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Chapter 7

Using Lone Star Ticks, *Amblyomma americanum* (Acari: Ixodidae), in *in Vitro* Laboratory Bioassays of Repellents: Dimensions, Duration, and Variability

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The *in vitro* laboratory bioassay is an important tool in tick repellent discovery and development, with a variety of bioassays used in recent years. Several factors, such as size and configuration of test surfaces and duration of tick exposure, can influence the outcome of bioassays. We tested two tick repellents, *N,N*-diethyl-3-methyl benzamide (deet) and (-)-isolongifolenone, in seven different bioassays or configurations. All bioassays used ≥ 4 concentrations of repellent and an ethanol control applied to filter paper against lone star tick nymphs, *Amblyomma americanum* (L.). Climbing bioassays included a 22 × 1 cm vertical filter paper strip and a 4 × 7 cm vertical filter paper strip plus four modifications of the basic 4 × 7 cm configuration. We used a moving object bioassay (MOB), in which a strip of filter paper treated with test solution was affixed to a rotating heated brass drum and ticks allowed to transfer to the paper. A horizontal bioassay in which ticks were confined between two filter paper discs that had one half treated with repellent was also used. For

each bioassay, deet and (-)-isolongifolenone were similarly effective, but in some bioassays ticks were repelled by lower concentrations of both repellents than in other bioassays. The 22 × 1 cm strip proved impractical for regular bioassay use, but showed that a height of 8-9 cm and ~6 min duration were optimal for climbing bioassays. When a loop of treated paper was added to untreated lower portion of the 4 × 7 cm filter paper, as alternative escape for ticks responding to repellents, more ticks were on the loop and lower untreated area of the strip at 10 min (end of the test) than were on the lower untreated area of the basic 4 × 7 cm strip. However, with the ethanol controls more ticks fell from 4 × 7 cm strips with loops than those without loops. Several important behaviors associated with host acquisition (contacting, transferring to and remaining on a moving surface) were recorded in the MOB, but we only found significant differences between treatment and control for the proportion of ticks that transferred to the filter paper and the length of time the ticks remained on paper. The petri dish bioassays lasted longer than other bioassays (2h compared to 10 min for the vertical filter bioassays) and allowed detection of a decline in repellency over time. Individual variation among ticks and fatigue (change in response) in repeatedly tested ticks were assessed in a vertical paper strip bioassay using deet. The responses of ticks tested twice on one day (morning and afternoon) did not differ between tests. However, continued repeated daily testing compromised results. A hiatus of about a week between tests allowed ticks to return to their initial response profiles.

Keywords: deet; (-)-isolongifolenone; dose response; repellency

Tick-borne diseases are a serious and increasing problem in United States and elsewhere in the habitable world (1). A variety of tick control measures have been developed and implemented (2), but repellents remain an important means of personal protection against tick bite (3). Repellent products, such as deet and permethrin, used on skin and clothes respectively, have been available for decades. However, there is a rising demand for novel, effective, safe, inexpensive tick repellents (4). The recent discovery of olfactory receptor neurons for repellents in *Drosophila* may lead to novel approaches for repellent testing (5), but *in vitro* and/or *in vivo* behavioral bioassays will probably remain a fixture in the discovery, development and registration of repellents for the foreseeable future. Behavioral bioassays should yield reliable, meaningful data that accurately represent the efficacy of a test compound or essential oil.

The lone star tick, *Amblyomma americanum* (L.), has grown in importance as a nuisance biter and vector of pathogens, such as *Ehrlichia chaffeensis* Anderson, Dawson, Jones and Wilson, the causative agent of human monocytic ehrlichiosis (6). Stromdahl et al. (7) reported a high prevalence of spotted fever group rickettsiae in lone star ticks from Maryland. *Amblyomma americanum* occurs from the south-central and southeastern United States northward along the Atlantic seaboard to New England (8). The distribution of *A. americanum* has been expanding northward along the Atlantic Coast (6, 9, 10). Although *A. americanum* lacks the cachet and attention of the blacklegged tick, *Ixodes scapularis* Say, the principal vector of the Lyme disease pathogen, there are some advantages to using *A. americanum* in repellent bioassays. First, it is easier to rear *A. americanum* on a large scale than *I. scapularis*, so the former are obtainable in greater quantities and, if purchased, at lower prices. Second, behavioral bioassays of repellents depend on the arthropod subjects moving about; *A. americanum* do so more readily and rapidly than *I. scapularis*. Although *A. americanum* are active host seekers whose strategy tends toward the hunter type (11), in nature host contact may often occur while ticks are on questing sites on vegetation. Unlike *I. scapularis*, however, *A. americanum* readily abandon questing sites and will move several meters toward a host. The lone star tick is well known for its proclivity to move rapidly toward sources of CO₂ (12). Laboratory-treated *A. americanum* nymphs appear to be suitable replacements for field-collected nymphs, as demonstrated by Carroll et al. (13) who found that laboratory-treated nymphs from Texas and Oklahoma responded similarly to field collected nymphs from Maryland in dose response bioassays using deet and racemic 220.

The characteristic responses of *A. americanum* to repellents are epitomized in the bioassays reported by Carroll et al. (14), in which *A. americanum* and *I. scapularis* nymphs were subjected to the same tests using deet and SS220. When host-seeking *A. americanum* nymphs were encircled by a 1-cm wide ring of test solution on a horizontal filter paper disc, they routinely crossed concentrations of deet and SS220 that repelled all *I. scapularis* nymphs, confining the latter within the repellent-treated ring. However, concentrations that did not repel *A. americanum* nymphs on the horizontal filter paper, repelled them on a vertical surface from which they could drop. When the middle 4 × 5 cm of a 4 × 7-cm filter paper strip was treated with test solution, and the paper dried and suspended vertically, ticks were allowed to mount the lower untreated edge. As depicted in Carroll et al. (14), the dose response curve of *A. americanum* nymphs to deet in the vertical bioassay slopes gradually compared to the steep curve for *I. scapularis* to deet. Many *A. americanum* dropped from the vertical papers treated with repellent, whereas *I. scapularis* would either not enter the treated portion of the vertical paper or shortly after entering retreat to the lower untreated zone.

This difference in the behavior of *A. americanum* and *I. scapularis* was also observed in fingertip tests with elemol (15). In responding to repellents, few *A. americanum* tend to remain near but not on the treated surface (15). Instead, they crawl away or release their hold on a vertical surface and fall. When an *A. americanum* nymph rushes onto a barrier treatment a few centimeters wide, there is some chance that it might continue completely across the treatment because it can no longer detect a repellent gradient associated with the edge of the treatment.

However, a tick that tends to approach the repellent slowly and penetrates the treatment only slightly if at all, is less likely to cross a barrier treatment by chance. Physical and temporal parameters, such as the width of barrier treatments (the distance a tick must cross to be considered not repelled), influence the outcome of tick repellent bioassays. Sometimes ticks may cross a repellent-treated surface only after entering and retreating a few times, so a bioassay that ends without allowing a sufficient yet reasonable time for a tick to reencounter the repellent would overestimate the repellent's protective capacity.

