

## ABSTRACT

CARR, ANN LOUISE. Response of the Ixodid Ticks *Amblyomma americanum* and *Dermacentor variabilis* to Hematophagous Insect Attractants in Laboratory Assays and Field Trials. (Under the direction of R. Michael Roe and Coby Schal.)

Through a literature survey, some candidate odorant compounds that attract hematophagous arthropods were identified for testing against adults of two tick species, *Amblyomma americanum* and *Dermacentor variabilis*. Carbon dioxide, 1-octen-3-ol, acetone, ammonium hydroxide, L-lactic-acid, dimethyl trisulfide and isobutyric acid were tested in laboratory Y-tube olfactometer bioassays for attraction and to establish dose-response curves for the active compounds. Only carbon dioxide, 1-octen-3-ol, acetone and ammonium hydroxide elicited a significant preference by *A. americanum* and only carbon dioxide elicited similar behavior in *D. variabilis*. In field studies, 1-octen-3-ol, acetone, and ammonium hydroxide were separately evaluated against a wild *A. americanum* population. Three release rates of each semiochemical were concurrently tested with carbon dioxide from dry ice and water used as controls. Carbon dioxide consistently attracted the highest number of host-seeking *A. americanum* nymphs and adults. However, for the first time, acetone and ammonium hydroxide were shown to attract high numbers of *A. americanum* ticks. The mean numbers of ticks attracted to acetone were not significantly different from the mean numbers of ticks attracted to carbon dioxide. Further research is needed to determine the utility of acetone and ammonium hydroxide as attractants in tick surveillance and area-wide management programs.

Response of the Ixodid Ticks *Amblyomma americanum* and *Dermacentor variabilis* to  
Hematophagous Insect Attractants in Laboratory Assays and Field Trials

by  
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## **DEDICATION**

To my parents, Richard and Marie Carr.

## **BIOGRAPHY**

Ann Carr was born and raised in Washington DC making her a 6<sup>th</sup> generation washitonian. She attended Holton-Arms School for Girls, and at graduation made her way down south to attend Texas A & M University. During her freshmen and sophomore years she interned at Walter Reed Army Institute of Research investigating genetic variances between SHIV positive and negative rhesus monkeys. Her junior year and senior years she worked as a lab technician for Dr. Albert Mulenga in the Texas A & M medical and veterinary entomology lab aiding in research on tick salivary proteins. Ann graduated from Texas A & M in 2009 with a BS double major in biomedical science and entomology. She arrived here at NC State University and began research evaluating tick host kairomones for her master's and will be continuing on for a pHD, sequencing a transcriptome for the tick's Haller's organ and investigating knock down therapies. In addition to her research, Ann is actively involved in EGSA student outreaches and is currently a Level I PSIA certified ski instructor and still enjoys teaching lessons every now and then.

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# 1. LITERATURE REVIEW

## INTRODUCTION

Ixodid ticks vector pathogens that cause diseases in both human and animal populations. The prevalence and geographic distribution of tick-borne diseases is largely dependent on the tick vector. Tick surveillance provides information regarding population densities, geographic spread, and infection rates with pathogens that may be transmitted to future hosts. Surveillance data are useful in interpreting trends in tick-borne diseases and to assess the risk of disease for humans and animals (Jameson & Medlock, 2011). Tick surveillance data are also used to instigate management strategies and to monitor the effectiveness of control programs (Haemig *et al.*, 2011). To date, there have been surveillance studies for ticks vectoring pathogens causing Lyme disease, Rocky Mountain spotted fever, anaplasmosis, ehrlichiosis, cattle fever and southern tick associated rash illness (STARI) (Bacon *et al.*, 2008; Hall-Baker *et al.*, 2009; Bisgard, 2009). Tick surveillance is an important tool in assessing the health risks for humans and animals spending time in tick infested areas. Similar surveillance techniques are available for hematophagous insects, such as mosquitoes, that exploit their host-seeking behavior (Service, 1993). These techniques involve baiting traps with host-associated kairomones that increase the efficiency of the traps in an inexpensive manner. Some surveillance methods are also used for control, such as trapping and removing hematophagous insects, including tabanids, eye gnats, tsetse flies, and stable flies, rather than conducting mass insecticide spray programs (Day & Sjogren, 1994). For tick surveillance, host-associated chemicals are also used to bait ticks, using dry ice or

wildlife trapping, and examining the animals for ticks. These surveillance techniques can be labor intensive, biased or require equipment that is expensive or not readily available. More research is needed to evaluate host-associated chemicals for use in tick surveillance programs that are easy to handle, inexpensive and efficient.

## TICK SENSORY RECEPTION

Sensory stimuli that induce tick questing and feeding behavior convey information about host family, proximity and directionality. Specific auditory and olfactory stimuli indicate the presence of a distant host and provide information on host type and basic directionality. Non-specific mechanical, tactile and thermal stimuli indicate the presence of a proximal host and the host's position in reference to the tick. Physical contact with host pelage and gustatory stimuli identify host type (Obenchain & Galun, 1982). In ticks, the main external apparatus responsible for olfaction is the Haller's organ. The Haller's organ, present on the foreleg tarsi, is a localization of olfactory sensilla. It consists of two main parts, the posterior capsule and the anterior pit. Chemosensation is ascribed mainly to the posterior capsule whereas the anterior pit is more associated with heat and humidity reception (Sinitsina, 1974; Hess & Vlimant, 1986). The posterior capsule contains 4-12 multiporous sensilla and additional pleomorph structures. Each olfactory sensillum contains four or five bipolar receptors cells that process information received through distal cilia (Gothe *et al.*, 1991; Balashov, 1983). The sensilla detect volatile chemicals, water vapor and carbon dioxide, all indicative of proximal hosts. The Haller's organ is also responsible for pheromone recognition. A few sensilla in the anterior pit detect 2,6-dichlorophenol (2,6-DCP) and other

volatile phenols and substituted phenols, which constitute the composition of multiple sex pheromones across Ixodid species (Sonenshine, 2006). There is a limited number of high specificity sensilla in the Haller's organ, which suggests that varying compositions and concentrations of compounds induce tick questing behavior and host selectivity (Waladde & Rice, 1982; Sonenshine, 1991). The ratio of signals from more generalized sensilla may provide the tick with a systematic method of host identification and pheromone recognition (Osterkamp *et al.*, 1999).

### ASSAYS FOR TICK ATTRACTANTS

Historically, the development of tick bioassay systems has been focused on testing repellents. There are limited assay methods for evaluating tick attractants, but the few assays that are utilized, provide information on short-range attraction, long-range attraction and olfactory sensilla responses. The three main assay types used for evaluating attractants are: laboratory choice bioassays, field trials, and electrophysiological assays. Despite the availability of limited research methods, there is much variability in tick attractant testing involving selection of tick species, life stage, and formulation and delivery of test chemicals. Laboratory choice bioassays provide information about the short-range attractive properties of a chemical. Choice bioassays provide ticks the opportunity to choose between chemicals, typically a test compound and a control substrate. Two-choice assays expose ticks to 1 or 2 chemical options, whereas four-choice assays expose ticks to 1, 2, 3 or 4 chemical options. A few different experimental designs have been used for conducting choice assays. One method is the glass rod experimental design, in which two glass rods, stabilized in a clay

block, are placed into an arena and one rod is coated with the test substance and the other rod is coated with control material. The assay takes advantage of the natural questing behavior of ticks on vegetation. Ticks placed into the arena have the option of remaining on the clay base or questing up the glass rods. Ticks questing up the rod treated with the test material would indicate an attractive behavioral response (Carroll, 1999, 2001). A second experimental method is the two-choice or four-choice quadrant design. The quadrant design uses treated filter paper to expose ticks to test chemicals in an enclosed Petri dish. The filter paper is divided into quadrants and 1, 2, 3 or 4 chemicals are applied to the quadrants. An attractive behavioral response would be indicated by ticks that arrest on chemically treated portions of the filter paper (Allan & Sonenshine, 2002; Arlian & Vyszenki-Moher, 1995). Both the glass rod and quadrant experimental design evaluate olfactory and gustatory responses. A third laboratory method involves use of olfactometers to test chemicals for olfactory mediated attraction. Olfactometers can have two ports or four ports, and allow testing of 1, 2, 3, or 4 chemicals. Olfactometers are closed systems that use either a push, or push-pull airflow system to move chemicals in a continuous airstream. Attraction of ticks into ports is indicative of an attractive behavioral response (Nana *et al.*, 2010). Field assays are effective in determining the long-range attractive properties of a chemical and its duration of attractive activity. There are multiple experimental designs for conducting field assays. The most common design involves releasing marked ticks at various distances from the chemical source and recording the number that return to the chemical source (Carroll, 2006). Electrophysiological assays provide information on olfactory cell stimulation. There are also field assay designs that merely count ticks attracted to the chemical source (Bissinger *et al.*,

2011). Electrodes inserted into an olfactory sensillum record action potentials when olfactory neurons respond to a behaviorally active compound (Holscher, 1980; Waladde, 1982; Steullet & Guerin, 1992). Electrophysiological assays enable rapid screening and quick identification of active compounds and the sensillum types responsible for odorant detection.

### SEMIOCHEMICAL ATTRACTANTS

Semiochemicals are individual chemicals or chemical mixtures that convey information from one individual to another. They are divided into three categories: allomones, kairomones and pheromones. Allomones are chemicals that convey information from one species to another while benefiting the emitter, not the receiver. Kairomones are chemicals that convey information between species that benefit the receiver and not the emitter. Pheromones are chemicals that convey information between two individuals of the same species and are typically beneficiary for both the emitter and receiver (Nation, 2002). Ixodid ticks exhibit attractive behavior to both kairomones and pheromones. Currently there are six identified tick pheromones (Sonenshine, 2006). Three have potential for incorporation as attractants in a lure and kill system. These pheromones are the assembly aggregation attachment pheromone, attractant sex pheromone and arrestment pheromone. Host kairomones are also important tick attractants for consideration as lures in tick surveillance programs.

### *Attraction Aggregation Attachment Pheromone*

Attraction-aggregation-attachment pheromone (AAAP) is a pheromone secreted by feeding males to attract feeding or non-feeding conspecific females, males and nymphs forming large feeding clusters on bovine or large ungulate hosts. The pheromone is secreted by large, ventrally located dermal secretory cells (Bowman & Nuttal, 2008). AAAP composition varies by species and is only produced by a small subset of species in the genus *Amblyomma* that feed on large animals. AAAP alerts questing ticks of nearby feeding ticks and strategically assembles reproductives together on the host (Sonenshine, 2006). Currently AAAP is known to be produced by *Amblyomma variegatum*, *A. haebrium*, *A. americanum*, *A. cajennense*, *A. marmoreum*, *A. lepidum*, and *A. gemma* (Bowman & Nuttall, 2008; Sonenshine, 2006; Diehl *et al.*, 1991). Although the composition varies by species, AAAP typically consists of 1-2 phenols with an attached fatty acid. AAAP of *A. variegatum*, the bont tick, is the most studied and well known. It is composed of methyl salicylate, *o*-nitrophenol and nonanoic acid in a 2:1:8 ratio (Schoni *et al.*, 1984). The sex pheromone, 2,6 dichlorophenol, that attracts female ticks has also been identified in emitting male tick extracts (Price *et al.*, 1994). This pheromone in combination with CO<sub>2</sub> emissions from the host creates a strong bioactive mixture capable of attracting host-seeking ticks from a distance of 5 to 10 m (Norval *et al.*, 1991; Barré *et al.*, 1997). Maranga *et al.* (2003) reported that 35% of released *A. variegatum* ticks were attracted to 6.6mg of synthetic AAAP and 90% of released *A. variegatum* ticks were attracted to 6.6mg of synthetic AAAP in the presence of CO<sub>2</sub> (500 and 50 g of dry ice). Additionally, Barré *et al.* (1997) reported that adding pheromone to 500g of dry ice increased tick attraction by 70 fold. Nchu *et al.* (2009)

conducted laboratory two-choice bioassays to test the bioactivity of AAAP against *A. variegatum* and reported that 0.022mg of synthetic AAAP had a relative attraction of 50%. In field assay Nchu *et al.* (2009) combined 0.022mg of synthetic AAAP with CO<sub>2</sub> and 1-octen-3-ol increasing attraction up to 94% of ticks released from distances of 6m.

