N O T E

Responses of Lone Star Tick (Acari: Ixodidae) Nymphs to the Repellent Deet Applied in Acetone and Ethanol Solutions in *In Vitro* Bioassays[1]

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Thousands of cases of tick-borne ailments, such as Lyme disease, human monocytic ehrlichiosis, and Rocky Mountain spotted fever, infect Americans annually. Repellents are considered an effective means of personal protection against tick bite and their use is recommended by the CDC (CDC 2002, Lyme disease, Department of Health and Human, Services, Centers for Disease Control and Prevention, Ft. Collins CO, 12 p; Vaughn and Meshnick 2011, Vector-Borne Zoon. Dis. 11: 869 - 875; Vazquez 2008, Emerg. Infect. Dis. 14:210 - 216). For decades, N,N-diethyl-3-methyl benzamide (Deet) and permethrin have dominated the repellent market for uses on human skin and clothing, respectively (Bissinger and Roe 2010, Pestic. Biochem. Physiol. 96: 63 - 79; Schreck et al. 1982, J. Med. Entomol. 19: 143 - 146). However, an interest by the public in alternative repellents, particularly natural products, has added impetus to the search for new, effective, safe and affordable repellents.

Behavioral bioassays remain an important tool in evaluating repellent efficacy. Typically repellent bioassays use a solvent, such as acetone or ethanol, to dispense desired concentrations of the active solute evenly on a substrate. Time is usually allowed for the solvent to evaporate before test organisms are exposed to the treatment. In cases where the repellent is especially volatile, drying times may be <5 min. Deet is often considered the standard against which other repellents are measured. Ethanol is a preferred solvent for Deet. We have evaluated Deet, SS220, (-)-isolongifolenone, and other compounds against ticks with filter paper as the substrate and 10 - 15 min drying times (Carroll et al. 2004, J. Med. Entomol. 41: 249 - 254; 2011, Pg. 97 - 120 *In* Recent developments in invertebrate repellents [eds. Paluch, G. and Coats, J. E.], ACS Symposium Series, vol. 1090, American Chemical Society, WA, DC. The lone star tick, *Amblyomma americanum* (L.), is receiving increased attention

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as a nuisance biter and vector of rickettsial diseases such as human monocytic ehrlichiosis (Childs and Paddock 2003, Annu. Rev. Entomol. 48: 307 - 337; Goddard and Varela-Stokes 2009, Vet. Parasitol. 160: 1 - 12; Stromdahl et al. 2011, Vector-Borne Zoon. Dis. 11: 969 - 977). Whereas testing Deet among a panel of compounds (all applied in acetone solutions to filter paper with a 10 - 12 min drying time) against nymphs of *A. americanum*, we realized that a range of concentrations of Deet that was proving to be highly repellent had been largely ineffective in previous dose response tests using ethanol solutions of Deet against *A. americanum* in the same bioassay (e.g., Carroll et al. 2008, J. Entomol. Sci. 43:426 - 430). Our first reaction was to prepare new solutions from other sources of acetone. The new solutions were similarly repellent in bioassays. We wished to obtain a distinct profile of *A. americanum* responses to acetone and ethanol solutions of Deet. Therefore, we tested nymphs against a range of concentrations of Deet in ethanol and acetone solutions in a vertical filter paper bioassay and determined the extent of the solvent related discrepancy for that bioassay protocol.

Ticks. Nymphal *A. americanum* from a colony at USDA, ARS Knipling-Bushland, U. S. Livestock Insects Research Laboratory, Kerrville, TX were held at 23 - 24°C, ≈RH and a photoperiod of 16:8 h (L:D). The *A. americanum* nymphs were tested 3 - 5 mo after molting.

Chemicals. Deet was purchased from Aldrich, Sigma-Aldrich, Inc., St. Louis, MO.