Variation, perhaps associated with the "dash through or drop" reaction of *A. americanum* to repellents, is observed less often in bioassays when weakly or strongly repellent test solutions are tested, but is manifested in dose response studies. For example, dose response results for (-)-isolongifolenone and deet in fingertip bioassays against *A. americanum* nymphs were similar (16), but the results for (-)-isolongifolenone were notably more variable, with some higher concentrations repelling fewer ticks than lower ones. The sesquiterpene (-)-isolongifolenone occurs naturally in *Humiria balsamifera* St. (Aubl.) Hill (Humiriaceae), a tree found in South America (17) and is dissimilar in structure from deet. Variation in responses is expected, but excessive variation requires extra replicates and muddles interpretation of bioassay outcomes. Excess variability can limit which different compound/concentrations can be discriminated.

Acknowledging variation in behavior among tick species, to chose to keep keep matters simple and compare results from different bioassay systems using the same tick species and life stage. We examined the responses of *A. americanum* to two repellents, deet and (-)-isolongifolenone, in several bioassays to ascertain the strengths, weaknesses, and reliability of the various methods and to define optima for test time and physical dimensions. Specifically, we wanted to answer the following questions: (1) In vertical filter paper tests, how long should the paper strip be?; how long should the test last?; (2) does adding a bottom loop to vertical filter paper tests help prevent ticks from dropping off?; if so, is it better for the ticks to climb onto the strip (loop) near the part with the repellent challenge or further from it?; (3) how do moving object bioassays compare to other repellent bioassays for ticks?; (4) how do choice experiments (ticks confined to a petri dish where they must choose between substrates with and without a repellent) compare with other repellent tick bioassays?; and (5) since purchasing ticks is expensive, can they be reused in repellent bioassays?

Methods

Ticks

Host-seeking *A. americanum* nymphs were obtained from colonies at the USDA, ARS, Knipping-Bushland U. S. Livestock Insects Research Laboratory, Kerrville, TX and Oklahoma State University, Stillwater, OK. The ticks were held at 23-24° C, ~97% RH and a photoperiod of 16:8 h (L:D), and tested 3-6 mo after molting.

Chemicals

(-)-Isolongifolenone was efficiently prepared as a sole major product from (-)-isolongifolene (Sigma, St. Louis, MO) utilizing *tert*-butyl hydroperoxide as the oxidant, chromium hexacarbonyl as the catalyst, and acetonitrile and benzene as the solvent in high isolated yield ($\geq 90\%$) with high purity ($\geq 99.9\%$) in a short reaction time (~2h) (Wang and Zhang 2008). Deet was purchased from Aldrich, Sigma-Aldrich, St. Louis, MO, 95% Ethanol (Sigma-Aldrich, St. Louis, MO) was used as the blank control and the solvent to make deet and (-)-isolongifolenone solutions for the assays.

Composite Scores

A method we developed (18) to optimally combine the various behaviors typically exhibited by ticks as they navigate a test paper strip into a single score was used on the moving object bioassay experiment described below, and works well when many concurrent (behavioral) measures are taken on each individual animal in an experiment and one wants to create a single composite score for the individual animal. An outline of this method follows, detailed information is provided in Kramer et al. (18). The basic idea is to use the behavioral differences observed as ticks are tested on different compounds to find optimal weightings of these behaviors (that best discriminate among the compounds) using canonical discriminant analysis. Compounds to which ticks responded similarly (in theory, compounds that ticks do not discriminate between) will produce similar composite scores, those where behaviors differed will have different scores.

In addition to variables measuring duration or counts of behaviors, indicator variables were created with a value of 1, if the behavior was performed, and 0, if not. This was done so that all variables could be included in the analysis, even if not performed by all ticks. Useful variables to create the scores were determined in a stepwise discriminant selection procedure. One dimensional composite scores were created by first fitting canonical discriminant functions, which consisted of the sum of these variables with weights (referred to as 'loadings') that best separated the compounds, and using scores from the first canonical discriminant function. Although, in theory, the scores could have more than one dimension (or axis), in no case did we find more than the first discriminant axis was useful. Thus, a composite score was created for each individual tick, and it consisted of a single number.

Experiments on the repeated testing of ticks also made use of composite scores, though the loadings used came from an earlier study (see 'Variation and repeated use of ticks' below). Part of this methodology was used in the '22-cm filter paper strip' experiment (see below) to identify behaviors that discriminated among the compound-concentration combinations, although the final creation of the composite score was not necessary.

22 × 1-cm Vertical Filter Paper

A 1 × 22-cm strip of Whatman No. 4 filter paper was marked with a lead pencil at 1 cm intervals and 165 µl of test solution evenly applied by pipettor to all but the terminal 1-cm sections. Concentrations of 103, 206, 413, 825, 1238, 1650 nmol deer or (-)-isolongifolenone/cm² and an ethanol control were tested. The strip was allowed to dry for ~10 min, and suspended vertically from a bulldog clip attached to a clip on a work holder (Aptex Corp., Bethel, Connecticut). A vial containing ticks was opened in a moated petri dish. An active (crawling or waving its forelegs) tick was allowed to mount the lower untreated end of the strip by holding the vial close to the filter paper or letting a tick mount a section of bamboo barbecue skewer from which the tick transferred to the filter paper. The locations of the tick were recorded at 1-min intervals, as were whether it dropped from the strip or climbed through the area that received the repellent or ethanol treatment. The time and location at which a tick fell from the strip and the highest location the tick attained were also recorded. A strip was reused with other ticks until 30 min after the first tick climbed on the strip. A moated petri dish beneath the strip confined ticks that fell from the strip. Twenty nymphs were tested for each concentration of (-)-isolongifolenone and deer. Nymphs were tested with an ethanol control each day repellents were tested.

The interest in this experiment was to determine both the optimal time for a single trial and the optimal length of the paper strip. We employed a stepwise discriminant analysis (SAS Proc Stepdisc) for both variables using modified data sets (similar to the methodology used to create composite scores). These were created using a Perl program which recoded the data as if the paper strip had been shorter. For example, if the paper strip sheet had been 10 cm rather than 20 cm and a tick dropped off at the 12 cm mark, that tick would have been recorded as completing the test by walking to the top of the strip. For each potential height (starting at 3 cm to the full 20 cm) we noted the average squared canonical correlation (these increase with improved discrimination) with the set of variables selected by the stepwise procedure (these sets could be different for different heights) and which location times (tick location at 1 min, 2 min, etc.) were most often included. We also analyzed subsets of the data (by dropping one of the compound-concentration combinations in turn) to make sure that results were not driven by a single combination.

