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Repellency of deet and SS220 applied to skin involves olfactory sensing by two species of ticks

J. F. CARROLL[1], J. A. KLUN[2] and M. DEBBOUN[3]

[1]Animal Parasitic Diseases Laboratory, [2]USDA, ARS, Chemicals Affecting Insect Behaviour Laboratory, Beltsville Agricultural Research Center, Beltsville, MD and [3]United States Army Center for Health Promotion and Preventive Medicine-South, Fort McPherson, GA, U.S.A.

Abstract. Responses of host-seeking nymphs of the blacklegged tick, *Ixodes scapularis* Say and lone star tick, *Amblyomma americanum* (Linnaeus) (Acari: Ixodidae) to the repellents N,N-diethyl-3-methylbenzamide (deet) and (1S, 2′S)-2-methylpiperidinyl-3-cyclohexene-1-carboxamide (SS220) were studied using fingertip laboratory bioassays. Ethanol solutions of both compounds applied to the skin strongly repelled both species of ticks at 0.8 and 1.6 μmole of compound/cm^2 skin. The ticks were also repelled when two layers of organdie cloth covered the portion of a finger treated with either deet or SS220. Gas chromatographic analyses of the outer layer of cloth that had covered skin treated with 1.6 μmole compound/cm^2 skin revealed only 0.1 nmole SS220/cm^2 cloth and 2.8 nmole deet/cm^2 cloth. However, in bioassays in which a single layer of cloth was treated with a dose of deet or SS220 equivalent to the amount found in the outer layer of cloth, ticks were not repelled. Results unequivocally demonstrated that these ticks responded to the repellents in the vapour phase when repellent treated skin was covered with cloth to obviate tactile contact with them, and made it clear that the ticks detect the repellents by olfactory sensing. Heretofore, the mode of action of deet and SS220 was unclear.

Key words. *Amblyomma americanum*, *Ixodes scapularis*, blacklegged tick, deet, fingertip bioassay, lone star tick, N, N-diethyl-3-methylbenzamide, SS220, (1S, 2′S)-2-methylpiperidinyl-3-cyclohexene-1-carboxamide.

Introduction

In the United States, the blacklegged tick, *Ixodes scapularis* Say, is of human health importance, the principal vector of the agent causing Lyme disease, and is involved in the transmission of babesiosis and human granulocytic ehrichiosis (Spielman *et al*., 1985; Dumler & Bakken, 1995). The lone star tick, *Amblyomma americanum* (Linnaeus), is also a major pest species that readily bites humans and is considered a vector of human monocytic ehrlichiosis (Walker & Dumler, 1996). Recently, more effective technologies have been developed for area-wide control of tick populations (Pound *et al*., 2000). For various reasons, such area-wide control measures will not be implemented everywhere they are needed, and thus repellents remain a last alternative for protection for persons entering most tick habitats (CDC, 2002). Tick repellents can be loosely classified as belonging to either of two function-based categories, those used on skin and those used on clothing. Repellent products containing permethrin are widely used on clothing, and permethrin has been shown to be effective when used in this fashion (Schreck *et al*., 1982; Lane & Anderson, 1984; Evans *et al*., 1990). Deet (N,N-diethyl-3-methylbenzamide) is the active component of most tick repellents marketed for use on skin, although it can be used on clothing (Mount & Snoddy, 1983; Schreck *et al*., 1986; Evans *et al*., 1990). Using a fingertip bioassay, Schreck *et al*. (1995) evaluated deet against *I. scapularis* and *A. americanum* and found it to be more effective against the latter species. Pretorius *et al*. (2003) also reported that deet was effective in fingertip bioassays with the bont tick,

Correspondence: J. F. Carroll, USDA, ARS, APDL, BARC-East, Building 1040, Beltsville, MD 20705, U.S.A. Tel.: +1 301 504 9017; fax: +1 301 504 5306; e-mail: jcarroll@anri.barc.usda.gov

