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SHORT COMMUNICATION

Comparative Activity of Deet and AI3-37220 Repellents Against the Ticks *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) in Laboratory Bioassays

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ABSTRACT The repellents *N,N*-diethyl-3-methylbenzamide (deet) and racemic 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220) were evaluated using two different laboratory bioassays to determine their relative effectiveness against host-seeking nymphs of the blacklegged tick, *Ixodes scapularis* Say, and the lone star tick, *Amblyomma americanum* (L.). In a petri dish bioassay, ticks were released within a ring of repellent on a horizontal filter paper disk. In the second bioassay, ticks were allowed to climb a vertical strip of filter paper whose central portion was treated with a repellent. Deet and AI3-37220 were more effective against *I. scapularis* than *A. americanum* nymphs. In the petri dish bioassay, none of the concentrations of deet or AI3-37220 tested confined *A. americanum* within the treated ring. However, in the vertical bioassay, both species exhibited avoidance of the repellents, and *I. scapularis* was repelled by much lower concentrations than *A. americanum*. *I. scapularis* were repelled by lower concentrations in the vertical bioassay than in the petri dish bioassay. Deet was slightly more effective against *I. scapularis* than AI3-37220 in both bioassays, but AI3-37220 was significantly more effective than deet against *A. americanum* in the vertical bioassay.

KEY WORDS bioassay, blacklegged tick, lone star tick, personal protection

TICK BITES AND TICK-BORNE diseases continue to be a serious public health problem in many parts of the United States, and elsewhere in the world. Although progress has been made in recent years in the development of effective tick population control technologies, personal protective measures remain a critical element in preventing tick bites to humans. The blacklegged tick, *Ixodes scapularis* Say, and *Amblyomma americanum* (L.) are of considerable medical importance in the United States. The former species is the principal vector of the causative agent of Lyme disease in the eastern and central United States, and is also involved in the transmission of babesiosis and human granulocytic ehrlichiosis (Spielman et al. 1985, Dumler and Bakken 1995). The lone star tick readily bites humans and is associated with transmission of monocytic ehrlichiosis (Walker and Dumler 1996).

Recommended personal protective measures against ticks usually include the use of repellents on clothing, exposed skin, or both (CDC 2002). Permethrin-based tick repellents are marketed for use on clothing (Schreck et al. 1982, Lane and Anderson 1984, Evans et al. 1990). Most tick repellents sold for use on the skin contain *N,N*-diethyl-3-methylbenzamide (deet) in a broad range of concentrations. The efficacy of deet-treated clothing has been evaluated by several investigators (Mount and Snoddy 1983, Schreck et al. 1986, Evans et al. 1990). Using a fingertip bioassay, Schreck et al. (1995) tested 29 candidate repellents, including deet, on human skin against host-seeking nymphs of *A. americanum* and *I. scapularis*, and found that deet provided 2.7-h protection against the former, but <1 h against the latter. Pretorius et al. (2003) also used a fingertip bioassay to test 20% formulations of deet and the piperidine compound KBR 3023 against the bont tick, *A. hebraeum* Koch. In a field test, Solberg et al. (1995) found that at 0.5 mg/cm^2 on volunteers' legs, the piperidine compound racemic 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220) was more effective than deet in repelling adult and nymphal lone star ticks, *A. americanum*. AI3-37220 is a promising multitarget arthropod repellent (Klun et al. 2001).

In vitro bioassays are an integral step in the discovery and development of new repellents, and are antecedent to evaluations involving human volunteers. The purposes of this study were to: 1) develop in vitro

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bioassays for evaluating repellents; 2) compare the relative efficacies of deet and AI3-37220 in repelling *I. scapularis* and *A. americanum* nymphs; and 3) establish efficacy data for comparison in future evaluation of candidate repellents.

Materials and Methods

Ticks. Unfed larvae of *I. scapularis* were obtained from the laboratory colony of J. Bowman, Oklahoma State University (Stillwater, OK), and fed to repletion on white rats (Beltsville Area Animal Care and Use Committee protocol 99-011). These fed larvae, and the nymphs that eclosed from them, were maintained at 24°C, ≈97% RH, and a photoperiod of 18:6 h (L:D). Host-seeking nymphs of *I. scapularis* were tested at 4–6 and 10–11 wk after eclosion (hereafter referred to as young and old ticks, respectively). Unfed nymphs of *A. americanum* were obtained from the laboratory colony at the United States Department of Agriculture, Agricultural Research Service, Knipling-Bushland United States Livestock Insects Research Laboratory (Kerrville, TX); maintained at 24°C, ≈97% RH, and a photoperiod of 18:6 h (L:D); and tested 8–11 wk after eclosion. Only ostensibly intact and healthy, host-seeking ticks were used in tests. Ticks were considered host seeking if they raised their forelegs or began crawling when exhaled upon by an investigator.

