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Characterization of botanical terpene activity in arthropods

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Characterization of botanical terpene activity in arthropods

by

Gretchen Elizabeth Paluch

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Botanicals have a long history of use for protection against biting arthropods and reducing the risk of exposure to arthropod-transmitted disease agents. The aim of these studies was to characterize the activity of the plant derived sequiterpenes and further develop these compounds as long-lasting botanical repellents and insecticides. Studies addressed the utility of sesquiterpene-rich essential oils from Amyris, *Amyris balsamifera*, and Siam wood, *Fokienia hodginsii*, and showed a broad spectrum of activity against arthropods including ticks, cockroaches, house flies and mosquitoes. Identification of the most active components of the Amyris and Siam wood essential oils guided the development of quantitative structure-activity relationship (QSAR) models for predicting repellency to the yellow fever mosquito, *Aedes aegypti*. The final QSAR models showed good similarity in the trends of selected descriptors, providing support for the importance of physicochemical and electronic parameters in influencing the activity of sesquiterpenes. The effects terpenes and other chemical repellents have on *Ae. aegypti* mosquito host-seeking behavior were studied in a wind-tunnel system incorporating three different host attractant cues: CO₂, lactic acid, and 1-octen-3-ol. Results and methods for expansion of this approach are discussed. Preliminary research on the olfactory neural mechanisms mediating repellency effects in *Ae. aegypti*, specifically the olfactory receptor gene sequences OR43b and OR83b (VectorBase), is reported.
CHAPTER 1. INTRODUCTION

Perspective on the Current Status of Insect Repellents Research

Even with a long history of research investigating how chemicals can influence insects, there are many challenges for the advancement of insect repellent science. With the large accumulation of information, it has become increasingly difficult to interpret the significance of repellents tested under different laboratory conditions, or explain how compounds with a wide variety of structures can influence insect behaviors under field conditions. Much of our knowledge of repellents was acquired in the United States during the 1940s with research on the development of synthetic chemicals to repel arthropods, which resulted in products containing dimethyl phthalate (dimethyl benzene orthodicarboxylate), Indalone (butyl-3,3-dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate), and ethyl hexanediol, also known as Rutgers 612 (2-ethyl 1,3-hexanediol). It was also around this time that DEET (N,N-diethyl-3-methylbenzamide) the gold standard of repellents was first tested by the Orlando Laboratory and further developed for use in topical applications in the 1950s.

Since that time there have been significant efforts in academia, government, and the private sector to look for new insect repellents. These have been largely driven by reports of DEET toxicity, minimal efficacy against some arthropod vectors (e.g., Anopheles spp. of mosquitoes), high incidence of arthropod-borne diseases, decreasing consumer acceptance, and the potential for insect resistance to develop to certain chemicals. Recent partnerships between the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) and the Department of Defense’s Deployed War-Fighter Protection Program
(DWFP) in 2004 have funded research in the areas of novel insecticide chemistries and formulations including repellents, application technology, and personal protective systems. The Centers for Disease Control and Prevention have actively supported on-going research efforts and provide continual recommendations based on efficacy data for use of current insect repellents. Government support for more basic research has also come from the National Science Foundation and the National Institutes of Health, specifically from the National Institute of Allergy and Infectious Diseases, and the National Institute on Deafness and Other Communication Disorders. Other substantial contributions toward repellents research include private funding from the Bill & Melinda Gates Foundation, through their Grand Challenges Grant Program. These sources represent new and relatively large funding contributions toward research on insect olfaction and behavior. As a result, there are significant advances in basic research that have occurred within the last 10 years. We have increased knowledge of several components in the insect olfactory and neural pathways and more tools are available for future studies, including genomic and proteomic data. There are efforts towards improved standardization of behavioral repellency bioassays, and development of new synthetic and botanical chemistries with repellent activity.

In recent years, universities have played an important role in the advancement of insect science by providing verification of new product efficacy for registration, as well as explore novel repellent chemistries. One example is the development of the BioUD active, 2-undecanone, which was identified as a mosquito repellent by Dr. Michael Roe at North Carolina State University and then licensed to HOMS, LLC, a North Carolina-based biotech company. The active compound was identified from a wild tomato, *Lycopersicon hirsutum* Dunal f. *glabratum* C. H. Müll, and is thought to play a role in natural plant defense.
mechanisms against insect herbivores (Farrar and Kennedy 1987, Kennedy 2003). Dr. Gregg Henderson at Louisiana State University has been working with the insecticidal and repellent properties of vetiver grass, *Vetiveria zizanioides* Lynn Nash, essential oil. One of the primary components, a sesquiterpene called nootkatone, was also investigated by Dr. Joseph Karchesy at Oregon State University as one of the repellent components of Alaska yellow cedar oil, *Chamaecyparis nootkatensis* (D. Don) Spach. The work in this dissertation, and other studies conducted in the Pesticide Toxicology Laboratory at Iowa State University under Dr. Joel Coats, serve as other examples where academia has contributed to the discovery and development of new repellent technologies.

Many of the new repellent technologies that offer alternatives to the older synthetic chemistries have to undergo a long developmental process, which includes thorough laboratory and field studies. Careful consideration of these data becomes essential as companies need to address questions about registration and marketability prior to final formulation and product development. With the current status of the regulatory requirements for topical repellents being reviewed by the Environmental Protection Agency (EPA), and proposed alterations of some biopesticide/pesticide guidelines (cases where the final product is not applied topically, but may be registered for use in a lawn, home, or garden.), there is substantial risk for any company looking to introduce a new repellent product in the market. The uncertainty met by companies looking to invest in new repellent technologies and the regulatory bodies governing registration, are faced with several challenges. These challenges include the evaluation of new repellent chemistries, improvement of standardized efficacy testing guidelines for registration and comparison against other classes of repellents, development of new application technologies and improved personal protection,
understanding of mosquito species-specific differences dictating behaviors involved in haematophagy, and ultimately, complete elucidation of the mechanism of repellency. The motivation for the studies conducted in the following chapters was to highlight some of these challenges: 1) address the utility of a novel class of plant-derived compounds, the sesquiterpenes, and relate potency of select compounds to structural features of the molecule, 2) expand our knowledge of the basic biochemical processes underlying host-seeking, and 3) interpret these findings with a mechanistic study of mosquito host-seeking behavior.

Evidence of the repellent activity contained in sesquiterpene-rich essential oils and their purified isolates and/or compounds (from heartwood, bark, leaves, etc.) has appeared in the literature over the last 10 years, with a recent focus on sesquiterpenes containing alcohol, aldehyde, ketone, and acid moieties from extractions of white cypress pine (*Callitris glaucophylla* Thompson et Johnson), Japanese cedar (*Cryptomeria japonica* (L. f.) D. Don), the American beautyberry bush (*Callicarpa americana* L.), Alaska yellow cedar (*Chamaecypars nootkatensis* D. Don), *Goniothalamus uvariodes* King, Osage orange (*Maclura pomifera*), Amyris (*Amyris balsamifera*), and Siam-wood (*Fokienia hodginsii*). Availability of these essential oils and extracts can be limited, but some are supplied by commercial sources. Improvement on the activity of this class of naturally occurring repellents requires basic studies on the spectrum of activity and structure-activity relationships; these topics are addressed in the following chapters.

Indeed, there are basic laboratory studies needed to improve understanding of the mechanisms by which even the most successful repellents affect host-seeking behaviors. This is largely apparent as there are many questions still surrounding the mode of action DEET. There are multiple hypotheses ranging from specific olfactory receptors in the antennae that
influence chemosensory response to lactic acid and 1-octen-3-ol (Ditzen et al. 2008), to the potential binding to a JH receptor (Bhattacharjee et al. 2000). Even with well designed mechanistic experiments like these, validation of the mechanism is needed at the mosquito level. A recent study of DEET’s mode of action proposed that mosquitoes are able to detect the DEET molecule, and supported this with findings of a DEET-sensitive olfactory neuron in the antenna (Syed and Leal 2008). However, authors noted that there were changes in the chemical emanations from human skin following topical applications of DEET. This might also significantly influence insect attraction to a host.

Studies of mosquito behavior are absolutely essential to identify the relevance of different modes of action of chemical repellents. There is often limited attention paid to the importance of connecting data from the organismal to the sub-organismal studies of mosquito-host interactions. The term “phagomone” was introduced recently and approaches the functionality of repellents with a broader perspective, i.e., “a chemical that affects feeding behavior, negatively or positively, by any mode of action”. The advantage of this approach reiterates the fact that an insect bite is not an independent event, but rather encompasses a larger set of behaviors and events over time and space (e.g. host-seeking and feeding behaviors). The final chapter presented here focuses on this issue by expanding on the initial laboratory studies with sesquiterpene and DEET repellency, to include a behavioral assay that was designed to measure influences on mosquito host-seeking.
Diversity of Chemical Repellents

Early Research and Identification of Repellents

Plants and their extracts have been used for millennia to protect against or repel arthropods. Some of the earliest recorded uses of repellents are noted in the writings of Herodotus (484 BCE – ca. 425 BCE) detailing the use of a plant oil by an Egyptian fisherman to repel mosquitoes (Herodotus 1996) (Charlwood 2003). Evidence of the bioactivity of plant extracts is apparent in their continued use today. The neem tree, *Azadirichta indica* is an excellent example, as it has a long history of use in a number of agricultural applications. As a repellent, leaves from the neem tree were also used to repel mosquitoes in Africa, South America, and Sri Lanka (Palsson and Jaenson 1999, Sears 1996, Konradsen 1997). The active properties (including antifeedant activity, growth regulation, repellency, fecundity suppression, and toxicity) of this tree are still utilized today in numerous commercial formulations. It is also interesting to note that oil of citronella was one of the earliest recommendations made by J. B. Smith (1901) for repellent protection against insects. This oil is still used today in a variety of applications and has historically served as a standard for comparison of new repellents (Dethier 1956). Other early essential oils and household remedies included those from pennyroyal (Bishop 1935), cassia oil (Bacot and Talbot 1919), cedar, lavender, eucalyptus, peppermint, castor oil, menthol, nutmeg, crushed pepper, lemon juice, and leeks (Bunker and Hirschfelder 1925). An initial compilation of these mixtures and their application as repellents and insecticides was summarized by Howard (1917) and Howard and Bishop (1928).

Prior to the start of WW II, there were limited data available on candidate repellents, or methods for evaluating repellents both in the laboratory and field. Some reports even
called into question the validity of earlier work due to issues of chemical or essential oil purity and variability in testing procedures (Bunker and Hirschfelder 1925, Christophers 1947). Testing conducted by Bacot and Talbot (1919), Bunker and Hirschfelder (1925), Rudolf (1930), Moore (1934), and Granett (1940) represent some of the first documented work on evaluating chemicals and mixtures for insect repellency. At the time, there were limited reports on activity of individual compounds since many recommended repellents were mixtures or plant extracts. Bunker and Hirschfelder (1925) were among the first to approach these questions from a chemical perspective by testing a series of simple hydrocarbons, aldehydes, ketones, ethers, esters, alcohols, and phenols. From this study they concluded that alcohols, esters, ketones, and aldehydes were more effective than the hydrocarbons and phenol ethers tested. Moore (1934) made another early attempt to decipher relationships between chemical structure and repellency. Many of the compounds evaluated by Moore were terpenes and phenols. The study concluded that esters improved on corresponding alcohol moieties and that unsaturated alcohols were better repellents compared to saturated alcohols. Much of the early work summarizing the structural basis of repellent compounds was later summarized in reviews by Roadhouse (1953), and Garson and Winnike (1968).

Dimethyl phthalate, indalone, and ethyl hexanediol were the primary synthetic repellents available prior to the start of the war. Dimethyl phthalate and dibutyl phthalate were some of the earliest synthetic repellents synthesized and were identified as fly repellents in 1929 by Moore (U. S. Patent 1,727,305). Later synthetics included indalone, which was patented by Kilgore in 1937 (U. S. patent 2,070,603) and Rutgers 612, which was developed by Granett at Rutgers University. With the outbreak of war, there was an immediate need for
repellents to protect soldiers against arthropod-borne disease overseas. This threat eventually translated into a total of 821,184 cases of malaria and 302 deaths reported among troops overseas (Mowrey 1963). Traditional repellents such as oil of citronella and various formulations of pyrethrum were tested but found inadequate (Christophers 1947). Much work and progress in the identification and evaluation of efficacious compounds and mixtures was put forth by Granett at Rutgers University (Granett and Haynes 1945), the Naval Medical Research Institute, Bethesda, MD, and largely the Orlando Laboratory, Bureau of Entomology and Plant Quarantine, FL. from 1942 to 1947 (Morton et al. 1947), which was supported by the Office of Scientific Research and Development and the U.S. Army (Travis 1947). The large-scale screening effort evaluated approximately 6,000 chemicals (Morton et al. 1947, Travis 1947, Travis et al. 1949, Dethier 1956) against several dipteran species (the yellow-fever mosquito, *Aedes aegypti*, a malaria mosquito, *Anopheles quadrivittatus*, and the stable fly, *Stomoxys calcitrans*) and found only a small percentage to equal that of existing standards. A report summarized the most active chemicals tested (59 total) in the Orlando Laboratory. Compounds that provided more than five hours of protection against *Ae. aegypti* yielded a percentage distribution of 35.6% nitrogen compounds (amides and imides), 16.9% alcohols, 16.9% esters and lactones, and 10% ethers and acetals (Travis et al. 1949) (Table 1). Another interpretation was published by Roadhouse in 1953 that sectioned compounds into chemical groups and then calculated a class average (Table 2). Using this approach, Roadhouse identified monoalcohols, hydroxyl acid esters, glycols, aldehydes and ketones, and a select number of miscellaneous compounds to evaluate. This study also reiterated the importance of the oxygen with respect to most of the compounds tested, first noted by Bunker and Hirshfelder (1925). Work by McCabe et al.
(1954) and Gilbert et al. (1955, 1957) on $N,N$-diethylamides and more specifically, $N-N$-diethyltoluamide, was also largely guided by the data generated by the Orlando Laboratory and is discussed below. It is interesting to note that this group also approached their study from a structural perspective and recognized $N,N$-dialkylamide and diol groups as the two “promising leads as a source of mosquito repellents” (McCabe et al. 1954).

In 1947, following this large screening effort, initial recommendations by the Bureau of Entomology and Plant Quarantine still included the early synthetics: dimethyl phthalate, indalone, Rutgers 612, dimethyl carbate, as well as mixtures (e.g. Rutgers 6-2-2 containing 60% dimethyl phthalate, 20% indalone, and 20% Rutgers 612) for protection against biting arthropods (Travis 1947). Other mixtures developed during this period included M-2020 (40% dimethyl phthalate, 30% Rutgers 612, and 30% dimethyl carbate), M-1960 (30% benzyl benzoate, 30% n-butylacetanilide, and 30% 2-butyl-2-ethyl-1,3 propanediol), and NMRI-448 (70% 2-phenylcyclohexanol and 30% 2-cyclohexylcyclohexanol). Other commonly used components of insect repellent mixtures included MGK R11 (2,3:4,5-bis(2-butylene) tetrahydro-2-furaldehyde), MGK 264 (dipropyl pyridine-2,5-dicarboxylate), and MGK 326 (di-n-propyl-isocinchomeronate), with the prefix of each representing the initials of the manufacturer, McLaughlin Gormley King Co. These were reported as effective general repellents since they maintained repellent activity against multiple species of insects tested. In addition to variability in repellency response across species, selection of candidate repellents was guided by toxicity issues that were investigated by the U.S. Food and Drug Administration.

Major contributions from this era also include the development and standardization of measures of repellency such as “time until the first bite” (Granett 1938), which is still used as
a repellency index in testing today. As a result of the extensive screening process there was an incredible amount of data generated on individual compounds, both natural and synthetic. Mixtures containing some of the most active compounds were developed for use.

Perhaps one of the most significant advances from this time period was work conducted by McCabe et al. (1954), which looked at structural improvements of \( N,N \)-dialkylamides based on the performance of propyl \( N,N \)-diethylsuccinamate, \( o \)-chloro-\( N,N \)-diethylbenzamide, and \( o \)-ethoxy-\( N,N \)-diethylbenzamide. Testing of different diethylamides prepared from a variety of aromatic acids (e.g. succinic, toluic, and cyclohexane-carboxylic acid) showed that the ring-substituted diethyl benzamides resulted in higher repellency. Different substituents on the ring were synthesized and tested (alkoxy, bromo, and chloro), but it was the alkyl substitutions that performed the best. The first \( N,N \)-diethylbenzamide was patented as an insect repellent by Gertler in 1946 (U.S. Patent 2,408,389), while the first report of mosquito repellency to ortho-, meta-, and para- \( N,N \)-diethyltoluamide isomers was made by McCabe et al. 1954. Soon after, validation of DEET’s repellent activity was observed in laboratory and field trials with multiple insects (Gilbert et al. 1955, Gilbert et al. 1957). Around this time additional studies were published related to optimization of the amide structure for repellency including: Gouck et al. 1957, Gertler et al. 1962, Alexander and Beroza 1965, and Johnson et al. 1967.

