2003

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Daniel E. Sonenshine
Old Dominion University, dsonensh@odu.edu

Trevor Adams

Sandra A. Allan

John McLaughlin

Francis X. Webster

Original Publication Citation

Sonenshine, D. E., Adams, T., Allan, S. A., McLaughlin, J., & Webster, F. X. (2003). Chemical composition of some components of the arrestment pheromone of the black-legged tick, *Ixodes scapularis* (Acari : Ixodidae) and their use in tick control. *Journal of Medical Entomology, 40*(6), 849-859. doi:10.1603/0022-2585-40.6.849

Chemical Composition of Some Components of the Arrestment Pheromone of the Black-Legged Tick, *Ixodes scapularis* (Acari: Ixodidae) and Their Use in Tick Control

DANIEL E. SONENSHINE,[1] TREVOR ADAMS, SANDRA A. ALLAN,[2] JOHN MCLAUGHLIN,[3] AND FRANCIS X. WEBSTER[4]

Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529

J. Med. Entomol. 40(6): 849–859 (2003)

ABSTRACT Chemical analysis (high-performance liquid chromatography) and bioassay demonstrated the presence of compounds that seem to be components of the *Ixodes scapularis* arrestment pheromone. Only two purines, guanine and xanthine, were found in acidified saline extracts made from cast skins after molting of fed nymphs, fed larvae, and fecal/excretory exudates deposited by unfed adults on substrates in their environment. The ratio of guanine to xanthine was 10.6:1 in an extract from the nymphal skins versus 0.95:1 in an extract from the larval skins. Guanine, xanthine, and traces of a third purine, tentatively identified as 8-azaguanine, were found in extracts made from filter paper strips or washings from glass vials contaminated with tick feces and excreta left by unfed adults. 8-azaguanine may be a product of microbial degradation of the other purines rather than a natural product from the ticks. Low concentrations of ammonia also were detected in saline extracts of excreta from feeding ticks. Hematin also was found in NH_4OH extracts of the black fecal/excretory exudates deposited by the unfed ticks. Hematin was tentatively identified by comparison of spectra with that of the authentic standard. Bioassays demonstrated a strong positive arrestment response to cast skins found to contain a mixture of guanine and xanthine and to black fecal/excretory exudates containing guanine, xanthine, the putative 8-azaguanine, and hematin. A Noldus video tracking system using a CCD video camera and Ethovision Pro tracking software showed statistically significant increases in the frequency of visits to the treated zone versus the control. Ticks were significantly more likely to assemble in response to the tick exudates within as little as 3 h compared with the controls. Previous bioassay studies also showed strong positive responses to guanine, xanthine, other purines, and hematin. Comparisons with the arrestment pheromones of other tick species are described. The inclusion of the pheromone components in a permethrin-impregnated oily matrix, Last Call, increased the lethal activity of the product to 95% compared with only 65% in the formulation with permethrin alone. More detailed knowledge of *I. scapularis* arrestment pheromone may be useful for improving the efficacy of this tick-killing technology even further.

KEY WORDS pheromone, *Ixodes scapularis*, hematin, purines

THE BLACK-LEGGED TICK, *Ixodes scapularis* L. is the primary vector of disease-causing agents in humans in North America, especially Lyme disease, human granulocytic ehrlichiosis, and human babesiosis (Walker and Dummler 1996, Childs et al. 1998). Consequently, efforts to control this tick vector as a means of disrupting disease transmission has aroused considerable interest. Among the various strategies that have been considered is the use of tick pheromones to facilitate targeted delivery of acaricides in the tick's natural habitat.

Ticks use a variety of different pheromones to regulate their behavior, especially for sexual activity and the assembly of clusters of individuals in their natural environment. Assembly results from a type of behavior known as arrestment, defined as the cessation of kinetic activity. This response reduces the distance between individuals that perceive a particular stimulus in their environment (Cardé and Baker 1984) and leads to clusters of individuals in locations favorable to mate-finding success, questing for hosts and long-term survival. The clusters themselves form a microenvironment that may enhance survival (Allan and Sonen-

All use of animals in this research was done in accordance with protocols 01-006 and 01-007 as approved by the Old Dominion University Institutional Animal Care and Use Committee on January 31, 2003, and the United States Department of Agriculture Protocol Number 9553.

[1] E-mail: dsonensh@odu.edu.

[2] Agricultural Research Service, U.S. Department of Agriculture, 1600 SW 23rd Avenue, P.O. Box 14565, Gainesville, FL 32604..

[3] IPM Technologies, Inc., Centennial Campus Partners Building II, 840 Main Campus Drive, Suite 3590, Raleigh, NC 27606.

[4] Department of Chemistry, College of Environmental Sciences and Forestry, Syracuse, NY 13210.

0022-2585/03/0849–0859$04.00/0

shine 2002). These pheromones are widespread in ticks, having been found in both hard ticks (Ixodidae) and soft ticks (Argasidae) (Leahy et al. 1973, 1975, Göthe and Kraiss 1982, Neitz and Göthe 1984, Otieno et al. 1985, Dusbàbek et al. 1991a, b, Dobrotvorskii et al. 1991, Grenacher et al. 2001, Allan and Sonenshine 2002). Studies done on different tick species suggest that the primary components of the arrestment pheromone are purines, especially guanine. Guanine alone was found to induce arrestment behavior in soft ticks (Neitz and Göthe 1984, Hassanali et al. 1989) and in several species of hard ticks (Otieno et al. 1985), while xanthine and hypoxanthine were found to be the components of the arrestment pheromone in the excreta of several species of soft ticks (Dusbàbek et al. 1991a). In the sheep tick, *Ixodes ricinus* (L.), four compounds found in tick excreta, guanine, xanthine, uric acid, and 8-azaguanine (the latter a bacterial breakdown product of guanine) induced an arrestment response. However, the mixture of these compounds was 100-fold more effective in stimulating the arrestment response than any individual compound. According to Grenacher et al. (2001), 8-azaguanine, xanthine and uric acid are not natural products of tick metabolism and their presence in tick excreta may vary depending on the extent of bacterial contamination and bacterial digestion of the original purines. The identity of the composition of the arrestment pheromone in other prostriate Ixodidae, including *I. scapularis*, is unknown. Previous studies with *I. scapularis* showed statistically significant responses to mixtures of purines standards, but no studies were done to determine whether any of these compounds were naturally present in cast skins or tick excreta (Allan and Sonenshine 2002).

