POTENTIAL APPLICATION OF A GULF COAST TICK, *Amblyomma maculatum* Koch, AGGREGATION-ATTACHMENT-PHEROMONE FOR SURVEILLANCE OF FREE-LIVING ADULTS

A Thesis

by

HEE JUNG KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Entomology

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Approved as to style and content by:

Pete D. Teel (Chair of Committee)	Michael Longnecker (Member)
Jimmy K. Olson (Member)	Leon H. Russell (Member)
Kevin M. Heinz (Head of Department)	

December 2004

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ABSTRACT

Potential Application of a Gulf Coast Tick, *Amblyomma maculatum* Koch, Aggregation-Attachment-Pheromone for Surveillance of Free-Living Adults. (December 2004)

Hee Jung Kim B.S., Texas A&M University

Chair of Advisory Committee: Dr. Pete D. Teel

The aggregation-attachment-pheromone (AAP) of two geographic strains of the Gulf Coast tick, *Amblyomma maculatum* Koch, was investigated to evaluate practicality of using solid-phase-microextraction (SPME) in an AAP study of Gulf Coast tick. Solid-phase microextraction was used to compare the AAP production in two strains of fed male Gulf Coast tick and demonstrate and confirm the presence of AAP in bioassays.

A solid-phase-microextraction (SPME) headspace collection technique was sufficient to capture volatile organic compounds produced by fed and unfed male Gulf Coast ticks. Gas chromatography analysis revealed three major volatile organic compounds were produced in significantly greater amounts ($p < 0.05$) by fed males than those produced by unfed males. These volatile compounds were produced in significantly higher amount by the third day of feeding by male ticks. However, two of these volatiles remained relatively constant in their production while the primary volatile compound increased in its production until the eighth day of feeding by male Gulf Coast ticks. Also, the relative abundances of these three volatile organic compounds were different between Oklahoma and Texas strains of Gulf Coast ticks.

The activity of AAP from fed male Gulf Coast ticks was confirmed using two bioassay techniques. A petri dish bioassay revealed significantly higher numbers of female Gulf Coast ticks attracted to fed-males which also produced significantly greater amounts ($p < 0.001$) of volatile organic compounds detected by GC analysis. The Y-tube olfactometer bioassay revealed that significantly higher numbers of females responded to fed-males or to CO_2 when compared to purified air ($p < 0.001$), but the differences in female response to fed-males and CO_2 were not significant ($p < 0.391$ in Oklahoma strain and $p < 0.458$ in Texas strain). However, female responses to stimuli containing both fed-males and CO_2 were significantly higher when compared to either stimulus alone ($p < 0.001$).

To you almighty GOD, and my savior Jesus Christ,
apart from you I can accomplish nothing and I am nothing…

To my parents Joo-Myung and William McNamee,
If I ever am blessed enough to become courageous and loving as you are…

To my mentor Dr. Pete Teel,
For your ten years of nurturing and guidance.
I will consider my life to be a successful one if I only become a man who can reach out
to others with your sincerity and caring heart.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank GOD for his amazing grace upon me to send his only son, my savior, Jesus Christ to sacrifice his life in place of my sin. All that I do, and all that I will become, may you always lead the way.

I would like to show my deepest gratitude and thanks to my chair of committee, Dr. Pete Teel. Dr. Teel, aside from your teachings in academia, you have taught me so much more about both professional and personal people skills, not to mention the unearthly amount of patience you have shown me during my career as your graduate student. Thank you for your sincere guidance and friendship that started ten years back.

Dr. Olson, thank you for your always interesting lectures and life stories. I have never met anyone who showed so much love and passion for teaching as you do. You have inspired me to become a military entomologist who can serve others in need of help and I only hope that I can successfully carry on your amazing legacy throughout my career in the military.

Dr. Longnecker, you have saved me from several panic attacks during my research with your always calm and gentle presence. Thank you for all your earnest assistance and help for the past two years and I hope to work with you again someday.

Dr. Russell, thank you for your remarkable contributions to my academic career. Your classes taught me to think “outside the box” and gave me the confidence I needed to incorporate my own imagination into my research.

Otto Strey, some might refer to you as “one” of many lab technicians we have in our department. However, none (and I mean NONE) of my experiments would have been possible without your assistance on….well, literally, everything. So, you are “the” most essential lab technician in my book and always will be. Also, I sincerely thank you for all your support and friendship for the past two years. I surely am going to miss our conversations.

Dr. Howard Williams, thank you for your help and enormous assistance during my research and also for allowing me to freely work and use your lab facility and equipment. I hope to work with you someday to further search out the findings from this research.

I would like to thank my parents, Joo-Myung and Bill McNamee, and my baby sister, Soo Kim. Thank you for all your support throughout my career in college and graduate school. You are my safe haven and paradise on earth and for that, I am truly blessed. To all my friends all of whose names I cannot possibly list, I thank you for all the laughter and good times we had. And Jessica! Thank you for coming into my life at the most critical moment. I needed you to help keep my sanity.

Glené, I am grateful that you are here with me today, and it is a blessing to know that you will be here with me tomorrow.

Finally, I would like to thank each and every faculty and staff member in the Department of Entomology at Texas A&M University. You were my home away from home and my family away from my family for the past 6 years. I thank God for all of you and know that you will always be in my prayers.

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CHAPTER I

INTRODUCTION

Background

Ectoparasitic arthropods produce tremendous amount of damage to livestock throughout the world. The Gulf Coast Tick, *Amblyomma maculatum* Koch, is one of the most problematic ectoparasitic arthropods, especially in pastured cattle in United States, Caribbean region, and Central & South America (Bram et al. 2002). The Gulf Coast tick is a 3-host tick. Larvae and nymphs typically feed on small rodents and ground dwelling birds (Barker et al. 2004). Adults primarily feed on the ears of large mammals such as cattle. The lesions resulting from these feeding sites often cause irritation and secondary infections by other microorganisms and/or arthropods (e.g. primary and secondary screwworms). These adverse conditions may cause decreased feed consumption, and milk production and weight loss in infested cattle (Williams et al. 1977). In addition, the Gulf Coast tick is known as a one of the primary vectors of a protozoan parasite *Hepatozoon americanum,* which may cause a lethal disease in dogs (Ewing et al. 2002) and *Rickettsia parkeri*, a newly-recognized causal agent of spotted fever rickettsiosis in the United States (Paddock et al. 2004).

Gulf Coast tick and heartwater in U.S. Animal health concerns in the U.S were recently escalated due to the laboratory confirmation that the Gulf Coast tick is able to transmit the causal agent of heartwater (Suman et al. 2000). Heartwater is an acute

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disease of ruminants, such as cattle, sheep, goats, and deer, caused by the rickettsial organism *Ehrlichia* (formally known as *Cowdria) ruminantum*. The disease is characterized by pyrexia, followed by signs of nervous disorders, hydropericardium, and often-severe gastroenteritis (Suman et al. 2000, Bram et al. 2002). Mortality rates can be up to 90% in susceptible cattle, and there are no satisfactory treatments nor have reliable antemortem diagnostic protocols been developed (Uilenberg 1983, Camus et al. 1996).

The current recommended management tactic for adult Gulf Coast ticks on cattle is acaricide treatment of ears using dust, foam, or ear tag formulations. There are currently no tactics available for the surveillance of free-living adult ticks (Hoelscher et al. 2000). The only general population suppression strategies currently available are area-wide prophylactic acaricide application, host control, (i.e. population density reduction for wild deer) and vegetation modification (Mount et al. 1999). There are several complicating factors that decrease the efficiency of this control strategy. The geographic range of the Gulf Coast tick has significantly expanded into inland states such as Oklahoma and Kansas, (Williams 2002) and the seasonal activity of adult ticks is early spring in Oklahoma and Kansas, but late summer to fall in the coastal region of Texas, requiring different seasonal approaches for pastured cattle systems. Finally, the threshold for Gulf Coast tick management has been based on number of ticks on cattle after damage has already been initiated (Teel, personal communication). Thus, the development of an effective surveillance tactic for free-living adult Gulf Coast tick is important in order to establish an economic threshold for Gulf Coast tick management that does not involve host animals.

Surveillance and control of Gulf Coast tick in U.S. Surveillance tactics for the Gulf Coast tick have been attempted using CO_2 tick traps. The CO_2 tick traps were originally developed and used to provide temporal and spatial distribution data for the lone star tick, *Amblyomma americanum* (Bram 1978). These traps provided promising results and have been frequently used as the standard surveillance method of ticks (Bengaly 1987, Anderson et al.1998). Both excitation and orientation responses of *A. americanum* can be seen when using the CO_2 tick traps. In excitation responses, ticks raise the first pair of legs to use Haller's organ to detect for CO_2 and/or other stimuli in their surroundings and walk around in a circular and/or zigzag fashion. An orientation response is described as ticks directing their movement toward the source of CO_2 (Sonenshine 1991). However, using the conventional CO_2 tick traps alone presents a complicated problem for adult Gulf Coast ticks because CO_2 only seems to elicit excitation without orientation. This observation is supported by Bengaly (1987), where conventional CO_2 tick traps were shown as an unsatisfactory surveillance strategy for free-living Gulf Coast tick adults, with mark-recapture rates being as low as 2% in some cases.

Aggregation-Attachment-pheromone (AAP) of Gulf Coast tick. In field and laboratory settings, the presence of AAP produced by male tick plays a vital role in attracting other members in the population. AAP is a chemical signal that is emitted by the feeding-male ticks, which trigger aggregation responses in both male and female ticks (Gladney 1971). Recent studies involving ticks that are closely related to Gulf Coast tick, such as *A. variegatum* and *A. hebraeum,* showed that modifications of

conventional CO_2 tick trap by incorporating AAP extracts from a corresponding species resulted in a drastic improvement in capturing free-living ticks (Barré et al. 1997, Bryson et al. 2000). In addition, questing female Gulf Coast ticks have been observed to discriminate against cattle without feeding males, only attaching to cattle in a herd that have feeding males (Teel, unpublished). However, the AAP of Gulf Coast tick has not been isolated and characterized, nor has it been applied as a tactic in Gulf Coast tick management.

Research objectives. The overall objective of this research was to systemically characterize and analyze the AAP of Gulf Coast tick in terms of its chemical and biological properties. The three specific objectives were to: (1) Evaluate the practicality of using solid-phase-microextraction in AAP study of the Gulf Coast tick; (2) Compare and contrast AAP production in two strains of fed-male Gulf Coast tick in terms of the duration of feeding and; (3) Demonstrate and confirm the presence of AAP in bioassays.

CHAPTER II

APPLICATION OF SOLID PHASE MICROEXTRACTION WITH GAS CHROMATOGRAPHY ANALYSIS TO CHARACTERIZE THE AGGREGATION-ATTACHMENT-PHEROMONE OF FED AND UNFED ADULT GULF COAST TICKS

Introduction

The fundamental problems and challenges when conducting experiments with pheromone are finding the proper way to detect, capture, and analyze the target pheromone. Chemical characterizations of tick pheromones in general, present new challenges for developing a reliable method to collect volatile compounds for analysis. One common method of choice to obtain volatile compounds in ticks is the "whole body" extraction (Sonenshine 1991). The "whole body" extraction is recommended when the exact source and/or location of pheromones is not identified. It is a primitive but logical method whereby crude volatile mixtures from ticks are made by washing the ticks with an appropriate solvent. This "crude" solution is then separated by several different steps of an extraction procedure (Sonenshine 1991). This method has been utilized for the study of geographical effects on AAP in tropical bont tick, *Amblyomma variegatum* (Sonenshine et al. 2000), a closely related species of Gulf Coast tick. However, it has not yet been successfully applied in Gulf Coast tick pheromone studies. Therefore, the application of "whole body" extraction to study the AAP components of

Gulf Coast ticks may not be the most plausible method in chemical characterization of AAP compounds of Gulf coast tick since that there are no reliable methods of "whole body" extraction that have been established for this species. Consequently, a new technique that can provide faster and more reliable means of characterizing the volatile compounds of Gulf Coast tick was needed in the current study.

Solid phase microextraction (SPME) solved this problem by introducing the new ways to capture and analyze the tick pheromone. SPME is a relatively new technique that can be used to detect, extract, and collect a wide range of volatiles and semi volatiles organic compounds from impure matrices such as air, water, and soil (Hinshaw 2003). The mechanism of SPME relies upon the extraction of solutes from a sample into the polymer-coated, fused silica fiber SPME absorptive layer. SPME can capture sample by directly immersing the fiber into the appropriate liquid compounds (Hinshaw 2003). SPME also can be used to capture samples from the headspace of liquid and solid compounds (Agelopoulos and Pickett 1998). Finally, SPME has been successfully used for volatile compound studies in insects (Consôli et al. 2002 and Monnin et al. 1998). However, there is currently no reference in the literature confirming the use of SPME in tick pheromone studies. This study evaluates the practicality of using the solid-phase-microextraction technique to collect and analyze the volatile compounds of Gulf Coast tick. The objectives of this study were to: (1) Evaluate the application of the SPME headspace technique in differentiating the retention times of volatile compounds between fed and unfed Gulf Coast tick males, and (2) determine the optimum window of

AAP collection time from feeding male ticks using the SPME headspace collection technique.