4 × 7-cm Vertical Filter Paper

A 4 × 7-cm strip of Whatman No. 4 filter paper was marked with a line 1 cm from and parallel to each end. The area between the lines (4 × 5 cm) received 165 µl of test solution evenly distributed by pipettor and was allowed to dry for ~10 min. Concentrations of 206, 413, 825, and 1650 nmol deer or (-)-isolongifolenone/cm² filter paper and an ethanol control were tested. The strip was suspended vertically from a bulldog clip attached to a clip on a work holder over a moated petri dish. A vial containing ticks was opened in a moated petri dish and 10 nymphs were allowed to climb onto the lower untreated edge of the filter paper. As the situation

dictated, the vial was held close to the filter paper or the filter paper (attached to the bulldog clip) was held close the vial in the petri dish to allow ticks to transfer. Tick locations were recorded at 1, 3, 5, 10 and 15 min after the tenth tick mounted the filter paper. Ticks were considered repelled if they fell from the filter paper without having crossed into the upper untreated area or were on the lower untreated area at 15 min after the tenth tick mounted the filter paper. Three replicates of 10 nymphs each were tested for each concentration of (-)-isolongifolenone and deer and ethanol control.

We tested the proportion of ticks repelled at the end of each trial for differences between the two compounds by fitting a generalized linear model, assuming that the proportions were samples from an over-dispersed binomial distribution, where the dependent variable is modeled as the logit of the proportion repelled. We used a square root transformation on concentration as it produced a more linear relationship with the logit of proportion repelled. We tested for difference between the two compounds by including a compound and compound by concentration interaction terms in the model, and noted the estimated over-dispersion for the compounds modeled separately.

4 × 7-cm Filter Paper with Loop

In order to provide *A. americanum* nymphs a third option (other options are dropping off or remaining in untreated area) for responding to a repellent barrier, the basic 4 × 7-cm filter paper was modified with two lateral extensions (1 × 6 cm, 1 × 5 cm) of the lower untreated zone that were curved to overlap 1 cm and were joined with transparent tape forming a ring or loop (Figure 1). The loop allowed ticks to move away from the 4 × 5-cm treated area with the possibility of returning and repeatedly challenging the repellent barrier. Two configurations of the avoidance loop were used. In both configurations, the upper 1 × 4 cm of the rectangle and the loop were untreated, as was a 1 × 4 cm approach tab that extended below the level of the loop. Ticks were allowed to climb onto the approach tab to start the bioassay. In the first configuration (Figure 1, panel A), the approach tab was directly below the 4 × 5 cm treated area and ticks could climb in a completely vertical route without interruption. In the second configuration (Figure 1, panel B) the tab was offset so that the left lateral margin of the approach tab was almost directly below the right margin of the rectangle. With the offset tab, ticks had to adjust their paths to continue to ascend, perhaps slowing their momentum as they encountered the treated section of the filter paper. The second configuration was tested with the loop on the near side and on the opposite side of the rectangle to the investigator, who was seated 0.6 m distant. Tick locations were recorded as in the 4 × 7-cm filter paper test. Three replicates of 10 nymphs each were tested for each concentration (206, 413, 825, and 1650 nmol/cm² filter paper) of (-)-isolongifolenone or deer, and an ethanol control.

We followed methodology similar to that given for the 4 × 7-cm filter paper trials to test for compound differences for each of the three paper configurations. In addition, we merged the datasets and, after removing the control (ethanol) trials, developed a generalized model to fit the proportion of ticks that fell (main

effects were paper configuration, concentration, and compound). We used the step function in R for this (similar to stepwise regression, using an AIC estimate to determine relative model fit). We also looked to see if there were differences in the control proportions that fell for the different paper configurations.

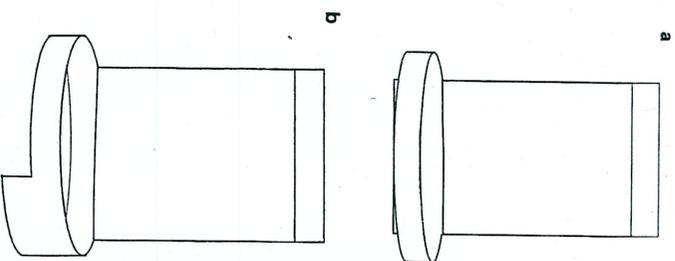


Figure 1. Loop configurations based on 4×7 -cm vertical filter paper. A) Loop (1 cm wide, ~ 4 cm diam) with 1 cm extension directly below 4×7 -cm rectangle. B) Loop same dimensions as A, but with extension offset so that ascending ticks could not go directly onto rectangle. Test solutions were applied to area between horizontal lines 1 cm from top and bottom of rectangle.

Moving Object Bioassay (MOB)

The moving object bioassay (MOB), described in detail by Dauel et al. (19, 20), is an *in vitro* system that features heat and motion, stimuli associated with the presence of a host. The system has been used primarily against *Ixodes* spp. (21). Douglas et al. (22) used a version of it with *A. americanum* nymphs. Briefly, a brass cylinder (drum) contained water warmed by an immersion heater (Tempco, Wood Dale, IL) heated to maintain temperatures of ~ 34 – 36°C on the drum's outer surface. A rotisserie motor rotated the drum horizontally at 13–15 rpm. A strip of Whatman No. 4 filter paper with a 2×10 cm section treated with 165 μl was affixed closely to the side of the cylinder over a brass plate soldered in place. Concentrations of 206, 413, 825, and 1650 nmol deet or (-)-isolongifolenone/cm²

filter paper, and an ethanol control were tested. The plate caused the paper to protrude slightly from the surface of the drum. A petri dish half (2 cm deep, 6 cm diam) containing a silicone island and water (to confine ticks to the island) was held in place at the level of the drum. An inverted L-shaped wire projected from the silicone island. A small platform fashioned from clay for placement of a tick was affixed at the bend of the wire. The tip of the wire (nearly perpendicular to the side of the drum) was positioned 1–2 mm from the surface of the filter paper, just close enough for a nymph to catch hold of the filter paper with its forelegs as the paper passed by on the rotating drum. We recorded whether a tick contacted the filter paper, transferred to the paper and dropped from the paper. The time elapsed until the tick reached a mark 1 cm from the tip of the wire, reached the tip of the wire, transferred to the filter paper and crawled or dropped off the paper were also recorded.

We used the composite score method, explained above, to create linear discriminate functions that best separated the compound-concentration combinations. Because we found very poor separation, we tried a number of modifications by subsets the data to improve the separation. Since the resulting composite scores appeared to be close to normally distributed, we used ANOVA to estimate which compounds differed and to estimate R^2 .

Petri Dish Choice Bioassay

One half of each of two Whatman No. 4 filter paper discs (9.0 cm diam) marked into halves with a lead pencil was evenly treated (by pipettor) with 200 μl ethanol, which was allowed to dry for 10–15 min. When the ethanol application dried, an equal volume of test solution was applied to the other half of each filter paper disc and was allowed to dry for 10–15 min. Concentrations of 157, 315, 629, and 1258 nmol deet or (-)-isolongifolenone/cm² filter paper and an ethanol control were tested. One filter paper disc was placed in a disposable plastic petri dish lid (9.3 cm diam). A piece of wire (1.0 cm long, 0.1 cm diam) was placed on the ethanol treated half of the filter paper disc and similar piece of wire on the repellent-treated half of the disc. Five nymphs were dumped from a Fluon™-coated centrifuge tube (0.4 cm inner diam, truncated to a length of 3.5 cm) on a disc of paraffin (0.6 cm diam) affixed by pressure on the center point of the filter paper. A second filter paper disc was placed on top of the disc in the petri dish, so that repellent and ethanol treated halves aligned. A Mason jar (0.94 l) lid ring (8.8 cm outer diam) placed on the filter paper and held in place by two rubber bands confined the ticks between the filter papers, a method used by Crystal and Derrilo (23) to confine mites in toxicant bioassays. The locations of the ticks were recorded at 10, 30, 60 and 120 min after the ticks were released on the filter papers. To aid in counting the ticks between the filter paper discs and discerning the diameter line, a flashlight (0.15 m distant) beam was shone briefly through the layers of paper.

