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Pheromonal Composition of Two Species of African *Amblyomma* Ticks: Similarities, Differences and Possible Species Specific Components

Thomas Lem Price Jr.
Old Dominion University

PHEROMONAL COMPOSITION OF TWO SPECIES OF AFRICAN *AMBLYOMMA* TICKS: SIMILARITIES, DIFFERENCES AND POSSIBLE SPECIES SPECIFIC COMPONENTS.

by

Thomas Lem Price Jr.
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Approved by:

Daniel E. Sonenshine (Director)

Raymond W. Alden, III

Frank E. Scully Jr.

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Abstract

Two species of bont ticks, *Amblyomma hebraeum* and *Amblyomma variegatum*, common to Africa have been studied to compare types and quantities of compounds known or believed to serve as components of the attraction-aggregation-attachment pheromone (AAAP). A complex of attraction, aggregation and attachment pheromones are used by these ticks to detect hosts, mates and perhaps minimize interspecific breeding. Solvent extraction of pheromone emitting ticks followed by Gas Chromatography and Mass Spectrometry revealed little difference in the composition of the AAAP in these two species. However, subtle differences in the relative makeup of the pheromonal blend are noted, suggesting that such differences may facilitate species-specific discrimination during aggregation and attachment. Differences in the relative abundance of benzaldehyde and methyl salicylate in the males of the two species were especially noteworthy. Possible methods by which such differences in phenolic compound composition may affect the behavior of these ticks are discussed.

Dedication

This long delayed work is dedicated to several important people; first always is my wife Janet who has provided me with two wonderful daughters during the course of this work and has also made this an extraordinary life. Also to my parents, two long suffering souls who, I'm sure, often wonder just how and what I think I'm doing. The last person is of course Brigham who neither started nor finished this period with me yet through his efforts has provided me with the continuity which makes all things possible.

Acknowledgements

Many of you have assisted both in the production of this thesis and completion of this degree program. Many thanks to Dan Sonenshine whose constant efforts have brought this thesis to completion. Thanks to Ray Alden and Frank Scully for remarkable patience. And to many others (in no particular order) thank you for time, words and works: Michael Ehret, Paul Homsher, Lytton Musselman, Nancy Wade and Michael Helmstetter.

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Introduction

Pheromones belong to the family of semiochemicals or information bearing compounds that allow chemical communication between individuals. When released by an individual of a species, pheromones affect the behavior of other individuals of the same species in a manner favorable to the survival of that species. An excellent example is the attractant sex pheromone. Used widely in both vertebrates and invertebrates alike, the attractant enables scattered members of a species to attract the opposite sex at a time appropriate to mating.

The term "pheromone" gained acceptance in the early 1960's as the study of insect communication compounds intensified. First identified in the silk moth, pheromones were attributed to as many as 40 species of moths by the 1970's. The first tick pheromone, also functioning as a sex attractant, was identified as 2,6-dichlorophenol by Berger in 1972. Since that time this same pheromone have been found in as many as 14 species of metastriate (hard) ticks, including five genera (Sonenshine, 1985). Other substituted phenols function as components of the attractant-aggregation-attachment pheromone (Norval et al., 1993).

The major classes of tick pheromones may be defined as: 1) assembly pheromones, 2)sex pheromones, and 3)attractant-aggregation-attachment pheromones. Assembly pheromones provide a weak attractant stimulus which produces clustering of unfed ticks. This response may be overridden by hunger or sexual needs. It serves to concentrate populations in protected microenvironments which enhance survival. Sex pheromones (SP), divided into several

subgroups, serve as the regulating mechanism for mating. Often consisting of three components, the sex pheromones first attract prospective mates with an attractant sex pheromone (ASP). The attracted mate then identifies a receptive partner via a mounting sex pheromone (MSP). Location of the gonopore and copulation is stimulated in some species by the presence of the genital sex pheromone (GSP). Attractant-aggregation-attachment pheromones (AAAP) are known to occur only in the genus *Amblyomma*. These pheromones regulate the attraction of adults (both sexes) and juveniles to a feeding site usually initiated by pioneer males. Response to the AAAP in this genus was once thought to be species specific (Rechav, 1978); however, more recent investigations dispute this hypothesis and demonstrate strong interspecific attraction (Yunker et al., 1990; Norval et al., 1993).

The species *A. hebraeum* and *A. variegatum* are African ticks with somewhat overlapping ranges of distribution and a common primary host. Feeding mostly on large ungulates, especially buffalo and domestic cattle, the species ranges are roughly divided on the basis of geography. In Zimbabwe, both species occupy a low veld environment with *A. hebraeum* generally distributed to the south of *A. variegatum*. Some overlap of these ranges is present with the occurrence of *A. hebraeum* noted to be increasing between 1975 and 1980 (Norval, 1983). Control of these ticks is affected by dipping commercial livestock in dilute insecticide baths on a regular basis in order to reduce infection by *Cowdria ruminantium*, the causative agent of the deadly heartwater disease. This disease, prevalent in much of Africa and the Caribbean, impedes development of the cattle industry, reduces their economic value and can be fatal in conjunction with other environmental or physical stresses. The host finding strategy

of these two species differs from that of other metastriate hunter ticks. These ticks are first alerted to the presence of potential hosts by carbon dioxide and are "activated", i.e., brought to a state of high excitement (Yunker and Norval., 1991). The activated ticks emerge from sheltered areas in the ground litter and run back and forth seeking hosts. If they also encounter the AAAP from male-tick infested hosts, they orient, quickly run towards those animals and attack them. Ticks will rarely attach to a host that has not been previously infested by male ticks (Norval et al., 1989). Recognition takes place at reported ranges of 9 and 11 meters. This behavior is so profound that several researchers refer to infested hosts as "baited" animals. Once on the host the ticks form clusters around the previously attached member (Rechav et al., 1977). In both species, males having fed for 4-6 days are responsible for production of the AAAP. Males, females and nymphs are all attracted to the AAAP (Rechav et al., 1976).

The advantages of this behavior are obvious; rather than chance acquisition of a host lacking members of the opposite sex, ticks are stimulated to locate hosts already infested with likely mates. In that ticks attached for several days are the producers of the AAAP, sites for aggregation are probably outside the grooming area of the host; thus, the ticks are less likely to be removed. A further advantage may be realized in the sheltering behavior of the ticks while awaiting hosts. These ticks avoid wasteful expenditure of energy or risk of desiccation until stimulation by CO_2 and AAAP. The advantages of the AAAP are balanced by the need to discriminate between the species utilizing this mechanism. Although geographically isolated throughout most of their species range (allopatric), some geographic overlap occurs. When confined together *A. hebraeum* and *A. variegatum* will interbreed and produce eggs. However,

when given a choice, conspecific matings outnumbered heterospecific pairings (Rechav et al., 1982). Interspecific mating between *A. hebraeum* and *A. variegatum* results in inviable offspring (Rechav et al., 1982). These findings suggest the possibility that some subtle differences in their chemical signals may facilitate species-specific discrimination. Attraction of ticks by the AAAP combined with pesticide sources has demonstrated potential for the use of pheromones in the control of these organisms (Rechav and Whitehead, 1978; Norval et al., 1991c).