### *2,6-Dichlorophenol Attractant Sex Pheromone*

Ixodid tick adults only become sexually mature with a blood meal. In adult female ticks, consumption of a blood meal initiates production of the sex pheromone 2,6-dichlorophenol (2,6-DCP). 2,6-DCP is secreted by foveal glands located on the dorsal alloscutum and causes nearby conspecific males to detach from feeding and search for emitting females to begin courtship and mating (Sonenshine *et al.*, 1985; Sonenshine, 2006). 2,6-DCP is currently known to be produced by seven genera and 16 species of ticks (Sonenshine, 1991). Females primarily utilize 2,6-DCP as a sex pheromone, though *A. variegatum* and *A. hebraeum* males will secrete 2,6-DCP to stimulate attraction and attachment of mature and immature ticks on the host (Norval *et al.*, 1992; Price *et al.*, 1994; Yoder & Stevens, 2000). 2,6-DCP is an extremely potent pheromone for male ticks. Tick decoys impregnated with 50 ng of 2,6-DCP induced 100% of tested *Anocentor nitens* ticks to engage in mate-seeking behavior and mounting behavior with the impregnated decoys (Borges *et al.*, 2002). Similar behavior was observed in *A. cajennense* when exposed to 500ng of 2,6-DCP (Louly *et al.*, 2008) and *A. americanum* when exposed to 100ng (Berger, 1972). 2,6-DCP is also known to elicit attraction behavioral responses in *D. variabilis* and *D. andersoni* (Sonenshine, 1976). The pheromone was also identified in all life stages of *Rhipicephalus microplus* though there

is no associated behavioral response (de Bruyne & Guerin, 1998). Electrophysiological studies determined that sensilla in the anterior pit of the Haller's organ of *A. variegatum* and *R. appendiculatus* mature and immature males responded to amounts of 2,6-DCP ranging from 0.04-1.6 ng (Waladde, 1982).

### *Arrestment Pheromone*

Arrestment pheromones, previously known as assembly pheromones are contact specific chemicals that cause a cessation of locomotor activity. They are found in both Ixodid and Argasid tick species. Arrestment pheromones cause clustering of conspecific individuals in sheltered areas, aiding in moisture retention, host finding and strategically assembles reproductives (Sonenshine, 1985). Arrestment pheromones are species specific and are active on unfed larvae, nymph and adult ticks (Bowman & Nutell, 2006). These pheromones are known to be produced by at least 14 species of Argasid ticks and by multiple Ixodid species including *Ixodes ricinus* (Graf, 1978), *I. scapularis* (Allan *et al.*, 2002; Sonenshine *et al.*, 2003), *I. uriae* (Benoit *et al.*, 2008), *I. holocyclus* (Treverrow *et al.*, 1977), *A. cohaerans* (Otiene *et al.*, 1985), *Hyalomma dromedarii* (Leahy *et al.*, 1981), *Rhipicephalus appendiculatus* (Otiene *et al.* 1985), *R. evertsi* (Gothe & Neitz, 1985), and *Aponomma concolos* (Treverrow *et al.*, 1977). Arrestment pheromones are found in excreta, caste skins and on emitting individuals (Sonenshine, 2006). Fecal deposits on filter paper caused 67% of larvae, 66% of nymphs, 64% of males, and 71% of females of *I. uriae* ticks to arrest. *I. uriae* ticks also elicited 68% arrestment behavior in response to soil collected from previously clustering individuals (Benoit *et al.*, 2008). *I. ricinus* ticks also exhibit significant arrestment

behavior in response to fecal matter with 48% of tested individuals ceasing activity and congregating (McMahon, 2003). GC-MS analysis of fecal material, caste skins and whole tick body washes has identified the primary components of arrestment pheromone to be hematin, guanine, xanthine, uric acid and several other purines (Sonenshine *et al.*, 2003; Grenacher *et al.*, 2001; Benoit *et al.*, 2008). Synthetic mixtures of these components elicit arrestment behavior in both adults and nymphs. Similar responses were observed when *I. uriae* was tested against varying concentrations of guanine and uric acid. For *I. scapularis*, a 25:1:1 mixture of guanine, xanthine and adenine caused 28-48% of adults and nymphs tested to engage in arrestment behavior (Sonenshine *et al.*, 2003).

#### *Host Kairomones*

The main determinant of tick host-seeking behavior can be attributed to the presence of host kairomones (Carroll, 2002). Ticks utilize host kairomones to detect the proximity of hosts and to identify host type. Behavioral assays have demonstrated the attractive quality of host breath, dermal and gland pelage and urine (Garcia, 1962; Carroll, 1999, 2001, 2002). Carbon dioxide (CO<sub>2</sub>), ammonia (NH<sub>3</sub>), and acetone are all attractive components of human and animal breath (McMahon & Guerin, 2002). CO<sub>2</sub> has long been recognized as an attractant for multiple species of ixodid ticks (Garcia, 1962). Ixodid ticks have both a long-range CO<sub>2</sub> sensillum that responds to small amounts of CO<sub>2</sub> and a short-range CO<sub>2</sub> sensillum that responds to large amounts of CO<sub>2</sub>. Sensilla cell responses to CO<sub>2</sub> have been recorded in *A. americanum*, *A. maculatum*, *A. variegatum*, and *D. variabilis* ticks (Holscher *et al.*, 1980; Steullet & Guerin, 1992). Sensory cell responses to NH<sub>3</sub>, and acetone have also been

recorded in *A. variegatum* ticks (Steullet & Guerin, 1992, 1994; McMahon & Guerin, 2002). 1-Octen-3-ol is an important attractive component specifically associated with bovine breath and bovine odors (Ostenkamp *et al.*, 1999). 1-Octen-3-ol is attractive to *A. hebraeum* and *R. microplus* ticks, which both exclusively parasitize bovine hosts (Norval, 1987; Norval *et al.*, 1988; Osterkamp *et al.*, 1999)

Nitrogenous wastes, ammonia and ammonium hydroxide are major components of dermal pelage and urine. *D. variabilis* exhibits arrestant behavior in response to canine urine (Smith *et al.*, 1946). *A. americanum* and *D. variabilis* both exhibit arrestant behavior in response to domestic canine ear pelage and deer tarsal gland extracts (Carroll, 1999, 2002). Deer tarsal pelage and its odor is created by bacterial fermentation of urine that is retained in the gland after urination. Lipids secreted by sebaceous glands provide a habitable environment for the microbial organisms (Osburn *et al.*, 2000; Alexy *et al.*, 2003). Canine ear pelage consists of dog earwax, or cerumen, lipids from sebaceous glands and a natural fauna of bacteria that live in the external ear canal (Harvey *et al.*, 2001). *D. variabilis* does not parasitize white-tailed deer, but this tick species has been reported to congregate around deer carcasses, increasing the likelihood of encountering canine scavengers, its primary host type. Its attraction to deer tarsal gland extracts reflects a means of feeding behavior with tick aggregation in high host traffic areas, identified by cervid odors (Carroll & Grasela, 1986). *D. variabilis*, *I. scapularis*, and *I. persulcatus* also exhibit arrestant behavior in response to canine fur odors (Smith *et al.*, 1946; Dobrotvorsky *et al.*, 2000). *A. variegatum* exhibits attractive behavior in response to rabbit and bovine dermal odors. Electrophysiological studies paired with GC-MS analysis identified the attractive components of the rabbit and

bovine odors to be aliphatic and aromatic aldehydes as well as lactones (Steullet & Guerin, 1993). Aliphatic aldehydes were detected by three different sensilla in the Haller's organ. Aliphatic aldehydes are produced in the anal and chin gland of the rabbit as well in dermal secretions of bovine (Steullet & Guerin, 1993; Burger *et al.*, 1981; Goodrich, 1983). Aromatic aldehydes are also produced in the chin glands of the rabbit (Goodrich, 1983). Sensory cell responses to lactones in human and bovine dermal pelage have also been recorded for *I. ricinus* ticks (Leonovich, 2004). Notably, statistically significant data identifying attractive chemical compounds associated with host kairomones are lacking. More research is needed to validate previous behavioral studies and to identify new attractive chemicals.

#### *Attractive Plant Extracts*

A few plant extracts have been found to be attractive to *Rhipicephalus* species. Leaf extracts from the *Acalypha fruticosa* plant were attractive to *R. appendiculatus* larvae (Hassan *et al.*, 1994). Dry leaf extracts of *Calpurnia aurea* were attractive to both *R. appendiculatus* and *R. pulchellus* ticks. A dose of 100 mg/ml of *C. aurea* extract attracted 52.2% of *R. pulchellus* and 44.4% of *R. appendiculatus* ticks from a distance of 1 m (Nana *et al.*, 2010). The active ingredients in plant extracts are currently unknown. It has been hypothesized that plant odors may mimic pheromones, or that plant odors represent safe habitat selection and attract ticks searching for protected arrestment locations. Further research is required to understand this phenomenon and to determine if plant extracts would be useful chemicals to evaluate for tick surveillance or lure and kill strategies.

## USE OF ATTRACTANTS IN TICK CONTROL STRATEGIES

Current tick control methods are heavily dependent on the use of acaricides and repellents to protect human and domestic animal hosts. Chemically based management strategies can effectively prevent tick attachment and control populations when used in area-wide applications. However, repetitive use of chemical acaricides and repellents often has the potential to cause development of resistant tick populations (George, 2000). These issues have driven a search for new tick control methods. Long lasting protective measures are being evaluated involving chemically controlled, slow release lure and kill strategies. Lure and kill strategies combine attractive chemicals and toxicants together in slow-release devices. The attractive chemicals lure the tick towards the impregnated object. The ticks are then killed when coming in contact with the toxins impregnated on the object (Allan *et al.*, 1998). Because the attractants and toxins are incorporated into a slow release delivery device, the attractive and fatal effects of the chemicals remain active for a period of up to 14 weeks (Norval *et al.*, 1994). The containment of the chemicals into a singular object allows for easier application with decreased environmental chemical exposure. Arrestment pheromone, 2,6-DCP attractant sex pheromone, and AAAP have all been determined to be effective chemical attractants. Allan *et al.* (2002) patented a device that incorporated *I. scapularis* arrestment pheromone and permethrin acaricide into an oil formulation (Last Call™, IPM technologies, Portland OR) that was applied to ground cover and vegetation with a pump sprayer. The pheromone-acaricide mixture resulted in 95% *I. scapularis* tick mortality in treated areas (Allan *et al.*, 2002). 2,6-DCP acts as a chemical lure and a chemical confusant that disrupts mating behavior. Two delivery devices have been evaluated

incorporating 2,6-DCP and acaricides. The first involves a water emulsion of gelatin-microencapsulated 2,6-DCP combined with propoxur. The emulsion was applied to dogs infested with *D. variabilis* ticks where the slow-release microcapsules continuously released the pheromone-acaricide mixture for multiple days. Tick mortality was observed, with a higher percentage of male ticks killed by the treatment than female. Mating disruption was also observed, resulting in a 90% decrease in oviposition (Sonenshine *et al.*, 1985). The second delivery device involved impregnating tick decoys with the pheromone-acaricide mixture. Tick decoys were plastic spherules made from polyvinyl chloride plastic. 2,6-DCP, cholesteryl oleate, and propoxur were impregnated into tick decoys and applied to rabbits infested with *D. variabilis* ticks. 100% mortality of male ticks was observed and there was no oviposition (Hamilton & Sonenshine, 1989). Similar results were observed when the impregnated tick decoys were placed onto tick-infested cattle (Sonenshine *et al.*, 1992) and camels (Adbel-Rahman *et al.*, 1998). AAAP is also effective when incorporated into a chemical lure. Plastic tags were impregnated with AAAP and pyrethroids and placed on the tails of cattle infested with *A. variegatum* and *A. hebraeum* ticks. Levels of tick control for a 3-month trial averaged 94.9% (Norval *et al.*, 1992, 1994). Because of the successful tick control demonstrated utilizing pheromone-acaricide mixtures, new research should focus on searching for alternative chemical lures associated with host kairomones and attractive plant extracts.

## TICK SURVEILLANCE METHODS

There are two main types of tick surveillance methods, passive surveillance and active surveillance. Passive surveillance involves collecting ticks submitted by the public that are typically found attached to a person or animal (Johnson *et al.*, 2004). Passive surveillance requires minimal labor though it can provide biased or incomplete data or misestimate the size tick populations and their geographic distribution. Ticks collected from people are representative only of the areas where the persons submitting ticks have travelled. Also, since this surveillance method is dependent on submissions made by the public, it can be affected by the ethnic composition of the population, education level, socio-economic status, and local attitudes regarding the importance of the surveillance program (Stone *et al.*, 2005). Active tick surveillance includes techniques that actively search for and collect ticks in the environment and have the potential to be extremely laborious and expensive. There are three main techniques utilized (Gray, 1985). The first is the use of sentinel animals such as canines, cervids, and other small mammals. These animals are more likely to become exposed to ticks in heavily infested area and represent indicators of tick population numbers (Lindenmayer *et al.*, 1991). Wild animal hosts, such as small mammals and cervids, are trapped or sedated and the ticks collected, counted and identified. Canines are released into tick-infested areas, re-captured and the attached ticks collected, identified and counted (Lindenmayer *et al.*, 1991; Johnson *et al.*, 2004). The second method involves simulating a host by physically flagging or dragging a piece of cloth over vegetation to collect ticks in the environment (Johnson *et al.*, 2004; Yu *et al.*, 2011). The third method uses carbon dioxide released from dry ice or a compressed gas cylinder to attract ticks to the area surrounding the

release point of the gas where they are collected (Garcia, 1962; Oliveira *et al.*, 2000; Guedes *et al.*, 2005). All of these active surveillance techniques have been reported to be effective in collecting ticks. Although flagging methods are labor intensive and may provide biased data because ticks are only collected from areas that are flagged, flagging does not require any specialized equipment. Use of sentinel animals requires costly trapping equipment and chemical sedatives to catch and restrain wildlife so that ticks can be collected off the host animals. Animal trapping can also be a laborious process. There is also the potential for collection of biased data because ticks are not collected from a proper representation of the wild mammal population or if trapping is not randomly conducted throughout the testing area. Dry ice is difficult to handle and is not always readily available in some communities, plus it can be expensive and problematic to ship in because of its sublimation properties (Cançado *et al.*, 2008). In areas where dry ice is unavailable, carbon dioxide chemical reaction kits have been utilized, though these kits also can be expensive and require proper training to be used effectively and safely (Norval *et al.*, 1987, 1988).