Test Repellents. Deet was purchased from Sigma-Aldrich (St. Louis, MO), and racemic AI3-37220 was obtained from Morflex (Greensboro, NC). The compounds were 98–99% chemically pure, according to gas chromatographic analyses. Stock solutions of the required solutions were prepared gravimetrically using 95% ethanol as solvent.

Petri Dish Bioassay. Concentric circles 1.6, 3.2, and 6.0 cm in diameter were drawn with a lead pencil on a disk of Whatman No. 4 filter paper (9 cm diameter; Whatman International, Maidstone, UK). To the 20-cm^2 ring between the 3.2- and 6.0-cm circles, 165 μl 95% ethanol containing repellent or ethanol alone (the control) was applied evenly with an adjustable volume pipettor. Care was taken to ensure that visible diffusion of samples in the filter paper went as close as possible to the marked boundaries of the treatment ring without crossing them. The filter paper was in a glass petri dish at the time of application to minimize the transfer of repellent from the filter paper onto the substrate. Each dried filter paper disk was placed in a plastic petri dish containing a clean disk of filter paper of the same size. The plastic petri dishes had been glued in a larger plastic petri dish (15 cm diameter) with water filling the space between the walls of the inner and outer petri dish. The water moat prevented escape of ticks released on the filter paper. Ten active ticks were removed from storage vials and placed in a plastic test tube whose inner surface had been coated with Teflon to inhibit the ticks from climbing. When 5 groups of 10 ticks had been placed in the test tubes and the treated filter papers had dried for 15–20 min, the ticks were tapped from the test tubes onto the central untreated circles of the filter paper disks, one tube (10 ticks) per petri dish. Bioassays were conducted at 23–26°C and 17–56% RH, and were illuminated by fluorescent ceiling lights and light entering through the windows. During both types of bioassays, an observer was 0.5–1 m from the vertical strip or the petri dish to record tick locations. The proximity of the observer to the host-seeking ticks probably provided stimuli (carbon dioxide in exhalations, shadows) that enhanced locomotion in the ticks.

The locations of the ticks were recorded at 3, 5, and 10 min after they were released, noting whether a tick was in the untreated central circle (drop zone), the inner untreated ring, the treated ring, or the outer untreated ring, including the petri dish. Only those ticks that were recorded outside the drop zone were considered active and included in the analysis of the data. Ticks that crossed beyond the treated ring were carefully removed using forceps, so that they could not return to the inner rings. Ticks on the treated ring at the time tick locations were recorded, plus those that crawled beyond the treated ring were considered as not repelled. Ticks recorded as having entered the treated ring and returned to the drop zone were considered active and repelled.

To estimate the EC_{50} and EC_{95} for deet against *I. scapularis*, concentrations of 1.574, 0.787, 0.394, 0.197, 0.099, and 0 μmol/cm^2 paper were used, and for AI3-37220, concentrations of 1.574, 0.787, 0.394, 0.296, 0.197, and 0 μmol/cm^2 paper were used. In tests on *I. scapularis*, each replicate consisted of four concentrations each of deet and AI3-37220 and two ethanol controls. Because a maximum of five petri dishes could be carefully observed at one time, each replicate was done in two sessions. Two concentrations of each compound were randomly assigned to either the first or second session, and an ethanol control was included in each session. The five petri dishes were arranged in line on the laboratory bench with each treatment, and the control randomly assigned to a particular position in the line for each session within each replicate. Against *A. americanum*, each compound was tested separately with three concentrations of the compound plus an ethanol control in each replicate. Only the three highest concentrations (described above) were used. Positions of the doses in the line of petri dishes were similarly randomized and completed in two sessions per replicate.