In both *A. hebraeum* and *A. variegatum* mating occurs when the female locates and moves close (within 2 - 5 cm) to a preattached pheromone emitting male (Norval, 1974). The male, remaining attached, raises his body perpendicular to the host surface in response to mechanical stimulation and awaits the female. The female pauses for several seconds to minutes raising and waving her first pair of legs on which the Haller's organ, responsible for olfactory response, is located. This pause is the apparent moment of decision by the female as to the acceptability of the male as a suitable mate. The pause and decision to accept the male appears to be stimulated by one of the short-range components, i.e., the aggregation component, of the AAAP. If acceptable, the female moves to the male who clasps her, wrapping his legs about her, and the female attaches to the host to begin feeding. In contrast to the female response, the male clasping response appears to be contact mediated (Norval, 1974). The male later detaches from the host for copulation, reattaching nearby afterwards. The female feeds to engorgement and drops off to oviposit. The male remains attached to feed and potentially mate again.

Previous studies (Schoni et al., 1984; Apps et al., 1988; Diehl et al., 1991; Lusby et al., 1991) have suggested the following as components of the AAAP:

Benzyl Alcohol	Benzaldehyde
Benzothiazole	2,6-Dichlorophenol
2-Methyl Propanoic Acid	Methyl Salicylate
2-Nitrophenol	Nonanoic Acid

Of these compounds, 2-nitrophenol appears to be main component in long range, interspecific attraction and aggregation. Studies with both species demonstrate strong response to either 2-nitrophenol alone or in combination with other components (Schoni et al., 1984; Hess and de Castro, 1986; Norval et al., 1991a; Norval et al., 1991b). In quantitative comparisons 2-nitrophenol has been demonstrated to be the major component of the pheromonal mix, however its role appears to be concerned primarily with attraction and aggregation. This suggests that first, another stimulus is necessary to complete attachment and mating and second that a reasonably well defined sequence of events must be followed to drive the response to completion. Field studies have shown that attraction of ticks is relatively poor if exposed only to 2-nitrophenol or authentic AAAP extracts without activation by CO_2. Thus the behavioral sequence may be defined as 1) activation by CO_2, 2) detection and attraction by 2-nitrophenol and 3) detection of one or more of the other components which may cause species segregation, attachment and mating. Sequencing of behavior, controlled by pheromones, has been described for other hard ticks as illustrated by the multi-component sex pheromone mechanism

(Sonenshine, 1991).

Obvious gaps exist in the understanding of the pheromonal systems of these two closely related species. Some basic questions may be answered by a comparative study of the extractable pheromone content of the two species. These questions, simply put, are:

1. Are there qualitative differences in the pheromonal content between species?
2. Are there quantitative differences in pheromonal content between species?
3. Are there differences in the relative availability of known pheromonal constituents between species?
4. If differences exist in the pheromonal content, is there evidence of a species segregation mechanism mediated by pheromones?

Materials and Methods

Ticks of both species were allowed to feed on cattle until pheromone production was evident. The ticks were then removed by hand, sorted by sex and extracted to remove the pheromonal component. Five types of extracts, as described below, were produced to provide a basis for comparison of surface availability of the pheromone. Extracts were then analyzed against reference materials to provide both quantitative and qualitative determinations of constituent components.

Sample Preparation: At the Veterinary Research Laboratory, Harare, Zimbabwe, laboratory reared *A.hebraeum* and *A.variegatum* were fed on cattle before collection and extraction. Two sets of samples were collected, each containing up to five types of extracts for each species. Type One extracts were produced by detaching individuals of the same species and sex and placing them into precleaned glass ampoules, covering them with cold (-70°C) hexane / diethyl ether (9:1) and sealing them for shipment to the United States (Long Duration Extract). Type Two extracts followed the same general procedure but after 16 hours of exposure to solvent, the tick bodies were removed and the ampoules resealed for shipment (Short Duration Extract) Type Three extracts followed the same protocol with the exception that tick bodies were removed after 30 seconds of exposure to the solvent mixture (Rinse). Type Four and Five extracts, available only for sample Set Two, are re-extractions of Type Two and Three samples; ticks previously extracted for 16 hours or 30 seconds were placed in solvent filled ampoules for three to four weeks during shipment as a secondary extraction. This enabled us to

determine how much material remained after the initial extraction. Samples of males and females of each species were prepared in accordance with these extraction procedures as summarized in Table 1. The samples received in 1991 (Set 1) contained 87-100 ticks/sample; those received in 1992 (Set 2) contained 45-50 ticks/sample. Upon receipt at ODU each sample was centrifuged to remove particles and concentrated under nitrogen. Samples were held at -70°C during concentration to reduce loss of volatile components. The volumes of extract were reduced to an amount representing 0.1 male equivalent (ME) or 0.1 female equivalent (FE) per microliter (ul). All materials were stored in the dark at -10°C after receipt.

TABLE ONE
Samples, Extract Types and Number of Ticks/Sample
Analyzed for AAAP Components

Set One (1991)		Set Two (1992)			
A. hebraeum ♂	A. variegatum ♂	A.h. ♂	A.h. ♀	A.v. ♂	A.v.♀
Type 1 87	96	45	50	45	50
Type 2 100	100	45	50	45	50
Type 3 100	100	45	50	45	50
Type 4 n/a	n/a	45	50	45	50
Type 5 n/a	n/a	45	50	45	50

All standard reference materials (Table 2) were purchased from Aldrich Chemical Company, Milwaukee, WI (substituted phenols) and Excel Scientific, Kingston, Rhode Island, (Fatty Acid Methyl Esters). Pesticide analysis grade solvents were obtained from Burdich and Jackson, Muskegon, Michigan and the esterification reagent (boron trifluoride) was purchased from Supelco Inc., Belefonte, PA.

TABLE TWO	
Substituted Phenols	Fatty Acid Methyl Esters*
Benzaldehyde	2-Methyl Propanoic Acid M.E.
Benzyl Alcohol	Butanoic Acid M.E.
Salicylaldehyde	2-Methyl Butanoic Acid M.E.
2-Nitrophenol	Pentanoic Acid M.E.
2,5-Dimethylphenol	4-Methyl Pentanoic Acid M.E.
2,4-Dichlorophenol	Hexanoic Acid M.E.
Methyl Salicylate	Heptanoic Acid M.E.
2,6-Dichlorophenol	Octanoic Acid M.E.
Benzothiazole	Nonanoic Acid M.E.
Nonanoic Acid	
2,4,6-Trichlorophenol	
4-Nitrophenol	
Phenylacetaldehyde*	

*Phenylacetaldehyde and FAME standards used only for 1992 sample set.

Analysis: Analyses were carried out in two phases. The first consisted of analysis by Gas Chromatography under Electron Capture (GC/ECD) or Flame Ionization Detection (GC/FID). Primary quantitation and tentative (retention time matching only) identification were provided during this analysis. The second phase consisted of confirmation of identification and tentative identification of unknown compounds by Gas Chromotography / Mass Spectrometry (GC/MS).