To use the arrestment pheromone of *I. scapularis* as an aid in tick control, the chemical composition of the pheromone must be identified. In this paper, we report the finding of two purines, guanine and xanthine, in the cast skins and excreta of these ticks, confirmed by chemical analysis and mass spectrometry. In addition, we report evidence for the occurrence of hematin and traces of another purine, tentatively identified as 8-azaguanine, and their possible contribution as components of the *I. scapularis* arrestment pheromone. Finally, this paper reports evidence showing the improved efficacy of a pheromone-acaricide impregnated matrix (Last Call, IPM Technologies, Inc., Portland, OR) compared with formulations without pheromone for attracting and killing deer ticks.

Materials and Methods

Ticks

A colony of blacklegged ticks, *I. scapularis*, was established from adult specimens originally collected from the Calder Research Station and Pound Ridge State Park in Westchester Co., NY. After feeding of the adults on New Zealand white rabbits (*Oryctolagus cuniculus*), the F_1 generation was reared from larvae to adults to eliminate infection with *Borrelia burgdorferi* and expand the population. All life stages were fed on tick-naive rabbits. Except when feeding, all life stages were held in an incubator at 26 ± 1°C and 96 ± 1% RH under a 16L:8D photoperiod. American dog ticks, *Dermacentor variabilis*, were taken from a colony originated from wild-caught adults collected near Suffolk, VA.

Extracts

Three different types of extracts were prepared. Solvents used for preparing extracts were 2 M HCl/0.5 M NaCl, hereafter termed acid-saline, and 10% NH_4OH.

Cast Skin Extract

A nymphal cast skin (CS) extract was prepared in acidified saline from 696 nymphal cast skins, weighing a total of 206.7 μg. The extract was centrifuged at 14,000 × *g* for 30 min, and the supernatant recovered. The extract was concentrated to 1.77 ml, containing 0.39 cast skins/μl or 0.117 μg/μl of soluble components. A larval CS extract was prepared in the same solvent from 500 cast skins, containing 0.44 μg/μl of soluble components.

Fecal/Excretory Exudates

Hereafter termed FE extract, this was prepared by collecting contaminants deposited by unfed ticks as they crawled over glass or plastic surfaces. The total weight of the contaminants was 175 μg. The contaminants were extracted in acidified saline and concentrated to 100 μl, containing 1.75 μg/μl. An FE extract also was collected from ticks while feeding on rabbit hosts, extracted in acidified saline, and concentrated to 100 μl for analysis by high-performance liquid chromatography (HPLC). Other samples of these same source materials were extracted in 0.95% saline for detection of ammonia or 10% NH_4OH to test for the presence of hematin. An FE extract also was collected from *D. variabilis* adults while feeding on rabbit hosts and extracted as described above.

Whole Body Extracts

An extract was prepared by washing 50 unfed adult females in acidified saline and concentrating the wash fluid to 500 μl. A similar extract was prepared from 50 unfed males.

HPLC

HPLC was done using a Waters model 680 automated gradient controller, two model 510 pumps, a Rheodyne model 7725i manual injector (Waters, Milford, MA) and a Shimadzu model SPD-M10A Photodiode Array detector (Shimadzu Scientific Instruments, Columbia, MD). The column was a 250 mm by 4.6 mm reversed phase C-18 column containing 5-μm silica particles (60 E; Alltech, Deerfield, IL). For pu-

rines, the solvent was 50 mM $NH_4H_2PO_4$ at 0.8 ml/min. Injections of sample ranged from 10 to 50 μl. For comparison, authentic purine standards (Sigma, St. Louis, MO; >99% purity) were prepared in acidified saline and analyzed by HPLC under identical conditions. In addition to their retention times, a library of the spectra (UV and visible range) was established. Standards used included guanine, 8-azaguanine, xanthine, hypoxanthine, inosine, adenine, adenosine monophosphate, uric acid (all from Sigma; >99% pure). Tentative identifications of chemical compounds found in the extract were made by comparison of peak retention times with that of known standards and by comparison of the UV spectra of the peaks with the spectra of the standards. Compounds showing similar retention times and >99% spectral matches were tentatively considered identical. Samples of peaks separated by HPLC were collected for further study by bioassay or confirmation by mass spectrometry using a Frac-100 fraction collector (Pharmacia-Biotech, Piscataway, NJ).

Mass Spectrometry

Fractions from the tick extracts containing purines tentatively identified as guanine and xanthine separated by HPLC were sent to Dr. F. X. Webster at the State University of New York, Syracuse, NY. The fractions were analyzed by LC-MS using an HP Series 1100 LCMS. The LC portion of the instrument was equipped with a reversed phase HPLC column (a Waters C18 Novapak, 3.9 by 150 mm) and eluted with the previously described ammonium phosphate buffer. The mass spectrometry detector was run in the atmospheric pressure ionization electrospray mode (positive ion) with an applied voltage of 60 V. The HP Series 1100 is also equipped with a UV photodiode array detector for comparison. Under these conditions, the purines give essentially their M + 1 ions (excluding isotope peaks). These ions and their corresponding retention times provide overwhelming evidence of identity.

Further confirmation of the pheromone components was done using NMR. The extract was spiked with a minimum of D_2O and analyzed by ^{13}C NMR spectrometry on a Bruker 600-MHz spectrometer in a 5-mm tube. A 24-h run was sufficient to match carbon resonances of the extract with standards.

Thin Layer Chromatography

This was done using Whatman (Hillsboro, OR) $KC_{18}F$ 10 by 10 cm, 200 μm thin layer chromatography (TLC) plates, impregnated with a fluorescent dye for viewing under UV. Before TLC, the plates were washed in solvent, isopropanol:methanol:acetic acid, 95:5:1 and heated (95°C). Samples, 25 μl (0.39 cast skin equivalents) and purine standards were spotted at the origin with a micropipette, dried, and the plates run in the TLC tanks with the same solvent. After exposure, heating, and drying, the plates were viewed with an UV lamp and photographed with a Kodak DC120 digital camera. The spots formed by the migration of the compounds were marked (pencil) and photographed again. After photo documentation, the sample spots were removed by scrapping the silica and collecting it in a microcentrifuge and re-extracting the contents in 0.5 M NaCl/2 M HCl for identification of the purines by HPLC as described above.

