ABSTRACT

ZHU, JIWEI. Identification of the Mevalonate-Farnesal Biosynthesis Pathway in Ticks and Development of Methyl Ketones as Fumigants for Pest Control. (Under the direction of R. Michael Roe and Charles S. Apperson.)

JH III biosynthesis. Ticks are blood feeding ectoparasites. They are vectors to humans and animals of many disease causing organisms. Our understanding of the molecular endocrinology of ticks has been greatly influenced by our understanding of insect systems. In insects, juvenile hormone (JH) controls the growth, development, metamorphosis, and reproduction. For many years, the general assumption has been that JH regulates tick development and reproduction the same as in insects. The characterization of the genes or enzymes involved in the synthesis of JH was fundamental to understanding the developmental biology of insects but has never been investigated in ticks. The tick synganglion contains regions which are homologous to the corpora allata, the biosynthetic source for JH in insects. Using the latest Next-gen sequencing technologies, three synganglion transcriptomes were constructed separately for adults of the American dog tick, *Dermacentor variabilis*, the deer tick, *Ixodes scapularis*, and the relapsing fever tick, *Ornithodoros turicata*, to determine whether ticks produce the messages for the enzymes that in insects are involved in the synthesis of JH or its precursors. All of the enzymes that make up the mevalonate pathway from acetyl-CoA to farnesyl diphosphate were found in at least one of the ticks studied but most were found in all three species. In the JH branch, we found a putative farnesol oxidase and methyltransferases (MTs). However, when the tick MTs were compared to the known insect JHAMTs at the amino acid level, the former lacked the farnesoic acid binding motif typical in insects. The P450s shown in insects to add the C10,11 epoxide to methyl

farnesoate are in the CYP15 family; this family was absent in our tick transcriptomes. The transcriptomic data *in toto* suggest that ticks do not synthesize JH III but have the mevalonate pathway and early parts of the JH III branch.

The mevalonate-JH pathway also was investigated as part of the first dampwood termite, *Zootermopsis nevadensis* genome analysis. Next-gen sequencing technology and comparative analysis with insects and ticks was used to identify the messages for the enzymes involved in the mevalonate-JH pathway in termites. With the identification of all of the transcripts for the enzymes involved in the mevalonate-JH pathway, it provides a new and easy to use tool to examine JH biosynthesis relative to termite development using qPCR or RNAseq.

Methyl ketone fumigants. The red imported fire ant (RIFA), *Solenopsis invicta*, is native to South America and was first detected in the United States in Mobile, Alabama during the 1930s. Since then it has become a major pest in the southern U.S. and has expanded north. Fire ants in hay pastures can cause equipment breakdowns and ants in hay is a worker hazard and is harmful to livestock. There is an important need to develop methods to stop their further dispersal without affecting non-target animals including native ants. Fumigants like phosphine, methyl bromide are highly effective and efficient for the control of structural, storage and agriculture pests. Unfortunately, many of these synthetic compounds are highly toxic to applicators and consumers, and methyl bromide has been de-registered because of its depletion of the stratospheric ozone layer. Several plant species synthesize and emit a variety of natural n-aliphatic methyl ketone fumigants to prevent plant herbivory. By using RIFA as model, an easy to use and inexpensive bioassay system was developed in this study to screen fumigants in the laboratory which

could be used for fire ant control and a variety of pest species including arthropods, seeds, microorganisms and nematodes. Structure-mortality studies were conducted using the RIFA as a model to examine the use of methyl ketones as a novel, arthropod fumigant. The LC_{50}s determined from probit models for heptanone, octanone, nonanone and undecanone were 4.27, 5.11, 5.26 and 8.21 μg/cm^3 compared to a lower and upper range of 48.08 and 112.2 μg/cm^3, respectively, for methyl bromide. In dose-response field studies, it was discovered that 2-undecanone formulated as an emulsion in water and injected into mounds, was effective as low as 12.5 ml of active achieving 100% kill of fire ant mounds in 4 days with no re-establishment of ants after 3 days.

Molecular approaches to fire ant control. A RIFA specific RNA interference (RNAi) approach was investigated. RNAi produces a sequence specific RNAi mediated down regulation of complementary mRNA, resulting in a reduced synthesis rate of the protein produced by a specific message. This approach of control takes advantage of the specific genetics of the RIFA which can result in minimal non-target effects. Double stranded RNA (dsRNA) complementary to the receptor of the yolk protein (VgR) and morpholinoes targeted the 28S ribosomal gene were synthesized. The dsRNA and morpholino constructs were fed to the ants and mortality was evaluated. The results were inconsistent. Typically, the preferred delivery method of dsRNA into insects has been microinjection, since insects lack the genes encoding RNA dependent RNA polymerase (responsible for amplification of dsRNA). Also there is the possibility of gut degradation of dsRNA and poor gut penetration when a feeding delivery method is used. An RNAi approach for fire ant control may be potentially viable but will require screening a large number of target genes to find the optimum target and studies of *per os* for ants.

Identification Of The Mevalonate-Farnesal Biosynthesis Pathway In Ticks And Development Of Methyl Ketones As Fumigants For Pest Control.

by

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DEDICATION

This dissertation is dedicated to my parents for all the sacrifices they made in order to send their only child to study in America. I also want to thank my uncle and aunt for their guidance when I first came to America. At last, to my wife Yuan for her support, encouragement and inspiration.

BIOGRAPHY

Jiwei Zhu was born in Shanghai, China. He moved to the United States of America at the age of 12. He learned English at University Presbyterian Church at Chapel Hill. In 2008, he joined Dr. Michael Roe's lab as an undergraduate technician. There he researched arthropod repellents and discovered the amazement of entomology. From that point on, he was determined to become an entomologist. In that same year, he received a scholarship from North Carolina Japan Center to study Japanese and bio-physics at Nagoya University, Japan for one year. Soon afterwards, he came back to U.S. and received a Bachelor of Science degree in Biological Sciences, along with minor in Japanese and Japanese literature from North Carolina State University in 2010. To fulfill his dream to become an entomologist, he started his graduate program in Entomology at North Carolina State University and continued in Dr. Michael Roe's lab to pursue a Doctor of Philosophy degree.

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I also like to thank Dr. Roe for his knowledge and guidance and most importantly, for giving me the opportunity to join the lab as an undergrad, and continuing to mentoring me throughout the Ph. D program. This would never have happened without him. Dr. Roe has taught me to think critically and the importance of team work. I am grateful to Dr. Brooke Bissinger, Dr. Jaap Van Kretschmar, Dr. Ana Cabrera and Dr. Kevin Donohue for mentoring me when I was an undergrad. I would especially like to thank Dr. Sayed Khalil for mentoring me in the world of molecular biology and bio-informatics. I thank Dr. Brooke Bissinger for mentoring me in arthropod repellents. I'm grateful to Mr. Robert Mitchell, Mr.Aniruhd Dhammi, Mrs. Haley Sutton, Ms. Ann Carr, Mr. Nick Travanty and Dr. Loganathan Ponnusamy for their assistance, knowledge and friendship.

I would also like to thank the tick expert Dr. Daniel Sonenshine for his knowledge, expertise and all the help, education, and support that I received from him. I am thankful to Dr. David Oi for providing fire ant queens for my RNAi work.

Finally, I appreciate my parents for their unconditional support and love, and my wife Yuan for advising me how to make beautiful PowerPoint presentations. Without her I would be nowhere.

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Chapter 1

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Mevalonate-Farnesal Biosynthesis in Ticks: Comparative Synganglion Transcriptomics and a New Perspective

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Abstract

Juvenile hormone (JH) controls the growth, development, metamorphosis, and reproduction of insects. For many years, the general assumption has been that JH regulates tick and other acarine development and reproduction the same as in insects. Although researchers have not been able to find the common insect JHs in hard and soft tick species and JH applications appear to have no effect on tick development, it is difficult to prove the negative or to determine whether precursors to JH are made in ticks. The tick synganglion contains regions which are homologous to the corpora allata, the biosynthetic source for JH in insects. Next-gen sequencing of the tick synganglion transcriptome was conducted separately in adults of the American dog tick, *Dermacentor variabilis*, the deer tick, *Ixodes scapularis*, and the relapsing fever tick, *Ornithodoros turicata* as a new approach to determine whether ticks can make JH or a JH precursor. All of the enzymes that make up the mevalonate pathway from acetyl-CoA to farnesyl diphosphate (acetoacetyl-CoA thiolase, HMG-S, HMG-R, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and farnesyl diphosphate synthase) were found in at least one of the ticks studied but most were found in all three species. Sequence analysis of the last enzyme in the mevalonate pathway, farnesyl diphosphate synthase, demonstrated conservation of the seven prenyltransferase regions and the aspartate rich motifs within those regions typical of this enzyme. In the JH branch from farnesyl diphosphate to JH III, we found a putative farnesol oxidase used for the conversion of farnesol to farnesal in the synganglion transcriptome of *I. scapularis* and *D. variabilis*. Methyltransferases (MTs) that add a methyl group to farnesoic acid to make methyl farnesoate were present in all of the ticks studied with

similarities as high as 36% at the amino acid level to insect JH acid methyltransferase (JHAMT). However, when the tick MTs were compared to the known insect JHAMTs from several insect species at the amino acid level, the former lacked the farnesoic acid binding motif typical in insects. The P450s shown in insects to add the C10,11 epoxide to methyl farnesoate, are in the CYP15 family; this family was absent in our tick transcriptomes and in the *I. scapularis* genome, the only tick genome available. These data suggest that ticks do not synthesize JH III but have the mevalonate pathway and may produce a JH III precursor.

Introduction

Ticks are ectoparasites and important vectors of human and animal diseases. They ranked second only to mosquitoes as vectors of life-threatening or debilitating human and animal diseases and transmit a larger variety of pathogen-borne diseases than any other arthropod [1]. Pathogens harbored by ticks cause Lyme disease, Rocky Mountain spotted fever, tick paralysis, tick toxicoses, heartwater disease, irritation, tick bite allergies, immune responses and others diseases and cause economic losses in animal production due to blood loss and disease. How ticks regulate their development and reproduction is of special interest because of their unique life style as obligatory blood feeders and their ancient divergence from crustaceans and insects [2]. Also, understanding the endocrinology of ticks like that in insects will provide new approaches to their control.

Insect molting, development through instars and stages, metamorphosis and reproduction are regulated by two hormones, ecdysteroids and juvenile hormone (JH). The presence of ecdysteroids and JH at the same time produces a larval-larval molt, ecdysteroids in the absence of JH initiate metamorphosis, and in most insects studied, JH initiates the synthesis and deposition of yolk protein in the eggs, a process known as vitellogenesis [3-6]. Crustacea (also a mandibulate group like insects) use ecdysteroids and methyl farnesoate but not JH, to regulate their development [6-12].

Because ticks and mites as terrestrial arthropods are similar in appearance and have similar developmental stages as insects, the general assumption for many years has been that ticks were like insects in the hormones used for regulating reproduction [1]. This was especially supported by Pound and Oliver [13] in studies with ticks and Oliver

et al. [14] in mites where they found that JH *in vivo* could rescue anti-JH effects and initiate egg development. However, since these initial reports, a number of subsequent studies have not supported a role for JH in egg development in ticks. Taylor et al. [15] and Chinzei et al. [16] found JH topically applied to ticks did not affect egg production; Sankhon et al. [17] showed that ecdysteroids initiated vitellogenin synthesis in fat body in organ culture; Friesen and Kaufman [18] showed that ecdysteroids did the same *in vivo*; and Thompson et al. [19, 20] and Khalil et al. [21] showed that ecdysteroids and not JH *in vivo* resulted in the expression of the vitellogenin (Vg) message, the appearance of Vg protein in the hemolymph, and the production of brown (vitellogenic) eggs. In addition, Neese et al. [22] were unable to find any of the common insect JHs in a species of both hard and soft ticks by highly sensitive radiometric biosynthesis and EI SI GC/MS.

The synthesis of JH III in insects occurs in the stomatogastric nervous system (in the insect head) and consists of two parts, the mevalonate pathway from acetate to farnesyl pyrophosphate followed by the JH branch ending in JH III. There are two well characterized enzymes in the JH branch shown to be involved in JH III biosynthesis in insects, i.e., a JH methyltransferase which adds a methyl ester to produce methyl farnesoate or JH III and a P450 in the family CYP15 which adds a C10*R*,11 epoxide [1, 23]. The characterization of the genes, messages or enzymes in the mevalonate pathway and JH branch, fundamental to the developmental biology of crustaceans and insects, has never been investigated in ticks. With the advancement in high throughput, highly repetitive DNA sequencing and bioinformatics, a tick transcriptome can be examined for the presence or absence of every enzyme in the mevalonate-JH pathway. In the current study, this approach was used to examine separate synganglion transcriptomes of two

hard tick species and one soft tick species with comparative work with the first tick genome to provide evidence whether there is a potential role for the mevalonate and at least parts of the JH branch in tick development.

Materials and Methods

Ticks

American dog ticks, *Dermacentor variabilis*, were reared as described by Sonenshine [24] and were colonized at the Old Dominion University Animal Facility in Norfolk, VA, USA. The colony originated from ticks originally collected near Richmond, Virginia, USA. Immature stages were fed on Norway rats, *Rattus norvegicus*. Adult male and female ticks were allowed to feed and mate naturally within a plastic capsule attached to their host, the New Zealand white rabbit, *Oryctolagus cuniculus*, or withheld from blood feeding and/or mating depending on the conditions of the particular experiment. Black-legged ticks, *Ixodes scapularis*, were reared as described also by Sonenshine [24]. The ticks were collected near Armonk, NY, USA. Adult ticks were confined within plastic capsules attached to New Zealand white rabbits and allowed to feed to repletion. Larvae and nymphs were allowed to feed on albino mice, *Mus musculus*. Relapsing fever ticks, *Ornithodoros turicata*, were obtained originally from Dr. James H. Oliver, Georgia Southern University, Statesboro, GA, USA. This particular colony originated from burrows of the gopher tortoise in FL, USA. They were maintained in wood chip litter. Adults and nymphs were fed on albino mice. The rearing conditions were 26 ±1°C, 92 ± 1% relative humidity, and 14:10 light versus dark for all ticks.

Ethics statement

In this study, we strictly followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health to minimize pain and discomfort of the animals. The protocols were approved by the Old Dominion University Institutional Animal Care and Use Committee (Animal Welfare Assurance Number: A3172-01). These protocols (#10-018 and #10-032) are on file at the Office of Research, Old Dominion University, Norfolk, VA, USA.

Tissue dissection, total RNA isolation, cDNA synthesis and sequencing

Synganglia from different adult ticks under study were isolated by dissection and included the pedal nerves and lateral segmental organs. All lab bench surfaces, petri dish dissecting containers and instruments used in the dissections were treated with RNaseZap (Ambion, Austin, TX, USA) to limit RNA degradation. All containers used were autoclaved and kept sterile until needed and never re-used. Because of the challenges of establishing and maintaining three different tick species in the laboratory, generating the number of ticks needed to collect enough tissue for transcriptome construction, developing different aged adults which was dependent in part on the developmental biology which differed between different tick species, the dissection of large numbers of synganglia from different species and different developmental stages, and the need to obtain RNA that was not degraded, the transcriptomes in this study were generated over an extended time period. During this period, there was a rapid evolution in the sequencing and bioinformatics technologies available in our core facility at North Carolina State University. Also for the sake of time and cost, it was not always possible

or necessary to standardized all aspects of the transcriptome construction and data analysis to achieve the goals of our research. The 454 and Illumina sequencing was conducted in the Genome Sequencing Laboratory at North Carolina State University.

RNA purity-- The quality and purity (260/280 nm) of the total RNA was first determined using a Nanodrop 2000 spectrophotometer (Thermofisher, Wilmington, DE, USA). Samples with low purity (<1.8) were discarded. A Bioanalyzer 2100 (Agilent Technologies) was used next to determine the integrity of RNA samples; samples that did not meet minimum requirements (RNA integrity ≥ 8) were discarded [25].

D. variabilis transcriptome--The *D. variabilis* synganglion 454 transcriptome was made from approximately 50 synganglia each from unfed, part-fed virgin (attached to the host for 4-5 days), part-fed virgin forcibly detached from the rabbit host and held in culture for 4–5 days, part-fed mated (allowed to mate for 1 day), and replete (1 day post drop off from the host) females. Tissues from each feeding stage were homogenized separately in TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA pellets were rehydrated in 100 mM aurintricarboxylic acid to prevent degradation [26]. Approximately 3µg total RNA from each group was pooled. The mRNA was isolated using an Oligotex mRNA isolation kit (Qiagen, Valencia, CA, USA). Purified mRNA was precipitated using ethanol then rehydrated and combined with 10 pmol of modified 3′ reverse transcription primer (5′ATTCTAGAGACCGAG GCGGCCGA CATG T(4)GT(9)CT(10) VN-3′) [27] and 10 pmol SMART IV oligo (5′-AAGCAGTGGTATCAACGC AGAGTGGCCATTACGGCCGGG-3′) [28]. The mRNA and primers were incubated at 72 °C for 2 min and then combined with the following reagents on ice: 1 ml RnaseOut (40 U/µl), 2 µl 5× first strand buffer, 1 µl 20 mM

dithiothreitol (DTT), 1 µl deoxynucleotide triphosphate (dNTP) mix (10 mM each) and 1 µl Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction was incubated at 42 °C for 90 min, then diluted to 30 µl with Tris-EDTA (TE) buffer (10 mM Tris HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid) and stored at -20 °C until further use. Second strand cDNA was synthesized by combining 5 µl first-strand cDNA with 10 pmol modified 3′ PCR primer (5′ATTCTAGAGGCCGAGGCGGCCGACAT G(4)GTCT(4)GTTCTGT(3)CT(4)VN-3′) [27], 10 pmol of 5′ PCR primer (5′AAGCAGT GGTATCAACGCAGAGT-3′) [28], 5 µl 10× reaction buffer, 1 µl dNTP mix, 2 µl $MgSO_4$, 0.4 µl Platinum Taq DNA polymerase High Fidelity and 34.6 µl H_2O (Invitrogen). Thermal cycling was conducted as follows: 94 °C for 2 min followed by 25 cycles of 94 °C for 20s, 65 °C for 20 s and 68 °C for 6 min. The first PCR reaction was conducted, and 5 µl aliquots from cycles 18, 22 and 25 were analyzed on a 1% agarose gel to optimize the number of cycles. Five additional reactions were conducted to produce sufficient quantities of cDNA for 454 library preparation. The contents were combined, and the cDNA was purified using a PCR purification kit (Qiagen) according to the manufacturer's recommendations. The cDNA library was prepared with appropriate kits (Roche, Indianapolis, IN, USA; Qiagen) for pyrosequencing on the GS-FLX sequencer (Roche) according to the manufacturer's recommendations described previously by Margulies et al. [29]. The only deviation from the protocol was prior to titration sequencing where following emulsification PCR, DNA-positive beads were enriched to increase the number of reads collected during titration [30].

I. scapularis transcriptomes--A 454 and two different Illumina synganglion transcriptomes were made from adults of *I. scapularis*. For the 454 pyrosequencing,

tissue processing, RNA extraction and sequencing were conducted as described earlier for the *D. variabilis* synganglion transcriptome and also described by Donohue et al. [30] and Egekwu et al. [31]. The dissections of *I. scapularis* ticks for the Illumina transcriptomes were conducted in phosphate-buffered saline on ice (pH 7.0, 10mM NaH_2PO_4, 14 mM Na_2HPO_4, and 150 mM NaCl). The synganglia were homogenized in Qiagen RLT buffer and total RNA extracted following the manufacturer's recommendations (Qiagen). RNA samples were stored at -80°C until needed. The total RNA was 3.25 µg from 30 part-fed virgin females synganglia for 454 pyrosequencing, 5.1 µg from a mixture of 50 unfed, part-fed virgin and replete female synganglia for the first Illumina transcriptome (**I**) and 3.28 µg from 45 part-fed virgin females for the second (**II**) Illumina transcriptome.

For Illumina sequencing, the Illumina TruSeq RNA Sample Prep Kit v2 (Part No. 15026495, Illumina, Inc. San Diego, CA, USA) was used. Following PCR amplification, adapters were included for sequencing with paired ends. For paired ends, Illumina GA-II sequencing adapters were ligated to the fragments as described by the Illumina's Paired-End Sample Preparation Guide (catalogue number PE-930-1001).

O. turicata transcriptome--For the *O. turicata* Illumina transcriptome, 50 synganglia from replete female adult ticks were dissected in phosphate-buffered saline (described earlier) and the synganglia homogenized in Qiagen RLT buffer. Total RNA was extracted according to the manufacturer's protocol using the RNeasy mini kit (Qiagen). A total of 6.97µg of total RNA was obtained and sequencing conducted using the Illumina Truseq RNA Sample Prep Kit v2 (Part No. 15026495, Illumina). After RT-PCR amplification, paired end sequencing was conducted using Illumina GA-II

sequencing adapters ligated to the fragments as described by the Illumina's Paired-End Sample Preparation Guide (catalogue number PE-930-1001) and Egekwu et al. [32].

Bioinformatics

For each transcriptome, duplicate reads, primer sequence contamination, adapter sequences and ambiguous base calls were trimmed. The assembly of the *D. variabilis* synganglion 454 transcriptome was done with GS Assembler (Roche) using default parameters. The other three transcriptomes (*I. scapularis* 454; Illumina transcriptome and *O. turicata* Illumina transcriptome) were assembled with the CLC-BIO program [33]. The assembled contiguous sequences will be referenced in this study as "contigs." The putative functions of contigs in each transcriptome were identified (annotated) by the Basic Local Alignment Search Tool (BLAST) against the GenBank non redundant database and EST database [34] with an e-value of E-06 (or lower) for the Illumina assemblies and E-10 for 454 pyrosequencing. Additional BLAST searches were done for selected contigs of interest against the conspecific *I. scapularis* genome, (www.vectorbase.org).

BLASTx and BLASTp searches of the *D. variabilis*, *I. scapularis* and *O. turicata* synganglion transcriptomes as well as transcriptomes generated for the *D. variabilis* male reproductive system and midgut described elsewhere [35, 36] were conducted for insect specific matches using the insect enzymes described before in JH III biosynthesis by Noriega et al. [37] and Belles et al. [38]. BLASTp searches were also conducted of the *I. scapularis* genome (www.vectorbase.com) using the CYP15A1 message from *D. punctata* (AAS13464) proven to catalyze the formation of the C10,11 epoxide of JH [23].

The search revealed 500 CYP genes in the *I. scapularis* genome; 20 selected matches based on being full length and having the lowest e-value were utilized to construct an optimal neighbor joining phylogenic tree using the Molecular Evolutionary Genetics Analysis (MEGA) program [39]. Sequence alignments of CYP15A1 were performed with ClustalW (www.ebi.ac.uk/clustalw). Sequence alignments of farnesyl diphosphate synthase, methyltransferase and farnesol oxidase were performed with Jalview Version 2.8.2 [40].

Results and Discussion

Tick transcriptomes

All of the enzymes that comprise the JH biosynthetic pathway in insects (the mevalonate pathway followed by the JH branch from farnesyl PP to JH III) (Fig. 1) are found in the corpora-allata, which is part of the stomatogastric nervous system located ventral and dorsal to the insect brain [41]. The insect brain and ventral nerve cord comprise the central nervous system (CNS) and along with the stomatogastric nervous system regulates neuroendocrine and endocrine functions. Typically, the mevalonate pathway in most animals would lead to the synthesis of steroids, but arthropods, including insects and ticks, have lost the ability to synthesize steroids *de novo* (Figure 1-1). JH biosynthesis is regulated by neuropeptides allatotropins and allatostatins produced in the brain that regulate JH biosynthesis in the stomatogastric nervous system and more specifically in the corpora allata in insects.