The type of surveillance strategy utilized depends on the life stage and species of the tick being surveyed. Surveillance strategies also reflect the goals of the survey. For example a survey searching for tick species typically found on white tail deer would utilize white tail deer trapping to collect ticks. Or a survey screening for ticks in acaricide treated areas might utilize flagging or carbon dioxide bait to survey for ticks. Specific surveillance methods are also more efficient for some ticks species and life stages than others. Carbon dioxide is very effective in attracting *A. Amblyomma* ticks though is not as effective in attracting *I. scapularis* ticks. Furthermore, Petry *et al.* (2010) determined that significantly more *A.*

*Amblyomma* nymphs were collected with carbon dioxide baited traps and significantly more *A. Amblyomma* larvae were collected with flagging. Additionally, because tick species vary by habitat type it is important to utilize surveillance techniques that incorporate environmental settings. Because of the demonstrated success with hematophagous insects using chemical baits, more research is needed testing host kairomones for tick bioactivity across species and life stages.

### HEMATOPHAGOUS INSECT SURVEILLANCE METHODS

Hematophagous insect surveillance only utilizes active collecting techniques. The majority of these techniques involve baiting traps with host-associated chemicals, light systems, and netting to attract and trap target insects. There are several baited trap systems used to collect and survey hematophagous insects and each contain different chemical or chemical mixtures.

Artificial baits are used to survey mosquitoes to identify local species, estimate populations, and locate possible disease vectors. Jawara *et al.* (2011) demonstrated that an artificial bait containing L-lactic acid, ammonia, tetradecanoic acid and carbon dioxide in a Mosquito Magnet X trap was significantly more attractive to mosquitoes than traditional CDC light traps. They also state that this simple, artificial combination attracted almost as many mosquitoes as human odors contained in worn socks. 1-octen-3-ol can also be used to lure mosquitoes, though its activity is increased when used in combination with carbon dioxide (Laporta & Sallum, 2011).

Artificial baits are also used to survey for biting flies such as tabanids, tsetse and ceratopogonid midges. Tsetse flies and tabanids can be collected using visual traps, though adding acetone as a chemical lure increased the catch of visual traps by 1.6-2.4 times (Mihok *et al.* 2007; Vale *et al.*, 1985). 1-Octen-3-ol is used to bait Nzi traps and increased attraction and collection rates of *Tabanus quinquevittatus* and *Stomoxys calcitrans* by 3.5-3.6 times compared to unbaited traps (Mihok *et al.*, 2007). In Canada, 1-octen-3-ol also increased the attraction and collection rates of *T. similis*, *T. quinquevittatus*, *Hybomitra lasiophthalma*, *Chrysops univittatus*, *C. aberrans* and *S. calcitrans* by 1.2-2.1 times compared to unbaited traps (Mihok *et al.*, 2007). Krcmar (2005) also determined that malaise traps baited with 1-octen-3-ol collected 21 times as many tabanids as unbaited traps, and malaise traps baited with acetone and ammonium hydroxide collected 8-9 times as many tabanids as unbaited traps. Carbon dioxide and 1-octen-3-ol has also been used to lure and trap ceratopogonid midges (Mands *et al.*, 2004). 4-methylphenol (*p*-Cresol) is an additional secondary attractant that can be combined with 1-octen-3-ol to increase trapping efficiency of tsetse flies and ceratopogonid midges (Phelps & Holloway, 1992; Vale *et al.*, 1988; Cilek *et al.*, 2003).

Chemical lures are also used to survey for bedbugs to determine if eradication or control protocols are needed. Wang *et al.* (2009) reported that pitfall traps baited with dry ice and a chemical lure containing L-lactic acid and 1-octen-3-ol captured 86.7% of bed bugs over 6hrs in laboratory settings. The same chemical lure with dry ice was tested with heat in four bed bug infested apartments to monitor for bed bugs and captured 12.0% of bed bugs over 12-14hrs. The significant decrease in bed bug responses between laboratory and field

settings may be due to the significant increase in test period, and subsequent depletion of dry ice or chemical lure sources decreasing the efficacy of the baited trap.

Preliminary lab studies have demonstrated the attraction of triatome bugs to chemical lures containing L-lactic acid, isobutyric acid, and 1-octen-3-ol in combination with dry ice and heat though the baited system has not been evaluated in field settings (Milne *et al.*, 2009).

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## **2. SCREENING OF CHEMICALS USED IN HEMATOPHAGOUS INSECT SURVEILLANCE TRAPS AGAINST *AMBLIOMMA AMERICANUM* AND *DERMACENTOR VARIABILIS* (ACARI: IXODIDAE) IN LABORATORY AND FIELD ASSAYS**

### INTRODUCTION

Ixodid ticks transmit bacteria, viruses and protozoa that cause severely debilitating diseases in humans and animals (Mullen & Durden, 2009). Tick surveillance provides information that locates tick populations and maps out their distribution in the environment. This information allows us to assess the risk of tick-borne diseases in local communities, provide educational resources and initiate control programs if deemed necessary. Similar surveillance programs are used in management programs for hematophagous insects. A review of the literature reveals that the majority of these surveillance investigations have involved blood-feeding diptera and some common attractive host chemicals: carbon dioxide, L-lactic acid, acetone, carboxylic acids, nitrogenous wastes, sulfides and 1-octen-3-ol. Future examination of host-associated chemicals is necessary to identify potential chemical lures for tick collecting and surveillance

Carbon dioxide is a universal attractant for blood feeding arthropods and is the main semiochemical used in the form of dry ice for tick surveillance (Garcia, 1962; Oliveira *et al.*, 2000; Guedes *et al.*, 2005). Carbon dioxide is also an attractant for a variety of hematophagous insects including mosquitoes, tabanid flies, tsetse flies, culicoides midges, bed bugs and triatome bugs (Jawara *et al.*, 2011; Krcmar, 2005; Torr *et al.*, 2011; Milne *et al.*, 2009; Wang *et al.*, 2009; Mands *et al.*, 2004). Short-chain carboxylic acids, the end products of microbial digestion of plant carbohydrates and aliphatic amino acids, are found in

host pelage, axillary secretions and ruminant rumen fluid (Fenner *et al.*, 1966). *Culex* mosquitoes, sandflies, ticks and triatome bugs all exhibit attractive behavior to carboxylic acids (Puri *et al.*, 2006; Dougherty *et al.*, 1999; Donzé *et al.*, 2004; Guerenstein & Guerin, 2001). In particular isobutyric acid was determined to be attractive to triatomine bugs (Guerenstein & Guerin, 2001). Additionally isobutyric acid is a known tick attractant (Yoder *et al.*, 1999). Lactic acid and acetone are components of human skin emanations (Bernier *et al.*, 2000). L-lactic acid is a well-established attractant for mosquitoes in the genus *Aedes* and *Anopheles* (Acree *et al.*, 1968; Dekker *et al.*, 2002; Bernier *et al.*, 2003). The “Kissing Bugs” *Triatoma dimidiata*, *T. infestans* and *Rhodnius prolixus* can also detect and are stimulated to host-seek by L-lactic acid (Guerenstein & Guerin, 2001; Barrozo and Lazzari, 2004). However, *T. dimidiata*, *T. infestans* and *R. prolixus* remain behaviorally unresponsive to L-lactic acid unless other host cues are present, such as heat or CO<sub>2</sub> (Milne *et al.*, 2009). Wang *et al.* (2009) also determined that traps baited with L-lactic acid, heat and CO<sub>2</sub> were effective in attracting and collecting bed bugs, *Cimex lectularius*, over short distances.

Acetone is attractive to tabanid flies and mosquitoes when combined with a secondary attractive compound, such as L-lactic acid and ammonium hydroxide, and exhibits the same excitation effect as CO<sub>2</sub> (Bernier *et al.*, 2001, 2003; Krcmar, 2005). Also, addition of acetone to tsetse fly visual traps greatly increased trap catch by acting as an effective olfactory lure (Vale *et al.*, 1985). Nitrogenous wastes such as ammonia and ammonium are components of human sweat and vertebrate urine. Ammonia has been shown to be attractive to *Aedes* and *Anopheles* mosquitoes, tabanids and triatomine bugs (Geier *et al.*, 1999; Smallegange *et al.*, 2005; Krcmar 2005; Taneja & Guerin, 1997). Sulfides are derived from

the metabolism of the amino acids cysteine and methionine and are found in human emanations and livestock waste (Mackie *et al.*, 1998; Bernier *et al.*, 2000). Tabanids, *Musca domestica* and *Amblyomma variegatum* are all attracted to sulfides (Steullet & Guerin, 1992; Cossé & Baker, 1996; Jeanbourquin & Guerin, 2007). Baiting traps with dimethyl trisulfide increased the catch of *Culex* mosquitoes and calliphorid flies (Allan *et al.*, 2006; Nilssen *et al.*, 1996). 1-Octen-3-ol is a product of bacterial fermentation and is a component of bovine emanations. The chemical is attractive to a variety of Dipterans including tsetse flies (Vale & Hall, 1985; Steullet & Guerin, 1994; Bursell *et al.*, 1988), *Aedes* and *Anopheles* mosquitoes (Takken & Kline, 1989; Kline *et al.*, 1991), tabanids (French & Kline, 1989; Nilssen, 1998) bot flies (Anderson, 1989), and ceratopogonid midges (Bhasin *et al.*, 2001). 1-Octen-3-ol has been used to increase the trap catch of triatomines (Barrozo & Guerin, 2001). It was also determined that the cattle tick *Boophilus microplus* exhibited an increased preference for bovine odors when presented with a variety of host odorants containing 1-octen-3-ol.

This study evaluated the attractiveness to ticks of semiochemicals determined to be attractive to other blood-feeding arthropod species. These chemicals were tested as attractants of two species of ixodid ticks, *Amblyomma americanum* and *Dermacentor variabilis*, to identify specific chemicals and/or chemical combinations for potential use as attractants for tick surveillance.

## MATERIALS AND METHODS

*Tick species Adult Amblyomma americanum* ticks were purchased from the Oklahoma State University Tick Rearing Facility, Stillwater, Oklahoma. Adult *Dermacentor variabilis* ticks were obtained from laboratory colonies of Dr. D. E. Sonenshine at Old Dominion University (Norfolk, VA). Ticks were housed in an insectary that is maintained at approximately 28°C, and 75% humidity with a photoperiod of 14-hour light: 10-hour dark, with dusk and dawn periods of one hour each at the beginning and ending of the scotophase. Ticks arrived at the laboratory facilities and were housed for 72 h prior to use in laboratory bioassays.

*Response to CO<sub>2</sub> in Olfactometer Assays* Preliminary Behavioral assays were conducted with carbon dioxide (CO<sub>2</sub>) using a glass Y-tube olfactometer (Fig. 1) to establish that this system could be used to measure tick response to an established tick attractant. Tests were conducted during the daytime between the hours of 1100-1500 (L-on) at 23 ± 1°C with relative humidity of 40% under ambient (fluorescent) lighting. CO<sub>2</sub> (3% in breathing quality air) was administered into one port of the Y-tube from a compressed gas tank (AirGas, NC, USA). The medical grade air (AirGas, NC, USA) was introduced into the second port of the Y-tube from a compressed gas tank. Gas flow rates were regulated with a #11 Compact Shielded flow meter (Gilmont Instruments, Barrington, IL, U.S.A). The response of *A. americanum* and *D. variabilis* adults were separately evaluated to the following CO<sub>2</sub> flow rates: 25, 50, 75, 100, 125 and 150 ml/min. Flow rates of breathing quality air released into the second port were symmetrically adjusted. A vacuum pump was used to remove gases from the downwind end of the Y-tube at a rate equal to the total flow through the

olfactometer. Gases removed from the olfactometer were exhausted out of the test area. Test odorants were rotated between the two ports to prevent positional bias. All equipment was washed with hot water and 95% ethanol between trials to prevent contamination. All ticks were acclimated to the experimental setting 30 min prior to being transferred into the olfactometer. Six unfed adult male and female ticks of the specified species were placed into the Y-Tube exhaust outlet, beyond a 2.5cm starting marker. Each trial was conducted for 5 min and replicated six times on separate dates with ticks not previously tested. Ticks that were recorded as positive responders were ticks that made a choice in the Y-tube bioassays, moving 1cm past the choice point.