Assay for Ammonia

Traces of ammonia in 0.95% saline extracts were detected using Quick Dip paper strips sensitive to 0.25 μg/ml (Jungle Laboratories, Cibolo, TX). Color changes in the test strip were compared with a color chart to determine the level of ammonia concentration.

Assay for Hematin

Samples of the fecal exudate were extracted in 10% NH_4OH and placed in a 1-ml cuvette, and the absorbance spectrum recorded with a Sequoia-Turner model 690 spectrophotometer (Diversified Equipment, Lorton, VA). Comparisons were made with authentic hematin (Sigma) also dissolved in 10% NH_4OH.

Bioassays

Bioassays were done as described previously (Allan and Sonenshine 2002) with modifications with *I. scapularis* nymphs and adults and *D. variabilis* adults. Plastic petri dishes (150 mm diameter) were used; the plastic nubs were removed so as to prevent tick escape but still allow influx of humid air. The petri dishes were fitted with filter paper discs (same size as the interior of the dish) on the underside of the bottom of the dish. Similar filter paper discs saturated with 0.95% saline were taped onto the inside of the lid. The bottom of each dish was subdivided into eight sectors. A sample was placed in sector one (treatment) or left empty (control). Ten adult or nymphal ticks were placed into the center of the dish and allowed to disperse. Observations were made at 1-, 2-, 3-, 4-, 6-, and 24-h intervals. Disposable plastic gloves were worn throughout the bioassay procedure. High humidity was maintained by placing the petri dishes in a large, sealed glass or plastic dessicator chamber filled with wet paper towels. Similar procedures were used with the 100-mm petri dishes, except that six sectors were used instead of eight. A clean Teflon disc, with a diameter of 1.6 cm, was placed into sector 1 of the petri dish. The disc was treated with test substance or left untreated as a control.

In addition to direct visual observations, assays also were done using the Noldus video tracking system (Noldus Information Technology, Leesburg, VA) with CCD camera (Panasonic model DP 330 digital video camera, Panasonic Consumer Electronics Co., Secaucus, NJ) and a Dell Dimension 2100 computer (Dell Computer Co., Dallas, TX) with Ethovision Pro version 3.0 software (Noldus, Leesburg, VA) installed

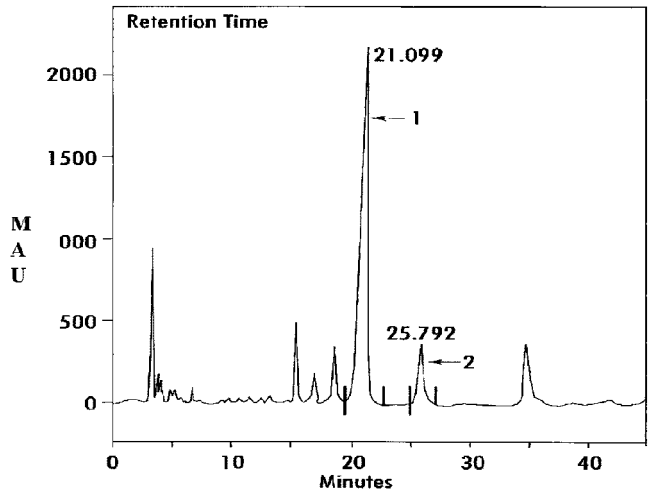

Fig. 1. HPLC chromatogram of a 10-μl aliquot of the cast skin extract analyzed with 50 mM $NH_4(H_2)PO_4$ at 0.8 ml/min showing evidence of guanine and xanthine in an extract of *I. scapularis* nymphal cast skins collected in acidified saline. Peak 1 = guanine; peak 2 = xanthine. MAU, milli-absorbance units.

under Windows XP (Microsoft Corp., Bellevue, WA). The experimental design was similar to that described by Noldus et al. (2002). This system was used to record simultaneously from four petri dishes: one control and three experimental dishes, with two ticks, one male and one female, per dish. Each petri dish was subdivided electronically into a treated zone containing the Teflon disc versus the untreated zones. Video tracking continuously recorded the movements of the ticks during the entire trial. Recordings were made for 30 min every 3 h over a 24 h trial period for a total of five trials. Measurements included the mean and SD for (1) frequency of visits to the disc, (2) duration of occurrence on the disc, and (3) duration of occurrence in the treated zone. In addition, trials also were done with 10 ticks per petri dish. In this case, the system could not retain the identity of the tracks of the individual ticks but accumulated all of the track observations as if they were from a single individual.

Test substances included 25 *I. scapularis* cast skins, 10 mg FE extract, 10 mg 8-azaguanine: xanthine mixture (6:4), and 10 mg guanine:xanthine mixture (25:1), 10 mg guanine, and 10 mg hematin. Tests also were done with discs treated with a spectrum of ammonium salts and with 100 μl NH_4OH solutions at dilutions of 10^{-4}, 10^{-5}, and 10^{-6} mol/liter. In addition, tests were done with different formulations of the formulated product, Last Call. Four formulations were tested: (1) Last Call impregnated with different percentages of DEET (range, from 10 to 0.001%) but no pheromone components or acaricide; (2) Last Call impregnated with guanine:xanthine:adenine (25:1:1) or ammonium carbonate; (3) Last Call impregnated with Permethrin (an acaricide) but no pheromone, and (4) Last Call impregnated with guanine:xanthine:adenine (25:1:1) plus Permethrin (6% by weight). Controls were done with the Last Call matrix, without Permethrin or pheromone.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using Microsoft Excel software. ANOVA was used to test the significance of the distribution of the ticks in the different sectors of the petri dishes. Statistically significant differences between means were determined by the Student's *t*-test. Best fit regression analysis, e.g., $\log(x)$, exponential, or linear regression (Microsoft Excel) was used to plot the changes in the movements of the ticks obtained over the defined time period with the Ethovision Pro video tracking recordings.