Since the time DEET entered the market in the 1950s, it has offered good residual protection against a broad spectrum of insects including mosquitoes, black flies, chiggers, ticks, bedbugs, and fleas (Moore and Debboun 2007). The EPA registered DEET for public use in 1957 and completed a re-registration eligibility decision in 1998 (EPA 1998) for use in households, and in topical applications to human body and clothing, cats, dogs and horses.
Complications with DEET toxicity have been reported in some circumstances amongst children and elderly people (Clem et al. 1993; Goodyer and Behrens 1998; Veltri et al. 1994). Significant amounts of this chemical can be absorbed through the skin (Qiu 1998), and there is some evidence of neurotoxicity resulting from high-level exposure of DEET in combination with permethrin and pyridostigmine bromide (all of which were commonly used by soldiers during the Persian Gulf War) (Cherstniakvoa 2006). In addition, DEET also can dissolve plastics, and has been described by some to have an unfavorable odor or to feel greasy. Even with these concerns, estimates say that DEET is used by approximately 30% of the U.S. population (ATSDR 2003). According to a recent survey by HealthStyles, 40.3% of respondents had repellents containing DEET in the household (Zielinski-Gutierrez et al. 2008). For over 50 years now, DEET has maintained the status of a general use insect repellent with minimal toxicity and safety issues.

**Recent Developments in Insect Repellent Chemistries**

Since the 1950s, DEET has dominated the market for topical insect repellents and as a result, there have been few improvements on alternative repellent chemistries. This is apparent in the literature as there is only a limited amount of data available for select groups. Significant efforts toward advancing many of these ‘alternative’ chemistry groups is discussed below including synthesis of new derivatives and modeling structure-activity, according to similarities in chemical structure for amides, piperdines, diols, and phthalates.
**Amides.**

The amides represent the most commercially successful class of modern insect repellents. The early work by Gertler on the insect repellency of \(N,N\)-diethylbenzamides (U. S. Patent 2,408,389 in 1949) and then McCabe et al (1954) with ortho-, meta-, and para- \(N,N\)-diethyltoluamide isomers, initiated the development of today’s most widely used active repellent. Some of the early work with derivatives included ring-substitutions with hydroxyl, alkoxy, and di-halogen that were less effective. It was the alkyl-substituted diethylbenzamides that were more active repellents (McCabe et al. 1954, Gilbert et al. 1955, Gertler et al. 1962). A trend was also seen with the number of carbons between the ring and amide group, which showed that one carbon was optimal and that an increase in the number of carbons decreased repellent activity (Gouck et al. 1957). Further exploration of this class of compounds was conducted on acid amides (Piper et al. 1951). Other promising amides that were made included DEPA (\(N,N\)-diethyl phenyl acetamide) (McCabe et al. 1954), MGK 264, MGK 326, and 3-(\(N\)-acetyl-N-butyl) aminopropionic acid ethyl ester (IR 3535 or MERCK 3535), made by Merck in 1975 (Klier and Kuhlow 1976).

In addition to these early studies on chemical structure, there have been more recent efforts to identify specific physical-chemical and electronic descriptors that can predict mosquito repellency of the diethylbenzamides (Ma et al. 1999, Suryanarayana et al. 1991, Katritzky et al. 2006). One of the first attempts made to use a quantitative structure-activity relationship approach was made by Suryanarayana et al. (1991) and yielded a model containing log lipophilicity (\(P\)), log vapor pressure (\(V_p\)), and log molecular length (\(ML\)). Although the predictive value of this model was not high (\(R^2 = 0.304\)), it provides a quantitative approach to measuring the importance of select physical-chemical properties that
had appeared in the repellents literature, including vapor pressure (Johnson and Skinner et al. 1968). More recent investigations, including those by Ma et al. (1999) suggest that the van der Waals surface, dipole moments, electrostatic potential and charge of the amide nitrogen, as well as the electrostatic potential of the amide oxygen, are essential for activity within this class of repellents. Interpretation of the electrostatic potential and dipole moment descriptors were that the intrinsic electrophilicity of the amide is important, and that there is an optimal range for lipophilicity or hydrophobicity (3.25 to 3.82 debye) (Gupta and Bhattacharjee 2007). Further, a more negative charge on the nitrogen atom yields a lower level of protection time.

**Piperdines.**

These cyclic amines have been studied by the USDA Gainesville and Beltsville laboratories since the 1970s (Moore and Debboun 2007). One of the more recent piperidine repellents developed is SS220 (1S,2S)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide), which was derived from an earlier repellent comprised of a racemic mixture of four isomers, AI3-37220 (1-[3-cyclohexen-1-ylcarbonyl]-2-methylpiperidine) (Klun et al. 2000, Moore and Debboun 2007, Klun et al. 2001). Interestingly, the 1S,2S stereoisomer (SS220) is the most effective isomer against mosquitoes (2001). Some of the early synthesis work on derivatives of cyclic amines includes a work by Alexander and Beroza (1963) on repellency of aliphatic amides of cyclic amines (C4, C5, and C6). It was McGovern et al. that first synthesized and tested AI3-37220 and AI3-35765 (1-[3-cyclohexen-1-ylcarbonyl] piperidine) (1978), in addition to a series of N-acyl and N-alkylsulfonyl derivatives of heterocyclic amines, and N,N-dialkylalkanesulfonamides (McGovern et al. 1974, 1975, 1978). Other significant
advances from the work conducted through the USDA laboratories included a recent publication on the development of an artificial neural network model to predict the repellent activity of series of $N$-acylpiperdines (Katritzky et al. 2008).

Another major piperdine repellent was developed by Bayer in the 1990s called picaridin, or KBR 3023 (1-piperdine carboxylic acid-2(2-hydroxyethyl)-methylpropylester) (Boeckh et al. 1996). Using a molecular overlay approach, Natarajan et al. (2005) compared topological descriptors to develop a stereochemical structure-activity relationship. In this model, Picaridin, SS220, and DEET shared similar structural motifs that might be useful for developing new repellents. Further, a three-dimensional QSAR was developed to improve on this initial model (Basak et al. 2007). Given that these models will serve as accurate predictors of insect repellency, there is good potential for continued optimization of the piperdine structure.

**Diols.**

In McCabe et al. (1954), two structural groups were identified from the screening program conducted by the Orlando Lab; “certain diols and the $N,N$-dialkylamides, especially diethylamides furnish very promising leads as a source of mosquito repellents”. Even with the focus of this paper on amides, the importance of diols as repellents was mentioned. In an earlier review, Christophers (1947) cited diols (and other high alcohols with a high boiling point) as one of the three types of compounds that “especially exhibit repellency”. This was likely due to the success of early repellents like ethyl hexanediol and 2-butyl-2-ethyl-1,3-propanediol. In particular, the ethyl hexanediol was a very effective repellent against mosquitoes developed by Granett and Haynes (1945) as part of a screening initiated in 1935.
Some amino analogs of the ethyl hexandiol were synthesized and evaluated against *Ae. aegypti*, but did not offer large improvements to the parent compound (Quintana et al. 1972). Beroza et al. (1966) looked at comparative repellency of the ethyl hexandiol diastereoisomers and did not show any differences between the threo- and erythro- isomers.

Another commercially successful diol is p-menthane-3,8-diol, which is a primary component of Quwenling or oil of lemon eucalyptus, *Corymbia citriodora*. Development of this compound was delayed until work in the 1990s revisited this oil (Curtis et al. 1990, Curtis et al. 1991, Schreck and Leonhardt 1991, Trigg 1996, Trig and Hill 1996), which is used widely in China as a mosquito repellent. In the United States, products containing oil of lemon eucalyptus are recommended by the CDC as a long-lasting plant-based repellent. However, lemon eucalyptus oil products are not approved for use on children under 3 years (CDC 2008). Since then there has been some interest in optimizing the structural activity of p-menthane-3,8-diol. Barasa et al. (2002) looked at the comparative activity of four stereoisomers and found that all were active repellents. Both the historical significance and efficacy of select structures within this group suggest that there is good potential for development of new potent repellents.

**Phthalates.**

The initial work on phthalate-repellent chemistries was with dimethyl phthalate in 1929 by Moore (U. S. Patent No.1,727,305). Activity within this class was first described with regard to dibutyl and dimethyl esters of phthalate acid, but was later expanded with dioctyl phthalate, and dimethyl carbate or dimalone (dimethyl bicyclo[2.2.1]hept-2-ene-5,6-dicarboxylate) (Goldenson and Sass 1948). In a later paper, Moore (1934) also evaluated
repellency of compounds and concluded that esters, primarily cyclic esters, were superior to alcohols and hydrocarbons. Other than these early studies, there is minimal data available on optimization of phthalate structure for use as repellents.

Miscellaneous Repellents.

As a result of the large scale screening efforts, many compounds have been evaluated for insect repellent activity. Much of the work on optimizing repellent structure has focused on the amides, piperdines, and to lesser extent, diols. However, there are a variety of other structural groups that have not been thoroughly investigated. Some that have a limited literature base include benzofurans (e.g. MGK 11), mandelic acid esters (Barthel et al. 1954; Leon et al. 1954), hydroxyethers, glycols, hydroxyesters, hydroxyketones, phenols, and monoalcohols (Garson and Winnike 1968, Jachowski and Pijoan 1946).

Botanicals.

A large amount of literature exists on the repellent activity of botanicals and has been summarized thoroughly in some reviews (Peterson and Coats 2001; Isman 2006). These compounds have been shown to protect against pest arthropods including mites, lice, and mosquitoes. A few studies have shown that certain species of birds will incorporate botanical chemical defenses into their nests including citrus peels and marigold flowers (Clark and Mason 1985; Clayton and Vernon 1993). Additionally, there is evidence these compounds benefit the host plant, aiding in pollination and seed production. One recent study examined the effects of repellents in the floral composition reduced the levels of florivory and nectar robbing (Kessler et al. 2008). Repellents have also been shown to reduce the amount of
nectar removed by hawk moth pollinators, and increase the number of visits to flowers, all of which would serve as an advantage for the flowering plant (Euler and Baldwin 1996).

There exist several diverse classes of plant-based chemistries that consistently show some insect repellent or deterrent activity, including alkaloids, phenols, terpenes, quinones, nitriles, furans, and lactones (Barasa et al. 2002). Of these groups, terpenes make up a large portion of repellent-active plant essential oil composition. The essential oils of citronella, *Cymbopogon nardus*, and lemon eucalyptus are examples of commercially successful terpenes. The historical significance of these oils and their major components (citronella - citronellal, citronellol, and geraniol; lemon eucalyptus – *p*-menthane-3,8-diol) is discussed above. Evidence of optimization and formulation of these plant essential oils can be seen in the current scientific and patent literature, including synergistic effects identified by EcoSMART Technologies (U.S. Patent No. 7,238,726). Structural activity of other terpenes still remains an area for development of new repellents, in particular the sesquiterpenes (Paluch et al. 2009; and Paluch et al. in preparation).

In order to more fully utilize these natural chemistries, more studies are needed to explore what features of the molecule are important for insect repellency. A study by Wang et al. (2008) is one attempt to develop a predictive model for mosquito repellent activity of pinene-related terpenes. With more studies such as these, it may be possible to synthesize new compounds that improve on the activity of the parent compound, potentially increasing repellent potency, broadening the spectrum of activity, as well as increasing residual effects.
Importance of Mosquito-Host Interactions

One of the constant challenges facing research on repellents is how exogenous chemicals can influence insect behavior. Such studies require attention toward the larger set of behaviors and events over time and space, associated with host-seeking and feeding. Thus, mosquito-host interactions are the basis for the connecting of the sub-organismal studies to the organismal studies of chemical repellents.

Mosquito Host-Seeking Behavior

There are considerable risks associated with haematophagy because detection by the host can in many cases be fatal to the arthropod. A successful mosquito is able to locate and approach a potential host, evaluate the quality of the food source, and begin feeding without alerting the host to its presence. Careful execution of this process is needed for mosquito growth and reproduction, and is controlled on different levels. Life history characteristics of the female mosquito are important for consideration as these details often pertain to host-seeking ability (Roitberg and Friend 1992). One major distinction is that of autogeny vs. anautogeny, where some female mosquitoes emerge from the pupal state with enough protein for egg development while others require a blood meal to provide the nutritional requirements for egg maturation. Witness the yellow fever mosquito, *Ae. aegypti*, which is an example of an anautogenous female. In this species, there is a time period required for maturation and increased sensitivity of the receptors needed for host location (1984 Davis).

The literature on mosquito host-seeking behavior broadly outlines the following sequence of events (Gillies 1980, Sucliffe 1987, Klowden 1996) in the progress to a host.
[Appetitive Search] -> Activation -> Orientation -> [Attraction] -> [Host Acceptance]

A mosquito’s appetitive search state varies accordingly with endogenous circadian rhythms and hunger state, which are dependent on the length of food deprivation (Roitberg and Friend 1992). An appetitive search behavior is viewed as an undirected or sometimes random search that terminates when a host stimulus is detected. This single event is termed activation. There is sufficient evidence that supports the role of one particular host cue, CO$_2$, in both the activation and orientation response. It is very possible that other host cues, in addition to CO$_2$ are also important in the activation response. Differences are likely due to interspecific variation, particularly across anthropophilic and zoophilic mosquito species.

Following exposure to CO$_2$, individual mosquitoes will take off and begin a sustained flight that is governed by a programmed flight behavior known as optomotor anemotaxis (Gillies 1980). Patterns of mosquito upwind flight closely mimic those of other insects that follow a pulsed chemical gradient toward a source. This process is referred to as orientation. Once at close range, mosquitoes are thought to depend on visual, motion, heat, and olfactory cues from their potential host for attraction.

There is surprisingly minimal behavioral data available on patterns associated with this event and those that direct host acceptance for mosquitoes. A variety of host surface volatiles and chemicals have been tested and identified as attractants, but the underlying behavioral mechanisms that guide this short-range approach are poorly understood. Interestingly, it’s during this short-range approach where the mosquito would encounter the greatest diversity of host cues, including a complex volatile blend comprised of semi- and
highly volatile compounds. In order to appreciate the multitude of chemicals emitted by the host, one must consider 1) primary odors that do not change regardless of changes in an individual’s diet; 2) secondary odors that are related to an individual’s diet and interaction in the environment; 3) tertiary odors that arise from outside sources (e.g. lotions, hair products, make-up, etc.) (Curran et al. 2005). Researchers have identified a variety of compounds prevalent in skin emanations, armpit odor, and even released while breathing that include short-chained (C4-C12) acids, aldehydes, alcohols, esters, and ketones. It has been noted in multiple publications that there is significant variation in these components within individuals of the same species (Bernier et al. 2007, Curran et al. 2005).

Once the mosquito lands on the host, it must locate an optimal place to feed. An individual may make multiple probes before finally committing to a feeding location and accepting a host as suitable. This is an important decision, as the mosquito must choose an optimal location for blood-feeding and avoid detection by the host.

**Importance of Mosquito Olfaction**

Within the last 10 years, there has been a large effort to dissect the underlying mechanisms of insect olfaction, and it is summarized in several recent review articles (Jacquin-Joly and Merlin 2004; Rutzler and Zwiebel 2005; Hallem et al. 2006). Building on these studies, researchers are starting to utilize more mechanistic approaches to understand the mode of action of repellents.
Insect Olfaction

Insect olfaction begins with a molecule, usually hydrophobic, entering the sensilla on either the antennae and/or maxillary palpi. These chemosensilla have been characterized into three morphological types: basiconic, trichoid, and coeloconic. Olfactory binding proteins (OBP) are secreted by accessory cells and then transport these molecules through the aqueous environment surrounding the olfactory receptor neurons. Current research suggests that the pheromone binding protein (PBP)-ligand binding mechanism might be pH-dependent (Wogulis et al. 2006). However, it is still unclear whether these OBPs are required for binding to the olfactory receptor (OR) or if their function is primarily in transport. Studies with silk moths have demonstrated that different concentrations and combinations of PBPs can affect neuron sensitivity (Pophof 2004). It is possible that OBPs play an important role in the binding of the ligand to the OR (Große-Wilde et al. 2006). One hypothesis is that OBPs interact with sensory neuron membrane proteins (SNMPs) and enhance delivery of the odorant/pheromone (Rogers et al. 1997). These 2 trans-membrane domain membrane proteins were first identified in insect olfactory neurons (ONs) and their role in the olfactory pathway is still under investigation (Nichols and Vogt 2008). More recently, it was shown that SNMP was required for sensitivity to a pheromone, 11-cis-vaccenyl acetate, in *Drosophila* (Jin et al. 2008). The role and specificity of these OBPs is still debated, as there are studies in *Drosophila* that show many different OR genes can maintain their specific response spectra after interaction with a single set of OBPs (Laissue et al. 1999; Kreher et al. 2005). Other studies report highly specific responses from OBPs (Xu et al. 2005).

ORs are more specifically G-protein-coupled receptors with seven transmembrane domains that, upon binding to their ligand, cause a conformational change and activate
enzymes including adenyl cyclase and phospholipase. Downstream messengers are cyclic AMP (cAMP) and inositol-3-phosphate (IP3) + diacyl glycerol (DAG); cAMP and IP3 are both capable of opening Na$^+$ or Ca$^{++}$ channels for ion influx, after which depolarization of the neuron occurs.