The tick's synganglion, which is the tissue source for our transcriptomics research, is located in the center of the body in the tick hemocoel and is homologous in insects with (i) the CNS (brain and ventral nerve cord) and (ii) the stomatogastric nervous system which in insects would include the corpora allata, (the site of biosynthesis of JH) [1]. A retrocerebral organ complex is located on the dorsal side of the supraesophageal region of the synganglion in what should be the same region as the insect stomatogastric nervous system. It has also been hypothesized that the lateral segmental organs located in the lateral nerve plexus between the pedal nerves leading from the synganglion may be the site of JH synthesis in ticks, primarily based on the abundance of smooth endoplasmic reticulum in these glands and the histological similarity of these organs to the insect corpora allata [5]. By developing transcriptomes to the entire synganglion including as much of the pedal nerves as possible and the lateral segmental organs, our dissections should include at least the tick equivalent of the insect corpora allata and essentially includes all of the tick CNS and stomatogastric nervous system. The only exceptions for the latter may be the tick equivalent of the insect ingluvial ganglia and nerves leading to the ingluvial ganglia (if present in ticks). For our research, we generated and examined synganglion transcriptomes from three different adult tick species, *D. variabilis* and *I. scapularis* (hard ticks) and *O. turicata* (soft tick). Transcriptomes from two other tissues, *D. variabilis* adult midgut and male reproductive system where JH biosynthesis would not be expected based on insect systems, were used for comparisons.

D. variabilis synganglion transcriptome--The transcriptome generated from the adult American dog tick synganglia contained 532,136 filtered and vector-trimmed reads and the average length of the reads was 229 base pairs (bp) (Table 1-1). Redundant

sequences were assembled with the GS Assembler algorithm (Roche Indianapolis, IN, USA) and 21,119 contigs were obtained; the Basic Local Alignment Search Tool or BLAST analysis of the National Center for Biotechnology Information (NCBI) database was conducted to predict putative functions of the contigs. A search against the GenBank nonredundant (nr) database by BLASTx using a translated nucleotide query with an *e*-value cut-off of e-10, resulted in at least one match for 13,344 sequences (63.2% of the expressed genes). There were 6,045 transcripts that had an expected value (*e*-value) of 1e-06 or lower when compared to the GenBank nr database using BLASTx [30].

I. scapularis synganglion transcriptome--Three separate transcriptomes were constructed for the black-legged tick (Table 1-1). For the first Illumina transcriptome (I, Table 1-1) mixed unfed, part-fed and replete female synganglia were extracted to create a cDNA library. Sequencing of this library produced a total of 34,520,330 reads with an average read length of 68 bp. After vector trimming, the raw reads were assembled using CLC-BIO [33] into a total of 41,249 contigs with an average length of 480 bp. BLASTx against the NCBI database was used to predict putative functions of the contigs. Searches against the GenBank nr database using BLASTx were conducted with an *e*-value cut-off of 1e-06. For the second *I. scapularis* Illumina transcriptome (II, Table 1-1), part-fed adult female synganglia total RNA was extracted and used to synthesize a cDNA library. After Illumina sequencing, a total of 117,900,476 raw reads were produced with an average length of 72 bp (Table 1-1). Assembly with the CLC-BIO [33] algorithm produced a total of 30,838 contigs with an average length of 655bp. The putative functions for these contigs were predicted with BLASTx using the NCBI database. The third transcriptome was constructed using 454 pyrosequencing. Part-fed female

synganglia total RNA was extracted and a cDNA library constructed followed by 454 pyrosequencing on a GS-FLX sequencer (Roche). Pyrosequencing generated 456,073 total reads with an average length of 267 bp per read (Table 1-1). The reads were assembled with the CLC-BIO algorithm [33] which produced 20,630 contigs with an average length of 523 bp. BLASTx against the NCBI database was used to predict putative functions of the contigs. Searches for protein function were conducted against the GenBank nr database with BLASTx using a translated nucleotide query, with an *e*-value cut-off of e-10.

O. turicata synganlion transcriptome--Synganglia were dissected from blood fed female *O. turicata*, total RNA extracted and a cDNA library constructed. After Illumina sequencing, a total of 63,528,102 raw reads was generated. The assembly of raw reads was conducted with the CLC-BIO [33] assembly program (CLC BIO, Cambridge, MA) and yielded 132,258 contigs with an average contig length of 438 bp. Batch BLAST from Blast2go pro [42] was used to compare these contigs with the nr database from NCBI to establish putative functions with an e-value cut off of e-6.

Additional comparative transcriptomes—Our research results with synganglion transcriptomes were compared with transcriptomes from two additional tissues exclusive of the CNS, namely, the male reproductive system and midgut of the American dog tick. The former was constructed using 500 part fed adult males accessory glands, testes and vas deferens and 454 pyrosequencing produced 563,093 reads which averaged 300 bp per read and assembled into 12,804 contigs (Table 1-1). Putative functions were determined by BLASTx against the NCBI database. A total of 3,951 contigs were found to match known genes, with an e-value cut-off of e-10, when compared to the GenBank nr

database by BLASTx [35]. The midgut transcriptome was developed from unfed and fed male and female adults and was conducted by our group prior to the availability of 454 or Illumina sequencing. We obtained 23,045 reads which were assembled using the CAP2 assembler [43] into 1,679 contigs (Table 1-1) [36]. The contigs were initially identified with BLASTx, BLASTn, and ROS-BLAST using the nr NCBI protein database. Then, the characterized proteins were compared to a custom-prepared ACARI database, the Gene Ontology (GO) database, and to the NCBI conserved domains database (CDD) including KOG, PFAM and SMART for putative functional assignments.

Putative mevalonate (farnesyl-PP) pathway in the tick synganglion

The initial steps in JH biosynthesis comprise the mevalonate or farnesyl-PP pathway (Figure 1-1). This pathway is important in many organisms leading to the steroid (cholesterol) biosynthesis pathway. Steroids are essential in membrane function and comprise the structure of hormones essential for life. Insects do not synthesize cholesterol de novo [38,44], and we unable to find squalene synthase, squalene monoxygenase and lanosterol synthase involved in steroid synthesis (Figure 1-1) in any of our transcriptomes. The mevalonate pathway uses acetyl-CoA as a starting material which undergoes condensation with another acetyl-CoA subunit via acetoacetyl-CoA thiolase to form acetoacetyl-CoA. Then acetyl-CoA condenses with acetoacetyl-CoA to form 2-hydroxy-3-methylglutaryl-CoA (HMG-CoA) through HMG-CoA synthase (HMG-S). HMG-CoA then is reduced to mevalonate by NADPH with the enzyme HMG-CoA reductase (HMG-R) (Figure 1-1), which is the rate limiting step in cholesterol synthesis. HMG-R is an important regulator step in steroid biosynthesis and has been the focus of research into the development of anti-cholesterol drugs. Mevalonate is

phosphorylated by mevalonate kinase (MK) to 5-phosphomevalonate, also known as phosphomevalonic acid. Then the enzyme phosphomevalonate kinase phosphorylates 5-phosphomevalonate to 5-pyrophosphomevalonate (mevalonate-5-PP). The enzyme, diphosphomevalonate decarboxylase metabolizes mevalonate-5-PP to isopentenyl diphosphate (IPP) (Figure 1-1). The C-5 isoprene unit is used by prenyltransferases to build prenyl chains whose carbon atom numbers are typically in multiples of five. Biogenesis of the sesquiterpene precursor of JH III, farnesyl diphosphate (FPP), is achieved by FPP synthase (FPPS, a prenyltransferase) which catalyzes the head to tail condensation of three isoprene units. In the latter reaction, the chain initiator is the allylic isomer of IPP, dimethyl allyl diphosphate (DMAPP), the production of which is catalyzed by an IPP isomerase (IPPI) [37, 38, 41, 45].

In the past, identification of precursors of JH or JH itself in ticks were conducted by the chemical isolation of the products in the mevalonate-JH biosynthetic pathway [22, 24, 46]. This work was challenging in insects because of the small size of the corpora allata, the source of JH, the difficulty of obtaining a clean dissection of the corpora allata, and the extremely low production levels of the hormones. Tracer studies using radiolabeled acetate have also been used in insects and ticks [22]; this approach is problematic because of variations in enzymatic rates at each step of biosynthesis (which makes detection of intermediates difficult), organ culture methods are needed that mimic *in vivo* conditions and again, there is the need to dissect the corpora allata. These problems are even more challenging for the study of ticks because (i) their small size compared to many insects, (ii) ticks do not have a distinct head region, (iii) the stomatogastric and CNS are condensed into a single synganglion, and (iv) tick rearing

itself is difficult requiring feeding on a live animal host at each stage of its development. For these reasons and as an alternative approach, we conducted in this study the first global analysis of gene expression in ticks for the enzymes in the mevalonate-JH III biosynthesis pathway examining the synganglion of three tick species representing hard and soft ticks; the goal was to determine whether adult ticks have the potential of making JH III or its precursors by the detection of the enzymes that comprise the JH III biosynthesis pathway (Figure 1-1). Our focus was JH III biosynthesis since this pathway has been well characterized in insects [38] including our recent identification of the entire pathway in the genome of the termite [47]. Also, the synthesis of JH III is exclusive to the lower insects and therefore most likely to also be found in ticks.

A BLASTx and BLASTn search of the synganglion transcriptomes of *D. variabilis*, *I. scapularis* and *O. turicata* (Table 1-1) for the insect enzymes involved in the synthesis of JH III (Figure 1-1) was successful in identifying all of the transcripts of the enzymes involved in farnesyl-PP biosynthesis (Table 1-2). The putative messages characterized were acetoacetyl-CoA thiolase (60% identical to insects), hydroxymethylglutaryl-CoA synthase (60% identical to insects), hydroxymethylglutaryl-CoA reductase (50% identical to insects), mevalonate kinase (38% identical to insects), phosphomevalonate kinase (52% identical to insects), diphosphomevalonate decarboxylase (54% identical to insects), and farnesyl diphosphate synthase (64% identical to insects) among the three transcriptomes examined. The top three insect BLAST matches for *D. variabilis*, *I. scapularis* and *O. turicata* in the mevalonate pathway are listed with contig number, length (bp), e-value (ranging from 0.0 to 6.3), and percent identity (Table 1-2). For *O. turicata*, all of the enzymes in the mevalonate

pathway was found except farnesyl diphosphate synthase (the last step for the synthesis of farnesyl-PP); however, this enzyme was found in both the *D. variabilis* and *I. scapularis* transcriptomes with the lowest e-values in the e-11 and e-12 range, respectively. Matches for many of the other enzymes in the mevalonate pathway were found in *D. variabilis* and *I. scapularis* with the best matches occurring in the forming ranging from e-14 to e-42 (Table 1-2). A BLASTx and BLASTn search of the *I. scapularis* genome for the insect enzymes involved in the synthesis of juvenile hormone (JH) III [48] revealed the presence of all but two of the enzymes involved in the farnesyl-PP pathway. The genes found were acetoacetyl-CoA thiolase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase and farnesyl diphosphate synthase. The top insect BLAST results from these *I. scapularis* messages had *e*-values ranging from *e*-44 to 0.0. Isopentenyl diphosphate isomerase and geranyl diphosphate synthase were not found. Figure 1-2 shows the farnesyl diphosphate synthase (FPPS) contig 9824 from the *I. scapularis* transcriptome and FPPS (XP_002408650) from the *I. scapularis* genome (www.vectorbase.org) aligned with FPPS described in *Bombyx mori* and *Drosophila melanogaster* [49]. In the alignments, seven prenyltransferase conserved regions previously identified by Koyama et al. [50] are highlighted in boxes. Only the last two conserved regions were found for contig 9824 because this contig from the *I. scapularis* transcriptome is not a full length sequence. But all seven prenyltransferase conserved regions were found in the FPPS sequence from the *I. scapularis* genome; also the region II and VI two aspartate-rich motifs (marked with "X" on top) were identified (Figure 1-2), further evidence for the presence of FPPS in the

synganglion and genome of *I. scapularis*. The FPPS sequence from the *I. scapularis* genome shares 43% identity with the FPPS found in *B. mori* (e-value of e-85); the FPPS (contig 9824) from the *I. scapularis* transcriptome shared 27% identity with an e-value of 6e-05. Even with 27% sequence identify, both messages share a high level of amino acid conservation. FPPSs are unique and diversified. There are three different copies of FPPS in *B. mori*, six in the honey bee, *Apis mellifera*, two in the monarch butterfly, *Danaus plexippus*, and one each in the fruit fly, *Drosophila melanogaster*, the malaria mosquito, *Anopheles gambiae* and red flour beetle, *Tribolium castaneum* [51]. Two FPPS messages were found between the *I. scapularis* synganglion transcriptome and genome (Figure 1-2).

The mevalonate pathway is an important metabolic pathway that exists in all higher eukaryotes and many bacteria and is involved in multiple metabolic functions. For example, it can play a role in cholesterol synthesis and maintaining membrane structure. The pathway is also involved in membrane-bound protein prenylation, the addition of hydrophobic molecules to proteins, and important in cell signaling and carcinogenesis. The mevalonate pathway contributes to the synthesis of heme A and ubiquinone that participate in electron transport across cellular membranes and the synthesis of isopentenyl adenine, which is present in some transfer RNA. The pathway can also produce hormonal messengers such as cytokinins and phytoalexins in plants, steroid hormones in mammals [53], defensive secretions, pheromones, and JH in insects [54], [38]. Since its final product is typically cholesterol that functions in maintaining cell membranes, most of the research on the mevalonate pathway has focused on vertebrate systems and its close association with human cardiovascular diseases [55].

Insects cannot synthesize cholesterol since they lack squalene synthase, squalene monooxygenase and lanosterol synthase in the sterol branch (Figure 1-1). We have identified all of the enzymes involved in the mevalonate pathway from acetyl-CoA to FPP in the three tick transcriptomes that were examined (Table 1-2) and part of the pathway in the *I. scapularis* genome [48]. The mevalonate pathway was also found in the spider mite, *Tetranychus urticae*, genome, a close relative to ticks [56]. Although the mevalonate pathway has other functions such as ubiquinone synthesis, dolichol synthesis and protein prenylation [44], we were not able to find any of the enzymes that make up this pathway in the *D. variabilis* male reproductive or midgut transcriptomes examined. Because of the ubiquitous distribution of fat body in ticks and its deposition on trachea, it is unlikely that fat body messages were not present in any of the transcriptomes that were examined. The evidence so far suggests that the mevalonate (farnesyl-PP) pathway is present only in the synganglion which is inclusive of the stomatogastric nervous system and the likely tick equivalent of the insect corpora allata. Its presence only in the synganglion further suggests that like in insects, the pathway is involved in a regulatory function and possibly is contributing to the production of a JH precursor beyond farnesyl-PP (further discussion follows).

Putative farnesal branch in ticks

Following the mevalonate pathway, there are two metabolic routes possible: the steroid biosynthetic pathway and the JH branch (Figure 1-1). In most animals, the final product of the mevalonate pathway is the synthesis of steroids like cholesterol. Insects lack the genes required for the production of cholesterol from farnesyl-PP; this include squalene synthase which catalyzes the reductive condensation of farnesyl-PP [44]; [57];

[38]. Searching through our tick transcriptomes (Table 1-1), we were not able to find any of the enzymes that make up the steroid biosynthesis pathway.

The JH branch (farnesyl-PP to JH III) found in insects involves five enzymes: farnesyl diphosphate pyrophosphatase, farnesol oxidase, farnesal dehydrogenase, JH methyl transferase and JH epoxidase (Figure 1-1). A BLASTx and BLASTn search of our synganglion transcriptomes of *D. variabilis*, *I. scapularis* and *O. turicata* (Table 1-1) for the insect enzymes involved in the JH branch (Figure 1-1) was successful in identifying (i) farnesol oxidase, a short chain dehydrogenase implicated in the conversion of farnesol to farnesal, and (ii) a methyltransferase that potentially could add a methyl ester to farnesoic acid or JH acid to produce methyl farnesoate or JH III, respectively (Table 1-3). We did not search for farnesyl diphosphate pyrophosphatase and farnesal dehydrogenase in the JH branch since these enzymes have not been identified yet in insects [38].

Absence of a JH epoxidase (CYP15A1) in ticks

The last step in JH biosynthesis in insects is the epoxidation of methyl farnesoate or farnesoic acid by a P450 which adds a C10,11 epoxide with a specific *R*-enantiomeric C10 asymmetric carbon to produce JH III or JH III acid, respectively. The JH epoxidases so far characterized in insects are in the P450 family CYP15A1. An absolute functional assignment has been demonstrated in insects to this P450 family, where the CYP15A1 message was cloned from the corpora allata of the cockroach, *Diploptera punctata*, and shown to add an epoxide at the proper location with C-10*R* high stereo selectivity [23]. All members of the CYP15 family share >40% identity at the amino acid level and those with >55% identity are in the CYP15A subfamily [58]. We were not able to find any JH

epoxidases in the CYP15A1 family in any of our tick transcriptomes (Table 1-3) or in the *I. scapularis* genome [48]. We found two hundred P450 transcripts in the *I. scapularis* genome that varied in length (not all were complete transcripts), but none were specific to the CYP15A1 subfamily. Figure 1-4 shows the alignment of XP_002410454 from the *I. scapularis* genome with CYP15A1 from *D. punctata* and *S. gregaris* (the latter also shown to add an epoxide to make JH III; [23]). XP_002410454 is a full-length sequence we identified in the *I. scapularis* genome that shared the highest identity and lowest e-value with the JH epoxidase CYP15A1 in *D. punctata*. The signature heme-binding region (F**G***C*G) was found in all of the P450s aligned as expected including XP_002410454, suggesting the latter is a P450. A phylogenetic tree was constructed for the top 20 (based on the lowest e-values compared to the cockroach JH epoxidase), full-length sequences in the *I. scapularis* genome to CYP15A1 from *D. punctata* and *S. gregaria* (Figure 1-3). The *D. punctata* and *S. gregaria* enzymes are in the same clade as would be expected. XP_002410454 also had the closest relationship with CYP15A1 but shared only 28% identity with *D. punctata* JH epoxidase placing it outside of the CYP15A1 family and subfamily. CYP15A1 also was not found in the spider mite genome [56].

Neese et al. [22] was unable to find that ticks could synthesize JH either *in vivo* or *in vitro* using different tissues, including the synganglion, using a highly sensitive radiotracer method in hard and soft ticks. In addition, these authors could not find any of the insect JHs in the hemolymph of vitellogenic hard and soft ticks by EI GC-MS nor could they find JH in hard ticks using the insect *Galleria* bioassay for JH. Furthermore, there is no consistent evidence that JH or JH mimics when topically applied affect tick

development or will induce vitellogenesis [1]. The lack of CYP15A1 in multiple synganglion transcriptomes from hard and soft ticks (Table 1-3), in the genome of *I. scapularis* [48], and in the genome of the spider mite [56], further supports the hypothesis that ticks do not make JH III or use JH in the regulation of their development.

JH acid methyltransferase (JHAMT) in ticks

The enzymes involved in the last two steps in JH biosynthesis in insects, JH acid methyltransferase (JHAMT) and JH epoxidase (Figure 1-1), have been successfully validated at the functional level through cloning, expression and substrate metabolism for their role in the synthesis of JH III [60]; [61]; [62]; [63]. The order of metabolism appears to be variable depending on the insect; in the Lepidoptera, epoxidation precedes esterification by JHAMT [64] and in the Orthoptera, Dictyoptera, Coleoptera and Diptera the reverse occurs [65-69]. JHAMT transfers a methyl group from S-adenosyl-L-methionine (SAM) to farnesoic acid or JH acid depending on the insect. Thus, JHAMTs must have a conserved SAM binding motif which is typical of the general SAM-dependent methyltransferase family [38].

JHAMTs have been found and characterized in insects but not in ticks. We found over 100 different methyltransferases in each of our tick synganglion transcriptomes, and there is evidence from the study of the *I. scapularis* genome of a large expansion of this gene family [48]. The expansion of this gene family is not found in any insect so far studied. The methyltransferases with the top two highest matches to putative insect acid methyltransferases based on e-values are shown in Table 1-3 along with the matches to functionally proven JHAMTs (shown in bold). For the latter for *D. variabilis*, the e-values range from e-25 to e-18, for *I. scapularis* e-24 to e-19 and for *O. turicata* from e-

34 to e-25 (Table 1-3). These data alone suggest that ticks have a JHAMT in their synganglia. Methylases including methyltransferases are responsible for transferring a methyl group from a donor to an acceptor substrate, and there are many types of methyltransferases in insects, e.g., DNA methyltransferase, histone methyltranferase, JHAMT and others. The putative association of tick methyltransferases as JHAMTs in Table 1-3 based on low e-values and high identity has to be considered with caution, since most methyltransferases identified in insects have been associated with JH biosynthesis. Also note in Table 1-3, there were matches ranging from e-32 to e-17 for acid methyltransferases which have not been functionally proven to be involved in JH synthesis. Selected methyltransferases of maximum length from the transcriptomes of *D. variabilis, I. scapularis* and *O. trunicata* were further aligned with insect acid transferases that are reported in the literature to be JHAMTs from *Bombyx mori* (NP_001036901), *Drosophila melanogaster* (NP_609793), *Tribolium castaneum* (NP_001120783), and *Aedes aegypti* (XP_001651876). Recombinant JHAMTs from *Bombyx mori* [60], *Drosophila melanogaster* [61], *Tribolium castaneum* [62], and *Aedes aegypti* [63] were shown to convert farnesoic acid into methyl farnesoate, as well as JHA into JH III and therefore are functionally proven JHAMTS which have the ability to metabolize both farnesoic acid and JHA [70]. The substrate (farnesoic acid or JH acid)-binding site is highlighted with "+" in Figure 1-5, along with the residues (labeled with X) that interact with the methyl donor (S-adenosylmethionine) and the carboxylic acid of the substrate methyl acceptors, farnesoic acid or JH acid. Interestingly, most of the amino acids are important in interactions with the methyl donor (SAM) and the substrate (farnesoic acid or JHA) are highly conserved in the insect JHAMT sequences. The insect

JHAMTs and the methyltransferases from the *D. variabilis* transcriptome have the classical SAM binding motifs, suggesting they are all methyltransferases. Two critical (conserved) residues near SAM, Gln-14(Q) and Trp-120 (W), bind the carboxyl group of farnesoic acid or JHA and place them in a suitable conformation for catalysis. In alignment (Figure 1-5), those two sites were labeled with a "+". Both syn19251 and syn18552 from the *D. variabilis* transcriptome lack the Gln-14(Q) and Trp-120 (W). Instead they have Lys (K) and Phe (F) in the place of Gln (Q). Amino acid Gln has a polar uncharged (neutral) side chain, but Lys has a positively charged side chain and Phe (F) has a hydrophobic ring on its side chain. Since they have different physical and chemical properties compare to Gln-14, they are structurally not conserved compared with the insect JHAMTs. Overall, the tick acid methyltransferases do not share the same key motifs as for the insect JHAMTs in the substrate binding site. We can confirm the presence of the key SAM-binding motifs in almost every tick methyltransferase we examined (Figure 1-5) but we are unable to locate a complete substrate-binding site for the JH precursors in the tick acid methyltransferases.