Results

Chemical Findings

Cast Skin Extract. When a 10-μl aliquot of the *I. scapularis* nymphal CS extract, containing ≈3.9 cast skin equivalents/μl, was analyzed for purines, HPLC showed up to seven peaks from ≈15 to 45 min (0.8 ml/min). Comparison of peak retention times with authentic known standards suggested that the largest peak at ≈21.10 min was guanine, representing an estimated 5.8 μg/μl; the later, smaller peak at 25.79 min was xanthine, representing an estimated 0.55 μg/μl. The ratio of the differences in the area comprised by the guanine versus the xanthine peak was 10.55:1 (Fig. 1). Spectral matching using the library of known standards showed that the earlier, dominant peak had a 99.88% match with guanine, whereas the second, later peak had a 99.95% match with xanthine. To facilitate spectral matching, larger quantities of the CS extract were injected into the HPLC, the individual peaks were collected and concentrated, the purified fractions were reinjected, and the process was repeated until pure fractions were obtained. Comparison of the purified candidate peaks for guanine and xanthine

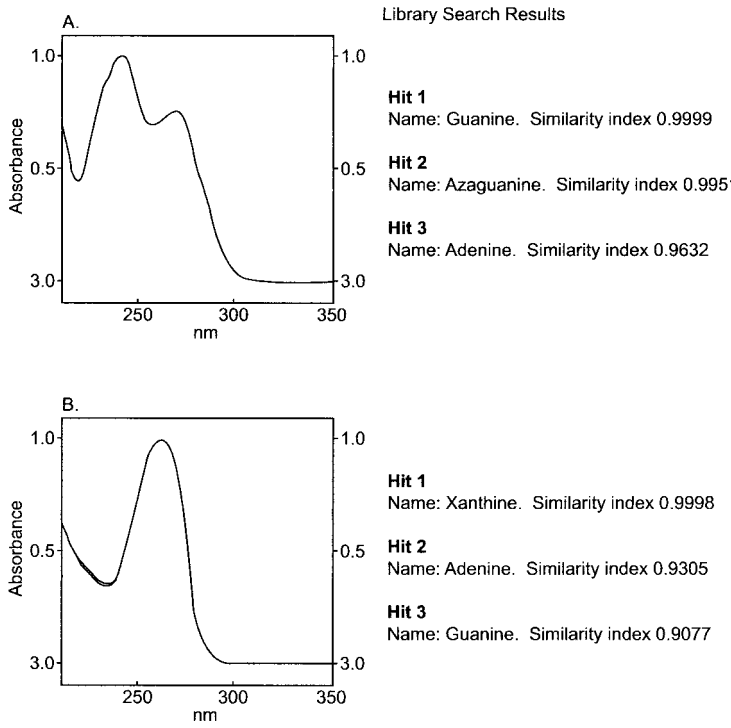

Fig. 2. (A) Spectral match for peak 1 with the spectrum of the authentic guanine standard. (B) Spectral match for peak 2 with the spectrum of the authentic xanthine standard.

showed a spectral match of 99.99% and 99.98%, respectively, with the known standard for these compounds (Fig. 2, A and B). None of the other peaks found in this HPLC chromatogram matched or were similar to any of the purine standards we tested.

In addition to HPLC retention times and UV spectrum (see Fig. 1), the identity of guanine and xanthine were confirmed by LCMS and 13C NMR. The LC peak corresponding to guanine's retention time gave a base peak of 152 m/z (M + 1) and no other significant peaks. Similarly, the LC peak corresponding to xanthine's retention time gave a base peak of 153 m/z (M + 1) and no other significant peaks. Peak matching of the ^{13}C resonances found in the ^{13}C NMR spectrum of the natural samples correspond to each of the resonances found in the standard spectra for guanine and xanthine.

When a 50-μl aliquot of the larval CS extract was analyzed by HPLC, peaks for guanine (0.18 μg/μl) and xanthine (0.19 μg/μl) were found. The ratio of the two compounds was 0.95:1.

Fecal/Excretory Exudates. When adult ticks were allowed to crawl over paper or glass surfaces, they frequently deposited black-colored material and iridescent white granules. In addition, the ticks were observed to accumulate in large numbers on the filter paper strips (Fig. 3). As many as 12 individual adults were found clustered on strips 0.5 cm wide by 2 cm long. When the ticks were removed, the filter paper was observed to be contaminated with black exudates, presumably from the ticks. HPLC of an extract of this material revealed three peaks. The largest peak proved to be guanine (spectral match 99.99%). One of the smaller peaks was found to be xanthine (spectral match 99.93%). A third peak comigrated with the retention time for 8-azaguanine, but the spectral match was inconclusive (<95%).

HPLC of an extract made from the *D. variabilis* fecal exudate showed a single large peak that comigrated with guanine. Spectral matching gave a 99.99% match for this compound. No evidence of other purines was found in this extract.

Evidence of hematin in the tick fecal exudates (FE extract) also was determined by comparing the absorbance spectra with that of an authentic hematin standard obtained with the spectrophotometer. A

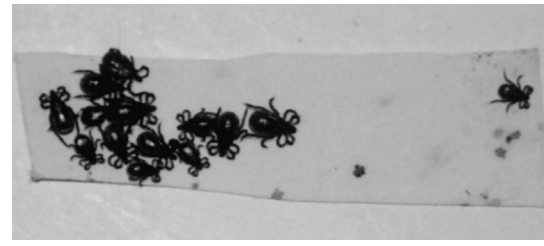

Fig. 3. Photograph showing a group of *I. scapularis* unfed adults clustered around tick fecal deposits on a strip of Whatman No. 1 filter paper.

Table 1. Responses of *I. scapularis* adults and nymphs to fecal/excretory exudates (tick hematin exudates)