Because the concentration of CO<sub>2</sub> in the tank was fixed, dose-response studies required that the flow rates be adjusted. To eliminate the possibility that the response of ticks was stimulated by increased air flow rate and not by the increased amount of CO<sub>2</sub>, ticks were tested for response to flow rates of 25, 50, 75, 100, 125 and 150 ml/min of only breathing quality air from both arms of the olfactometer.

*Putative Attractants* The following reagent grade chemicals (Sigma Aldrich, U.S.A) were screened: 1-octen-3-ol (>98%), acetone (>99.9%, HPLC), L-lactic-acid (>98%), isobutyric acid (99%), ammonium hydroxide (28-30% NH<sub>3</sub> in water, ACS) and dimethyl trisulfide (>98%, FG). Chemicals were diluted with distilled water. 1mL dilutions were made based on density 1h prior to bioassay. All chemicals used were purchased 2 weeks prior to laboratory bioassays and bottles stored accordingly in acid or flammable materials cabinets.

*Y-tube Assay of Putative Attractants* Putative attractants were tested for bioactivity in the glass Y-tube olfactometer as described above for CO<sub>2</sub>. All semiochemicals were first evaluated against *A. americanum* ticks. Only chemicals eliciting significant responses were tested against *D. variabilis*. Airflow, based on results of CO<sub>2</sub> assays, was set to 100 ml/min/port for *A. americanum* trials and to 75 ml/min/port for *D. variabilis* trials. Each chemical dilution (22.5 µl) was applied to cellulose filter paper (1.5 cm<sup>2</sup> square), which was immediately placed into one of the Y-tube ports with forceps. Distilled water was used as a control and was applied to a second piece of filter paper (1.5cm<sup>2</sup>), which was immediately placed into the second Y-tube port. Control filter paper was always placed into Y-tube ports first to prevent cross contamination of chemicals during handling and assay set-up.

Treatments and controls were rotated between the two ports to prevent positional bias. All equipment was washed between trials with hot water and 95% ethanol to eliminate contamination. All ticks that made a choice in the Y-tube bioassays were recorded as positive responders. Chemicals eliciting significant attraction by *A. americanum* were tested in combination and with carbon dioxide for synergism in the Y-tube behavioral assays.

*Field trials* Semiochemicals exhibiting statistically significant attraction in laboratory assays were tested at a field site. Acetone, ammonium hydroxide and 1-octen-3-ol were compared with CO<sub>2</sub> (from dry ice) and water controls. Field trials were conducted in Harris Lake County Park in Newhill NC, USA (35°37'30.92" N, -78°55'35.24" W) during June 2011. Field trials were set-up during 11:00-12:00 h and conducted from 12:00-13:00 h. Air temperatures ranged between 32.2-35° C with relative humidity ranging from 40-70%. The

ground cover at test site consisted of oak leaf and pine needles litter. Trials were never conducted on days with wet or damp ground cover. The three semiochemicals and water were dispersed by placing 2 ml of undiluted chemical into open-mouth vials (1.4 cm i.d., 5.5cm h.). Amounts of each chemical released were increased by increasing the number of vials. Carbon dioxide was dispersed using 0.68 kg of pelleted dry ice. Vials and dry ice pellets were placed onto individual 60 x 60 cm, white cloth sheets (60% cotton, 40% polyester) that were placed on the ground at the field site. Prior to use, all cloth sheets were cleaned in a washing machine without detergent, in hot water on a regular, cotton cycle and dried on high heat in a dryer. After washing, sheets were stored in plastic bags until used. Nitrile gloves were worn when handling cloths.

At the field site, wire surveyor's flags were used to mark out 5 x 5 grid of sampling cells; each cell within a column measured 7.6 x 7.6 m. For each semiochemical, one trial was conducted per day with test and control compounds in subsequent trials rotated through all 5 cells in each row of cells (Table 2). Five trials were completed for each semiochemical. When a trial was conducted, one sheet was placed on the ground in the geometric center of each cell and vials containing Semiochemicals, water or dry ice were placed onto the centers of the sheets. Gloves were worn during trial set-up to prevent contamination of vials and sheets with skin odorants. One hour after placement of vials and dry ice on each cloth, ticks were collected and stored in labeled 50 ml conical tubes (BD Falcon, NJ, U.S.A.). Subsequently in the laboratory, ticks were identified to species and life stage and counted. Release rates of test chemicals, water and carbon dioxide were determined by weighing the vials or dry ice immediately before and after field trials.

*Data Analysis* Olfactometer assays in <2 of the 6 ticks responded and made a choice were excluded from analysis. Response data for each semiochemical were converted to percentages of the total number of tick making a choice of test and control substances. Percentages were compared in a chi-square analysis using the PROC FREQ procedure of SAS software (SAS for Windows, ver. 9.2, SAS Institute, Cary, NC) with an expected probability for Y-tube choice of 0.5 for both test and control materials.

Field trial results for each semiochemical were analyzed separately by analysis of variance with the Glimmix procedure in SAS. Response was modeled through a Poisson regression with over-dispersion parameter to account for larger variation than expected under the assumption that tick counts followed a Poisson distribution. It was expected that the mean of each tick count was a function of *Treatment*, *Day* and *Position*, with *Day* and *Position* accounting for the Latin Square experimental design used to study *Treatment* effect on the number of ticks attracted to each semiochemical. The model in Poisson regression assumes that the expected mean value for the number of ticks observed in a plot (experimental unit) at a particular position and at a given day receiving a given treatment is represented as a linear model for the natural log of the expected mean,  $\log(\mu) = \text{Overall constant} + \text{Day\_effect} + \text{Position\_effect} + \text{Treatment\_effect} + \text{uncontrolled\_random\_effect}$ . Treatment effect differences were analyzed with Tukey's multiple range test for pair wise least squares means at a 0.05 significance level, and Dunnett-Hsu test to compare treatment least squares means against water (negative control) least squares means and also to compare treatment least squares means against CO<sub>2</sub> (positive control) at a 0.05 significance level.

## RESULTS

*Response to CO<sub>2</sub> in olfactometer Assays* Air flow rate had no impact on tick response showing that all observed movement up the Y-tube olfactometer was in response to the CO<sub>2</sub> (Fig. 2). *A. americanum* and *D. variabilis* adults were stimulated by CO<sub>2</sub> to move up the Y-tube olfactometer. Choice responses for all CO<sub>2</sub> flow rates are shown in Figs. 3 and 4. *A. americanum* exhibited significant attraction to all flow rates except 25 ml/min, with the highest response to CO<sub>2</sub> flow rate of 100 ml/min ( $\chi^2 = 21.16$ ; df = 1; P < 0.0001) (Figure 4). In contrast, *D. variabilis* was significantly attracted to CO<sub>2</sub> flow rates of 50 ml/min ( $\chi^2 = 13.7619$ ; df = 1; P = 0.0002) and 75 ml/min ( $\chi^2 = 17.1905$ ; df = 1; P < 0.0001).

*Response to semiochemicals in olfactometer assays* Of the 6 semiochemicals tested, only 1-octen-3-ol, acetone and ammonium hydroxide elicited movement of *A. americanum* towards the odorants. Ticks showed a preference for distilled water at three higher concentrations of isobutyric acid (0.05, 1.00 and 10.00%) (Fig. 4c). The choice of distilled water was significant at 0.05% ( $\chi^2 = 10.89$ , df = 1, P = 0.0010) and 10.00% isobutyric acid ( $\chi^2 = 22.53$ , df = 1, P < 0.0001), but not at 1.00% isobutyric acid ( $\chi^2 = 2.58$ , df = 1, P = 0.11). Lower concentrations isobutyric acid were less repellent to ticks, though no preference was observed for either distilled water or isobutyric acid (0.10%  $\chi^2 = 0.11$ , df = 1, P = 0.74; 0.01%  $\chi^2 = 0.82$ , df = 1, P = 0.37). Responses to dimethyl trisulfide were similar to those seen with isobutyric acid (Fig. 5d and Fig. 5e). Ticks showed a significant preference for distilled water when tested against 10.0% dimethyl trisulfide ( $\chi^2 = 9.94$ , df = 1, P = 0.0016). Lower concentrations were less repellent, but differences in the responses to distilled water

and dimethyl trisulfide were not statistically significant (1.0%:  $\chi^2 = 0.20$ , df = 1, P = 0.65; 0.10%:  $\chi^2 = 0.11$ , df = 1, P = 0.74; 0.01%:  $\chi^2 = 0.11$ , df = 1, P = 0.74). When testing L-lactic acid against distilled water in the Y-tube, ticks did not exhibit a preference for any of the lactic acid concentrations tested (Fig. 5f). Significant repellency was observed at 1.0% L-lactic acid ( $\chi^2 = 11.27$ , df = 1, P = 0.0008). At lower concentrations of lactic acid more ticks chose distilled water, but none of the responses were statistically significant (10.0%:  $\chi^2 = 0.14$ , df = 1, P = 0.71; 5.0%:  $\chi^2 = 2.00$ , df = 1, P = 0.16; 0.05%:  $\chi^2 = 1.14$ , df = 1, P = 0.29; 0.01%:  $\chi^2 = 1.60$ , df = 1, P = 0.21). When testing 1-octen-3-ol against distilled water, higher numbers of ticks chose all 1-octen-3-ol concentrations tested except 10.0% (Fig. 5a). However, preference for 1-octen-3-ol was only statistically significant for 2.5% ( $\chi^2 = 9.00$ , df = 1, P = 0.0027). The 10.0% concentration was significantly repellent ( $\chi^2 = 10.29$ , df = 1, P = 0.0013). Ticks showed significant preference for acetone at concentrations of 5.0% ( $\chi^2 = 5.44$ , df = 1, P = 0.02), 1.00% ( $\chi^2 = 7.14$ , df = 1, P = 0.0075) and 0.5% ( $\chi^2 = 3.76$ , df = 1, P = 0.05) (Fig. 5b). No preference for either distilled water or acetone was evident at concentrations of 10.0% ( $\chi^2 = 0.33$ , df = 1, P = 0.56) or 0.1% ( $\chi^2 = 0.28$ , df = 1, P = 0.59). Ticks exhibited significant preference for ammonium hydroxide at concentrations of 12.5% ( $\chi^2 = 4.57$ , df = 1, P = 0.032), 10.00% ( $\chi^2 = 6.25$ , df = 1, P = 0.012), 5.0% ( $\chi^2 = 7.14$ , df = 1, P = 0.0075) and 1.0% ( $\chi^2 = 4.45$ , df = 1, P = 0.035) but not at concentrations of 25.0% ( $\chi^2 = 2.00$ , df = 1, P = 0.15), 7.5% ( $\chi^2 = 3.00$ , df = 1, P = 0.083) and 0.1% ( $\chi^2 = 0.67$ , df = 1, P = 0.41) (Fig. 5c).

*D. variabilis* were not attracted to any of the semiochemicals (Fig. 6). Ticks showed a significant repellency to 1-octen-3-ol at concentrations of 10.0% ( $\chi^2 = 9.80$ ,  $df = 1$ ,  $P = 0.0017$ ) and 1.0% (1.00%  $\chi^2 = 9.53$ ,  $df = 1$ ,  $P = 0.002$ ). No preference for 1-octen-3-ol or water was observed at concentration of 0.5% ( $\chi^2 = 0.69$ ,  $df = 1$ ,  $P = 0.41$ ) or 0.10% ( $\chi^2 = 0$ ,  $df = 1$ ,  $P = 1.00$ ). A higher percentage of ticks showed preference for distilled water compared to acetone for both concentrations of acetone tested. However, choice differences at 10.0% ( $\chi^2 = 3.60$ ,  $df = 1$ ,  $P = 0.0578$ ) and 1.00% acetone ( $\chi^2 = 0.50$ ,  $df = 1$ ,  $P = 0.48$ ) were not statistically significant. A higher percentage of ticks preferred ammonium hydroxide compared to distilled water though none of the ammonium hydroxide concentrations elicited statistically significant responses (10.0%:  $\chi^2 = 1.33$ ,  $df = 1$ ,  $P = 0.25$ ; 1.0%:  $\chi^2 = 2.91$ ,  $df = 1$ ,  $P = 0.088$ ; 0.5%:  $\chi^2 = 1.14$ ,  $df = 1$ ,  $P = 0.29$ ; 0.1%:  $\chi^2 = 2.78$ ,  $df = 1$ ,  $P = 0.96$ ).

Additional bioassays were carried out with *A. americanum*. 1-Octen-3-ol, acetone and ammonium hydroxide were tested individually in combination with 3% carbon dioxide at a flow rate of 100 ml/min carbon dioxide. These three chemicals were also combined and tested without and with carbon dioxide (100 ml/min). There was no significant increase in responses ( $P > 0.05$ ) to the semiochemicals when added separately to carbon dioxide or in combination in ratios of 2:1, 1:1 or 1:2 with and without carbon dioxide.