Materials and Methods

Ticks. Gulf Coast ticks originating from Refugio County, Texas and Payne County, Oklahoma, and maintained in colony at the Tick Research Laboratory, Veterinary Medical Research Park, Texas A&M University were used in this project. These ticks were fed as immatures on commercial chickens and as adults on cattle under Animal Use Protocol No. 2002-208: Gulf Coast Tick Management Tactics for Pastured Cattle. Fed ticks were then placed in one-liter jars and covered with Chiffon Georgette fabric and secured using the accompanying lid with the center removed. The jars were placed in a glass incubator maintained at 20°C, 85% RH and 10:14 hour photophase.

Source of AAP. Fed adult males were used as the sources of AAP. Unfed 4 to 8 weeks old ticks from each strain were used as described in Animal Use Protocol No. 2002-208 Gulf Coast Tick Management Tactics for Pastured Cattle. In summary, cotton sleeves were glued around clipped areas on the backs of *Bos taurus* calves creating secure feeding sites for adult ticks. Then 30 unfed male ticks from each strain were released inside of two separate sleeves and allowed 24 hours to attach. Ticks were then allowed to feed for eight full days, duration previously found to result in AAP

production to insure the production of AAP (Gladney et al. 1974).

SPME collection. Ten Gulf Coast tick males from each strain were manually removed from a calf a full eight days after attachment and transferred into a 2-ml glass vial with a Teflon-lined septum screw cap. Control ticks were unfed Gulf Coast ticks from the maintenance incubators. Control ticks were used concurrently with fed counterparts and vials were prepared in a same manner for SPME collections. The SPME manual holder and sample vials were held in place using a ring stand and test tube clamp. The SPME manual holder was equipped with Carbowax®/Divinylbenzene (Supelco, Bellefornte, Pennsylviania, USA). The SPME holder was securely placed onto the stand so that the SPME fiber was immediately above the ticks inside of the vial when the fiber was extended (Fig 1). Three SPME collections of two hours duration each were sampled from each test and control vial. The collection intervals were 0-2 hours, 2-4 hours and 4-6 hours after tick removal from the calf. Separate SPME fibers were used for each collection, and the vials of both control and fed ticks were placed under the SPME apparatus and the collections were taken at the same time.

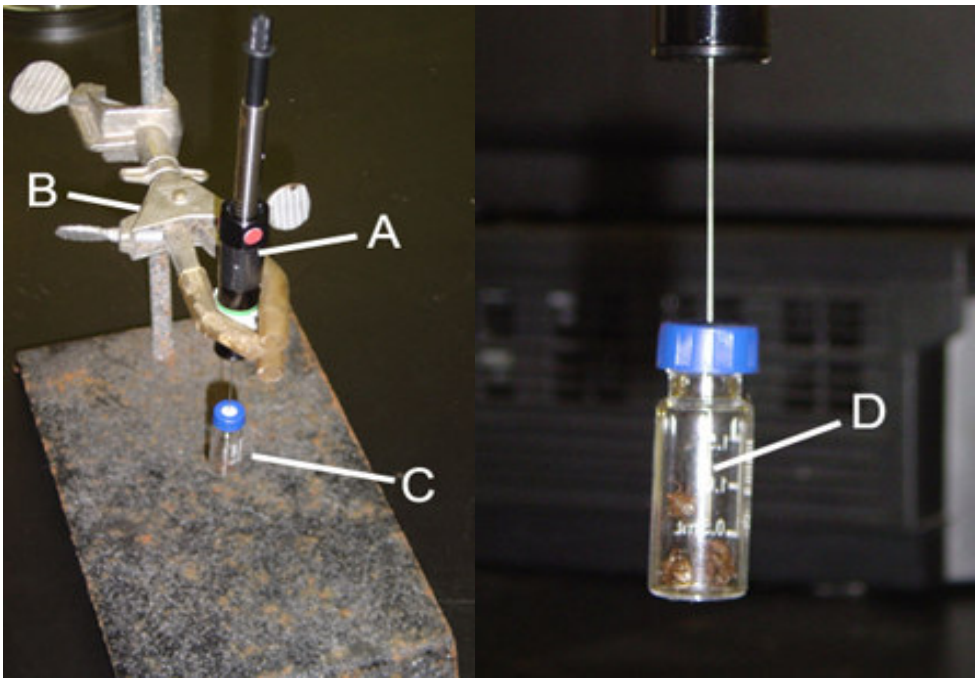


Figure 1. Solid phase microextraction headspace collection apparatus used for AAP Gulf Coast tick Koch, *Amblyomma maculatum.* The Solid phase microextraction manual holder for fiber (A) is held by the test tube holder on a ring stand (B), directly above the glass vial (C). The fiber is extended immediately above the ticks inside of the vial (D).

Gas chromatography. At the end of SPME collection, the SPME sample was immediately injected in splitless mode into a HP 6980 GC, equipped with a BP-20 carbowax column (25 m x .25 mm ID) (SGE, Ringwood, Australia) (Initial temperature = 50 °C for 5 min, to 150 °C at 15 °C /min, to 185 °C at 5 °C /min, to 225 °C at 20 °C /min, with 138 KPa He as the carrier gas).

Data analyses. The retention time and the area under detected peaks were used as the primary parameter signals to indicate the presence of the volatile compounds, since the nature of AAP, especially in terms of its composition (i.e. single compound vs. multi-blend of compounds) is undetermined.

Results

Volatile chemical compounds were obtained with retention times ranging from 8 to 24 min. The materials with retention time greater than 18 min. were likely long-chain fatty acids and compounds with low vapor pressure and were, therefore, not likely to be associated with pheromone activity. Thus, large peaks detected between retention times greater than 8 min. and less than 20 min. for fed ticks and not recorded for unfed ticks were used in the comparison analyses.

There were several large peaks that were only associated with the fed males of both tick strains included in this study. These peaks were found at retention times of 9.7, 10.7, 11.0, 11.1, 11.4, 12.6, 15.0, 15.1, and 15.6 min. The most dramatic differences obtained by gas chromatography were among 3 compounds with peaks at retention times of 10.7, 11.4 and 12.6 min. The most dominant peak occurring in both the Oklahoma and Texas strain of ticks was at the retention time of 11.4 minutes (Table 1). However, the value of these three peaks found at retention time of 10.7, 11.4 and 12.6 minutes and their relative abundances (ratio of amount produced) were different for each tick strain. These peaks had greater areas under their respective curves for the Texas strain ticks as compared to those for the Oklahoma strain ticks. The corresponding rates of the peaks found at retention time of 10.7, 11.4 and 12.6 min. were approximately 1:17:1.5 for the Oklahoma strain ticks and 1:5.3:1.7 for the Texas strain ticks (Figure 2).

Table 1. Gas chromatography retention time and area under curve (pKa) for peaks of interest associated with volatile compounds collected from unfed and fed male Gulf Coast ticks, *Amblyomma maculatum* Koch, by a solid phase microextraction headspace collection technique.

RETENTION TIME (Minute)	TEXAS Unfed (pA*s)	TEXAS Fed (pA*s)	OKLAHOMA Unfed (pA*s)	OKLAHOMA Fed (pA*s)
9.7	0	3402	533	0
10.7	8622	41859	5014	9264
11.0	3786	10253	4012	15675
11.1	0	0	2029	3568
11.4	3097	223050	0	156455
12.6	0	72769	2660	13991
15.0	1139	1326	1392	0
15.1	1103	0	2606	2715
15.6	0	0	1811	2936

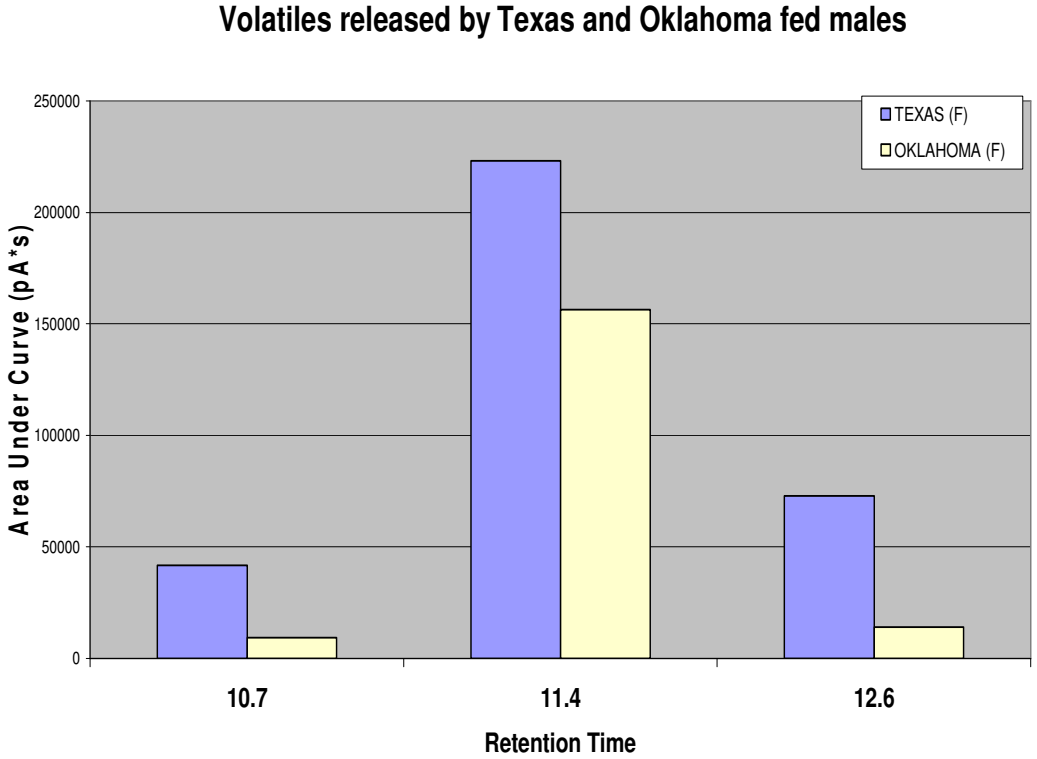


Figure 2. Volatile compounds captured at gas chromatography retention times of 10.7, 11.4 and 12.6 min. from fed Oklahoma and Texas Gulf Coast ticks, *Amblyomma maculatum* Koch.

There was an inverse relationship between areas under curve obtained in the GC analysis and sample collection times. Results summarized in Table 2 show that the volatile compounds at the largest peaks declined in its amount produced as the length of the time after a fed tick was removed from host increased. Figure 3 shows for Texas strain, that a decline in the major peak (retention time 11.4 minute) occurs very rapidly with the second interval (2-4 hour) collection with approximately 10 % of initial collection (0-2 hour). In contrast, the major peak (retention time 11.4 minute) declined more slowly with the third interval collection (4-6 hour) at approximately 50 % of the second interval collection (2-4 hour). In Oklahoma strain, the major peak (retention time 11.4 minute) declined more slowly and approximately 50 % of initial collection was captured by the third interval collection.

Table 2. Gas chromatography retention times and areas under curves (pKa) for peaks of interest associated with volatile compounds collected from fed male Gulf Coast ticks, *Amblyomma maculatum* Koch, at three different collection intervals.

	Texas Strain			Oklahoma Strain		
Retention time	SPME Collection 0 to 2 hrs	SPME Collection 2 to 4 hrs	SPME Collection 4to 6 hrs	SPME Collection 0 to 2 hrs	SPME Collection 2 to 4 hrs	SPME Collection 4 to 6 hrs
10.7	41859	4694	4214	9264	8830	7634
11.4	223050	25789	17207	156455	124561	84841
12.6	72769	2121	1175	13991	6789	5615

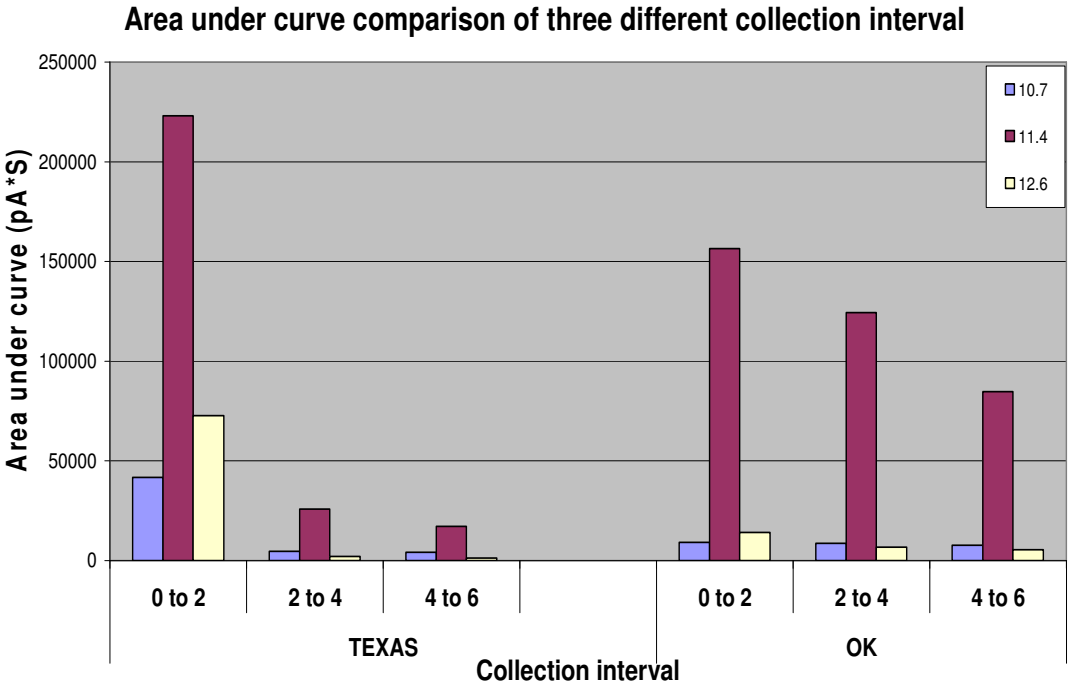


Figure 3. Comparison of areas under curve for volatile compounds found at retention time 10.7, 11.4 and 12.6 minutes of Texas and Oklahoma fed Gulf Coast ticks, *Amblyomma maculatum* Koch, at three collection intervals.