ONs converge on the antennal lobe, and sensory processing is believed to be combinatorial (one molecule can stimulate multiple classes of ONs, and individual ONs can respond to more than one chemical). Information conveyed by ONs can include chemical identity, concentration, and chemical dynamics (fluctuations in concentration). ONs contained in the basiconic sensillum of *Drosophila* have been extensively studied and can serve as an example of odor neuron-processing (Fishilevich and Vosshall 2005, Hallem et al. 2004, de Bruyne and Warr 2005). The ONs in the antennal basiconic sensillum have been studied and fall into 24 functional classes, designated by their responses to different chemicals (de Bruyne et al. 1999, Elmore et al. 2003). Some classes are more narrowly tuned than others, as is seen with some pheromone ONs in moths.

Currently, there appears to be a numerical relationship between OR genes, the different functional classes of ONs, and the olfactory glomeruli, as is shown in a study by Couto et al. 2005. Much information on the connection between ORs and ONs has come from studies with *Drosophila Δhalo* mutants and *Xenopus laevis* oocytes (Hallem et al. 2004, Nakagawa et al. 2005). These studies demonstrate that removal of the OR genes results in loss of the ONs capacity to respond to a range of chemical stimuli, but does not eliminate ON firing activity (Hallem et al. 2004). Additionally, OR genes have been shown to relay the same response spectra when expressed in mutant neurons, even ONs normally expressed in morphologically distinct sensilla. This change in the response spectra includes differences in
ligand specificity, firing rate, response dynamics and signaling mode. Many uncertainties still exist, as there are still ORs with unique response spectra that do not match known ONs. It is also known that multiple ORs can be expressed in a single ON, which leads to more questions about OR gene expression and olfactory coding (Bruyne and Warr 2005).

Studies of sequence similarities between OR genes show that these are highly divergent within and across species. This excludes the Or83b co-receptor, which appears to be a highly conserved gene, expressed in the majority of olfactory neurons, and is necessary for proper neuron function. It is currently hypothesized that this OR plays a role in localizing other proteins and might also assist in odorant binding and signal transduction (Larsson et al. 2004).

Once the ON has fired, signaling is believed to cease due to signal cascades commonly associated with G-protein receptors. These include protein kinases and arrestins which uncouple the receptor and cease the signaling cascade. Some arrestin functions include interference with G-protein binding (termination of ligand signal), interaction with dynamin and other proteins to trigger endocytotic internalization of the receptor, and sometimes stimulation of the mitogen-activated protein (MAP) kinase pathway.

Recent Developments on Insect Repellent Mode of Action

After more than 50 years of use, the mode of action of DEET is still debated in the scientific literature. Some of the most recent hypotheses involve interactions within the olfactory pathway. Research in the 1970s suggested that DEET affects mosquito sensitivity to lactic acid, as studied in the ONs in the antennae of *Ae. aegypti* (Davis and Sokolove 1976), which was later supported by behavioral data (Dogan et al. 1999). More recent work
with ORs in *Drosophila* showed that DEET blocks sensitivity to certain food odors by inhibiting the OR +OR83b (co-receptor) complex in select neurons located in the antennae (Ditzen et al. 2008). A component of this study also showed *An. gambiae* electrophysiological data with inhibition of the ON response to 1-octen-3-ol, a component of human breath, following applications of DEET. This relates to the previous finding in *Drosophila* because the mosquito response is mediated by (gustatory receptors) GPOR8+GPOR7 (co-receptor), which shares similarity with *Drosophila* ortholog OR83b. The most recent finding of a DEET-sensitive olfactory neuron in the antennae and maxillary palps of *Culex quinquefasciatus* suggests that mosquitoes are able to respond directly to the DEET molecule and that the repellency effect is not due to inhibition of a co-receptor/odorant complex (Syed and Leal 2008).

**Summary**

Within the last 10 years there has been some progress with developing alternative insect repellents including other chemicals that fall into the amide, piperdine, diol, and terpene classifications. The newer repellent compounds recommended by the Centers for Disease Control and Prevention that fall into these groups include IR3535, picaradin, and p-menthane-3,8-diol. It is encouraging to follow the advancement of some of these repellent chemistries, particularly in the under-studied chemical classes such as piperdines and terpenes. There are also still large efforts toward standardization of efficacy testing for registration and comparison to other candidate repellents, as well as new application technologies. However, one of the most intriguing questions remains, by what mode of action
do repellents work? Schreck (1977) stated that following the development of DEET, there was interest in understanding repellent mode of action and the functionality of repellents.

It is the opinion of the author that to explaining the mode of action of a chemical repellent, it is necessary to first identify which component of the of insects’ behavior is influenced by an exogenous stimuli, or candidate repellent, and then interpret this behavior with a knowledge of how these types of compounds can affect insect olfactory mechanisms. In the following chapters, the repellent activity of a specific class of botanical repellents, sesquiterpenes, is investigated, including spectrum of activity and toxicity. Further, attempts to explain how sesquiterpenes influence insect orientation behavior are addressed.

Role of Co-Authors

Dr. Joel Coats served as the primary PI, over-seeing the progression of the research reported in this dissertation. Additional advising and support was issued by the co-PI, Dr. Lyric Bartholomay. In Chapter 2, Dr. Junwei Zhu acted as a collaborator and contributed execution of sample analysis on GC-MS. Dr. Justin Grodnitzky is listed as a co-author on Chapter 3 for his assistance in interpretation of the sesquiterpene QSAR model. All of the co-authors credited in the following chapters were given the opportunity to comment on the methodology, data analysis, and final interpretation of results, prior to the submission of this dissertation.

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Table 1. Chemical class percentage distribution of 59 materials tested by the Orlando Laboratory (Morton et al. 1947) that were effective repelling the yellow fever mosquito, *Aedes aegypti*. Table adapted from Travis et al. 1949.

<table>
<thead>
<tr>
<th>Chemical Class</th>
<th>Time Effective</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 to 5 Hours</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>0</td>
</tr>
<tr>
<td>Acids and anhydrides</td>
<td>1.9</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>1.7</td>
</tr>
<tr>
<td>Esters, lactones</td>
<td>30.4</td>
</tr>
<tr>
<td>Ethers, acetals</td>
<td>17.8</td>
</tr>
<tr>
<td>Ketones</td>
<td>1.7</td>
</tr>
<tr>
<td>Alcohols (including phenols)</td>
<td>14.2</td>
</tr>
<tr>
<td>Nitrogen compounds</td>
<td></td>
</tr>
<tr>
<td>a. Amides, imides</td>
<td>18.5</td>
</tr>
<tr>
<td>b. Amines</td>
<td>4.9</td>
</tr>
<tr>
<td>c. Nitriles</td>
<td>2.6</td>
</tr>
<tr>
<td>d. Nitro compounds</td>
<td>1.3</td>
</tr>
<tr>
<td>e. Any other (azo, azoxy, hydrazo,</td>
<td>1.3</td>
</tr>
<tr>
<td>nitroso, thiocyanates, oximes, etc.)</td>
<td></td>
</tr>
<tr>
<td>Halides</td>
<td>1.7</td>
</tr>
<tr>
<td>a. Sulfur compounds</td>
<td>0.6</td>
</tr>
<tr>
<td>b. Phosphorus compounds</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Table 2. Relative repellency of chemical classes calculated by Roadhouse (1953) from screening trials conducted by Morton et al. 1947. Table was adapted from Roadhouse 1953.

<table>
<thead>
<tr>
<th>Chemical Class</th>
<th>Number tested</th>
<th>Class average</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyethers</td>
<td>134</td>
<td>2.5</td>
</tr>
<tr>
<td>Glycols</td>
<td>112</td>
<td>2.3</td>
</tr>
<tr>
<td>Amides, imides, amide-esters</td>
<td>270</td>
<td>2.2</td>
</tr>
<tr>
<td>Hydroxyesters</td>
<td>221</td>
<td>2.2</td>
</tr>
<tr>
<td>Monoalcohols</td>
<td>196</td>
<td>1.9</td>
</tr>
<tr>
<td>Aldehydes, ketones and hydroxy-ketones</td>
<td>254</td>
<td>1.8</td>
</tr>
<tr>
<td>Ether esters and epoxy esters</td>
<td>200</td>
<td>1.7</td>
</tr>
<tr>
<td>Nitriles</td>
<td>137</td>
<td>1.6</td>
</tr>
<tr>
<td>Esters and keto-esters</td>
<td>1600</td>
<td>1.5</td>
</tr>
<tr>
<td>Ethers</td>
<td>349</td>
<td>1.3</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>100</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen heterocyclic compounds</td>
<td>70</td>
<td>2.1</td>
</tr>
<tr>
<td>Acid anhydrides</td>
<td>50</td>
<td>2.1</td>
</tr>
<tr>
<td>Acetals</td>
<td>70</td>
<td>2.0</td>
</tr>
<tr>
<td>Amines</td>
<td>52</td>
<td>2.0</td>
</tr>
<tr>
<td>Organosulphur compounds</td>
<td>69</td>
<td>1.4</td>
</tr>
<tr>
<td>Natural oils</td>
<td>67</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Group 1, more than 100 tested.
** Group 2, 50-100 tested.

Example calculation of class average by Roadhouse 1953:

of 196 substances in the monoalcohol group tested by Morton et al., 30 had been rated at “4”, 14 at “3”, 52 at “2”, and 100 at “1”; the value of this chemical group was therefore

\[
\frac{(30\times4) + (14\times3) + (52\times2) + (100\times1)}{196} = 1.87
\]
Figure 1. Structures of common insect repellents. A) Amides and Imides, B) Phthalates.

A)

DEET
Diethyl toluamide
CAS No. 134-62-3

DEPA
N,N-diethyl-2-phenyl-ethanamide
CAS No. 243196-1

N-butylacetanilide
CAS No. 91-49-6

IR3535
Ethylbutylacetylaminopropionate
CAS No. 52304-36-6

MGK Repellent 326
CAS No. 136-45-8

MGK Repellent 264
CAS No. 113-48-4

Methylneodecanamide
CAS No. 105726-678

B)

Dibutyl phthalate (DBP)
CAS No. 84-74-2

Dimethyl phthalate (DMP)
CAS No. 131-11-3

Dimethyl carboxylate (DMC)
CAS No. 5826-73-3

Diocetyl phthalate
CAS 117-81-7
Figure 2. Structures of common insect repellents. A) Piperdines, B) Miscellaneous.
CHAPTER 2. AMYRIS AND SIAM-WOOD ESSENTIAL OILS: 
SESQUITERPENE ACTIVITY AGAINST INSECTS

A chapter in press, American Chemical Society, Symposium Series, Household, Structural and Residential Pest Management

Gretchen Paluch, Junwei Zhu, Lyric Bartholomay, and Joel Coats

Abstract

Recent investigations on the sesquiterpene-rich Amyris (Amyris balsamifera L.) and Siam-wood (Fokienia hodginsii L.) essential oils revealed significant arthropod repellency and toxicity responses. Amyris essential oil and one of its major components, elemol, were evaluated in laboratory bioassays and identified as effective mosquito repellents, specifically characterized by high levels of contact and minimal spatial repellency. Mosquito responses to catnip (Nepeta cataria L.) essential oil are characterized with high spatial activity, but lack significant contact repellency. Sampling within the static-air bioassay chamber with solid-phase microextraction provided measurements of the relative concentration and distribution of volatiles. These results supported the differences observed in repellency between essential oil treatments. Essential oil mixtures containing both spatial (catnip) and contact (Amyris) repellents were made and showed high levels of residual control via both modes of action. Siam-wood essential oil scored high in both spatial and contact efficacy against mosquitoes. Observations during this study included signs of toxicity. Two of the primary components of Siam-wood essential oil were tested for 24-hour house fly (Musca domestica L.) topical
mortality. Trans-nerolidol and fokienol were found to possess similar insecticidal activity (topical LD$_{50}$ values ranged from 0.17-0.21 μmol/fly). Amyris essential oil was selected for additional testing with brown dog ticks (*Rhipicephalus sanguineus* Latreille) in a ‘barrier’ repellency assay. Individuals were observed repeatedly avoiding and moving away from surfaces treated with Amyris essential oil.

**Introduction**

Nature holds a diversity of terpenoid structures, and the functionality of these compounds is still poorly understood. Only a small number actually serve a primary metabolic function (ex. carotenoids, sterols, etc.). In the 1970s, researchers started to identify other terpene bioactivities including toxicity, attraction, and repellency (Langenheim 1994). The challenges today still include the characterization of terpene function, but also improvement of our understanding of their ecological roles. A variety of living organisms are known to utilize terpenes for coordinating antagonistic and beneficial interactions, such as inter- and intraspecific communication, and defense (Gershenzon and Dudareva 2007).

Terpenoid compounds are classified into groupings based on the number of isoprene units: hemiterpenes C5, monoterpenes C10, sesquiterpenes C15, diterpenes C20, sesterterpenes C25, triterpenes C30, tetraterpenes C40, and polyterpenes (terpene polymers). In plants, terpene biosynthesis pathways are either via the formation of a mevalonic acid intermediate or the pyruvate pathway. Mono-, sesqui-, and diterpenes are formed by continual addition of 5-carbon units, whereas other larger terpenes require joining of large carbon units, e.g. two sesquiterpenes to form a triterpene.
Bioactivity of Sesquiterpenes

Sesquiterpenes are produced in a number of plant families and appear in different concentrations in the essential oil composition. In many of these cases sesquiterpenoids make up only a small percentage of the essential oil blend, however there are examples of oils containing large amounts of these compounds with similar ring structures and specific functional groups. There is evidence of essential oils, and the actual plant tissues (heartwood, bark, and leaves), containing sesquiterpenes with alcohol, aldehyde, and acid moieties, possessing high levels of insecticidal or repellent activity. The essential oil obtained from the bark of *Goniothalamus uvariodes* King, a small tree endemic to Borneo, is one example. Both the bark and leaves from this plant are used by several local groups including the Kedayan and Iban communities in Sarawak and the Sungai in Sabah as an insect repellent. The chemical constituents of the bark includes sufficient amounts of nerolidol (5.2%), α-eudesmol (5.6%), hedycaryol (13.6%), γ-eudesmol (16.0%), and β-eudesmol (31.5%) (Ahmad and Jantan 2003). These compounds and other closely related structures (farnesane, eudesmane, eremophilane, and elemene derivatives) appear in other reports detailing insect response to essential oils.

Several eudesmol isomers, and a eudesmane sesquiterpene acid and methyl ester derivatives were isolated from *Callitris glauca* Thompson et Johnson and identified as termite repellents (Watanabe et al. 2005). The *Cryptomeria japonica* (L. f.) D. Don essential oil contains elemol as its major component (18.2%), and was recently identified as a repellent to silverfish (Wang et al. 2006). Another interesting study investigated the essential oil composition of *C. japonica* cultivars that varied in susceptibility to the *Cryptomeria* bark borer (*Semanstus japonicus* Lacordaire). Attractant and repellent responses of the
Cryptomeria bark borer were used to assay select chemical components of the essential oils, and quantitative comparisons were made across the different cultivars. There were notable differences in the essential oil compositions of the resistant and susceptible cultivars, with the bark oils showing great diversity in structures and amounts of terpene hydrocarbons in particular, pinene (16-52%), limonene (7-12%), and δ-cadinene (4-8%). Many of the terpene hydrocarbons, e.g. β-pinene, camphene, sabinene, β-phellandrene, β-caryophyllene, and longifolene, were found to be attractants for the Cryptomeria bark borer. Four compounds were found to occur in significantly higher levels in the resistant cultivars and identified as repellents in the laboratory bioassay. These included three oxygenated sesquiterpenes α-terpineol, nerolidol, and β-eudesmol (Yatagai et al. 2002).

Callicarpenal and intermedeol were isolated from the American beautyberry bush (Callicarpa americana L.) and recently tested for insect activity. Researchers used a finger tip climbing assay and found both to be effective tick repellents. At an application rate of 155 nmol/cm² deer tick (Ixodes scapularis Say) nymphs were repelled 98 and 96%, respectively. These compounds were compared with commercial standard N,N-diethyl-m-toluamide (DEET) , and there was no significant difference with DEET (callicarpenal, EC₅₀ 14.2 nmol/cm²; intermedeol, EC₅₀ 17.4 nmol/cm²; DEET, EC₅₀ 23.9 nmol/cm²) (Carroll et al. 2007).