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14. ABSTRACT

Responses of host-seeking nymphs of the blacklegged tick, Ixodes scapularis Say and lone star tick, Amblyomma americanum (Linnaeus) (Acari Ixodidae) to the repellents N,N-diethyl-3-methylbenzamide (deet) and (1S, 20S)- 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (SS220) were studied using fingertip laboratory bioassays. Ethanol solutions of both compounds applied to the skin strongly repelled both species of ticks at 0.8 and 1.6 mmole of compound/ cm2 skin. The ticks were also repelled when two layers of organdie cloth covered the portion of a finger treated with either deet or SS220. Gas chromatographic analyses of the outer layer of cloth that had covered skin treated with 1.6 mmole compound/cm2 skin revealed only 0.1 nmole SS220/cm2 cloth and 2.8 nmole deet/cm2 cloth. However, in bioassays in which a single layer of cloth was treated with a dose of deet or SS220 equivalent to the amount found in the outer layer of cloth, ticks were not repelled. Results unequivocally demonstrated that these ticks responded to the repellents in the vapour phase when repellent treated skin was covered with cloth to obviate tactile contact with them, and made it clear that the ticks detect the repellents by olfactory sensing. Heretofore, the mode of action of deet and SS220 was unclear.

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Amblyomma hebraeum Koch. Whether the repellent is applied to skin or cloth, the desired result is to prevent ticks from biting the skin.

Although they lack antennae, ticks have sensitive chemoreceptors, particularly on their forelegs, which they wave in the air like antennae (Waladde & Rice, 1982; Sonenshine, 1991). Because ticks often wait on vantage points on vegetation and catch hold of passing (sometimes from downwind) hosts, until recently little attention has been paid to the way in which ticks detect repellents. Using a locomotion compensator, McMahon *et al.* (2003) found that neither of two doses of deet affected the attraction of *A. variegatum* Fabricius to its aggregation-attachment pheromone, when both were co-presented in an air stream. Dautel (2004) reviewed methods for testing repellents against ticks. Where tick repellency has been tested, it has generally been impossible to discern whether repellent action was due to olfactory or tactile chemoreception of the repellent compounds. However, an *in vitro* method used by Dautel *et al.* (1999) allowed them to observe distance (albeit only mm) responses of *Ixodes ricinus* (Linnaeus) to deet. The primary purpose of the present study was to clarify which of these sensory modalities was involved in the repulsion of ticks. We conducted bioassays to elicit responses to skin treated with repellent and to skin treated with repellent covered with cloth to ascertain the mode of action of the repellents.

Klun *et al.* (2003) reported that the (1S, 2′S) stereoisomer of 2-methylpiperidinyl-3-cyclohexene-1-carboxamide was the most effective repellent against *Aedes aegypti* (Linnaeus) and *Anopheles stephensi* Liston. In two *in vitro* laboratory bioassays (Carroll *et al.*, 2004) and in a field test (Solberg *et al.*, 1995), the piperidine compound racemic 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220) was more effective than deet in repelling nymphal and adult *A. americanum*. A secondary purpose of the present study was to determine the effectiveness of deet and SS220 in an *in vivo* (fingertip) bioassay at concentrations that might be used in the field.

Materials and methods

Ticks

Ixodes scapularis nymphs were reared from larvae obtained from the laboratory colony of J. Bowman, Oklahoma State University, Stillwater, OK, and fed on rats (Beltsville Area Animal Care and Use Committee protocol #02–015) at the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Beltsville Agricultural Research Center, Beltsville, Maryland. Host-seeking *A. americanum* nymphs were obtained from a colony at the USDA, ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, Kerrville, TX. Both species of ticks were maintained at 24°C, ~97% r.h. and LD 16 : 8 h until testing.

Repellent compounds

Deet was obtained from Morflex, Inc. (Greensboro, NC) and SS220 was synthesized at the USDA, ARS Chemicals Affecting Insect Behaviour Laboratory (Klun *et al.*, 2003). The compounds were at least 98% chemically pure according to gas chromatographic (GC) analyses. The stereoisomeric composition of SS220 was 95% SS with traces of the other three isomers (Klun *et al.*, 2003). Stoichiometrically equivalent stock 95% ethanol solutions of the compounds were prepared: 55.7 μg deet/μL, 111.4 μg deet/μL, 60.3 μg SS220/μL, and 120.6 μg SS220/μL for use in all bioassays in which compounds were applied to human skin. The volumes of the respective solutions were used to generate 0.8 μmole and 1.6 μmole repellent doses/cm^2 skin based on the dimensions of the index finger of the corresponding author. The volume of the treated solutions required to give the desired μmoles/cm^2 skin dosages was calculated (area = πdh) from the diameter (d) and length (h) of the described treatment area.

