*Biopestic. Int.*13(1):1-12 (2017) ISSN 0973-483X / e-ISSN 0976-9412

Effects of Two Commercial Neem-based Insecticides on Lone Star Tick, *Amblyomma Americanum* (L.) (Acari: Ixodidae): Deterrence, Mortality, and Reproduction

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KEY WORDS

Azadirachtin
Bioactive compounds
Egg hatchability
Fecundity
Insect growth regulator
Sublethal effects
Toxicity

ABSTRACT The lone star tick, *Amblyomma americanum* (L.), is a widely distributed three-host obligate blood-feeding ectoparasite in the United States and Mexico. It mostly attaches to white-tailed deer, *Odocoileus virginianus* (Zimmerman) and wild Turkey, *Meleagris gallopavo* L., as well as a wide variety of other, domestic and wild hosts such as cattle, dogs, horses, goats, quail, squirrels, opossums, hares, coyotes, and humans. Diseases known to be transmitted by *A. americanum* include ehrlichiosis, rickettsiosis, tularemia, and protozoan infections. Two commercial neem-based products registered for home and garden use, Neemix (a viscous liquid) and AzaSol (a powder), containing 4.5 and 6% azadirachtin (a limonoid), respectively, as the labeled active ingredient, were assessed for contact toxicity, fumigant toxicity, deterrence, and sublethal effects on egg laying and hatching. We determined that Neemix also contained high concentrations of three additional bioactive limonoids: Nimbolide, nimbin, and salannin. When concentrations of azadirachtin were approximately the same, the two neem-based formulations caused similar contact mortality against larvae. High concentrations of Neemix-induced complete mortality from volatiles when larvae and adults were exposed and nonazadirachtin compounds appeared to be the cause. Neem-based products can induce multiple bioactive effects that differ from other neem-based products because the products might be comprised different bioactive constituents and because the products might have the same constituents but in substantially different amounts. Only Neemix was deterrent against larvae, which might have resulted from the presence of the bioactive compounds other than azadirachtin. Growth regulator and sublethal effects on egg laying and egg hatchability were not observed. The feasibility of protecting hosts against *A. americanum* using the neem-based products is discussed.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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INTRODUCTION

The lone star tick, *Amblyomma americanum* (L.), widely distributed across Eastern, Southeastern, and Midwestern regions of the United States and south into Mexico (James and Harwood, 1969; Childs and Paddock, 2003), is an obligate blood feeder that attaches to three different hosts during the larval, nymphal, and adult stages (Holderman and Kaufman, 2014). It is typically found in secondary growth in woodland habitats (Kollars, 1993) where white-tailed deer, *Odocoileus virginianus* (Zimmerman), and wild Turkey, *Meleagris gallopavo* L., are common hosts, as well as a wide variety of other, domestic and wild, hosts (e.g., cattle, dogs, horses, goats, quail, squirrels, opossums, hares, and coyotes), and humans (Cooley and Kohls, 1944; Bishopp and Trembley, 1945; James and Harwood, 1969; Kollars *et al.*, 2000). Several days after a blood-engorged adult female drop from its host, ~5,000 eggs are deposited in a protected location (e.g., leaf litter) (Patrick and Hair, 1979). After larvae hatch and undergo a quiescent period, they "quest" for a host (James and Harwood, 1969). Questing involves climbing up an object, such as a blade of grass, and waiting for a host to brush past the larva which moves onto the host, seeks a favorable feeding site, inserts its chelicerae into the skin and ingests blood for 1-3 days, drops from the host to digest its blood meal, and molts into a nymph. Nymphs repeat the process and become adults. The life cycle under natural conditions is ~2 years (Troughton and Levin, 2007). Seasonal population peaks occur mainly during late spring to early fall (Semtner and Hair, 1973; Kollars *et al.*, 2000).

A. americanum is the most frequently reported species of tick to bite humans in the Southeastern and South Central United States (Masters *et al.*, 2008). Diseases known to be transmitted by *A. americanum* include ehrlichiosis, rickettsiosis, tularemia, and protozoan infections (James and Harwood, 1969; Holderman and Kaufman, 2014).

Control practices for ixodid ticks sometimes include insecticide treatment of deer (Solberg *et al.*, 2003), reduction of vegetative undergrowth (Allan *et al.*, 2010), and exclusion of deer from tick management areas (Bloemer *et al.*, 1990; Holderman and Kaufman, 2014). For protection of humans from *A. americanum*, repellents applied to clothing are recommended.