Another collection of sesquiterpenoids from the heartwood of the Alaska yellow cedar (Chamaecypars nootkatensis D. Don), include nootkatone and valencene-13-ol. Both of these compounds were just as repellent to I. scapularis as DEET (nootkatone, RC₅₀ 0.0458% wt/vol solution; valencene-13-ol, RC₅₀ 0.0712% wt/vol solution; DEET, RC₅₀ 0.0728% wt/vol solution) (Dietrich et al 2006).
Amyris Essential Oil

West Indian sandalwood or Amyris oil (Amyris balsamifera L.) is produced from the heartwood of a small tree (3-6 m, 75-150 DBH) in the Rutaceae. Some of the identifying features of this tree include three to seven ovate, opposite and compound leaflets, white flowers in lateral clusters, and a black drupe fruit. Trees are described as having a smooth grayish bark, with a rounded crown of aromatic foliage. Its distribution is mostly limited to the Caribbean islands, but is also found in some South American countries. Amyris is also referred to as bois chandelle (candlewood) in Haiti, torchwood in Jamaica, tigua in Venezuela, but in the United States as Amyris, balsam amyris, or West Indian sandalwood. Interestingly, this species is not closely related to the other sandalwood (e.g. Indian or Australian sandalwoods), which are highly valued, wood-scented essential oils derived from trees in the Santalales. The sandalwood oils and other byproducts (including incense, pastes, and wood-carvings) have a rich history of being used in religious and social ceremonies. Some other common uses for the Amyris heartwoods have included torches, firewood, fence posts, and ancient wood-carvings mosaics (Weiss 1997). This is not surprising considering the soft-quality of the heartwood and its use in carving. Also, there are studies citing the antimicrobial activity of Amyris extracts. Amyris essential oil is an effective inhibitor of Klebsiella pneumonia growth, and minimally effective against Staphylococcus aureus (gram-positive), Escherichia coli (gram-negative), and Pseudomonas aeruginosa (Jirovetz et al. 2006). Such properties would no doubt be beneficial for maintaining the integrity of the wood in several of the uses listed above.

In most regions where Amyris is commercially grown, it is used for essential oil production. Steam distillation is estimated to yield 2-4%, depending on the portions of wood
used. The essential oil is a viscous amber liquid composed mostly of oxygenated sesquiterpenes (80%) and sesquiterpene hydrocarbons (20%). Its woody scent is used in perfumery, soaps, and cosmetics and is also believed to be used by the cosmetic and perfume industries to dilute more expensive sandalwood oils such as that from East Indian sandalwood, Santalum album L. (Howes et al. 2004). There are also pharmaceutical and nutraceutical benefits from Amyris chemistries. Anti-mutagenic activity has been shown with β-eudesmol, one of the primary components. This compound suppressed SOS-inducing activity of furylfuramide, in addition to suppression of gene expression (ID$_{50}$ 0.09 µmol/ml) in Salmonella typhimurium TA1535/pSK1002 with the furylfuramide mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide. Additional suppression activity was seen against the Trp-P-1 mutagen 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Miyazawa et al. 1996).

Previous studies in the Pesticide Toxicology Laboratory at Iowa State University, Ames, IA identified the repellent activity of Amyris essential oil against mosquitoes (Schultz and Coats 2005). Amyris was one of forty essential oils recently screened for repellency of Aedes, Anopheles, and Culex spp. mosquitoes using the human-bait technique (Schreck and McGovern 1989; WHO 1996). The Amyris essential oil formulation provided a 480-minute protection period against Anopheles and Culex and 240 minutes for Aedes. Percentages of landing and biting mosquitoes reported was also low (Anopheles, 0% landing and biting; Culex, 0% landing and biting; Aedes, 9.6% landing and 0.8% biting). These levels were comparable to the Bayrepel and DEET formulations (Amer and Mehlhorn 2006a). Studies with Amyris essential oil as a potential mosquito larvicide were conducted using the yellow fever mosquito, (Aedes aegypti L.). With fresh preparations, researchers found 100% mortality of the mosquito larvae at 6 h following application, at a rate of 50 ppm (Amer and
Efficacy following storage of this preparation showed that it was not effective after 1 week in a dark environment.

**Siam–wood Essential Oil**

Siam-wood (*Fokienia hodginsii* L.), which is also known as Vietnamese pemou, produces a highly prized oil from the heartwood of a tree in the Cupressaceae. These cypress trees are the only living species in the genus *Fokienia* and are adapted to growing at higher altitudes (600-1800m) in regions of Southern China, Northern Lao PDR, and Vietnam (World Wildlife Fund 2007). Some of the people in these regions, such as the Greater Annamites, utilize the wood for housing and furniture construction. This is due to the longevity of the wood and its ability to handle many climatic factors and resist insect injury. The essential oil is extracted from the stumps and roots. Constituents of the essential oil were reexamined by Weyerstahl et al., and they found only sesquiterpenes. The major components identified were (E)-nerolidol (34.8%) and fokienol (25.7%); minor components were multiple cadinene isomers (6.5%), eudesmol isomers (7.4%), α-cadinol (1.9%) and daucadiene (14),11-dien-9-ol (3.1%) (Weyerstahl et al. 1999). There is limited literature available on the insect activity of Siam-wood extracts. Only one citation was found that mentioned that the wood is resistant to termites and moths (Weyerstahl et al. 1999).

The intent of this study was to characterize the bioactivity of two sesquiterpene-rich essential oils, Amyris and Siam-wood. In the initial screening trials, both oils showed evidence of repellency against a mosquito (*Ae. aegypti*). One area of particular interest was observation of residual repellency effects (including both contact and spatial repellency), which were supported by the relative concentration of volatiles measured inside the bioassay.
chambers. These essential oils were evaluated against actives contained in commercial natural products, and then incorporated into mixtures to test for improvements of natural product residual efficacy. The results of this study show that Amyris and Siam-wood significantly repel arthropods, are superior to other natural products in today’s market, and could potentially be utilized to improve residual control in repellent formulations.

**Materials and Methods**

**Mosquito Repellency Bioassay**

Bioassays were conducted in a static-air apparatus (9 x 60-cm section of glass tubing) at a controlled temperature of 26°C. Yellow fever mosquitoes (*Aedes aegypti*), a Costa Rican strain, were from an established laboratory colony in the Iowa State University, Medical Entomology Laboratory, Ames, Iowa. Eggs were hatched in deoxygenated water, and larvae were fed Tetramin fish food (Melle, Germany). Pupae were sorted from the larvae and placed in paper cups with mesh lids until emergence. Newly emerged adults were fed a 10% (0.3 M) sucrose solution and aged for at least 5-days before testing. Incubator conditions were set at 80% relative humidity and held at 27 °C under a 16 hr light/8 hr dark cycle. Only female mosquitoes were used in the testing.

Essential oils and mixtures included catnip (*Nepeta cataria* L.) oil, which was produced from a steam distillation in the laboratory (Pavia et al. 1988). Amyris oil was purchased from Sigma Aldrich, St. Louis, Missouri; Siam-wood essential oil was purchased from Oshadhi, Petaluma, California. Elemol, a sesquiterpene found in both Amyris and Siam-wood essential oil, was purified from a crude commercial source (Augustus Oils, New Hampshire, England) using column chromatography techniques with silica gel. Several of the
commercial repellent active compounds were available for purchase: DEET, citronella oil, 2-undecanone, and cis/trans p-menthane-3,8-diol (Sigma Aldrich, St. Louis, Missouri).

Test solutions were made up in a carrier solvent (either acetone or hexane), applied to 9-cm diameter round filter papers (63.6 cm$^2$), and then the solvent was evaporated off prior to testing. The resulting rate of exposure was 78.6 $\mu$g/cm$^2$. Treated filter papers were placed inside the lids of 9-cm glass petri dishes, and the dishes were placed over the ends of the glass chamber. A group of 20 female mosquitoes were anaesthetized with CO$_2$ and introduced through a 2-cm hole drilled at the midpoint of the chamber. Mosquito distribution inside the static-air choice-test apparatus was observed over a total of 360-minutes. The experimental design was a completely randomized design using three replications of each treatment. Data generated by this study was used to examine two measures of mosquito repellency, **percentage (spatial) repellency** and **contact repellency**. Percentage repellency was calculated with the following formula to provide an indication of spatial repellency:

\[
\text{Percentage Repellency} = \left( \frac{\text{Number of Individuals in Untreated Half} - \text{Number of Individuals in Treated Half}}{20} \right) \times 100
\]

Contact repellency was defined in this assay as 100% avoidance of the treated filter paper (no contact) throughout the 360 minute observation period. Observations were made at the individual mosquito level. The resulting contact repellency was compared with control treatments, using Fisher’s Exact Test.
Collection of Volatiles Using Solid-Phase Microextraction

Relative concentrations of volatiles were sampled inside the static-air glass apparatus used in the repellency bioassays. Test solutions were applied to filter papers at a rate of 78.6 µg/cm² and then enclosed in the system. Catnip essential oil, elemol, and DEET were selected, based on the differences in mosquito repellency (contact vs. spatial activity) observed in the previous bioassay. Temperature and light were held constant throughout the study. Solid-phase microextraction (SPME) field samplers containing a 100 µm PDMS fiber (Supelco, St. Louis, Missouri) were conditioned in a GC inlet held at 250 °C for 30 minutes before sampling. Holes were drilled in the center of equally-spaced quadrants of the static-air chamber and covered with a small amount of parafilm, to allow placement of the four SPME fibers in each volatile sampling replicate (Figure 1). Prior to the start of the study, static-air chambers were sampled with SPME fibers and identified a minimal level of background contamination.

SPME fibers were exposed inside the treated chambers for one of two 15-minute time periods; collection of volatiles was conducted immediately following treatment (0-15 min.), or 15 minutes after treatment (15-30 min.). Differences in the time to reach equilibrium were not determined for each compound in this study. Volatile samples were replicated three times for each test solution and time period. Relative concentrations of volatile samples were measured by GC-FID. Quantitative standards were made up for DEET (Sigma Aldrich), as well as elemol (≥0%), Z,E-nepetalactone (≥90%), and E,Z-nepetalactone (≥90%), which were purified in the laboratory by column chromatography. Theoretical vapor pressures were calculated using ACD/Lab Boiling Point software, Version 8.0.
**House Fly Toxicity Test**

Toxicity bioassays were performed with adult house flies (*Musca domestica* L.), from an established laboratory colony in the Iowa State University, Pesticide Toxicology Laboratory, Ames, IA. Individuals were chilled on a cooled surface and dosed with one μl of test solution on the ventral abdominal surface. Test solutions consisted of five different concentrations of the active ingredient in an acetone solvent along with an acetone-only control, dispensed using a topical applicator (Model PB-600, Hamilton Co., Inc., Whittier, California). Each concentration was applied to a population of 10 house flies and then placed in a screen-covered glass mason jar containing a cotton wick soaked in a saturated sucrose solution. Mortality was recorded after 24-hours. All treatments were replicated three times.

**Tick Repellency Bioassay**

Tick responses to candidate repellent essential oils and compounds were evaluated in a climbing arena. Positive controls consisted of DEET and a 20% pyrethrum solution (Sigma Aldrich). Brown dog ticks (*Rhipicephalus sanguineus* Latreille) were purchased from EL Lab, Soquel, California. Four individuals were placed in a glass Petri dish arena (area of 10.2 cm²) surrounded by water, maintained at 23-24 °C. In the center of the arena, a braided cotton wick was suspended. Treatments were made up as solutions in acetone and applied evenly across a “barrier”, designed at 2.54 cm from the bottom of the arena. The solvent was allowed to evaporate off the cotton wick (1-2 minutes) prior to the start of the test period. Ticks were allotted 60 minutes to search the arena and begin climbing behavior. The total number of ticks that attempted to climb the cotton wick was recorded. Individuals that passed the treated barrier were removed from the arena and recorded. If a tick approached the
chemical barrier and either circled or turned around, the activity was noted and then the individual was allowed to continue movement in the arena until the 60 minutes had concluded. Five replications were completed for each treatment.

Results

Results for Amyris essential oil and for a mixture (1:1), containing a potent spatial repellent, catnip essential oil, are shown in Table 1. The difference between Amyris and catnip oils can be seen in the comparison of their percentage repellency values (measure of spatial repellency) and avoidance frequency (contact repellency). Amyris yielded a significant degree of spatial repellency compared to the control, but this percentage repellency value was lower than for the catnip oil. There was also a noticeable difference in avoidance frequency of Amyris and catnip. Amyris avoidance frequency accumulated over the 3-hour test period was 0.97, i.e. only one mosquito came in contact with the treated filter paper. The Amyris and catnip essential oil mixture resulted in significant levels of both spatial repellency and contact repellency.

Elemol makes up approximately 10% of the Amyris essential oil, along with a collection of other oxygenated sesquiterpenes (eudesmols, valerianol, etc.). Our laboratory has previously reported the mosquito repellent activity of elemol (Schultz et al. 2006). When tested for spatial and contact mosquito repellency, elemol showed similar characteristics to its parent essential oil; significant spatial repellency that, on average is lower than catnip essential oil, but with higher levels of contact repellency. The elemol/catnip essential oil mixture provided a combination of highly significant spatial and contact repellencies.
The differences observed in spatial and contact repellency are also highlighted by the relative concentrations of these volatilized compounds inside the repellency bioassay chamber (Table 2). Higher amounts of Z,E- and E,Z-nepetalactone isomers (ratio in this sample of catnip essential oil was 75:25 Z,E / E,Z-nepetalctone) distributed quickly inside the repellency chamber, which would be expected of a good spatial repellent. Elemol and DEET, both highly significant contact repellents did not distribute as far, or as quickly as the nepetalactone isomers inside the chamber. Out of the four compounds tested, the lowest level of volatiles collected were in the DEET applications.

Siam-wood essential oil was tested for efficacy in the short-term residual mosquito repellency bioassay. Results for these tests showed good residual spatial and contact repellency (Table 3).

Some Siam-wood toxicity effects were observed in the repellency screening trials and motivated a house fly LD₅₀ toxicity test with the two major components in its essential oil, fokienol and trans-nerolidol (Table 4).

Amyris (good contact repellent) and catnip (good spatial repellent) essential oils were selected for further testing against active components that are presently used in commercial topical mosquito products. Amyris and catnip essential oils, and p-menthane-3,8-diol were the only three actives to significantly differ in percentage repellency from the control in this study.

A small-scale ‘barrier’ test was used to study brown dog tick repellency. Amyris essential oil was evaluated against an untreated control, and two positive standards DEET and pyrethrum (20%).
The resulting tick climbing activity in the untreated control treatment was 65%. Amyris essential oil and DEET significantly repelled brown dog ticks. Out of 20 ticks that were exposed to Amyris essential oil, only one tick climbed past the Amyris essential oil barrier after repeatedly turning around and climbing down to the arena. No ticks crossed the DEET-treated barriers.

**Discussion**

Plant essential oils are a rich source of sesquiterpenes that can both affect insect behavior and cause mortality. In particular, this study focused on essential oils that contain a select number of closely related sesquiterpenes. Amyris and Siam-wood essential oils were both tested and identified as effective mosquito repellents in a laboratory bioassay. Amyris essential oil was also an effective barrier against brown dog ticks. The majority of these essential oil compositions include oxygenated derivatives of farnesane, eudesmane, eremophilane, and elemene sesquiterpenes. Some of these also are present as primary components of other essential oils (American beautyberry bush, Alaska yellow cedar, for example) that posses repellent properties. However, interpretation of the sesquiterpene functionality is often times confounded by differences of chirality. One such example is the study of gossypol (+) and (-) enantiomers, found in the cotton plant. These enantiomers have been shown to differ in toxicity to herbivores and pathogens (Stipanovic et al. 2005; Gonzalex-Garza et al. 1992).

The mosquito laboratory assay in this study allowed for differentiation between contact and spatial repellent activities. High percentage repellency values were observed from mosquitoes exposed to catnip essential oil. The majority of individuals preferred to stay
≥30 cm away from the treated surface, representing a significant level of spatial repellency when compared to the control. This observed behavior was not surprising considering the relative concentration of the \( Z,E:E,Z \)-nepetalactone isomers that distributed inside the static-air chamber. Spatial repellency of Amyris essential oil, although lower than catnip, was significantly different from the control treatment and comparable with actives contained in commercial mosquito repellents. Contact repellency, which was measured by cumulative observations of mosquito avoidance of the treated surfaces, was highly significant with Amyris oil. Throughout the 3-hour test period, only one individual came in contact with the treated surface. Similar results of high contact and minimal spatial repellency were seen when testing efficacy of elemol. Relative volatility of elemol, one of the primary components of the Amyris essential oil, was also sampled inside the static-air chamber and did not distribute throughout the chamber as quickly as the nepetalactone isomers. These results show that a chemical’s volatility can be an important factor for spatial repellency, affecting the concentration that reaches the insect (Johnson et al. 1967; Davis et al. 1976). Experimental data on the terpene gas diffusion coefficients would be useful in future studies to discern the role of volatility in this system. Interestingly, this significant spatial repellency did not always align with effective contact repellency. In the catnip trials there were several mosquitoes that came in contact with treated surfaces and there was no significant difference when compared to the control. These results are consistent with previous studies that have noted the minimal residual effects of catnip essential oil (Schultz et al. 2006). This end result is similar to residual effects often observed with many of the first-generation natural repellents. Fradin and Day (2002) evaluated the protection time of several commercially available repellent formulations, including citronella, peppermint oil,
cedar oil, lemongrass oil, and geranium oil. On average, these products provided from 1 to 60 min. of protection whereas DEET formulations scored in a range of 200 to 360 min.

Comparison of catnip and Amyris essential oil shows that volatility is not the only factor contributing to the repellent activity. Studies that explored the activity of vetiver essential oil found that the individual component’s volatility was inversely related to termite repellency (Zhu et al. 2001). Based on the characteristic differences in mosquito repellent activity, a mixture containing catnip essential oil (which provided good spatial activity) and the sesquiterpene-rich Amyris essential oil (good contact repellency) was tested. This mixture gave excellent mosquito repellency values via both contact and spatial modes of action. One of the major components in Amyris essential oil, elemol, was also made up in a mixture with catnip essential oil and found effective.