*Field Trials of Selected Semiochemicals* Based on results of laboratory olfactometer experiments for *A. americanum*, three semiochemicals (1-octen-3-ol, ammonium hydroxide and acetone) were chosen for evaluation against field populations of ticks. In these field trials, only *A. americanum* was collected with nymphs and adults comprising 90% and 10%,

respectively, of the collections. An analysis of variance was carried out for each putative attractant using the combined data for nymphs and adults. *Treatment* was the only significant main effect variable for all three semiochemicals. There were no significant differences in the numbers of ticks collected between days during the field trials of Semiochemicals (Table 2).

1-Octen-3-ol was tested in field assays at release rates of  $10 \pm 0.78$  (mean  $\pm$  SE) (1 release vial), 20 (2 release vials) and 30 mg/hr (3 release vials) separately against distilled water ( $10.1 \pm 7.0$  mg/h) and CO<sub>2</sub> ( $547 \pm 23$  g/h) controls. 1-Octen-3-ol was not attractive under field conditions (Fig. 7a). There was no significant difference between the number of ticks attracted to distilled water and 1-octen-3-ol at the 10 ( $t = 1.44$ ,  $df = 12$ ,  $P = 0.18$ ), 20 ( $t = 1.03$ ,  $df = 12$ ,  $P = 0.32$ ) and 30 mg/h ( $t = 2.00$ ,  $df = 12$ ,  $P = 0.07$ ) release rates. The number of ticks attracted to CO<sub>2</sub> was significantly different from distilled water ( $t = 2.73$ ,  $df = 12$ ,  $P = 0.018$ ), 10 mg/h 1-octen-3-ol ( $t = -2.81$ ,  $df = 12$ ,  $P = 0.015$ ), and 20 mg/h release rate of 1-octen-3-ol ( $t = -2.94$ ,  $df = 12$ ,  $P = 0.012$ ). Also, the numbers of ticks attracted to the three release rates of 1-octen-3-ol were not significantly different ( $P > 0.05$ ).

Acetone was also tested in field assays at three release rates (300, 600 and  $900 \pm 10.8$  mg/h). Acetone was considerably more attractive than that 1-octen-3-ol (Fig. 7b). Significant differences were observed for the number of ticks responding to 600 mg/h acetone ( $t = 3.11$ ,  $df = 12$ ,  $P = 0.0089$ ), 900 mg/h acetone ( $t = 2.71$ ,  $df = 12$ ,  $P = 0.019$ ), and CO<sub>2</sub> ( $t = 3.65$ ,  $df = 12$ ,  $P = 0.0034$ ) compared to distilled water. No differences were observed between the response of 300 mg/h acetone and distilled water ( $t = 1.77$ ,  $df = 12$ ,  $P = 0.10$ ). Significantly more ticks responded to CO<sub>2</sub> than 300 mg/h acetone ( $t = -3.83$ ,  $df = 12$ ,

$P = 0.0024$ ), 900 mg/h acetone ( $t = -2.64$ ,  $df = 12$ ,  $P = 0.024$ ) or distilled water ( $t = 3.65$ ,  $df = 12$ ,  $P = 0.0034$ ). No differences were found between the numbers of ticks responding to  $\text{CO}_2$  and 600 mg/h acetone ( $t = -1.57$ ,  $df = 12$ ,  $P = 0.14$ ). There were no significant differences between the numbers of ticks responding to any of the three acetone treatments ( $P > 0.05$ ).

Ammonium hydroxide was tested in field trials at three release rates (0.6, 1.2 1.8  $\pm$  0.013 g/h). Similar to acetone, ammonium hydroxide attracted field *A. americanum* in the field (Fig. 7c). The numbers of ticks attracted to 1.8 g/h ammonium hydroxide ( $t = 4.60$ ,  $df = 12$ ,  $P = 0.0006$ ) and  $\text{CO}_2$  ( $t = 5.84$ ,  $df = 12$ ,  $P < 0.0001$ ) were significantly greater than the numbers attracted to distilled water. No difference was observed for the number of ticks attracted to distilled water and 0.6 g/h ammonium hydroxide ( $t = -0.97$ ,  $df = 12$ ,  $P = 0.35$ ) or distilled water and 1.2 g/h ammonium hydroxide ( $t = 0.42$ ,  $df = 12$ ,  $P = 0.68$ ). Significantly more ticks were attracted to  $\text{CO}_2$  than to all ammonium hydroxide release rates (0.6 g/h:  $t = -4.91$ ,  $df = 12$ ,  $P = 0.0004$ ; 1.2 g/h:  $t = -5.32$ ,  $df = 12$ ,  $P = 0.0002$ ; 1.8 g/h  $t = -2.90$ ,  $df = 12$ ,  $P = 0.01$ ). When comparing release rates of ammonium hydroxide, only the highest release rate of 1.8 g/h attracted significantly more ticks than the 0.6 g/h ( $t = -4.06$ ,  $df = 12$ ,  $P = 0.0016$ ) and 1.2 g/h ( $t = -4.22$ ,  $df = 12$ ,  $P = 0.0012$ ) release rates.

## DISCUSSION

Laboratory bioassay results showed for the first time that several host-associated kairomones were attractive to *A. americanum* but not to *D. variabilis* adults. *A. americanum* was attracted to various concentrations of acetone and ammonium hydroxide. Acetone and ammonium hydroxide are waste products of microbial metabolism from lipid metabolism

and nitrogen removal and are emitted in host breath, dermal secretions and waste products (Barreto *et al.*, 2011). Similar attractive behavior was also exhibited by *Amblyomma variegatum* and *Rhipicephalus sanguineusticks* (Haggart & Davis, 1981; McMahon & Guerin, 2002). *A. americanum* exhibited a strong attraction to 1-octen-3-ol whereas *D. variabilis* showed no such behavior. 1-Octen-3-ol is a waste product of microbial metabolism of linoleic acid specifically associated with bovine odors that is emitted through breath and waste products (Ostenkamp *et al.*, 1999; Barreto *et al.*, 2011). The attractive response of *A. americanum* to a low range of concentrations of 1-octen-3-ol is similar to findings by McMahon & Guerin (2002) with *A. variegatum* ticks. 1-Octen-3-ol has also been determined to be attractive to *A. hebraeum* and *R. microplus* ticks (Norval, 1987; Norval *et al.*, 1988; Osterkamp *et al.*, 1999). The responses of the *A. americanum* and *D. variabilis* ticks suggest that differences in their host preference can be partially explained by the differential bioactivity of host-associated semiochemicals. Naturally preferred hosts of *Amblyomma* species include large ungulate species that are known to emit 1-octen-3-ol in breath. *D. variabilis* preferred host that are medium-sized mammals such as canines. *D. variabilis* ticks do not feed on large ungulate hosts.

The bioactivity of 1-octen-3-ol, acetone and ammonium hydroxide in attracting *A. americanum* ticks was tested for the first time under field conditions. Only acetone and ammonium hydroxide attracted ticks in the field trials. The intermediate release rate of acetone (600 mg/h) attracted a mean of approximately 22.8 ticks/h, which was not significantly different ( $P > 0.05$ ) from the mean of 26 ticks/h attracted by carbon dioxide. The highest release rate of ammonium hydroxide (1.8 g/h) attracted on average about 21

ticks/h, which was significantly ( $P < 0.05$ ) lower than the mean of 29 ticks/h attracted by carbon dioxide. Despite positive laboratory bioassays, 1-octen-3-ol attracted on average 10 ticks/h at the high release rate (30 mg/h), which was not significantly different from carbon dioxide or from water. However, it should be noted that the release rate of carbon dioxide from dry ice (547 g/h) was 900X, 300X, and 18,000X greater than the release of acetone, ammonium hydroxide, and 1-octen-3-ol, respectively, based on the loss of weight of these chemicals during the field trials. On the basis of chemical molarity, the three semiochemicals were more potent tick attractants than carbon dioxide. Carbon dioxide captured an average of 2.3 ticks collected per mole of gas released per hour compared to 2,800, 736, and 42,735 ticks collected per mole of semiochemical released per hour for acetone, ammonium hydroxide and 1-octen-3-ol, respectively.

Field results for the dose-response trials suggest that higher release rates of 1-octen-3-ol and ammonium hydroxide would attract more ticks. Since the intermediate release rate of acetone captured the largest number of ticks, it is unlikely that releasing acetone at a higher rate would increase the catch of ticks. It is likely, however, that a longer exposure time would increase the number of ticks attracted to all three of these semiochemicals. Additionally, because carbon dioxide has been shown to synergize the activity of hematophagous insect traps baited with chemical lures, the semiochemicals tested should be re-evaluated in combination with carbon dioxide against field populations of *A. americanum*.

The field sampling design that involved a grid of sampling cells was effective in evaluating the bioactivity of the test chemicals. However, future experiments should include more replicates and incorporate larger sampling areas to avoid any possibility of the

treatments interfering with each other. Touré (2005) demonstrated attraction to a semiochemical lure at a maximum distance of 8 m, which is larger than previous studies reporting attraction at a maximum distance of 5 m (Maranga *et al.*, 2003). Field trials of tick attractants could also be conducted by releasing cohorts of marked ticks into woodlands rather than testing semiochemicals against wild tick populations. This approach would have the advantage of reducing sampling variability observed between trials, since tick populations are not distributed evenly throughout the environment but exist in clusters (Carroll *et al.*, 2006). It would also account for the differences observed in the attraction of adult and nymph ticks. In the field bioassays it is not known whether the differences in attraction between nymphs and adults is due to population difference or behavior differences. Additionally, testing should be conducted during variant seasons and in different habitats to compare bioactivity of acetone and ammonium hydroxide to different tick species and life stages. The strong behavioral response of *A. americanum* to these semiochemicals in laboratory bioassays and field trials suggests that these chemicals may be attractive to other ixodid species.

In conclusion, acetone, ammonium hydroxide and 1-octen-3-ol were shown for the first time to be bioactive in attracting *A. americanum* adults in laboratory bioassays and nymphs in field trials.

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Table 1. Rotation of putative tick attractants and control chemicals in field trials.

For each test chemical, 5 trials were carried out with each trial completed a different day.

Day	Position				
	A	B	C	D	E
1	Dry ice	Distilled Water	1 Vial	2 Vials	3 Vials
2	3 Vials	Dry ice	Distilled Water	1 Vial	2 Vials
3	2 Vials	3 Vials	Dry ice	Distilled Water	1 Vial
4	1 Vial	2 Vials	3 Vials	Dry ice	Distilled Water
5	Distilled Water	1 Vial	2 Vials	3 Vials	Dry ice

Table 2. Analysis of variance for response of ticks to putative attractants in field trials.

Source	DF	F Value	Pr > F
1-octen-3-ol			
Treatment	4, 12	5.08	0.0145
Position	4, 12	0.49	0.7433
Day	4, 12	0.39	0.8113
Acetone			
Treatment	4, 12	6.90	0.004
Position	4, 12	5.23	0.011
Day	4, 12	2.54	0.0942
Ammonium Hydroxide			
Treatment	4, 12	18.37	<0.0001
Position	4, 12	5.67	0.0084
Day	4, 12	2.76	0.077

\*Type III test of fixed effects.