Discussion

This study demonstrated that the SPME headspace collection technique was sufficient for detection of volatile compounds from ticks by GC analysis. The SPME headspace collection technique was able to detect difference in volatile compounds between unfed and fed ticks, and further more, demonstrated that the amount of primary volatile compounds decline the longer fed males are held after removal from the host blood source. Also, the SPME headspace collection technique may be ideal for a field study because it is highly mobile and compact. Most importantly, the SPME technique does not kill the ticks used when sampling for volatile compounds. This advantage may

provide additional opportunities for research such as sampling for the release of volatile compounds and revealing physiological development using same individual tick specimen.

There were three peaks found in the SPME sample collection that were distinctively higher in fed males. In both strains peaks found at the retention time 10.7, 11.4, and 12.6 appear to be higher in their areas under the curve when compared to their corresponding peaks in unfed male ticks. However, further study with much higher replication was needed in order to establish whether differences between these peaks are statistically significant. The Chapter III will discuss in-depth study of volatile compounds released by the fed and unfed male Gulf Coast tick.

Volatile compounds released by the Gulf Coast tick may not have a constant rate of release. Figure 3 shows that quantities of volatile compounds detected at retention times of 10.7, 11.4 and 12.6 minutes decreased as time of tick separation from the blood meal source increased.

The data from SPME head space collection samples of Gulf Coast ticks raised additional topic of interest that are investigated in Chapter III. First, the productions of volatile compounds detected is actually due to feeding of blood by ticks or simply ticks being in contact with the host animal needed to be distinguished. Also, repeatable detection of the volatile compounds from replicated sets of ticks should be demonstrated in order to validate results from this study. The influence of the duration of feeding times (days allowed to feed after attachment) and the amount and blend of volatile compounds produced were examined in the study described in Chapter III.

CHAPTER III

STIMULUS AND DYNAMICS OF VOLATILE ORGANIC COMPOUND PRODUCTION IN TWO STRAINS OF FED MALE GULF COAST TICK

Introduction

The prior study described in the previous chapter (Chapter II) demonstrated the presence of detectable volatile organic compounds specific to fed adult male Gulf Coast ticks and determined that these volatile compounds decline two hours after tick removal from the host. The SPME headspace collection technique with GC analysis was determined as the valid method to capture and detect compounds that are potentially involved in the aggregation attachment pheromone (AAP) of the Gulf Coast tick. This success provided the method and background to pursue questions regarding the stimulus and dynamics of volatile compound production in Gulf Coast tick strains from Oklahoma and Texas.

The studies in this chapter focused on the questions raised in the previous study (Chapter II). One of these questions dealt with the demonstration that the volatile compounds found at the retention times of 10.7, 11.4 and 12.6 min. could be repeatedly-detected in fed ticks in statically viable numbers to further establish the associations between these volatile compounds and feeding male ticks. Also, in the prior study (Chapter II), the possibility of production of detected volatile organic compounds due just to male ticks being in contact with host animal but not feeding on them was not addressed. Therefore, an investigation focusing on the production of these volatile

compounds by the male ticks that were exposed to host animals but unfed was conducted. Additionally, the production and the dynamics of these volatile compounds are investigated in this study.

Results from previous studies (Gladney et al. 1974. and Rechav et al. 2000) indicate that 8 days of feeding after the male tick has been attached to its host are required in order for the production of AAP in Gulf coast tick. In the current preliminary studies of the use of SPME headspace sample technique described in chapter II indicated that volatile compounds at retention times 10.7, 11.4 and 12.6 min. were also detected from male tick after only 3 days of feeding. Consequently, SPME headspace collections from 3- and 8-day fed male ticks were compared.

Furthermore, the profiles of volatile organic compounds described for GC analysis by using the SPME headspace collection technique (Chapter II) and from fed male Gulf Coast ticks seemed different between Oklahoma and Texas tick stains. The volatile compound found at retention time of 11.4 min. was the most dominate peak found in both strains, but the amount collected was different by the factor of approximately 12 X and the ratio of these three peaks (found at retention times of 10.7, 11.4 and 12.6 minutes) seem different in each strain. Difference in pheromone composition is a phenomenon often found among “intra-species” of insects and other arthropods (Roberts and Uetz. 2004, Coates and Hellmich 2003, and Regnier and Law 1968). However, there are no studies that suggest such differences occur in ticks. In fact, Sonenshine et al. (2000) reported that the geographic variation of in tropical bont tick, *Amblyomma variegatum,* a closely-related species of Gulf Coast tick, and there is

no evidence of geographic intra-specific variation in pheromone composition for this particular tick species.

This chapter reports results of studies designed to test the hypothesis that (1) volatile compounds of interest (found at retention times of 10.7, 11.4 and 12.6 min. by SPME headspace collection and GC analysis) are produced in response to tick feedings, (2) production of these volatile compounds in ticks fed for 3 and 8 days are different, and (3) that the ratio of these volatile compounds vary between Oklahoma and Texas tick strains.

Materials and Methods

Ticks. Gulf Coast ticks originating from Refugio County, Texas, and Payne County, Oklahoma, and maintained in colony at the Tick Research Laboratory, Veterinary Medical Research Park, Texas A&M University were used in this project. These ticks were fed as immatures on commercial chickens and as adults on cattle under Animal Use Protocol No. 2002-208: Gulf Coast Tick Management Tactics for Pastured Cattle. Fed ticks were then placed in one-liter jars and covered with Chiffon Georgette fabric and secured using the accompanying lid with the center removed. The jars were placed in a glass incubator maintained at 20°C, 85% RH and 10:14 hour photophase.

Host animals. Two calves were used for each strain of Gulf Coast ticks for a total of four *Bos taurus* calves. Seven orthopedic stockinet sleeves were glued to the surrounding hair of clipped circular area on the backs of each calf creating secure feeding sites for the Oklahoma strain adult male ticks. For the Texas strain, five sleeves

were prepared on the backs of the remaining two cows creating secure feeding sites for adult male ticks. Each host animal served as a block variable in the experimental design. Five treatment groups were established for each tick strain as described in the next subsection.

Tick treatment groups. To obtain unfed adult male ticks held in close proximity to the host but not allowed to feed, a double screened petri dish was used to hold male ticks from being able to feed on calves while placed on the calf (Figure 4). In general, a five cm diameter holes were made on both top and bottom of ten cm diameter plastic Petri dish. Chiffon Georgette fabrics was glued on to each openings of Petri dish from both sides (from inside and outside) to ensure close contact to host animal by ticks was possible without their being able to feed. One hundred eighty Oklahoma strain and 100 Texas strain unfed males were placed on each corresponding calves by confining ticks in the double screened Petri dishes and holding them next to the calves in a stockinet sleeves. To obtain fed males, ticks were placed in stockinet sleeves on host skin. The total infestations were comprised of 30 unfed Oklahoma stain ticks released in 6 stockinet (Figure 5) and 50 unfed Texas stain ticks in 4 stockinet sleeves per (Figure 6). Two calves were used per study of each strain of ticks and all ticks were allowed 24 hours to attach. For both stains of ticks, half of the ticks were allowed to feed for 3 days and remaining ticks were allowed to feed for 8 days before there were manually removed from the host for analyses.

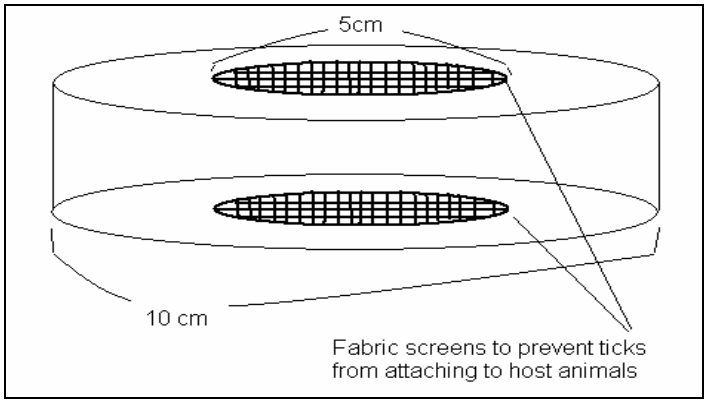


Figure 4. Diagram of double screened petri dish for exposed unfed male ticks used for solid-phase microextraction headspace collection.

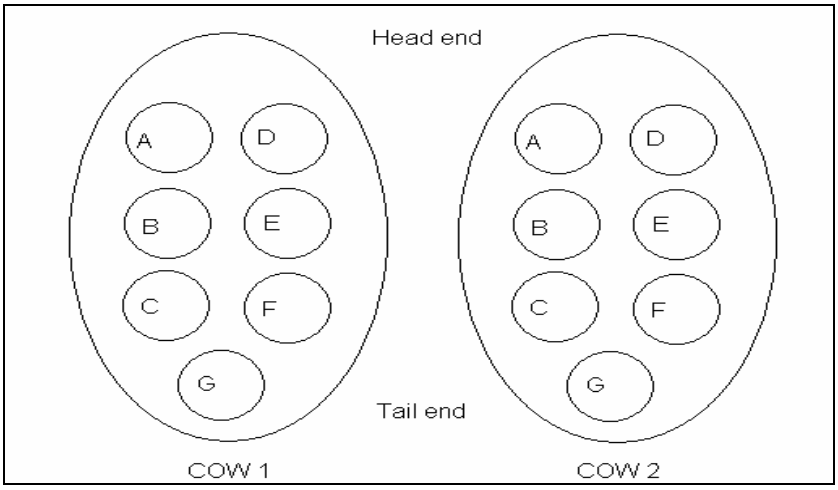


Figure 5. Host infestation arrangements of Oklahoma strain Gulf Coast tick, *Amblyomma maculatum* Koch, for solid-phase-microextraction headspace collections. Thirty unfed, 4- to 8-week old Oklahoma strain Gulf Coast ticks were placed in the stockinet sleeves at locations A to F for each cow. Ninety unfed 4- to 8-week old Oklahoma strain Gulf Coast ticks were placed inside of double-screened Petri dish and placed in stockinet sleeve at the location G for each cow to generate the condition where ticks were able to maintain close contact with the host animal but not able to feed on it (exposed unfed ticks). Ticks at locations A, B, and C were allowed to feed for 3 days and ticks at locations D, E and F were allowed to feed for 8 days before solid-phase-microextraction headspace collection samples were taken.

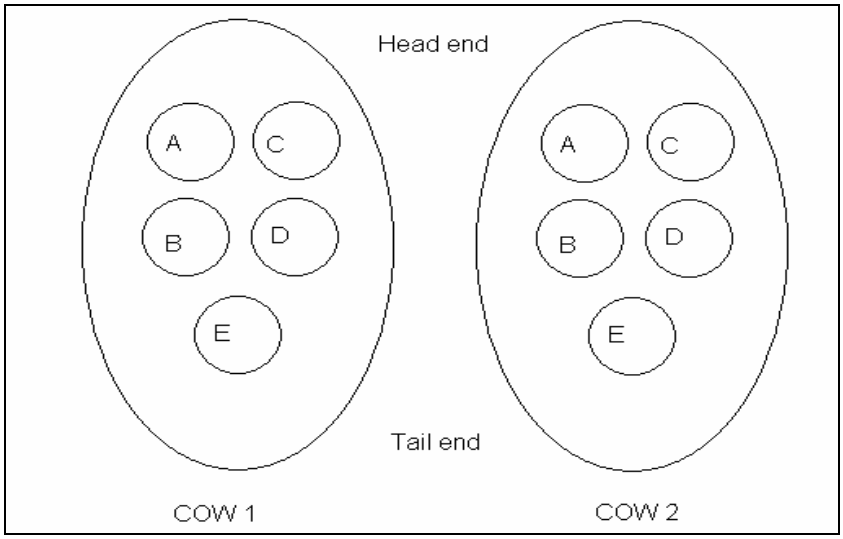


Figure 6. Host infestation arrangements of Texas strain Gulf Coast ticks, *Amblyomma maculatum* Koch, for solid-phase-microextraction headspace collections. Fifty unfed 4-to 8-week old Texas strain Gulf Coast ticks were placed in the stockinet sleeves at locations A to D for each cow. One hundred unfed 4-to 8-week old Texas strain Gulf Coast ticks were placed inside of double-screened Petri dish and placed in stockinet sleeve at the location E for each cow to generate the condition where ticks were able to maintain close contact with the host animal but not able to feed on it (exposed unfed ticks). Ticks at locations A and B were allowed to feed for 3 days and ticks at locations C and D were allowed to feed for 8 days before solid-phase-microextraction headspace collection samples were taken.