Chromatographic Analysis

Chromatographic analysis was performed using a Shimadzu GC-14A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with both ECD and FID. Results were recorded on a Shimadzu C-R6A integrator. The column was a DB-5 fused silica capillary column 30m long by 0.32mm i.d. containing a bonded, 0.25um thick, 5% phenyl : 95% dimethylsilicone

stationary phase (J&W Scientific Co., Folsom, CA.). For ECD analysis, the operating conditions were as follows: injector temperature 180°C, detector temperature 200°C, and oven temperature isothermal 110°C. The column flow was 2.7 ml/min of the carrier gas, helium. The injector was operated in the split mode at 85:1. Detector flow was set to 40 ml/min with nitrogen (make up gas). For FID analysis the operating conditions were injector temperature 200°C, detector temperature 300°C, oven temperature 60°C for 0.25 min, then increased 5°C/min to 250°C. Column flow was 1.5 ml/min of the helium (carrier gas). The injector split was operated in the splitless mode for 0.25min, then split at 100:1. Detector makeup flow was set to 40 ml/min total with helium.

Identification of compounds recognized by GC was done by matching peak retention time with that of authentic standard materials and by coelution of peaks with authentic standard materials. Quantities of analyte were calculated using the response factor generated by the standard material. Linearity of response for the instrument was demonstrated for quantities between 6.25 to 100 ng of material via GC/FID. All identifications by GC were considered tentative until they were confirmed by mass spectral analysis, as described below.

Mass Spectral Analysis

Quadrapole GC/MS analyses were conducted at The Applied Marine Research Lab, ODU (Norfolk, VA). These analyses were performed using two instruments, a Finnigan-MAT OWA-30 and an INCOS-50 from the same manufacturer (Finnigan-MAT, San Jose, CA). For maximum reproducability, both instruments were operated under identical GC and EI

parameters. All separations were done with a J&W Scientific DB-5 capillary column, 30m by .32mm i.d. x .25um, with a 5% phenyl / 95% dimethylsilicon. Ion source temperature for both instruments was maintained at 180°C with an ionization energy of 70 eV. Before analysis, each instrument was tuned to provide a fragmentation spectra for decafluorotriphenylphosphine recommended by the United States Environmental Protection Agency. This procedure enhances matching against the U.S. National Bureau of Standards / National Institute of Health (NBS/NIH) spectral library. Chromatographic conditions were as follows: injector temperature 175°C, oven temperature from 60°C to 250°C at 5°C/min then 20°C/min to 300°C. Column flow was measured at 1.7 ml/min for helium (carrier gas). The injector was operated in the splitless mode for one minute and at 100:1 split for the remainder of the analysis.

Identification and quantitation followed a two tiered approach. First, a library of standard reference materials was created to provide both authentic spectra and retention time. This library was then calibrated for quantitation of each material. All identified peaks had associated authentic retention time, spectra and quantitative values. Linearity of response for each instrument was demonstrated for quantities between 6.25 and 200 ng of each standard material in this tier.

In the second tier, the spectrum generated from each peak in each sample was compared to the three compounds in the NBS/NIH library having the best fit. These fits were examined to establish tentative (spectral only) identification and were quantitated against the response of an internal standard material, 2-Fluorobiphenyl.

Results

Confirmed Identifications (Males)

Qualitative and quantitative analysis of the Type One Male extracts for each species yielded the following identified compounds: 2-nitrophenol, methyl salicylate, benzaldehyde, 2,6-dichlorophenol and nonanoic acid. Additionally, heptanoic and octanoic acid were also found in *A. hebraeum*. With the exception of methyl salicylate, found only in *A.hebraeum* Set 1, and benzaldehyde, found only in *A.variegatum* Set 2, each compound was found in both sets for each species. To provide a statistical analysis of the total amount of each compound produced, the mean was taken from four "total extract values". The first two values of this mean are the Type One extract quantitations from Sets One and Two. The second two values are the result of summing the short duration or rinse extracts with their respective secondary extraction. These totals should represent the same value: the net amount of pheromone available at the time of extraction. However, there is potential for the presence of an additional variable, the extraction efficiency. Figure 1 presents mean and standard deviation values for the five compounds identified above. Of these compounds 2-nitrophenol was the most abundant in each species. *A. hebraeum* produced significantly less 2-nitrophenol and methylsalicylate than *A. variegatum*[1]. Nonanoic acid was also significantly less abundant in *A. hebraeum*; however,

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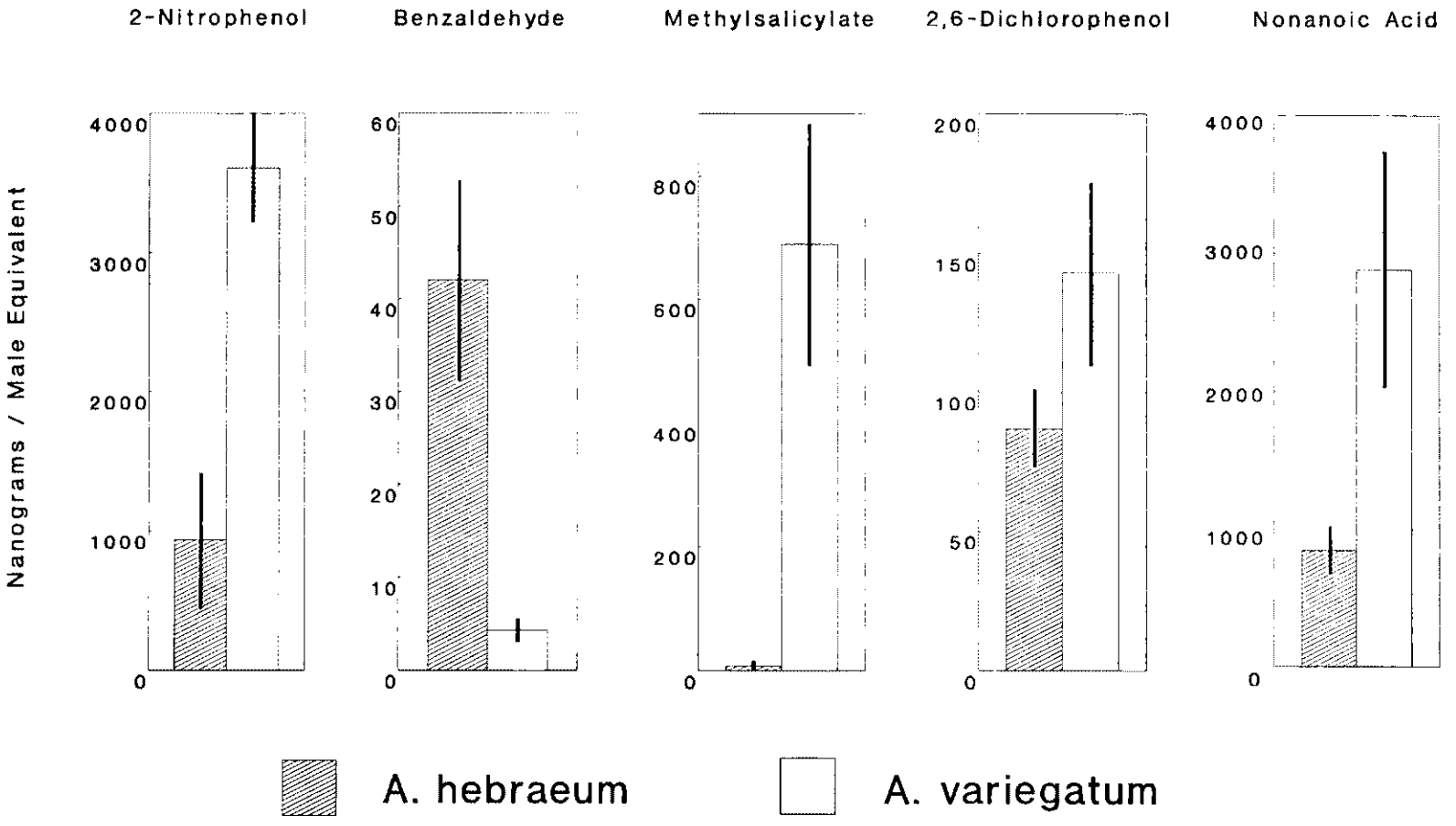
[1] 2-nitrophenol $t_{[.05,6]} = 4.31$
benzaldehyde $t_{[.05,3]} = 2.48$ (note: unequal variance demanded loss of degrees of freedom)
methylsalicylate $t_{[.05,6]} = 3.54$
2,6-dichlorphenol $t_{[.05,6]} = 1.61$
nonanoic acid $t_{[.05,6]} = 5.09$