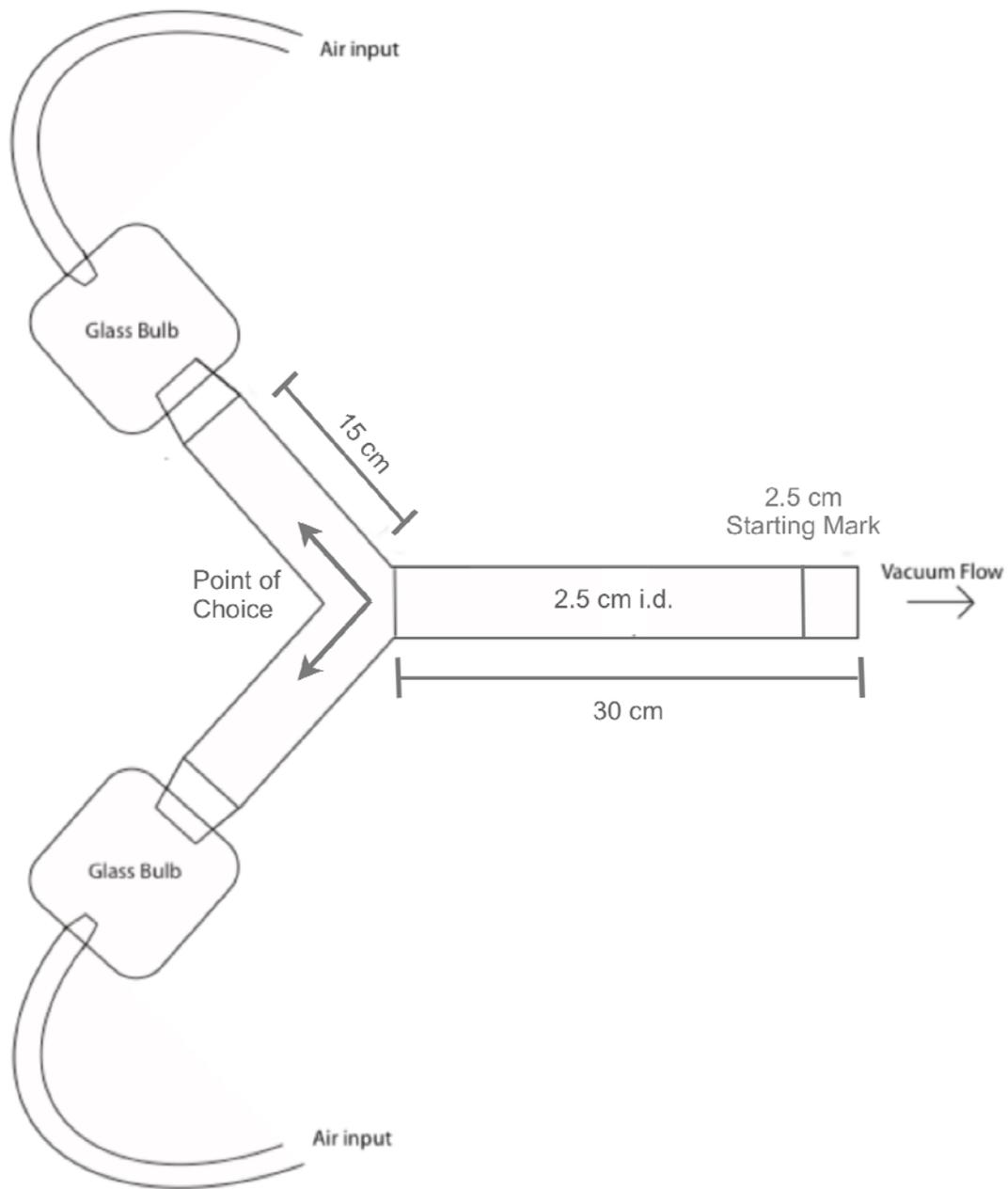


Figure 1. Diagram of Y-tube apparatus used in laboratory bioassays of putative attractants

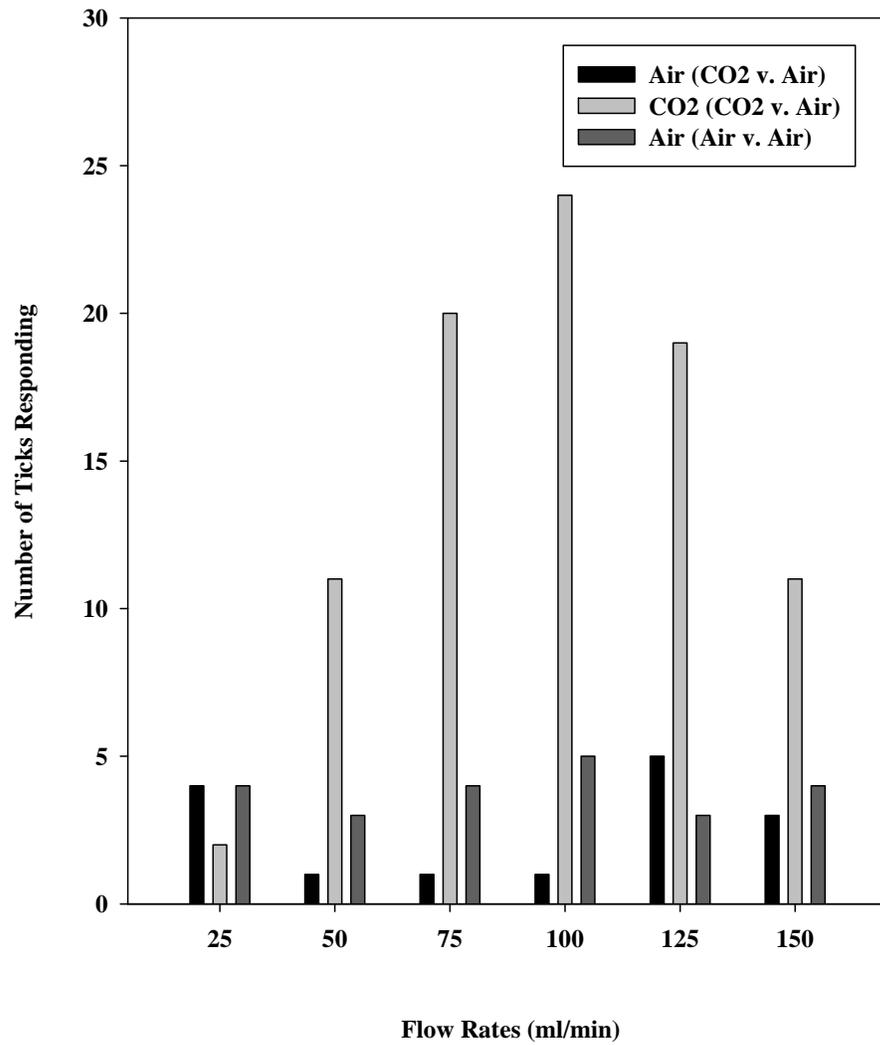


Figure 2a. Number of responding ticks to 3% CO<sub>2</sub> and humidified air in a Y-tube olfactometer compared to number of responding ticks to only humidified air in a Y-tube olfactometer for *Amblyomma americanum* adults.

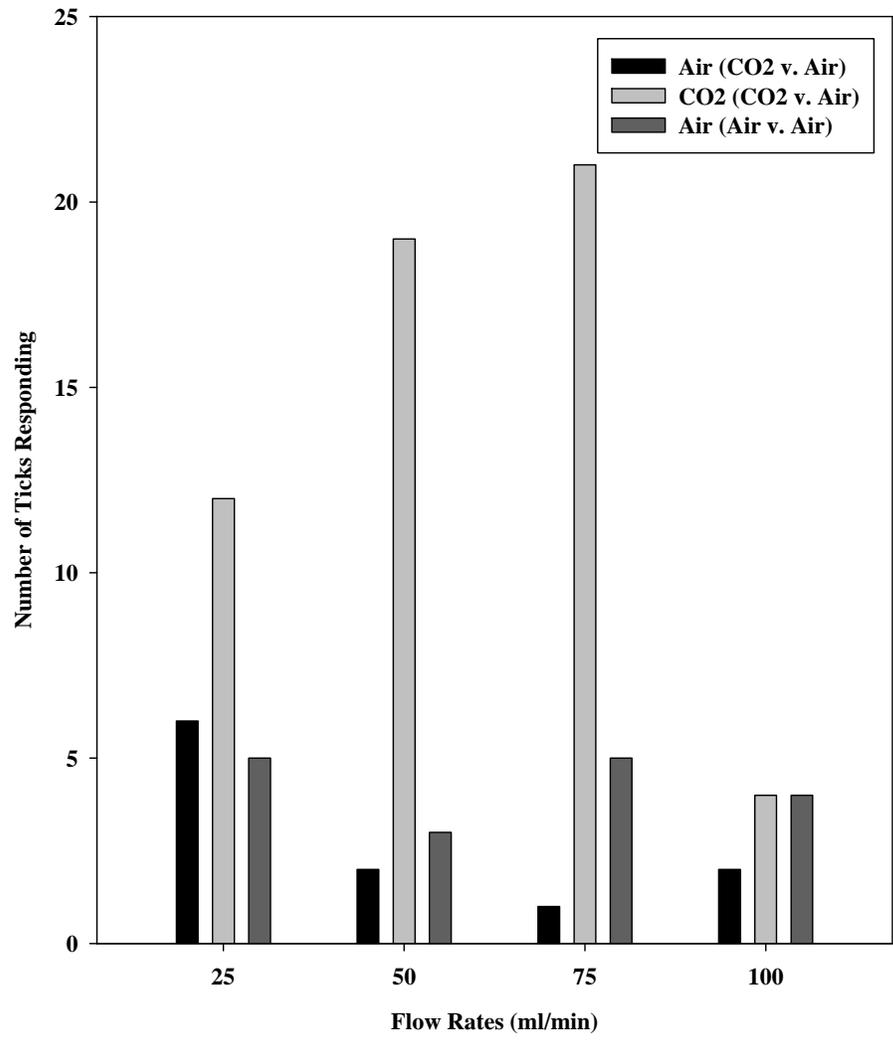


Figure 2b. Number responding ticks to 3% CO<sub>2</sub> and humidified air in a Y-tube olfactometer compared to number of responding ticks to only humidified air in a Y-tube olfactometer for *Dermacentor variabilis* adults .

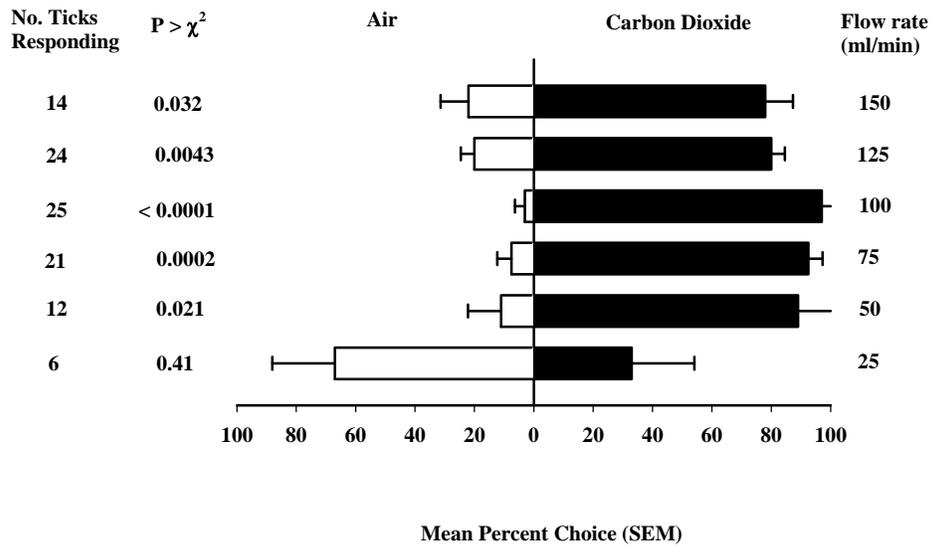


Figure 3. Response of *Amblyomma americanum* adults to 3% CO<sub>2</sub> and humidified air in a Y-tube olfactometer.

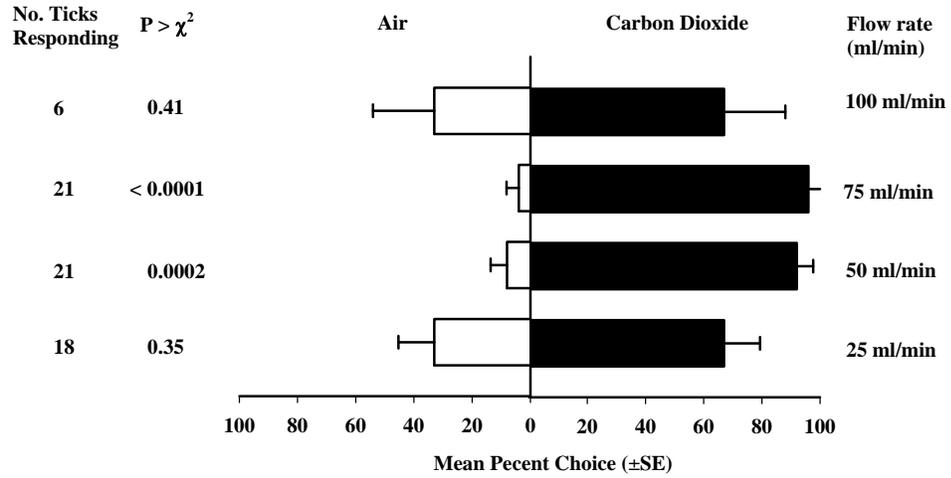


Figure 4. Response of *Dermacentor variabilis* adults to 3% CO<sub>2</sub> and humidified air in a Y-tube olfactometer.