Neese et al. [22] incubated synganglion from the hard tick, *D. variabilis*, and the soft tick, *Onithodoros parkeri* in organ culture with a high specific activity, ^{3}H-methyl methionine; no ^{3}H-methyl JH or ^{3}H-methyl farnesoate could be found in the synganglion or culture media. These studies also were conducted with and without the addition of farnesoic acid. In both cases, radiolabeled methyl farnesoate and JH were not found while the same experimental conditions were successful in producing these products in a positive, insect corpora allata control. Furthermore, Neese et al. [22] were unable to find methyl farnesoate as well as the insect JHs in the hemolymph of vitellogenic ticks of both

of these same tick species by EI GC-MS. Interestingly, methyltransferases were also found in the spider mite genome and these methyltransferases also lacked the insect substrate binding residues like in ticks (data analysis not shown), but methyl farnesoate was reported by GC-MS in the spider mite. Acid methyltransferases were not exclusive to the synganglion in ticks but were also found in the male reproductive organs and midgut transcriptomes from *D. variabilis* (data not shown). Based on the current evidence in ticks and mites, we cannot conclude that the methyltransferases in ticks are involved in the synthesis of methyl farnesoate; however, more work is needed to re-examine this question of whether ticks can synthesize methyl farnesoate. It is unlikely the tick methyl transferases are involved in the synthesis of JH in ticks, since there are multiple lines of evidence that ticks do not make or have JH and there is a lack of any biological effect when ticks are treated with JH (discussed earlier).

Farnesol oxidase in ticks

Two of the enzymes in the early part of the JH branch, farnesyl diphosphate pyrophosphatase and farnesal dehydrogenase (Figure 1-1), have not been characterized in insects and were not studied in our transcriptomes (Table 1-1) or in the *I. scapularis* genome [48]. At this juncture, there is no direct evidence the enzymes are present in ticks. Short-chain dehydrogenases are needed for the conversion of farnesol to farnesal (a farnesol oxidase) and then from fanesal to farnesoic acid (a farnesal dehydrogenase)(Figure 1-1). Mayoral et al. [63] characterized the amino acid sequence and function of an NADP+-dependent farnesol dehydrogenase (AaSDR), a farnesol oxidase, in the corpora allata of the mosquito, *Aedes aegypti*, which was shown to transform farnesol into farnesal. The AaSDRs have several conserved motifs that placed

them in the SDR family of proteins and in the subfamily cP2, and orthologues have been found in another mosquito species, *Anopheles gambiae,* where they shared 61-67% identity with the *A. aegypti* AaSDR. Table 1-3 shows the BLASTp matches for a putative tick synganglion farnesol oxidase to the top three matches based on e-values in insects which were annotated as farnesol oxidases and to the functionally validated farnesol oxidase (AsSDR) in *A. aegypti* (shown in bold). For *D. variabilis*, the e-value was e-12 for the mosquito that was functionally proven as a farnesol oxidase with the top other putative farnesol oxidase matches ranging from e-102 to e-99 and for *I. scapularis*, was e-21 for the *A. aegypti* farnesol oxidase with top other matches ranging from e-102 to e-99. No matches were found for the soft tick. We reverse BLASTed the AaSDR sequence from *Aedes aegypti* against our *D. variabilis* synganglion transcriptome and found orthologues that matched the mosquito AaSDR sequence. The *D. variabilis* contig 5964 shared 24% identity and an e-value of 2e-9 with AaSDR. Based on the alignment in Figure 1-6, it shared several conserved motifs with AaSDR, but not all the motifs could be found due to the short contig length. From BLASTp searches conducted on the *I. scapularis* genome using the *A. aegypt*i farnesol oxidase, we found 79 dehydrogenases that shared 23%-30% identities with AaSDR (e-values ranging from e-21 to 0.001). We aligned the top match (EEC12752) from the *I. scapularis* genome with the *A. aegypti* AaSDR (Figure 1-7), and we found they shared 33% identity, with an e-value of 4e-22, and all the conserved motifs were present (Figure 1-7). It appears from both our transcriptomic studies in two hard tick species, *D. variabilis* and *I. scapularis*, and an analysis of the *I. scapularis* genome, ticks have the enzyme farnesol oxidase which is involved in the conversion of farnesol to farnesal. A direct functional analysis will be

needed in the future to confirm this finding. Based on this discovery, a reasonable hypothesis is that the presence of farnesol oxidase argues for also the presence of farnesyl diphosphate pyrophosphatase (Figure 1-1) since the latter is responsible for the synthesis of the substrate, farnesol. It is also possible that a farnesal dehydrogenase is present to make farnesoic acid based on some evidence of methyl transferases in ticks and mites and the report of methyl farnesoate in mites. However, the evidence is conflicting that supports the presence of insect JHAMTs in ticks or that ticks can synthesize methyl farnesoate as already discussed. Our data based on the presence of a farnesol oxidase is that at least the early parts of the JH branch but not JH is present in ticks, and more work will be needed to understand what JH precursors in the JH branch are synthesized by ticks.

Model for the regulation of adult reproduction in ticks and a role for the mevalonate-farnesal pathway

The most complete understanding of the endocrine regulation of tick development is associated with female reproduction, and the most advanced understanding of this process is in the American dog tick, *D. variabilis*.

Figure 1-8 is a model summarizing our current understanding of this process in *D. variabilis* and the potential role of the mevalonate-farnesal pathway in reproduction. In the initial steps for reproduction, both males and females must find a host together. The females feed to a part-fed condition, stop feeding and remain attached. Once the males fully feed, they detach from the host, find the part-fed female, mount the female and then insert their mouthparts into the female genital pore. This stimulates the production of the spermatophore and along with gonadotropin is transferred to the female genital tract [1].

The transfer initiates the synganglion to release epidermal trophic hormone (EDTH), stimulates rapid engorgement in the female to the replete (fully fed) condition, initiates synthesis of the insect molting neuropeptides (the tick function is unknown), and release of allatostatins and allatotropins (which may stimulate or inhibit the mevalonate-farnesal pathway; Figure 1-8). these peptides can also have other function in insects. EDTH initiates the production of ecdysteroids by the epidermis, which results in the synthesis of VgR in the ovaries and Vg in the fat body and midgut; Vg is then secreted into the hemolymph. Vg moves into developing oocytes via VgR-receptor mediated endocytosis and is deposited as vitellin.

It appears that the enzymes in the mevalonate pathway are present in adult synganglia in at least the three tick species examined, *D. variabilis*, *I. scapularis* and *O. turicata*. There was no evidence of the P450 family CYP15A1 in either our transcriptomes or in the genome of *I. scapularis* which in insects adds the C10,11 epoxide to make JH. Along with the lack of biochemical evidence for JH in ticks and lack of JH biological activity in ticks, it appears ticks do not make JH. However, we provide evidence that an earlier part of the JH branch is present for the synthesis of farnesal from farnesol by the enzyme farnesol oxidase. Methyltransferases were also found in the synganglia, but they lacked the JH or farnesoic acid binding motif found in insects, and biochemical studies did not show ticks could synthesize methyl farnesoate or had methyl farnesoate in their hemolymph. We hypothesize that ticks synthesize JH precursors found in the JH branch at least at the level of farnesal but the presence of methyl farnesoate is equivocal and more work is needed. It is likely that the mevalonate-farnesal pathway has some regulatory role in adult development potentially being

regulated by neuropeptides that in insects regulate JH biosynthesis, allatotropins and allatostatins, and which are also found in adult ticks. The exact role of the mevalonate-farnesal pathway is unknown and the products from this pathway have not yet been identified in ticks.

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Author Contributions

Conceived and designed the experiments:JZ RMR. Performed the experiments: JZ. Analyzed the data: JZ SMK RMR. Contributed reagents/materials/analysis tools: DES RMR BWB NE. Wrote the paper: JZ RDM RMR. Revising and rewriting of manuscript and approved final version to be published: RMR DES.

Table 1-1: Comparison of transcriptomes from different tick species, sexes, tissues and sequencing methods.

Tick	Sequencing method	Sex/Feeding stage/Tissue	Number of reads	Average read length (base pairs)	Number of contigs
D. variabilis	454 pyrosequencing (GS-FLX)	50 synganglia each from unfed, part-fed virgin and part-fed mated	532,136	229	21,119
I. scapularis	Illumina Truseq-I	50 synganglia mixed from unfed, part-fed, and replete females	34,520,330	68	41,249
	Illumina Truseq-II	45 part-fed female synganglia	117,900,476	72	30,838
	454 pyrosequencing (GS-FLX)	30 part-fed female synganglia	456,073	267	20,630
O. turicata	Illumina Truseq	50 replete female synganglia	63,528,102	438	132,258
[1]*D. variabilis*	454 pyrosequencing (GS-FLX)	500 eac from male accessory glands, testes vas deferens	563,093	300	12,804
[2]*D. variabilis*	Sanger	[3]unfed and fed ticks of both sexes	23,045	Not Available	1,679

[1]*D. variabilis* male reproductive system transcriptome from Sonenshine et al. (35).
[2]*D. variabilis* (midgut) transcriptome from Anderson et al. (36).
[3]Number of ticks unknown.

Table 1-2: Longest contig from the synganglion transcriptomes of *D. variabilis*, *I. scapularis*, and *O. turicata* with the lowest e-values for matches in the mevalonate pathway (leading to the synthesis of juvenile hormone III) in insects[1].

Substrate / Enzyme	*Dermacentor variabilis* Contig # (length, bp)	top 3 insect matches (Accession #)	e-value (% identity)	*Ixodes scapularis* Contig # (length, bp)	top 3 insect matches (Accession #)	e-value (%identity)	*Ornithodoros turicata* Contig # (length, bp)	top 3 insect matches (Accession #)	e-value (%identity)
Acetyl-CoA		*M. hirsutus*	4e-22		*A. darlingi*	8e-93		*A. aegypti*	5e-122
		ABN11931.1	(61%)		ADMH01001403.1	(56%)		XP_001657918.1	(65%)
Acetoacetyl-CoA	19096	*N. vitripennis*	2e-21	11699	*A. aegypti*	2e-92	74522	*D. simulans*	5e-119
Thiolase	(298)	XP_001606375.1	(60%)	(222)	XM_001654701.1	(57%)	(773)	XP_002076898.1	(66%)
		A. pisum	3e-21		*A. aegypti*	4e-92		*D. mojavensis*	6e-119
		XP_001943983.1	(61%)		DQ440481.1	(57%)		XP_002009553.1	(65%)
Acetoacetyl-CoA		*A. gambiae*	1e-13		NA	NA		*G. morsitans*	0.0
		XP_315872.3	(57%)					ADD19375.1	(62%)
HMG-S	11805	*D. jeffreyi*	2e-13	NA[2]	NA	NA	5140	*B. germanica*	0.0
	(231)	AAF89580.1	(50%)				(2758)	P54961.1	(62%)
		B. germanica	2e-13		NA	NA		*N. vitripennis*	0.0
		P54961.1	(59%)					XP_003426942.1	(62%)
HMG-CoA		*I. Paraconfusus*	5e-42		*N. vitripennis*	0.44		*G. viridula*	3e-114
		AAD20975.2	(77%)		XP_001601546.1	(28%)		ABO37161.1	(45%)
HMG-R	4404	*C. floridanus*	1e-41	4746	*A. pisum*	2.2	112681	*P. cochleariae*	4e-114
	(525)	EFN61977.1	(70%)	(306)	XP_003245001.1	(23%)	(1851)	ABO37160.1	(45%)
		N. vitripennis	2e-41		*D. yakuba*	2.2		*B. impatiens*	7e-114
		XP_001601404.1	(70%)		XP_002099595.1	(30%)		XP_003492098.1	(43%)
Mevalonate								*B. impatiens*	7e-04
								XP_003492382.1	(38%)
Mevalonate Kinase	NA	NA	NA	NA	NA	NA	67236	*D. simulans*	9e-04
							(313)	XP_002107342.1	(37%)
								A. gambiae	9e-04
								XP_313644.5	(48%)
Mevalonate-5-P		*B. mori*	5e-14		*D. pseudoobscura*	0.43		*S. invicta*	4e-52
		BAF62110.1	(46%)		XP_002138748.1	(60%)		EFZ11390.1	(50%)
phosphomevalonate	3143	*H. saltator*	2e-13	12685	*D. persimilis*	0.43	42225	*A. echinatior*	5e-50
Kinase	(330)	EFN87008.1	(44%)	(383)	XP_002016604.1	(60%)	(1020)	EGI69273.1	(47%)
		C. floridanus	2e-12		*D. yakuba*	2.1		*A. florea*	9e-50
		EFN64677.1	(44%)		XP_002095199.1	(36%)		XP_003695278.1	(48%)
Mevalonate-5-PP		NA	NA		*D. mojavensis*	2.8		*D. ponderosae*	5e-141
					XP_002003397.1	(56%)		AFI45055.1	52%
Diphosphomevalonate	NA	NA	NA	9701	*A. darling*	6.3	5162	*A. mellifera*	2e-140
Decarboxylase				(208)	EFR19559.1	(34%)	(1544)	XP_001121619.2	51%
		NA	NA		*T. castaneum*	6.3		*A. aegypti*	3e-139
					EEZ98110.0	(37%)		XP_001648384.1	23%

Tables continues

Substrate / Enzyme	*Dermacentor variabilis* Contig # (length, bp)	top 3 insect matches (Accession #)	e-value (% identity)	*Ixodes scapularis* Contig # (length, bp)	top 3 insect matches (Accession #)	e-value (%identity)	*Ornithodoros turicata* Contig # (length, bp)	top 3 insect matches (Accession #)	e-value (%identity)
Isopentenyl-PP→Dimethylally-PP Geranyl-PP←┘									
↓		*D. jeffreyi*	7e-11		*A. darling*	5e-12			
		AAX78435.1	(57%)		EFR25052.1	(71%)			
Farnesyl Diphosphate	4375	*D. yakuba*	3e-10	10790	*A. gambiae*	5e-12	NA	NA	NA
Synthase	(241)	XP_002090289.1	(62%)	(461)	XP_316306.3	(71%)			
		D. sechellia	3e-10		*S. invicta*	7e-12			
		XP_002033374.1	(62%)		EFZ14302.1	(60%)			

[1] *Acromyrmex* echinatior, Leaf-cutter ant; *Acyrthosiphon pisum*, Pea aphid; *Aedes aegypti*, Yellow fever mosquito; *Anopheles darlingi*, American malaria mosquito; *Anopheles gambiae*, African malaria mosquito; *Apis florea*, Red dwarf honey bee; *Apis mellifera*, Western honey bee; *Blattella germanica*, German cockroach; *Bombus impatiens*, Eastern bumble bee; *Bombyx mori*, Silkworm; *Camponotus floridanus*, Carpenter ant; *Dendroctonus ponderosae*, Mountain pine beetle; *Dendroctonus ponderosae*, Mountain pine beetle; *Drosophila mojavensis*, Fruit fly; *Drosophila persimilis*, Fruit fly; *Drosophila pseudoobscura*, Fruit fly; *Drosophila simulans*, Fruit fly; *Drosophila yakuba*, Fruit fly; *Gastrophysa viridula*, Green dock beetle; *Glossina morsitans*, Savannah tsetse fly; *Harpegnathos saltator*, Indian jumping ant; *Ips* paraconfusus, Bark beetle; *Maconellicoccus hirsutus*, mealybug; *Nasonia vitripennis*, Jewel wasp; *Phaedon cochleariae*, Mustard leaf beetle; *Solenopsis invicta*, Red imported fire ant; *Tribolium castaneum*, Red flour beetle.

[2]NA, no match found.

Table 1-3: Longest contig from the synganglion transcriptomes of *D. variabilis*, *I. scapularis*, and *O. turicata* with the lowest e-values for matches in the JH branch in insects[1].

Substrate	*Dermacentor variabilis*			*Ixodes scapularis*			*Ornithodoros turicata*		
Enzyme	Contig # (length bp)	top 3 insect matches[2] (Accession #)	e-value (% identity)	Contig # (length bp)	top 3 insect matches[2] (Accession #)	e-value (%identity)	Contig # (length bp)	top 3 insect matches[2] (Accession #)	e-value (%identity)
Farnesyl-PP									
↓ Farnesyl Diphosphate Pyrophosphatase	NA[3]	NA	NA	NA	NA	NA	NA	NA	NA
Farnesol		*N. vitripennis*	1e-102		*C. floridanus*	1e-102			
		XP_001606362.2	(53%)		EFN72306.1	(58%)			
		D. ananassae	5e-101		*S. invicta*	5e-101			
↓ Farnesol Oxidase	5964	XP_001960084.1	(54%)	8342	EFZ18513.1	(54%)			
	(758)	*D. willistoni*	1e-99	(440)	*C. capitata*	1e-99	NA	NA	NA
		XP_002068852.1	(56%)		XP_004523325.1	(56%)			
		A. aegypti[4]	**3e-12**		**A. aegypti**	**8e-21**			
		D2WKD9	**(25%)**		**D2WKD9**	**(32%)**			
Farnesal									
↓ Farnesal Dehydrogenase	NA	NA	NA	NA	NA	NA	NA	NA	NA
Farnesoic acid		***B. mori***	**4e-25**		*A. darlingi*	4e-24		***A. aegypti***	**4e-34**
		NP_001036901	**(30%)**		ETN59754.1	(39%)		**XP_001651876.1**	**(38%)**
		T. castaneum	**2e-24**		***T. castaneum***	**2e-24**		*S. litura*	1e-32
		NP001120783	**(33%)**		**NP001120783**	**(32%)**		BAF63629.1	(37%)
		A. aegypti	**1e-18**		***D. melanogaster***	**9e-24**		***B. mori***	**5e-30**
↓ Juvenile Hormone Methyltransferase	19251	**XP_001651876.1**	**(32%)**	24500	**NP_609793**	**(35%)**	16407	**NP_001036901**	**(35%)**
	(1014)	*A. pisum*	1e-18	(649)	*S. gregaria*	5e-23	(757)	*H. armigera*	1e-28
		NP_001156251.1	(35%)		ADV17350.1	(39%)		BAF63630.1	(35%)
		D. melanogaster	**2e-18**		***A. aegypti***	**8e-23**		***D. melanogaster***	**4e-26**
		NP_609793	**(25%)**		**XP_001651876.1**	**(32%)**		**NP_609793**	**(34%)**
		C. floridanus	2E-17		***B. mori***	**1e-19**		***T. castaneum***	**2e-25**
		EFN68634.1	(28%)		**NP_001036901**	**(31%)**		**NP001120783**	**(38%)**
Methyl farnesoate									
↓ JH epoxidase	NA	NA	NA	NA	NA	NA	NA	NA	NA
Juvenile hormone III									

[1] *Acyrthosiphon pisum*, Pea aphid; *Aedes aegypti*, Yellow fever mosquito; *Anopheles darlingi*, American malaria mosquito; *Bombyx mori*, Silkworm; *Camponotus floridanus*, Carpenter ant; *Ceratitis capitata*, Mediterranean fruit fly; *Drosophila ananassae*, Fruit fly; *Drosophila melanogaster*, Fruit fly; *Drosophila Willistoni*, Fruit fly; *Nasonia vitripennis*, Jewel wasp; *Schistocerca gregaria*, Desert locust; *Solenopsis invicta*, Red imported fire ant; *Spodoptera litura*, Leafworm/Noctuid moth; *Tribolium castaneum*, Red flour beetle; *Tribolium castaneum*, Red flour beetle

[2] The order of BLAST results are listed based on e-value.

[3] NA, no match found.

[4] Results highlighted in bold are for insect messages that when expressed, the enzyme could metabolized the substrate as shown

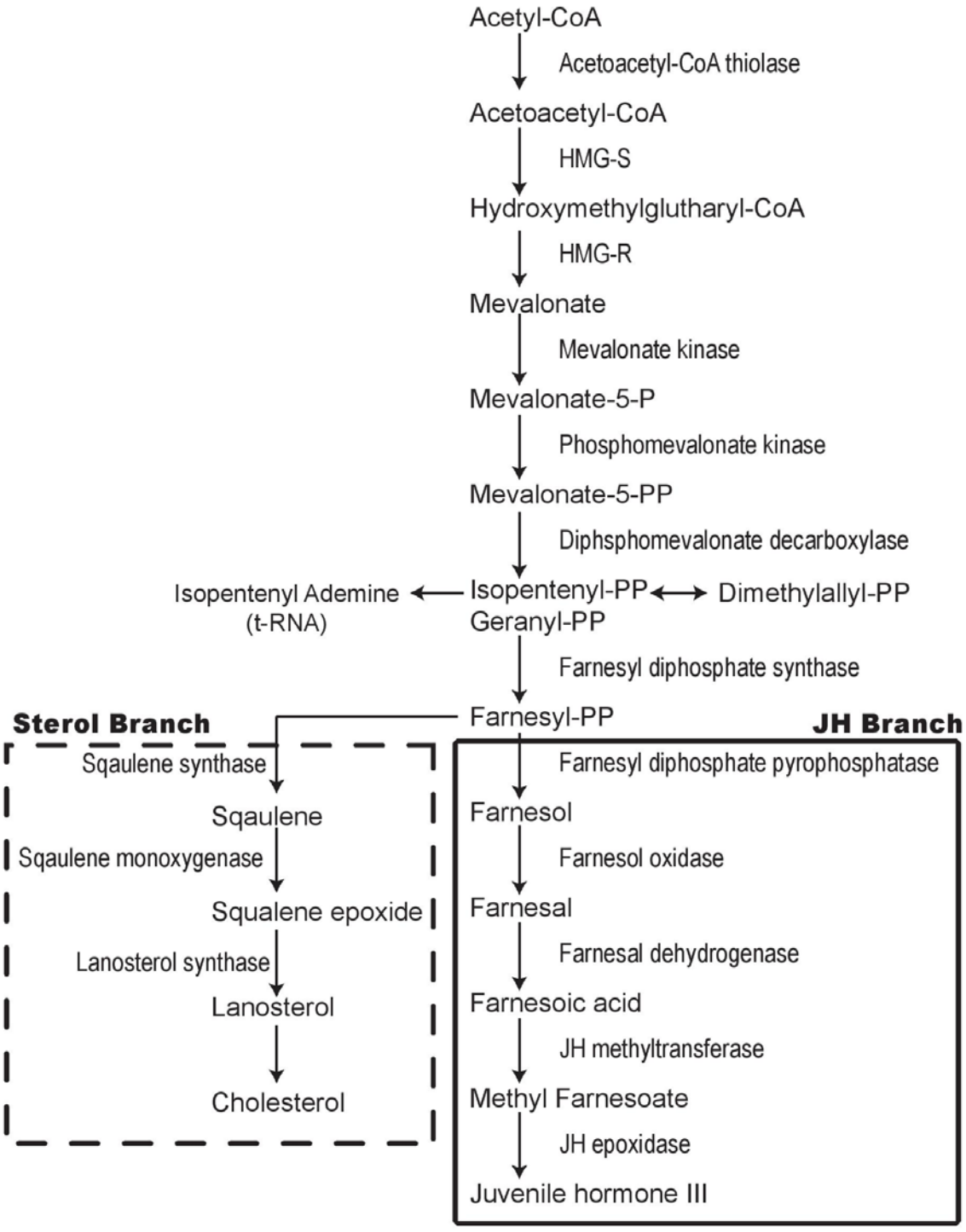

Figure 1-1: Juvenile hormone III and cholesterol bio-synthesis pathways in insects, modified from Bellés et al. (38).