Life stage	Time (h)	(mean ± SE) Number of ticks in each sector[a]								
		1	2	3	4	5	6	7	8	Side of dish
Adults[b]	1	28 ± 0.58	8 ± 0.20	10 ± 0.32	8 ± 0.58	8 ± 0.37	10 ± 0.45	18 ± 0.20	4 ± 0.25	6
	3	28 ± 0.37	4 ± 0.25	8 ± 0.37	8 ± 0.49	6 ± 0.40	6 ± 0.60	14 ± 0.51	12 ± 0.74	14
	4	30 ± 0.55	6 ± 0.40	10 ± 0.32	10 ± 0.32	6 ± 0.25	10 ± 0.44	10 ± 0.32	12 ± 0.58	6
	6	32[e] ± 0.37	4 ± 0.25	10 ± 0.45	8 ± 0.25	6 ± 0.40	4 ± 0.40	14 ± 0.51	10 ± 0.45	12
	24	38[f] ± 0.49	8 ± 0.37	6 ± 0.40	8 ± 0.58	2 ± 0.20	8 ± 0.58	12 ± 1.2	2 ± 0.20	14
Nymphs[c]	1	12 ± 0.80	6 ± 0.40	4 ± 0.25	4 ± 0.25	4 ± 0.25	4 ± 0.25	10 ± 0.55	2 ± 0.20	54
	3	20 ± 0.32	2 ± 0.20	4 ± 0.25	4 ± 0.25	4 ± 0.25	4 ± 0.25	4 ± 0.25	2 ± 0.25	56
	4	20 ± 0.55	2 ± 0.20	6 ± 0.40	6 ± 0.25	2 ± 0.20	2 ± 0.20	4 ± 0.25	2 ± 0.25	56
	6	28[g] ± 0.58	0	8 ± 0.49	2 ± 0.20	4 ± 0.25	2 ± 0.20	2 ± 0.20	4 ± 0.25	50
	24	40[h] ± 0.63	4 ± 0.25	4 ± 0.25	6 ± 0.25	6 ± 0.25	4 ± 0.25	8 ± 0.37	4 ± 0.25	26
Control[d] (nymphs)	1	4	0	8	8	2	10	4	6	58
	3	2	2	6	8	6	4	10	6	56
	4	8	2	4	4	6	4	4	4	64
	6	4	2	6	4	6	6	4	6	62
	24	6	6	4	4	2	6	6	4	62

Controls were done with 0.95% saline.
[a] Individuals that remained on the sides of the petri dishes are not included in the sector values.
[b] $N = 5$ trials with 10 ticks/trial.
[c] $N = 10$ trials with 10 ticks/trial.
[d] No significant differences.
[e] $F = 5.05$; 3 df, $P = 0.10$
[f] $F = 4.19$; 3 df, $P = 0.10$
[g] $F = 7.33$; 3 df, $P = 0.05$
[h] $F = 13.7$; 3 df, $P = 0.01$

sample of Sigma standard hematin dissolved in 10% NH_4OH produced an E_{max} absorbance at 575 nm and another between 675 and 700 nm. A sample of *I. scapularis* FE extract and the contaminated vial extract showed absorbency peaks with similar wavelengths. Similarly, the FE extract from *D. variabilis* also showed absorbance peaks at 575 and 675–700 nm. No absorbance at or near 410 nm, the E_{max} for heme, was detected in any of the tick samples.

Ammonia in excess of 6 μg/ml was detected in saline extracts of tick FE extracts using the Quick Dip colorimetric test.

Whole Body (Female) Tick Extract. When aliquots of female and male body washes made in 0.9% saline were assayed by HPLC [with the $NH_4(H_2)PO_4$ buffer], no evidence of guanine, xanthine, or other purines was found. However, when aliquots of whole body extracts made in acidified saline were assayed with the same HPLC buffer, a single large peak was observed that comigrates with guanine, estimated at 1.3 μg/female and 1.1 μg/male. Spectral matching showed a 99.3% match with the spectrum for guanine. There was no evidence of xanthine or other purines in these extracts.

TLC. When the CS extract (25-μl aliquots) was analyzed by TLC, most of the material in these extracts remained at the origin. However, compounds that did migrate advanced to two discrete spots that could be detected under UV illumination. The upper spot comigrated with the purine standards, guanine, hypoxanthine, xanthine, and adenine. The lower spot did not match any of the purines, and its identity remains unknown. Differences in the identification of the purine standards were determined by staining with bromphenol blue/silver nitrate. When examined by UV illumination, a light blue color in the upper spot characteristic of guanine was observed. However, no evidence of hypoxanthine, xanthine, or adenine, which appear brilliant blue, was observed. When the two spots were reextracted and analyzed by HPLC, the upper spot showed a peak that coeluted with guanine. Spectral matching showed a 99.98% match for guanine. Guanine was also found in the lower spot, confirmed by spectral matching, presumably as a result of imprecise separation on the TLC plate. These findings showed evidence of guanine in the CS extract but not any of the other purines.

Bioassay Results

Bioassays reported previously (Allan and Sonenshine 2002) showed that *I. scapularis* adults assembled in response to guanine and cast nymphal skins. In the current studies, bioassays showed a strong statistically significant response by adults and nymphs to the adult tick FE extracts containing hematin. Adults and nymphs seemed to accumulate in increasing numbers in the treated zone within as little as 1 h after placement in the petri dishes. The differences between the experimental and control distributions were statistically significant by 6 h (adults, 32%; nymphs, 28%) and highly significant by 24 h (adults, 38%; nymphs, 40%) after tick release (Table 1). There was no difference in the tick arrestment response to fresh fecal exudates (<24 h) versus exudates 4–6 wk old (data not shown). However, bioassays with fecal exudates from feeding *D. variabilis* (Say) did not elicit a significant response by *I. scapularis* adults or nymphs (data not shown).