For analysis, we used methodology similar to that described above, fitting a generalized linear model based on a quasi-binomial (over-dispersed binomial) distribution, and estimating means and a 95% confidence interval about the mean for each compound-concentration combination at each of the four time points.

Variation and Repeated Use of Ticks

To assess variation among ticks in how they respond to repellents, individually identified ticks were tested repeatedly within a day and over days in a vertical paper strip bioassay similar to those described above. Unlike the other bioassays we describe which used filter paper, this bioassay used recycled bond paper. Briefly, 15 μ l of test solution was applied evenly with a pipettor to the area (4 cm²) between the 2 and 6-cm marks of a 1 \times 8-cm strip of paper marked transversely at 1-cm intervals. Acetone was the solvent. The concentration (0.016 mg deet/cm² paper) was determined by preliminary testing to repel 40-60% of the ticks. After the paper had dried for 10 min, it was suspended vertically, and a tick was allowed to crawl onto the lower untreated portion of the strip. Observations lasted until the tick climbed past 6 cm, fell from the paper without climbing past 6 cm or 10 min elapsed from the time the tick crawled onto the paper. The behaviors recorded (some are presence/absence, some are duration) are listed in Table 1. One group ($n = 15$) of *A. americanum* nymphs was tested twice a day for three consecutive days. A second group ($n = 15$) of nymphs was tested twice a day for four consecutive days and, after a hiatus of 3 d, tested twice a day for two consecutive days. Thirty nymphs were tested once a day and at intervals of 5, 13, 3, and 4 d thereafter.

Table 1. *In vitro* bioassays discussed in this chapter. All had test solutions applied to Whatman No. 4 filter paper

22 x 1-cm vertical filter paper strip
4 x 7-cm vertical filter paper strip
4 x 7-cm vertical filter paper strip, loop extended, direct
4 x 7-cm vertical filter paper strip, loop extended, offset near observer
4 x 7-cm vertical filter paper strip, loop extended, offset near observer
moving object bioassay (MOB)
petri dish choice

Although several behaviors were recorded during a trial, since ticks were always tested with the same concentration of repellent, we could not employ the methods in Kramer et al. (18) which used different compounds to create a composite score. Instead, we created the composite score from the weights (loadings) used in Weldon et al. ((24), Table 1 in that paper), which used the same testing method and produced clear discrimination among many compounds, ranging from those with little repellent activity to those with considerable repellent activity. We reasoned that if a tick's performance deteriorated over time by repeated testing, it would show similar changes to being tested on a more effective repellent. For example, after many tests it might be more likely to drop off the paper strip earlier, be more reluctant to cross the area with repellent, etc. Preliminary analyses suggested that, at least for ticks tested frequently over many

days, the composite score was a good summary, with values increasing with the number of repeated tests (consistent with increasing values for more repellent compounds in Weldon et al. (24).

We fit the data with mixed models using the nlme package in R (25), with the composite score as the dependent variable, and test day, time of day (AM or PM) as fixed independent variables, and individual tick as a random block effect. Test day was treated as either a regression variable (with a linear and quadratic component) or as a factor; typically a better fit (judged using AIC) resulted from using test day as a regressor. The basic model was altered as appropriate for the different experiments (e.g. in one of the experiments ticks were tested only in the morning). Residuals were inspected for autocorrelation (for an individual tick, it is possible that residuals from sequential trials would be more alike than residuals separated by more time), but none was found.

Results

For each type of bioassay, deet and (-)-isolongifolenone were similarly repellent to *A. americanum*, indicating that for the purpose of comparing the efficacy of the two compounds (deet generally considered the standard of repellent activity), the various filter paper bioassays yielded the same conclusions.

22 x 1-cm Vertical Filter Paper

We found that the optimal height of the filter paper strip for testing *A. americanum* was approximately 8-9 cm, which resulted in the highest canonical correlation (Table 2), with correlations decreasing as one moved away from that distance. This was the optimal paper strip height for all subsets of compounds, as well as the full set. We found that tick locations after 6 min were not selected for the 8-9 cm height, and rarely selected for other heights. Tick locations at 6 min were marginally or not significant (though selected to be in the model), so perhaps tests could be even shorter.

4 x 7-cm Vertical Filter Paper

We found no statistical difference in the slope of deet and (-)-isolongifolenone for the logit of the proportion of repelled ticks regressed on the square root of concentration ($p = 0.423$, *t*-test, 26 d.f.). Thus, the two compounds appear to have similar repellent activity at the same concentrations (regression equation: logit (p) = $-3.799 [0.562] + 0.106 [0.018] \times \text{sqrt}(\text{conc.})$, standard error of estimates in square brackets, concentration in mmol/cm², p is the proportion repelled) (Figure 2 illustrates the data and fitted model). The over-dispersion parameter was larger for deet (2.239 versus 1.287), though both are well within the range commonly seen for experiments of this kind, with responses to deet indicating moderate over-dispersion.

Table 2. Results from a stepwise selection on useful behaviors to discriminate among compounds for filter paper strip heights of 6-10 cm. Behaviors abbreviations are: Locxmin = tick location at x min, lxd = 0 if tick was still on strip at x min, 1 if tick dropped off the strip at or before x min, DropLoc = height (cm) where tick dropped off strip

Height (cm)	Behaviors, in order of entry	Average squared canonical correlation
6	Loc1min, Loc5min, l4d	0.0560
7	Loc1min, Loc1min, l1d, Loc5min	0.0644
8	Loc1min, Loc3min, Loc2min, Loc6min, l5d	0.0762
9	Loc1min, Loc5min, Loc3min, l2d, DropLoc	0.0797
10	Loc1min, Loc5min, DropLoc	0.0628

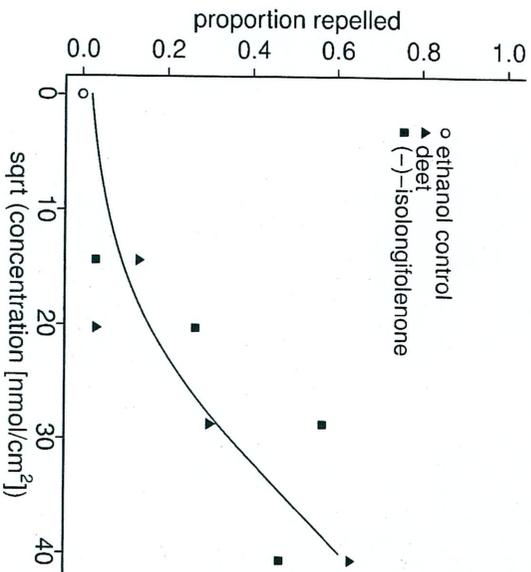


Figure 2. Points represent the proportion of ticks (aggregated over trials) that did not successfully crawl above the treated area in the 4 × 7-cm vertical paper test. The line represents the model fit to these data (note: this is a straight line on the logit scale).