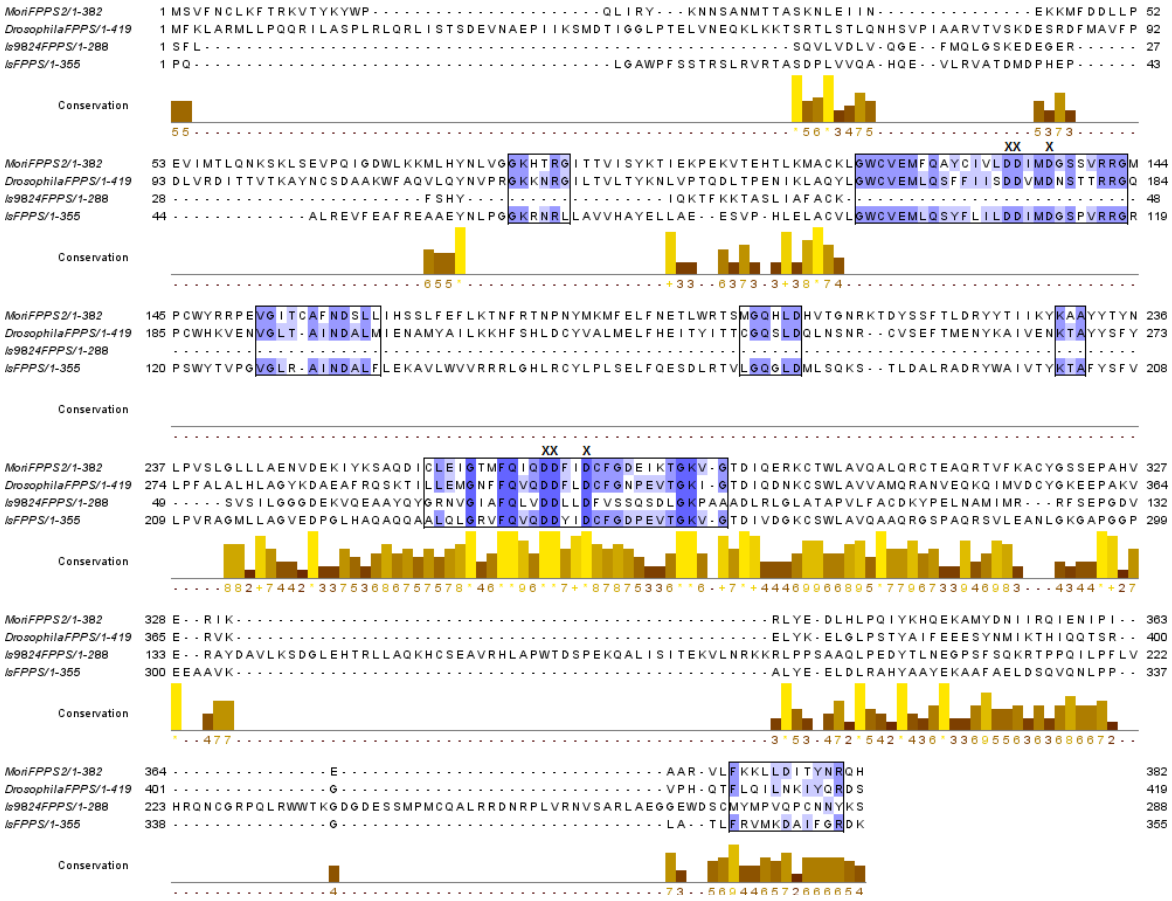

Figure 1-2: Farnesyl diphosphate synthase (FPPS2) described from *Bombyx mori* and *Drosophila melanogaster* aligned with contig 9824 from the *Ixodes scapularis* transcriptome and FPPS (XP_002408650) from the *Ixodes scapularis* genome. All the boxed regions are the seven prenyltransferase conserved regions previously identified by Koyoma et al. (50). "X" above the amino acids indicates position of Asp residues within the two aspartate-rich domains. Below the sequence alignment is the conservation panel which is measured as a numerical index (9 – 0) reflecting the conservation of physicochemical properties in the alignment. * (asterisk) denotes the highest identity score (identical residues in all species) followed by a score of 9 for the next most conserved group of residues containing substitutions by amino acids included in the same physicochemical class as described by Livingstone and Barton (69).

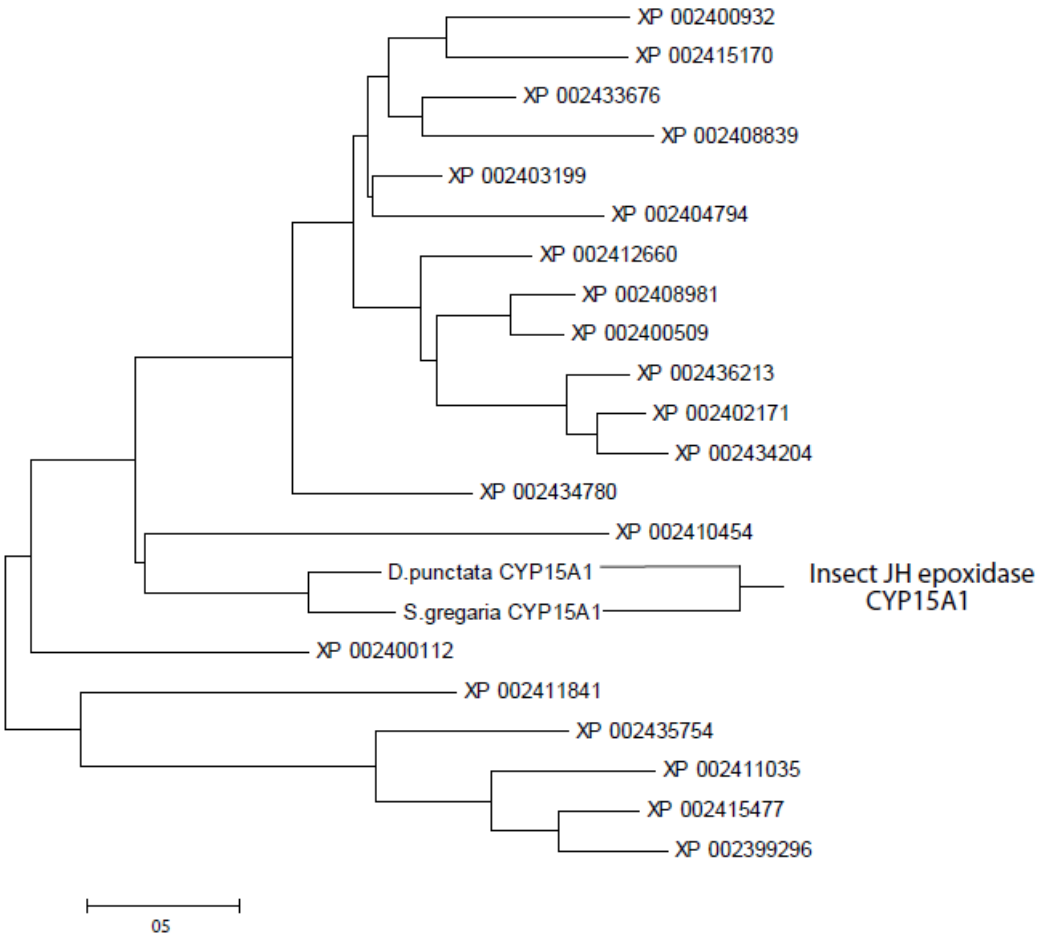

Figure 1-3: Phylogenetic tree comparing the P450 CYP15A1 known in *D. punctata* (AAS13464) and *S. gregaria* (HQ634703) to add the C10,11 epoxide to methyl farnesoate to make JH III to the top BLASTp matches (lowest e-values) to the *D. punctata* CYP15A1 found in the *I. scapularis* genome and which had the longest length in base pairs. The 20 full length CYP messages with lowest e-values and which were length from *I. scapularis* were not in the CYP15A1 family (were in a different clade). The optimal neighbor-joining phylogenic tree was constructed by the Molecular Evolutionary Genetics Analysis (MEGA) program. All of the accession numbers labeled with XPs are CYP messages from the *I. scapularis* genome. The recombinant expressed CYP15A1s from *D. punctata* and *S. gregaria* are labeled insect JH epoxidase. Percent identity was determined by BLASTp. The highest identity message XP_002410454 shares 28% identity with *D. punctata* and 30% identity with *S. gregaria*. No members of the Cyp15A family were found in the *I. scapularis* genome (48). An alignment of the closest sequence XP002410454 to the CYP15A1 family is shown in Figure 1-4.

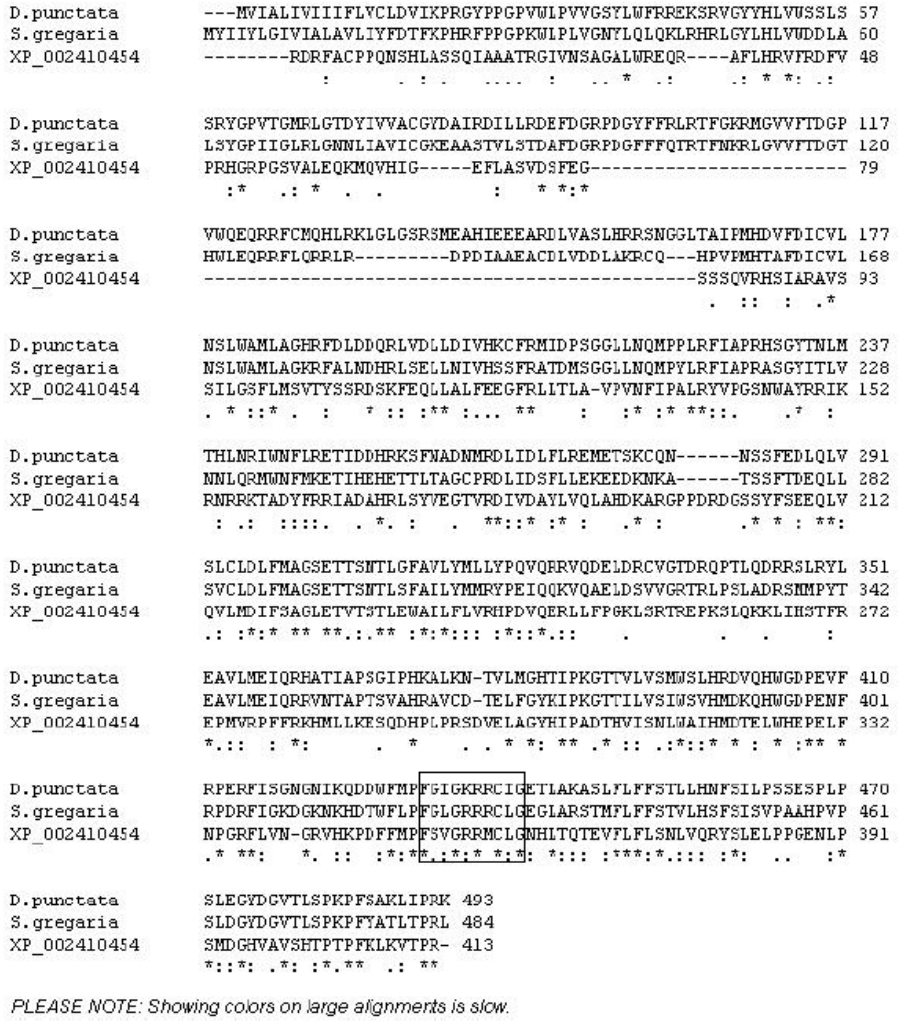

Figure 1-4: Sequence alignment (pairwise) for XP_002410454, the top BLAST match from the *I. scapularis* genome to that of the published sequences (GenBank) for CYP15A1 from D. *punctata* AAS13464, (23) and *S. gregaria* HQ634703, (70). The JH epoxidase is a member of the CYP15A1 family and has been cloned and characterized from the corpora allata of *D. punctata* and *S. gregaria*. It has been demonstrated this enzyme can add an epoxide to the C10,11 position of methyl farnesoate to produce JH III with high stereo selectivity (10*R*). All members of the family CYP15 share >40% identity at the amino acid level and >55% identity at the subfamily level (57). The boxed sequence on the alignment is the signature heme-binding motif of P450 (F**G***C*G). An * (asterisk) indicates positions which have a single, fully conserved residue. A colon indicates conservation between groups of strongly similar properties, scoring > 0.5 in the Gonnet PAM 250 matrix. A period indicates conservation between groups of weakly similar properties, scoring ≤ 0.5 in the Gonnet PAM 250 matrix (Larkin et al., 2007). Based on our alignment, the *I. scapularis* sequence XP_002410454 only shares 28% identity with *D. punctata* and 30% identity with *S. gregaria*. Therefore XP_002410454 is not a member of the CYP15 gene family.

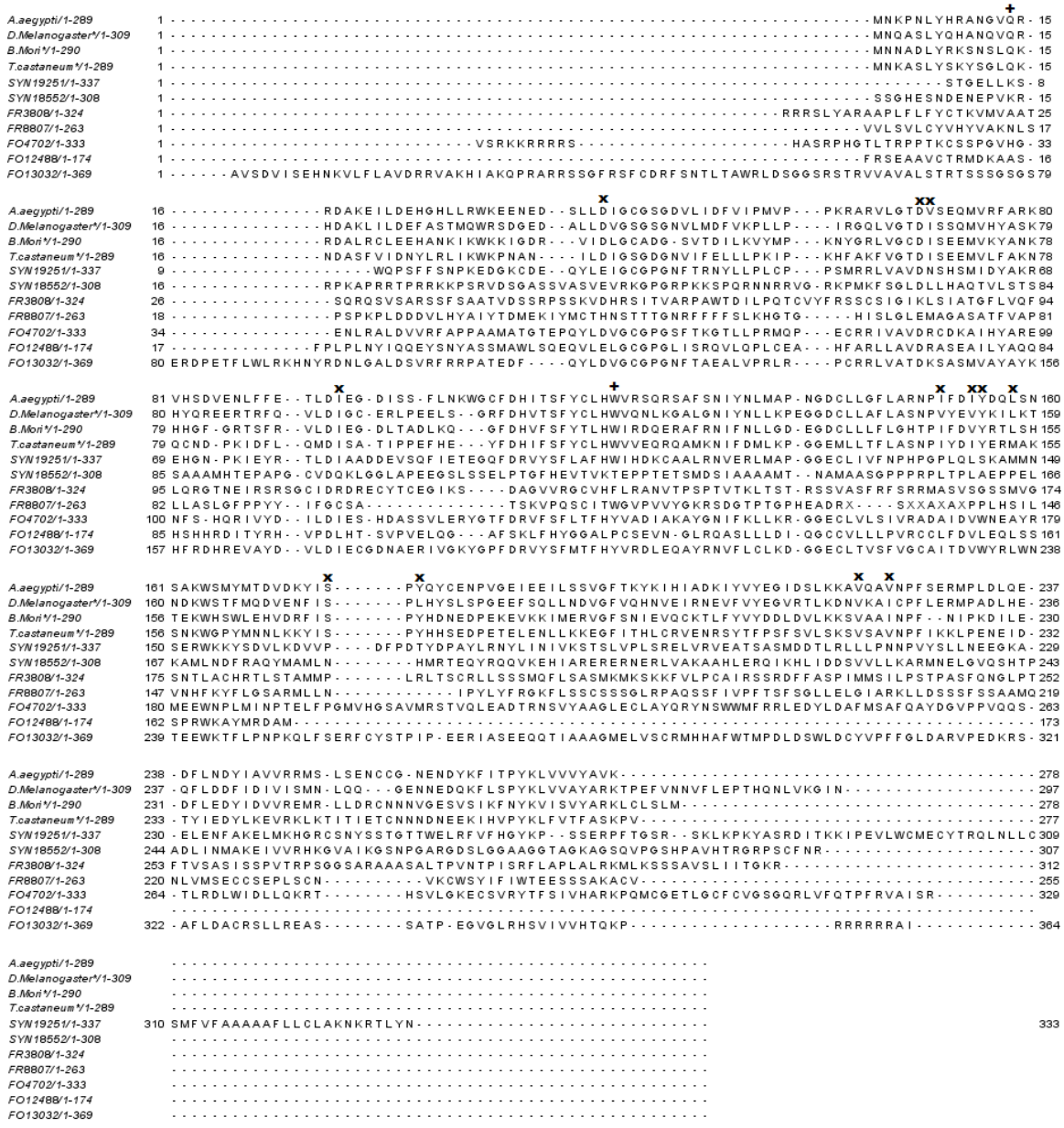

Figure 1-5: Alignment of methyltransferases from ticks with that of known JH methyltransferases from insects (based on published direct demonstration of function or by bioinformatics). Contains the Gln-14 and Trp-120 residues which are important for farnesoic acid or JH acid interaction, and part of the SAM binding motif. Both Gln-14 and Trp-120 residues are labeled with **+** on the top of the sequence. The SAM binding motif and ligand interactions are marked with **X** on the top of the sequence. Selected methyltransferases with the maximum length in bps from the transcriptomes of *D. variabilis*, *I. scapularis* and *O. trunicata* were aligned with known insect JH methyl transferases from *Bombyx mori* (NP_001036901), *Drosophila melanogaster* (NP_609793), *Tribolium castaneum* (NP_001120783), and *Aedes aegypti* (XP_001651876).

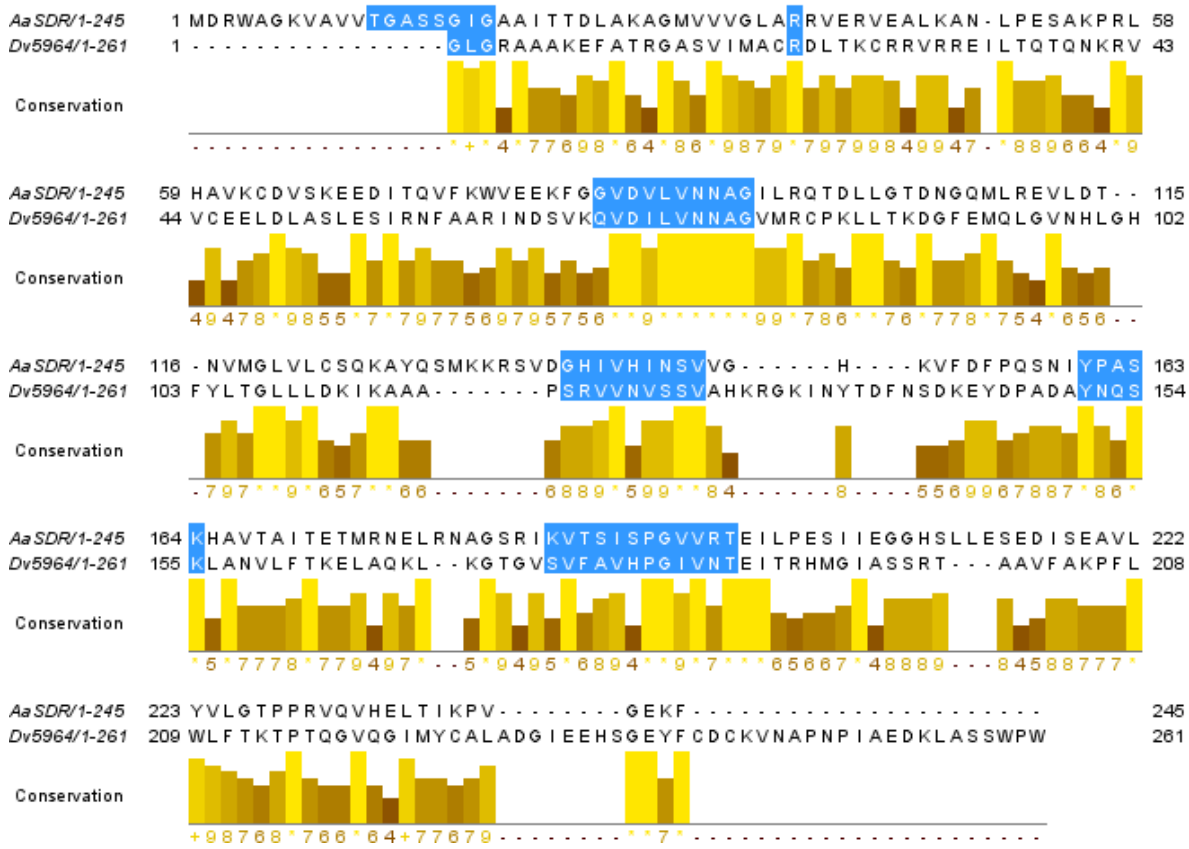

Figure 1-6: Farnesol dehydrogenase (AaSDR) from *Aedes aegypti* aligned with contig 5964 from the *D. variabilis* synganglion transcriptome. Highlighted residues on both alignments show the conserved motifs that place them in the SDR family and the subfamily cP2. Below the sequence alignment is the conservation panel which is measured as a numerical index (9 – 0) reflecting the conservation of physicochemical properties in the alignment. * (asterisk) denotes the highest identity score (identical residues in all species) followed by a score of 9 for the next most conserved group of residues containing substitutions by amino acids included in the same physicochemical class as described by Livingstone and Barton (69).

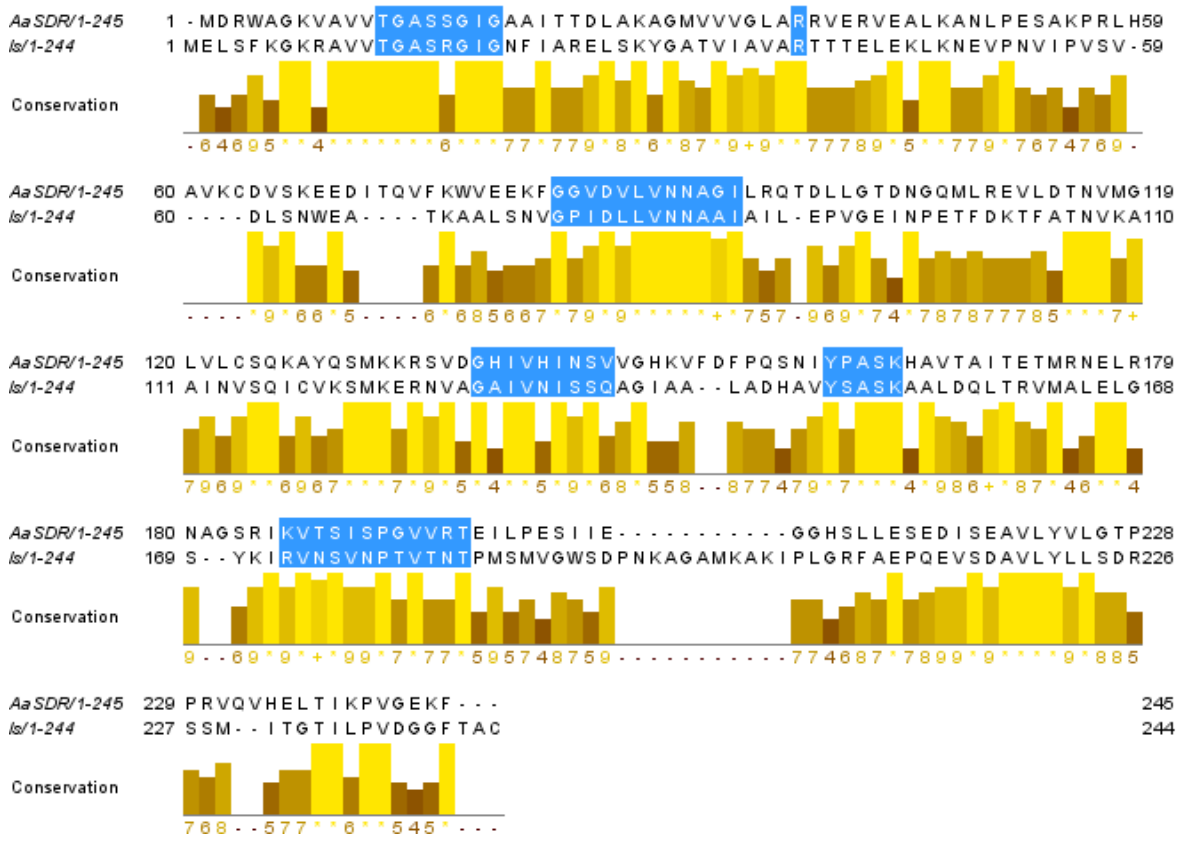

Figure 1-7: Farnesol degydrogenase (AaSDR) from *Aedes aegypti* is aligned with sequence EEC12752 from the *I. scapularis* genome. Highlighted residues on both alignments shows the conserved motifs that place them in the SDR family and the subfamily cP2 . Below the sequence alignment is the conservation panel, which is measured as a numerical index (9 – 0) reflecting the conservation of physicochemical properties in the alignment. * (asterisk) denotes the highest identity score (identical residues in all species), followed by a score of 9 for the next most conserved group of residues containing substitutions by amino acids included in the same physicochemical class as described by Livingstone and Barton (69).