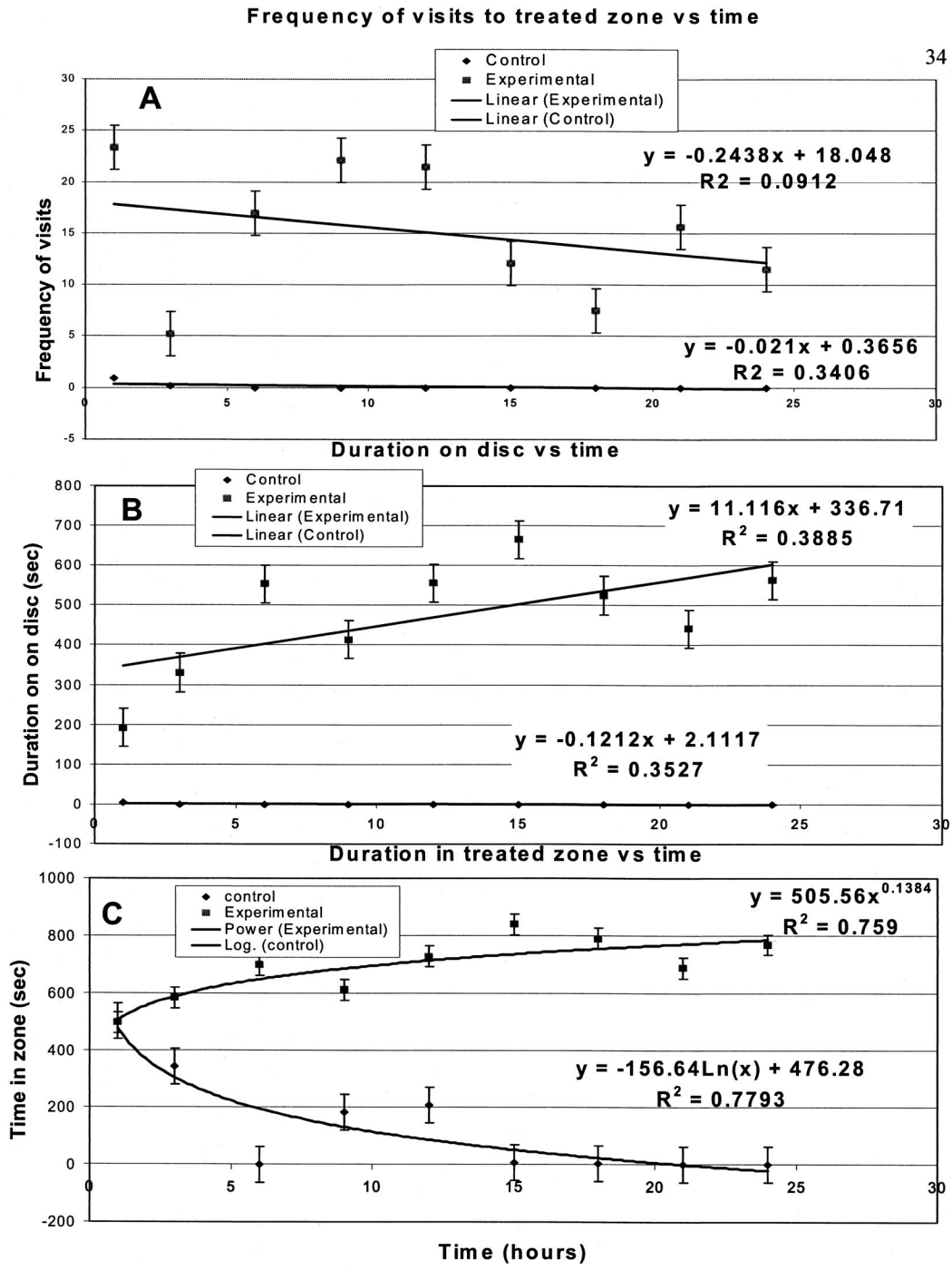

Fig. 4. Graphs showing rate of assembly of adult tick, *I. scapularis*, on discs containing tick fecal/excretory exudates (tick excreta) as determined by computerized video tracking and software (Ethovision). (A) Frequency of visits to and from the treated zone containing the tick excreta (experimental) or blank disc (control) in relation to time. (B) Duration of tick occurrence in the treated zone (experimental) or control zone versus time. (C) Tick occurrence (assembly) on the treated disc (experimental) versus untreated disc (control).

Video tracking of the tick movements in response to the FE stimulus revealed additional aspects of tick behavior not evident from periodic visual observations as described above (Fig. 4). Ticks exposed to the experimental treated zone (containing the FE extract) entered that zone much more frequently than

Table 2. Comparison of responses of *I. scapularis* to various formulations of DEET in Last Call (% weight) matrix

Formulation	Percent nymphs in sector[a]			Percent adults in sector[b]		
	15 min	60 min	120 min	15 min	60 min	120 min
10%	4.0 (1.6) a	4.0 (2.2) a	4.0 (1.3) a	0 a	0 a	0 a
1%	7.0 (1.3) a	2.0 (1.3) a	2.0 (1.3) a	0 a	0 a	0 a
0.1%	14.0 (2.2) b	13.0 (3.7) b	14.0 (4.0) b	30.0 (10.8) b	5.0 (5.0) a	15.0 (8.2) b
0.01%	17.0 (2.6) b	18.0 (2.5) c	23.0 (4.0) c	25.0 (9.9) b	10.0 (7.1) ab	0 a
0.001%	18.0 (3.9) b	12.0 (2.9) bc	12.0 (5.7) b	20.0 (9.1) b	30.0 (10.5) b	30.0 (10.5) b
Matrix only	17.0 (2.8) b	17.0 (3.5) bc	20.0 (3.0) c	10.5 (7.1) ab	10.0 (6.8) ab	15.7 (8.4) b
Solvent	12.5 (2.2) b	11.0 (2.0) b	12.5 (1.2 b	10.0 (6.9) ab	10.0 (6.8) ab	15.0 (8.2) b

Bioassays were conducted as petri dish bioassays with resulted presented as percentage of ticks present in the treatment sector of the petri dish (100 μg of Last Call used per assay).

Means within each column and tick stage followed by different letters are significantly different (paired t-test, $P < 0.05$).

[a] $N = 10$ replications with 10 nymphs, except for controls which have 20 replications.

[b] $N = 20$ replications with one female per bioassay.

ticks exposed to the control treated zone (empty Teflon disc). However, although ticks continued to walk in and out of the experimental treated zone, this response declined gradually, from a mean of 23.3 visits/0.30 min at 1 h to as low as 7.5 visits/0.30 min by 18 h. In contrast, the ticks in the control petri dish averaged only one visit/0.30 min at 1 h and none thereafter (Fig. 4A). The differences in the frequency of visits was highly significantly different ($t = -3.97$, $P < 0.001$, 16 df). Tick occurrence in the experimental treated zone increased gradually during the 24-h observation period and became significantly greater ($t = 6.55$, $P < 0.001$, 16 df) than tick occurrence in the control-treated zone (Fig. 4B). Tick assembly on the experimental disc (FE exudates) was significantly greater than tick assembly on the control disc (no stimulus), which in the latter case, was zero. In addition, tick assembly on the experimental disc increased gradually during the 24-h observation period (Fig. 4C). The duration of tick occurrence increased significantly in the experimentally treated disc ($t = 3.48$, $P > 0.05$, 6 df) and treated zone ($t = 2.90$, $P < 0.05$) versus the control within as little as 3 h. The statistical difference in tick occurrence increased during the remaining observation periods (6 h, $t = 7.09$, $P < 0.01$, 6 df) and was highly significantly different from the controls at five of the eight observation periods in the experimental treated zone and three of the eight observation periods for the treated disc. Repeating the trials with 10 ticks per petri dish gave similar results. Ticks were in the experimental treated zone 48.8% of the time versus 6.41% of the time in the control ($t = 6.82$, $P < 0.01$, 16 df). Similarly, ticks were on the experimental treated disc 33.3% of the time versus 1.2% of the time in the control ($t = -3.99$ $P < 0.05$, 16 df).