SPME collection. Ten feeding males of each tick strain were collected after 3 and 8 days attachment and transferred to a 2-ml silanized glass vial with Teflon-lined septum screw cap. Correspondingly, 10 male control ticks from the incubator cohort and from the exposed-contact-but-unfed cohort were prepared in a same manner. Eighteen SPME headspace collection samples were generated per each treatment (incubator/ 3 day fed/ 3 day exposed unfed/ 8 day fed/ and 8 day exposed unfed) were prepared for the Oklahoma strain ticks and 10 SPME headspace collection samples were generated per

each treatment for the Texas strain ticks (The number of replications were larger in the case of the Oklahoma strain due to the short supply of ticks for the Texas strain).

The SPME manual holds and sample vials were held in place using a ring stand and test tube clamp. The SPME manual holder was equipped with Carbowax®/Divinylbenzene (Supelco, Bellefornte, Pennsylviania, USA). The SPME holder was securely placed onto the stand so that the SPME fiber was immediately above the ticks inside of the vial when the fiber was extended. The SPME collection period was 2 hours immediately after the ticks were manually removed from the host animal as described in Chapter I.

Gas chromatography. At the end of SPME collection, each SPME sample was immediately injected in spliteless mode into a HP 6980 GC, equipped with a BP-20 carbowax column (25 m x .25 mm ID) (SGE, Ringwood, Australia) (Initial temperature = 50 °C for 5 min; to 150 °C at 15 °C /min; to 185 °C at 5 °C /min; to 225 °C at 20 °C /min; 138 KPa He as the carrier gas). The data were collected for the 3 peaks of interest defined in Chapter II (i.e. volatile compounds found at retention times of 10.7, 11.4 and 12.6 min.).

Data analyses. Treatments were: (1) unfed control ticks from the incubator, (2) 3-day-exposed-unfed ticks, (3) 3-day-fed ticks, (4) 8-day-exposed unfed ticks, and (5) 8-day-fed ticks. Data for the 5 treatment levels were arranged as a randomized block design with calves as the blocking variable. One way ANOVA test in the Statistical Analysis System (SAS) program (SAS Institute Inc.) was used to analyze the data and test the following hypothesis: (1) The production of three volatile organic compounds

described in Chapter II (detected at retention time of 10.7, 11.4 and 12.6 minutes) are due to feeding, (2) The three volatile organic compounds of interest are produced insignificantly higher amount after 3 days of feeding rather than at 8 days of feedings and, (3) the relative amounts (ratio) of three volatile compounds are different between the Texas and Oklahoma strain of ticks.

Results

Tables 3, 4 and 5 show the differences in production of volatile organic compounds produced by an Oklahoma stain of Gulf Coast tick between all combinations of treatments. Significantly greater amounts three volatile compounds were produced by 3- and 8-day fed males when compared to unfed incubator ticks and 3- and 8-day exposed unfed ticks. In addition, the amounts of these volatile compounds produced were not significantly different ($p < 0.05$) among unfed-incubator ticks and 3- and 8-day exposed unfed ticks (Fig. 7).

Table 3. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at gas chromatography retention times of 10.7 minutes in five treatment groups of an Oklahoma strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Treatment comparison	Difference between mean area under curve (pA * S)	Simultaneous 95% Confidence Limits (pA * S)
3-day fed and 8-day fed	20.53	9.00 32.06 ***[3]
3-day fed and 8-day exposed [1]	32.18	20.65 43.71 ***
3-day fed and unfed [2]	33.79	23.81 43.78 ***
3-day fed and 3-day exposed	37.36	25.83 48.89 ***
8-day fed and 8-day exposed	11.65	0.12 23.18 ***
8-day fed and unfed	13.26	3.28 23.25 ***
8-day fed and 3-day exposed	16.83	5.30 28.36 ***
8-day exposed and unfed	1.611	-8.374 11.596
8-day exposed and 3-day exposed	5.181	-6.349 16.710
Unfed and 3-day exposed	3.57	-6.415 13.554

[1] Ticks were keep in close contact with the host animal but not allowed to feed bloodmeal. [2] Ticks were never exposed to host animal and kept in a incubator. [3] Comparisons were significant at the 0.05 level

Table 4. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 11.4 minutes in five treatment groups of an Oklahoma strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Treatment comparison	Difference between mean area under curve (pA * S)	Simultaneous 95% Confidence Limits (pA * S)
3-day fed and 8-day fed	101.86	49.62 154.10 ***[3]
3-day fed and 8-day exposed [1]	90.13	37.89 142.37 ***
3-day fed and unfed [2]	87.94	42.70 133.18 ***
3-day fed and 3-day exposed	88.13	35.89 140.36 ***
8-day fed and 8-day exposed	192	139.76 244.23 ***
8-day fed and unfed	189.80	144.56 235.04 ***
8-day fed and 3-day exposed	189.99	137.75 242.23 ***
8-day exposed and unfed	2.2	-43.04 47.44
8-day exposed and 3-day exposed	2.01	-50.23 54.25
Unfed and 3-day exposed	0.19	-45.05 45.43

[1] Ticks were keep in close contact with the host animal but not allowed to feed bloodmeal. [2] Ticks were never exposed to host animal and kept in a incubator. [3] Comparisons were significant at the 0.05 level

Table 5. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 12.6 minutes in five treatment groups of an Oklahoma strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Treatment comparison	Difference between mean area under curve (pA * S)	Simultaneous 95% Confidence Limits (pA * S)
3-day fed and 8-day fed	22.94	9.47 36.40 ***[3]
3-day fed and 8-day exposed [1]	32.51	19.05 45.98 ***
3-day fed and unfed [2]	21.02	9.36 32.68 ***
3-day fed and 3-day exposed	18.85	5.39 32.32 ***
8-day fed and 8-day exposed	9.58	-3.89 23.04 ***
8-day fed and unfed	1.92	-9.74 13.58 ***
8-day fed and 3-day exposed	4.09	-9.38 17.55 ***
8-day exposed and unfed	11.50	-0.16 23.16
8-day exposed and 3-day exposed	13.66	-0.2 27.13
Unfed and 3-day exposed	2.17	-9.50 13.83

[1] Ticks were keep in close contact with the host animal but not allowed to feed bloodmeal. [2] Ticks were never exposed to host animal and kept in a incubator. [3] Comparisons were significant at the 0.05 level

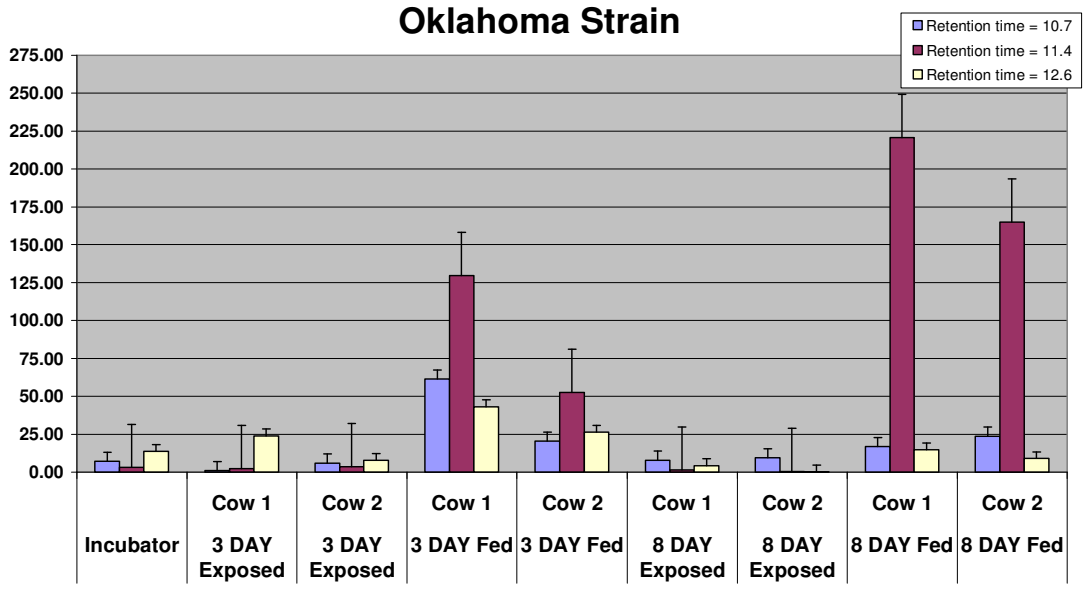


Figure 7. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 10.7, 11.4 and 12.6 minutes in five treatment groups of an Oklahoma strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Tables 3, 4 and 5 show the differences in production of volatile organic compounds produced by the Texas stain of Gulf Coast ticks between all combinations of experimental treatments. Significantly greater amounts three volatile compounds were produced by 3- and 8-day fed males when compared to unfed incubator ticks and 3- and 8-day exposed unfed ticks. In addition, the amounts of these volatile compounds produced were not significantly different among unfed-incubator ticks and 3- and 8-day exposed unfed ticks (Fig 8).

Table 6. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 10.7 minutes in five treatment groups of a Texas strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Treatment comparison	Difference between mean area under curve (pA * S)	Simultaneous 95% Confidence Limits (pA * S)
3-day fed and 8-day fed	7.873	-1.075 16.821
3-day fed and 8-day exposed [1]	17.363	8.415 26.311 ***[3]
3-day fed and unfed [2]	17.198	6.239 28.156 ***
3-day fed and 3-day exposed	17.477	8.529 26.425 ***
8-day fed and 8-day exposed	25.236	16.288 34.297 ***
8-day fed and unfed	25.071	14.112 36.029 ***
8-day fed and 3-day exposed	25.35	16.402 34.297 ***
8-day exposed and unfed	0.166	-10.793 11.125
8-day exposed and 3-day exposed	0.113	-8.835 9.061
Unfed and 3-day exposed	0.279	-10.680 11.238

[1] Ticks were keep in close contact with the host animal but not allowed to feed bloodmeal. [2] Ticks were never exposed to host animal and kept in a incubator. [3] Comparisons were significant at the 0.05 level

Table 7. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 11.4 minutes in five treatment groups of a Texas strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Treatment comparison	Difference between mean area under curve (pA * S)	Simultaneous 95% Confidence Limits (pA * S)
3-day fed and 8-day fed	124.05	79.30 168.79 ***[3]
3-day fed and 8-day exposed [1]	10.46	-34.28 55.21 ***
3-day fed and unfed [2]	10.9	-43.90 65.71 ***
3-day fed and 3-day exposed	11.29	-33.45 56.04 ***
8-day fed and 8-day exposed	134.51	89.77 179.26 ***
8-day fed and unfed	134.95	80.15 189.75 ***
8-day fed and 3-day exposed	135.34	90.60 180.09 ***
8-day exposed and unfed	0.44	-54.36 55.24
8-day exposed and 3-day exposed	0.83	-43.91 45.58
Unfed and 3-day exposed	0.39	-54.41 55.19

[1] Ticks were keep in close contact with the host animal but not allowed to feed bloodmeal. [2] Ticks were never exposed to host animal and kept in a incubator. [3] Comparisons were significant at the 0.05 level

Table 8. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 12.6 minutes in five treatment groups of a Texas strain Gulf Coast tick, *Amblyomma maculatum* Koch.

