DEET applied at 12.5%, which is lower than the concentrations in most commercial products, was effective in repelling ticks immediately after application in the porcine skin bioassay and the *in vitro* bioassay. The results imply an alternative effective concentration for DEET used as a tick repellent. The Centers for Disease Control and Prevention (CDC) states that when 20% DEET (typically described as applied at 1–2 $\mu$l/cm$^2$) is applied to skin it can protect humans from ticks for several hours (CDC 2015). This statement is supported by data collected previously from *Ixodes* nymphs (Büchel et al. 2015), and is similar to our findings for 40% (1.11 $\mu$l/cm$^2$) and 50% DEET solutions (1.39 $\mu$l/cm$^2$) at 4 h post application. Our results from the *ex vivo* bioassay suggests that DEET solutions lower

than 20% (12.5% or 0.34 μl/cm$^2$) may repel ticks when used as a short-term repellent, such as for field workers, short trail hikes, or short periods of clearing brush around a household. Using different solutions of DEET depending upon the task may aid in reduction of human exposure to DEET. Equally important is that when DEET is applied to clothing or another surface with decreased tick motivation similar to the *in vitro* bioassay, lower solutions of DEET may be applied for prolonged durations of potential tick exposure. Admittedly, field work may create conditions which impact the rate of DEET degradation which were not present in our current study. Future studies addressing these factors are warranted.

There are 120 products containing DEET that are currently registered by the EPA (US EPA 2016). Many tick repellents have been compared for their effectiveness and safety. Among them, permethrin is regarded as one of the most effective tick repellents (Bissinger & Roe 2010). Other chemicals, such as dodecanoic acid (DDA)-formulations and DEPA (N, N-diethyl penylacetamide), have also been widely studied for their effectiveness as repellents (Kalyanasundaram & Mathew 2006, Schwantes et al. 2008). Our study demonstrates that DEET is an effective repellent using the *in vitro* filter paper bioassay. However, a higher concentration of DEET is required to provide similar repellency when using the *ex vivo* porcine skin bioassay. We also found lower tick repellency of both bioassays 4 h after application of DEET solutions. Future studies should focus on utilizing the *ex vivo* bioassay as a potential model for comparing alternative repellents, testing additional tick genera and developmental stages, and modifying the *in vitro* filter paper bioassay to more closely mimic clothing and field conditions.

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