Botanical products that contain bioactive compounds are desirable for pest management when they are effective and complement natural enemy activity (Ascher, 1993; Schmutterer, 1990, 1995; Koul, 2005, 2016). Bioactive plant-derived compounds, in general, are considered to be minimum-risk pesticides and are often exempt from Environmental Protection Agency registration under section 25(b) of the Federal Insecticide and Rodenticide Act (Cloyd *et al.*, 2009). Neem - *Azadirachta indica* A. Juss (Meliaceae) - based insecticides containing azadirachtin can control >400 species of insects (Schmutterer, 1990; Isman, 1999; Walter, 1999; Greenberg *et al.*, 2005), and some neem-based insecticides have negligible effects on beneficial insects (Schmutterer, 1995; Haseeb *et al.*, 2004). Although neem extracts include a variety of bioactive compounds, efficacy is most often attributed to azadirachtin, a nortriterpenoid (type of limonoid), that acts as an insect growth regulator (Kraus *et al.*, 1985; Schluter *et al.*, 1985; Prabhaker *et al.*, 1986; Mordue and Blackwell, 1993; Greenberg *et al.*, 2005; Khater, 2012). Other bioactive compounds, some with insecticidal properties, detected in neem extracts include salannin, salannol, nimbinen, gedunin, and dirachtin derivatives (Jones *et al.*, 1989; Walter, 1999); therefore, antifeedant and deterrent effects of neem against some herbivorous insects might be attributable to such compounds (Redfern *et al.*, 1981; Rice *et al*., 1985; Showler *et al.*, 2004; Greenberg *et al.*, 2005). An important advantage of multiple modes of action is delayed resistance development by pest populations (Feng and Isman, 1995).

Research on natural products, including neem-based formulations, against ticks has been increasing particularly against species that infest pets and cattle (Denardi *et al.*, 2010, 2011, 2012; Flor-Weiler *et al.,* 2011; Benelli *et al.,* 2016), but relatively little has been conducted for control of *A. americanum.* Because *A. americanum* is encountered primarily in wooded habitats, if tick control is needed, widespread application of conventional pesticides will likely be unacceptable because of environmental concerns. Similarly, acaricides, deterrents, and repellents for topical use on humans, livestock, and pets must conform to stringent safety standards. Hence, less toxic approaches, such as using botanically-based pesticides, might provide alternative means of control. While neem and other azadirachtin-containing solutions have been tested against a variety of ixodid ticks (Mulla and Su, 1999; Handule *et al.*, 2002; Landau *et al.*, 2009; Broglio-Micheletti *et al.*, 2009; De Sousa *et al.*, 2014), *A. americanum* has not been fully assessed as a target. The purpose of this study was to determine lethal, deterrent and repellent, and sublethal effects of two commercial neem-based home and garden pest control products on *A. americanum*.

MATERIALS AND METHODS

Insect

All experiments were performed at the USDA-ARS Knipling-Bushland United States Livestock Insects

Research Laboratory (KBUSLIRL) in Kerrville, Kerr County, Texas. *Amblyomma americanum* used in the experiments was obtained from a closed colony of 10th and 11th generation of originally wild-caught (August 2006) ticks maintained at KBUSLIRL. Engorged female *A. americanum* were fed on living cows, *Bos taurus* L. Bioassays mostly involved larvae because they constitute a free living stage that becomes attached to the host.

Neem Products

Two commercial neem products for home and garden pest control were tested. Neemix 4.5 (Certis, Columbia, MD, USA) is an emulsifiable concentrate containing 4.5% azadirachtin labeled as the active ingredient. AzaSol (SoluNeem, Sausalito, CA, USA) is a wet table powder with 6% azadirachtin labeled as the active ingredient. Both products are sold as insect growth regulators that kill during molting and during egg eclosion, pupation, and adult eclosion. Nondiluted Neemix was too viscous to spray but it could still be used in our bioassays. The highest aqueous concentration of the formulated product that, although viscous, was feasible for bioassays was 16.7%. Neemix and AzaSol bioassays were conducted and analyzed separately. Deionized water was used for preparing treatment dilutions and as negative controls. In all bioassays, treatments were arranged in a completely randomized design.

Selected bioactive limonoid compounds that are commonly found in neem extracts, nimbolide, nimbin, and salannin and were quantified in Neemix and AzaSol by high-performance liquid chromatograph-(HPLC) electrospray ionization mass spectrometry (MS) on an Agilent (Agilent Technologies, Atlanta, GA, USA) model 6224 ESI-time-of-flight MS in conjunction with an Agilent model 1260 Infinity binary pump HPLC system. HPLC conditions were: Column, ZORBAX Extend-C18 Rapid Resolution HT (2.1 mm × 50 mm, 1.8 µm); mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in acetonitrile; flow rate, 0.2 ml/min; gradient, 0% B to 100% B in 20 min. MS conditions were: Detection, positive ion, profile mode; mass range, m/z 100-m/z 3200; scan rate, 0.7 spectra/s. A standard curve was constructed over the range of 10 ng to 3,000 ng/mL from analysis of authentic nimbolide (m/z 467.207; retention time 14.75 min), nimbin (m/z 541.243; 15.30 min), and salannin (m/z 597.306; 15.7 min). Samples for analysis in organic solutions were diluted directly with acetonitrile. Aqueous samples were concentrated by solid-phase extraction (C18 ZipTip; Millipore) and eluted in acetonitrile/0.1% formic acid.

Contact Toxicity

Lethality of each dilution and the controls were assessed using >3-week-old larvae according to the immersion method described by Klafke *et al*. (2006). This involved releasing 78-172 larvae into each of 36 10 mL glass tubes for the Neemix bioassay and 89-234 larvae into each tube for the AzaSol bioassay (the variability is because larvae are small, fast, and difficult to handle in precise numbers). The tubes were immediately sealed with a cap.