Treatment comparison	Difference between mean area under curve (pA * S)	Simultaneous 95% Confidence Limits (pA * S)
3-day fed and 8-day fed	0.817	-15.253 16.886
3-day fed and 8-day exposed [1]	16.785	0.716 32.854 ***[3]
3-day fed and unfed [2]	15.864	3.817 35.545 ***
3-day fed and 3-day exposed	20.094	4.025 36.164 ***
8-day fed and 8-day exposed	15.968	0.101 32.038 ***
8-day fed and unfed	15.047	4.634 34.728 ***
8-day fed and 3-day exposed	19.277	3.208 35.347 ***
8-day exposed and unfed	0.921	-18.760 20.602
8-day exposed and 3-day exposed	3.309	-12.760 19.379
Unfed and 3-day exposed	4.23	-15.451 23.911

[1] Ticks were keep in close contact with the host animal but not allowed to feed bloodmeal. [2] Ticks were never exposed to host animal and kept in a incubator. [3] Comparisons were significant at the 0.05 level

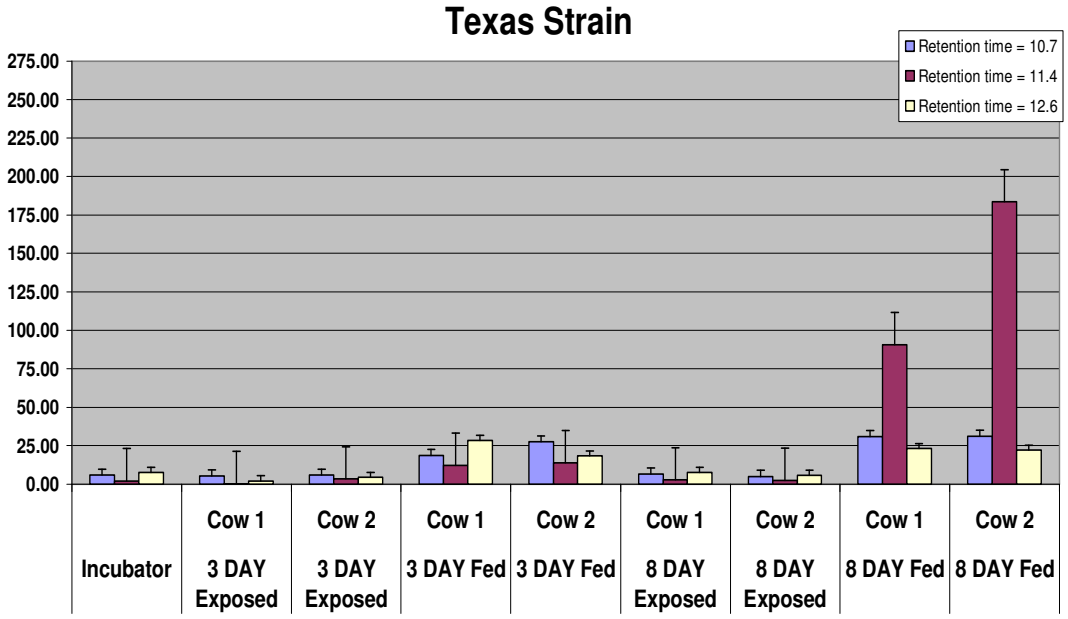


Figure 8. Comparisons of a volatile organic compound captured by solid-phase-microextraction and detected at a gas chromatography retention time of 10.7, 11.4 and 12.6 minutes in five treatment groups of a Texas strain Gulf Coast tick, *Amblyomma maculatum* Koch.

In addition, the volatile compound found at the retention times of 10.7 and 12.6 min. were produced in significantly greater amounts by 3-day fed ticks than those produced by 8-day fed ticks in Oklahoma strain ticks (Table 4 and 9). Also, the differences between the amounts produced were significant (Table 3 and 5). The volatile compound found at retention time 11.4 minutes was produced significantly greater amounts by 8-day fed ticks than that produced by 3-day fed ticks (Table 9). The difference in the amount produced was also significant (Table 4). The relative abundance of the volatile compound detected at the retention time of 11.4 min. was produced up to

13 times (8-day fed ticks on cow 2) greater than the other volatile compounds detected at the retention times of 10.7 and 12.6 min. (Table 9).

Table 9. Volatile compound produced by 3-day and 8-day fed Oklahoma strain male Gulf Coast tick, *Amblyomma maculatum* Koch.

		Mean area under curve (pA*s ± S.E.)		
Days allowed to feed	Host animal	10.7 minute	11.4 minute	12.6 minute
3-day fed	cow 1	61.31 ± 35.48	129.58 ± 116.71	43.14 ± 23.17
3-day fed	cow 2	23.83 ± 20.59	52.52 ± 43.78	26.33 ± 17.79
8-day fed	cow 1	16.77 ± 13.03	220.78 ± 181.73	14.70 ± 8.46
8-day fed	cow 2	20.35 ± 27.07	165.05 ± 170.02	8.90 ± 6.90

The volatile compound detected at the retention time of 11.4 min. was produced in significantly greater quantities by the 8-day fed ticks than those produced by the 3-day fed ticks in the Texas strain Gulf coast tick (Tables 5 and 10). The productions of volatile compounds detected at retention times of 10.7 and 12.6 min. by the 3-day fed ticks were not significantly different (Tables 6 and 8). Finally, the relative abundance of the volatile compound detected at retention time of 11.4 was produced up to 6 times (e.g. 8 day fed ticks on cow 2) greater then other volatile compounds detected at retention time of 10.7 and 12.6 min. (Table 10).

Table 10. Volatile compound produced by 3-day and 8-day fed Texas strain male Gulf Coast tick, *Amblyomma maculatum* Koch.

		Mean area under curve (pA*s ± S.E.)		
Days allowed to feed	Host animal	10.7 minute	11.4 minute	12.6 minute
3-day fed	cow 1	18.78 ± 9.82	12.33 ± 6.42	23.24 ± 25.38
3-day fed	cow 2	27.70 ± 12.72	13.94 ± 8.74	18.50 ± 18.50
8-day fed	cow 1	30.93 ± 17.71	90.73 ± 80.65	28.54 ± 31.23
8-day fed	cow 2	31.30 ± 16.92	183.63 ± 117.78	22.16 ± 26.06

Discussion

Only the fed ticks in this set of experiments produced significantly increased amounts of the three volatile organic compounds described in Chapter II (detected at GC retention times of 10.7, 11.4 and 12.6 minutes). The amounts of volatile compounds produced were significantly greater in ticks after 3 days of feeding in both strains of the Gulf Coast tick when compared to the amount of volatile compounds produced by unfed and exposed unfed ticks. These data indicate increased production of three volatile compounds is associated with blood feeding by ticks.

Production of the volatile compounds detected at retention times of 10.7 and 12.6 min. did not increase with the increased period of feeding by ticks. In fact, the volatile compounds detected at retention times of 10.7 and 12.6 minutes produced by the Oklahoma strain were detected in smaller quantities in 8-day fed ticks when compared to the quantities produced by 3-day fed ticks. The Texas strain Gulf Coast ticks did not show significant differences in the quantities of these two volatile compounds produced between 3- and 8-day fed ticks. These data indicate that the production of volatile compounds detected at retention times of 10.7 and 12.6 min. reached maximum production by the third day of feeding. The volatile compound found at retention time of 11.4 minutes showed increased production with increased duration of feeding by ticks in both strains. The production of this compound was significantly higher by the eighth day of feeding in both strains. However, the relative abundance of this volatile compound was higher in Oklahoma than Texas Strains of Gulf Coast tick.

The data from the experiments described in this chapter associate the significantly greater production of the volatile compounds described in Chapter II with blood feeding by ticks. The production of these compounds was determined to be the highest by the 8-day fed males in both strains Gulf Coast ticks. Also, the difference in the relative abundance of these volatile compounds produced by each strain of Gulf Coast tick was significant. Evidence of biological activity is needed to associate these volatile compounds as the part of the AAP composition in the Gulf Coast tick. The association of these volatile compounds with the aggregation response by the female Gulf Coast tick was investigated using two bioassay techniques as described in Chapter IV.

CHAPTER IV

BIOASSAY TECHNIQUES FOR ASSESSMENT OF AGGREGATION-ATTACHMENT-PHEROMONE ACTIVITY IN THE GULF COAST TICK

Introduction

Evidence of the presence of an AAP in Gulf Coast tick was first demonstrated by strategically placing the crude body wash from 10-day fed male Gulf Coast ticks obtained by "whole body extraction" technique on a host animal followed by releasing of numbers of females and tracking them (Gladney et al. 1974). This study revealed that the behavior of female Gulf Coast ticks on host is strongly influenced by a chemical (or chemicals) produced by feeding males.

Current studies now show that volatile organic compounds with GC retention times of 10.7, 11.4 and 12.6 minutes are associated with fed male ticks but not with unfed ticks. A reliable bioassay technique is needed to evaluate the response of female Gulf Coast ticks to male, extract preparations, and eventually isolated chemical compounds involved. Among many bioassays developed for the ticks, two bioassay techniques have been used to evaluate tick pheromones, and these techniques offer different assessments of behavioral response to stimuli.

Petri dish bioassay. A petri dish bioassay technique has been the conventional technique for pheromone bioassays of ticks (Rechac et al. 2000, 1977a and 1977 b). In general, a petri dish was divided into pie-shaped sectors with only one sector containing

target stimuli. Test ticks were released at the center of Petri dish and location was observed after a given time elapsed. This bioassay technique was used to test AAP activity in the male Bont tick, *Amblyomma hebraeum* (Rechav et al. 1977a and 1977 b). This technique was also used to study the response of female to artificially fed Gulf Coast ticks (Rechav et al. 2000). The study revealed that the significantly higher numbers of females were found at the sector of the petri dish where fed males were placed when compared to sector with unfed males.

Y-tube olfactometer bioassay. The use of a Y-tube olfactometer is a common bioassay technique applied in attraction studies of parasitoids and other highly mobile insects (Demas et al. 2000). In general, target insects are released at the base of a Y-shaped glass tube while air, or air containing test stimuli, are being passed through the system. The locations where the released insects are found after specific time indicate the relative attraction to the stimuli inserted into the test arm of the Y-tube. The Y-tube olfactometer bioassay has two potentially advantageous features when compared to the petri dish bioassay technique. First, the Y-tube olfactometer bioassay apparatus can easily sustain constant condition by pushing or pulling the air in and out of the system. This feature eliminates the problem caused by the diffusion of stimuli (saturation and/or equilibrium of the stimuli within the bioassay system) that are commonly observed in closed bioassay systems such as the petri dish technique. In addition, the Y-tube olfactometer can test the effects of multiple stimuli by inserting numbers of stimuli through two different entry points (arms). This feature presents opportunities to study

not only tick attraction to certain stimuli, but also to perform comparative studies that will determine the preference of stimuli when more then one stimulus is present.

This chapter summarizes studies that test the hypotheses that fed male Gulf Coast ticks producing volatile organic compounds with retention times of 10.7, 11.4 and 12.6 minutes are attractive to females of this tick species.

Materials and Methods

Ticks. Gulf Coast ticks originating from Refugio County, Texas and Payne County, Oklahoma, and maintained in colony at the Tick Research Laboratory, Veterinary Medical Research Park, Texas A&M University were used in this project. These ticks were fed as immatures on commercial chickens and as adults on cattle under Animal Use Protocol No. 2002-208: Gulf Coast Tick Management Tactics for Pastured Cattle. Fed ticks were then placed in one-liter jars and covered with Chiffon Georgette fabric and secured using the accompanying lid with the center removed. The jars were placed in a glass incubator maintained at 20°C, 85% RH and 10:14 hour photophase.

Petri dish bioassay. The procedure described by Rechav et al. (1977a and b, 1978) was used with the following modifications. An uncontaminated Petri dish (washed with acetone and oven dried) was divided into six sectors. Ten 8-day fed males were held in a 1 ml centrifuge tubes that was cut 1 cm from the top then sealed with fabric screens and placed in sector one and empty centrifuged tubes were placed in the remaining sectors as controls. Ten unfed female Gulf Coast ticks were released at the center of the petri dish. At the end of 3 min., the number of female ticks found in each

sector was recorded. The procedure was also repeated with unfed males as the treatment. The petri dish bioassay apparatus was then placed over the heat pack to maintain the temperature at (38ºC ± 2 ºC) to simulate the surface temperature of a cow. All replication was recorded with the camcorder in order to confirm the location and the count of females at the end of the experiments (Fig 9). Both Oklahoma and Texas strains of Gulf Coast ticks were examined using their con-specific strain of male ticks. There were total of thirty replications for each combination of fed and unfed males of each tick strain.

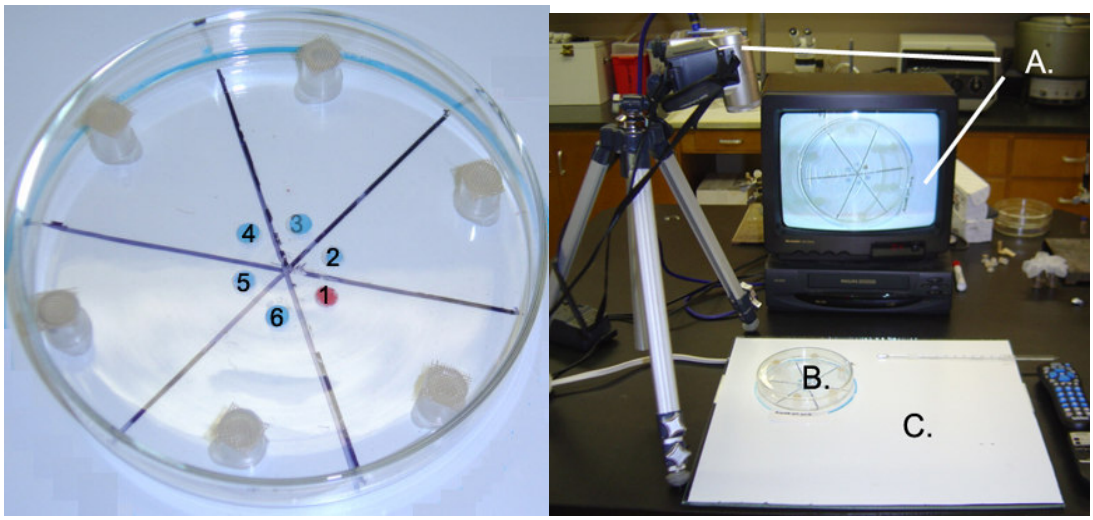


Figure 9. Petri dish bioassay setup for Gulf Coast tick, *Amblyomma maculatum* Koch. Each petri dish was divided into six sectors. Sector 1 held the treatment (10 unfed or fed male gulf Coast ticks), sector 2-6 served as controls. Each trial was recorded via a camcorder (A) and the petri dish (B) was placed on top of heat pack (C) to maintain a constant temperatures.