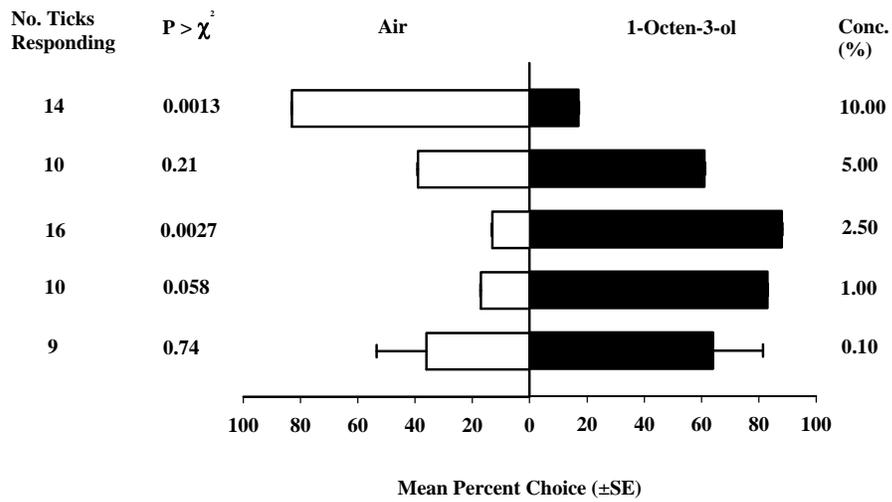


Figure 5a. Response of *Amblyomma americanum* adults to 1-octen-3-ol concentrations and water in a Y-tube olfactometer.

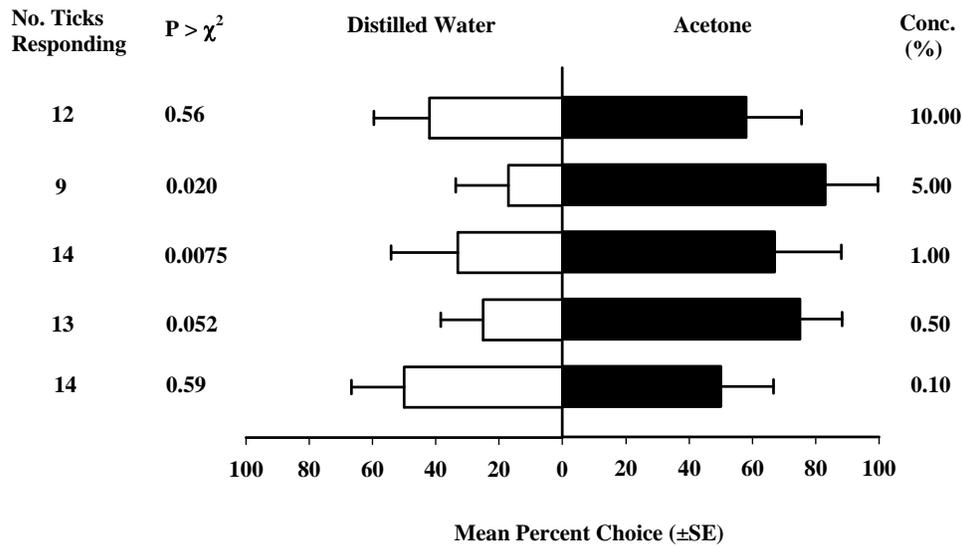


Figure 5b. Response of *Amblyomma americanum* adults to acetone and water in a Y-tube olfactometer.

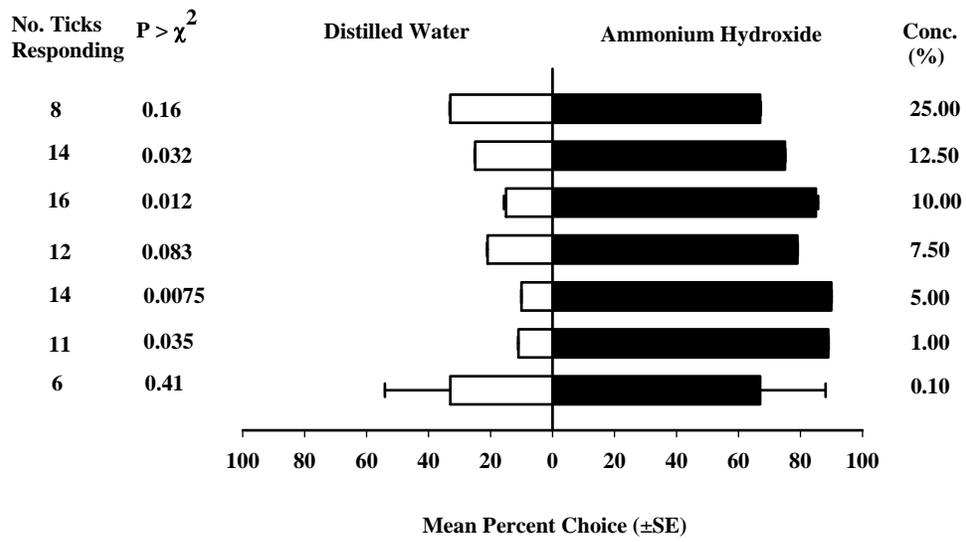


Figure 5c. Response of *Amblyomma americanum* adults to ammonium hydroxide and water in a Y-tube olfactometer.

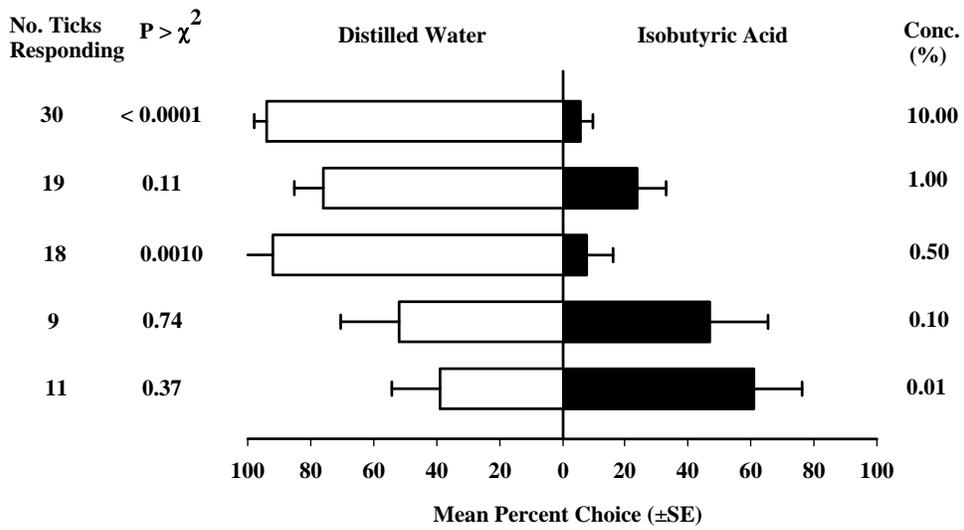


Figure 5d. Response of *Amblyomma americanum* adults to isobutyric acid and water in a Y-tube olfactometer.

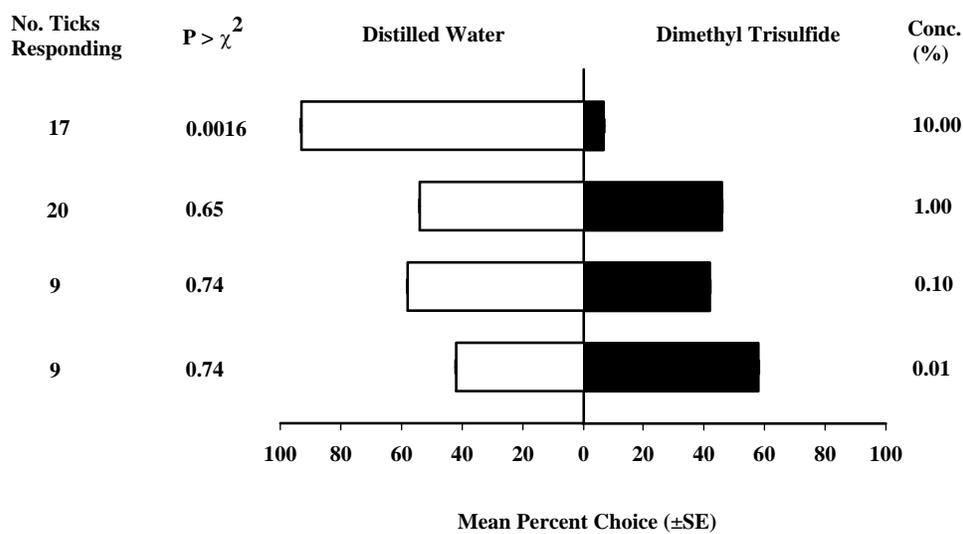


Figure 5e. Response of *Amblyomma americanum* adults to dimethyl trisulfide and water in a Y-tube olfactometer.

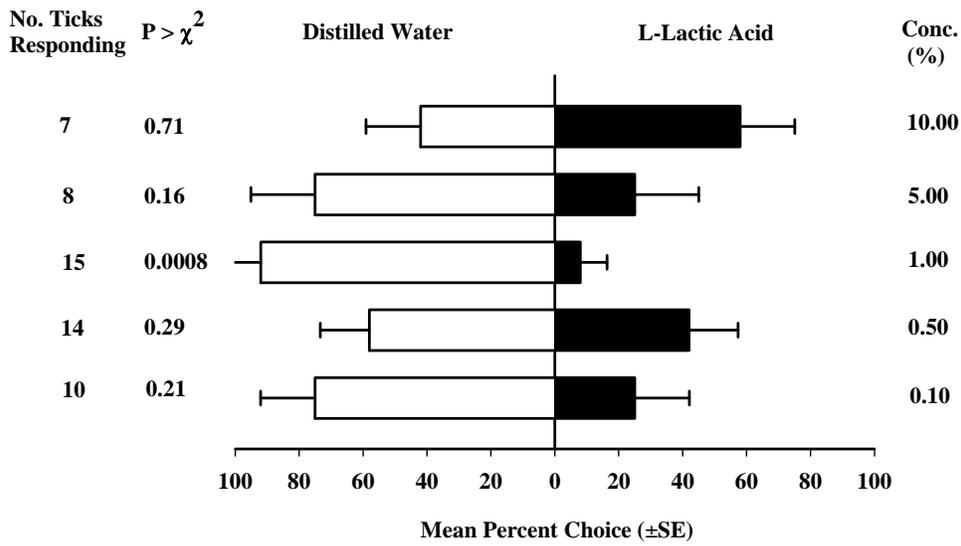


Figure 5f. Response of *Amblyomma americanum* adults to L-lactic acid and water in a Y-tube olfactometer.

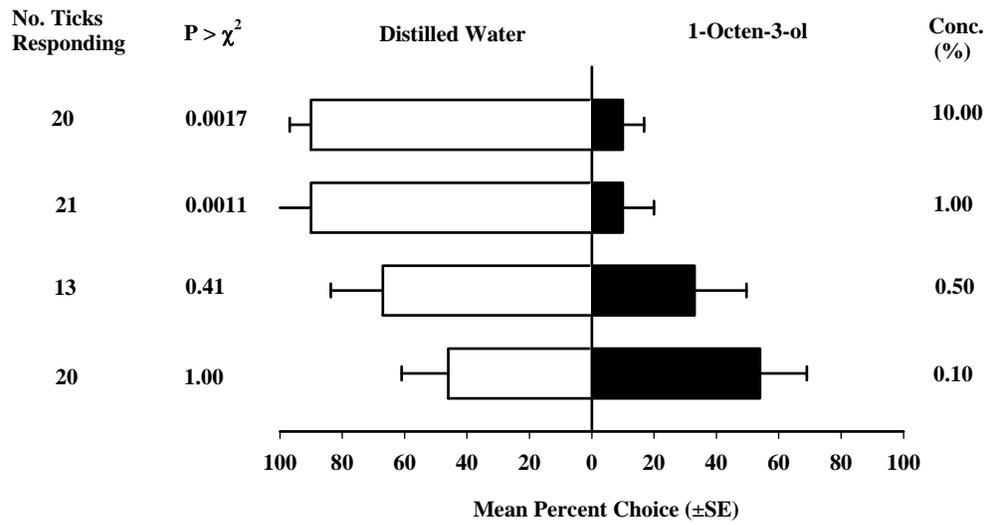


Figure 6a. Response of *Dermacentor variabilis* ticks to 1-octen-3-ol and water in a Y-tube olfactometer.

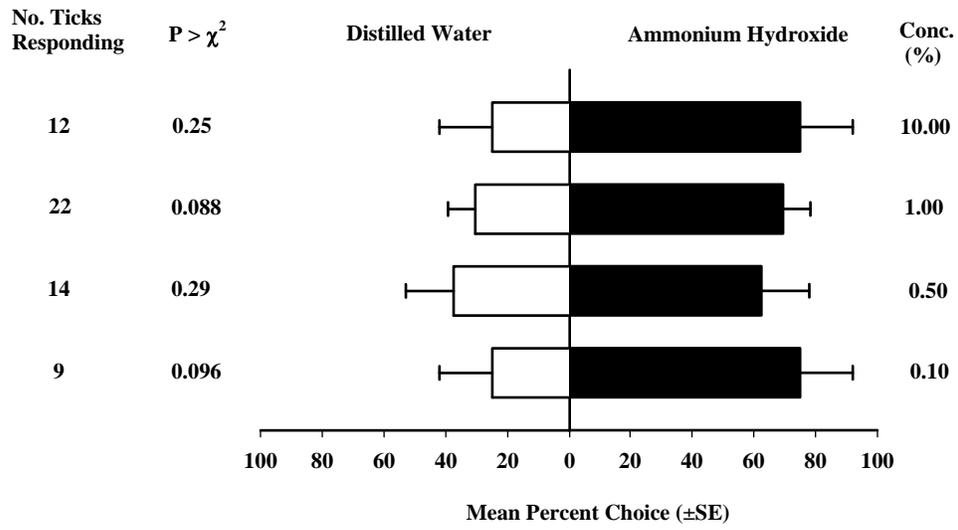


Figure 6b. Response of *Dermacentor variabilis* ticks to ammonium hydroxide and water in a Y-tube olfactometer.