4 × 7-cm Vertical Filter Paper with Loop

The data (proportion repelled and proportion that fell off the paper) are illustrated in Figure 3 for all three kinds of added loops and the two repellent compounds. Results for the paper configuration with no offset above the ring ('direct') were very similar to those of the previous experiment; no significant differences ($p = 0.428$, t -test, 28 d.f.) were found between the two compounds for the number of ticks repelled (regression equation: $\text{logit}(p) = -1.631 [0.280] + 0.0837 [0.0122] \times \text{sqrt}(\text{conc.})$, standard error of estimates in square brackets, concentration in nmol/cm^2). In this experiment, the over-dispersion parameter was smaller for deet (1.040 versus 1.530). Results for the paper configuration where the offset was near the researcher were similar to those from no offset; no significant differences ($p = 0.192$, t -test, 35 d.f.) were found between the two compounds (regression equation: $\text{logit}(p) = -0.337 [0.194] + 0.0541 [0.0103] \times \text{sqrt}(\text{conc.})$, standard error of estimates in square brackets, concentration in nmol/cm^2). In this experiment, the over-dispersion parameter was larger for deet (1.749 versus 1.174). Similar results were again obtained when the offset was opposite the researcher (far), (regression equation: $\text{logit}(p) = -2.087 [0.440] + 0.0808 [0.0178] \times \text{sqrt}(\text{conc.})$, standard error of estimates in square brackets, concentration in nmol/cm^2). In this experiment, the over-dispersion parameter was larger for deet (3.816 versus 2.384).

The data sets were combined to determine if there were differences in the proportion of ticks that fell, and a higher dimension model was fit with a stepwise procedure. The model produced suggested that the offset paper configuration, with the researcher far from the loop, differed from the other two configurations in that far fewer ticks fell at lower concentrations (Figure 3, panel B), but with a more positive slope (so that falling rates were similar at high concentrations). There was also a systematic larger difference (about 3 times as large, on the logit scale) in falling rates between the direct and near configurations for (-)-isolongifolenone than for deet (i.e. on Figure 3, panel B, the points for (-)-isolongifolenone for the direct and near paper configurations are mostly far apart at the same concentrations). However, there was no significant difference between the paper configurations for the ethanol controls ($p = 0.100$, t -test, 13 d.f., over-dispersion parameter = 2.210).

For the two highest doses, 825 and 1650 nmol/cm^2 filter paper, of (-)-isolongifolenone, proportions of 0.17 and 0.07 ticks ($n = 30$) remained below the treatment at 10 min in the offset ring (near) compared to proportions of 0.03 and 0 ticks ($n = 30$) in the basic 4 × 7-cm bioassay. For the same doses of deet, proportions of 0.13 and 0.17 ticks ($n = 30$) remained below the treatment in the offset loop (near) compared to proportions of 0.07 and 0.10 ticks ($n = 30$) in the basic 4 × 7-cm bioassay. No ticks ($n = 30$) fell from ethanol controls of the basic 4 × 7-cm bioassay, whereas proportions of 0.15 ($n = 40$), 0.40 ($n = 70$), and 0.18 ($n = 50$) ticks fell from the controls of the direct, offset (near), and offset (far) ring 4 × 7-cm bioassays respectively.

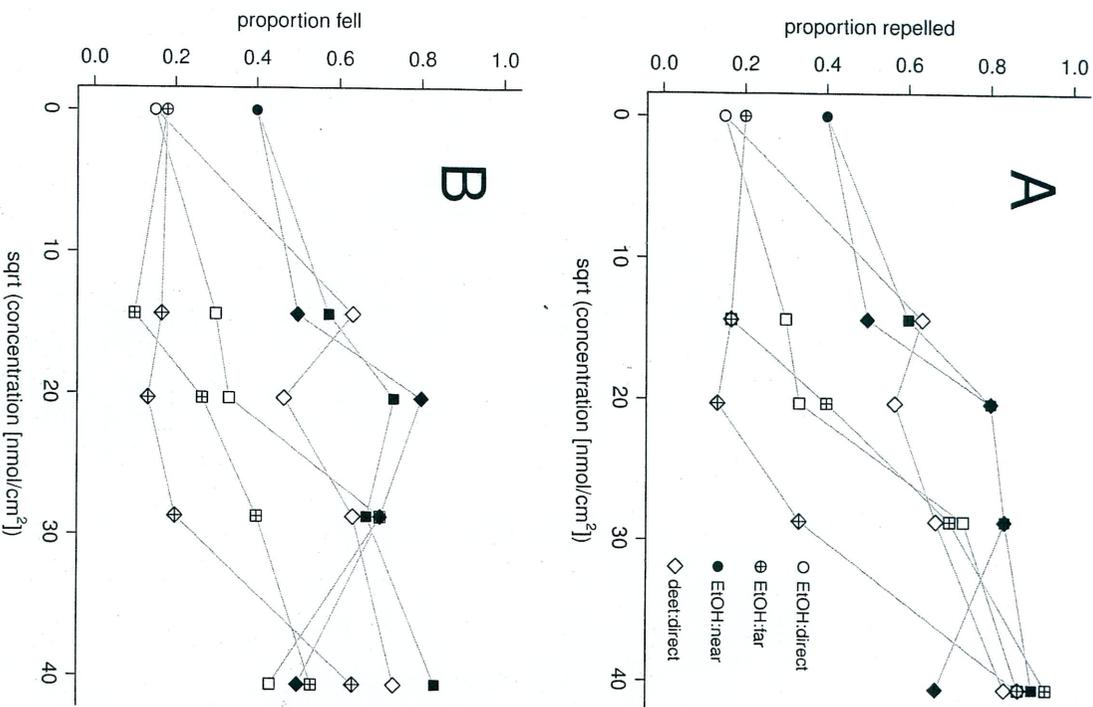


Figure 3. Points represent the proportion of ticks (aggregated over trials) that did not successfully crawl above the treated area (panel A) or fell (panel B) in 4×7 -cm vertical paper tests with three different types of added loops and two compounds. The gray lines are an aid for following individual compound-loop combinations over the concentrations. Without including the effect of over-dispersion, the s.e. for each point would be 0.1. Thirty ticks were tested for each compound concentration and control.

Thus, there is an effect of the configuration of the paper used in these tests. All added loop configurations tended to increase drop rates of ticks, even in control conditions. This affects the proportion 'repelled', since a tick that drops is considered to be 'repelled', as one can readily observe by noting the similarity between the two panels in Figure 3. Of the three configurations with loops, the best appears to be 'far', but this configuration does not seem to improve on the vertical paper without a loop in vertical repellent tests.