Treatments, each replicated six times, were 100 (undiluted), 75, 50, 25, and 12.5% aqueous dilutions of formulated Neemix (4.5, 3.375, 2.25, 1.125, and 0.56% a.i., respectively), and, in a separate bioassay, 16.7, 12.5, 8.35, 4.15, 2.75, 1.6, 0.8, 0.4, and 0.2% formulated AzaSol (1, 0.75, 0.5, 0.25, 0.16, 0.096, 0.048, 0.024, and 0.012% a.i., respectively). Deionized water was used as the control for each bioassay. After 1 ml of each dilution was deposited in separate tubes of larval ticks, each tube was shaken vigorously by hand for 10 s, and gently shaken using a mechanical rocker for 10 min. The larvae in each tube were transferred using a #5 1.6-cm camel hair paint brush (Charles Leonard, Glendale, NY) to a 13 cm diameter filter paper disc and air dried at room temperature for 10 min. The larvae from each treatment replicate were placed in a packet constructed by folding filter paper (#1 Whatman, GE Healthcare, Little Chalfont, Buckinghamshire, England) into 8 cm × 8 cm packets with the sides folded so that each of the folded sides was closed tightly with a No. 3 Bulldog clip (Hunt Manufacturing, Statesville, NC). The packets were stored in an environmental chamber at 28°C and 80-90% RH for 24 h. Numbers of living and dead tick larvae were counted. Ticks were considered to be dead if they were unable to move on their own, even after prodding with a needle.

Fumigant Toxicity

About 10>1 month old *A. americanum* larvae were placed in 6 cm dia × 1.5 cm deep Kimax glass dishes (Kimble Chase, Vineland, NJ) with well-fitting lids. Nondiluted, and 87.5, 75, and 62.5% aqueous dilutions of Neemix (4.5, 3.94, 3.38, and 2.81% a.i., respectively), and, in a separate bioassay, a 16.7% (1% a.i.) aqueous dilution of AzaSol and deionized water were used as the controls. 1 mL of each treatment solution and the control was soaked into a 1 mL ball of cotton at the bottom of a 1.5 mL plastic micro test tube (Bio-Rad Laboratories, Hercules, CA) with a 3 mm diam hole punched in the closed lid. One micro test tube was placed inside each glass dish, and each treatment was replicated five times. Dead larvae were counted at 30 min, and 1, 2, 3, and 4 h. The same

bioassay was used to assess toxicity of volatiles against engorged adults.

Growth Regulatory Effects

A. americanum larvae used in this bioassay were used after they had fed on a cow host and dropped off for 1-5 days. Aqueous formulated Neemix dilutions of 3.125, 6.25, 12.5, and 25%, and AzaSol dilutions of 0.3, 0.612, 1.25, and 2.5% were used with a deionized water-only control in this assay. The dilutions were each anticipated to be marginally or completely sublethal because they were based on results from the contact toxicity bioassay. Larval *A. americanum* was immersed in each dilution in groups of 25 per dilution in the same way as described for the contact toxicity bioassays. After placing the larvae on filter paper for 10 min to dry, the groups of larvae were transferred to a 50 ml vial until they molted. Dead ticks were counted 20 days later (before molting began), and dead ticks were counted again 37 days later (after molting was complete). Each treatment was replicated six times.

Deterrent Effects

Aqueous formulated Neemix dilutions of 50, 45.8, 41.6, 37.4, 33.2, 29, 25, and 21% (2.25, 2.06, 1.87, 1.68, 1.49, 1.3, 1.12, and 0.94% a.i., respectively), and, in a separate bioassay, a 16.7% aqueous solution of formulated AzaSol (1% a.i.), and deionized water controls, were applied to 30 cm long, 2 cm wide filter paper strips in a 1 cm wide band across the strip (10 cm from one end). The upper and lower edges of the "barrier" were marked lightly with a pencil. The treatment "barrier" was air dried for 5 min, and each strip of paper was suspended from the top crossbar of a simple wooden frame 35 cm high such that the treatment "barrier" was 10 cm from the crossbar.

In the Neemix bioassay, 10-17 larvae were released 2 cm from the bottom of each paper strip, and 10-16 larvae were released on each strip in the AzaSol bioassay. Ixodid ticks are negatively geotropic, crawling upward on vertical surfaces (Kroeger *et al.*, 2013; Romaschenko *et al.*, 2013). At 1, 10, and 30 min, the numbers of larvae that crossed the treatment "barrier" were counted. The strips were exchanged for new ones between each of six replications.

Effects on Fecundity and Egg Hatchability

Replete F24 colony-reared adult females were collected from their bovine host over 3 days. All of the females were weighed, and those that were between 0.45 and 0.75 g were set aside for use in the experiment. Of 221 ticks collected, 44.8% were within this weight range.