Amyris essential oil was selected for further testing against the brown dog tick. In a climbing arena, individuals that were exposed to an Amyris essential oil barrier would not cross it and frequently avoided contact. These findings were compared with results from a DEET-treated barrier, which successfully prevented ticks from climbing past the chemical barrier. A pyrethrum solution was also tested, but did not significantly prevent ticks from climbing past the barrier.

Siam-wood oil, which contains nerolidol and fokeinol, was also tested for efficacy and evaluated in a mixture with catnip essential oil. Results for these tests showed high levels of both spatial and contact mosquito repellency. Additionally, some mosquito mortality was observed at the concentrations tested inside the static-air chamber. The two major components of Siam-wood were identified as significantly toxic to house flies. To our
knowledge, this is the first documented report of insect repellency and toxicological investigation of Siam-wood essential oil.

These findings highlight the potential use of catnip, Amyris, and Siam-wood essential oils for arthropod management. Although the specific repellency mode action of these oils appears to differ in terms of contact and spatial activity, formulated combinations of these did show improvement in a controlled laboratory setting. It is possible that similar mixtures might increase protection efficacy of other natural products.

Acknowledgements

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References


   Essential oil from the leaves of *Cryptomeria japonica* acts as a silverfish (Lepisma saccharina) repellent and insecticide. J. Wood Sci. 52: 522-526.


Table 1. The 15-minute spatial repellency and 3-hour contact repellency of yellow fever mosquitoes (*Aedes aegypti*) exposed to 78.6 µg/cm² rate of Amyris and catnip essential oils and mixtures (1:1) in the static-air repellency chamber.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage Repellency (^a)</th>
<th>Std. Dev</th>
<th>Avoidance Frequency (^c)</th>
<th>Contact Rep. (^d) (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catnip Essential Oil</td>
<td>77.7*</td>
<td>14</td>
<td>0.19</td>
<td>0.218</td>
</tr>
<tr>
<td>Amyris Essential Oil</td>
<td>55.2*</td>
<td>23</td>
<td>0.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Catnip/Elemol Mixture</td>
<td>93.0*</td>
<td>11</td>
<td>0.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Catnip/Amyris Mixture</td>
<td>82.6*</td>
<td>20</td>
<td>0.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elemol</td>
<td>63.6*</td>
<td>53</td>
<td>0.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>6.8</td>
<td>17</td>
<td>0.19</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Percentage repellency was determined at 15 minutes.

\(^*\) Significantly different from control (\(\alpha = 0.05\)) in LS means comparison.

\(^c\) Avoidance frequency = average of mosquito contact repellency over 3-hour time period.

\(^d\) Contact repellency comparison of avoidance frequency between control and treatment.
Table 2. Volatile collections (in nmol) of Z,E and E,Z-nepetalactone from catnip essential oil, elemol, and DEET (78.6 µg/cm² application rate) in the static-air glass apparatus using solid-phase microextraction with a 100 µm PDMS fiber.

<table>
<thead>
<tr>
<th>Volatiles</th>
<th>Time</th>
<th>8 cm</th>
<th>23 cm</th>
<th>38 cm</th>
<th>53 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z,E-nepetalactone*</td>
<td>15 min</td>
<td>113</td>
<td>29</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>(V.P. = 1.75 mmHg)</td>
<td>30 min</td>
<td>116</td>
<td>24</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>E,Z-nepetalactone*</td>
<td>15 min</td>
<td>34</td>
<td>10</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>(V.P. = 1.75 mmHg)</td>
<td>30 min</td>
<td>36</td>
<td>9</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Elemol</td>
<td>15 min</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(V.P. = 0.24 mmHg)</td>
<td>30 min</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>DEET</td>
<td>15 min</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(V.P. = 0.58 mmHg)</td>
<td>30 min</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

V.P. = vapor pressure (100°C) calculated by ACD Boiling Point software, Version 8.0.

*Isomer measurements made from surfaces treated with catnip essential oil.
Table 3. Spatial and contact repellency of yellow fever mosquitoes (*Aedes aegypti*) exposed to 78.6 µg/cm² application rate of Siam-wood and catnip essential oils and mixtures in the static-air repellency chamber.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage Repellency over Time</th>
<th>Avoidance Frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Contact Rep.&lt;sup&gt;b&lt;/sup&gt; (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>2 hr</td>
<td>3 hr</td>
</tr>
<tr>
<td>Catnip Essential Oil</td>
<td>20.3</td>
<td>100%</td>
<td>------</td>
</tr>
<tr>
<td>Siam-wood Oil</td>
<td>82.2</td>
<td>92.9</td>
<td>96.3</td>
</tr>
<tr>
<td>Catnip/Siam-wood Mixture (1:1)</td>
<td>74.1</td>
<td>74.1</td>
<td>100%</td>
</tr>
<tr>
<td>Control</td>
<td>7.4</td>
<td>-14</td>
<td>-18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Avoidance frequency = average of mosquito contact repellency over 3-hour time period.

<sup>b</sup> Contact repellency = 100% of the individuals off treated surface.
Table 4. House fly 24-hour toxicity to *trans*-nerolidol and fokienol, two major components in Siam-wood essential oil.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LD$_{50}$</th>
<th>95% C. I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerolidol</td>
<td>0.17 µmol/fly</td>
<td>0.14 - 0.21</td>
</tr>
<tr>
<td>Fokienol</td>
<td>0.21 µmol/fly</td>
<td>0.12 - 0.34</td>
</tr>
</tbody>
</table>
Table 5. Spatial and contact repellency tests with yellow fever mosquitoes (*Aedes aegypti*) to surfaces treated with active ingredients (78.6 µg/cm² application rate) of commercially available botanical-based repellent candidates and our targeted essential oils in a static-air repellency chamber.

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Active Ingredient</th>
<th>15 min.</th>
<th>30 min.</th>
<th>1 hr.</th>
<th>Avoidance Frequency</th>
<th>Contact Rep. b (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFF® Botanicals (SC Johnson)</td>
<td>p-menthane-3,8-diol</td>
<td>40</td>
<td>80*</td>
<td>78*</td>
<td>1.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Skin So Soft Bug Guard (Avon)</td>
<td>2-undecanone</td>
<td>41</td>
<td>19</td>
<td>16</td>
<td>0.33</td>
<td>0.093</td>
</tr>
<tr>
<td>SCENT OFF TWIST-ONS (ScentOff Corp)</td>
<td>Citronella Oil</td>
<td>52*</td>
<td>44</td>
<td>13</td>
<td>0.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Technical Grade</td>
<td>Catnip Oil</td>
<td>74*</td>
<td>59*</td>
<td>54*</td>
<td>0.66</td>
<td>0.001</td>
</tr>
<tr>
<td>Technical Grade</td>
<td>Amyris Oil</td>
<td>27</td>
<td>40</td>
<td>62*</td>
<td>0.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Solvent Control</td>
<td>Control</td>
<td>-11</td>
<td>-7.4</td>
<td>3.7</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

*Significantly different from control (α = 0.05) in LS means comparison.

a Avoidance frequency = average of mosquito contact repellency over 1-hour time period.

b Contact repellency = 100% of the individuals off treated surface.
Table 6. Climbing activity of the brown dog tick (*Rhipicephalus sanguineus*) when exposed to barrier-treated surfaces.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application Rate</th>
<th>Percentage Climbing Past Barrier</th>
<th>Std. Dev.</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<td>0</td>
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<td>65</td>
<td>22.3</td>
</tr>
</tbody>
</table>

*Significantly different from control (α = 0.05) in LS means comparison.*
Figure 1. Schematic of volatile sampling in the static-air glass apparatus using solid-phase microextraction with a 100 µm PDMS fiber.
CHAPTER 3. QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP OF BOTANICAL SESQUITERPENOIDS: SPATIAL AND CONTACT REPELLENCY TO THE YELLOW FEVER MOSQUITO, AEDES AEGYPTI

An article for submission to the Journal of Agricultural and Food Chemistry

Gretchen Paluch, Justin Grodnitzky, Lyric Bartholomay, and Joel Coats

Abstract

The plant terpenoids encompass a diversity of structure and functional roles in nature, including protection against pest arthropods. Previous studies in our laboratory have identified naturally occurring sesquiterpenes contained in essential oils from two plants, Amyris (Amyris balsamifera) and Siam-wood (Fokienia hodginsii), that are significantly repellent to a spectrum of arthropod pests. In efforts to further examine the biological activity of this class of compounds we isolated and purified 12 of these plant-derived sesquiterpenes and assayed them for spatial and contact repellency against the yellow fever mosquito, Aedes aegypti. These data were used to develop a quantitative structure-activity relationship that identified key properties of the sesquiterpene molecule, including electronic and structural parameters that were used to predict optimal repellent activity. There were notable similarities in the models developed for spatial repellency over five time-points, and for contact repellency. Vapor pressure was an important component of all repellency models.
Initial levels of spatial repellency were also related to polarizability of the molecule and lowest unoccupied molecular orbital (LUMO) energy, while the equation for late spatial repellency was dependent on other electronic features, including Mulliken population and electrotopological state descriptors. The model identified for contact repellency was the best fit and most significant model in this analysis, and showed a relationship with vapor pressure, Mulliken population, and total energy.

**Introduction**

Since its development in 1953, the synthetic repellent, DEET (N,N-diethyl-m-methylbenzamide), has been a highly effective topical repellent against mosquitoes and other disease-carrying arthropods. (Fradin and Day 2002); however, complications with toxicity have been reported in some circumstances, especially amongst children and elderly people (Clem et al. 1993; Goodyer and Behrens 1998; Veltri et al. 1994). Significant amounts of this chemical can be absorbed through the skin (Qiu et al. 1998), and there is some evidence of neurotoxicity resulting from high-level exposure of DEET in combination with permethrin and pyridostigmine bromide (all of which were commonly used by soldiers during the Persian Gulf War) (Cherstniakvoa et al. 2006). Another limitation is the minimal efficacy against the primary vectors of human malaria parasites, *Anopheles* spp. (Frances et al. 2004). Further, with continued use of only a select number of insecticides and repellents, it is possible that arthropod populations will develop resistance mechanisms. The existence of a DEET-insensitive mutant has been recently documented in *Drosophila melanogaster* (Reeder et al. 2001). For all of these reasons, it is advantageous to invest efforts in the development of new technologies for management of arthropods affecting human and animal health.
The practice of using plant derivatives, or botanical-based insecticides and repellents in agriculture dates back at least two to five millennia in ancient China, Egypt, Greece, and India. Even in Europe and North America, the documented use of botanicals extends back more than 150 years, dramatically predating discoveries of the major classes of synthetic chemical insecticides. Recent studies have further proven the effectiveness of botanicals as alternatives for arthropod repellents (Schultz et al. 2004; Sukumar et al. 1991; Barnard 1999; Zhu et al. 2006). Although a limited number of botanical repellents are commercially available, there is intense interest in developing these compounds because of increasing regulation for, and negative public perception of, synthetic chemicals (Coats 1994; Isman 2006). Research and development of alternative repellent compounds for mosquito control would also serve a valuable role in the overall movement toward integrated management of arthropods that transmit diseases.

Early studies on the chemical, structural, and physical properties of mosquito repellents showed that measures of volatility (Davis 1985; Christophers 1947; Garson and Winnike 1968; Johnson et al. 1968), such as vapor pressure and boiling point, correlated with repellent responses. Other properties have been examined including partition coefficients, melting points, infrared absorption, viscosity, molecular weights, surface tension, polarizability, and Hammett’s substituent constants (Davis 1985). Considerations have been made regarding functional groups as well; Garson and Winnike (1968) noted that compounds containing amides, imides, phenols, alcohols hydroxyethers, glycols, and hydroxyesters were active, whereas the parent hydrocarbons were poor repellents (Bunker and Hirschfelder 1925; Roadhouse 1953). Christophers (1947) reported the repellent activity of alcohol groups, along with aldehydes and phenols, and hypothesized that the activity was related to the
positioning of the –OH groups. Another study using terpenes found that conversion of the alcohol group to the acetate, minimized repellency (Piper et al. 1951), while the corresponding ester was superior in activity. (Moore 1934).

A selection of plant essential oils that contain significant levels of sesquiterpenes, most notably eremophilane, eudesmane, and germacrane derivatives, have appeared in the literature as significant arthropod repellents (Dietrich et al. 2006; Paluch et al. 2009). Recent studies with a collection of sesquiterpenoids from the heartwood of the Alaska yellow cedar (Chamaecyparis nootkatensis D. Don) include testing of nootkatone and valencene-13-ol. Both of these compounds were equally repellent to I. scapularis as DEET (nootkatone, $RC_{50} 0.0458\%$ wt/vol solution; valencene-13-ol, $RC_{50} 0.0712\%$ wt/vol solution; DEET, $RC_{50} 0.0728\%$ wt/vol solution) (Dietrich et al. 2006). Examination of nootkatone derivatives showed that the ketone group was important for repellent activity to the Formosan subterranean termite (Coptotermes formosanus Shiraki). Modification to a 1,10-dihydro- and tetrahydronootkatone derivative, by reducing the 1,10 double bond, also improved repellency (Zhu et al. 2003). Other recently identified sesquiterpenes with repellent activity include callicarpenal and intermedeol, which were isolated from the American beautyberry bush (Callicarpa americana L.) and evaluated for activity against mosquitoes and ticks (Carroll et al. 2007; Cantrell et al. 2005). Also, research in our lab has reported on the mosquito repellent properties of elemol, a major component of the Osage orange essential oil (Maclura pomifera (Raf.) Schneid.) and two sesquiterpene-rich essential oils, Amyris (Amyris balsamifera L.) and Siam-wood (Fokienia hodginsii L.) (Schultz et al. 2006; Paluch et al. 2009). Further characterization of the bioactivity of these botanical sesquiterpenes by
examination of quantitative structure-activity relationships (QSAR) provides insight into the mechanism of action of repellents, as well as guide selection of most potent compounds.

In this study, we selected 12 sesquiterpenes that share structural similarities and represent a range of mosquito repellent activity. Individual compounds were tested in a standardized laboratory bioassay and measures of spatial and contact repellency were observed. These data were analyzed with molecular descriptors, which encompass physical-chemical properties discussed in the literature, as well as structural and electronic features relevant to ligand-receptor interactions.

**Materials and Methods**

**Chemicals**

Nootkatone (≥ 99%) (Sigma Aldrich), elemene (≥ 80%) (Augustus Essential Oils), farnesol (≥ 95%) (Sigma Aldrich), α-bisabolol (≥ 95%) (Sigma Aldrich) and trans-nerolidol (≥ 98%) (Fluka Chemie GmbH, Buchs, Germany) were purchased from commercial sources. Sufficient quantities of β-eudesmol, elemol, 10-epi-γ-eudesmol, valerianol, elemol, α-santalol, turmerone, and fokienol sesquiterpenes were isolated from either technical grade materials or essential oils, and then purified in the laboratory. A supply of technical grade, 55% purity elemol (Augustus Essential Oils, Ltd., Hampshire, England) was further purified to ≥ 95% via column chromatography with silica gel, 40-140 mesh (J.T. Baker, Phillipsburg, NJ), using a hexane/diethyl ether (9:1) mobile phase. A similar approach was used with hexane/acetone/diethyl ether (7:2:1) and hexane/diethyl ether (95:5) solvents to isolate and purify fokienol (≥ 85%) from Siam-wood essential oil, similarly with purified α-santalol (≥ 85%) from East Indian sandalwood (Santalum album L.) essential oil and turmerone (≥ 70%)
from turmeric (Curcuma longa Linn.) essential oil. β-Eudesmol, 10-epi-γ-eudesmol, and valerianol were isolated from Amyris essential oil (Sigma Aldrich, St. Louis, MO) with argentation column chromatography techniques. Multiple columns with 10% silver-nitrate impregnated silica gel, +230 mesh (Sigma-Aldrich, St. Louis, MO), were required to attain purity levels ≥ 80%. Purity of samples was assessed on a Hewlett Packard 5890 Series II gas chromatograph with a 30-m x 0.25-mm i.d. x 0.25 µm film thickness DB-WAX column (Alltech, Deerfield, IL) with flame ionization detection. The injector temperature was 250 °C, and the split valve was opened 1 min after injection. The oven initial temperature was set at 120 °C for 1 min, and then increased at 4 °C/min to 236 °C. Confirmation of compound identity was completed on a Hewlett Packard 5890 Series II gas chromatograph interfaced to a Hewlett Packard 5972 Mass Selective Detector (MSD). Mass spectra were recorded from 30 to 550 a.m.u. with electron impact ionization at 70 eV. The assignments of chemical identities to the chemical compounds detected were confirmed by comparison of the retention indices with reference spectra in a mass spectral library (Wiley 138K, John Wiley and Sons, Inc.) and comparison to literature sources (Van Beek et al. 1989; Weyerstahl et al. 1999). For select compounds, commercially available analytical standards were used for comparison (β-eudesmol, Sigma Aldrich, St. Louis, MO).