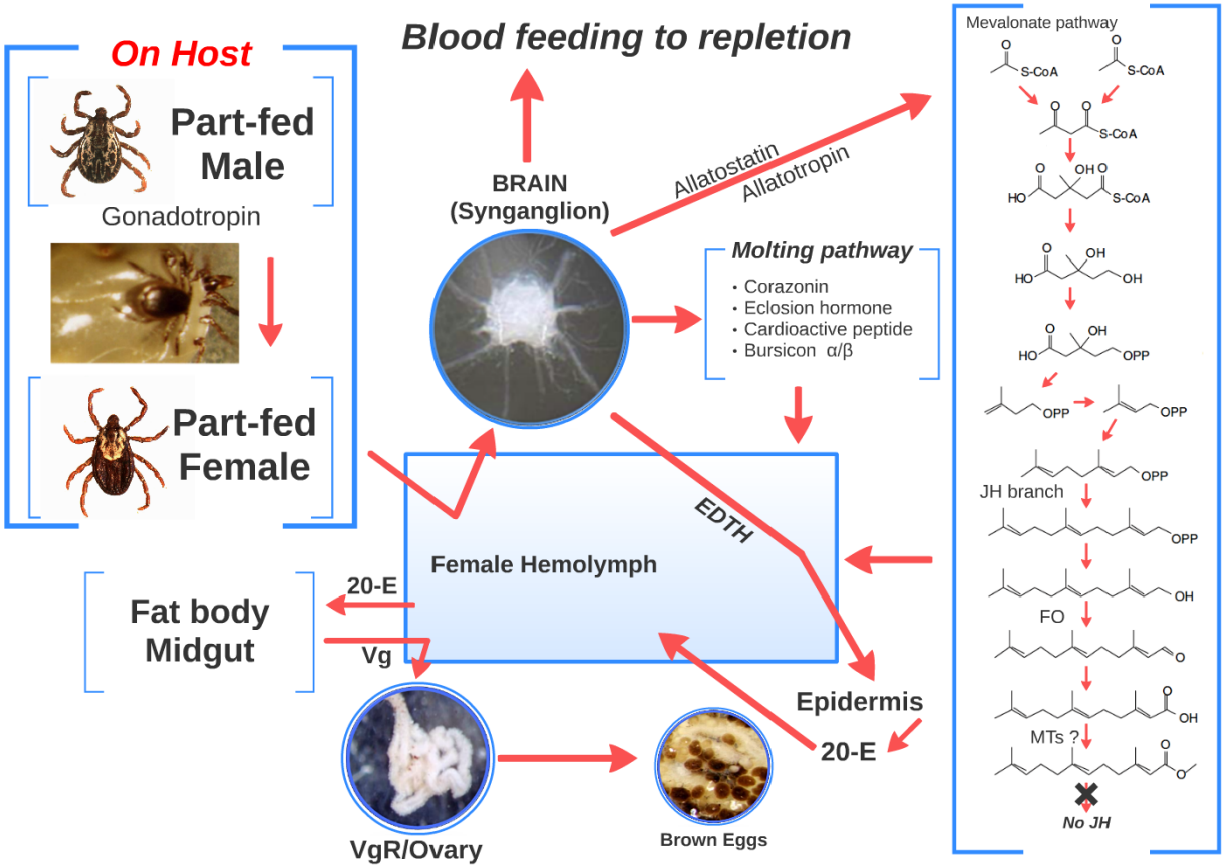

Figure 1-8: Model for the endocrine regulation of vitellogensis in *D. variabilis* and potential role of the mevalonate-farnesal pathway. See text for description. Abbreviations in figure: EDTH, hypothesized epidermal trophic hormone; Vg, vitellogenin; VgR, Vg receptor; 20-E, 20-hydroxyecdysone

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Chapter 2

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Novel use of aliphatic n-methyl ketones as an alternative to methyl bromide for fumigation of the red imported fire ant, *Solenopsis invicta* and other insects

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Abstract

Fumigants like phosphine, methyl bromide and sulfuryl fluoride are highly effective and efficient for the control of structural, storage and agriculture arthropod pests. Unfortunately, many of these synthetic compounds are highly toxic to applicators and consumers, many pests have developed resistance to these compounds such as flour beetles to phosphine, and methyl bromide has been de-registered because of its depletion of the stratospheric ozone layer. Several plant species synthesize and emit a variety of natural n-aliphatic methyl ketone fumigants to prevent plant herbivory. To examine the use of methyl ketones as a novel, arthropod fumigant, structure-mortality studies were conducted using the red imported fire ant, *Solenopsis invicta* Buren, as a model. The compounds were screened with a bioassay system that was developed in this study to screen fumigants in the laboratory. The bioassay system is easy-to-use and inexpensive and could be used for a variety of pest species including arthropods, seeds, microorganisms and nematodes. The LC_{50}s determined from probit models for heptanone, octanone, nonanone and undecanone were 4.27, 5.11, 5.26 and 8.21 µg/cm^3 of ambient air, respectively, compared to a lower and upper range of 48.08 and 112.2 µg/cm^3, respectively, for methyl bromide. Although the former three methyl ketones were more effective than undecanone, subsequent research was conducted with 2-undecanone since this compound already has an US EPA registration as a biopesticide for use on human skin as an arthropod repellent. In dose-response field studies, undecanone formulated as an emulsion in water and injected into mounds, was effective at a low dose of 12.5 ml of active achieving 100% kill of fire ants 4 days post treatment with no re-establishment of ants after 7 days post treatment. Undecanone at a concentration that

achieved 100% kill in our lab bioassay was more cost effective than methyl bromide for fire ants, German cockroaches, and tobacco budworm eggs, but slightly more costly for flour beetles.

Keywords: fumigant, methyl bromide, methyl ketone, 2-undecanone, *Solenopsis invicta*.

Introduction

Fumigation is an effective and efficient method for controlling structural, storage, and agricultural pests, and methyl bromide (MeBr) has been the fumigant of choice for many years (Thompson et al. 1966). MeBr has a broad spectrum of activity against insects and other pest species including nematodes, plants and bacteria and has been used in many different applications including soil, storage and transport of goods, and for structural and agriculture pests prior to planting and for post-harvest products. One important attribute of MeBr is a low absorption rate; this allows for rapid and efficient penetration into the substrate being treated but at the same time rapid dissipation after ventilation with MeBr-free air. There are also some negative aspects to this technology. For example, applicators must be thoroughly trained for safe use of the product, and permits are required for applications. Because MeBr is an odorless and colorless gas, it cannot be detected readily. Consequently, safe handling is critical to avoid accidental exposure. Accidental injury from the use of MeBr continues to occur regardless of safety precautions. Tracer (warning) compounds such as chloropicrin that are eaily detected by smell or tearing at low levels are required as an addition to MeBr to treat some commodities or areas. However, additives produce transient symptoms that can be quite uncomfortable. The major shortcoming of MeBr is its contribution to the depletion of the stratospheric ozone layer. Under the Montreal Protocol and the Clean Air Act, the production and usage of MeBr was to be phased out after January 1, 2005 (EPA). There are limited MeBr alternatives available, i.e., sulfuryl fluoride and phosphine, but these too are EPA restricted use compounds because of safety concerns. All of these fumigants are synthetic chemistries. The concerned public is looking for solutions that come from

natural sources, which are safer, but are at least as efficient and cost effective as current synthetic technologies for fumigation.

The red imported fire ant, *Solenopsis invicta* Buren, the model insect pest used in this study, was introduced into the United States of American in Mobile, Alabama in the 1930s (Lofgren et al. 1975). Since then, *S. invicta* has spread and currently occupies a much greater area, now occurring in nine states from the Carolinas to Texas and in California. Fire ants are known for their aggressive behavior and are notorious for their potent sting, inflicting pain and inducing hypersensitivity reactions in humans. They also form large colonies at high densities; are capable of damaging agriculture machinery and interfering with crop production; and can displace native ant species, affecting the ecology of new areas where they become established (Vinson 1986; Lard et al. 2006). The economic impact from the red imported fire ant is estimated to be $5 billion USD in the United States (McDonald 2006). The warm, wet weather of the South is ideal for fire ants, and they flourish in crop lands, roadsides, parks and residential lawns (Whitcomb et al. 1972). Fire ants in hay pastures can cause equipment breakdowns and in hay is a worker hazard and is harmful to livestock. Infested hay is also regulated and cannot be shipped to areas of the US where fire ants are not yet established. Shipment of regulated items from infested areas under federal quarantine must be fumigated before shipping, but as discussed earlier, desirable fumigants for this application are not available.

Historically, plant based insecticides have been used for centuries (Ebeling 1971; Coats 1994). Many of them are secondary plant substances such as quinones and essential oils. (Raven et al. 1992). Surprisingly, many of the same compounds are found in cosmetics, foods, and pharmacological additives where they provide flavors and

fragrances. The glandular trichomes of the wild tomato plant, *Lycopersicon hirsutum* Dunal f. *glabratum* C. H. Müll, produces an aliphatic methyl ketone, 2-undecanone, that protects the plants from herbivorous catipillars (Witting-Bissinger et al. 2008). A variety of methyl ketones are produced by other plants. For example, 2-nonanone, the main constituent of Algerian oil of rue can also be found in the essential oil of cloves, *Eugenia curyophyllata*, and 2-heptanone was found in cinnamon oil and in oil of cloves. Nonanone, undecanone and tridecanone were identified in the essence of coconut oil. Many plant species produce volatile essential oils where 2-undecanone or a combination of methyl ketones occur mixed with other organics to protect the plant from herbivory by arthropod pests (Forney and Markovetz 1971; Dimock and Kennedy, 1983; Farrar and Kennedy, 1987; Kennedy, 2003).

The compound 2-undecanone has strong repellent activity against mosquitos and ticks, and it has been developed into an EPA-registered compound in the commercial arthropod repellent, BioUD® (HOMS LLC, Clayton, NC, U.S.A.), for use on skin and clothing to control mosquitoes and ticks. BioUD was found to be two to four times more repellent than *N*,*N*-diethyl-*meta*-toluamide (DEET) against three species of ixodid ticks, *Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis*, in in vitro two-choice assays using filter paper as a substrate (Bissinger et al. 2009) and showed >90% repellency for 5 weeks on cotton cheesecloth against *A. americanum* (Bissinger et al. 2009). When applied to human skin under field conditions, BioUD also provided excellent repellency against mosquitoes, more efficacious than 30% DEET for 6 h (Witting-Bissinger et al. 2008). Like many spatial repellents, 2-undecanone also demonstrated insecticidal activity as a vapor which has not been previously investigated.

Because 2-undecanone is registered by the US EPA as a biopesticide, it has both repellent and insecticidal activity as a vapor, it has a safe toxicity profile including being approved by the EPA for use on human skin, and undecanone is found in nature (produced by plants), we examined the potential use of this compound and its related chemistry as a fumigant and possible alternative to MeBr using the red imported fire ant as a model insect system with selected comparisons to other insect pests.

Material and methods

Insects

Red imported fire ants, *Solenopsis invicta*, were collected with an insect aspirator (model number 1135A, Bioquip, CA, U.S.A) from colonies within a 50 m radius of the Dearstyne Entomology Building (35°47'18.9"N 78°41'56.7"W; Figure 2-1) on the North Carolina State University Campus, Raleigh, North Carolina, U.S.A. Approximately 500 fire ants were transferred to nest material (a plastic Petri dish 14 cm in diameter and 1.5 cm in height, the bottom dish filled with dental plaster up to 8 mm in height) and one or more collections placed into plastic trays (40 cm by 52 cm by 10 cm) painted along the top (inside) open edge of the container with a Teflon emulsion (PTFE-30, Dupont, Wilmington, DE, U.S.A.) to prevent the ants from escaping. The 20% Teflon emulsion was made of fluon in distilled water (vol./vol.). Only the top 6 cm of the tray sides was coated using a cotton ball. The petri dish (14 cm diameter and 1.5 cm height with the bottom dish filled with dental plaster up to 8 mm in height) provided the ants with an artificial nesting site. Ants were held at 25±3ºC and 60±5% RH with a photoperiod of

12:12h (L:D) and were supplied *ad libitum* a 10% (wt./vol.) sucrose (99.7% purity, Acros Organics, Fisher Scientific, Pittsburgh, PA, U.S.A.) solution made in distilled water (provided via a cotton wick). Tobacco budworm, *Heliothis virescens*, eggs were purchase from Benzon Research Inc. Carlisle, PA, U.S.A. At the start of experiment, the eggs were 1 day old. A German cockroach, *Blattella germanica*, colony was established from insects provided by TyraTech Inc., Morrisville, NC, U.S.A. The colony was maintained in a plastic bucket (22cm in diameter, 24cm in height). The top 6 cm from the opening on the inside of the container was treated with Teflon emulsion as described before. The opening was covered with cheese cloth (26 cm x 26 cm, Uline, WI, U.S.A) and held in place with 2 rubber bands to prevent roaches from escaping. The colony was held at 25±3ºC and 60±5% RH with a 12:12h (L:D) cycle and provided *ad libitum* Home 360® premium dog food (Hannaford, Portland, ME, U.S.A) and distilled water (using cotton wicks). A red flour beetle, *Tribolium castaneum*, colony (North American GA-1) was a gift from Dr. Marcé Lorenzen, Department of Entomology, NCSU. This colony was originally collected in a farmer's corn bin in Georgia, U.S.A in 1980 (Haliscak and Beeman, 1983). The beetles are reared in a mason jar (8 cm in diameter, 13 cm in height) at 25±3ºC and 60±5% RH with a 12:12h (L:D) cycle (Beeman and Stuart, 1990) and fed Pillsbury wheat flour (The Pillsbury Company, Minneapolis, MN, U.S.A):brewer's yeast (MP Biochemicals, Salon, OH, U.S.A (95:5; wt./wt.).

Methyl ketones

Technical methyl ketones were purchased from Sigma-Aldrich, Saint Louis, MO, U.S.A. The four methyl ketones were 2-undecanone, 2-nonanone, 2-octanone and 2-

heptanone. All four methyl ketones were a liquid at room temperature. The 2-undecanone (CAS number 112-12-9) was 99% pure and appeared colorless to light yellow with a density of 0.825g/mL at 25 ºC; 2-nonanone (CAS number 821-55-6) was ≥99% pure and colorless to faint yellow with a density of 0.82 g/mL at 25 ºC; 2-octanone (CAS number 111-13-7) was ≥98% pure and colorless to pale yellow with a density of 0.819 g/mL at 25 ºC; and 2-heptanone (CAS number 110-43-0) was ≥98% pure and colorless with a density of 0.82 g/mL at 25 ºC. These methyl ketones are volatile compounds at room temperature; the bottles in which they were received were sealed between the plastic cap and bottle with Parafilm (Fisher Scientific, Ithaca, IL, U.S.A.) and kept underneath the fume hood at room temperature until needed. All methyl ketones were used within 6 month of receipt from Sigma-Aldrich.

Fire ant fumigation bioassay and dose response

A fumigation chamber was constructed with 2 plastic Petri dish bottoms (8.6 cm in diameter and 1.8 cm in height each), stacked lip-to-lip to create a total inside volume of 210 cm^3 as shown in Figure 2-2. The two petri dish halves were separated by a 10 × 10 cm section of cheese cloth. The technical grade methyl ketone was applied with a 5 µl Hamilton syringe (Part Number 80016, Sigma-Aldrich) to the upper surface of a glass cover slip placed in the lower chamber bottom for test volumes ≤5 µl. For testing volumes greater than 5µl, a pipette (PIPETMAN P20, Gilson Inc. Middleton, WI, U.S.A) was used to dispense the appropriate volume of methyl ketones. Equivalent volumes of distilled water were used as controls in a separate fumigation assay, and controls were always run at the same time as treatments. For each assay, 25 adult ants of varying size

were first transferred from their rearing container (described earlier) to a 30 ml, transparent polypropylene cup (Solo Cup Company, Lake Forest, IL, U.S.A.), chilled on ice for 15 sec, and then transferred to the chamber above the cheese cloth (Fig. 2) using a fine camel hair paint brush. A 2 cm cotton wick saturated with 10% sucrose (wt./vol.) had been transferred to the lid of the upper chamber and was held in place by a 1.5 × 2.5 cm strip of Scotch tape (Scotch). The two petri dish bottoms were held together and sealed with Parafilm (Fisher Scientific), and the insects were held at 25±3ºC and 60±5% RH for 24 h. After the exposure period, mortality for the treatment and control was recorded where death was defined as no movement of the insect when touched with a blunt probe. Only the 2 petri dish bottoms of the fumigation chamber were re-used; the inside was rinsed with 95% ethanol to remove any residual chemicals, then washed with dish soap (Dawn, P&G, Browns Summit, NC, U.S.A), rinsed in distilled water and air dried in a fume hood for at least 12 h.

Range finding to determine an approximate dose (=volume) of the methyl ketones to add to the fumigation chamber to obtain 50% morality in 24 h was conducted first with 10 ants per test. Once the dose range was obtained, more careful studies were conducted with 25 ants per assay and replicated at least 3 times at each dose. All the methyl ketones tested were liquid when applied but had fully evaporated by the end of the 24 h incubation period. The fumigant concentration ($\mu g/cm^3$) was calculated based on the weight applied to the cover slip (calculated from the volume applied and density of the methyl ketone) per unit volume of the fumigation chamber, assuming that no methyl ketone escaped from the sealed chamber during the 24 h incubation. Compounds (concentrations) tested were as follow: 2-undecanone (7.86, 8.25, 8.64, 9.04, 11.79

µg/cm^3), 2-nonanone (3.9, 5.00, 5.86, 6.64, 7.81 µg/cm^3), 2-octanone (3.90, 5.56, 6.64, 7.03, 7.81 µg/cm^3) and 2-heptanone (1.95, 3.90, 5.86, 7.81, 11.71 µg/cm^3).

Field Tests

Red imported fire ant colonies (mounds) within a 50 m radius of the Dearstyne Entomology Building (35°47'18.9"N 78°41'56.7"W) were flagged on 16 April 2014 (Figure 2-1). Mounds ranged from 10 cm in length by 12 cm in width by 3 cm in height to 45 cm in length by 38cm in width by 9 cm in height. Each fire ant mound was disturbed using a wooden dowel and classified as described in Table 2-1 according to the rating system of Harlan (1981). An active mound would contain worker brood during the warm season, which is strong evidence that the colony contains an egg laying queen. In contrast, the absence of worker brood would indicate that the colony lacked a queen. A rating index of 0 to 10 (Table 2-1) was assigned depending on the estimated number of worker ants and brood. Any mound with an activity rating less than 5 was not used in our field tests. A mound injection tool (Figure 2-3) was developed for this study. It consisted of a 20 cm length of rubber tubing with a 10 ml glass pipette attached on one end and a 100 ml plastic syringe at the other. Different concentrations of 2-undecanone were emulsified in distilled water (100%, 50%, 25% and 15%; vol./vol.) with a handheld milk frother (Aerolatte, model number 56AL3SAT, Aerolatte Ltd. U.S.A) for 60 sec. A volume of 10 ml of test solution or a water control was drawn into the glass pipette, inserted into the mound to a depth of 10-15 cm, and then dispensed over a 5 sec period while slowly pulling the pipette out of the mound; this allowed for an even distribution of the 2-undecanone throughout the depth of the ant nest. This application was repeated five times at different random positions over the surface of the mound approximately

equal distance from each other but also with injections that spanned the entire mound surface (for a total application of 50 ml). Mounds chosen for injection were a minimum distance of 10 m apart to limit inter-mound treatment effects. Also a distance of 50 m was maintained between control mounds and 2-undecanone treatmented mounds. Three replicate mounds for each concentration and control were conducted. The condition and activity of each mound were evaluated daily after treatments also according to the rating system of Harlan et al. (1981, Table 2-1). We categorized the activity of the mounds for 4 days post treatment.

Fumigation bioassay for other insect pests

The bioassay system described above for fire ants was also used to evaluate the fumigant efficacy of 2-undecanone against adult males of the German cockroach, *B. germanica*, adults of mixed sex red flour beetles, *T. castaneum*, and the tobacco budworm, *H. virescens* eggs (1 day old). Three German cockroaches or 10 red flour beetles were assayed in each experimental trail. Approximately 1000 tobacco budworm eggs were obtained from Benzon Research Inc. (Carlisle, PA, USA). The eggs, deposited on a 36 × 36 cm piece of cloth, were cut with scissors into quarters (12.5 × 12.5 cm). Each quarter of cloth contained approximately 250 budworm eggs and was folded and place into the upper fumigation chamber for each test. The minimum 2-undecanone concentration achieving 100% mortality of the German cockroach, red flour beetle and 0% egg hatch were determined by a range finding approach similar to that for the fire ant (described earlier). The volume of 2-undecanone applied to the funmigation chamber was increased until all insects were dead or did not hatch at 24 h post-treatment. Once this threshold volume was obtained, the results were duplicated, showing 100% mortality

for both replicates. After exposure to the fumigant for 24 h, hatch rate of the tobacco budworm eggs were monitored daily for 5 d; otherwise, the exposure period (fumigation period) was 24h for the other insects. The concentrations obtained for 100% mortality were 41g/m^3 for the German cockroach, 101.2 g/m^3 for tobacco budworm eggs, and 165.8 g/m^3 for the red flour beetle (Table 2-3). For the former two insects, mortality was defined as described earlier in this paper for fire ants. Egg hatch was defined as the appearance of a budworm larva separate from the egg shell regardless of whether the larva was alive or dead at the time of the observation.

Data analysis

Abbott's correction (Abbott, 1925) was applied to all data in the dose–response experiments. Median lethal doses were calculated by plots of probit mortality versus log dose (Finney, 1971). The variance-covariance matrix and 95% confidence intervals for the toxicity ratios were estimated by the methods of Steel and Torrie (1980) and Robertson and Preisler (1982). Formulas and calculations were made in Microsoft Excel spreadsheets (Microsoft, 2013) developed by Young et al. (2003). One way ANOVA analysis was done using JMP 12 (SAS).

Results and Discussion

New bioassay system for evaluating fumigants

An easy to use and inexpensive bioassay system was developed to evaluate fumigants against a variety of pest organisms and with these organisms in or on a variety of substrates (Figure 2-2). The assay was designed for fumigants that are a liquid or solid at room temperature with volatility low enough to allow time for compound transfer to the glass cover slip in the bottom chamber (Figure 2-2), transferring of insects to the top chamber, and sealing of the container with Parafilm (Fisher Scientific) before a significant amount of the fumigant has evaporated. Because the assay is constructed from plastic petri dishes, the operator has the option of using the assay device only once at a minimal cost per assay. Since the plates are transparent and the surface area to depth ratio is high, observations on the test organism can be viewed easily by eye or under a dissecting microscope.