Legend: Figure 1

Figure 1 demonstrates mean concentration values for five compounds found in both species of male tick extracts. Concentrations are expressed in nanograms per Male Equivalent (ng/ME) as determined by Gas Chromatographic quantitation. Values presented have been calculated from four replicates and are displayed with standard deviations as the solid bar within the histogram.

Figure One

Mean Concentration of Identified Compounds
Male Extracts



this result is based only on the three analyses from Set Two.

The data described in Figure 1 demonstrated insignificant differences in variance between species (F_{max}) for all compounds, with the exception of benzaldehyde. Analysis of these data via principal component analysis yielded a moderate separation of the two species as shown in Figure 2. Discriminant analysis, however, indicates that this difference is insignificant at the 95% confidence interval ($F_{[.05,4,3]} = 2.65$). Raw data for GC and GC/MS quantitation are provided in Appendix 1. Tentatively identified compounds are listed in Appendix 2.

Tentative Identifications (Males)

Compounds found by matching peak spectra from GC/MS chromatograms against spectra in the NBS library are considered tentatively identified. Estimated quantitations are based on the response factor of 2-Fluorobiphenyl, an internal standard, for compounds identified by spectral match only. These tentative identifications are selected based on structural similarity with known pheromonal compounds and indication of potential activity. Characteristics for selection of compounds and their quantitation are presented in Table 3.

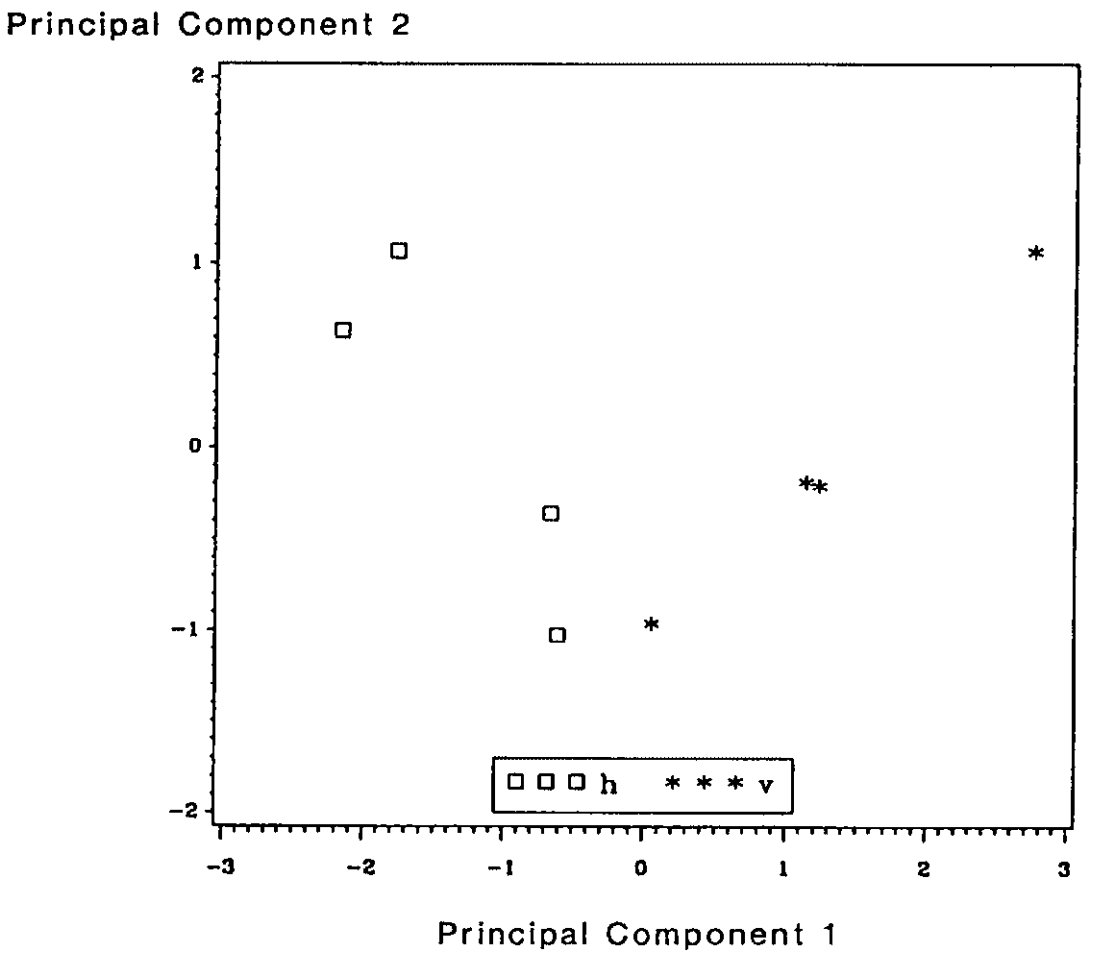
Legend: Figure 2

Figure 2 demonstrates a plot of four replicates for each species as separated by principal component analysis. Separation of the species occurs along the axis of principal component one which is responsible for 68% of the difference between the two species. The compound primarily responsible for the separation is 2-nitrophenol.