Y-tube olfactometer bioassay. The Y-tube olfactometer apparatus were assembled based on previous studies of tick parasitoid *Ixodiphagus hookeri* (Demas et al. 2000). A custom-made Y-tube (made by: Department of Chemistry, Texas A&M University) was used as an observation field, with the starting chamber located at the base of the stem. The stem of Y-tube extended 10 cm then branched into 15 cm arms (Fig 10). The "end point" was 2 cm above the point where each stem begins. One of the arms of Y-tube was capable of incorporating additional known amount of stimulant at any given time and the flow of added stimulant was monitored using a flowmeter (Cole Parmer Instrument Co. Vernon Hills, Illinois). The Y-tube olfactometer was placed in warm water (38 ºC ± 2 ºC) bath in such a manner that the surface of the water touched the entire bottom surface of observation field. The water bath temperature simulated the surface temperature of a cow. A vacuum pump (Curtin Matheson Scientific Inc. Houston Texas) was used to push the air (through activated charcoal) at the rate of 1200 ml per min. (600 ml per each arm of Y-tube olfactometer) per minute. The Y-tube olfactometer was thoroughly washed with distilled water and acetone then oven dried between each trial to minimize the risk of contamination.

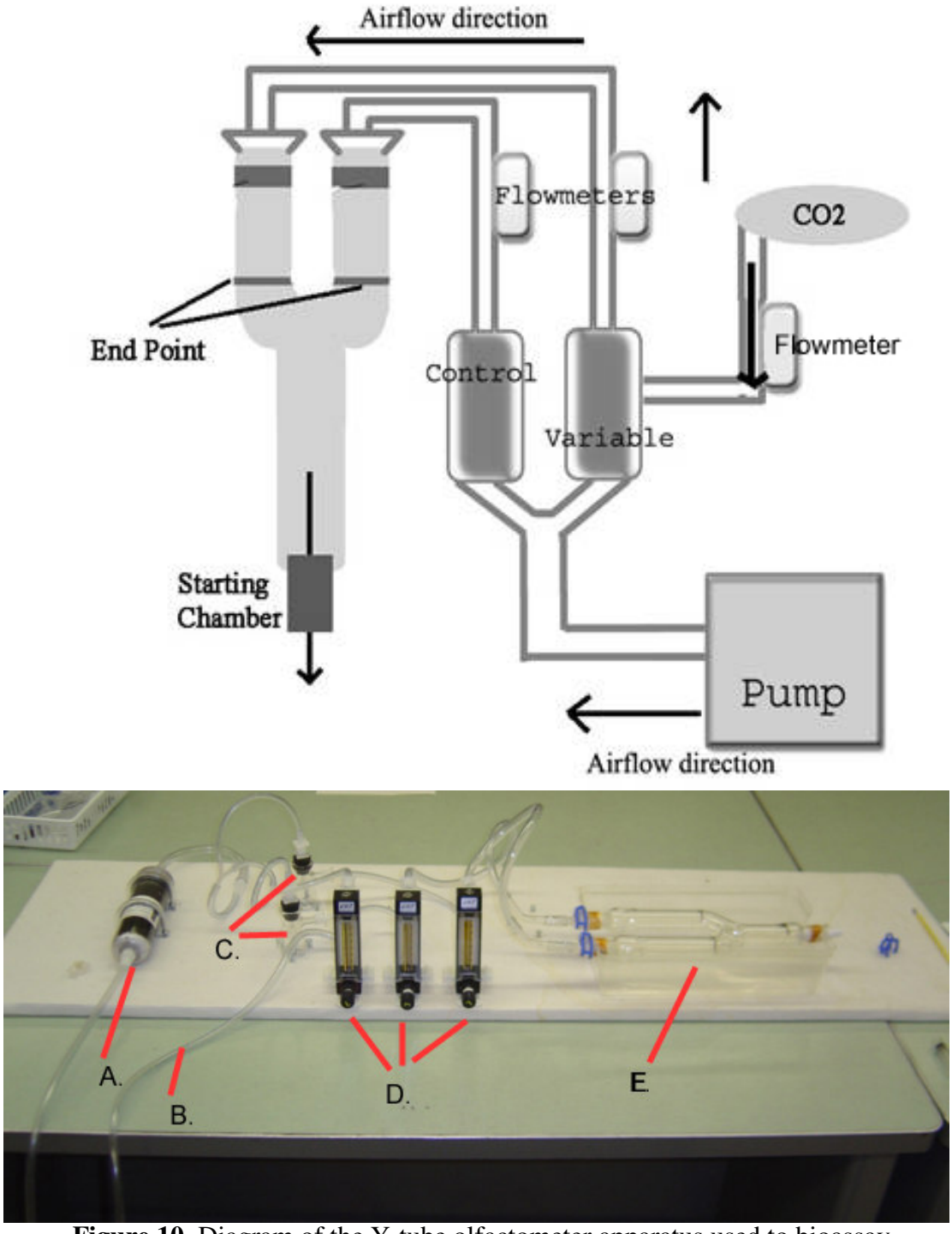


Figure 10. Diagram of the Y-tube olfactometer apparatus used to bioassay female Gulf Coast tick, *Amblyomma maculatum* Koch, attraction to various sets of paired stimuli. (A) Activated charcoal, (B) Source of CO2, (C) Treatment sets, (D) Flowmeters (E) Y-Tube Olfactometer.

Fifty female ticks were released into the starting chamber and air was pushed through the system for 10 minutes. At the end of ten minutes, ticks were counted in four locations (starting chamber, stem, control arm, and treatment arm). The five treatment sets with stimuli inserted into each arm were: (1) No airflow (no air was pushed through the system), (2) Air flow with out any additional stimuli (600 ml per each arms of Y-tube olfactometer), (3) Air verses, Air plus Carbon dioxide (CO_2 flow rate = 30 ml/min) (4) Air verses, Air plus ten 8 day fed male ticks and (5) Air verses Air plus carbon dioxide (CO_2 flow rate = 30 ml/min) plus ten 8 day fed male ticks.

Verification of the presence of volatile organic compounds. Three randomly selected paired sets of unfed and fed ticks from each strain of Gulf Coast tick used in both petri dish and Y-tube olfactometer bioassay were also used to generate SPME headspace collection data to observe the production of volatile organic compounds described (detected by GC analysis at retention time of 10.7, 11.4 and 12.6 minutes) in previous studies (Chapter II and III).

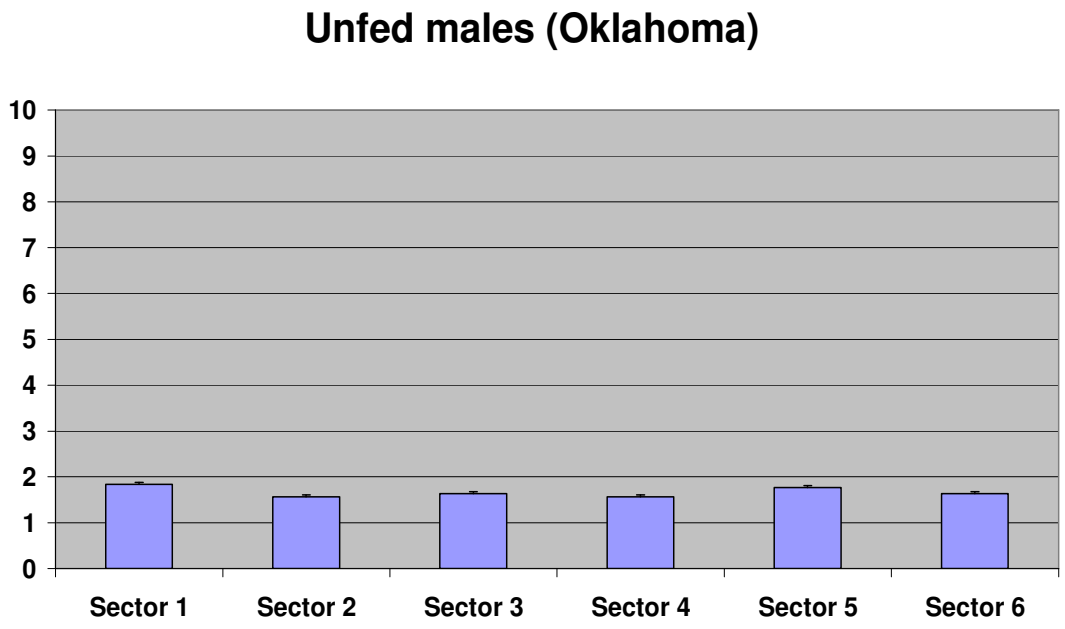
Data analyses. The petri bioassay setup had 5 control sectors and 1 treatment sector. The unfed male ticks (from incubator) and 8 day fed male ticks were used as the treatment in their designated bioassay and total 30 replications were done for each treatment. The Y-tube olfactometer bioassay setup had 5 sets of paired treatments that were inserted to the arms of the Y-tube olfactometer. These sets of treatments were (1) No airflow, (2) Air Vs. Air, (3) Air Vs. Air + CO_2 (4) Air Vs. Air + Ten 8-day fed male ticks and (5) Air Vs. Air + CO_2 + Ten 8-day fed male ticks. Three replications were done for each set of treatments and the data were recorded in terms of the number of

female ticks found at 4 locations (starting chamber, stem, control and treatment arms) of Y-tube Olfactometer.

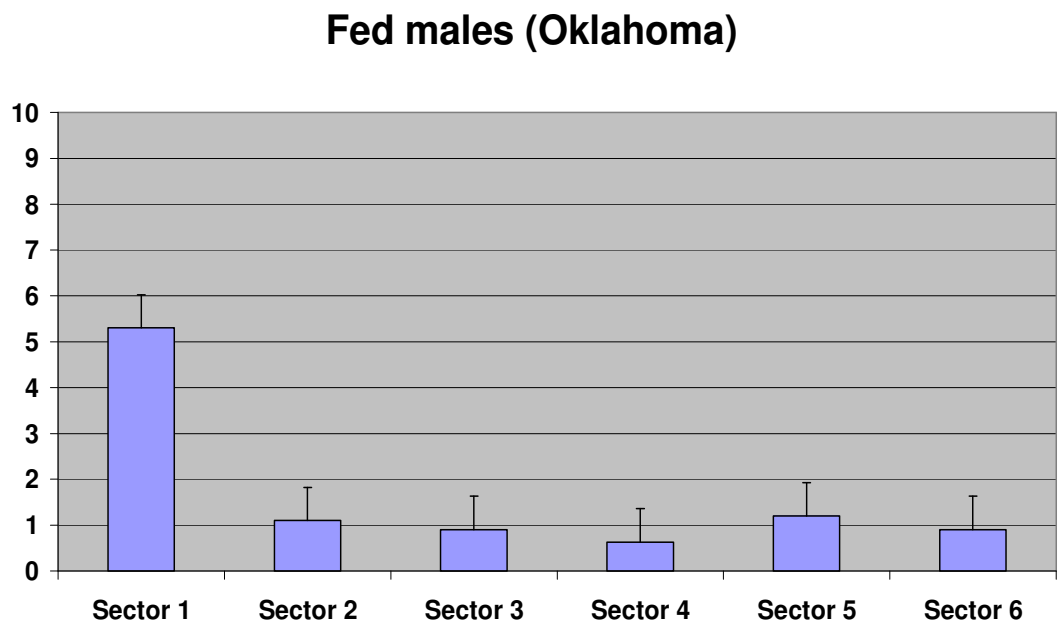
One way ANOVA test in the Statistical Analysis System (SAS) program (SAS Institute Inc.) was used to analyze the data and test the following hypothesis: (1) Female tick attraction (number of female ticks found at sector) to fed male ticks is higher than that of unfed male ticks in the petri bioassay, and (2) Female tick attraction to sets of stimuli that include fed males is significantly higher than that witch do not includes fed males.

Results

Petri dish bioassay. For both Oklahoma and Texas strain Gulf Coast ticks, there were no significant differences (Oklahoma strain, $p < 0.254$; Texas strain, $p < 0.568$) found between numbers of female ticks responding to sectors containing unfed males compared to sectors containing empty centrifuge tubes (Figs 11 and 12). In contrast, the sector containing fed males attracted significantly higher numbers of females ($P< 0.001$ for both strains) for both tick strains (Figs 11 and 12).