Hedycaryol was synthesized in the laboratory using elemol as a starting material, in 20% silver nitrate solution and ethyl acetate (Jones and Sutherland 1968). The reaction was run under ice for 48 hours, and the hedycaryol product was confirmed by TLC and GC-FID.
**Repellency Bioassay**

Bioassays were conducted in a static-air chamber (9 x 60-cm section of glass tubing) at a controlled temperature of 26 °C, over a period of 5 days. Yellow fever mosquitoes, *Aedes aegypti* (Liverpool strain), were used from an established laboratory colony. Newly emerged adults were maintained under standard incubator conditions (80% relative humidity and held at 27 °C) and fed a 10% (0.3 M) sucrose solution. Only female mosquitoes were used in testing and were at least 5 days old.

Test solutions were made up in acetone and applied to 9-cm diameter Whatman No.1 round filter papers (63.6 cm$^2$). A solvent-only (acetone) control was used for comparison to treatments. The acetone was allowed to evaporate off the filter paper for 5 minutes prior to testing. A 78.6 μg/cm$^2$ rate of exposure was used as it has been used in past studies to effectively measure and compare repellency effects of terpenoids (Schultz et al. 2006; Paluch et al. 2009). Treated filter papers were placed inside the lids of 9-cm glass petri dishes, and the dishes were placed over the ends of the glass chamber. A group of 20 female mosquitoes was anaesthetized with CO$_2$ and introduced into the chamber through a 2-cm hole drilled at its midpoint. Mosquito distribution inside the static-air choice-test apparatus was observed at five time points over a total of 180 minutes. The experimental design was a completely randomized design using five replications of each treatment. Data generated by this study were used to examine two measures of mosquito repellency, percentage (spatial) repellency and contact repellency. Percentage repellency was calculated with the following formula:

\[
\text{Percentage Repellency} = \frac{(\text{Number of Individuals in Untreated Half} - \text{Number of Individuals in Treated Half})}{20} \times 100
\]
Contact repellency was defined in this assay as the avoidance frequency of the treated filter paper (no contact=100% avoidance) throughout the 180-minute observation period and was compared with control treatments, using Fisher’s Exact Test.

**QSAR Calculations and Model Development**

Descriptors were selected to represent molecular properties and features relevant to receptor-ligand interactions, and physicochemical properties that could be correlated with repellent activity. Molecular connectivity, total valence connectivity, molar refractivity, molecular topological index, and Wiener index, were used to account for size and shape of the molecule. Both classical and quantum parameters were also examined, including log P (octanol-water partition coefficient), Henry’s constant, highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), dipole moment, Mulliken population, and polarizability. Descriptors were calculated in GAMESS, through an interface with ChemBio3DUltra 11.0 (CambridgeSoft Corporation, Cambridge, MA). Vapor pressures were calculated at 111 °C using ACD/Boiling Point 8.0 (Advanced Chemistry Development, Inc., Ontario, Canada) to distinguish small differences in select compounds. The energy and geometry of each molecule was optimized with a split valence basis set and a polarization function (6-31*d) calculation. Electrotopological state descriptors (E-state) were calculated in E-Calc (SciVision, Inc. Burlington, MA).

In order to achieve an accurate model for sesquiterpene repellency, descriptors were analyzed for evidence of intercorrelation, using Pearson and Spearman rank correlation procedures. Parameters that were highly correlated were noted, and only one was used in the final model. A stepwise regression procedure was used to identify key descriptors, prior to
final selection of the overall best model selected from a subset regression. Overall fitness of the model was based on AIC values and potential bias of descriptors was examined by ridge regression. The number of descriptors in the final model was also based on an overall improvement of $\Delta R^2 < 0.02-0.04$ (Katritzky et al. 2006). Validation of the best fit models was completed with the leave-one-out method (Gramatica 2007; Grodnitzky and Coats 2002):

$$Q^2_{\text{LOO}} = 1 - \frac{\text{PRESS}}{\text{SSTO}}$$

where

$$\text{PRESS} = \sum_y (Y_{\text{predicted}} - Y_{\text{actual}})^2$$

All multiple and linear regression procedures were performed on SAS 9.1. Best-fit models were completed for spatial and contact repellency values, independently. Spatial and contact repellency, along with sesquiterpene vapor pressures were analyzed following log transformation. Predictability of repellency models were also interpreted with an external validation test using a structurally similar sesquiterpene, turmerone.

**Results and Discussion**

A selection of 12 sesquiterpenes, including eudesmane, elemene, eremophilane, bisaboe, and germacrane compounds, including acyclic and bridged systems (Figure 1) were evaluated for repellent activity against *Ae. aegypti*. Overall, hedycaryol and 10-epi-$\gamma$-eudesmol showed the highest levels of repellent activity, and elemene and trans-nerolidol were the lowest. Repellency values for 10-epi-$\gamma$-eudesmol were the most consist of the compounds tested in this assay. In particular, these compounds show the range observed in
spatial and contact activity that was important for the construction of QSAR models for sesquiterpene insect repellency. The molecular descriptors considered in this analysis represent physical-chemical properties, especially those that have been indicated as important for repellent activity, and structural and electronic features relevant to ligand-receptor interactions. Electrotopological descriptors were also considered in model development. Models were developed for each of the spatial repellency time-periods.

Both spatial and contact mosquito repellency data (Table 1) were used to develop QSAR models capable of predicting the repellent activity of sesquiterpenes. These data show a range of spatial repellency values over multiple time-points, as well as contact repellency, and were analyzed separately to select parameters for the best-fit models and validation. The final models for each spatial repellency time-point and the contact repellency (shown in Table 2) shared several similarities in the final parameters that were selected. Log vapor pressure was identified as an important parameter in all of the models reported, and the Mulliken population of carbon 1 (Figure 1) was also important in the majority of the repellency models. Notable differences in final parameters selected for the spatial repellency models are seen in the comparison of the initial spatial repellency time (15 minutes) against the later time-points (60, 90, 120, and 180 minutes). Models for the early measure of spatial repellency (REP) contained vapor pressure (VP), polarizability (POL), and LUMO parameters (15 minute: \[ \text{LOG (REP)} = 0.94(\pm 0.09) - 1.01(\pm 0.16)[\text{LOG(VP)}] + 0.09(\pm 0.03)[\text{LUMO}] - 0.64(\pm 0.37) [\text{POL}] \]). Internal and external validation, as well as fitness of the model, provided good evidence of the spatial repellent predictability with the 15-minute model \((N = 12, F = 16.93, R^2 = 0.86, \hat{Q}^2_{\text{LOO}} = 0.61)\). Models for the later spatial repellency time-points, starting at 60 minutes, all contained the same selection of parameters
including log vapor pressure, Mulliken population of carbon 1, and E-state of carbon 7 (Figure 1) (equations listed in Table 2). Fitness and validation of these models provided good evidence of sesquiterpene spatial repellent predictability (60 minute, \( N = 12, F = 12.20, R^2 = 0.82, Q^2_{LOO} = 0.73 \); 90 minute, \( N = 12, F = 15.61, R^2 = 0.85, Q^2_{LOO} = 0.69 \); 120 minute, \( N = 12, F = 13.99, R^2 = 0.83, Q^2_{LOO} = 0.72 \); 180 minute, \( N = 12, F = 23.88, R^2 = 0.89, Q^2_{LOO} = 0.75 \)).

Comparison of the calculated vs. observed spatial repellency of turmerone, used for external validation, resulted in residuals ranging from 6.1-19.7, with the highest residual resulting from the 90-minute model. This was in line with the results from internal validation of the model (i.e. weakest late spatial repellency model, \( Q^2_{LOO} = 0.69 \)). In addition to the spatial repellency time-point models, data collected on mosquito continual avoidance of the sesquiterpene-treated surfaces were also analyzed and used to develop a best-fit model for contact repellency (AVOID). The final model contained vapor pressure, Mulliken population of carbon 1 (MULP-C1), and total energy (TENG) ([LOG(AVOID)] = -7.42( ± 0.79) – 0.15( ± 0.05)[LOG(VP)] + 0.12( ± 0.06) [MULP-C1] - 0.01( ± 0.001) [TENG]). Fitness and cross validation of this model was the most significant of all the repellency models reported \( (N = 12, F = 112.89, R^2 = 0.97, Q^2_{LOO} = 0.87) \), and the residual resulting from the difference in calculated vs. observed contact repellency of turmerone was 0.0 (Table 3).

The similarities observed in the parameters selected for the best-fit models of sesquiterpene repellency appear to fall into three categories: early spatial repellency, late spatial repellency, and contact repellency. The prediction models in each of these categories differ in terms of either one or two parameters, but all maintain a negative relationship between mosquito repellency and vapor pressure, which is representative of a compounds’
volatility. Volatility is widely recognized as an important factor for mosquito repellency as it can impact mosquito responses via chemical contact with the mosquito chemosensory structures. Our results show that repellency increased as vapor pressure of sesquiterpenes decreased, which suggests that minimal volatility within this class of compounds provided optimal repellency. Experimental data on sesquiterpene gas diffusion coefficients would be useful in future studies to discern the role of volatility in this system. The trend in vapor pressure is maintained even with consideration of any outliers in the dataset, including those compounds with a higher vapor pressure. We believe that this trend is most likely a product of our bioassay system and that interpretation of these results should also include consideration of the repellency assay design (size and static-air) used in this study. Size of the bioassay chamber places constraints on the space available for volatilization of candidate repellents. Compounds with lower volatility will be able to maintain a consistently higher concentration on the treated side of the chamber. However, this particular system limits the influence of a number of physical factors on repellency, as well as removes the potential for bias due to other attractants/chemicals involved in mosquito host-seeking. Thus, our bioassay offers more sensitivity to the inherent deterrent activity of sesquiterpenes, and allows us to examine electronic and structural properties that are important for receptor-ligand interaction. Other studies that utilize a larger air flow-through system or that incorporate host-generated attractants might show a different relationship, especially with regard to the relationship between spatial repellency and vapor pressure (volatility).

Another interesting outcome of the repellency models is the differentiation in electronic properties between spatial repellency at the early time-point, as compared to the late. The electronic parameters selected in the early time-point model (15-minute) included
polarizability and LUMO. In these models, the repellent activity increased as polarizability decreased and LUMO increased.

Two specific electronic parameters were identified as important for measures of repellency. One parameter that was present in the late spatial repellency and contact repellency models was Mulliken population at carbon 1. In the 60, 90, 120, 180-minute spatial repellency models and the contact repellency model, repellent activity increased as the Mulliken population (electron density) around carbon 1 (Figure 1) increased. The other parameter that appeared in multiple models, primarily the late spatial repellency time-point models (60, 90, 120, and 180-minute models), was the electrotopological state descriptor on carbon 7 (E-state, carbon 7) (Figure 1). These equations show that repellency increases as the electronic accessibility of carbon 7 decreases. In the collection of sesquiterpenes tested, there were different arrangements of the functional/substituent groups at carbon 7, including a hydroxyl group attached to a tertiary carbon that resulted in active repellency. The importance of these two areas on the sesquiterpene molecule share some similarity to other findings with East Indian sandalwood odor-active groups (Dimolglö et al. 1995), which also contained sesquiterpenes with an electron-donor group (hydroxyl) on the quaternary/tertiary carbon atom. This structural feature was essential for olfactory activity of sandalwood oil, along with the presence of a bulky fragment on the molecule located a distance from the hydroxyl group that serves an electron-acceptor function.

The contact repellency model (AVOID), which is a measure of continual mosquito avoidance of the treated surfaces over the 180-minute observation period, showed the highest level of significance in both internal and external validations (Tables 2 and 3). Parameters selected for this model shared similarities with spatial repellency including trends with vapor
pressure and Mulliken population of carbon 1. Compounds with lower vapor pressure maintain a higher concentration on the treated filter paper and therefore provide higher contact repellency (avoidance). Increased Mulliken population at carbon 1 again resulted in enhanced repellency. However this model was unique with respect to one parameter, total energy. Total energy has been interpreted in other QSAR studies to measure stability (Bello-Ramírez et al. 2003), as well as non-specific interactions (such as solute interactions or membrane flux) (Ośmialowski et al. 1986; Agatonovic-Kustrin et al. 2001). The significance of total energy might relate to the difference in stability of the sesquiterpene 10-carbon ring structure. Other studies have noted the germacrene Cope-rearrangement to form elemenes (Terada and Yamamura et al. 1982; Takeda 1974) and highlighted the importance of steric energies in the transition state (CC). Improved understanding of the contribution of sesquiterpene total energy, as it relates to mosquito repellency might include further examination of steric energy, rotational energy barriers, flexibility of active conformers (Mekenyan et al. 2003), as well as other structural and physicochemical parameters, which are relevant to the formation of stable ground states and possible transition state conformations of selected molecules.

Results from these models highlight the importance of electronic properties, especially on two specific areas of the sesquiterpene structure (carbons 1 and 7) that can affect *Ae. aegypti* repellent activity by possibly interacting with an odorant receptor or another component of the olfactory pathway. It also suggests that several different electronic properties of the molecule can help explain the relationship between structure and repellent activity.
Conclusions

The mode of action of mosquito repellency offers numerous challenges, as there are many mechanisms and processes involved in mosquito host-seeking and feeding behaviors. In this study we examined repellency under controlled conditions, specifically a static-air bioassay, to provide information about mosquito avoidance of and movement away from candidate sesquiterpenes. The information generated from these studies was used to develop a QSAR model for repellency. Multiple models of different measures of mosquito repellency (spatial and contact) highlighted the importance of vapor pressure (volatility), as well as electronic and electrotopological descriptors, in predicting repellent activity. Vapor pressure has long been recognized as essential for mosquito repellency, and our study shows that in our static-air chamber and with this class of terpenes, minimal vapor pressure is optimal, since that allows for a sustained gradient in the chamber. Analysis of the electronic and electrotopological properties of the sesquiterpene structure revealed two areas on the molecule that were important to repellent activity and possibly involved in receptor-ligand interactions. The predictive value of this information can also be utilized to assist in the search for effective natural repellents and the optimization of sesquiterpene structure for insect repellency within a controlled laboratory setting. The main conclusions from this study include: 1) in a controlled static-air laboratory bioassay, a minimal sesquiterpene vapor pressure is optimal for *Ae. aegypti* repellency, 2) the electronic and electrotopological properties of carbon 1 and carbon 7 affect activity, and 3) of the 12 plant-derived sesquiterpenes evaluated, hedycaryol and 10-epi-γ-eudesmol resulted in the highest spatial and contact repellency values.
Acknowledgements

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References


Table 1. Spatial and contact repellency of botanical sesquiterpenes to the yellow fever mosquito (*Aedes aegypti*) at a concentration of 78.6 µg/cm² (from application of 1 ml of a 0.05% solution).

<table>
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<th>Treatment</th>
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</table>

* Significantly different from control (α = 0.05) in Dunnett’s test.

<sup>a</sup> Avoidance frequency = average of mosquito contact repellency over 3-hour time period (based on 6 time-points).

<sup>b</sup> Contact repellency = 100% of the individuals off treated surface. Comparison with control treatments were made using Fisher’s Exact Test.

<sup>c</sup> Sesquiterpene used for comparison in model validation.
Table 2. QSAR models for spatial and contact repellency* of botanical sesquiterpenes to the yellow fever mosquito (*Aedes aegypti*).

<table>
<thead>
<tr>
<th>Response</th>
<th>Best Fit Model</th>
<th>( R^2 )</th>
<th>( Q_{LOO}^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{LOG(Spatial Repellency)} )</td>
<td>\begin{tabular}{c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>( \text{LOG(Contact Repellency)} )</td>
<td>\begin{tabular}{c</td>
<td>c</td>
<td>c</td>
</tr>
</tbody>
</table>

*Spatial and contact repellency values reported in Table 1.

\( Q_{LOO} \) Leave-One-Out; 1-PRESS/SSTO

Abbreviations: VP – vapor pressure, LUMO – lowest unoccupied molecular orbital, POL – polarizability, TENG – total energy
Table 3. Comparison of QSAR calculated and observed values of turmerone for spatial and contact repellency of botanical sesquiterpenes to the yellow fever mosquito (*Aedes aegypti*).