Figure Two

Principal Component Analysis

A. hebraeum and A. variegatum Males



Variables = 2-Nitrophenol, Methylsalicylate, Benzaldehyde and 2,6-Dichorophenol

h = A. hebraeum, v = A. variegatum

Table Three			
Physical Characteristics of Known and Suspected Pheromonal Compounds			
Known Pheromonal Compounds			
Compound	MP[1]/ BP[2]°C	OH[3]Present	Activity
2,6-Dichloro Phenol	65° / 219°	Yes	Odor
Benzaldehyde	-56° / 179°	No	Odor/Taste
2-Nitrophenol	45° / 215°	Yes	Odor
Methyl Salycilate	-8° / 222°	Yes	Odor
Suspected Pheromonal Compounds			
Phenyl Acetaldehyde	33° / 195°	No	Odor
Hexadecanoic Acid	63° / na	Yes	Irritant
Dodecanal	na / na	No	Irritant
Methyl Phenol	30° / 191°	Yes	Odor
Bromochloro Phenol	47° / 233°	Yes	Odor
Naphthalene	82° / 218°	No	Odor
Nonanoic Acid	12° / 253°	Yes	Odor
Dimethylethyl Phenol[4]	50° / na	Yes	Irritant

[1]MP = Melting Point
[2]BP = Boiling Point
[3]OH = Hydroxyl Functional Group
[4]Dimethylethyl Phenol values are based on 5-Isopropyl-3-methyl Phenol

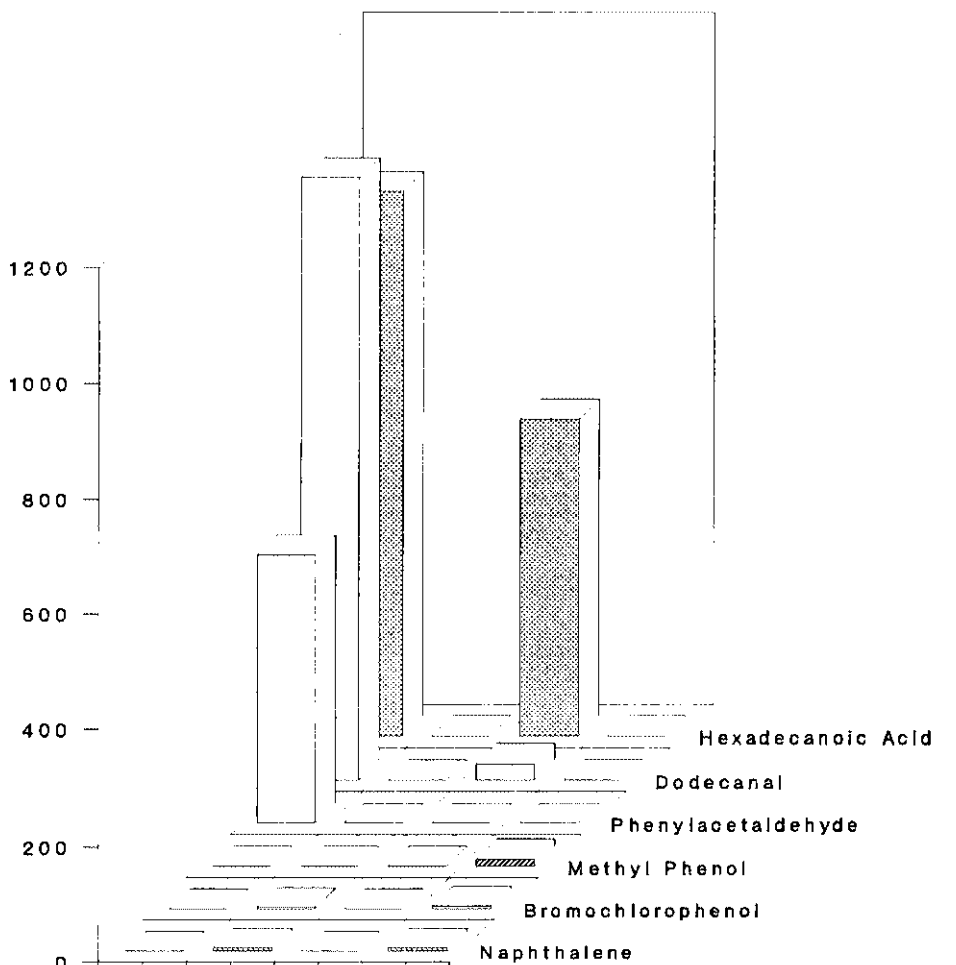
As demonstrated in Figure 3, none of the tentatively identified compounds were consistently found in both sets of extracts for either species.

Legend: Figure 3

Figure 3 shows relative concentrations of compounds, tentatively identified from male extracts by Gas Chromatography / Mass Spectroscopy. Concentrations are estimated based on the response factor of 2-fluorobiphenyl, an internal standard and are reported as nanograms per Male Equivalent. Each species is divided into two sets (1 & 2) based on the year of collection. Note that none of the compounds tentatively identified in Set 1 are present in Set 2.

Figure Three

Male Tentatively Identified Compounds[1] Estimated Quantities (ng/ME)[2] by GC/MS



	A.h.1	A.h.2	A.v.1	A.v.2
Hexadecanoic Acid	944	0	550	0
Dodecanal	1042	0	27	0
Phenylacetaldehyde	462	0	0	0
Methyl Phenol	0	0	0	12
Bromochlorophenol	0	3	0	5
Naphthalene	0	5	0	5

1 Tentative ID ▪ Spectral Match Only
2 ng/ME ▪ Nanograms / Male Equivalent

Relative Abundance (Males)

To rank relative availability of compounds as a potential pheromone, extract durations were compared, based on percent recovery by extract type. Figure 4 demonstrates recovery values from each extract type as a three bar histogram as follows:

Type 1 = 100%

Type 2a = Type 2 / (Type 2 + Type 4)[2]

Type 3a = Type 3 / (Type 3 + Type 5)[3]

Note that in *A. hebraeum*, O-nitrophenol and nonanoic acid are the compounds most abundant in Type 3 extracts as compared to O-nitrophenol, nonanoic acid and benzaldehyde in *A. variegatum*. Values in these figures are based on GC quantitation. Methyl salicylate was not detected for *A. hebraeum* by this method. These analyses are based on a single sample set (Set 2) and provide no statistical data.

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[2]Type 2 + Type 4 represents 100% of the extractable material of this type of extract.

[3]Type 3 + Type 4 represents 100% of the extractable material of this type of extract.

Legend: Figure 4

Figure 4 demonstrates the relative availability of five compounds based on extract type for males of each species. Short duration (type 2) and rinse (type 3) extracts quantitations are presented as a percent of the long duration extract value (type 1) based on Gas Chromatographic quantitation. Note that availability of compounds may be generally ranked as:

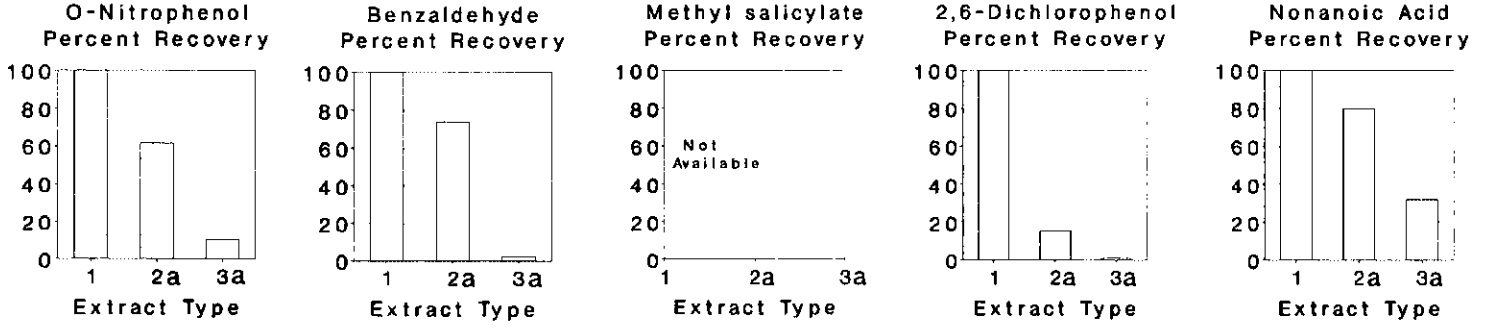
Readily available (2-nitrophenol, nonanoic acid)