Dionized water was used to dilute the two neem products. Neemix was diluted to make sublethal concentrations (based on results of the contact lethality bioassay) of 25, 12.5, and 6.25%, and AzaSol was diluted to 3, 1.5, and 0.75%. Ticks were immersed in the same way as in the contact lethality bioassay using enough solution to completely submerge the adult ticks. The ticks were placed on dry filter paper and each tick was gently blotted with a Kimwipe (Kimberly-Clark, Neenah, WI) to remove excess solution. Ticks were then placed in individual 30 mL shell vials which were kept in an environmental chamber at 22°C, 94% RH, and 12:12 (L:D) photoperiod. After 30 days, the females were removed from the glass vials which by that time contained 1-2 egg masses each. The vials had been weighed before use, and the weight of the empty vial was subtracted from the weight of the vial containing eggs to obtain egg mass weights. The vials were placed back into the environmental chamber for 7 weeks; then, two persons made percentage egg hatch estimates for each vial, and the two estimates were used to get an average percentage egg hatch. Each treatment was replicated 30 times.

Statistical Analyses

Treatment differences for the contact toxicity data and the fecundity and egg hatchability data were detected using a one-way ANOVA, and means were separated using Tukey's honestly significant difference (HSD) (Analytical Software, 2008). Fumigant toxicity and deterrence were analyzed using repeated measures to detect differences associated with treatments and with time and to detect treatment × time interaction. The fumigant toxicity and deterrency datasets were also analyzed using one-way ANOVA to detect differences between specific treatments at each sampling time, and Tukey's HSD was used to separate means. Percentage data were expressed as ratios before arcsine square root transforming data for statistical tests, but nontransformed (percentage) data are presented. Because normality and homogeneity of variance assumptions were not violated, data were not log(x+1)-transformed. Percentage data were arcsine square root transformed before analysis.

RESULTS

Neem Products

Neemix was found to contain 953.8 µg/mL of nimbolide, 2703.2 µg/mL of nimbin, and 26314.9 µg/mL of salannin. AzaSol had 9.3 µg/mL of nimbolide, 28.4 µg/mL of nimbin, and

205.9 µg/mL of salannin; hence, Neemix had 102.6-, 95.2-, and 158-fold more nimbolide, nimbin, and salannin, respectively, than AzaSol.

Contact Toxicity

The lowest concentration of Neemix, 12.5%, did not cause more mortality of larval *A. americanum* than the control which failed to kill any (Fig. 1). The 25, 50, and 75% concentrations, and nondiluted Neemix, however, killed 5-, 19.7-, 162.7-, and 212.7-fold more larvae than the lowest concentration ($F = 257.09$, df = 5, 17, $P < 0.0001$), although the maximum mortality was only ≈63% (Fig. 1). The lowest four concentrations of AzaSol did not cause significantly more mortality than the control (Fig. 2). The 2.75, 4.15, 8.3, 12.5, and 16.7% concentrations induced 66.7-, 79.5-, 224-, 265-, and 332-fold greater mortality than the control ($F = 158.86$, df = 9, 29, $P < 0.0001$), but the maximum mortality was only ≈66% (Fig. 2).

Fumigant Toxicity

Repeated measures analysis detected fumigant treatment ($F = 65.26$, df = 4, 124, $P < 0.0001$) (Fig. 3a) and time ($F = 138.31$, df = 4, 124, $P < 0.0001$) effects on larval *A. americanum*. In terms of the treatment effects, each consecutively greater concentration caused more mortality ($P < 0.05$) than the next lower concentration; the 67.5, 75, 87.5%, and nondiluted Neemix concentrations resulted in 4.7-, 7.3-, 15.7-, and 22.3-fold greater mortality than the control (Fig. 3a). Larval mortality increased at each time of observation until 3 h, but mortality at 4 h did not differ from 3 h.

Volatiles from nondiluted Neemix caused complete mortality among larval *A. americanum* by 3 h, and the mortality observed in the 87.5% concentration treatment was not statistically different from 100% by 2 h (Fig. 3b). By 3 h, the nondiluted Neemix had killed all of the larvae (Fig. 3b). In the lower concentration treatments and the control, 57.1 to 94%, and 51 to 92% less mortality was observed by 3 and 4 h, respectively, than in the two high concentrations ($F = 47.62$, df = 24, 124, $P < 0.0001$) (Fig. 3b). Mortality in the control, in the 67.5 and 87.5% concentrations, did not rise above 8, 30, and 48%, respectively, during the 4-h experiment (Fig. 3a).

When Neemix volatiles were used for killing adult *A. americanum*, repeated measures analysis detected treatment ($F = 97.23$, df = 4, 99, $P < 0.0001$) (Fig. 4a) and time ($F = 77.39$, df = 4, 99, $P < 0.0001$) effects. In terms of treatment effects, the two highest concentrations caused ≥81% more mortality than the two lower concentrations and the control (Fig. 4a).