Bioassay. We used an in vitro bioassay, described in detail by Carroll et al. (2004), that exploits the tendency of host-seeking *A. americanum* to climb. Briefly, a 4 × 7-cm rectangle of Whatman No. 4 filter paper was marked with a pencil into two 1 × 4-cm zones at the far ends of the paper and a central 4 × 5-cm zone. Using a pipettor, 165 µL of test solution was evenly applied to both sides of the central 4 × 5 cm of the filter paper. After drying 10 - 12 min the paper strip was suspended from a bulldog clip hung from a slender horizontal dowel held by an Aptex No. 10 double clip work holder (Aptex, Bethel, CT). A Petri dish (9 cm diam) glued in the center of a 15-cm Petri dish created a moat when water was added between their walls (1.5 cm high). The moated Petri dishes were placed beneath the suspended filter paper to confine ticks that dropped from the filter paper. When *A. americanum* nymphs had climbed to the rim of a storage vial opened in the center of moated Petri dishes (5.5 and 9 cm diam), the filter paper strip was removed from the dowel and held so that 10 ticks crawled onto the lower untreated zone. Only ticks that readily transferred to the filter paper were used. The locations of the *A. americanum* nymphs were recorded at 1, 3, 5, 10 and 15 min after the tenth nymph began clinging to the lower untreated zone of the filter paper. Ticks were considered repelled if they were in the lower untreated zone at 15 min or if they fell from the filter paper without having crossed the upper boundary of the treated zone.

Experimental design. Acetone solutions of Deet were tested at 0 (acetone only), 125, 250, 500, 1000, and 2000 nmol compound/cm^2 filter paper, with 110, 10, 60, 30, 40, and 30 ticks used per concentration, respectively. Ethanol solutions of Deet were tested at 0 (ethanol only), 250, 500, 1000, and 2000 nmol compound/cm^2 filter paper, with 60, 10, 20, 30, and 20 ticks used per concentration respectively. Controls were tested each day that bioassays were conducted.

Statistical methods. Because these are binomial data (proportion of ticks in a group of 10 that were repelled), we used a generalized linear model to estimate parameters and test them using the R software (often described as a logistic regression). We determined that over-dispersion was not an issue (for the ticks tested using an acetone solvent, the over-dispersion parameter was estimated as 1.30, for the ticks

tested using an alcohol solvent, the over-dispersion parameter was estimated as 1.06; for binomial data with no over-dispersion, this parameter is 1). We found that a square root transformation on Deet concentration was required, but also included the untransformed concentration in the model, the latter allows for the relationship between the logit of proportion repelled (binomial data are modeled using the logit link) and square root of concentration to be a two degree polynomial, rather than a straight line.

As depicted in Fig. 1 (the model has been back-transformed to the data scale [lines] with data points depicted), the dose-response relationship from the 2 solvents differed greatly, and was similar only for zero and high concentrations of Deet. This is demonstrated statistically (using a *z* statistic, based on the normal distribution), both interaction terms (solvent:sqrt(concentration), solvent:concentration) were significant (see Table 1 for parameter estimates and significance tests; note these are given on the logit scale). Thus, the slope coefficients for both the linear and quadratic terms differed between the solvents, as is readily seen in Fig. 1. The main effect of solvent is not significant (Table 1) because it is a test for intercept differences at zero concentration, where the 2 solvents produced similar proportions (see Fig. 1).

At 500 and 1000 nmol compound/cm^2 filter paper, Deet applied in ethanol solutions repelled 0 (n = 20) and 13.3% (n = 30) of the *A. americanum* nymphs, whereas 250, 500 and 1000 nmol Deet/cm^2 filter paper in acetone solutions repelled 78.3% (n = 60), 90% (n = 30) and 96.7% (n = 30) of the nymphs, respectively. At 2000 nmol Deet/cm^2 filter paper, both acetone and ethanol solutions were highly repellent. With both ethanol and acetone solutions of Deet, nearly all repelled ticks fell from the filter paper with few ticks on the lower untreated zone at 15 min after mounting the paper strip. Similarly, Carroll et al. (2011), using the same vertical filter paper bioassay protocol, reported no repellency of *A. americanum* nymphs by ethanol solutions of Deet at 413 nmol compound/cm^2 filter paper, and increasing to ~60% repelled at 1650 nmol Deet/cm^2 filter paper. Laboratory-reared and field-collected *A. americanum* nymphs were also little repelled by ethanol solutions of Deet at concentrations <800 nmol compound/cm^2 filter paper (Carroll et al. 2008)