Moderately available (benzaldehyde, methylsalicylate)

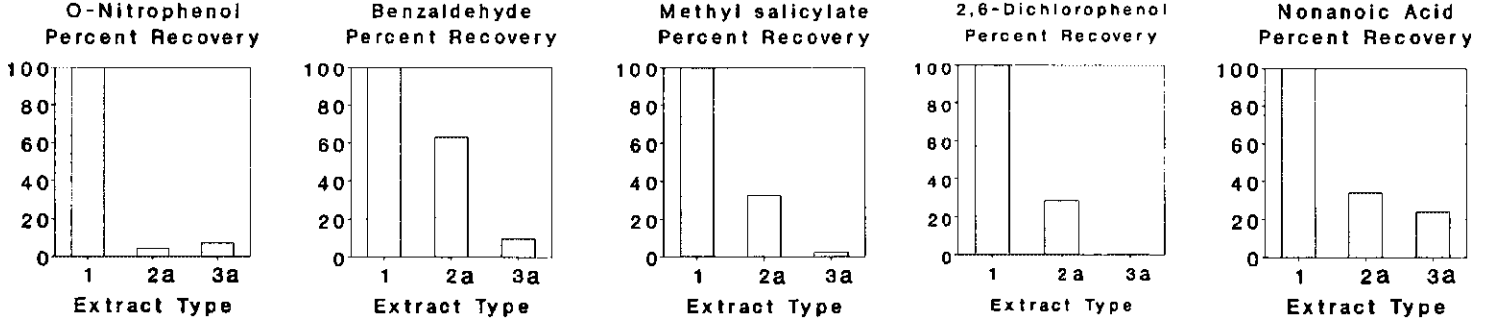
Poorly available (2,6-dichlorophenol).

Figure Four
Relative Availability of Compounds by Extract Type

A. hebraeum



A. variegatum



Female Tick Extracts

Figure 5 shows the results of Type One extracts of females for each species. These extracts yielded 2,6-dichlorophenol in concentrations nearly as great as those present in males. Additionally benzaldehyde and nonanoic acid were identified compounds in *A. hebraeum*, but not in *A. variegatum* females. Figure 6 compares the concentration of 2,6-dichlorophenol for female extracts. Note that 2,6-dichlorophenol was abundant in both species, but little is extracted by short duration or rinse extracts indicating limited surface availability. Also note that in the absence of Type 4 and 5 extracts for females, this comparison reflects true quantitative values rather than normalized values. Tentatively identified compounds for female extracts yielded two compounds not found in male extracts: dimethylethyl phenol and biphenyl. In both cases, these materials were very poorly represented and are not further discussed.

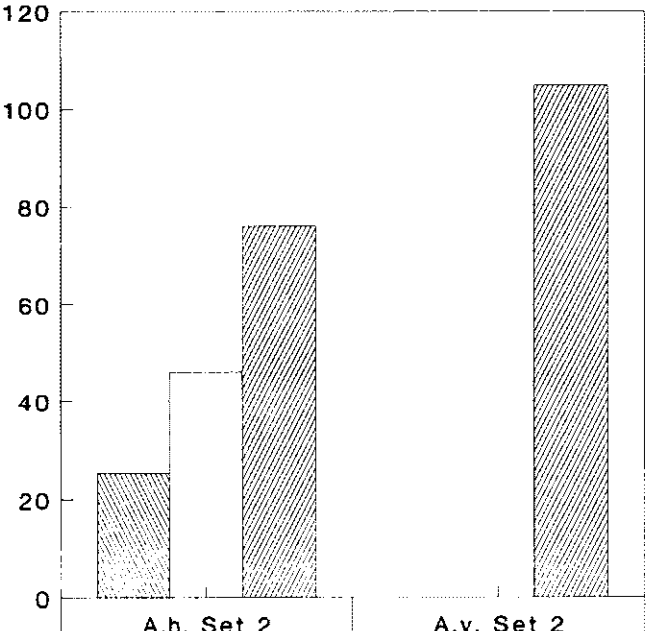
Legend: Figures 5 and 6

Figure 5 demonstrates relative concentrations of compounds identified and quantitated by Gas Chromatography / Mass Spectrometry for females of each species. Note that in both cases 2-nitrophenol is absent. Concentrations of 2,6-dichlorophenol are similar to those identified in males of each respective species and benzaldehyde, found only in the *A. hebraeum* female sample, is also similar in concentration to the amount found in males of this species.

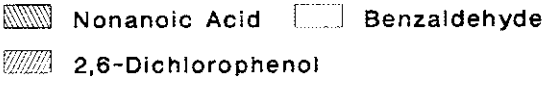
Figure 6 demonstrates the relative availability of 2,6-dichlorophenol for females in each species. Note that in the absence of type four and five extracts for females, these concentrations are presented as true values, rather than percents. In any case it appears that the availability of 2,6-dichlorophenol is low.

Figure Five

Female Type 1 Extracts[1]
Nanograms/FE[2], GC/MS Quantitation



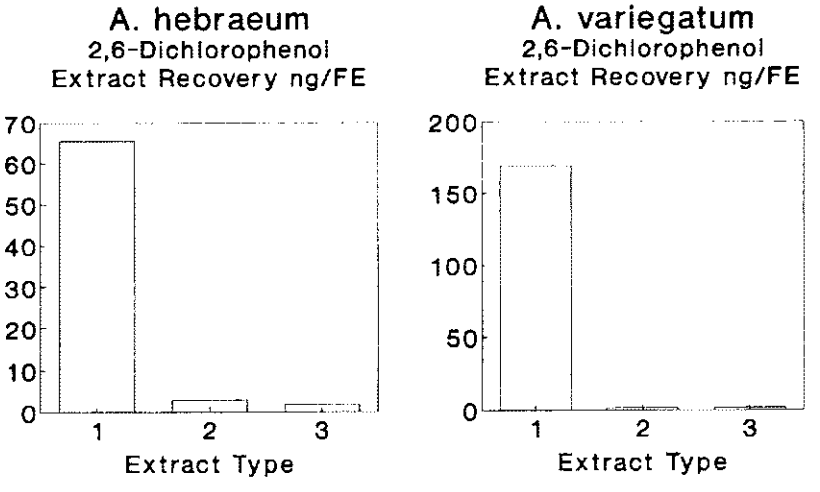
	A.h. Set 2	A.v. Set 2
Nonanoic Acid	25.4	0
Benzaldehyde	45.9	0
2,6-Dichlorophenol	76	105



1 Type 1 = Long Duration Extraction
2 FE = Female Equivalent

Figure Six

Extract Type Comparison, Females



Discussion

In examining the pheromonal components which may contribute to species specific host finding and mating behavior (if any) in these ticks, at least three possibilities may be considered. First, is there a single compound which by its presence or absence could provide an attractant or inhibitory effect on only one species? Second, is there a concentration related phenomenon demonstrated by compounds present in each species, i.e., ticks only respond when a certain threshold is reached. Finally, is there a unique blend of compounds which provides identification, attraction or inhibition to one species, a phenomenon well known in the Lepidoptera.