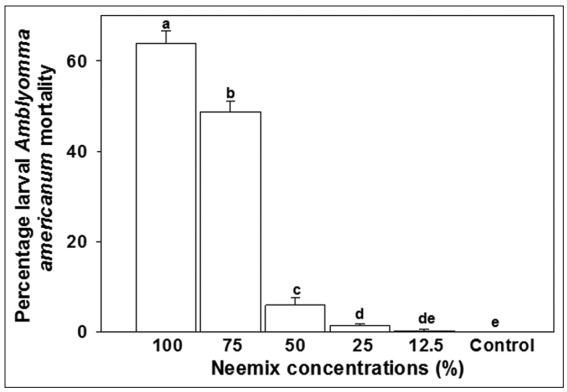

Fig. 1. Mean ± standard error percentages of *Amblyomma americanum* larval mortality after 10 min immersion in five Neemix concentrations, 78-172 larvae/replicate (one-way ANOVA, Tukey's honestly significant difference, $P < 0.05$).

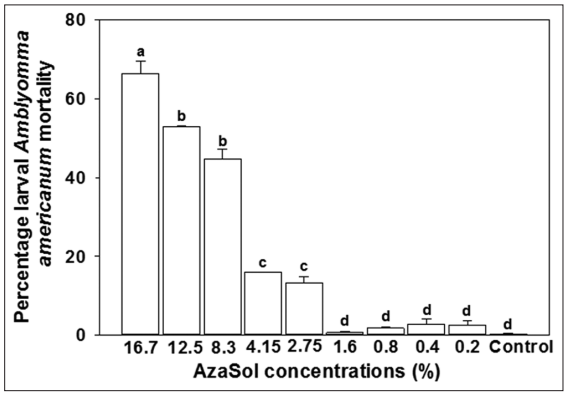

Fig. 2. Mean ± standard error percentages of *Amblyomma americanum* larval mortality after 10 min immersion in nine AzaSol concentrations, 78-172 larvae/replicate (one-way ANOVA, Tukey's honestly significant difference, $P < 0.05$).

Overall mortality was relatively low during the first hour, increasing 9-fold by 2 h, and by 11.8-fold by 3 h, leveling off thereafter at ≈4.8%. A treatment × time interaction was detected ($F = 18.40$, df = 16, 99, $P < 0.0001$).

Volatiles from nondiluted Neemix killed all of the adults by 2 h, and the mortality observed in 87.5% concentration treatment was not statistically different from the nondiluted concentration by 3 h (Fig. 4b). In the lower concentration treatments and the control, 80-100%, and 75-100% less mortality was observed by 3 and 4 h, respectively, than in the two high concentration treatments ($F = 63.59$, df = 24, 124, $P < 0.0001$) (Fig. 4b). Mortality in the control, in the 67.5 and 87.5% concentration treatments, did not rise above 0, 25, and 15%, respectively, during the 4 h experiment (Fig. 4b).

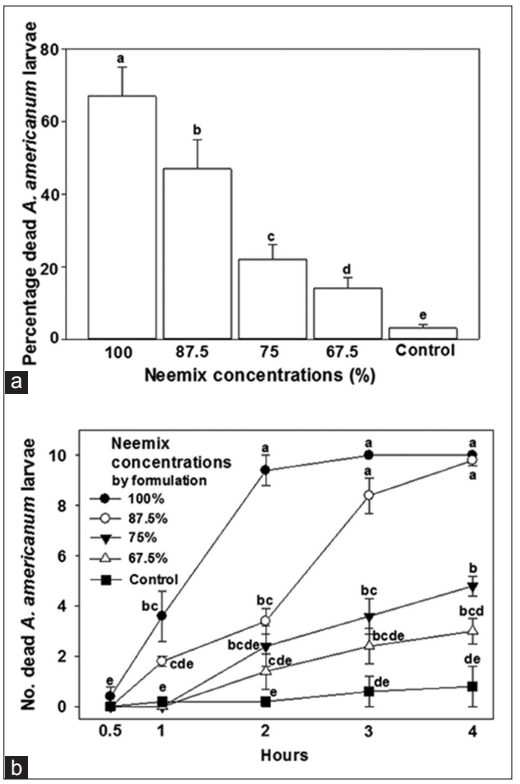

Fig. 3. Mean ± standard error percentage mortality of *Amblyomma americanum* larvae exposed to volatiles from Neemix at four concentrations, 10 ticks/replicate. (a) Repeated measures analysis; (b) one-way ANOVA (Tukey's honestly significant difference, $P < 0.05$).

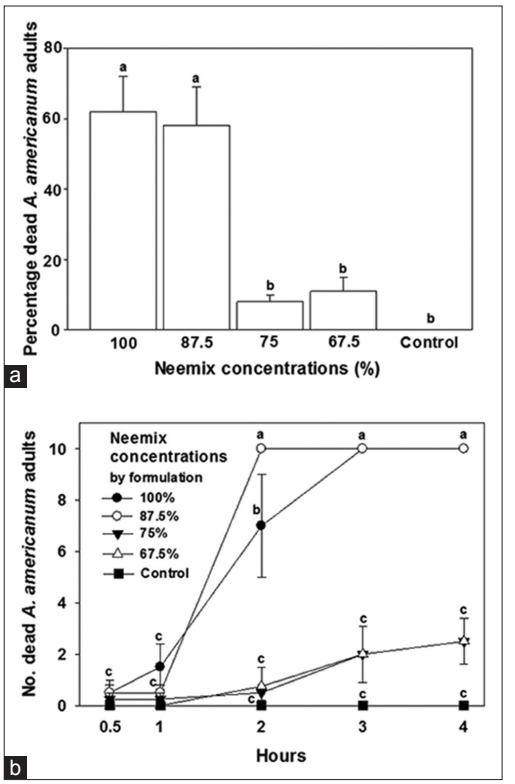

Fig. 4. Mean ± standard error percentage mortality of *Amblyomma americanum* adults exposed to volatiles from Neemix at four concentrations, 10 ticks/replicate. (a) Repeated measures analysis; (b) one-way ANOVA (Tukey's honestly significant difference, $P < 0.05$).