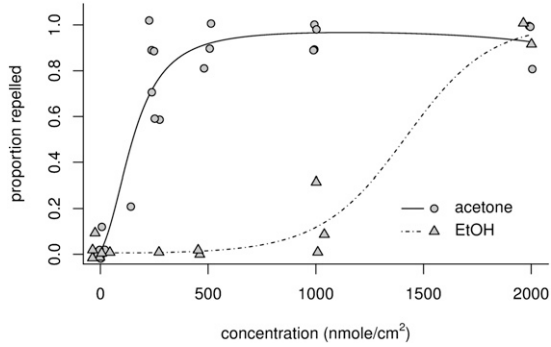

Fig. 1. Plot of the dose-response relationship between two different solvents (acetone and ethanol) used to create different concentrations of Deet to measure the proportion of repelled ticks. Lines give the models (parameter estimates are in Table 1), back-transformed from the logit to the data scale, data points (jittered to better separate them) are superimposed.

Table 1. Coefficient estimates, standard errors, and tests of significance for model parameters. The significant interactions of solvent:sqrt(conc) and solvent:conc indicate that the two solvents follow different repellency functions as the dose of deet is increased. The parameters represent contrasts between acetone and ethanol, i.e. the solvent:sqrt(conc) represents the difference in the linear slope component between acetone and ethanol.

Coefficients	Estimate	Standard Error	z-value	*P* value
intercept	–4.892	0.948	–5.16	<0.0001
solvent	0.740	1.406	0.526	0.599
sqrt(conc)	0.487	0.084	5.779	<0.0001
conc	–0.007	0.002	–4.577	<0.0001
solvent:sqrt(conc)	–0.648	0.129	–5.022	<0.0001
solvent:conc	0.014	0.003	5.194	<0.0001

Acetone has a high vapor pressure and evaporates more quickly than ethanol, but by 10 min after test solutions of Deet were applied to the filter paper, both the acetone solution-treated and ethanol solution-treated filter papers looked and felt dry. Among all the ticks exposed to acetone only (controls, $n = 110$ ticks) only 1 tick met the criteria for being repelled. Similarly a single tick exposed to ethanol only (controls, $n = 60$ ticks) was repelled. Thus, neither solvent in itself, when allowed to dry 10 - 12 min, appeared to be repellent to the *A. americanum* nymphs.

It is not clear why the acetone solutions of Deet were more repellent. The absorbency of ethanol and acetone preparations of Deet by skin has been studied by Stinecipher and Shah (1997, J. Toxicol. Environ. Health 52:119 - 135), Moody and Nadeau (1993, Toxic. in Vitro 7: 167 - 176), and others. Ethanol has been shown to enhance absorption of Deet by skin (Stinecipher and Shah 1997), but skin is quite unlike filter paper. Other substrates or drying times could produce different results. Solubility characteristics of the repellent compound may determine which solvent is used to prepare test solutions.

The difference in efficacy between acetone and ethanol solutions of Deet against *A. americanum* is of interest because comparisons must be made of tick bioassay data in the discovery, development and registration stages of a synthetic or natural repellent product. Comparisons of repellents tested within the same bioassay system that use the same test species, life stage, solvent, substrate, dimensions, and time parameters is not a problem. To attempt comparisons of efficacy among data from bioassays where just one of the variables differs, invites problems. Inasmuch as the solvent is generally considered to have evaporated before the treatment is exposed to ticks, there might be tendency to ignore solvent as an influential factor when comparing results. Our findings call that assumption into question. Further investigation of solvent based differences in the efficacy of Deet and possibly other repellents against ticks and other test organisms is warranted.

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