Moving Object Bioassay (MOB)

Results from following the composite score methodology (Table 3) yielded composite scores that were close to normally distributed but also with means close together (i.e. there was not a linear discriminant function that, based on the behaviors observed, could separate the compound-concentration combinations). Only deet at 413 nmol/cm² was significantly different than the ethanol control, and the ranked means did not correspond to the concentrations (which makes little sense). We then redid the composite scores using fewer concentrations (e.g. ethanol, deet at 1650 nmol/cm² filter paper, (-)-isolongifolone at 1650 nmol/cm²), then applying the loadings to all compound-concentration combinations to create new composite scores; also we eliminated some individual ticks that seemed to have unusual behaviors (producing an unusual composite score). This did not result in better (or more interpretable) separation, deet at 413 nmol/cm² filter paper was still the only one that significantly differed from ethanol and the ranked means did not match their respective concentrations. We also examined the individual behaviors' relation to the compound-concentration combinations using summary statistics and graphics and found no obvious pattern. In all models, R² was relatively small (about 10%), indicating that the model explained little of the variation in the composite scores. Whether or not ticks transferred to the filter paper differed significantly between repellent treatments of the highest concentration tested and the control ($p = 0.002$), but no difference was detected between compounds ($p = 0.395$). Thus, we conclude that our implementation of this test was not effective to test for compound or concentration differences with *A. americanum*.

Petri Dish Choice Bioassay

Both compounds were avoided at higher doses (Figure 4 gives model based means with a 95% confidence interval), with deet showing some decline in repellency with time (the positive linear time trend was significant; $p = 0.031$, t -test, 91 d.f.). The wide 95% confidence intervals are due to the relatively small sample sizes used. The over-dispersion parameter was estimated to be only about 1.4 (where 1.0 indicates no over-dispersion). The asymmetry in the confidence intervals is due to the back-transformation, the 95% confidence intervals are symmetric on the logit scale.

Table 3. Tick behaviors used to construct composite scores for moving object bioassay

Behavior	Loading, 1 st Principal Component
Reach final 1 cm of wire (yes/no)	1.66
Reach final 0.5 cm of wire (yes/no)	-4.29
Contact with paper (yes/no)	2.20
Transfer to paper (yes/no)	0.562
Drop from paper (yes/no)	1.19
Time to final 1 cm of wire	0.000526
Time to tip of wire	-0.0145
Time to transfer to paper	0.00340
Time left paper	-0.0150

Variation and Repeated Use of Ticks

To determine if ticks fatigue (change in response) with continuous testing, we tested each tick twice a day (tested on days 1, 2, 3, 4, 8, 9). The following mixed model was fit to the data (with variance estimates of 0.418 and 3.668 for the among tick and residual components, respectively): $y = -0.442 + 0.378 x_1 - 0.078 x_2 + 0.521 x_3$, where y = composite score, x_1 = day (with values 0, 1, 2, 3, 7, 8, s.e. = 0.0629); $x_2 = (x_1 - \text{mean}(x_1))^2$, s.e. = 0.0263; $x_3 = 0$ for AM and = 1 for PM (i.e. a dummy variable), s.e. = 0.290, p -value estimates for the coefficients of the x variables were 0.000, 0.004, and 0.074, respectively. The regression equation can be interpreted as the composite score generally increasing (ticks exhibiting reduced performance, more easily repelled) as day increases, though with some curvature due to the quadratic component, and with a marginally significant effect of time of day (composite scores generally higher in PM). Tick to tick variation was moderate, but the large residual variance indicates that, for each tick, there was considerable variability in composite score from one trial to the next, as shown in Figure 5 for a few example ticks. These results demonstrate that continuous testing adversely affects tick performance.

To determine if a less intense schedule ameliorated the repeated testing effect, we tested another group of ticks once per day (tested on days 1, 6, 19, 22, 26). The following mixed model was fit to the data (with variance estimates of 0.823 and 2.445 for the among tick and residual components, respectively): $y = -1.179 + 0.067 x_1$, where y = composite score, x_1 = day (with values 0, 5, 20, 23, 26, s.e. = 0.013), p -value estimate for the coefficient of x_1 was 0.000. A quadratic effect examined in a preliminary model was not significant. While the slope is shallower, the results suggest that there is still a repeated testing effect.

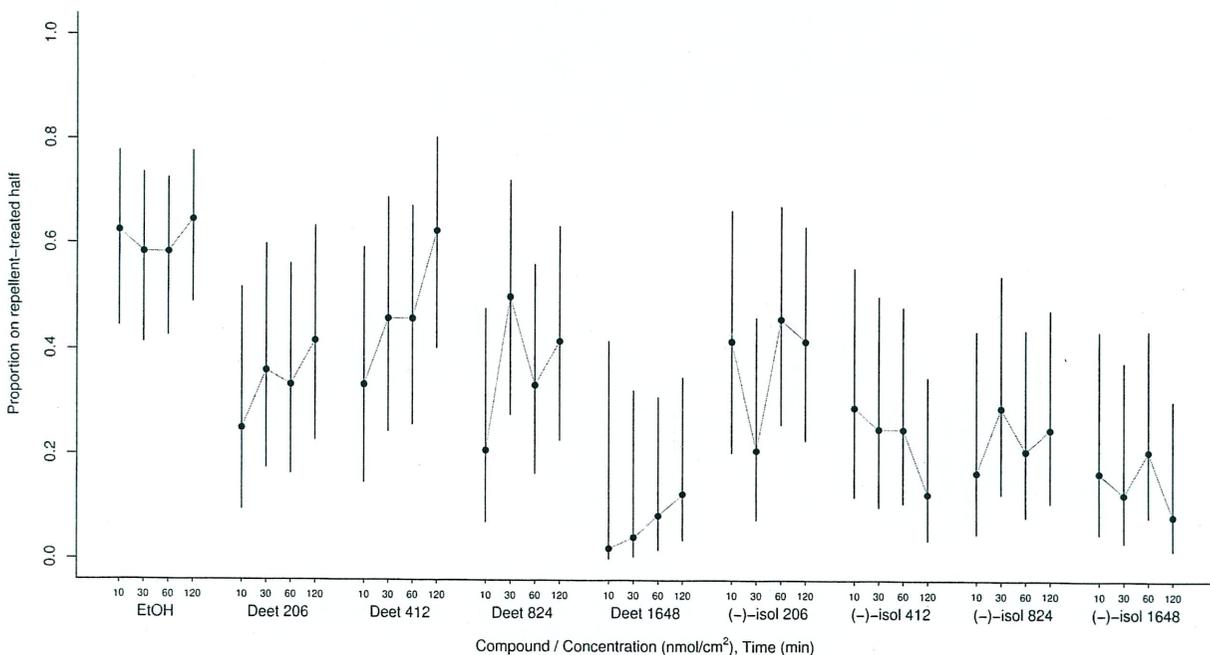


Figure 4. Points give the proportion of ticks on the repellent-treated half of a filter paper in a Petri dish at 10, 30, 60, and 120 min for various concentrations of deet and (-)-isolongifolenone. Vertical bars give 95% confidence intervals (asymmetric on the back-transformed proportion scale).