The results of the comparisons of the components in the extracts of males and females of the two *Amblyomma* species show that, in both species, the males are the primary producers of the AAAP, confirming the work of previous authors (Schoni et al., 1984; Apps et al., 1988; Lusby et al., 1991). In terms of chemical composition, the substituted phenols and organic acids produced by the males of the two species show a high degree of similarity: 2-nitrophenol, methyl salicylate, nonanoic acid, 2,6-dichlorphenol and benzaldehyde were identified in the extracts in both cases. The most abundant of these compounds, 2-nitrophenol, is the primary long-range attractant (Norval et al., 1991a), although it also stimulates aggregation (Norval et al., 1993) and attachment (Norval et al., 1991b). It is produced in greater quantities by *A. variegatum* than by *A. hebraeum*. Previous studies (Lusby et al., 1991) also support the conclusion that 2-nitrophenol is more abundant in *A. variegatum* than in *A. hebraeum*. Whether

these differences have any biological significance is unclear. In field trials in which ticks were exposed to test compounds at a distance of four meters, 2-nitrophenol attracted adults of *A. variegatum* and *A. hebraeum* more or less equally (40.7 and 44.8 percent respectively) (Norval et al., 1991a). Similar results were observed when natural extracts or synthetic AAAP was tested with adults of these two species (Yunker and Norval, 1991). Although possible dose-response relationships were not tested in this study, it appears that 2-nitrophenol serves as a general attractant. The observed differences in its concentrations may affect the range of pheromone signaling in the natural environment but not the behavior of the recipient ticks.

The identified compound 2,6-dichlorophenol which has been demonstrated to have long range attractant, aggregation and attachment stimulant activity (Norval et al., 1991a, 1991b, 1993) for both species. This compound presents no significant difference in concentration. Well known as a sex attractant pheromone in many other tick species, this compound is a poor candidate for species discrimination.

Methyl salicylate demonstrated a significant difference (when tested individually) in concentration at the 95 percent confidence level between the two species. The importance of this difference is supported by the very poor spectral match of its single identification in *A. hebraeum*. Although Apps et al. (1988) did not regard methyl salicylate as a significance component of the *A. hebraeum* pheromone, Lusby et al. (1991) reported this compound in *A. hebraeum*, although in lesser amounts than in *A. variegatum*. Lusby et al.'s (1991) findings

are consistent with our data. Despite the differences in relative and true concentrations between species, comparisons of attractiveness of compound mixes containing methyl salicylate show no conclusive preference between the species at least not at the concentrations tested (Norval et al., 1993).

Nonanoic acid, also identified in both species, was present at a significantly greater concentration in the *A. variegatum* samples as compared to the *A. hebraeum* samples. This compound is a long range attractant in both species (Norval et al., 1991a), but provokes a poor aggregation (Norval et al., 1993) and attachment response (Norval et al., 1991b) in either species. In addition to nonanonic acid, heptanoic and octanoic acid have also been identified from *A. hebraeum* in lesser amounts than the nonanoic acid. Heptanoic and octanoic acid have been previously identified by Apps et al. (1988) as volatile components associated with attached *A. hebraeum*. In that these compound have been identified only in one species, there is potential that these compounds may provide a segregating mechanism. However, the activity of these compounds is unknown. Like nonanoic acid, a known anti-microbial, (Norval, personal communication), these compounds may serve roles other than those of pheromones.

The last identified compound, benzaldehyde, was much more abundant in *A. hebraeum* than in *A. variegatum*. However, due to unequal variance between the species, this difference was not significant at the 95% confidence level. Nevertheless, the concentration data in

Figure 1 suggests that the differences may be significant in future studies with more replicates[4]. Benzaldehyde provokes a long range attractant response (Norval et al., 1991a) and also an attachment response (Norval et al., 1991b), especially for *A. hebraeum*. There is no apparent aggregation response to benzaldehyde alone in either species (Norval et al., 1993). However, a pheromone mixture described in the same study containing benzaldehyde was found to inhibit the aggregation of *A. variegatum*. Consequently, it is possible that small differences in the relative amount of benzaldehyde, acting synergistically with the other components, may function at the aggregating stage of the AAAP regulated behavior by inhibiting *A. variegatum*. Note that benzaldehyde is apparently more abundant in *A. hebraeum* than in *A. variegatum*. Also notable was the discovery that, in females, benzaldehyde was present only in the *A. hebraeum* sample. Species differentiation based on relative abundances of a single compound is a well known phenomena in metastriate ticks. The ticks *Hyalomma dromedarii* and *Hyalomma anatolicum excavatum* (Khalil et al., 1983; Silverstein et al., 1983) utilize the same host in the same geographic region and apparently rely on relative abundance of 2,6-dichlorophenol to prevent interbreeding. In that case, the concentration of 2,6-dichlorophenol produced by *H. dromedarii* females (approximately two to four times that of *H. anatolicum excavatum*) acts as a deterrent to interspecific males, inhibiting courtship and mounting. Examples of concentration based inhibition of similar components at very low concentrations are documented for the beetles *Ips pini* and *Ips paraconfusus (Coleoptera)* (Birch et al., 1980). In this case, as little as three percent of a pheromonal component of *I. paraconfusus* was identified as inhibiting the response

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[4] Number of replicates calculated to demonstrated an 80 percent difference at $\alpha=0.05$ is 35.

of *I. pini* to its own pheromone, an enantiomer of the inhibitor. It is also interesting to note that *I. paraconfusus* relies on a synergistic blend of three compounds, inactive as individuals, but strongly attractive to the insects when combined.

Relative availability of each of the identified compounds was ranked based on the presence of greater amounts in the type three extract. These data indicate that o-nitrophenol, benzaldehyde and nonanoic acid are of comparative importance. Due to lack of replication, no error component is available for the Type Three extract relative concentrations (Fig. 4); however, based on our single analysis, differences do appear to exist. Species specificity based on pheromone blend ratios has been demonstrated in the moths *Archips argyrospilus* and *Archips mortuanus (Tortricidae)* (Carde et al., 1977) . Inhabiting the same area, these two species affect reproductive isolation by detecting ratio differences in four shared pheromonal compounds.

With the exception of phenylacetaldehyde, none of the tentatively identified compounds have been evaluated as potential pheromones. Phenylacetaldehyde has been demonstrated as an aggregation stimulant (Norval et al., 1993) in both species, with somewhat greater activity in *A. hebraeum* than in *A. variegatum*. Another well represented, but tentatively identified, compound is dodecanal. This compound is much better represented in *A. hebraeum* and also strongly resembles known insect pheromones (Tumlinson and Teal, 1987). Tetradecanoic acid, found again only in *A. hebraeum*, is also similar in structure to many known insect pheromones; however, based on the observed effects of nonanoic acid, it is unlikely that this compound is a species discriminant. Other tentatively identified compounds, especially substituted phenols,

which are present in very low relative concentrations, are probably synthesis intermediates in the production of the primary pheromones. With the exception of phenylacetaldehyde, none of the compounds tentatively identified in this study have been consistently found between sample sets and none are known to affect tick behavior.