In the studies described in this paper, we examined a variety of insects and different insect stages using this method. In dose-response studies on fire ants, we demonstrated that probit models could be established for several different aliphatic methyl ketones (discussed later). The assay can be used for other pest organisms on or in different materials housed in the top chamber; some examples are seeds separate or in soil, nematodes on artificial media or in soil, bacteria and other microorganisms in a variety of media including soil, and other insects in and on a variety of media. Because of the small size of the bioassay system, the amount of the fumigant needed is minimal;

this is especially important in screening programs where the amount and/or cost of the test compounds might prohibit larger scale testing.

Fire ant fumigation structure-activity studies with methyl ketones

This is the first study to determine the structure-activity relationship for n-aliphatic methyl ketones as an insect fumigant. In these experiments, we used the red imported fire ants as a model insect and the bioassay system previously described (Figure 2-2). Four methyl ketones were studied, 2-heptanone, 2-octanone, 2-nonanone and 2-undecanone (Table 2-2); these were chosen over other methyl ketones because they are found naturally in different plant species and were available from commercial sources. Tridecanone is also an aliphatic methyl ketone produced by plants but is a solid at room temperature with a low volatility rate.

In the development of the environmental and incubation parameters for the bioassay, we examined the relative rate of evaporation of the C-7 to C-11 methyl ketones. In different experiments, different amounts of each methyl ketone were transferred separately to the surface of the cover slip (Figure 2-2), the fumigation chamber assembled as described earlier, and then the device incubated at room temperature (25±3ºC). In these experiments, all of the methyl ketones examined were completely evaporated by 24 h. For example, the length of time for 1 microliter of each methyl ketone to evaporate for 2-heptanone was 0.5 h; for 2-octanone, 4 h; for 2-nonanone, 10 h; and for 2-undecanone, 24 h. For the fire ant structure-activity studies, a total of 25 worker fire ants were randomly selected and placed in the fumigation chamber (Figure 2-2). Mortality was recorded 24 h after this transfer. 2-undecanone with the lowest vapor pressure was 100%

in vapor form by the time of the assay end point. It was assumed in these assays and in the development of probit models that there was minimal or no escape of the methyl ketone from the fumigation chamber, no absorption of the compound from either the liquid or vapor phase to any of the inner surfaces of the chamber and an even distribution of the fumigant in the device at 24 h. No visible changes in the plastic petri plates were observed during any of the assays.

A graphic presentation of the probit models generated for each methyl ketone is shown in Figure 2-4, and a summary of the LC_{50}s, LC_{80}s, and statistics is provided in Table 2-2. For all of the methyl ketones that we examined, there was significant variation in mortality with changes in concentration (for 2-undecanone, $F=8.14$, $df=4$, $p<0.05$; for 2-nonanone, $F=71.29$, $df=4$, $p<0.05$; for 2-octanone, $F=83$, $df=4$, $p<0.05$; and for 2-heptanone, $F=88.47$, $df=4$, $p<0.05$). An accurate measure of a population response to a toxicant typically would be expected at the LC_{50}. The trend was for the LC_{50} to decrease with carbon length, from 8.21 $\mu g/cm^3$ for 2-undecanone (C-11) to 5.26 $\mu g/cm^3$ for 2-nonanone (C-11) to 5.11 $\mu g/cm^3$ for 2-octanone (C-10) to 4.27 $\mu g/cm^3$ for 2-heptanone (C-7) (Table 2-2). Based on 95% confidence intervals, the LC_{50} for 2-undecanone was significantly higher than 2-nonanone and 2-octanone but was not higher than that of 2-heptanone (Table 2-2). One explanation for the lack of significant difference between 2-undecanone and 2-heptanone might be the much larger confidence interval of the latter (representing a greater variation between doses and replicates) than for any of the other methyl ketones tested. This trend of a lower LC_{50} with a decrease in the carbon length also occurred for the LC_{80}, from 9.85 $\mu g/cm^3$ for 2-undecanone (C-11) to 7.58 $\mu g/cm^3$ for 2-nonanone (C-11) to 6.38 $\mu g/cm^3$ for 2-octanone (C-10) to 5.37

$\mu g/cm^3$ for 2-heptanone (C-7). The LC_{80} for 2-undecanone was significantly higher than 2-octanone but was not higher than that of 2-nonanone and 2-heptanone based on the 95% confidence intervals (Table 2-2).

As discussed earlier, there was an increase in the volatility of the methyl ketones from C-11 to C-7 as measured by reduced time to total evaporation with C-chain length. Fire ant increased susceptibility (lower LC_{50}) to these methyl ketones was negatively correlated with carbon length and positively correlated with increased volatility. Since we only examined mortality at 24 h and since the concentration of fumigant by weight in the vapor phase was higher at an earlier time during the incubation period for the shorter methyl ketones, we are unable to determine what impact volatility alone had on toxicity of the compounds tested. It is possible that the lower molecular weight methyl ketones are more toxic to fire ants. However, it is also possible that because of the more rapid evaporation rate, the time of exposure of the full dose of vapor is longer as the C-chain is reduced and this is affecting the measure of susceptibility. There also could be a combination of effects based on toxicity differences between compounds and time to full evaporation. As we observed before, one microliter of 2-heptanone will vaporize in 30 minutes while one microliter of 2-undecanone required a much greater amount of time up to 24 h. An additional factor to consider are the differences in molecular weight for the compounds tested. For example, there is 1.6-fold statistically significant difference in the LC_{50} between 2-undecanone and 2-octanone correlated with a 1.3-fold difference in molecular weight difference between these two compounds. The latter results in a higher molar concentration of 2-octanone versus 2-undecanone in the vapor phase in our assay,

which could also be a factor contributing to differences in fire ant susceptibility to these compounds.

Additional studies will be needed to fully understand the mode of action of the methyl ketones as a fumigant on the red imported fire ant. The current hypothesis for the toxic action of these compounds is physical disruption of cuticular lipids resulting in water loss (a non-toxic mode of action). This hypothesis is partly derived from their apparent mode of action on plants, which when exposed directly with 2-undecanone or the vapor of 2-undecanone, in 30 min lose water, wilt and fall to the ground surface (Roe and Kennedy, 2011).

Field Tests

Although 2-heptanone, 2-nonanone and 2-octanone were more effective than 2-undecanone based on LC_{50}s (Table 2-2), field tests against fire ant mounds were conducted using 2-undecanone. There were several reasons for this decision. Undecanone already has a US EPA registration as a biopesticide for use on human skin as an arthropod repellent at concentrations as high as 8% (Roe et al. 2006; Bissinger et al. 2008; 2009; 2011; Witting-Bissinger et al. 2009). Therefore, there is considerable animal toxicology data and use on humans demonstrating the safety of 2-undecanone. In addition, there are commercial efforts to develop 2-undecanone as a herbicide (Roe and Kennedy, 2011), and the herbicide research suggests that this compound could have broad activity as a fumigant for weed, microbial plant disease and nematode control in soil. The current work here is to investigate its use on soil insects as well, in this case ants.

Flagged fire ant mounds (Figure 2-1) were examined for ant activity and rated according to the criteria shown in Table 2-1 using the system developed by Harlan et al. (1981). An active mound would contain worker brood during the warm season, and the presence of brood is strong evidence that the colony contains an egg laying queen. A rating number of 0 to 10 is given based on the estimated number of workers and the presence/absence of worker brood. Any mound containing more than 100 live ants was consider active, but only mounds with an activity rating ≥ 5 were used in this study. Five different concentrations of 2-undecanone in water emulsions (100%, 50%, 25% and 10% undecanone:water (vol. /vol.)) were tested. The treatments and control (distilled water only) were injected in a 50 ml total volume per mound by methods described earlier. We repeated the field tests 3 times on different mounds for each concentration of 2-undecanone as well as for the control. For each replicate we used a different mound but with the same colony activity rating system for all concentrations.

One day after treatment, mounds injected with 100% 2-undecanone were reassessed for activity and received a rating of 2 (no brood on the mound surface and the number of fire ants was reduced); this represented a 3 fold decrease in the colony activity rating (Figure 2-5). Mounds treated with 50% and 25% 2-undecanone also showed signs of a decreased population. The activity of the 50% 2-undecanone treated mounds declined from a mean rating of 7 to 4, and the 25% 2-undecanone treated mounds decreased from an average of 6 to an activity rating of 3. There was no ant activity (colony activity rating was 0) in mounds injected with 100% 2-undecanone from day 2 to day 4. Generally, hundreds of ants would rush out of the mound upon a slight disturbance of the tumulus, but this did not occur for mounds treated with 100% 2-

undecanone (Figure 2-5 and Figure 2-6D). Fifty percent and 25% 2-undecanone treated mounds eventually became inactive (a rating of 0) on day 3 and day 4, respectively (Figure 2-5 and Figure 2-6F). The control mounds and 10% 2-undecanone treated mounds showed no decrease in colony activity over the same 4 days; over the 4 d period, their colony activity rating of 7 and 5, respectively remained the same since day one.

Figure 2-6 shows a before and after comparison of the control mound (A and B, respectively) with mounds treated with 100% 2-undecanone (C and D, respectively) and 25% 2-undecanone (E and F, respectively). The surface of these mounds before treatments are granular in appearance from small peaces of soil that have been excavated from below the soil surface (Figure 2-6 A, C and E). Four days after treatment, only the control mound had active fire ants (Figure 2-6B) and maintained the granular appearance, while the 2-undecanone treated mounds were flat in appearance (Figure 2-6 D and F). The results shown in Figure 2-6 were typical for all of the control replicates and the replicates for both the 100% and 25% 2-undecanone treatments. Figure 2-7 shows a close up of an active mound on the left (colony activity rating of 5) next to a mound (right) that had been treated with 50% 2-undecanone after 4 days (colony activity rating 0). The mound treated with 2-undecanone showed no sign of ant activity (0 rating) and when disturbed appears as a shallow nest with no active workers while workers are shown rushing out of the active mound.

One week after the treatment with 2-undecanone for even the 25% 2-undecanone treatment representing an application of 12.5 ml of 2-undecanone per mound, there was no re-establishment of ants in the mound. Further research will be needed in the future to understand the impact of mound treatments by the methods shown here on overall mount

density and ant populations for a given treatment area and immediate areas outside of a treated area. Longer term studies are also needed to study mound re-establishment after a treatment. However, it is clear from our results that at least a short-term elimination of ant activity is possible with injection of low amounts of 2-undecanone emulsified in water. Future formulation research with 2-undecanone may also be useful to examine the relationship between volatility and longevity of ant disruption. It is possible the longer the 2-undecanone can be retained in the soil, the longer the ants will not be able to re-inhabit the treated location. It is clear just based on odor, that the 2-undecanone is being released into the atmosphere from the soil just above the treated area for at least 3 days.

Fumigation effect on other insect species and life stages

To better understand the utility of our fumigation bioassay and to determine the use of 2-undecanone as a fumigant for insects in general, we conducted additional studies with red flour beetle adults, adult male German cockroach and tobacco budworm eggs. Range finding experiments were conducted to determine the lowest 2-undecanone concentrations for 100% mortality for each species, and then at these concentrations, 100% mortality was repeated twice to validate 100% kill. For 10 fire ants per replicate, 4.1 g/m^3 was sufficient to produce 100% mortality in 24 h (Table 2-3; Figure 2-8). For five German cockroaches per replicate, an undecanone treatment of 41 g/m^3 resulted in 100% mortality; for tobacco budworms (n = 250 for each replicate) 101.6 g/m^3 resulted in 0% egg hatch; and for the red flour beetle (n=10 for each replicate) 165.8 g/m^3 produced 100% mortality (Table 2-3; Figure 2-9). Compared to the MeBr minimum and maximum recommended treatment ranges for most insects (48.08-112.2 g/m^3; MeBr USDA APHIS

treatment manual 2011), 2-undecanone on a weight basis per unit volume had similar insect activity to a variety of insects and different life stages (egg versus adults) to that of MeBr.

Cost comparisons of undecanone versus MeBr for insect fumigation

MeBr has been the gold standard within the fumigant industry because of its broad activity against insects and other pests for many different commodities and most important, its low price point. For common insect pests or commodities, the minimum dosage range for MeBr is 48.08 g/m^3 and the maximum dosage range is 112.2 g/m^3 (MeBr USDA APHIS treatment manual 2011). The cost per gram for MeBr for field use in 2012 was US$0.013. In comparison the cost for reagent grade 2-undecanone in 2013 from Sigma-Aldrich was US$0.025/gram. Although 2-undecanone is twice the price per gram of MeBr, the former is a reagent grade material sold in small amounts for research use while the MeBr price is for large scale commercial applications. Table 2-3 shows the calculation per gram for the control of different insects for 2-undecanone versus MeBr based on a minimum concentration needed for 100% mortality. In the cost comparison in Figure 2-8, MeBr at its minimum recommended concentration was 11.7 times more expensive and at its maximum recommended concentration was 27.4 times the cost of 2-undecanone for 100% fire ant control. Note that the effective dose for 100% control of fire ants with 2-undecanone could change depending on the substrate in which the ants might be located. Current studies so far suggest that 2-undecanone is a cost effective alternative to MeBr. For the other insects and insect stages examined (Figure 2-9) similar finding were made, i.e., that 2-undecanone is a reasonable alternative to MeBr based on

treatment cost for 100% insect control. The worst case scenario was for red flour beetles, where 2-undecanone was 1.5 times the cost of the maximum dose for MeBr (Figure 2-9). Considering that MeBr has been banned since 2005 because of its impact on the stratosphere ozone layer, the difficulty of its detection in treated areas (has no odor or color) and it represents a synthetic chemistry (not found in nature), 2-undecanone could be a reasonable substitute which needs further consideration. Some of the advantages of 2-undecanone are the following: (i) the compound is found in nature, (ii) undecanone is produced in plants which we eat, (iii) the compound has a known safety record, (iv) it has a moderate odor allowing easy detection, (v) it can be easily transported as a liquid at atmospheric pressure and ambient temperatures, (vi) undecanone has a non-biochemical or non-toxic (physical) mode of action with potential broad pest (arthropods, plant, nematode, and bacteria) and commodity uses, and (vii) it is currently safe enough to be used on human skin as an arthropod repellent without restrictions approved by the US EPA. Also the public acceptance of a natural fumigant should be much easier than synthetic compounds or compounds with high off-target toxic effects.

Use of methyl ketones as a fumigant

The mode of action of fumigants may vary greatly. Some fumigants kill rapidly, while others can be slow acting. Some fumigants may have a paralyzing effect on the pest at sub lethal dosages while others will not allow the pest to recover. Also in general, the toxicity of a fumigant depends on the respiration rate of the target pest, the lower the respiration rate of the organism the lower the susceptibility (MeBr USDA APHIS treatment manual 2011). The mode of action of 2-undecanone is unknown but one

hypothesis is that it acts directly on the cuticular surface of insect and plants, suggesting that respiration is not a target in its action. Even under cold environmental conditions, 2-undecanone was found to be fast acting as a herbicide (Roe and Kennedy, 2011).

With the complete phasing out of MeBr and the reduction in number of labeled fumigants overall, there are limited choices where fumigation is needed for pest control Factors such as the type of commodity to be treated, type of pest and developmental stages present, type of structure to be treated, the cost of the fumigant, and the safety to the applicator and the environment are important considerations. Also, for any control chemistry, public acceptance is becoming critical, and increasingly, the public is demanding "green technologies". Many natural essential oils have been explored for pest control (Kumar et al. 2012). For example, cinnamaldehyde, the main constituent of cinnamon oil, produced direct contact toxicity to both *T. castaneum* and the maize weevil, *Sitophilus zeamais* (Huang and Ho, 1998). Oil of clove was toxic to the rice weevil, *Sarocladium oryzae*, and the lesser grain borer, *Rhyzopertha dominica* (Sighamony et al. 1986). Extracts of the flower buds of clove, *Syzygium aromaticum*, and star anise, *Illicium uvrum*, were insecticidal to *T. castaneum* and *S. zeamais* and suppressed progeny production (Ho et al. 1994). Essential oil extract from the neem tree, *Azadirachta indica*, prevented adult insect emergence, reduced oviposition rates and prevented insect development (Babu et al. 1989).

The methyl ketone, 2-undecanone, is synthesized by the trichomes of the wild tomato plant, *Lycopersicon hirsutum* Dunal f. *glabratum* C. H. Müll. It has been used as a food addictive, in cosmetics and perfumes, and as a flavoring agent and can be derived from natural plant extracts (Bissinger et al. 2009). This methyl ketone has also been used

as an arthropod repellent (Bissinger et al. 2009; Witting-Bissinger et al. 2008). Undecanone has a relatively low mammalian toxicity, and it already has a US EPA registration as an ingredient in a biopesticide for application to human skin as an arthropod repellent.

In this study, we explored the fumigant potential of methyl ketones. In fumigation bioassays and field application experiments, four different methyl ketones (2-undecanone, 2-nonanone, 2-octanone and 2-heptanone) were found to be effective in eliciting mortality of fire ants in a dose dependent matter. Heptanone exhibited the lowest LC_{50} (4.27µg/cm^3) in comparison to 2-octanone, 2-nonanone and 2-undecanone (Table 2-2). Vaporization tests showed the number of carbon in the methyl ketone correlated to its volatility with shorter carbon chain methyl ketones being more volatile compared to long carbon chain methyl ketones. Undecanone also has fumigant effects against adult German cockroaches, adult red flour beetles and the eggs of the tobacco budworm. The concentration of 2-undecanone required to treat these pests fell within the lower to upper range for MeBr (48.08-112.2 µg/cm^3) and slightly more for the red flour beetle (Table 2-3). In field tests, the direct injection of 2-undecanone was 100% effective in controling the ants in mounds at concentrations as low as 25% representing a total treatment of technical 2-undecanone of 12.5 ml (Figure 2-6). Direct injections for ant mound control has been suggested before (Drees et al. 2009) using liquid nitrogen. Our studies suggest 2-undecanone could be used more effectively, would be easier to obtain and handle if commercially available, and would be much safer than handling liquid nitrogen. For example an aerosol can with a 10 to 20 cm long dispensing tip could be used by a home owner to treat individual mounds in a home lawn. Injecting 2-

undecanone is safe and directly injecting 2-undecanone into the ant mound was 100% effective in mound elimination. Methyl ketones also are candidate fumigants for the rapid control of insect pests in applications where current fumigants are not appropriate or preferred, including its use as a replacement for MeBr. Use of methyl ketones in soils and other substrates for arthropod, plant, nematode and disease control is suggested from our studies but more research is needed to understand their full potential.

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Table 2-1: Red imported fire ant, *Solenopsis invicta*, mound activity rating system used in current study adapted from Harlan et al. (1981).

Colony activity rating	Number of worker ants	Brood presence
0	No activity	NA*
1	<100	-
2	100 – 1000	-
3	1000 – 10000	-
4	10000 – 50000	-
5	>50000	-
6	<100	+
7	100 – 1000	+
8	1000 – 10000	+
9	10000 – 50000	+
10	>50000	+

*NA: not applicable

Table 2-2: Fumigation LC50, LC80, 95% confidence intervals, chi-square (χ^2), slope and R^2 values for heptanone, octanone, nonanone and undecanone against fire ants.

Methyl ketone	n	LC_{50}[a] ($\mu g/cm^3$)	95% CI[b]	LC_{80}[a] ($\mu g/cm^3$)	95% CI[b]	Chi-square	Slope±SE	R^2
Heptanone	75	4.27^{AB}	2.14 – 7.88	5.37^{AB}	3.50 –12.90	6.47	4.66±0.66	0.94
Octanone	75	5.11^{A}	3.66 – 6.64	6.38^{A}	4.86 – 8.62	4.46	8.74±1.36	0.93
Nonanone	75	5.26^{A}	4.24 – 6.39	7.58^{AB}	6.39 – 9.47	2.05	7.16±0.85	0.96
Undecanone	75	8.21^{B}	7.24 - 9.12	9.85^{AB}	8.85 – 11.15	1.47	10.65±1.17	0.96

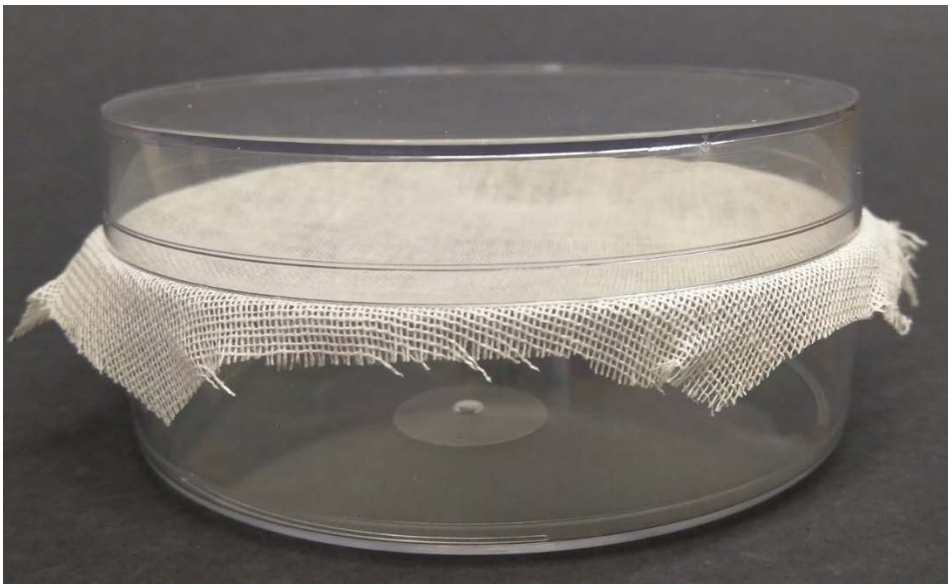
[a]LC, Lethal Dose, results were corrected for control mortality by Abbot's correction. LD50 and LD80 followed by capital letter is not significantly different based on the 95% confidence interval.
[b]CI, confidence interval.

Table 2-3: Cost comparison of 2-undecanone versus methyl bromide as a fumigant for 100% insect control.

Chemical	Target pest	g/m^3	US$/gram	(US$/m^3)
Undecanone	*S. invicta* (n[1]=10; r[2]=2)	4.1	0.025[3]	0.1025
	B. germanica (n=5; r=2)	41.0		1.025
	H. virescens eggs (n≈250; r=2)	101.6		2.54
	T. castaneum (n=10; r=2)	165.8		4.15
MeBr	Common pest insects[4]	Max dose 48.08[4]	0.013[5]	0.625
		Min dose 112.2[4]		1.458

[1]The total number of insect treated per replicate.
[2]The number of replicates.
[3]US dollars per gram, Sigma-Aldrich (2013).
[4] USDA APHIS Treatment Manual.
[5]US dollars per gram (2013).

Figure 2-1: Left: An aerial view area (35°47'18.9"N 78°41'56.7"W) of the site where fire ants were collected for the laboratory methyl ketone structure-activity toxicity studies and the field evaluation of 2-undecanone for fire ant mound control. Red dots indicate proximity of the actual mounds. Right: Close up of flagged fire ant mounds used for field studies (in same area shown on left).