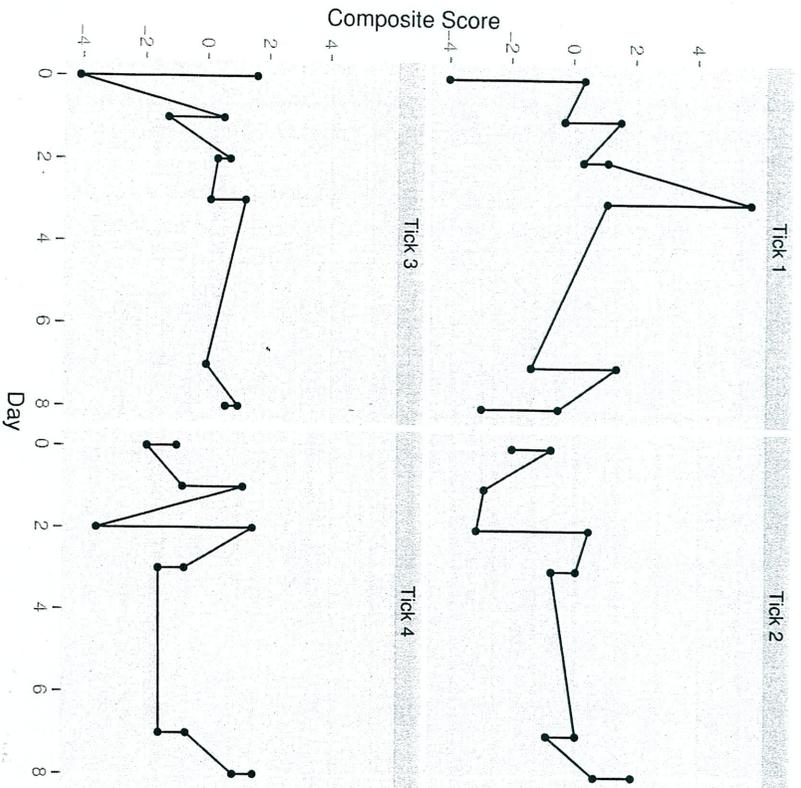


Figure 5. Composite scores over days (ticks tested twice a day) for four example ticks that were repeatedly tested on vertical filter paper. Note that the composite scores are rather erratic over time and the patterns among ticks dissimilar.

An alternative to testing each tick many times is to test it twice. We tested each tick twice on the same day, and analyzed the resulting composite scores in a mixed model using a factor with two levels (AM and PM). This factor was not significant, $p = 0.792$, suggesting that a second test on the same day does not decrease performance if each tick is only tested twice. Variance estimates were 0.000 and 2.861 for the among tick and residual components, respectively.

In our last set of trials we wanted to determine if a 2-wk 'recuperation' time (tested twice on day 1, tested once on days 15 and 16) would ameliorate the repeated testing effect. A factor was created with 4 levels (for the 4 trials per tick). In a mixed model (with variance estimates of 0.000 and 1.748 for the among tick and residual components, respectively), this factor was not significant, $p = 0.777$, suggesting that a two week recuperation time is sufficient for ticks to regain prior performance levels.

Thus, we found that repeatedly testing ticks does not decrease performance, if they are (1) tested twice a day only, and (2) they are allowed to 'recuperate' from the first day of testing for 2 wk before retesting. Thus, researchers can benefit from these results since they show that one can use one half to one fourth as many ticks to produce comparable results. The price paid for this is that the ticks must be held for a 'recuperation' period and that the statistical models used must allow for the correlation induced by repeated testing of the same tick. This correlation was estimated to be zero when testing ticks twice in the same day and when allowing for a 'recuperation' period (and was small in other tests), so that the repeated testing does not greatly affect effective sample size (if the correlation was high, then the effective sample size can be much smaller than the number of ticks actually used).

Discussion

Dautel (20) reviewed an array of methods used to assess the efficacy of tick repellents. He grouped the methods in three categories: 1) those using live hosts, 2) those using attractants associated with hosts, and 3) those using no attractants. The *in vitro* bioassays we examined fall into categories 2 and 3, with the petri dish bioassay essentially lacking host cues and the MOB using the simulated host cues, temperature and motion. In the MOB and the other bioassays, ticks are exposed to host cues in the form of chemical, vibrational and visual (*A. americanum* possess eyes) stimuli from an observer/experimenter situated nearby.

We tested the same stage of the same species of tick against mostly the same concentrations of two repellents tested under nearly the identical conditions (solvent, filter paper, temperature and RH range). The similarity in effectiveness between deet and (-)-isolongifolene reported by Zhang et al. (16) was confirmed in the various types of bioassays. In certain types of bioassays, higher proportions of *A. americanum* nymphs were repelled (e.g. 4×7 -cm offset ring configurations) compared with other bioassays (e.g. 4×7 -cm basic configuration). These findings provide more evidence that a panel of test compounds must include at least one 'standard' repellent, such as deet, to provide a common basis or link for comparing results from bioassays that use different methods.

The tendency of *A. americanum* and other ticks to climb has been used in several *in vitro* and *in vivo* bioassays. In testing fractioned compounds from *Chamaecyparis nootkatensis* (D. Don) Spach. essential oil, Dietrich et al. (26) allowed *I. scapularis* to climb a vertical cotton-tipped applicator with test solutions applied to its apical portion. The repellency of benzoquinone compounds secreted defensively by millipedes was tested by releasing *A. americanum* nymphs on a clay substrate, and encircled by a ~3-cm high cylinder of filter paper to which a 2-cm wide band of test solution had been applied (27). In fingertip bioassays (16, 27-29) used to evaluate repellent efficacy against *A. americanum*, the finger was held vertically with the untreated tip down. Cream, spray and lotion formulations of repellents were applied in a 5-cm wide ring encircling each ankle of human volunteers and challenged by ticks that were placed or crawled onto the volunteers' feet at 2-h intervals for 12 h (30, 31). A similar test using a treatment

on the wrist and forearm is the bioassay recommended by the EPA for obtaining data for registration of tick repellents (32).

The results of the 22 × 1-cm vertical filter paper bioassay indicate that in climbing type bioassays with *A. americanum* nymphs the vertical dimension need not be great, with 8-9-cm height optimal. When the strip was treated with ethanol alone, 21 of 54 (38.9%) of the nymphs never climbed the full 20 cm "treated" section in 10 min, with 28.1% of these ticks dropping from the strip. On ethanol treated 22 × 1-cm strips, 94.4% of 54 ticks climbed past 5 cm in 10 min, and 79.6% climbed past 8 cm. In the interest of having robust controls, a vertical treatment of 5 cm may be a good option.

The narrowness (1 cm) of the 22-cm strip allowed little lateral movement by ticks. A critical dimension in barrier type repellent tests is the minimum distance across the treated surface that a tick must traverse to defeat the treatment. A fast moving tick that enters an overly narrow barrier treatment might quickly detect a decreasing gradient of repellent toward the opposite border of the treatment and continue through the barrier, whereas a broader barrier would allow more opportunity for a tick to retreat from or drop off the treated surface. How narrow is too narrow? On the 22 × 1-cm strips treated with 1238 nmol/deet or (-)-isolongifolenone/cm² filter paper, >50% of the ticks did not climb above 1 cm. As the heights increased, the proportions of ticks not reaching those heights (repelled) increased, so heights approaching 8 cm would give better discrimination from controls.