Bioassays using a DEET-impregnated formulation of Last Call showed slight but significant increases in the occurrence of *I. scapularis* ticks in the treated sector. Low concentrations, i.e., from 0.1 to 0.001%, were mildly attractive compared with the acetone control. This was most evident with nymphs at 120 min (two-fold greater occurrences of ticks in the treated sector versus the acetone control). High concentrations, 1 and 10%, were strongly repellent (Table 2).

When the tests were repeated with formulations of guanine:xanthine:adenine in a ratio of 25:1:1 instead of DEET, a strong attractant response was found with female ticks exposed to the highest concentration, 20%, and at 1% (100 mg), reaching 45% by 1 h after tick exposure to the treated sector. The response persisted for 2 h but was greatly reduced (16.2–17.4%) by 24 h. Nymphal ticks also showed a weak positive response to the higher concentrations compared with the Last Call control. There was no response to the ammonium carbonate formulation (Table 3). When the tests were done with various ammonium compounds by them-

Table 3. Comparison of responses of *I. scapularis* nymphs and females in petri dish bioassays to formulations of assembly pheromone components (guanine:xanthine:adenine [25:1:1] or ammonium carbonate in the Last Call matrix)

Formulation[a]	Percent nymphs in treatment sector (SE)			Percent adults in treatment sector (SE)		
	15 min	60 min	120 min	15 min	60 min	120 min
20%	15.6 (2.5) a	21.6 (2.6) a	20.3 (2.3) a	20.0 (8.9) a	45.0 (11.1) a	45.0 (11.1) a
10%	21.0 (2.3) a	17.5 (2.9) ac	21.5 (3.2) a	20.0 (8.9) a	25.0 (9.6) ab	25.0 (8.9) b
1%	15.5 (2.7) bc	19.5 (3.1) a	18.5 (3.2) ab	45.0 (11.1) b	30.0 (10.2) ab	35.0 (10.6) ab
0.1%	15.5 (2.5) bc	19.5 (4.3) a	16.5 (3.3) ab	20.0 (8.9) a	25.0 (9.6) ab	20.0 (8.9) b
NH_4CO_3						
10%	12.0 (2.5) a	12.0 (1.7) a	12.0 (2.0) a	0.0 a	30.0 (5.4) b	20.0 (7.4) b
1%	19.0 (3.1) ab	20.0 (3.3) a	17.0 (3.1) a	10.0 (7.1) a	10.0 (7.1) a	10.0 (7.1) a
LC Control[b]	13.0 (1.6) c	12.0 (2.4) c	14.5 (2.4) b	10.0 (6.7) a	10.0 (6.7) b	15.0 (7.9) b

[a] Formulation by weight.

[b] LC Control = Last Call without pheromone.

Means within each column and tick stage followed by a different letter are significantly different (Paired t-test, $P < 0.05$). Assays were replicated 10 times with 10 nymphs per replicate except for controls that had 20 replications; assays were replicated or 20–30 times with one female per replicate.

Table 4. Comparison of responses of *I. scapularis* nymphs and females in petri dish bioassays to ammonium compounds (1 μg) presented on a disc in one sector of the petri dishes

Compound	Percent nymphs in treatment sector (SE)			Percent females in treatment sector (SE)		
	15 min	60 min	120 min	15 min	60 min	120 min
NH_4CO_3	21.3 (4.4) bc	13.2 (3.5) ab	13.9 (3.6) ab	15.0 (6.6) ab	40.0 (9.1) bc	33.3 (8.7) bc
NH_4Cl	18.0 (4.1) ab	11.2 (3.7) a	22.5 (5.0) bc	0.0 a	20.0 (9.1) ab	20.0 (9.1) ab
NH_4PO_4	18.0 (3.6) ab	11.1 (3.5) a	10.5 (3.6) a	20.0 (9.1) ab	10.0 (6.9) a	20.0 (9.2) ab
NH_4SO_4	16.6 (2.4) ab	22.6 (2.4) c	18.9 (2.7) bc	26.6 (8.2) b	26.6 (8.2) b	26.6 (8.2) bc
Allantoin	20.5 (2.1) bc	18.1 (2.9) bc	15.6 (2.8) ab	20.0 (9.1) ab	10.0 (6.7) a	15.0 (8.2) ab
Guanosine	16.0 (3.7) ab	12.4 (2.9) ab	12.5 (2.5) a	10.0 (6.9) a	15.0 (8.2) a	10.0 (6.9) a
Solvent control	12.5 (2.2) a	11.0 (2.0) a	12.5 (1.2) a	10.0 (6.9) a	10.0 (5.6) a	13.3 (6.3) a

Means within each column and tick stage followed by a different letter are significantly different (paired *t*-test, $P < 0.05$). Assays were replicated 10 times with 10 nymphs per replicate or 20–30 times with one female per replicate.

selves (1 $\mu g/cm^2$), adults showed a strong and persistent aggregation only in response to ammonium carbonate. There was no response to ammonium phosphate, the salt used (in solution) as the mobile phase for HPLC of the purine extracts (Table 4). The concentrations of the ammonium salts did not influence tick responses. There was no significant difference in response to concentrations ranging from 0.01 to 1.0 $\mu g/cm^2$ of a representative salt, ammonium sulfate (Table 5).

Tests of the formulated product, Last Call with and without pheromone, showed a significant increase in arrestment around the pheromone-impregnated matrix compared with the matrix without pheromone (Table 6). The results also show clear evidence of significantly increased mortality when pheromone is included in the formulation. For adults, ticks were up to four times as likely to aggregate in the sector treated with Last Call/Pheromone as the controls and mortality was 95% (increased from 70% with Permethrin alone). For nymphs, ticks were up to two times more likely to aggregate in the sector treated with Last Call/Pheromone as the controls, and mortality was 52% (increased from 30% for Permethrin alone). Clearly, the inclusion of the pheromone resulted in significantly improved performance of the product as a tick-killing device.