Fingertip bioassay

Responses of both species of ticks to both repellents were evaluated with a fingertip bioassay similar to those described by Schreck *et al.* (1995) and Pretorius *et al.* (2003). The boundaries of the treated area, which encircled the finger along the prominent basal and the middle dorsal creases of the first and second joints, were marked with a fine-tipped pen (Fig. 1a). By means of a pipettor, 52 μL of a repellent solution or ethanol control was evenly applied completely around the second phalanx of the corresponding author's left forefinger (only volunteer and finger used in all bioassays). The ink boundary lines not only aided in defining the treatment area during application of the solutions, but indicated if, and where, a solution may have spread slightly beyond the prescribed treatment area. After the solution dried for 10 min, a vial containing *A. americanum* nymphs was opened in a Petri dish (9 cm diameter, 1 cm high) that had been glued in the centre of a larger Petri dish (15 cm diameter, 1.5 cm high) and the intervening space filled with water to form a moat. The treated finger was held horizontally and 10 nymphs were transferred singly with forceps to the dorsal surface of the untreated distal segment of the finger between the base of the finger nail and the joint. Once all the ticks were clinging to the finger, it was tilted to vertical with the tip pointing down. The locations of the ticks were recorded at 10 min after the last nymph was released on the fingertip. Ticks on the untreated fingertip and those that fell or dropped from the finger onto the moated Petri dish 3–4 cm below were considered repelled, whereas ticks on the treated area and those that crossed it were considered not to have been repelled. Because *I. scapularis* nymphs were more apt to fall from untreated skin than *A. americanum* nymphs, we, similar to Schreck *et al.* (1995), screened the former for tenacity and readiness to climb. Just before each repellent or ethanol control was

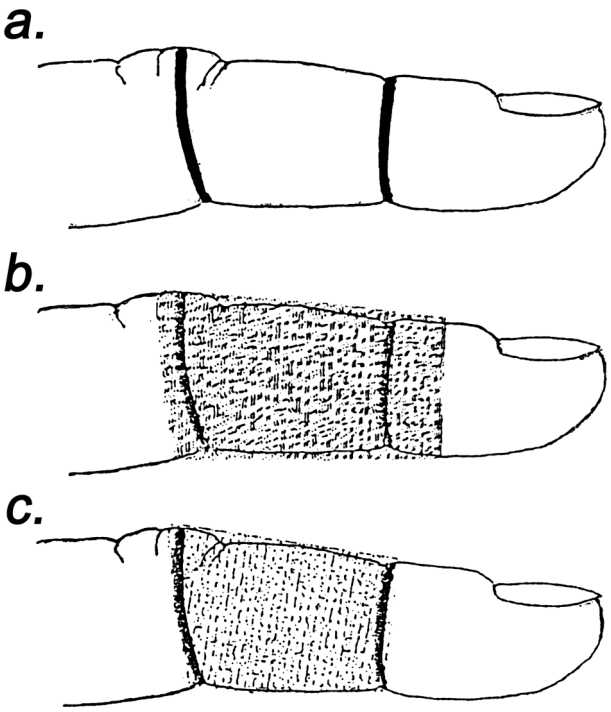

Fig. 1. Fingertip bioassays: (a) test solutions were applied to the second phalanx of the index finger between the ink lines drawn around the joints; (b) in the double-wrapped finger bioassay, an untreated cloth strip was wrapped twice around (completely covering) the second phalanx topically treated with a test solution (note that cloth extends beyond boundary lines of treatment ensuring that ticks do not contact treated skin); and (c) in the repellent containment bioassay, a cloth strip, the same width as the treatment area and treated with a test solution, was wrapped once around the untreated skin of the second phalanx.

applied, *I. scapularis* nymphs were placed on the tip of an untreated finger until 10 ticks climbed ~0.5 cm. The ticks that climbed were used in the bioassay that was otherwise the same as that described for *A. americanum*. Each of two concentrations (0.8 and 1.6 μmole of compound/cm^2 skin) of deet and SS220 was tested five times with each species of the ticks. Before each bioassay, the corresponding author thoroughly washed his forefinger with soap and rinsed with water. The repellent solutions and ethanol controls were tested randomly.