No differences in larval or adult mortality were detected between the greatest AzaSol concentration, 16.7%, and the control. Mortality never rose above 2% in the AzaSol fumigant toxicity bioassay.

Growth Regulatory Effects

While the 6.25, 12.5, and 25% Neemix concentrations caused 24.7-97.2% mortality before molting began, none of the concentrations showed growth regulator effects against larvae molting into nymphs. In the AzaSol bioassay, no premolting mortality was observed and growth regulator effects did not occur.

Deterrent Effects

Repeated measures analysis detected Neemix treatment ($F = 77.64$, df = 8, 134, $P < 0.0001$) (Fig. 5a) and time ($F = 20.30$, df = 2, 134, $P < 0.0001$) effects on *A. americanum* larvae. In terms of treatment effects, the low, 21%, concentration was no more deterrent than the control (Fig. 5a). The 25, 29, 33, 37.6, 41.6, 45.8, and 50% concentrations deterred 27.9, 35.7, 65.5%, 56.8, 63.8, 74.3, and 86%, respectively (Fig. 5a). The numbers of *A. americanum* larvae that crossed the treatment barrier increased by a relatively gradual 27.9% throughout the 30-min assay. A treatment × time interaction was not detected.

Percentages of larvae that crossed the treatment "barrier" mostly diminished with decreasing concentrations (Fig. 5b) ($F = 25.75$, df = 26, 134, $P < 0.0001$). The fewest, 3.6 and 2%, were in the 45.8 and 50% Neemix concentration treatments, respectively (Fig. 5b).

Repeated measures analysis and ANOVA did not detect AzaSol treatment differences. Repeated measures analysis detected a time effect ($F = 16.60$, df = 2, 29, $P = 0.0001$). A treatment × time interaction was not detected.

Effects on Fecundity and Egg Hatchability

The sublethal concentrations used in this assay did not affect egg mass weight or egg hatchability. The pooled averages for egg mass weight and for percentage egg hatch were 0.224 ± 0.003 g and 93% ± 6.4, respectively.

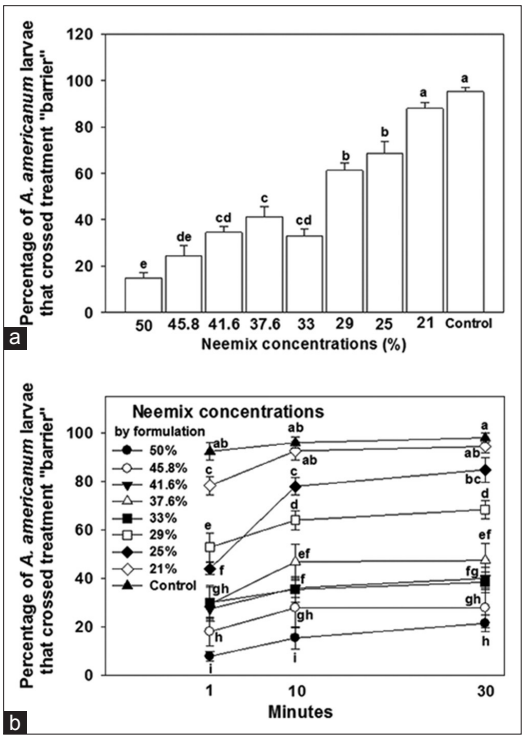

Fig. 5. Mean ±standard error percentages of *Amblyomma americanum* larvae that crossed the treatment "barrier" comprised eight different concentrations at 1, 10, and 30 min, 10-17 larvae/replicate. (a) Repeated measures analysis; (b) one-way ANOVA (Tukey's honestly significant difference, $P < 0.05$).

DISCUSSION

Neemix has a relatively weak and short (24 h) feeding and oviposition deterrent effects against boll weevil, *Anthonomus grandis grandis* Boheman, on cotton squares (Showler *et al*., 2004), but the product was not assessed for contact and volatiles lethality, or sublethal effects. Used against the beet armyworm, *Spodoptera exigua* (Hübner), Neemix showed relatively weak or moderate oviposition and feeding deterrence, and weak or moderate ovicidal and larvicidal efficacy (Greenberg *et al*., 2005). On the horn fly, *Haematobia irritans irritans* L., Neemix exhibited moderate adult knockdown capabilities, relatively low repellency, moderate or strong adult mortality from exposure to volatiles, no ovicidal activity, strong insect growth regulatory effects, and sublethal effects on egg production. AzaSol showed weak adult knockdown, no repellency, strong insect growth regulatory effects, and no sublethal effects (ATS, unpublished data).