In the 22 × 1-cm bioassay, the proportions of ticks reaching the height increments 1-10 cm tended to decrease as the concentrations of the two repellents increased to 1238 nmol repellent/cm² filter paper, but at the highest concentration (1650 nmol repellent/cm² filter paper) the proportions were similar to those for 413 and 825 nmol repellent/cm² filter paper. This variability is reminiscent of that observed in the Zhang et al. (16) data for (-)-isolongifolenone in fingertip tests with *A. americanum*. Analysis of the 22 × 1-cm strip tests, show that climbing-type bioassays need not last long with ~6 min duration capturing the critical data. Because adequate replication is essential, minimizing the duration of individual bioassays is important.

We thought that the addition of the loop (offset and direct) to the lower untreated area of the 4 × 7-cm filter paper might preempt the "run or drop" behavior of *A. americanum* by providing an alternative escape from the repellent, but such was not the case. The addition of the ring below the 1 cm untreated area at the lower end of the filter paper may have enhanced the likelihood of ticks dropping from the paper. We have used the basic 4 × 7-cm filter paper bioassay in several studies (e.g. (14, 15, 33)), and in our experience, only rarely ≥2 of 10 ticks fell from untreated controls. In the case of the basic 4 × 7-cm tests we report here, no ticks (n = 30 tested) fell from the controls. However, in 10 of 16 controls in the loop bioassays ≥2 ticks fell, with 0.40 of the ticks (n = 70) falling in the controls of the offset (near) loop bioassay. When the loop was on the same side of the 4 × 7-cm strip as the observer, it allowed ticks to approach as close as ~4 cm to the observer and away from the repellent. With this configuration, the highly active *A. americanum* ticks may have fallen from the loop in an attempt to reach the observer. For the two highest doses of deet and (-)-isolongifolenone (825 and

1650 nmol compound/cm² filter paper), higher proportions of ticks were repelled and remained below the treated area in the offset (near) loop bioassays than in the basic 4 × 7 cm bioassays. The apparent higher repellency may be due to a greater tendency of ticks to drop from the loop configurations, as seen in the controls, or to the escape option of the loop, manifested in ticks remaining below the treated area at 10 min. The problem with ticks falling from the paper makes the offset loop (near) bioassay unsatisfactory.

For the MOB, we recorded the same behaviors as Dautel et al. (19) who tested deet (0.11 mg/cm² filter paper) and ethanol controls against *Ixodes ricinus* (L.). Several important behaviors associated with host acquisition (contacting, transferring to and remaining on a moving surface) were recorded in the MOB, but like Dautel et al. (19) we only found significant differences between treatment and control for the proportion of ticks that transferred to the filter paper and the length of time the ticks remained on paper. While the behaviors recorded seemed well suited for the composite score analysis, separation of the treatments did not occur. Testing an additional higher concentration for this and the other bioassays might have improved discrimination. In our tests, at low concentrations of repellent ticks left filter paper treated with (-)-isolongifolenone more quickly than deet-treated paper. Two factors confound "time on paper" results. First, ticks move at different speeds, which is evident in untreated controls. Second, when a tick transfers to the moving filter paper, it is not equidistant to all the edges of the paper; the distance a tick travels to the edge of the paper depends on where it gets on the paper and the direction it crawls. With highly effective concentrations of repellent, *A. americanum* would be expected to quickly fall from the paper, which we observed. The petri dish choice bioassay differs from the other bioassays in this study in that it offered no opportunity for a tick to remove itself more than 4.5 cm from the repellent treatment. Larger petri dishes would allow ticks to escape further from the repellent. With an active tick like *A. americanum*, the absence of a complete escape option may create a stronger challenge to the repellent than vertical tests, but probably not as strong a challenge as placing the ticks within a horizontal ring of a repellent, as used by Carroll et al. (14). The no escape feature in petri dish tests has been used to force ticks to choose to contact (or avoid) either of two repellent treatments, when each half of the substrate received a different repellent treatment (34). Once set up, the petri dish bioassay does not require the constant attention of an observer. We recorded tick locations periodically for 2 h after placing the ticks between the filter papers, which allowed detection of a decline in efficacy of deet over time.

The highest concentration we used (1650 nmol compound/cm² filter paper) was rather effective, but even 3 times that concentration in 4 × 7-cm vertical filter paper bioassays under the same conditions does not repel all *A. americanum* nymphs (Carroll, unpublished data). The dose range used in these tests may have been adequate, but the addition of a higher (even more repellent) concentration would have given a more complete picture of dose response relationships. The highest concentration of (-)-isolongifolenone repelled 90% of *A. americanum* in the 4 × 7-cm strip in the offset ring (near) configuration, the highest concentration of deet repelled 86.7% of the ticks in the 4 × 7-cm strip in the offset ring (opposite)

configuration, and in the petri dish bioassay no ticks were in the treated half at 10 min.

Because rearing or purchasing ticks for laboratory use can be costly, reusing individual ticks in bioassays is a reasonable option if a similarity in response between reused ticks and naïve ticks could be assured. By retesting individually tracked *A. americanum* nymphs, we found that the ticks' performance was negatively affected by repeated testing. While testing the same ticks twice in one day (morning and afternoon) did not produce different results, based on our findings continued once or twice daily testing is inadvisable. Ticks allowed 2 wk to recover between testing responded similarly to naïve ticks. Eventually age related changes in tick activity and responses can be expected.

Although there are advantages to using *A. americanum* in repellent bioassays, some drawbacks exist. Perhaps, the greatest challenge in using *A. americanum* in behavioral bioassays involves transferring these particularly active and tenacious ticks into vials or bioassay arenas. In this regard, pump operated aspirators are useful in capturing ticks and putting them in vials. None of the climbing bioassays used in this study seemed to mitigate the variation observed in Zhang et al. (16). Further investigation is needed for a better understanding of the nuances of *A. americanum* responses to repellents.

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Chapter 8

Development of Space Repellents for Vector Control

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Arthropod-borne diseases impact large portions of the developing world and impart substantial economic and health burdens in these regions. Despite the burden these afflictions have on local populations, our tools for controlling the vectors responsible for pathogen transmission are limited. One critical component of any vector-borne disease management strategy is the use of chemicals in either indoor residual sprays, on bed nets or as topically applied repellents. The chemicals that are currently recommended for use, however, are quickly becoming inadequate to sustain disease control due in part to insecticide resistance. Evaluation of how mosquitoes respond to insecticides is an expanding field of study and the knowledge gained from these endeavors is paramount to advancing the development of new classes of chemistry to expand our current arsenal of effective compounds. One area of particular interest is the exploitation of behavior-modifying actions of chemicals in order to create vector free spaces and thereby reduce human-vector contact. Such chemicals could be used in various delivery platforms and in combination with other vector control interventions to enhance the effectiveness, affordability and sustainability of public health tools.