Vertical Bioassay. A second bioassay was used to assess the effectiveness of the repellents, largely because the petri dish bioassay showed that, in the concentrations used, neither deet nor AI3-37220 had meaningful repellent activity against *A. americanum*. We surmised that because the petri dish bioassay did not allow ticks to drop from repellent surfaces as would occur in the field, it might be inappropriate for the highly mobile *A. americanum*. A 4 × 7-cm rectangle of Whatman No. 4 filter paper was marked with a pencil into two zones (1 × 4 cm) at either of the far ends of the paper and a 4 × 5-cm zone between them. As with the petri dish bioassay, 165 μl ethanol containing the repellent or ethanol alone was evenly ap-

plied onto the 20-cm^2 middle zone of the filter paper strip. The strip was allowed to dry 15–20 min before testing, and suspended from a bulldog clip attached to one of the two untreated zones at opposite ends of the strip. The vertical strip hung over moated petri dishes (9 and 15 cm diameters). A storage vial containing ≈50 ticks was placed in the center of moated plastic petri dishes (5.5 and 9 cm diameters). The vial was opened. When 15–20 ticks had crawled onto the rim of the vial and the petri dish, the vial and petri dish were held beneath the lower end of the vertically suspended strip of filter paper, so that ticks could crawl onto the lower untreated zone of the filter paper. Approximately 10 ticks were allowed onto the filter paper. With *I. scapularis*, some ticks were placed onto the untreated end of the strip of filter paper.

The locations of the ticks were recorded at 3, 5, and 10 min after the tenth tick climbed or was placed onto the filter paper. Ticks were considered repelled if they remained on the lower untreated part of the strip or if they dropped off the strip. The moated petri dish below the filter paper confined ticks that dropped from the paper. Ticks that crawled onto the upper untreated zone or the bulldog clip were removed to prevent their return to the lower untreated zone. Three replicates of three concentrations of deet and AI3-37220 plus an ethanol control were tested in the vertical bioassay. The treatments and control in each replicate were tested in a randomized order. Concentrations used to estimate the EC_{50} and EC_{95} for deet and AI3-37220 for *A. americanum* were 1.574, 0.787, 0.394, 0.197, and 0 μmol/cm^2 paper, and for *I. scapularis* 0.787, 0.394, 0.295, 0.147, and 0 μmol/cm^2 paper were used.

Statistical Analysis. Because the data are binomial in nature (an individual tick is scored as either repelled or not repelled), we used a generalized linear model with a binomial link (McCullagh and Nelder 1989) to model the logit of the proportion of ticks repelled ($\log [p/(1 - p)]$), where p is the proportion of ticks repelled) using the R software package (Free Software Foundation, Boston, MA; http://www.gnu.org/). The models allowed for different slopes for deet and AI3-37220 for within-species and within-age comparisons (intercept estimates, represented by the control, should be identical). We also checked for curvature in the response to changes in concentration by including a quadratic term, but in no case did the quadratic model significantly improve the fit. Thus, results presented in this work are based on a linear relationship between concentration and the logit of the proportion of ticks repelled. EC_{50} and EC_{95} were estimated using inverse regression, and the standard errors for the concentration at these points using fiducial limits (Draper and Smith 1981); these are implemented in the R software with the MASS library developed by W. N. Venables and B. D. Ripley (http://lib.stat.cmu.edu/R/CRAN/). This methodology can provide estimates beyond the range of the data (extrapolation), although with increasing uncertainty as one departs from the vicinity of the data. For calculating EC_{50} and EC_{95}, and displaying the data graphically, an intercept and slope were estimated independently for 8 of the 10 data sets (these statistics were deemed inappropriate for 2 of the data sets). Because graphs on the original scale (proportion repelled) are easier to interpret, the modeled concentration-response relationship was back transformed from the logit scale for visually displaying the concentration-response relationship (corresponds to dose response). However, the straight line relating the logit to the concentration then becomes a curve.

Results

Nymphs of *I. scapularis* and *A. americanum* behaved differently in the bioassays and also exhibited different sensitivities to repellents. In the petri dish bioassay, some *I. scapularis* had often not yet started to move at 3 and 5 min after their release regardless of whether they were exposed to the repellent or ethanol control. Even 10 min after the ticks were released, deet continued to effectively prevent *I. scapularis* nymphs from crossing the treated ring of filter paper at a concentration of 0.787 μmol/cm^2 (Fig. 1A), and AI3-37220 was nearly as effective (Fig. 1*B*). In contrast, 87.8 and 52.2% of the *A. americanum* nymphs crossed the AI3-37220 and deet-treated rings, respectively, within the 3 min after they were released on the filter paper. At 1.574 μmol/cm^2 paper, deet had low efficacy (25% repelled) in restricting *A. americanum* movements and AI3-37220 had no apparent repellent effect on *A. americanum* in the petri dish bioassay (Fig. 1, C and D). Concentration-response curves were developed for deet and AI3-37220 for young and old *I. scapularis* nymphs (Fig. 1, A and B). Both young (4–6 wk posteclosion) and old (10–11 wk posteclosion) nymphs were significantly ($P < 0.01$) more sensitive to deet than to AI3-37220, and old nymphs tended to be more sensitive to deet ($P = 0.06$) than were the young nymphs (Table 1).