<table>
<thead>
<tr>
<th>Best-Fit Model</th>
<th>Calculated Percentage Repellency</th>
<th>Observed Percentage Repellency</th>
<th>Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spatial Repellency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 minute</td>
<td>34.0</td>
<td>43.4</td>
<td>9.4</td>
</tr>
<tr>
<td>60 minute</td>
<td>56.5</td>
<td>62.6</td>
<td>6.1</td>
</tr>
<tr>
<td>90 minute</td>
<td>54.5</td>
<td>74.2</td>
<td>19.7</td>
</tr>
<tr>
<td>120 minute</td>
<td>73.3</td>
<td>80.4</td>
<td>7.1</td>
</tr>
<tr>
<td>180 minutes</td>
<td>76.5</td>
<td>88.9</td>
<td>12.4</td>
</tr>
<tr>
<td><strong>Contact Repellency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoidance Frequency</td>
<td>0.8</td>
<td>0.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 1. Structures of sesquiterpenes and DEET (N,N-diethyl-m-methylbenzamide). A) Eudesmane, elemene, eremophilane, bisaboeone, and germacrane compounds, B) Acyclic; C) Bridged system; D) DEET.
Figure 2. Calculated vs. observed spatial and contact repellency values shown for three of the five sesquiterpene repellency models: A) spatial 15-minutes, B) spatial 180-minutes, C) contact repellency.
CHAPTER 4. INTERRUPTION OF HOST-SEEKING BEHAVIOR
OF THE YELLOW FEVER MOSquito, *Aedes aegypti*, WITH
CHEMICAL REPELLENTS

A paper to be submitted to the Journal of the American Mosquito Control Association

Gretchen Paluch, Lyric Bartholomay, and Joel Coats

Abstract

Candidate repellent terpenoids identified as effective spatial and contact repellents from previous studies in our laboratory were assayed for efficacy and residual activity against the yellow fever mosquito, *Aedes aegypti*, in a small-scale static-air system. Catnip essential oil (*Nepeta cataria* L.) and elemol, a naturally occurring sesquiterpene in Amyris essential oil (*Amyris balsamifera* L.), were identified as having differential activities in the small-scale assay and were selected for further study on impacts as short-range volatiles on mosquito flight orientation behaviors in a wind tunnel. These candidate repellents were compared against a commercial standard *N,N*-diethyl-*m*-toluamide (DEET), to assess potential effects of different structural classes of repellents on mosquito behavior. The overall repellent response was not significant to reduce flight behavior compared to the control (CO₂) and did not allow for pair-wise comparisons across treatments. A separate trial series investigated the influence of lactic acid and 1-octen-3-ol host attractant cues on mosquito flight behavior, which produced good levels of activation, orientation response, and
sustained flight. When DEET was added in combination with the lures, a significant repellency affect was observed relating to flight activation and contact with a warmed surface.

**Keywords:** *Aedes aegypti*, orientation, repellent, DEET, catnip, elemol

**Introduction**

Evidence of naturally occurring insect repellents, in particular the plant sesquiterpenes purified from heartwood, bark, and leaves, has recently appeared in the literature, including extracts of *Callitris glaucophylla* Thompson et Johnson (Watanabe et al. 2005), *Cryptomeria japonica* (L. f.) D. Don (Wang et al. 2006; Yatagai et al. 2002), the American beautyberry bush (*Callicarpa americana* L.) (Cantrell et al. 2005; Carroll et al. 2007), Alaska yellow cedar (*Chamaecypars nootkatensis* D. Don) (Dietrich et al 2006), *Goniothalamus uvariodes*, Osage orange (*Maclura pomifera*), Amyris (*Amyris balsamifera*), and Siam-wood (*Fokienia hodginsii*) (Schultz et al. 2006; Paluch et al. 2009). Additional study of these compounds, as well as the broader class of terpenes, is needed to improve on potency, spectrum of activity, as well as overall efficacy as repellents.

Selection of the repellents evaluated in this study was based on distinctions of ‘spatial’ repellents (catnip, *Nepeta cataria* L., essential oil; citronellal, both containing representative monoterpenes) and a ‘contact’ repellent (elemol, a naturally occurring sesquiterpene) that have been used in our laboratory in the past to screen for new candidate repellents (Schultz et al. 2006; Paluch et al 2009). Similar reports of the differential activity of topical repellents have been previously reported (Bernier et al. 2005; Grieco et al. 2005).
Results are presented on the activity of surfaces treated with spatial and contact terpenoid repellents of the yellow fever mosquito, *Aedes aegypti*, assayed in a small scale static-air system and wind tunnel.

Continued research is required to identify the effect of DEET or other repellents on distinct stages of mosquito host-seeking behavior, which can then support basic studies on the mechanism of repellency. The literature on mosquito host-seeking behavior broadly outlines the following sequence of events: appetitive search, activation, orientation, attraction, and host acceptance (Hocking 1971; Sutcliffe 1987; Klowden 1996). This study investigated the influence of specific host attractant cues in mosquito host-seeking stages by measuring the frequency of upwind flight (activation), orientation toward an attractant source, and landing on a warmed surface. The wind tunnel system was modified to introduce a repellent into the plume for comparison of the effects different structural classes of repellents impose on mosquito behavior.

Mosquitoes that are successful in host-seeking locate and approach a potential host, evaluate the quality of a food source, and begin feeding without alerting the host. This process carries considerable risk, pending detection by the host. Prior to the start of these events, the endocrine state and physiology of the mosquito must be optimal for blood-feeding. Several factors are known to adversely affect adult flight and feeding behaviors, as well as specific responses to long-range olfactory stimulants (Bowen 1991 and 1996). The theoretical basis for the mosquito response to volatile compounds from the host takes into consideration nutritional and somatic energy consumption as a basis for mosquito behavior (Roitberg and Friend 1992; Roitberg et al. 1994).
Appetitive search behavior is an undirected and random search, terminating when a host stimulus is detected. This initiates activation. Gillies’ work (1980) supports the role of one particular host cue, CO$_2$, in both the mosquito activation and orientation response. Exposure to CO$_2$ initiates the mosquito sustained flight behaviors governed by a programmed flight behavior known as optomotor anemotaxis (Colvin et al. 1989; Bowen 1991), following flight mechanisms similar to those of other insects in a pulsed chemical gradient. However, there are still many facets of mosquito flight behavior that warrant further study, including irregular mosquito flight patterns and variation in upwind flight response across species (Edman 1979; Omer 1979; Takken and Kline 1989). Other challenges are represented by the complexity of host volatiles emitted, which contain both inter- and intraspecific variation with daily fluctuations (Bowen 1996; Pappenberger et al. 1996; Meijerink et al. 1999; Bernier et al. 2001).

Integration with other host cues, alone or in combination with CO$_2$, produced synergistic effects relating to sustained flight and orientation response (Acree et al. 1968; Smith et al. 1970; Eiras and Jepson 1991). There is minimal quantitative data on how mosquito orientation behaviors differ between short-range to long-range flights. A variety of host surface volatiles and chemicals have been tested and identified as host attractants including lactic acid (Geier et al. 1996; Eiras and Jepson 1991; Gillies, 1980) and 1-octen-3-ol (Kline et al. 1990; Takken and Kline 1989).

Past studies of insect attraction to odorants have stressed the importance of chemical structure, conformation, and concentration in the environment for complete interpretation of the effect on host-seeking behaviors. The difference in laboratory and field plume structure can affect several of these factors and was discussed by Gillies (1980) in the context of
mosquito orientation as it relates to the role of CO$_2$. Some of these factors were addressed by Geier et al. 1999, through quantitative studies of *Ae. aegypti* activation and sustained upwind flight under different CO$_2$ and lactic acid concentrations in homogenous, filamentous, and turbulent odor plumes. Sustained upwind flight was more frequent with intermittent cues of CO$_2$ delivered by filamentous plumes, but this same trend was not seen with mosquito orientation to lactic acid concentrations. This result is not unexpected as there are likely differences in the temporal and spatial release of CO$_2$ and skin odors from potential hosts that account for changes in host-seeking behaviors. In comparison, skin odors continuously emitted from warm-blooded animals, following convection currents, provide a more homogenous plume structure compared with periodical release of CO$_2$ (Willemse and Takken 1994, Geier et al. 1999). Flight orientation behavior and the interaction of select host cues that mediate the short-attraction sequence are of particular interest and still require further study.

Application of mosquito host-seeking behavioral research is essential for improved understanding and evaluation of chemical repellents, including current synthetics as well as natural compounds. The mode of action of the most commonly used insect repellent developed in the 1950’s, DEET (*N,N*-diethyl-3-methylbenzamide), is still debated, but the majority of hypotheses involve interactions within the olfactory pathway. Work by Davis and Sokolove (1976) suggested that DEET affects mosquito sensitivity to lactic acid, as measured in the olfactory neurons in the antennae of *Ae. aegypti*. This finding was later supported by behavioral data from Dogan et al. 1999. More recent work with ORs (olfactory receptors) in a *Drosophila* system showed that DEET could block sensitivity to certain food odors by inhibiting the OR +OR83b (co-receptor) complex, in select olfactory neurons (Ditzen et al.
In *Anopheles gambiae*, electrophysiological data showed inhibition of the ON response to 1-octen-3-ol following applications of DEET (Ditzen et al. 2008). More recently, Syed and Leal (2008) identified a DEET-sensitive olfactory neuron in the antennae and maxillary palps of *Culex quinquefasciatus* and directly showed that mosquitoes are able to respond to the DEET molecule. Syed and Leal also reported a ‘fixative’ or ‘masking’ effect on the release of host cues (e.g. 6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranyl acetone) with topical applications of DEET that likely contributes to mosquito repellency.

**Materials and Methods**

**Insects**

Yellow fever mosquitoes, *Aedes aegypti* (Liverpool strain), were used from an established laboratory colony in the Iowa State University, Medical Entomology Laboratory, Ames, Iowa. Eggs were hatched in deoxygenated water, and larvae were fed Tetramin fish food (Melle, Germany). Pupae were sorted from the larvae and placed in paper cups with mesh lids until emergence. Newly emerged adults were maintained under standard incubator conditions (80% relative humidity and held at 27 °C) and fed a 10% (0.3 M) sucrose solution. Only female mosquitoes were used in testing and were at least 5 days old prior to the start of testing.

**Static-air Repellency Bioassay**

Laboratory bioassays (Paluch et al. 2009) were conducted in a static-air chamber (9 x 60-cm section of glass tubing) at a controlled temperature of 26 °C. Test solutions were
made up in acetone and applied to 9-cm diameter Whatman No.1 round filter papers (63.6 cm²). DEET (N,N-diethyl-3-methylbenzamide) (Sigma Aldrich, St. Louis, Missouri), Amyris essential oil (Amyris balsamifera) (Sigma Aldrich), citronellal (Sigma Aldrich), and elemol (technical grade) (Augustus Essential Oils, Ltd. Hampshire, England) were obtained commercially. Technical grade elemol was further purified to ≥ 95% via column chromatography with silica gel, 40-140 mesh size (J.T. Baker, Phillipsburg, NJ), using a hexane/diethyl ether (9:1) mobile phase (Paluch et al. in preparation). A solvent-only control (acetone) was included for comparison against treatments. Individually treated filter papers were then aged for 0, 30, 60, 180, or 360-minutes to allow for volatization off the filter paper. After the specified aging period, filter papers were placed on the inside of the 9-cm glass petri dish lids, and then placed over the ends of the glass tube. A 78.6 μg/cm² exposure concentration was used, as it has been used in past studies to effectively measure and compare spatial repellency effects of terpenoids (Schultz et al. 2006; Paluch et al. 2009). A group of 20 female mosquitoes was anaesthetized with CO₂ and introduced into the chamber through a 2-cm hole drilled at its midpoint. Mosquito distribution inside the static-air choice-test apparatus was observed after 15 minutes. The experimental design was a completely randomized design using three replications of each treatment. Data generated by this study were used to calculate percentage (spatial) repellency:

\[
\text{Percentage Repellency} = \left(\frac{\text{Number of Individuals in Untreated Half} - \text{Number of Individuals in Treated Half}}{20}\right) \times 100
\]

Analysis of variance was used to identify significant differences related to treatment and aging period and pair-wise comparisons made using LS means. Regression analysis was used to examine percentage repellency relationship to time and assess residual effects.
Wind Tunnel

Design and Integration with Chemical Cues.

The wind tunnel design was similar to that described by Miller and Roelofs (1978), with dimensions of 2.4 x 1 x 1 m, and maintained in a room at 3 lux red light intensity under 25 ± 2 °C with > 70% humidity. The wind speed was held constant at 40 cm/s and the light intensity inside the tunnel was 0.5 lux (mixture of red and white light). The floor of the tunnel was scattered with red dots (David 1982) to provide cues for visual feedback used by a mosquito in monitoring upwind progress (Marsh et al. 1978). CO₂ is a known activator for Ae. aegypti that induces upwind and sustained flight (Gillies 1980) and was used for this purpose in the wind tunnel design. A vacuum adaptor connected to a glass condenser was used to deliver a 4% concentration of CO₂ in clean moistened air, pumped at a rate of 1.3 L min⁻¹. The attractant and/or repellent source was placed on a platform 30 cm from the upwind end of the tunnel, such that the plume emanating from the CO₂ source was 23 cm above the tunnel floor. The CO₂ delivery system was also supplied with circulating water set at 38 °C to mimic the human body temperature and provide a warm surface for landing. This source was fixed by a metal stand that was placed 20 cm above the wind tunnel floor at the upwind (wind velocity at ~ 0.3 m/s) end of the tunnel (figure 1).

Lactic acid (≥ 90%) (Fluka, Gillingham, UK) and 1-octen-3-ol (98%) (Sigma Aldrich, St. Louis, Missouri) attractants were prepared in a 1% (wt/vol) stock solution in a hexane solvent. Ten microliters of solution were applied to a No. 1 Whatman filter paper (42.4 mm) and the hexane solvent was allowed to evaporate off prior to positioning in the attractant/repellent source in the wind tunnel. In cases where combinations of DEET (N,N-diethyl-3-methylbenzamide) (Sigma Aldrich, St. Louis, Missouri), elemol, or catnip essential
oil candidate repellents were tested, these were also made up in either 5% or 1% wt/vol stock solutions. The final application rates of these compounds were 35 and 7 µg/cm², respectively.

Catnip (Nepeta cataria L.) essential oil was produced from a steam distillation in the laboratory (Pavia et al. 1988). Confirmation of compound identity was completed on a Hewlett Packard 5890 Series II gas chromatograph interfaced to a Hewlett Packard 5972 Mass Selective Detector (MSD). Mass spectra were recorded from 30 to 550 a.m.u. with electron impact ionization at 70 eV. The assignments of chemical identities to the chemical compounds detected were confirmed by comparison of the retention indices with reference spectra in a mass spectral library (Wiley 138K, John Wiley and Sons, Inc.) and comparison with literature sources (Van Beek et al. 1989).

Treated filter papers containing combinations of attractant and/or repellents were immediately placed in a glass adapter positioned at the end of a glass condenser to deliver volatiles within the CO₂ plume. Adapters were removed upon completion of each mosquito flight observation and thoroughly cleaned. Care was taken to ensure minimal contamination of the wind tunnel with attractant or repellent volatiles, specifically cleaning the wind tunnel was thoroughly cleaned with ethanol (70%) after each observation.

**Testing Procedures.**

Experiments were executed using a randomized complete block design, with time as the block to identify changes in mosquito behavior associated with age and other potential changes in testing conditions within the chamber. Treatments were selected to compare mosquito attraction behaviors under different candidate repellents: DEET (contact repellent), elemol (contact repellent), and catnip essential oil (spatial repellent). Further work in the
wind tunnel assessed the effects of the repellent DEET on the short-range attraction to CO₂ and the host cues, lactic acid and 1-octen-3-ol. In both studies, effects were compared against a control containing only CO₂ for activation and sustained upwind flight. Observations for each treatment (attractant and/or repellent combination) were replicated eight times, i.e. eight individual mosquitoes were used per treatment. The duration of flight responses were recorded in a separate study and compared against mosquito flight behaviors previously reported (Dekker et al. 2005).

Individual females were released from a glass aspirator positioned at a height of 23 cm above the wind tunnel floor, 1.5 m downwind from the attractant source. Prior to release, females were maintained in a fume hood to avoid exposure to the volatile cues generated within the wind tunnel chamber. The time period allotted for each female to respond and initiate flight behavior was 5 minutes. If the individual did not respond within this time period, the observation was recorded as a ‘no-response’. Mosquito flight behaviors recorded included upwind flight (activation), orientation toward an attractant source (lock-on), contact with attractant source, and landing on a warm surface. The frequency of erratic and controlled flights was recorded as well as the time-lapse data for the control (CO₂–only) treatment.

Fitness of the actual time-lapse data and transformed data sets to potential distributions was assessed using the R statistical software (Version 2.6.1). Additional analysis included examination of the variability in flight response, as well as consideration of flight as a covariate. The overall effect of the repellent DEET on changes in mosquito flight behavior frequencies under different lure combinations was examined with pair-wise
comparisons of different treatment combinations were completed using Fisher Exact tests (PROC FREQ; SAS Institute)

**Results**

Previous work in our laboratory with the Northern house mosquito (*Culex pipiens*) has shown that surfaces treated with elemol or DEET exhibit higher levels of contact repellency compared to catnip essential oil (containing ≥ 80% nepetalactone monoterpenes), whereas catnip essential oil acts a potent spatial repellent (Schultz et al. 2006; Paluch et al. 2009). Results presented here with *Ae. aegypti* are consistent with these previous studies in that individuals responded to treated surfaces with high significant percentage repellency with the monoterpane citronellal (spatial repellent), and lower levels with DEET or elemol-treated surfaces (contact repellents). This high initial repellency to citronellal significantly decreased over the 180-minute test period. At the first time point, elemol and DEET had lower percentage repellency values than citronellal, but neither treatment showed a negative relationship between percentage repellency and time (citronellal, P=0.027; all other treatments, P>0.200), retaining significant activity throughout the different aging periods (Table 1). Catnip essential oil (spatial repellent), elemol (contact repellent), and DEET were further examined for impacts on mosquito flight orientation behavior using a wind tunnel system with the 4% CO₂ attractant source. Elemol resulted in lowest amount of individual flights with 63% of mosquitoes responding with flight activation, followed by catnip essential oil (88%) and DEET (100%) (Table 3). However at the concentration tested (10 µl of a 1% solution), the overall repellent response was not significant to reduce flight behavior beyond the CO₂ control and did allow for pair-wise comparisons across treatments.
The duration of flight responses are reported in Table 2. Examination of mosquito activation, upwind flight, and orientation (lock-on) fit well with a Weibull distribution, which has been previously described in the literature as fitting different insect (*Aedes aegypti*, *Glossinia* spp., and Coleoptera) activities including vertical response to attractants as well as resting behaviors (Crawley 1993; Dekker et al. 2005; Ogana 1998). The Weibull shape parameter ($\beta$) (activation response, $\beta=0.91$; orientation response, $\beta=0.71$), provides an indication of how activation varies with time, in this case showing that the probability decreases as time increases ($\beta \leq 1$), which is consistent with other studies (Dekker et al. 2005). These differed from the trend in upwind flight, where the probability increases with an increase in time ($\beta=1.37$).