Double-wrapped finger bioassay

To determine if deet and SS220 could repel *I. scapularis* and *A. americanum* via olfactory sensing alone (without contact chemosensing), we modified the fingertip bioassay as follows. A 7 × 7 mesh/mm strip of organdie cloth (Hancock Fabrics, Laurel, MD) was cut in the shape of a hockey stick (9 cm long section, 4.5 cm short section, 4–4.5 cm wide) so that it could be wrapped twice around the middle segment of the index finger (completely covering the treatment area described above, and extending 5–6 mm beyond the edge of the treated area onto the untreated tip area) with 2–3 mm overlap (Fig. 1b). A repellent solution (52 μL) or ethanol (control) of the same volume was applied to the designated treatment area on the finger. After the skin dried (10 min), the organdie was wrapped twice around the index finger. To keep the cloth wrapped around the finger, three small dabs of beeswax were smeared on the upper surface of the inner layer of cloth where they overlapped and pressure from another finger applied for 10 s. The ink lines marking the boundaries of treatment area were visible through the two layers of cloth. Two concentrations (0.8 and 1.6 μmole of compound/cm^2) of each repellent and ethanol were tested against four groups of 10 *A. americanum* nymphs and three groups of *I. scapularis* nymphs. Ticks were considered repelled if they had dropped from the finger or were on the untreated tip at 10 min after they were placed on the finger.

Repellent containment bioassay

To prove that neither deet nor SS220 wicked through from the skin to the outer layer of cloth in the double-wrapped finger bioassay and that the outer layer of cloth was not otherwise significantly contaminated, the organdie fabric was cleaned by a Soxhlet extraction using ethanol to remove any impurities that might interfere with GC analyses for deet and SS220. A repellent solution or ethanol (52 μL) was applied to the finger and allowed to dry for 10 min. A strip of extracted organdie cut as previously described (but narrower to match exactly with the boundary lines of the treated area), was wrapped twice around the repellent-treated middle of the index finger. The cloth strip was held in place by three small dabs of beeswax on the area of overlap. The cloth was removed after 10 min and cut into two pieces, designated as inner or outer layers. Each piece of organdie was soaked separately in 2 mL ethanol. The amount of repellent recovered from the inner and outer cloth layers that covered the repellent-treated finger was quantified by external standard GC analysis of the ethanol extract using a splitless sample injection mode with a Hewlett-Packard 6890 gas chromatograph with a flame ionization detector (FID) and fitted with a DB-Waxer, 60 m × 0.25 mm, film thickness 0.25 μm column (Agilent, Inc., Palo Alto, CA). The oven temperature was programmed at 120°C for 1 min, then to 185°C at 15°C/min and held for 16 min. Hydrogen was used as the carrier gas at a flow rate of 40 mL/min.

Doses of deet and SS220 at the mean amounts found on the outer layer of cloth by gas chromatography (Table 1) were subsequently applied to clean cloth strips and tested for repellency against nymphs of both species. Pieces of extracted cloth were cut (8.0 × 2.8 × 6.3 × 3.8 cm) to wrap once around the second phalanx of the index finger (treatment area) with a 2–3 mm overlap (Fig. 1c). A cloth strip was placed in a glass Petri dish, and 52 μL of test solution slowly applied to it by a pipettor, except for the overlap area

Table 1. Amounts of deet and SS220 found by gas chromatographic analyses of the outer layer of cloth wrapped twice for 10 min around finger treated with either repellent.*

	Skin treatment** (μmole/cm^2)	Repellent recovery from outer layer (mean nmole/cm^2 cloth)
Deet		
	0.8	0.02 ± 0.02
	1.6	2.80 ± 1.31
SS220		
	0.8	0.15 ± 0.17
	1.6	0.10 ± 0.11

*Soxhlet extraction using ethanol.
**Six replicates of each treatment of each repellent.

which had been marked off with a lead pencil. After the repellent-treated cloth dried for 10 min, it was wrapped around the index finger and secured by three dabs of beeswax on the overlapping portions of the cloth. Four groups of 10 *A. americanum* nymphs and four groups of 10 *I. scapularis* nymphs were tested against 0.1 nmole SS220/cm^2 cloth and 2.8 nmole deet/cm^2 cloth (average detectable levels of repellent contamination found on outer layer of double-wrapped cloth), 1.6 μmole deet/cm^2 of cloth and 1.6 μmole SS220/cm^2 cloth and an ethanol control (doses routinely applied to skin). Ticks were considered repelled if they dropped from the finger or were on the untreated finger tip at 10 min after they were placed on the finger.

All data were analysed using Chi square 2×2 contingency tables.