In contrast to the petri dish bioassay, in the vertical bioassay concentration-related responses to deet and AI3-37220 were observed in *A. americanum* nymphs (Fig. 1, G and H), although *I. scapularis* was far more sensitive to the repellents (Fig. 1, E and F). For *A. americanum*, AI3-37220 was the more effective repellent ($P = 0.04$), with all 30 nymphs repelled by a concentration of 1.574 μmol/cm^2 paper (Fig. 3H) compared with 67.7% repelled by the same concentration of deet (Fig. 1G). *I. scapularis* nymphs were repelled by significantly lower concentrations (0.295 μmol/cm^2 paper) of deet and AI3-37220 in the vertical test ($P < 0.01$) than in the petri dish test. The low concentration of 0.197 μmol/cm^2 paper of either compound repelled >90% of young *I. scapularis* in the vertical test (Fig. 1E), but only 20% of *I. scapularis* were repelled at the same concentration in the petri dish bioassay (Fig. 1A). On ethanol-treated (control) filter paper strips, 98.4% of *A. americanum* and 96.8% of *I. scapularis* nymphs ascended to the top of the strip within 10 min of crawling onto the strip.

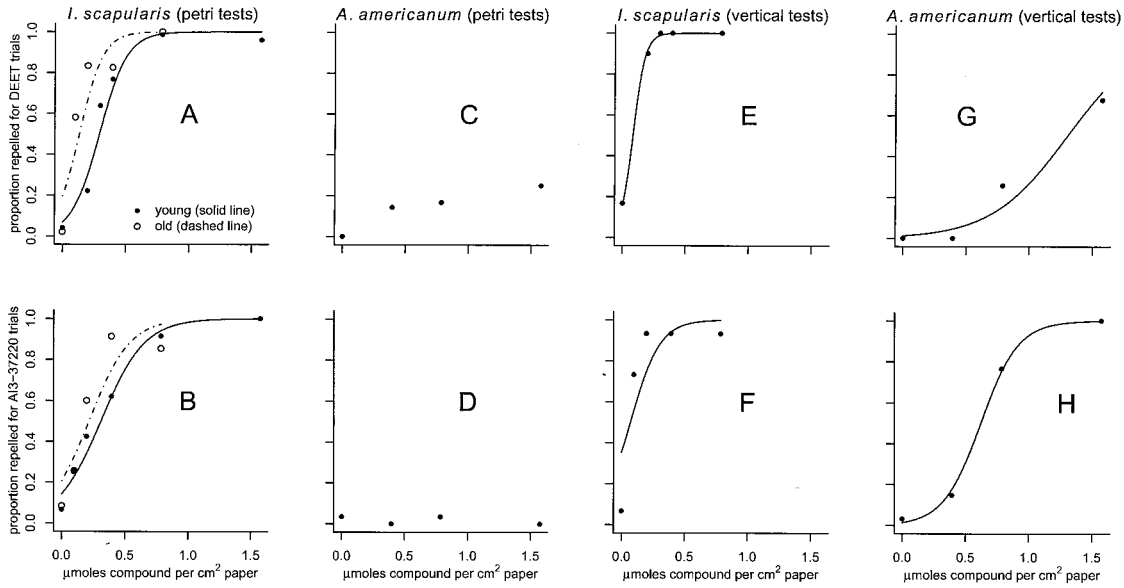

Fig. 1. Concentration responses of nymphs of *I. scapularis* and *A. americanum* to deet and AI3-37220 in petri dish and vertical bioassays. Graphs on top row are for deet, lower row for AI3-37220, and A and B are of young and old *I. scapularis* nymphs.

Discussion

The petri dish and vertical bioassays demonstrated that *I. scapularis* and *A. americanum* nymphs respond quite differently to the same concentrations of deet and AI3-37220. Even at the highest concentrations of AI3-37220 used in the petri dish bioassay, none of the active *A. americanum* remained in the untreated center of the filter paper at 10 min after the ticks were released, and with deet ≤25% were confined by the treated ring. In contrast, *I. scapularis* nymphs were strongly repelled by these concentrations (1.574 and 0.787 μmol compound/cm^2 paper) of these two compounds. When nymphs were presented with the option of dropping off the filter paper in the vertical bioassay, it was apparent that both repellents affected the behavior of *A. americanum.* Nonetheless, it again required higher concentrations (1.574 and 0.787 μmol compound/cm^2 paper) to repel *A. americanum* in the vertical bioassay than were needed to repel *I. scapularis.*