In terms of the labeled active ingredient, azadirachtin, the 12.5% and 25% Neemix concentrations contained 0.56% and 1.125% azadirachtin, respectively, roughly similar to AzaSol's 8.3% and 16.7% concentrations which contained 0.5 and 1% azadirachtin, respectively. The lethal contact potency of the two commercial products differed substantially with AzaSol producing 149.3- and 44.3-fold greater larval mortality, respectively, than the corresponding Neemix concentrations, suggesting that AzaSol might contain lethal bioactive compounds aside from nimbolide, nimbin, and salannin, which were found in substantially greater concentrations in Neemix than in AzaSol. Neem trees produce at least 35 bioactive compounds that include salannin, salannol, salannolacetate, gedunin, nimbin, nimbinen, nimbolide, dirachtin, and viselin (Jones *et al*., 1989; Mulla and Su, 1999; Walter, 1999; Jayaraj and Ignacimuthu, 2005; Babu *et al*., 2006). In neem-based emulsifiable formulations, other limonoids are often present in relatively low concentrations, unlike neem kernel extract in which the other limonoids are generally absent (Jayaraj and Ignacimuthu, 2005). Further, there is more than one kind of azadirachtin (azadirachtin A, azadirachtin B, and others) (Ramesh and Balasubramanian, 1999), and azadirachtin has more than one mode of action against arthropods (Kraus *et al.*, 1985; Schmutterer, 1990; Mordue and Blackwell, 1993; Khater, 2012).

Neemix contact lethality against *Dermacentor albipictus* was greater when compared to *A. americanum* using the same concentration, and decreasing concentrations of Neemix reduced larval *D. albipictus* mortality gradually (ATS, unpublished data) while efficacy against *A. americanum* dropped dramatically at concentrations <75%. These observations suggest that *D. albipictus* is more susceptible to Neemix than *A. americanum*, and that *A. americanum* appears to have a concentration threshold between 75 and 50%, over which susceptibility to Neemix greatly increases. AzaSol's contact lethality against *D. albipictus* (ATS, unpublished data) and *A. americanum* were roughly the same and abrupt changes in efficacy from one concentration to the next did not occur to the extent observed when Neemix was used against larval *A. americanum*.

Although the maximum concentration of AzaSol, 16.7%, was fluid enough to include in the bioassay, it was too viscous to apply as a spray. The 100% concentration of Neemix was also too viscous to use as a spray. Maximum spray able Neemix and AzaSol concentrations in our study were the 75 and 12.5% concentrations, respectively.

While azadirachtin can affect insects in different ways (Schmutterer, 1990; Suman *et al*., 2013), its utility is mostly as a growth regulator (Schluter *et al.*, 1985;

Prabhaker *et al*., 1986; Mordue *et al*., 1998; Showler *et al*., 2004, Greenberg *et al*., 2005; Khater, 2012). On *A. americanum*, however, relatively high concentrations failed to inhibit molting from the larval stage to the nymphal stage. Because of similarities between responses of *D. albipictus* (ATS, unpublished data) and *A. americanum* to each of the two neem-based products, it is possible that the products and azadirachtin do not affect other ixodid ticks in terms of growth regulation. Hence, the occurrence of more than one bioactive compound in the neem-based formulations and the variety of effects on *D. albipictus* suggests that the nonazadirachtin compounds exerted effects other than growth regulation (ATS, unpublished data). Neemix and AzaSol were deterrents and caused contact mortality against larvae, at least some of which might have resulted from bioactive compounds other than azadirachtin. A number of bioactive insecticidal compounds, some with insecticidal properties, have been detected in some neem extracts, including salannin, salannol, nimbinen, gedunin, and azadirachtin derivatives (Jones *et al*., 1989; Walter, 1999; Koul *et al*., 2003, 2004). The frequent presence of bioactive compounds in neem extracts and oils is often suggested as an explanation for observed ranges of activities caused by neem-based products against insects (Redfern *et al*., 1981; Rice *et al.,* 1985; Schmutterer, 1990; Showler *et al*., 2004, 2017; Greenberg *et al.*, 2005; Hasan and Ansari, 2011). In the instances of nimbolide, nimbin, and salannin, detected in relatively large quantities in Neemix (in contrast to substantially smaller quantities in AzaSol), nimbolide is cytotoxic; salannin can deter feeding, delay molting, and kill larvae and pupae; nimbin is an antifeedant (Cohen *et al*., 1996; Govindachari *et al*., 1996), but their ranges of bioactivity and efficacies against pest species have not yet been fully explored. Neem seed can contain varying amounts of nimbin and salannin from 18.2 to 636.8 mg/kg, and from 45.4 to 1830.3 mg/kg, respectively. Amounts of such bioactive compounds can vary as a result of genetic influence and from environmental factors (Sidhu *et al.*, 2004). The negligible mortality associated with AzaSol volatiles in contrast with the lethal effects of Neemix volatiles indicates that nimbolide, nimbin, salannin, or another nonazadirachtin bioactive compound (with greater volatility, perhaps, than typical limonoids), or a combination of them, had a lethal respiratory effect. Although limonoids are not particularly volatile, neem seed oil volatiles were reported to kill pulse beetle, *Callosobruchus maculatus* F., eggs, larvae, and adults (Reddy and Singh, 1998). The same concentrations showed that Neemix and AzaSol volatiles affected *D. albipictus* larvae (ATS, unpublished data) about the same as we observed for *A. americanum*, including the two higher concentrations having substantially greater potency than the lower concentrations. While the formulations we tested were not highly effective against *A. americanum*, enhanced potency against *A. americanum*, and possibly other tick species, might be obtained by purifying and applying bioactive compounds singly or in selected combinations.