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Appendix 1
Quantitation Data

Amblyomma hebraeum, GC Quantitation, Sample Set 2

Males by Extract Type (ng/ME)					
Compound	Type 1	Type 2	Type 3	Type 4	Type 5
Benzaldehyde	4.9	50.1	1.6	17.8	66.9
Benzyl alcohol	0.0	4.9	Trace	0.0	19.8
Phenyl acetaldehyde	0.0	0.0	0.0	0.0	0.0
2-Nitrophenol	339	212	49.2	132	421
2,5-Dichlorophenol	8.8	0.0	0.0	0.0	0.0
2,6-Dichlorophenol	130	10.2	1.0	55.6	108
Methyl salicylate	0.0	0.0	0.0	0.0	0.0
Nonanoic acid FAME	1227	579	185	147	397

Females by Extract Type (ng/FE)					
Compound	Type 1	Type 2	Type 3	Type 4	Type 5
Benzaldehyde	5.5	0.8	0.6	NA	NA
Benzyl alcohol	0.0	Trace	0.0	NA	NA
Phenyl acetaldehyde	0.0	0.0	0.0	NA	NA
2-Nitrophenol	0.0	0.0	0.0	NA	NA
2,5-Dichlorophenol	0.8	0.0	0.0	NA	NA
2,6-Dichlorophenol	65.5	2.9	1.8	NA	NA
Methyl salicylate	0.0	0.0	0.0	NA	NA
Nonanoic acid FAME	14.4	0.0	2.1	NA	NA

NA = Not analyzed.

Amblyomma variegatum, GC Quantitation, Sample Set 2

Males by Extract Type (ng/ME)					
Compound	Type 1	Type 2	Type 3	Type 4	Type 5
Benzaldehyde	0.8	1.9	0.6	1.1	5.5
Benzyl alcohol	0.0	0.0	0.0	1.7	0.0
Phenyl acetaldehyde	1.5	0.0	0.0	Trace	3.1
2-Nitrophenol	3900	130	231	2620	2950
2,5-Dichlorophenol	1.0	0.0	0.0	0.0	0.0
2,6-Dichlorophenol	174	22.7	0.4	56.9	105
Methyl salicylate	225	78.4	26.0	163	876
Nonanoic acid FAME	4730	452	617	876	1970

Females by Extract Type (ng/FE)					
Compound	Type 1	Type 2	Type 3	Type 4	Type 5
Benzaldehyde	0.0	0.0	Trace	NA	NA
Benzyl alcohol	2.2	0.0	0.0	NA	NA
Phenyl acetaldehyde	0.0	0.0	0.0	NA	NA
2-Nitrophenol	0.0	0.0	0.0	NA	NA
2,5-Dichlorophenol	0.6	0.0	0.0	NA	NA
2,6-Dichlorophenol	169	1.6	1.6	NA	NA
Methyl salicylate	0.0	0.0	0.0	NA	NA
Nonanoic acid FAME	0.0	7.9	25.4	NA	NA

NA = Not analyzed.

GC/MS Quantitation, Sample Set 1				
Males (ng/ME)	*A. hebraeum*		*A. variegatum*	
Compound	Type 1	Type 3	Type 1	Type 3
Benzaldehyde	173	12.0	8.4	0.0
Benzyl alcohol	0.0	0.0	0.0	0.0
Salicylaldehyde	0.0	0.0	0.0	0.0
2-Nitrophenol	2380	69.0	4420	420
2,5-Dimethylphenol	0.0	0.0	0.0	0.0
2,4-Dichlorophenol	0.0	0.0	0.0	0.0
Methylsalicylate	31.8	0.0	1100	108
2,6-Dichlorophenol	61.0	0.0	226	0.0
Benzothiazole	0.0	0.0	0.0	0.0
Nonanoic Acid*	189	34.2	616	329
2,4,6-Trichlorophenol	0.0	0.0	0.0	0.0
4-Nitrophenol	0.0	0.0	0.0	0.0

* Quantitation suspect due to poor resolution of the non-esterified acid.

GC/MS Quantitation, Type One Extracts, Sample Set 2				
(ng/ME) (ng/FE)	*A. hebraeum*		*A. variegatum*	
Compound	Males	Females	Males	Females
Benzaldehyde	20.2	45.9	0.0	0.0
Benzyl alcohol	0.0	0.0	0.0	0.0
Salicylaldehyde	0.0	0.0	0.0	0.0
Phenylacetaldehyde	0.0	0.0	0.0	0.0
2-Nitrophenol	522	0.0	4100	0.0
2,5-Dimethylphenol	0.0	0.0	0.0	0.0
2,4-Dichlorophenol	0.0	0.0	0.0	0.0
Methylsalicylate	0.0	0.0	516	0.0
2,6-Dichlorophenol	112	76.0	165	105
Benzothiazole	0.0	0.0	0.0	0.0
4-Nitrophenol	0.0	0.0	0.0	0.0
Fatty Acid Methyl Esters				
2-Methylpropanoic	0.0	0.0	0.0	0.0
Butanoic	0.0	0.0	0.0	0.0
2-Methylbutanoic	0.0	0.0	0.0	0.0
Pentanoic	0.0	0.0	0.0	0.0
4-Methylpentanoic	0.0	0.0	0.0	0.0
Hexanoic	0.0	0.0	0.0	0.0
Heptanoic	35.7	0.0	0.0	0.0
Octanoic	93.2	17.2	0.0	0.0
Nonanoic	581	25.4	2650	0.0

Appendix 2
Compound Identifications

Amblyomma hebraeum
Sample Set 1

Identified Compounds

Benzaldehyde
2-Nitrophenol
Methyl Salicylate
2,6-Dichlorophenol
Nonanoic Acid

Tentatively Identified Compounds

Phenylacetaldehyde
Hexadecanoic Acid
Dodecanal

Amblyomma variegatum
Sample Set 1

Identified Compounds

Benzaldehyde
2-Nitrophenol
Methyl Salicylate
2,6-Dichlorphenol
Nonanoic Acid

Tentatively Identified Compounds

Hexadecanoic Acid
Dodecanal

Amblyomma hebraeum
Sample Set 2, Males

Identified Compounds

Benzaldehyde
2-Nitrophenol
2,6-Dichlorophenol
Heptanoic Acid
Octanoic Acid
Nonanoic Acid

Tentatively Identified Compounds

Bromochlorophenol
Naphthalene

Amblyomma hebraeum
Sample Set 2, Females

Identified Compounds

Benzaldehyde
2,6-Dichlorophenol
Octanoic Acid
Nonanoic Acid

Tentatively Identified Compounds

Dimethylethylphenol
Biphenyl

Amblyomma variegatum
Sample Set 2, Males

Identified Compounds

2-Nitrophenol
Methyl Salicylate
2,6-Dichlorophenol
Heptanoic Acid
Octanoic Acid
Nonanoic Acid

Tentatively Identified Compounds

Methylphenol
Bromochlorophenol
Naphthalene

Amblyomma variegatum
Sample Set 2, Females

Identified Compounds

2,6-Dichlorophenol

Tentatively Identified Compounds

Dimethylethylphenol
Biphenyl