Schreck et al. (1995) used two fingertip bioassays to test repellents on *A. americanum* and *I. scapularis* nymphs. They applied 0.3 mg compound/cm^2 skin in ethanol on segments of a volunteer's index finger. They reported that deet provided 2.7 h of protection against *A. americanum* nymphs and <1 h of protection against *I. scapularis* nymphs. In both of our bioassays, *I. scapularis* were more sensitive to deet than were *A. americanum*, the reverse of the results obtained by Schreck et al. (1995). Although the concentration of deet used by Schreck et al. (1995) was approximately equal to 1.574 μmol compound/cm^2, our highest concentration, the many differences between the bioassays do not allow a direct comparison. Factors, such as differential reactivity of deet with skin and filter paper or differences in the quality and quantity of host-

Table 1. Petri dish and vertical bioassays of repellency of deet and A13-37220 against *I. scapularis* and *A. americanum* nymphs

Repellent	EC level	Mean μmol cm^2			
		Petri dish bioassay		Vertical bioassay	
		I. scapularis		*I. scapularis*	*A. americanum*
		Young	Old		
Deer	EC_{50}	0.288 (0.016)[a]	0.130 (0.018)	0.080 (0.019)	1.296 (0.91)
Deer	EC_{95}	0.610 (0.044)	0.404 (0.049)	0.222 (0.031)	2.177 (0.200)[b]
A13-37220	EC_{50}	0.310 (0.024)	0.216 (0.027)	0.070 (0.027)	0.622 (0.045)
A13-37220	EC_{95}	0.819 (0.068)	0.690 (0.080)	0.414 (0.069)	1.057 (0.101)

[a] Standard errors in parentheses.
[b] Extrapolated estimate (observations were not made near this concentration).

produced stimuli influencing tick behavior, could account for the different results. The discrepancy reinforces the need for a full range of in vitro, in vivo, and field evaluations of repellent compounds. Interestingly, the repellent that Schreck et al. (1995) reported to provide the longest protection against *A. americanum* was 1-(3-cyclohexen-1-ylcarbonyl) piperidine (AI3-35765), a compound differing only slightly from AI3-37220. In our tests, AI3-37220 was more effective than deet against *A. americanum.* The field trial of Solberg et al. (1995), tested both compounds at 0.5 mg compound/cm^2 on volunteers' legs against nymphs and adults of *A. americanum.* They found that AI3-37220 provided >90% protection 6 h after treatment, whereas deet never achieved >85% repellency.

Aging of the *I. scapularis* nymphs did not alter their comparative responses to deet and AI3-37220. Deet was more effective against both old and young ticks. Old *I. scapularis* nymphs (10–11 wk posteclosion) were marginally more sensitive to deet than were the young (4–6 wk posteclosion) nymphs. Therefore, the age of *I. scapularis* nymphs used in repellent tests should not exceed 10–11 wk, and data for ticks of different ages should not be combined.

The contrasting responses of *I. scapularis* and *A. americanum* in the petri dish tests may reflect differences in the basic behavioral characteristics of each species. Lone star ticks are known for their mobility and rapid kinesis toward host-produced stimuli (Wilson et al. 1972). Blacklegged ticks are more sedentary, slow moving, and more dependent on ambush contact in acquiring hosts (Falco and Fish 1991). *A. americanum* may rely on their mobility to cope with noxious stimuli, such as a repellent compound on the substrate and in the air. Hence, when on a horizontal surface, *A. americanum* will run across a substrate treated with repellent, and thereby distance themselves from the noxious stimulus. The slow-moving *I. scapularis* may minimize exposure to the noxious stimulus by avoiding the high concentration of the compound (the treated ring in the case of the petri dish bioassays). The petri dish bioassay may be a more rigorous indicator of the effectiveness of a repellent than the vertical test because stronger repellency is needed to confine these tick species on horizontal surfaces. For both species, the vertical bioassay is a more sensitive tool than the petri dish bioassay for detecting repellent properties of a compound. In nature, a tick contacting a person will encounter surfaces at various angles to the horizontal, although vertical slopes would predominate on a standing person.

The vertical bioassay appears to have wider applicability in evaluating repellents against different species of ticks. The results also demonstrate that a repellent evaluation program should not be based on testing only one species of tick. Field testing (the ultimate proof of a repellent's effectiveness) of deet and a stereoisomer of AI3-37220 (Klun et al. 2003) against *I. scapularis* and *A. americanum* is needed to ascertain the relative effectiveness of these repellents, and duration of repellent activity, in natural situations.

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