Extracts of azadirachtin-containing plants such as chinaberry, *Melia azedarach* L., have sublethal effects on aspects of southern cattle fever tick, *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), reproduction (De Sousa *et al*., 2014), but the concentrations and immersion times we used for Neemix and AzaSol failed to affect reproduction of *A. americanum*. Sublethal concentrations of Neemix and AzaSol also did not affect *D. albipictus* egg laying and hatching (AS, unpublished data).

Only moderate larval contact mortality against *A. americanum* larvae was achieved by Neemix even when the undiluted formulation was used; hence, the product appears to be of little use as a control tactic. Similarly, AzaSol used at the highest possible aqueous concentration, 16.6%, has moderate killing power against *A. americanum* larvae. Although at the highest concentrations of Neemix, volatiles were effective at killing *A. americanum* larvae and adults within hours, it is difficult to conceive of a way in which Neemix volatiles could be used for protection of humans and animals. However, the fact that ixodid ticks are relatively hardy and robust arthropods (particularly the adults), and *A. americanum* is susceptible suggests that Neemix contains volatile compounds that might be useful in terms of fumigant activity against insects in enclosed spaces.

Because Neemix, particularly at the highest concentrations, deterred *A. americanum* and AzaSol was not deterrent, azadirachtin likely played no role. We could not ascribe the relatively strong deterrent activity observed when the 50 and 45.8% Neemix concentrations were used to specific bioactive compounds. While limonoids are not particularly volatile, limited volatility might have been involved in the observed deterrence but it seems more likely that other, more highly volatile, components of Neemix that we did not detect were responsible.

While Neemix and AzaSol might not be practical and sufficiently efficacious in their present formulations for control of *A. americanum*, we did find attributes, particularly regarding the lethality of Neemix volatiles, that should be pursued as a fumigant for controlling pests in enclosed spaces, such as storage containers. Our study demonstrated that neem-based commercial products can have multiple effects against ticks,

and that different neem-based products can contain substantially different concentrations of a variety of bioactive limonoids that are not indicated on the label. The presence and abundances of those limonoids might confer a variety of modes of action to neem-based products. Although azadirachtin has received much attention for being an insect growth regulator, other modes of action by azadirachtin and other bioactive compounds (e.g. nimbolide, nimbin, and salannin) should be explored for pest management applications.

Our study showed that AzaSol is less active against larval *A. americanum* than Neemix, but even Neemix did not have appreciable effects unless it was used at relatively high concentrations (e.g., >35% for substantial fumigant effects). Observed responses of the larvae, however, indicate that Neemix, in particular, has biological activity that might be useful for tick control. The diversity of bioactive compounds that are found in neem extracts and in Neemix (compared to AzaSol) suggests that heightened efficacy might be attainable by isolating bioactive ingredients of Neemix, none of which, excluding azadirachtin, are identified on the commercial label. Isolation and testing of bioactive components in Neemix is a useful next step in assessing natural organic chemicals that, in more pure and concentrated formulations than those found in Neemix, have strong pest control properties at relatively low concentrations.

In terms of practical applications for the neem-based commercial products used in this study effective protection against *A. americanum* is possible. Control on large wild ungulates presents a challenge (ATS, unpublished data) but there is at least one method for applying acaricides to white-tailed deer (Pound *et al*., 2000). Because tick control is important in areas frequented by the public and in environmentally protected areas such as wildlife refuges that can harbor hosts of disease-vectoring tick species to wild host animals, livestock on adjacent rangeland, and humans, application of botanically-based (organic) compounds such as those that occur in Neemix (and possibly other neem-based commercial products) using devices such as the four-poster applicator (Pound *et al.* 2000) might become a viable management tactic. Further research on the bioactive constituents of commercial neem-based pesticides might yield important information about bioactive compounds that have previously not been assessed for use against ticks.

Acknowledgements. We thank Jessica Harlien, Wayne Ryan, Elizabeth Gomez, Margarita Barco, Elias Showler, Lizbet del Torres, Alyssa Poore, and Alyssa May for technical assistance. Mass spectrophotometry analyses were conducted in the UTHSCSA Institutional Mass Spectrometry Laboratory, supported in part by UTHSCSA and NIH grants 7P30CA54174-14 and IS10OD016417-01 to Susan Weintraub. The expert technical assistance of Sammy Pardo is gratefully acknowledged for the mass spectrophotometry.

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Accepted: 6 May 2017