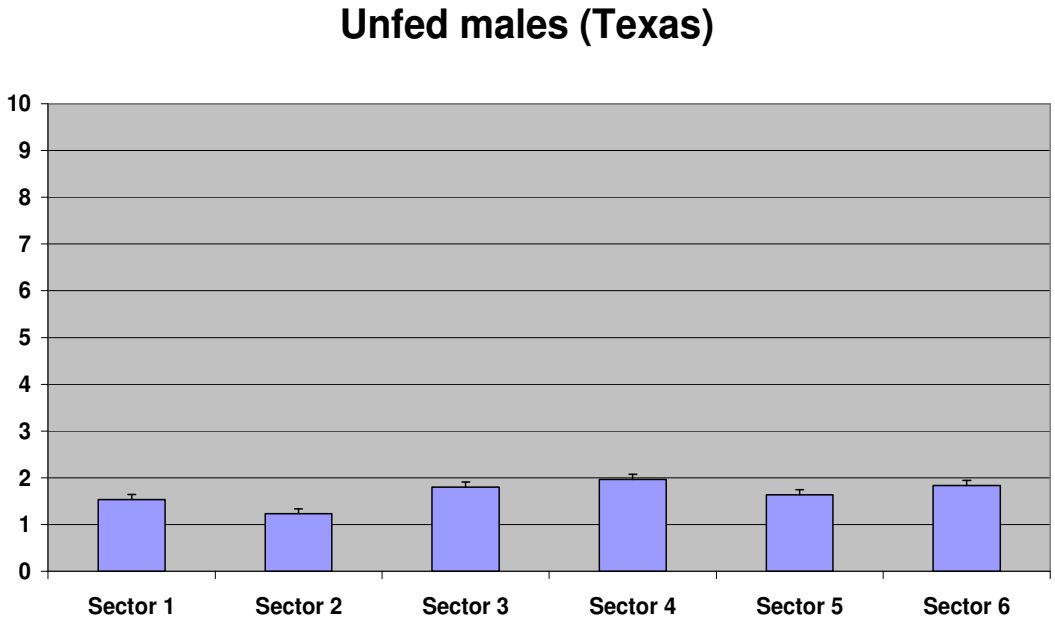


n = 30, d.f =5, x^2=9.57, p < 0.294

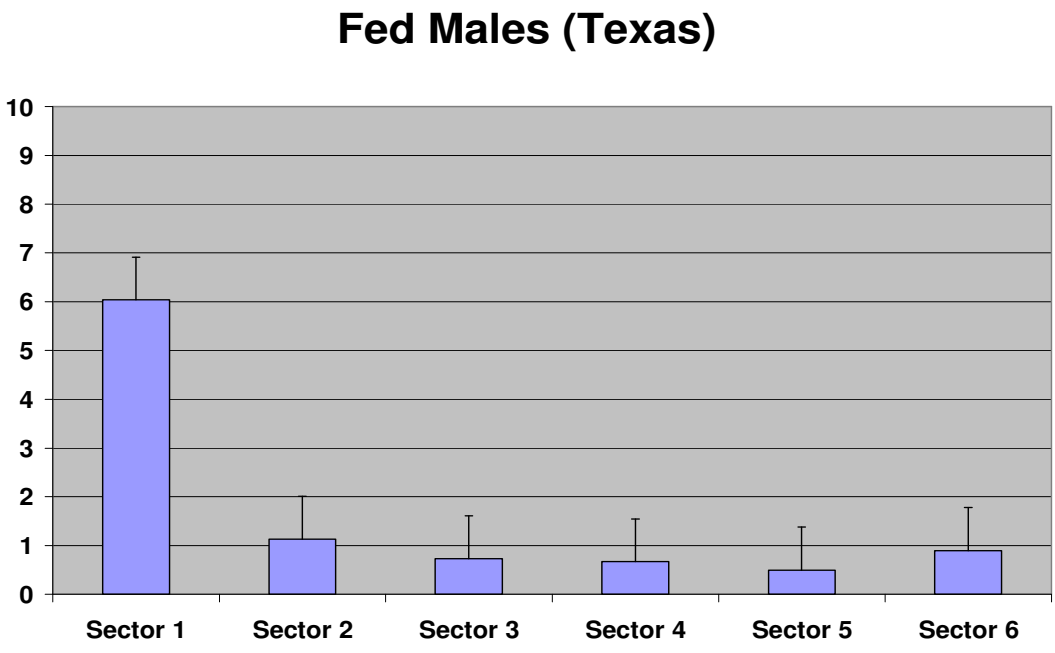


n = 30, d.f =5, x^2=139.63, p < 0.001

Figure 11. Mean attraction response (mean numbers of female ticks found in each sector ± SD) by female Oklahoma strain Gulf Coast ticks, *Amblyomma maculatum* Koch, to unfed and fed male Oklahoma strain Gulf Coast ticks placed in sector 1.



n = 30, d.f =5, x^2=4.35, p < 0.568



n = 30, d.f =5, x^2=90.37, p < 0.001

Figure 12. Mean attraction response (mean numbers of female ticks found in each sector ± SD) by female Texas strain Gulf Coast ticks, *Amblyomma maculatum* Koch, to unfed and fed male Texas strain Gulf Coast ticks placed in sector 1.

Y-tube olfactometer bioassay. For both Texas and Oklahoma strains, ticks showed little or no excitation or orientation when there was no airflow in the olfactometer. When purified air was pushed through the Y-tube olfactometer, the number of ticks found at stem, and each arm of olfactometer increased, but the majority of ticks still remained in the starting chamber (Fig 13). In addition, the numbers of female ticks found at either arm of olfactometer were not significantly different ($p < 0.001$) when purified air without any stimuli was pushed through the olfactometer, indicating that the surrounding environment of olfactometer (i.e. presence of people, and lighting in the lab) was not biasing selection for either arm of the Y-tube made by the female ticks.

Treatment sets "Air vs. Air +CO_2", "Air vs. Air +fed males", and "Air vs. Air + CO_2 + fed male ticks" demonstrated significantly higher numbers of females attracted to the stimulus arm of Y-tube when compared to the treatment set " Air vs. Air" in both strains of the Gulf Coast tick ($P < 0.001$ in all comparisons). However, the differences in female attraction between treatment sets "Air vs. Air +CO_2", "Air vs. Air +fed males" were not significant ($p < 0.391$ in Oklahoma strain tick, $p < 0.458$ in Texas tick). Most importantly, the treatment set "Air vs. Air + CO_2 + fed male ticks" for both strains of Gulf Coast ticks exhibited a significantly higher female attraction response ($p < 0.001$) than fed-males or CO_2 (Fig 13).

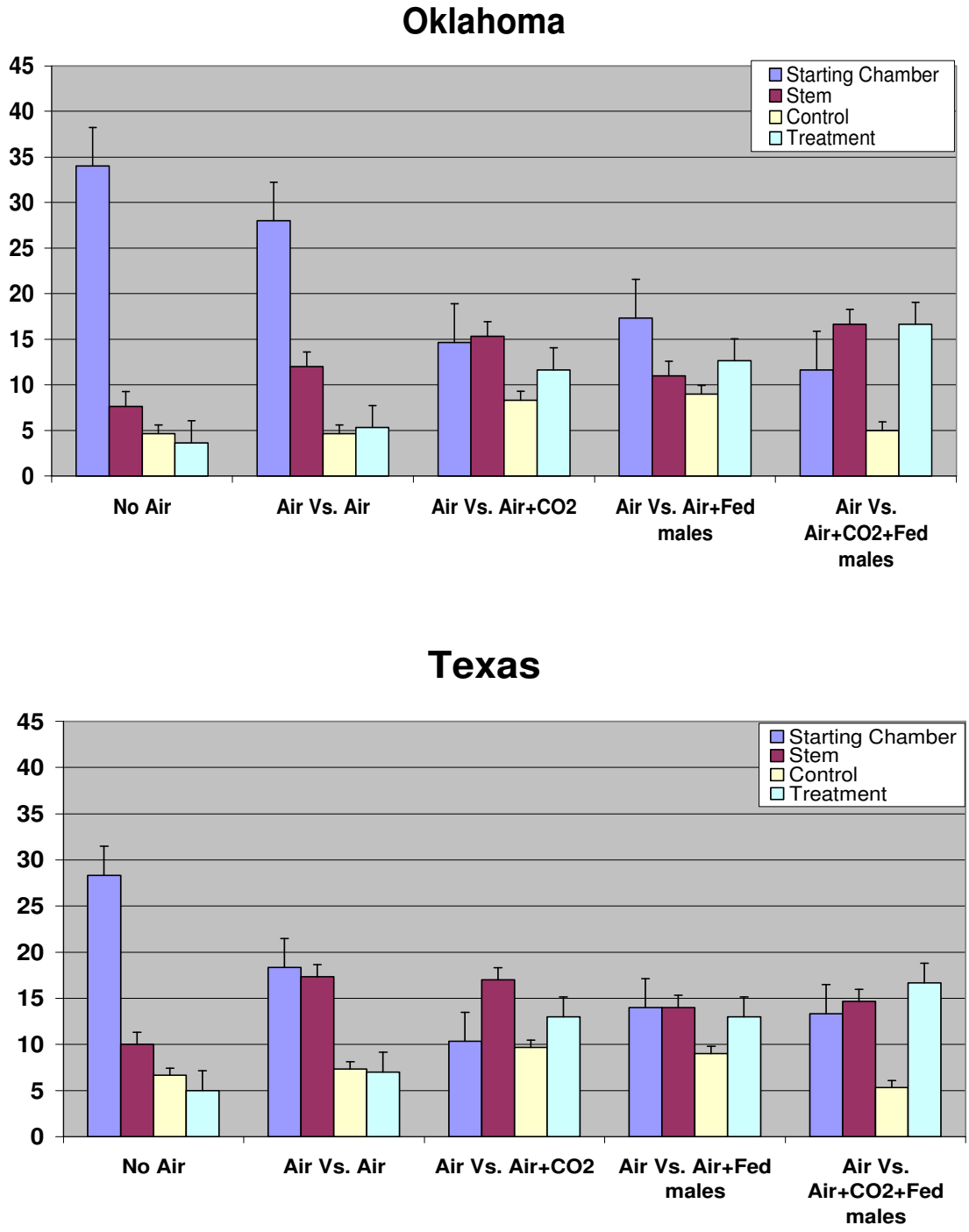
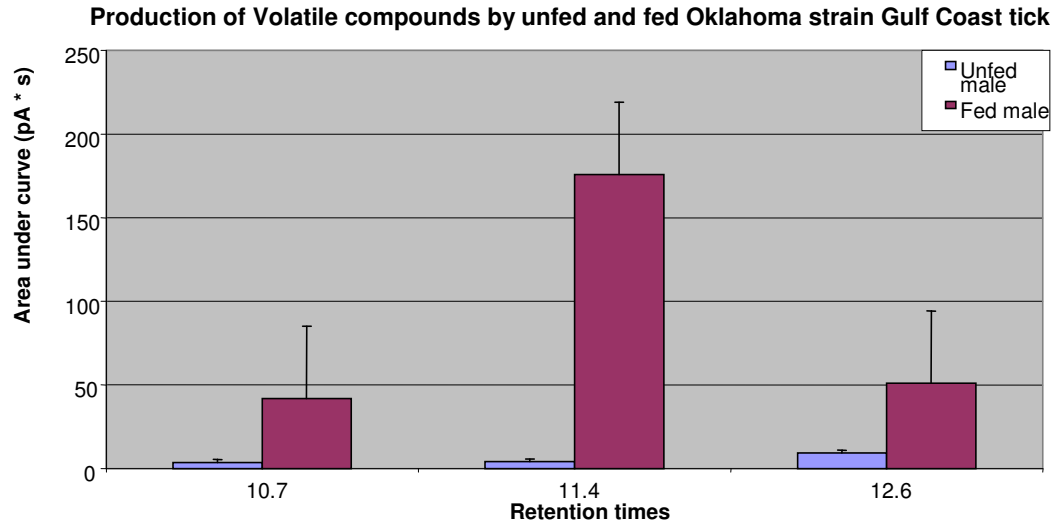
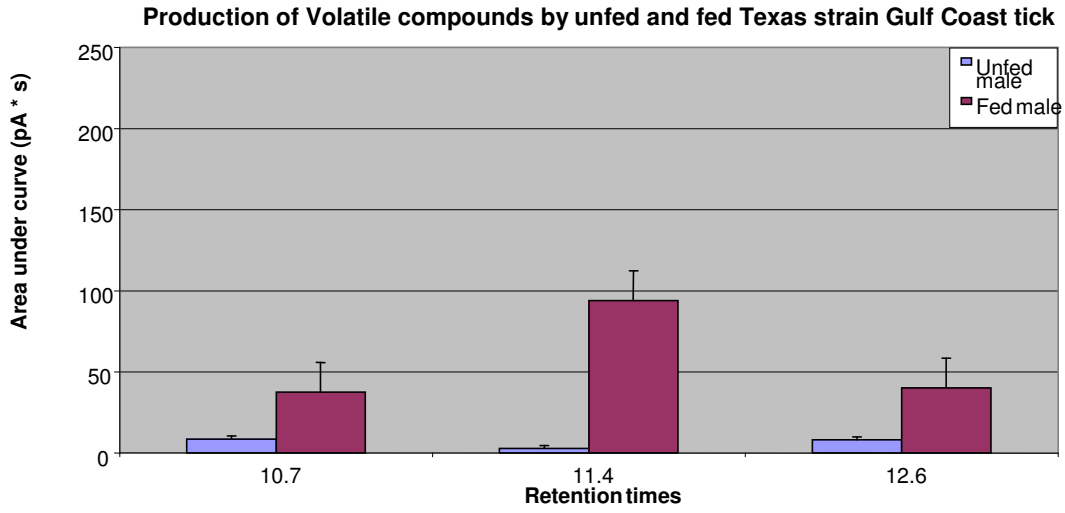


Figure 13. Mean attraction response (mean numbers of female ticks found in each sector ± SD) by Oklahoma and Texas strain female Gulf Coast ticks, *Amblyomma maculatum* Koch, to various stimuli introduced into the Y-tube olfactometer.