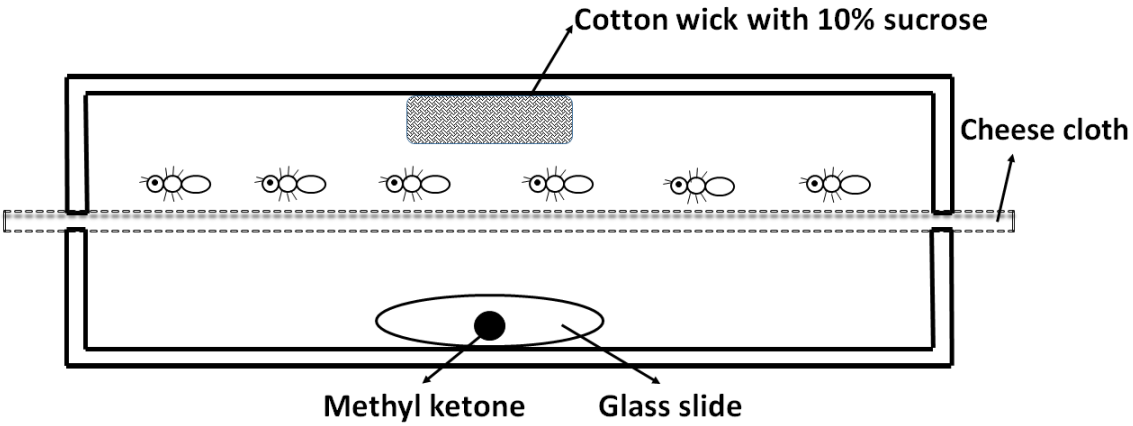


Figure 2-2: Fumigation chamber consists of 2 plastic petri dish bottoms which together as shown creates an inside space 8.6 cm in diameter by 3.6 cm in height and a total volume of 210 cm^3 (the test arena). The two petri dish halves were separated by a 10cm x 10cm section of cheese cloth. The technical methyl ketone to be tested is applied to the lower chamber on a glass cover slip. The chamber above the cheese cloth contains a 2cm cotton wick saturated with 10% sucrose; 25 fire ants of random size and caste were used for each test.

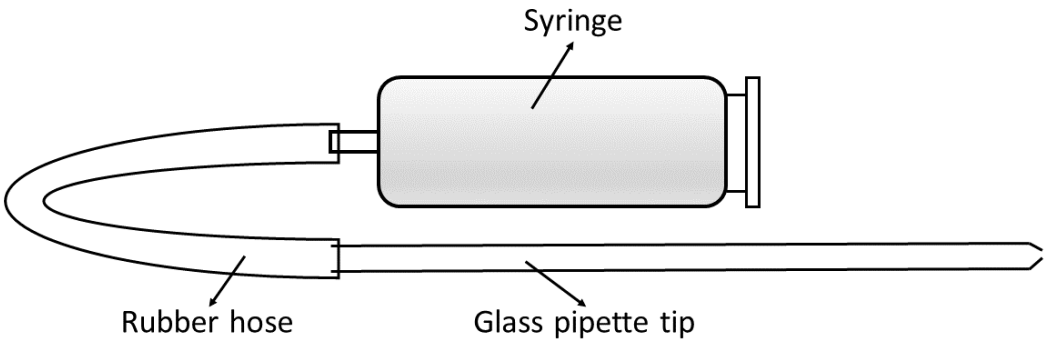


Figure 2-3: The injection tool to treat fire ant mounds consists of a 10 ml glass pipette attach to a 20 cm flexible hose on one end of the hose and a 100 ml plastic syringe installed on the other end of the hose.

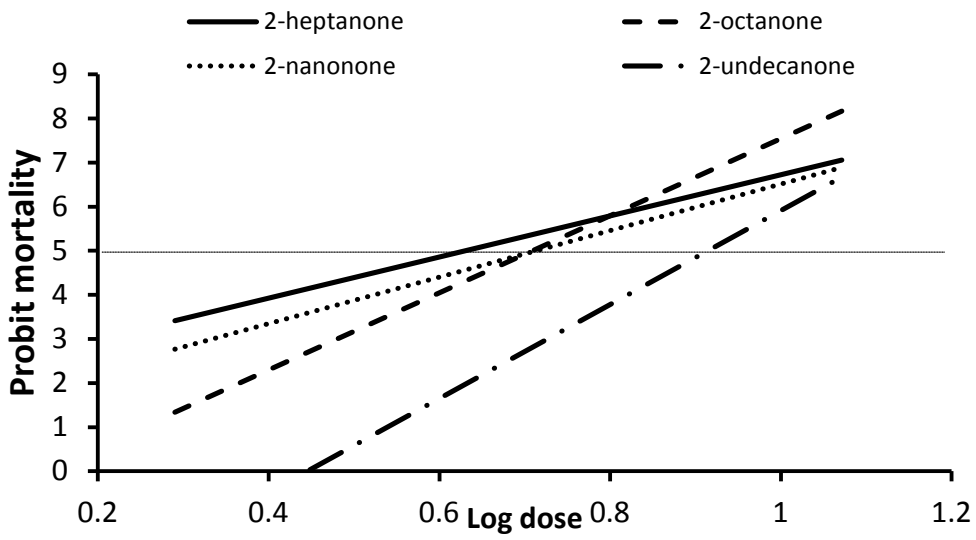


Figure 2-4: Probit models for log dose versus probit mortality for different methyl ketones (C-7 to C-11) as a fumigant against the red imported fire ant, *Solenopsis invicta*, using the bioassay system described in Figure 2-2. The LC50s and LC80s along with the slope and statistics are provided in Table 2-2. The horizontal line drawn on the graph represents 50% mortality.

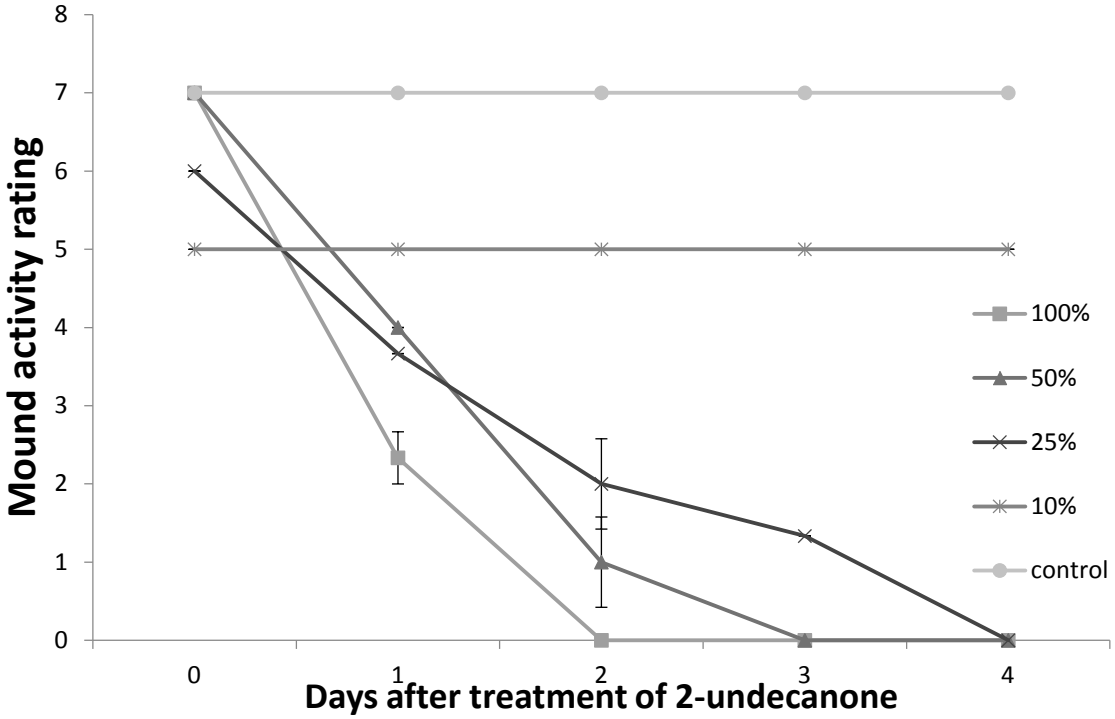


Figure 2-5: Activity rating of fire ant mounds after injected with different concentrations of 2-undecanone in a water emulsion using the injection tool described in Figure 2-3. The activity ratings are defined in Table 2-1. Points indicate means ± 1 SE of 3 replicate mounds. The location of these studies are shown in Figure 2-1. For the control and 10% treatment, there was no change in ratings during the course of the experiment.

Figure 2-6: A typical before and after comparison of the control mound (A & B, treated with 50 ml distilled water) to a mound treated with 50 ml of 100% 2-undecanone (C & D) and 50 ml of a 25% 2-undecanone in water emulsion (E & F) for the field results shown in Figure 2-5. The field study was replicated 3 times for each concentration.

Figure 2-7: Left: A swarm of fire ants rushed out of control mound with a colony activity rating of 5. Right: A mound treated with 50% 2-undecanone at day 4, now with a colony activity rating of 0 since there was no activity when disturbed.

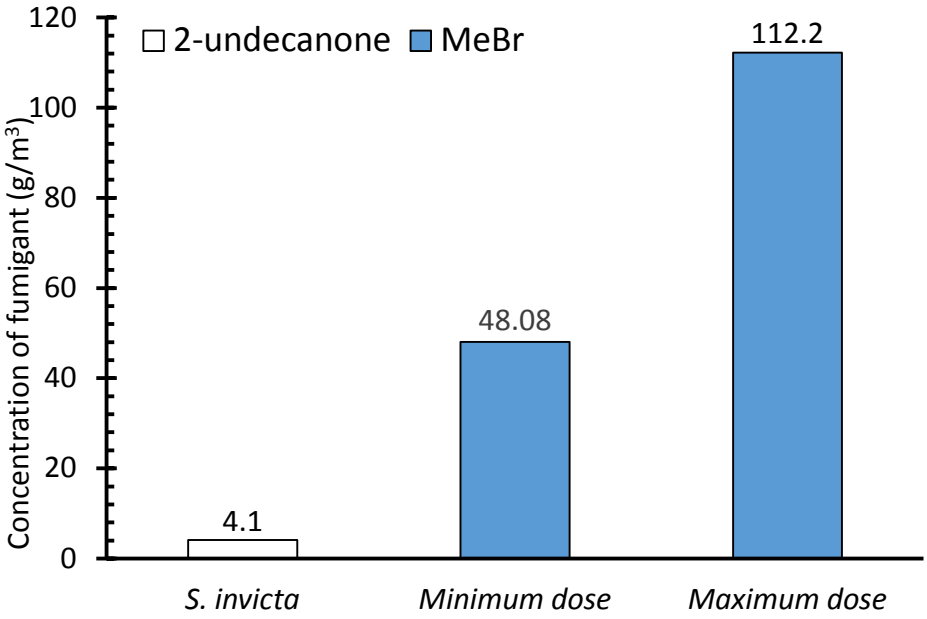


Figure 2-8: Concentration that produced 100% mortality by fumigation of the red imported fire ant, *Solenopsis invicta* (random castes and sizes) using the bioassay shown in Figure 2-2 and compared to the lower (48.08 g/m*3*) and upper (112.2 in g/m^3) concentrations for MeBr recommended in the USDA APHIS Treatment Manual. Cost comparisons are shown in Table 2-3.

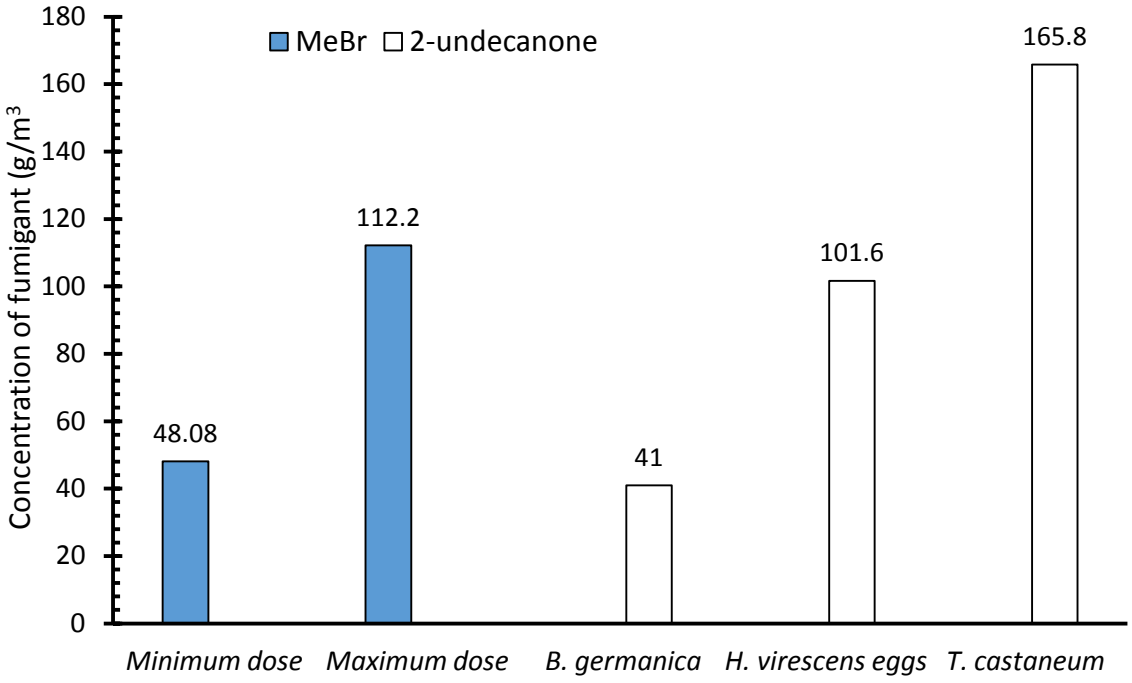


Figure 2-9: Concentration that produced 100% mortality by fumigation for the German cockroach, *Blattella germanica*, tobacco budworm egg, *Heliothis virescens*, and red flour beetle, *Tribolium castaneum*, using the bioassay shown in Figure 2-2 and compared to the lower (48.08 g/m^3) and upper (112.2 in g/m^3) concentrations for MeBr recommended in the USDA APHIS Treatment Manual. Cost comparisons are shown in Table 2-3.

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Appendix A

Molecular traces of alternative social organization in a termite genome

N. Terrapon, C. Li, H.M. Robertson, L. Ji, X. Meng, W. Booth, Z. Chen, C.P. Childers, K.M. Glastad, K. Gokhale, J. Gowin, W. Gronenberg, R.A. Hermansen, H. Hu, B.G. Hunt, A.K. Huylmans, S.M.S. Khalil, R.D. Mitchell, M.C. Munoz-Torres, J.A. Mustard, H. Pan, J.T. Reese, M.E. Scharf, F. Sun, H. Vogel, J. Xiao, W. Yang, Z. Yang, J. Zhou, J. Zhu, C.S. Brent, C.G. Elsik, M.A.D. Goodisman, D.A. Liberles, R.M. Roe, E.L. Vargo, A. Vilcinskas, J. Wang, E. Bornberg-Bauer, J. Korb, G. Zhang, J. Liebig, Molecular traces of alternative social organization in a termite genome, Nature communications, 5 (2014), pp. 1-12.

Termites are major structural pests around the world, their wood eating habits cause an annual cost of 40 billion dollars in damage and control (Rust and Su, 2012). Termites also play an important role in ecosystem by maintaining biodiversity, they are important in nutrient recycling. Their complex social structure have enhanced their environmental adaptability, contributing to their success. Termites shared a similar social structure with eusocial Hymenoptera, they divide labor among castes. A caste system including few individuals reproduce (queens and kings) and rest of colony consist of workers and soldiers, they perform tasks like foraging, brood care or defense (Korb, 2008). Even though they shared these similarities to eusocial Hymenoptera, phylogenetically termite societies have distinct origin and biological traits. They form a monophyletic clade nested within the Blattodea (Inward et al. 2007), indicating a single origin of termite eusociality. Termites are hemimetabolous, having several immature

stages that become more adult-like with each transition, while Hymenoptera have a holometabolous development in which the final larval stage develops via a pupa into adult hood. Because termites and Hymenopteran have similar social and physiological traits, yet they are in totally different phylogeny. A genome wide study in comparison to eusocial Hymenopterans will help us to understand the mechanism, selective pressures and specific adaptations to achieve the eursociality.

This is the first termite genome of the termite *Zootermopsis nevadensis nuttingi* (Termopsidae), along with transcriptomic data of various caste and developmental stages. The purpose of his genome wide study was to identify common and divergent associations of traits contribute to eusociality. The genome revealed a significant expansion of gene families related to male reproduction and chemoperception, indicating differences between *Z. nevadensis* and eusocial Hymenoptera in mating biology and communication, respectively. Also the genome identified genes in involved in endocrinology, immunity, reproductive development and caste differentiation, those gene are essential in maintenance of eusociality (Terrapon et al. 2014).

Juvenile hormone controls the growth, development, metamorphosis, reproduction and caste differentiation in insects. Since I have characterized the mevalonate-farnesal pathway in ticks, it was my honor to work with scientists around world to characterize juvenile hormone pathway in *Z. nevadensis.* Below are my contribution to the *Z. nevadensis* genome paper.

JH III Biosynthetic Pathways and Juvenile Hormone Binding Proteins

Juvenile hormones (JHs) control growth, development, metamorphosis, and reproduction in insects. These sesquiterpenoids are synthesized *de novo* in specialized

endocrine glands called the corpora allata (CA) (Schooley and Baker, 1985). In social insects, JH additionally regulates other developmental processes such as polyphenisms and caste determination (Hartfelder et al. 2012; Nijhout 1998). There are several forms of JH that have been fully characterized chemically and physiologically, with JH III found in termites as well as other insects.

The biosynthetic pathway of JH III is divided into two parts. The initial steps of JH biosynthesis follow the isoprenoid pathway from acetyl-CoA to farnesyl pyrophosphate (FPP) (Bellés et al. 2005). In the later part, FPP is hydrolyzed by a pyrophosphatase to farnesol, which is then oxidized to farnesal and farnesoic acid (FA; Cao et al. 2009; Mayoral et al. 2009; Baker et al. 1983). The order of the final two enzymes in JH synthesis differs depending on the insect. In Lepidoptera, epoxidation by the P450 monooxygenase precedes esterification by a juvenile hormone acid methyltransferase (JHAMT) (Reibstein et al. 1976). In Orthoptera, Dictyoptera, Coleoptera and Diptera, epoxidation follows methylation (Feyereisen et al. 1981; Tobe and Pratt, 1974; Hammock, 1975; Weaver et al. 1980; Li et al. 2003).

To identify and assign functionality to *Zootermopsis nevadensis* genes involved in the JH biosynthetic pathway, we used as a reference each enzyme from the insect JH pathway so far studied (Noriega et al. 2006). Using the amino-acid sequences of the enzymes in reference species (*D. melanogaster, A. aegypti and A. gambiae*), we identified orthologs and recent paralogs in the *Z. nevadensis* lineage using reciprocal BLAST searches. These assignments were confirmed using the orthoDB database and orthoMCL ortholog groups. Finally, gene models were refined when required and possible by analyzing Z. nevadensis assembly to match corresponding insect genes using

the Apollo Genome Annotation and Curation Tool (Lewis et al. 2002). The annotation allowed the assignment of putative functions to the complete list of the enzymes of the JH III biosynthetic pathway (Table A-1).

Every enzyme involved in the isoprenoid pathway from acetyl-CoA thiolase to FPP was characterized, which includes: an acetoacetyl-CoA thiolase, two hydroxymethylglutaryl-CoA synthases (HMG-S), a hydroxymethylglutaryl-CoA reductase (HMG-R), a mevalonate kinase, a phosphotransferase (that phosphorylates mevalonic acid to 5-phosphomevalonic acid), an isopentenyl-diphosphate delta-isomerase (that catalyses the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)), and a farnesyl pyrophosphate synthetase (a prenyltransferase that catalyses the condensation of isopentenyl pyrophosphate with geranylpyrophosphate to FPP, which is the last product of the isoprenoid pathway; Noriega et al. 2006).

Following the isoprenoid pathway, two metabolic routes are possible, i.e., the sterol branch and the JH branch, of which the sterol branch has been lost in insects. Insects and other arthropods do not synthesize cholesterol de novo (Clark and Bloch 1959), due to the absence of genes encoding squalene synthase and other subsequent enzymes of the sterol branch (Bellés et al. 2005). No squalene synthase homologs were found in our search of the Z. nevadensis genome suggesting that only the JH branch exists in this termite. On the other hand, the JH branch, which spans from FPP to JH III, appears to be conserved in Z. nevadensis since we found matches for all five major enzymes of the JH branch. However, it should be noted that no absolute functional assignments (based on their demonstrated synthesis of the precursors of JH III) are

currently available for insect farnesyl diphosphate pyrophosphatase, farnesol oxidase, and farnesal dehydrogenase (marked with an asterisk in Table A-1).

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Table A-1: Enzymes involved in the juvenile hormone III biosynthetic pathway

Isoprenoid pathway		JH branch	
Substrate ↓ Enzyme	*Z. nevadensis* protein ID	Substrate ↓ Enzyme	*Z. nevadensis* protein ID
Acetyl-CoA		Farnesyl-PP	
↓ acetoacetyl-CoA thiolase	Znev_06783	↓ farnesyl diphosphate pyrophosphatase*	Znev_13079
Acetoacetyl-CoA		Farnesol	
↓ HMG-S[1]	Znev_08283 Znev_14140	↓ Farnesol dehydrogenase*	Znev_12423†
HMG-CoA		Farnesal	
↓ HMG-R[2]	Znev_02974	↓ Farnesal dehydrogenase*	Znev_01798 Znev_03358
Mevalonate		Farnesoic acid	
↓ mevalonate kinase	Znev_14029	↓ JH methyltransferase	Znev_12145
Mevalonate-5-P		Methyl farnesoate	
↓ phosphomevalonate kinase	Znev_12655	↓ JH epoxidase	Znev_14299
Mevalonate-5-PP		Juvenile hormone III	
↓ Diphosphomevalonate decarboxylase	Znev_08133		
Isopentenyl-PP ← isopentnyl-diphosphate δ-isomerase → Dimethylally-PP	Znev_09747		
+Geranyl-PP ↓ farnesyl diphosphate synthase	Znev_02161		
Farnesyl-PP			

[1] HMG-S stands for 3-hydroxy-3-methylglutaryl-CoA synthase
[2] HMG-R – 3-hydroxy-3-methylglutaryl-CoA reductase
* No structural data are available to associate them specifically to JH III biosynthesis
† We are reporting here only the protein with highest E-value (best BLAST hit), while several paralogs are candidates and the true participant is unknown at this time. This was confirmed by the orthoDB database that clusters ~9 on average paralogs for all insect species, involving nine proteins (4 being adjacent) in *Z. nevadensis* genome: Znev_05770, Znev_05797, Znev_07337, Znev_12420, Znev_12421, Znev_12422, Znev_12423, Znev_17844 and Znev_17938.

Appendix B

Use of RNAi to control red imported fire ants, *Solenopsis invicta*

Introduction

The red imported fire ant (RIFA), *Solenopsis invicta*, is native to South America and was first detected in the United States in Mobile, Alabama during the 1930s. Since then it has become a major pest in the southern U.S. and has expanded north (APHIS). Fire ants are well known for their aggressive behavior and painful stings. For humans, the sensation of the sting is similar to walking bare foot on fire, hence their moniker. Each sting results in a pimple-like lesion that can take a week or more to heal, and some people have life threatening allergic reactions to the venom (McDonald 2006). In addition, their mound building activity can damage plant roots, which can eventually cause crop loss and interfere with mechanical cultivation. Finally, when fire ants invade a new territory, they displace indigenous ants potentially upsetting the ecological balance. Since fire ants are now wide spread in the US, there is an important need to develop methods to stop their further dispersal without affecting non-target animals including native ants.