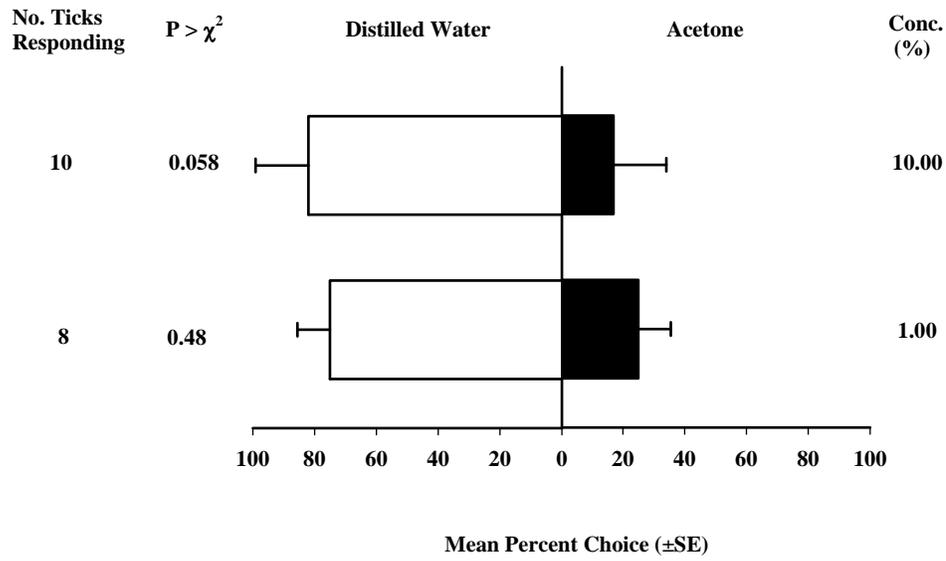


Figure 6c. Response of *Dermacentor variabilis* ticks to acetone and water in a Y-tube olfactometer.

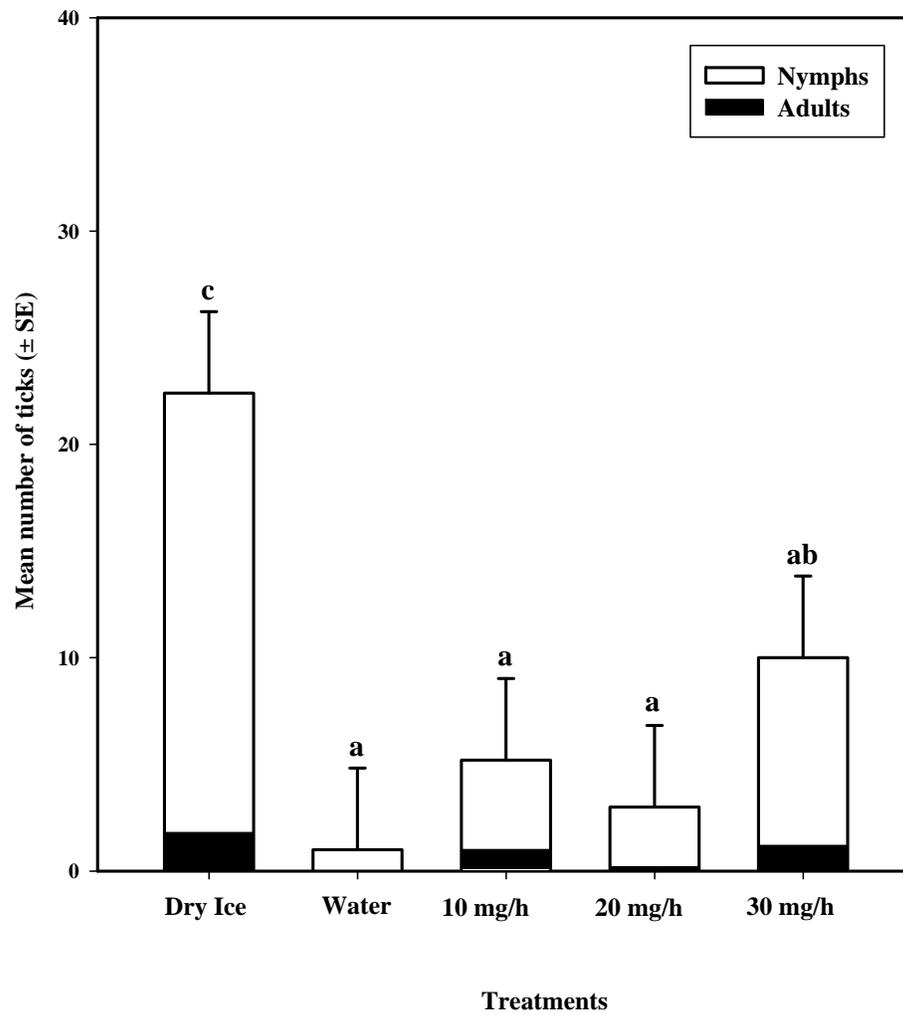


Figure 7a. Mean number ( $\pm$  SE) of *Amblyomma americanum* ticks collected in field trials of 1-octen-3-ol with water and CO<sub>2</sub> controls. Treatments were compared with pairwise t-tests. Treatments with the same letter are not significantly different ( $P > 0.05$ ).

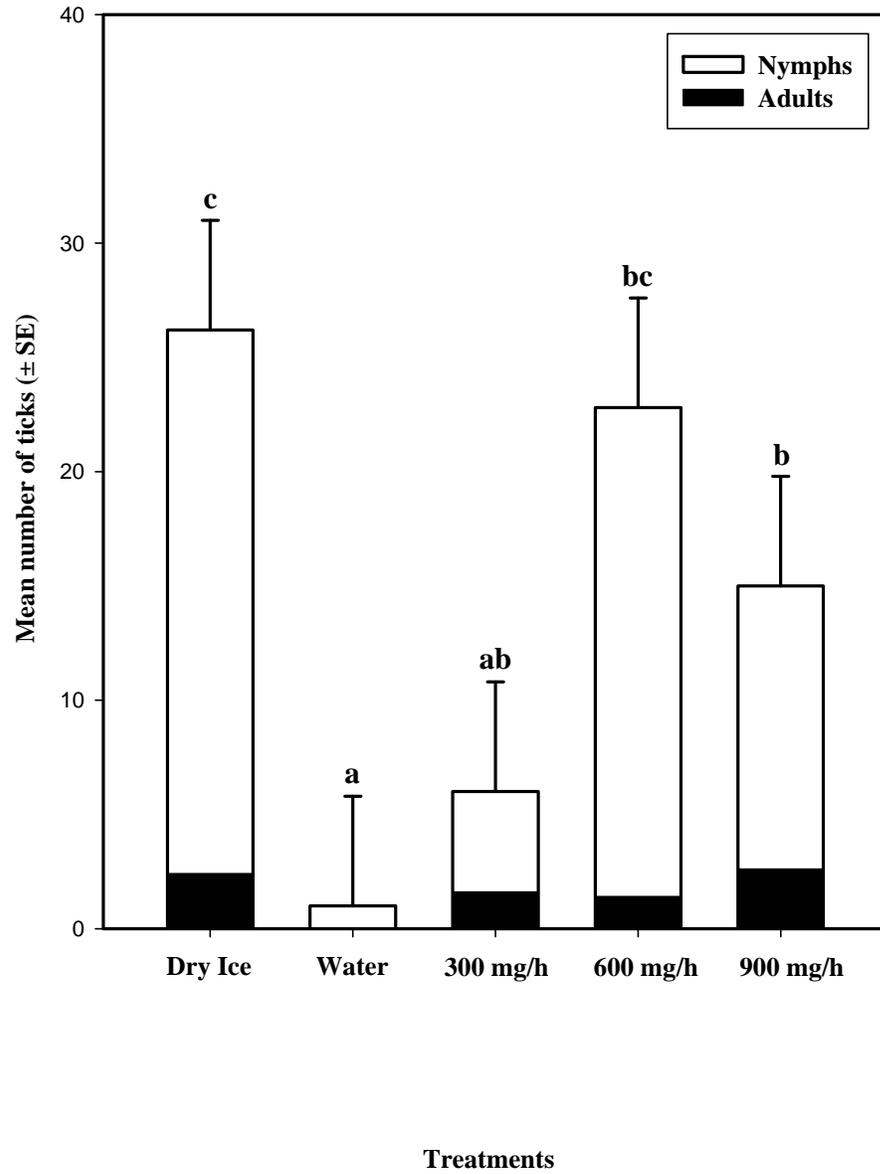


Figure 7b. *Amblyomma americanum* ticks collected in field trials of acetone with water and CO<sub>2</sub> controls. Treatments were compared with pairwise t-tests. Treatments with the same letter are not significantly different (P>0.05).

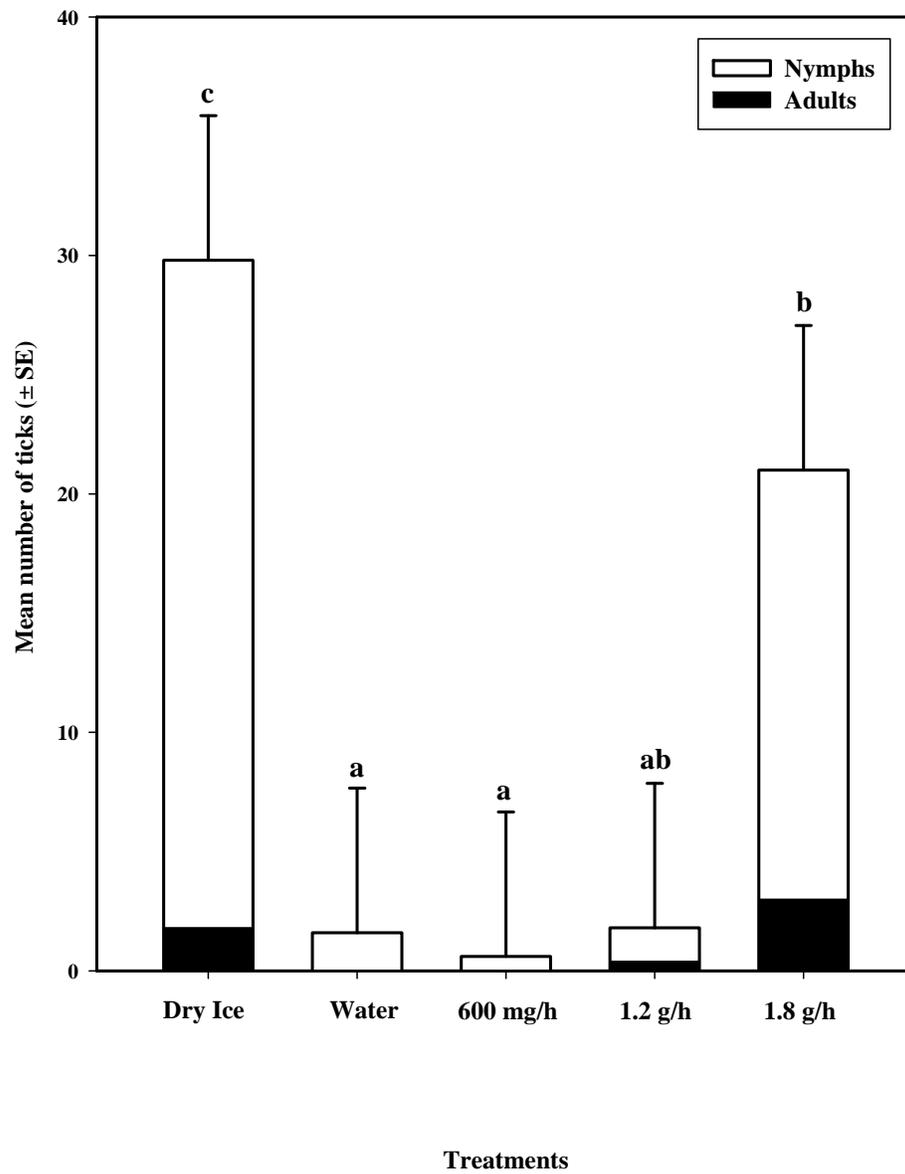


Figure 7c. *Amblyomma americanum* ticks collected in field trials of ammonium hydroxide with water and CO<sub>2</sub> controls. Treatments were compared with pairwise t-tests. Treatments with the same letter are not significantly different ( $P > 0.05$ ).