Results

Both deet and SS220 strongly repelled *I. scapularis* and *A. americanum* nymphs (Table 2). Ticks that fell from the finger or were on the untreated fingertip at 10 min after their release were considered repelled. When exposed to 0.8 and 1.6 μmole of deet/cm^2 skin, 88 and 96% of *A. americanum* nymphs, respectively, were repelled. In contrast, when ethanol was applied to the skin, 91% of *A. americanum* nymphs moved across the treated portion of the finger by 10 min after their release. All *A. americanum* nymphs were repelled by the 0.8 μmole of SS220/cm^2 skin and 94% by the higher concentration compared to 9% of nymphs exposed to the ethanol control. At the 0.8 μmole/cm^2 concentration, SS220 was more repellent than deet against *A. americanum* ($\chi^2 = 7.079$, 1 d.f., $P < 0.008$). With *I. scapularis*, 98% of the nymphs were repelled by both concentrations of deet, whereas only 4% of the nymphs were repelled by ethanol. Both concentrations of SS220 repelled 94% of *I. scapularis* nymphs, and with the ethanol control only 4% of the nymphs were considered repelled.

In bioassays where a strip of organdie was wrapped twice around the finger completely covering the treatment area, no *I. scapularis* crossed ethanol-treated cloth covering skin treated with 0.8 or 1.6 μmoles of deet/cm^2 of skin within 10 min after their release (Table 3). All *I. scapularis* were repelled when cloth covered treated skin (1.6 μmole of SS220/cm^2) at 10 min, and 86.7% of *I. scapularis* were repelled by 0.8 μmole of SS220/cm^2 beneath the cloth. Lone star tick nymphs were strongly repelled by both concentrations of SS220 through two layers of cloth (Table 3). Significantly more *A. americanum* nymphs were repelled by both concentrations of deet than by ethanol (Table 3), but at lower levels than by SS220.

The ethanol extractions of organdie strips doubly wrapped around a treated finger for 10 min showed extremely low concentrations of deet and SS220 (2.8 ± 1.3 and 0.1 ± 0.1 nmole/cm^2, respectively) on the outer layer of cloth when the skin was treated with 1.6 μmole/cm^2 (Table 1). When a strip of cloth treated with the contaminating concentrations was wrapped only once around the finger, the percentages of ticks of both species that crossed the cloth were not significantly different from percentages crossing cloth treated with ethanol (Table 4). Deet and SS220, at

Table 2. Percent of tick nymphs repelled by two concentrations of deet and SS220 in fingertip bioassays. Ticks that were on untreated fingertip or had fallen off the finger at 10 min after they were released on the finger were considered repelled.

Species	Repellent	Concentration (μmole/cm^2 skin)	% repelled (fell)*	χ^{2}**	*P*
A. americanum	Deet	0.8	88 (88)	57.76	< 0.0001
		1.6	98 (98)	71.0.1	< 0.0001
	SS220	0.8	100 (94)	88.68	< 0.0001
		1.6	94 (92)	77.44	< 0.0001
		0	9 (9)		
I. scapularis	Deet	0.8	98 (34)	84.78	< 0.0001
		1.6	98 (28)	84.78	< 0.0001
	SS220	0.8	94 (56)	84.78	< 0.0001
		1.6	94 (56)	84.78	< 0.0001
		0	4 (3)		

*Five groups of 10 nymphs of each species were tested for each concentration of each repellent, and 10 groups of 10 nymphs each species at 0 concentration (ethanol control). Numbers in parentheses indicate percent of ticks that fell from finger.
**Chi square comparison with ethanol control.

Table 3. Percent of tick nymphs that were repelled when test solutions applied to an index finger were covered by a strip of organdie wrapped twice around the finger. Ticks that were on untreated fingertip or had fallen off the finger at 10 min after they were released on the finger were considered repelled.

Species	Repellent	Concentration (μmole/cm^2)	% repelled*	χ^{2}**	*P*
A. americanum	deet	0.8	35.0	16.5	< 0.0001
		1.6	57.5	39.2	< 0.0001
	SS220	0.8	95.0	91.4	< 0.0001
		1.6	100	100	< 0.0001
	control	0	6.3		
I. scapularis	deet	0.8	100	47.1	< 0.0001
		1.6	100	47.1	< 0.0001
	SS220	0.8	86.7	32.5	< 0.0001
		1.6	100	47.1	< 0.0001
	control	0	23.3		

*Four groups of 10 *A americanum* nymphs and three groups of 10 *I. scapularis* nymphs were tested for each concentration of each repellent, and eight and six groups of 10 *A americanum* and *I. scapularis*, respectively, for the 0 concentration (ethanol control).
**Chi square comparison with ethanol control.