The traditional method of controlling fire ants is using synthetic, chemical baits. The active ingredient in these baits include such compounds as avermectins, oxadiazine, fipronil, boric acid and others (NC Cooperative Extension; Drees and Lennon 1998; McDonald 2006), some of which are quite toxic. For example, fipronil is cytotoxic to human cells in the low micromolar range. Also, in the past, fire ant baits were found to be harmful to wildlife and in some cases like mirex, caused cancer. There has been a

history of non-target effects using chemical insecticides including the killing of native ants (Overmyer et al. 2005; McCracken et al 1993), and the public today is looking for "green" technologies for pest control, especially in areas where humans and animals may come into contact with the compounds used to control fire ants. Biological methods that have been considered previously include use of phorid flies, protozoa, fungi and viruses, but in some cases the biological control agents once released have unintended effects. New, more effective approaches for fire ant control are needed with minimal off target effects and that will be more acceptable to the public, especially in urban environments.

RNAi has become a powerful tool for specific gene knockdown (Fire 1999). RNAi has shown its ability to deliver sequence specific RNAi mediated down regulation of a complementary mRNA, resulting in knocking down the production of the protein produced by a specific message (Kim et al. 2006). For one approach, we examined the use of double stranded RNA (dsRNA) complementary to the receptor of the fire ant yolk protein (VgR) located in the ovary of the queen. The absence of this receptor would prevent the uptake of yolk (vitellogenin, Vg) from hemolymph of the queen, which would then prevent egg development (Lu et al. 2009). This idea in our lab group originated with work in the tick, *Dermacentor variabilis*, where Mitchell et al. (2007) found that egg production could be prevented by the administration of dsRNA complimentary to the *D. variabilis* VgR mRNA. In addition to a dsRNA approach, we also evaluated the efficacy of a morpholino that targets the 28S ribosomal gene. Similar to dsRNA, morpholino oligomers are another class of antisense technology used to block access of other molecule to specific sequences with in mRNA. Structurally, morpholinos have the same nucleic acid bases, but with six membered morpholine rings which replace

dexoyribose rings and link through uncharged phosphorodiamidate groups instead of phosphates. They do not degrade their target RNA molecule like in a RNAi system, but instead, morpholinos act by steric blocking, binding to a target sequence within an RNA and getting in the way of molecules that might interact with the RNA. The goal of this study was to show the feasibility of using dsRNA or morpholinos to control the red imported fire ant by feeding.

Materials and Methods

Insects

Red imported fire ant, *Solenopsis invicta*, queens were provided to us by Dr. David Oi, USDA-ARS CMAVE, Gainesville, FL, U.S.A. The shipment included 3 tubes (50 ml centrifuge tube) of ants, where each tube included one fire ant queen and approximately 50 worker ants. We also established fire ants colonies in our lab. The ants were collected from mounds within a 50 m radius of the Dearstyne Entomology Building (35°47'18.9"N 78°41'56.7"W) on the North Carolina State University Campus, Raleigh, North Carolina, U.S.A. Approximately 500 fire ants and nest material (a petri dish 14 cm in diameter and 1.5 cm in height where the bottom dish was filled with hardened dental plaster up to 8 mm in height (nest material was a gift from Dr. Edward Vargo, Department of Entomology, NCSU)) were placed into Teflon emulsion lined plastic trays (40 cm by 52 cm by 10 cm). The 20% Teflon emulsion was made of fluon (PTFE-30, Dupont, Wilmington, DE, U.S.A.) in distilled water (vol/vol). Only the top 6 cm of the tray sides was coated with Teflon emulsion using a cotton ball and was applied in a circular motion. The ants were held at 25±3ºC and 60±5% RH, exposed to a photoperiod

of 12:12 (L:D) h, and supplied *ad libitum* with 10% sucrose solution via a wick system. The 10% sucrose (99.7%, Acros Organics, Fisher Scientific, Pittsburgh, PA, U.S.A.) was made in distilled water (wt/vol).

Total RNA extraction and cDNA library preparation

Three abdomens from fire ant queens were removed using a sterile stainless razor blade (Feather, Osaka, Japan). RLT Reagent (Qiagen, Valencia, CA, U.S.A) was added to the tissues and then homogenized using a Fisherbrand disposable pestle system (Fisher Scientific, Pittsburgh, PA). Total RNA was extracted from this homogenate with a Qiagen RNeasy mini kit according to the manufacturer's protocol in the presence of DNase I (Qiagen). Approximately 41 ng/µl of total RNA was collected in 50 µl of RNase free water provided by the kit. First strand cDNA synthesis was conducted using 10 µl of the total RNA generated from the extraction combined with 1 µl of oligo$(dT)_{12\text{-}18}$, 1 µl dNTP mix (10mM) and then heated at 72 °C for 2 min. Afterward, the tube containing the total RNA and primers was placed immediately on ice and the following reagents were added: 4 µl of 5X first-strand buffer, 2 µl of 20mM dithiothreitol (DTT), 1 µl RNAse Out (40 U/µl) and 1 µl of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA); this was followed by incubation at 42 °C for 90 min. The reaction was diluted to 30 µl with 1X TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5), heated at 70 °C for 15 min, and then stored at -80 °C until used.

VgR primer design and VgR gene isolation

Based on the published vitellogenin receptor (VgR) sequence (Accession number AAP92450) from Lu et al. (2009), forward primer (SiFP1): 5'-

CCAATTGCCATCAAACTCCT -3’ and reverse primer (SiRP1): 5’-TTCGGTGCTTGTGGATGATA -3’ was designed using the software, “Primer3” (Rozen et al. 1999) to amplify the *S. invicta*’s VgR gene by a polymerase chain reaction (PCR). The PCR reactions contained 2 µl of cDNA, 10 pmol of forward and reverse primers, 8.5 µl of water, and 12.5 µl of Green Master Mix (Promega, Madison, WI). The thermal cycler was set with the following program: 95 °C for 2 min, followed by 35 cycles with 95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 1 min, with a final cycle of 72 °C for 5 min. The final yield of VgR PCR product was 30 µl at 86.7 ng/µl. The PCR product was purified with a QIAGEN PCR purification kit (QIAGEN) and sequenced by Eton Bioscience Inc. (Durham, NC, U.S.A). Sequencing was conducted by primer extension sequencing on an ABI automated sequencer which translates the fluorescent signals into their corresponding base pair sequence. The basic local alignment search tool (BLAST, NCBI) was used to confirm the VgR sequence identity.

dsRNA

Following successful isolation of VgR from fire ants and its validation by sequencing was completed, 4 µl of cDNA was used as template for a PCR reaction under the following cycling conditions: 95 °C for 4 min followed by 5 cycles of 95 °C for 30 sec, 62 °C for 30 sec, 72 °C for 1 min; then 30 cycles of 95 °C for 30 sec, 72 °C for 30 sec, 72 °C for 1 min; and a final extension of 72 °C for 7 min. The forward PCR primer T7SiFP1 5’-TAATACGACTCACTATAGGGCCAA TTGCCATCAAACTCCT -3’and reverse primer T7siRP1 5’-TAATACGACTCACTATAGGG TTCGGTGCTTGTGGATGATA -3’were used to synthesize the 523 bp region of the VgR gene that includes a T7 promoter region on both the sense and antisense strands.

dsRNA was produced using the MEGAscript RNAi kit (Ambion, Austin, TX) according to the manufacturers recommendations. The targeted region was chosen because a BLAST search showed no significant homology to other genes in GenBank (NCBI), thereby decreasing the chances of off target effects. A total of 50 µl of dsRNA was obtained at concentration of 303.4 ng/µl. The dsRNA was sent to Dr. Oi for fire ant feeding assays.

Morpholino

The ribosomal 28s gene (ADC34225.1) from *S. invicta* was synthesized using the forward primer SiL1, 5'- GGCGAACAAAATCGACAGAT -3', and the reverse primer SiR3, 5'- GTGGGCAGCATTCTTCTAGG -3' from the *S. invicta* cDNA library constructed previously. The primer set was designed using "Primer3" (Rozen et al. 2000). The isolated gene product was amplified and purified using the same method as described before. Purified PCR product was sequenced by Eton Bioscience Inc., and BLAST (NCBI) was used to confirm the 28S ribosomal sequence identity. Following validation of sequence identity of the 28S ribosomal gene from *S. invicta*, approximately 3 µl of cDNA was sent to to the company, Gene Tool, LLC to synthesize the morpholino. Similar to dsRNA, morpholino oligomers are another class of antisense technology used to block access of other molecules to specific sequences within mRNA. Approximately 400 nmol of morpholino was obtained based on the 28S ribosomal gene (ADC34225.1) of *S. invicta* and synthesized by Gene tools LLC. (Philomath, OR, U.S.A). The synthesized morpholino had the sequence 5'- CTGGTTTGTCCATAATGATCGCCTC -3'.

Feeding assay

The knockdown of the VgR and 28S ribosomal gene was conducted using a feeding assay. The endpoint for dsRNA knockdown the Vg receptor message in queens was the cessation of egg production. The morpholino knockdown of the 28S ribosomal messages in all castes and stages of the fire ant should result in mortality. The feeding assays for dsRNA was conducted in Dr. Oi's lab, USDA-ARS CMAVE, Gainesville, FL, U.S.A. The morpholino target of the 28S ribosomal gene was conducted in our Dearstyne Entomology Laboratory, Department of Entomology, North Carolina State University, Raleigh, NC, U.S.A.

dsRNA feeding assay. The dsRNA was designed for targeting the VgR of the fire ant queen, so one of the challenges of this target is the possible dilution effect as the material is passed from workers to the queen. Successful feeding of the queens was only possible by starving them for 24 h before the feeding assay. Newly mated fire ant queens were starved for 24 h before each of the feeding assays; nine fire ant queens were fed 1 µl of dsRNA each at 100% concentration. Twelve queens were used in the control. Half of the control queens were fed distilled water, and the other half were fed elution buffer (the buffer that was used to elute the dsRNA). The assay end point is egg production, which should be reduced in the treatment versus control. Observation of actual egg oviposition was monitored daily.

Morpholino feeding assay. The morpholino was designed for targeting the 28s ribosomal gene; this gene is found in fire ants of any sex and caste, e.g., workers, soldiers, drones and queens. Ten fire ants of random size and caste were used in each feeding assay. The ants were starved for 24 h, then gently transferred to a clear petri dish (8.6 cm

in diameter, 1.5 in height) with a fine camel hair paint brush. Four microliter of morpholino: 10% sucrose mixture (1:1 mixture, vol/vol) was dispensed at the center of the Petri dish with a Pipetman (P20, Gilson Inc. Middleton, WI, U.S.A.). Similarly, one microliter of distilled water was pipetted at the center of the Petri dish for the control experiments. The ants were immediately observed crowding around the morpholino droplet (Figure B-1). After the ants fully consumed the 1 µl morpholino droplet, a piece of 2 cm cotton saturated with 10% sucrose was place in the Petri dish and the ants held at 25±3ºC and 60±5% RH, under a photoperiod of 12:12 (L:D) h. The 10% sucrose (99.7%, Acros Organics, Fisher Scientific, Pittsburgh, PA, U.S.A.) was made in distilled water (wt/vol). The assay end point was mortality, defined as no movement of the ant when touched with a blunt probe. The mortality of the ants was checked and recorded daily for 1 week for all feeding assays. Due to the unavailability of the buffer used to solubilize the morpholino (the manufacturer would not provide the buffer after repeated requests), we were not able to run a control with ants feeding on elution buffer.

Morpholino injections

A morpholino microinjection assay was performed in the laboratory of Dr. Marcé Lorenzen's (Department of Entomology, NCSU). The microinjection was performed under a dissecting stereomicroscope (Leica MX8). Fire ant workers were chilled on ice for 5 min, then aligned on a glass slide, and immobilized with double-stick tape. The morpholino was injected into the dorsum between the first and second abdominal segments using a standard Narishige micromanipulator with an HI-7 stainless steel needle holder and a 10 µl glass capillary tube pulled by a Narishige needle puller (Tomoyasu and Denell 2004). Approximately 0.5 µg of morpholino (1µg/µl) was injected into each

ant. Injected ants were removed from the slide and place into a Petri dish with a 10% sucrose saturated cotton wick as describe earlier. Similarly, 0.5 µl of distilled water was injected into worker ants for the control. Due to the unavailability of the elution buffer for the morpholino, we were not able to conduct a buffer injected control as described before. A total of 6 worker ants were injected with morpholino and another 6 workers ants were injected with distilled water (the control). The mortality of the ants was check and recorded daily, mortality defined as no movement of the ant when touched with a blunt probe.

Results and discussion

dsRNA targets VgR

dsRNA was fed to nine newly mated queens prior to oviposition. Four queens died and the remaining five queens laid eggs. Out of six queens in the control experiment (with distilled water and elution buffer) two died and rest of them laid eggs. Natural death is very common among newly mated queens initiating colonies. The number of eggs produced in the dsRNA, elution buffer and distilled water application to queens were the same. There are several disadvantages of using VgR as a target. First, it only targets the receptor of the fire ant yolk protein located in the ovary of the queen only. Therefore, it is necessary for the dsRNA to be obtained by the female from workers in the colony; this results in a significant dilution effect and potentially loss of the dsRNA in the workers from uptake and degradation in the worker system. The other problem with our bioassay approach, was the use of adult queens. VgR transcripts are first made during the pupal stage of the virgin female. This may delay any impact on the queen in our assays

depending on the age of the laying queen. Furthermore, dsRNA is found naturally in biological systems and is subject to biodegradation. Using artificial nucleic acids could potentially enhance their biological activity by reducing their turnover rate. For these reasons, we shifted our work to another target message in fire ants and to artificial nucleic acids.

Morpholino targeting the 28S ribosomal genes

Morpholinos are another class of antisense technology used to block access to specific sequences within mRNA and potentially prevent translation into protein. Gene Tool LLC synthesized for us the morpholino that targets the 28S ribosomal message with an additional delivery dendrimer attach. The 28S ribosomal genes are conserved in all castes of fire ants and was considered as an alternative to the VgR message as a target which is only expressed in the queen. The dendrimer attachment aids in the delivery of the morpholino into cells without damage or degradation. The Morpholino was fed to the ants as described before. In our feeding assay, the ants consumed 4 µl of the morpholino and sucrose mixture within 30 min, and mortality was observed over a period of 6 d. The experiment was replicated three times but results were not consistent. In the first replicate, the morpholino induced 100% mortality (Figure B-2) after 6 d of treatment, whereas no mortality was observed in the control. For the other two replicates, no mortality was found in the treated ants. The reason for this lack of reproducibility could not be determined but could be explained by a number of factors: (i) mortality in the treatment in the first replicate was by chance and was not the result of feeding on the morphilino; and (ii) the lack of mortality in the second two replicates was the result of degradation of the morphilino in storage. The cost of synthesizing the morphilino and the

reduction in our grant funding for supplies, prevented the synthesis of new morphilinos to test the second hypothesis. An obvious alternative approach would be to feed 28S ribosomal dsRNA, but time did not permit us to conduct these experiments.

Microinjection assays were conducted with the 28S ribosomal morpholino. Injecting a half microliter of morpholino into the ant's hemocoel was difficult, was time consuming and required a high skill level. As for the result of the microinjections that were completed (six worker ants injected with morpholino to knockdown their 28S ribosomal gene and another six worker ants injected with distilled water as a control), no differences in mortality between the morpholino injected group and distilled water injected group was found. The morpholino treatment group mortality was high and so was the control group. Half of the ants in both groups showed bleeding from the injection sites that could have caused the observed mortality.

Both of our dsRNA and morpholino *per os* results were inconsistent and inconclusive. dsRNA mediated gene silencing is a conserved mechanism in many eukaryotic organisms (Geley and muller, 2004; Hannon, 2002). Recently, RNAi has become a valuable tool to study functional genomics in insects. The preferred delivery method in the majority of insect studies is microinjection of microgram amounts of dsRNA into the insect hemoceol (Dzitoyeva et al. 2001). Lu et al. (2009) were able to inject dsRNA into the fire ant queen in the pupal stage and knockdown VgR. The absence of this receptor prevented the uptake of yolk (vitellogenin, Vg) from hemolymph of the adult queen. In the *C. elegans* model, gene knock down effects can be achieved by feeding *C. elegans* with bacteria expressing dsRNA (Timmons and Fire, 1998; Timmons et al. 2001) or by soaking *C. elegans* in a dsRNA solution. The genomic study of

Drosophila melanogaster revealed the reason why RNAi does not work as efficient and effective in insect systems. Insects are lacking the genes encoding RNA dependent RNA polymerase (RDRP). RDRP is the enzyme responsible for amplifying siRNA, which eventually leads to systemic RNAi effects (Sijen et al. 2001). Without RDRP in insects, any effects of RNAi will be limited to each cell where the dsRNA has been absorbed and will require continuous input of dsRNA to have a persistent knockdown effect.

The absence of dsRNA amplification implies the gene knockdown effects produced by feeding RNAi to insects would be difficult and limited. The effects would only be expected in cells exposed to the dsRNA; these cells would be those of the midgut and associated structures because these are the only regions of the insect not covered by the chitin exoskeleton. Degradation of dsRNA in the gut would require continuous administration of high levels of dsRNA. In the cotton bollworm, *Heliothis virescens*, a cytochrome P450 gene (CYP6AE14) is highly expressed in the midgut. Mao et al. (2007) found CYP6AE14 is responsible for detoxification of gossypol, a cotton secondary metabolite. Suppression of the expression of this gene could increase the sensitivity of the insect larvae to the plant's endogenous defense. Mao et al. (2007) engineered an *Arabidopsis* plant to produce dsRNA against CYP6AE14 gene. When the plant material was fed to larvae, effective knockdown of the CYP6AE14 transcript was observed, and the insects showed increased sensitivity to gossypol rich artificial diets. Choosing targets highly expressed in the fire ant's midgut maybe the solution for delivering dsRNA via feeding.

Baum et al. (2007) used a screening approach to identify one lethal phenotype in *Diabrotica virgifera virgifera* out of 290 poetential targets. This lethal target was a

midgut V-type ATPase A (V-ATPase). V-ATPases are highly expressed in the midgut of the insect. Knockdown of V-ATPase results in disruption of the electrochemical gradient across the gut epithelia, which causes high larval mortality. With the availability of the *S. invicta* genome, V-ATPases (ranging from 112 bp to 500 bp) have been found within the genome; the next step is to synthesis dsRNA for each V-ATPase and test for ant mortality via feeding. At last, the two most important steps for using RNAi to control insects via feeding is to first screen and identify potential targets and then delivery of sufficient amounts of intact dsRNA for uptake by the insect. These approaches are still viable for fire ant control and worth further research. Again the advanatage of an RNAi approach to fire ant control is the potential selective killing of the invasive pest without harming beneficial native ant species or even other organisms in general. This specificity is due to the specificity possible in base pair interactions for species specific sequences.

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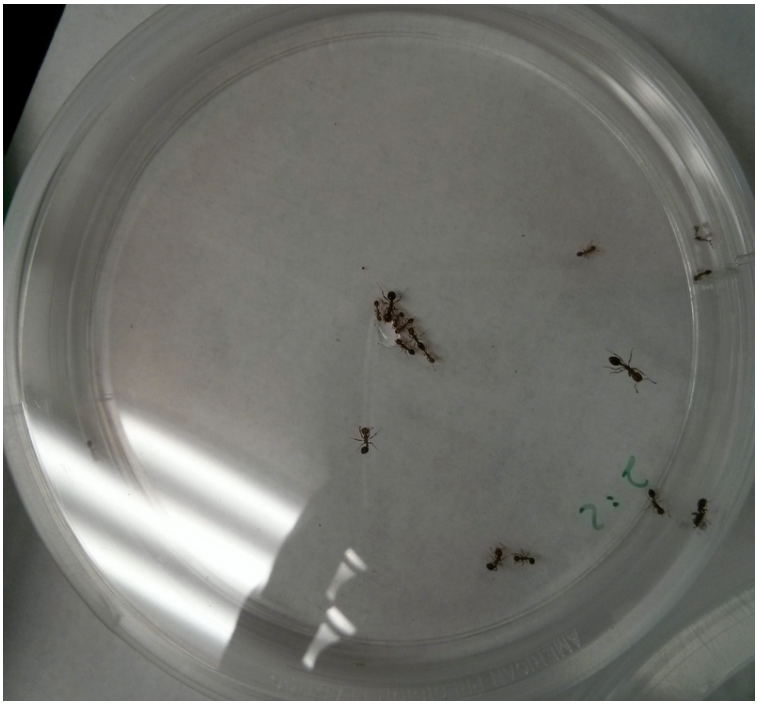

Figure B-1: Fire ants, *Solenopsis invicta*, of random caste and size were starved for 24h, then placed inside the clear petri dish. After dispensing 1 µl of morpholino in the center, the ants started crowding around the droplet and started feeding.

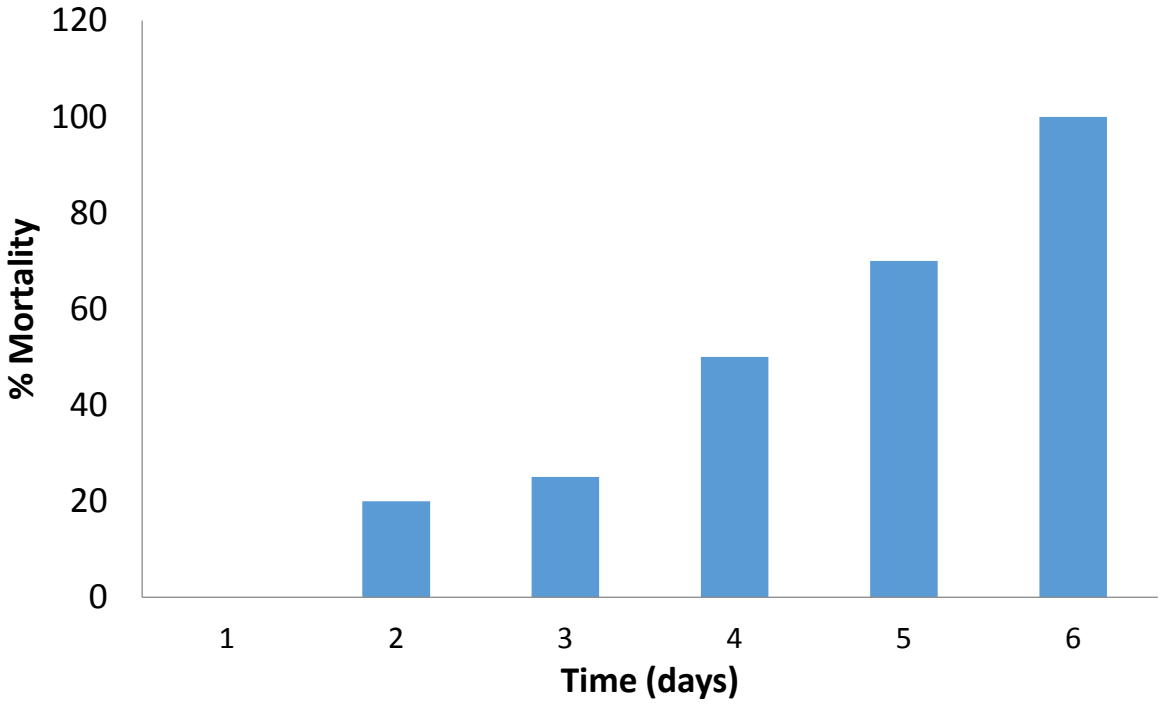

Figure B-2: Percentage mortality of ants after ingesting morpholino (targeted the 28s ribosomal gene) over 6 d. No mortality was observed in the control.