Discussion

These studies show the presence of guanine and xanthine in the crude extracts that induce assembly in the black-legged ticks, *I. scapularis.* Both compounds were found in constant ratios in both the nymphal and larval cast skin extracts, although guanine was much more abundant in the nymphal than in the larval cast skins. These findings suggest that both compounds are natural constituents of tick fecal/excretory wastes. The strongest responses were found when hematin was present. Guanine is a known constituent of the arrestment pheromone of many other ticks species, whereas guanine, xanthine, and uric acid were reported as constituents of the fecal wastes of the sheep tick *I. ricinus* (Grenacher et al. 2001). However, no conclusive evidence of 8-azaguanine, uric acid, or other purines were found in the *I. scapularis* fecal exudates or cast skins extracted in acidified saline. In *I. ricinus,* guanine was identified as a constituent in feces collected from engorged nymphs molting to adults "that had passed all stages of digestion" (Grenacher et al. 2001), suggesting that it was the only authentic purine metabolite from the tick's tissues. Urea also was identified in tick feces from *I. ricinus.* Other purines, specifically xanthine, uric acid, and 8-azaguanine, also identified in fecal exudates collected from *I. ricinus*–contaminated filter paper strips may have been produced by microbial activity. 8-azaguanine has been reported to result from bacterial digestion of guanine (Hirasawa and Isono 1978), whereas uric acid was reported to result from microbial digestion of purines in fecal deposits of *Argas walkerae* (Dusbàbek et al. 1978). We frequently observed fungal hyphae (species unknown) contaminating cast skins and tick fecal exudates deposited on filter paper strips. The extent to which microbial action plays a role in tick perception of the cast skin or fecal exudates is unknown. However, there is no evidence to support the hypothesis that bacterial digestion of cast skin or tick fecal exudates contributes to the tick's arrestment response. No bacteria were detected in the fecal exudates, and no differences were observed in the tick arrestment responses between freshly deposited versus aged fecal exudates. Nevertheless, the response is species-specific, because *I. scapularis* did not assemble in response to fecal exudates collected from feeding dog ticks, *D. variabilis.*

These findings suggest that the assembly pheromone of *I. scapularis* is a mixture of purines and other compounds, especially hematin. Guanine, xanthine, and hematin seem to constitute major components, while the role of 8-azaguanine and possibly other

Table 5. Comparison of responses of *I. scapularis* nymphs to different concentrations of ammonium sulfate in petri dish bioassays

Concentration (ug)	Percent nymphs in treatment sector (SE)			
	15 min	60 min	120 min	*N*
1.0	16.6 (2.4)	22.6 (2.4)[a]	16.9 (2.7)	30
0.1	22.8 (3.2)[a]	16.5 (2.6)	14.7 (3.6)	20
0.01	16.9 (2.8)	14.7 (3.2)	17.0 (4.2)	20
Solvent control	12.5 (2.2)	11.0 (2.0)	12.5 (1.2)	20

[a] Significantly different than solvent control in the same column, paired *t*-test ($P < 0.05$)

Table 6. Responses and mortality of *I. scapularis* females and nymphs in petri dish bioassays to treatment Last Call containing permethrin, purines (guanine:xanthine:adenine [25:1:1] 20%), both ingredients, or Last Call matrix alone

	Percent ticks in treatment sector (SE)			Percent mortality
	15 min	1 h	2 h	24 h
Adult females[a]				
Treatment type				
Permethrin/last call	10.0 (6.9) a	10.0 (6.9) a	10.0 (6.9) a	70
Permethrin and pheromone last call	40.0 (11.2) bc	35.0 (10.9) b	30.0 (10.5) ac	95
Pheromone/Last Call	25.0 (9.9) ac	20.0 (9.2) a	40.0 (11.5) a	0
Last Call (no pheromone or permethrin)	10.0 (6.9) a	10.0 (6.9) a	5.0 (5.0) b	0
Nymphs[b]				
Treatment type				
Permethrin/Last Call	6.0 (1.5) a	8.0 (2.2) a	10.0 (2.5) a	30
Permethrin and Pheromone/Last Call	20.0 (4.4) b	14.5 (2.8) a	24.0 (3.3) b	52
Pheromone/Last Call	16.1 (2.6) b	20.6 (2.8) b	22.3 (2.2) b	0
Last Call (no pheromone or permethrin)	10.0 (1.9) a	10.0 (1.9) a	8.5 (2.2) a	0

Means within each column and tick stage followed by a different letter are significantly different (paired *t*-test, $P < 0.05$).
[a] 20 replications of one female.
[b] 10 replications of 10 nymphs.

purines is uncertain. Ticks respond strongly to larval and nymphal cast skins, proven to contain guanine and xanthine, but no evidence of hematin or 8-azaguanine was found in these materials. Ticks also respond strongly to fecal exudates that were shown to contain guanine, xanthine, and hematin.

The results of the video tracking observations suggest that tick assembly is guided by a weak arrestment response, which may be reinforced by repeated contact with the stimulus rather than a single contact event. Ticks did not cease ambulatory activity immediately after contact with the source stimulus. However, although they left and returned, the duration of their time in contact with the stimulus increased; by 24 h, when observations were terminated, their time on the treated disc was almost three times what it was at the beginning of the trial period. The duration of time in the treated zone also increased significantly, whereas that of the controls declined, suggesting that the excursions from the treated zone gradually diminished during the trial period.

Another unresolved factor in the tick arrestment response concerns ammonia. This compound was shown to be present in *I. ricinus* fecal deposits and ticks assembled around a bronze mesh containing such deposits, presumably in response to the ammonia odors (Grenacher et al. 2001). In other tick species, ammonia-specific receptors have been reported on the tarsus of leg I (Haggart and Davis, 1979, 1980), suggesting a role for ammonia in host-finding behavior and assembly. Consequently, it is likely that low concentrations of ammonia may stimulate searching behavior, leading to assembly after contact with the fecal exudates.

These results are being used to enhance the performance of Last Call as a tick-killing technology for use in the natural environment where deer ticks, *I. scapularis*, are present. Studies to evaluate the efficacy of Last Call containing the artificial mixture of *I. scapularis* arrestment pheromone components under field conditions are planned.

Acknowledgments

The authors thank L. Ayash, IPM Technologies, Raleigh, NC, for assistance in carrying out some of the bioassays reported in this study. The results reported in this study were supported by a grant from the Small Business Innovative Research program of the National Institutes of Allergy and Infectious Diseases, NIH, Grant R44 AI47513-02 to IPM Technologies.

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Received for publication 3 April 2003; accepted 14 July 2003.