A subset of fed males for both tick strains used to conduct bioassays produced significantly greater amounts of volatile compounds at retention times of 10.7, 11.4 and 12.6 min. (Fig 14), when compared to its corresponding unfed males used for both petri dish and Y-tube Olfactometer Bioassays.



n=3, P < 0.001



n =3, p < 0.001

Figure 14. Comparison of mean production (mean area under curve ± SD) of volatile compounds (retention times of 10.7, 11.4 and 12.6 minutes) produced by unfed and fed male Gulf Coast ticks, *Amblyomma maculatum* Koch, by Oklahoma and Texas strain male ticks used in Petri dish and Y-tube olfactometer bioassay.

Discussion

The results from the petri dish bioassay reaffirm past studies that show female Gulf Coast ticks do demonstrate an attraction and aggregation behavior towards fed males (Rechav et al.1977a&1977b and Rechav et al. 1978). However, some observations made during the petri dish bioassay from this study revealed some lack of versatility of Petri dish bioassay. First, when female ticks were left in the petri dish for additional period of time after the end of each trial (< 3 minutes), the overall mobility of females decreased (less excitation and orientations) and females dispersed throughout the petri dish perhaps indicating the saturation of stimuli within the Petri dish. Also no systematic incorporation of additional stimuli (such as CO_2) was possible in Petri dish bioassay.

Results from the Y-tube olfactometer bioassay demonstrated female Gulf Coast ticks were more attracted to either the stimulus generated by fed-males or CO_2 than to purified air. However, the level of female attraction to either stimulus alone was significantly lower than that to the combined stimuli of fed male and CO_2. These findings provide an explanation for low mark-recapture rate of Gulf Coast ticks (as low as 2%) using a conventional tick trap in the field, which utilizes CO_2 as the sole source of attractant (Bengaly 1987). The CO_2 trap was originally developed/designed to provide temporal and spatial distribution data for the lone star tick, *Amblyomma americanum* (Bram 1978). Data from this study suggest that a new trap designed to incorporate CO_2 with Gulf Coast tick AAP could improve trapping efficiency.

Solid-phase-microextraction headspace samples of both strains of fed Gulf Coast ticks used in the bioassays of this study revealed significantly greater production of volatile compounds detected at retention times of 10.7, 11.4 and 12.6 minutes. In conclusion, the associations between the volatile compounds produced as the part of AAP components were established by the significantly higher female attraction responses to fed-male as demonstrated by both the Petri dish and Y-tube olfactometer bioassays.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

This research demonstrated the SPME headspace collection technique was sufficient for detection of volatile compounds produced by ticks using GC analysis. The SPME headspace collection technique was able to provide quantitative data to compare the production of volatile compounds by unfed and fed Gulf Coast ticks. Due to its versatile nature including compact design, high mobility of the apparatus, and salvage after collection, the SPME headspace collection technique can provide additional research opportunities in both field and laboratory settings.

There were three GC peaks found in the SPME sample collection that were distinctively higher in fed male ticks. Also, in both strains, these peaks were found at the retention times of 10.7, 11.4, and 12.6 min, and had greater area-under-curve of each peak when compared to their concurrent peaks produced by unfed male ticks. The production of volatile compounds observed at retention times 10.7 and 12.6 minutes were produced in significantly greater amounts by the 3 days of blood feeding but did not increase with further period of feeding (8 days). These two volatile compounds seemed to reach their maximum measurable production by the 3 days of feeding by the male ticks. The production of a volatile compound observed at the retention time of 11.4 min. increased with time of male blood feeding (3 days to 8 days) for both strains of Gulf Coast ticks. However, the production of a volatile compound observed at the retention time of 11.4 minutes was higher for Oklahoma ticks in both 3- and 8-day fed

ticks when compared to Texas strain ticks. Furthermore, relative abundance of these three volatile compounds were different in each strain of Gulf Coast ticks, with the greatest amount of volatile compound found at 11.4 min. retention time as produced by the Oklahoma strain.

Two bioassay techniques were used to establish that the three volatile compounds described above are associated with AAP activity in the Gulf Coast tick. Results of both a Petri dish test and Y-tube olfactometer test revealed higher attractions to fed-males by conspecific female Gulf Coast ticks. In addition, the Y-tube olfactometer bioassay results demonstrated higher attractions to stimuli composed of fed-males and CO_2 by the female Gulf Coast ticks compared to stimulus that was either fed-males or CO_2 alone. This provided a potential explanation for low mark-recapture rate of Gulf Coast ticks in field studies using the conventional trap with CO_2 as the sole attractant.

The findings from this research provide the foundation for future AAP studies of Gulf Coast ticks. The identification of the volatile compounds captured by the SPME headspace collection technique must be accomplished in order to either synthesize and/or incorporate the AAP into the surveillance and control tactic program for the Gulf Coast tick. Gas Chromatography-Mass Spectrum (GC-MS) analyses of these volatile compounds captured by the SPME headspace collection technique will be needed to identify these compounds. Finally, bioassays conducted in this research were done with intra-strain males and females. Currently, three Gulf Coast tick strains (Oklahoma, Kansas, and Texas) have been identified based on geographic source, seasonal differences in phonology of host seeking activity and population genetics. Biological

responses associated with the differences in the dynamics of pheromones across these three strains must be evaluated in order to develop a surveillance and control program that are suitable for all strains of the Gulf Coast tick.

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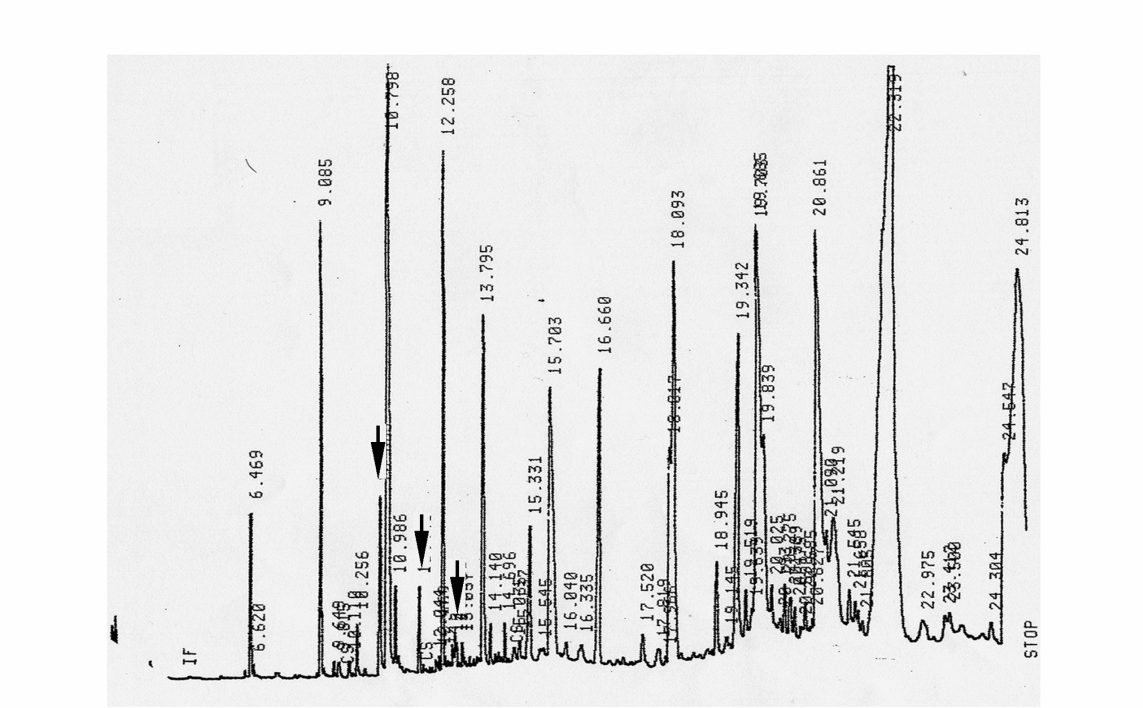
APPENDIX I

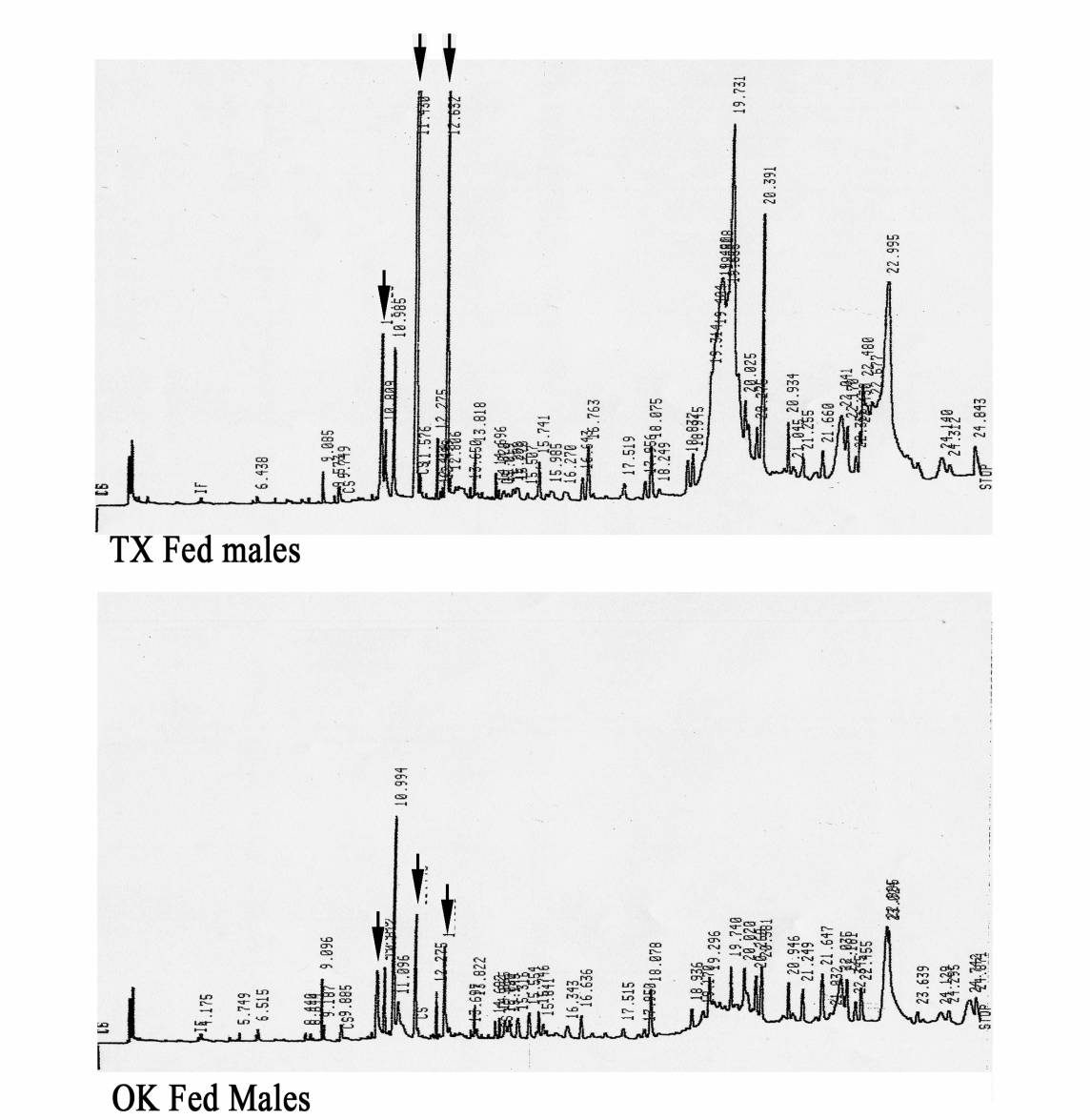
Example of Gas Chromatography output of solid phase microextraction head collection sample of unfed Gulf Coast tick, *Amblyomma maculatum* Koch.



HP 6980 GC, equipped with a BP-20 carbowax column (25 m x .25 mm ID) (SGE, Ringwood, Australia) (Initial temperature = 50 °C for 5 min, to 150 °C at 15 °C /min, to 185 °C at 5 °C /min, to 225 °C at 20 °C /min, with 138 KPa He as the carrier gas). Three peaks of interest (volatile compound found at retention times of 10.7, 11.4, and 12.6 min.) are indicated by arrows.

APPENDIX II

Example of Gas Chromatography output of solid phase microextraction head collection sample of fed Gulf Coast tick, *Amblyomma maculatum* Koch.



HP 6980 GC, equipped with a BP-20 carbowax column (25 m x .25 mm ID) (SGE, Ringwood, Australia) (Initial temperature = 50 °C for 5 min, to 150 °C at 15 °C /min, to 185 °C at 5 °C /min, to 225 °C at 20 °C /min, with 138 KPa He as the carrier gas). Three peaks of interest (volatile compound found at retention times of 10.7, 11.4, and 12.6 min.) are indicated by arrows.

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