Table 4. Percent of tick nymphs that were repelled when a strip of organdie treated with test solutions was wrapped around the middle of an index finger. Ticks that were on untreated fingertip or had fallen off the finger at 10 min after they were released on the finger were considered repelled.

Species	Repellent	Concentration (μmole/cm^2)	% repelled*	χ^{2}**	*P*
A. americanum	deet	2.8	17.5	0.03	0.863
		1.6	85.0	52.9	< 0.0001
	SS220	0.1	15.0	0.03	0.860
		1.6	100	75.9	< 0.0001
	control	0	16.3		
I. scapularis	deet	2.8	7.5	0.69	0.406
		1.6	95.0	75.6	< 0.0001
	SS220	0.1	5.0	1.67	0.197
		1.6	97.5	79.8	< 0.0001
	control	0	12.5		

*Four groups of 10 nymphs of each species were tested with each concentration of each repellent, and eight groups of 10 ticks of each species for the 0 concentration (ethanol control).
**Chi square comparison with ethanol control.

1.6 μmole/cm ^{2} on a single layer of cloth, strongly repelled ($\geq 85\%$) nymphs of both species of ticks.

Discussion

Nymphs of *I. scapularis* were strongly deterred from crossing untreated cloth covering skin treated with either concentration of deet or SS220. When covered by untreated cloth, deet was only moderately repellent to *A. americanum*, whereas SS220, at either concentration, repelled nearly all *A. americanum* nymphs. These doses (0.8 and 1.6 μmole compound/cm^2) were the same as those used by Carroll *et al.* (2004) in filter paper bioassays, and the higher dose was closely equivalent to that used by Schreck *et al.* (1995) in fingertip bioassays. The contrasting responses of *A. americanum* nymphs to deet and SS220 when covered with cloth were similar to those observed for this species in filter paper bioassays (Carroll *et al.*, 2004) with deet and the racemic piperidine compound, AI3-37220.

A difficult issue in ascertaining whether olfaction is involved in tick responses to repellents (in particular deet) has been the distance over which airborne repellents act in standard repellent bioassays, such as filter paper and fingertip bioassays. If ticks were observed to avoid approaching within even 1 cm of a repellent treatment, tick olfaction of repellents would not be questioned. McMahon *et al.* (2003) found that indalone (butyl 3,4-dihydro-2, 2-dimethyl-4-oxo-2*H*-pyran-6-carboxylate) in an air stream in a locomotion compensator caused *A. variegatum* to walk downwind. However, in an experiment in which airborne deet and the *A. variegatum* aggregation-attachment pheromone were co-presented to ticks in the locomotion compensator, McMahon *et al.* (2003) observed no repellent response. Our tests showed that ticks need not contact deet or SS220 to be repelled by them, but the double-wrapped cloth separated the ticks from the treated skin by ≤ 1 mm. Dautel *et al.* (1999) found that nymphs of *I. ricinus* in a Y-tube bioassay would approach to about 1–3 mm of deet-treated filter paper, but not contact it.

To be able to conclude that *I. scapularis* and *A. americanum* nymphs were reacting to deet and SS220 via olfaction, we had to be able to prevent any tactile contact of a repellent by the ticks. The cloth projected 5–6 mm beyond the boundary of the treated skin, so that ticks could move onto the cloth without contacting the repellent. In a few cases when a solution spread slightly across the ink-marked boundary of the treated area of the finger, the cloth extended at least 3 mm beyond the spread (indicated by the soluble ink). Most ticks crawled onto cloth covering repellent-treated skin and turned back when they reached the edge of the treated area (the ink line was visible through the cloth). Due to the unevenness of the skin on the finger, portions of the inner layer of wrapped cloth did not contact the treated skin, so at most a variegated pattern of contamination of the inner cloth was likely. Because of the density of organdie mesh and the shortness of the nymphs' legs, it is unlikely they touched the inner layer of cloth. The low concentrations of both compounds, found to contaminate the outer layer of organdie, had no repellent effects on *I. scapularis* and *A. americanum*, thus demonstrating that deet and SS220 in the vapour phase were responsible for the observed avoidance responses by the ticks and that the modality for repellent activity in these compounds is olfactory.

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