In a separate trial series, mosquito flight responses to combinations of 4% CO$_2$ with lactic acid or 1-octen-3-ol resulted in high levels of activation and orientation response (Table 4). The lactic acid+CO$_2$ combination and the CO$_2$ treatments initiated activation responses in all of the individuals tested, followed by 88 and 75% of the individuals that were able to orient to the odor source. The combination of 1-octen-3-ol+CO$_2$ had a lower amount of individual flights such that 63% of mosquitoes responded with flight activation. Table 4 also highlights the comparison of each lure combination with and without the repellent DEET. Overall, the addition of DEET resulted in a significant reduction in mosquito activation response at an alpha level of 0.10 ($P=0.09$) and contact with warmed surface ($P=0.08$). Pair-wise comparisons of mosquito flight responses among individual lure and DEET treatments showed no statistically significant differences.
Discussion

The design of experiments presented here attempted to identify the effect of candidate terpenoid repellents and DEET on stages of mosquito flight behavior including upwind flight (activation), orientation toward an attractant source, and landing on a heated surface. A wind tunnel system was used to minimize the complexity of the environment with incorporation of select short-range volatile cues. It should also be noted that other sources of variation include the irregular flight patterns, upwind flight responses to volatiles that exist across mosquito species, are not addressed in this study.

Previous work in our laboratory with C. pipiens has identified significant spatial repellent activity with monoterpenes, compared to oxygenated sesquiterpenes that provide potent contact repellency (Schultz et al. 2006; Paluch et al. 2009). The residual activity of surfaces treated with spatial and contact terpenoid repellents of Ae. aegypti, evaluated in a static-air laboratory bioassay, showed similar trends with respect to percentage (spatial) repellency and residual efficacy. High initial percentage repellency to citronellal-treated surfaces significantly decreased as the aging period increased, whereas the responses to Amyris essential oil, elemol, and DEET surfaces that resulted in overall lower percentage repellencies compared to citronellal, did not lose activity over the 3-hour time period.

Comparison of spatial and contact repellents was continued in the wind tunnel system, along with the commercial standard DEET. The concentration used in the study (10 µl of a 1% solution), was chosen based on a range-finding study with catnip essential oil because of its higher level of volatility (results not shown). However, this level was not sufficient to identify an overall repellent response in flight behavior beyond the control and did allow for direct comparisons across treatments. It is possible that the activity of these
compounds, in particular the sesquiterpenes, are not largely dependent on the flight behavior but other components of the host-seeking process such as the landing and feeding response. Examination of other concentrations, additional flight characteristics including flight speed and track angle (Dekker et al. 2005), and landing behaviors would be useful in future studies.

A separate trial series assessed the impact of DEET on mosquito flight responses to lure combinations of 4% CO$_2$ with lactic acid or 1-octen-3-ol. The concentration of DEET used in this study was higher than in the previous study (10 µl of a 5% solution), and resulted in a significant repellent effect ($\alpha=0.10$) on mosquito activation response and contact with the warmed surface. This trend is also visible in Table 4 where the proportion of individuals responding to lure combinations with DEET is reduced. Additionally, early observations from this study were that the introduction of DEET into the wind tunnel system continually affected mosquito flight responses in the untreated controls, which resulted in a decline in flight activation. To avoid any interference across treatments, careful cleaning of the wind tunnel and execution of each volatile combination was required prior to collecting the data shown (methods described above). Based on these findings, it is concluded that DEET can negatively influence the $\textit{Ae. aegypti}$ activation response. Further, there is some evidence that DEET can also impact mosquito contact with a warmed surface located at the attractant source.

The lactic acid+CO$_2$ and 1-octen-3-ol+CO$_2$ lure combinations resulted in good levels of activation, orientation response, and sustained flight. Comparisons of the different lure combinations were statistically non-significant. These results do not provide support for DEET affecting mosquito sensitivity to lactic acid or 1-octen-3-ol, both findings that were previously reported (Dogan et al. 1999; Ditzen et al. 2008) and shown by
electrophysiological data. Trends seen in this study more closely align with the ‘fixative’ or ‘masking’ effect described by Syed and Leal (2008).

The data presented in this study represent a first attempt to utilize the wind tunnel design and method to evaluate efficacy and compare activity of candidate chemical repellents. Future studies would benefit from an increased number of replications (N=30) for evaluation at a different alpha level. Alternatively, a useful measure for comparison of the treatments is the duration of each host-seeking behavior. With the addition of time-lapse data, it would also be possible to compare changes in activation, sustained flight, orientation, contact, and landing, by examining the Weibull shape parameter. These measures could potentially provide additional information about how different chemical repellent structures influence mosquito host-seeking behavior.

At present, there exist a limited number of active compounds for use as insect repellents. Efforts toward the advancement of alternative classes, particularly natural chemistries including terpenes, can offer substantial benefits. Investigations of the insect flight behaviors influenced by current and new chemical repellents are an important component in the interpretation of their mode of action. Results from this study provide a basis for future studies on the effects of chemical repellents on mosquito flight behavior as well as a report on the impact of DEET on Ae. aegypti flight activation and contact with treated surfaces.
Acknowledgements

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References

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33. **Sutcliffe, J. F. 1987.** Distance orientation of biting flies to their hosts. Insect Sci. Applic. 8: 611-616.


Table 1. Repellency of 0, 60, 120, 180-minute aged treatments of elemol, DEET, citronellal, and Amyris essential oil (*Amyris balsamifera*) to at a concentration of 78.6 µg/cm² (from application of 1 ml of a 0.05% solution) to the yellow fever mosquito (*Aedes aegypti*) evaluated in a static-air bioassay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Percentage (Spatial) Repellency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Elemol</td>
<td>56*</td>
</tr>
<tr>
<td>Citronellal</td>
<td>79*</td>
</tr>
<tr>
<td>DEET</td>
<td>52*</td>
</tr>
<tr>
<td>Amyris essential oil</td>
<td>56*</td>
</tr>
<tr>
<td>Control</td>
<td>16.0</td>
</tr>
</tbody>
</table>

* Significantly different from control (α = 0.05) in LS means comparison.
Table 2. Duration of flight responses of the yellow fever mosquitoes (*Aedes aegypti*) to 4% CO₂ (N=11) in a wind tunnel.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Average Time (seconds)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to Flight (Activation)</td>
<td>88.1</td>
<td>32.6</td>
</tr>
<tr>
<td>Upwind Flight</td>
<td>34.7</td>
<td>7.5</td>
</tr>
<tr>
<td>Orientation (Lock-on)</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Total Flight Time</td>
<td>37.2</td>
<td>7.6</td>
</tr>
</tbody>
</table>
Table 3. Frequency of responses of the yellow fever mosquitoes (Aedes aegypti) to select spatial and contact repellent compounds at a rate of 7 µg/cm² (application of 10 µl of a 1% solution) in a wind tunnel.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upwind Flight (Activation)</th>
<th>Orientation (Lock On)</th>
<th>Contact</th>
<th>Land</th>
<th>No Response</th>
<th>Erratic Flight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catnip</td>
<td>0.88</td>
<td>0.50</td>
<td>0.38</td>
<td>0.00</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>DEET</td>
<td>1.00</td>
<td>0.50</td>
<td>0.50</td>
<td>0.13</td>
<td>0.00</td>
<td>0.25</td>
</tr>
<tr>
<td>Elemol</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
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<tr>
<td>Control*</td>
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<td>0.75</td>
<td>0.50</td>
<td>0.00</td>
<td>0.00</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Four percent CO₂ was used to initiate mosquito activation and sustained upwind flight.
Table 4. Frequency of responses of the yellow fever mosquitoes (*Aedes aegypti*) to different combinations of DEET and the attractants/lures: 1-octen-3-ol and lactic acid at a rate of 35 μg/cm² (application of 10 µl of a 5% solution) in wind tunnel.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upwind Flight (Activation)</th>
<th>Orientation (Lock On)</th>
<th>Contact</th>
<th>Land</th>
<th>No Response</th>
<th>Erratic Flight</th>
<th>Controlled Flight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-octen-3-ol</td>
<td>0.63</td>
<td>0.25</td>
<td>0.38</td>
<td>0.00</td>
<td>0.13</td>
<td>0.38</td>
<td>0.50</td>
</tr>
<tr>
<td>DEET+1-octen-3-ol</td>
<td>0.62</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.37</td>
<td>0.12</td>
<td>0.50</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>1.00</td>
<td>0.88</td>
<td>0.88</td>
<td>0.63</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>DEET+Lactic Acid</td>
<td>0.88</td>
<td>0.63</td>
<td>0.63</td>
<td>0.38</td>
<td>0.13</td>
<td>0.13</td>
<td>0.75</td>
</tr>
<tr>
<td>Control*</td>
<td>1.00</td>
<td>0.75</td>
<td>0.75</td>
<td>0.37</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>DEET</td>
<td>0.63</td>
<td>0.38</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.13</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Four percent CO₂ was used to initiate mosquito activation and sustained upwind flight.
Figure 1. Schematic of attractant source and mosquito release position inside the wind tunnel system.
CHAPTER 5. GENERAL CONCLUSIONS

The need for alternatives to traditional synthetic chemicals for arthropod control is driven by several factors including increased consumer awareness, toxicity concerns, minimal efficacy against select arthropod vectors of disease (*Anopheles* spp.), environmental impacts, and the potential for insect resistance to develop to certain chemicals. All of these factors combined have generated an interest in academia, government, and the private sector to look for new arthropod repellents, along with the competition that often accompanies new patentable technologies. Careful review of the early literature on chemical repellents shows that there are a variety of structural compounds that can influence arthropod behaviors under field conditions, with a particular emphasis on classes of amides, piperdines, and diols. Only a small number of these structural classes have been investigated for determination of optimal potency and overall efficacy. The work in this dissertation (Chapters 2 and 3) represents a broad effort toward the development of a new class of chemical repellent, the terpenes.

Over the centuries, many plant-based chemistries have been identified as having repellent or deterrent properties, including alkaloids, phenols, terpenes, quinones, nitriles, furans, and lactones (Barasa et al. 2002). Of these groups, terpenes make up a large portion of repellent-active plant essential oil composition. Many researchers are investigating plant extracts referenced in folklore, but the relatively short persistence of some of these botanicals has limited their use in commercialized products. The focus on sesquiterpenes as a candidate repellent group was largely based on their extended duration of activity observed in laboratory bioassays. Building from the work presented here on this class of compounds, it
may be possible to synthesize new compounds that improve on the activity of the parent, potentially increasing repellent potency, broadening the spectrum of activity, as well as increasing residual effects.

Current scientific and regulatory advances are limited by the difficulties in the interpretation of repellent compounds and application technologies tested under laboratory and field conditions, which continue to maintain a high degree of variability in results. Substantial benefit could be drawn from a more complete understanding of how certain chemicals, such as topical repellents, influence mosquito behaviors associated with host-seeking and feeding. This information is also of particular interest in the interpretation of effects on the insect olfactory mechanisms, such as the case with \(N,N\)-diethyl-m-toluamide’s (DEET) mode of action (Davis and Sokolove 1976; Ditzen et al. 2008; Syed and Leal 2008).

Chapter 4 addresses the effects from DEET and terpenoid (both mono and sesquiterpenes) repellents imposed on mosquito flight behavior. Minimal effects on flight behavior were seen with the two terpenes, however DEET reduced the proportion of yellow fever mosquito, \textit{Aedes aegypti}, flight activation and contact with a warmed surface. Interpretations of these data suggest that the activity of terpenoid repellents, in particular the sesquiterpenes, is not largely dependent on the flight behavior but other components of the host-seeking process, possibly the landing and feeding response.

In recent years, academia has significantly contributed to the advancement of insect science by providing verification of new product efficacy for registration and exploration of novel repellent chemistries. Further development of repellent technologies, such as the sesquiterpenes, will require involvement from the private sector and government agencies in order to complete product development, produce efficacy data for registration, develop
registration guidelines, conduct marketing campaigns, and continue validation of new product efficacy. In many cases these collaborative efforts are difficult to build given the time and resource requirements, as well as the substantial risk involved. Continued efforts toward building collaborative projects are necessary for the development of effective alternatives as the populations of arthropod vectors and incidence of disease continues to expand.

References


ACKNOWLEDGEMENTS

Thanks to my colleagues in the Iowa State University Pesticide Toxicology Laboratory and fellow entomology graduate students for all of their encouragement and support. I am thankful to have Dr. Joel R. Coats and Dr. Lyric Bartholomay as advisors and mentors. They have provided me with guidance, motivation, and support through the duration of this research project. Lastly, I would like to thank my family, and especially Tim, for all of their patience and understanding over the past few years.
APPENDIX A. PREDICTION OF SESQUITERPENE REPELLENCY: APPLICATION OF THE QSAR MODEL

Introduction

Preliminary work on the repellent activity of the Amyris and Siam-wood essential oils has guided the development of a sesquiterpene quantitative structure-activity relationship (QSAR) model for predicting mosquito repellency. The final models, presented in Chapter 3, showed good similarity in the trends of selected descriptors, providing support for the importance of vapor pressure, total energy, and two electronic parameters (Mulliken population and E-state) in influencing the activity of sesquiterpenes. Results presented here are predictions of sesquiterpene activity using the described QSAR models.

Natural Sesquiterpenes

<table>
<thead>
<tr>
<th>Best-Fit Model</th>
<th>Calculated Percentage Repellency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spatial Repellency</td>
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</tr>
<tr>
<td>15 minute</td>
<td>2.1</td>
</tr>
<tr>
<td>60 minute</td>
<td>48.6</td>
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<td>44.3</td>
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<tr>
<td>120 minute</td>
<td>58.5</td>
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<tr>
<td>180 minutes</td>
<td>62.1</td>
</tr>
<tr>
<td>Contact Repellency</td>
<td></td>
</tr>
<tr>
<td>Avoidance Frequency</td>
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</table>

(Cantrell et al. 2005; Carrol et al. 2007)
<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS No.</th>
<th>Calculated Percentage Repellency</th>
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<td>intermedeol</td>
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<td>86.3</td>
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<tr>
<td></td>
<td></td>
<td>95.4</td>
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(Ahmad and Jantan 2003)

| Contact Repellency | Avoidance Frequency | 1.0 |

<p>| | | |</p>
<table>
<thead>
<tr>
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</tbody>
</table>

(Cantrell et al. 2005; Carrol et al. 2007)
<table>
<thead>
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<th>Best-Fit Model</th>
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</tr>
</thead>
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<td><strong>Spatial Repellency</strong></td>
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</tr>
<tr>
<td>15 minute</td>
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</tr>
<tr>
<td>60 minute</td>
<td>68.3</td>
</tr>
<tr>
<td>90 minute</td>
<td>85.8</td>
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<tr>
<td>120 minute</td>
<td>86.1</td>
</tr>
<tr>
<td>180 minutes</td>
<td>≥100</td>
</tr>
<tr>
<td><strong>Contact Repellency</strong></td>
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<tr>
<td>Avoidance Frequency</td>
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</table>

<table>
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<tr>
<td>15 minute</td>
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</tr>
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<td>60 minute</td>
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<td>90 minute</td>
<td>83.7</td>
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<tr>
<td>180 minutes</td>
<td>95.0</td>
</tr>
<tr>
<td><strong>Contact Repellency</strong></td>
<td></td>
</tr>
<tr>
<td>Avoidance Frequency</td>
<td>≥1.0</td>
</tr>
</tbody>
</table>

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(Weverstahl et al. 1999)

(Mata et al. 1987)

(Itokawa et al. 1986)
References


APPENDIX B. ODORANT RECEPTORS IN *Aedes aegypti* AS TARGETS FOR CHEMICAL REPELLENTS

Abstract

Basic studies on the biochemical processes that mediate mosquito behavioral responses to volatile compounds are valuable in numerous applications, including the arena of repellents where there is potential for influencing the olfactory stimuli or processing to negatively impact host-seeking or feeding activities of haematophagous insects. The focus of this project is the isolation of terpene olfactory receptors from the yellow fever mosquito, *Aedes aegypti*. Two olfactory receptor gene sequences (OR43b and OR83b) of interest were identified using VectorBase and examined for expression in adult female mosquito tissues. Efforts were also directed toward investigation of OR target gene silencing using double stranded RNA (dsRNA). Multiple primers were designed with a T7 RNA polymerase promoter for synthesis of a dsRNA with an approximate sequence length of 700 nucleotides to target *Aedes* OR83b. The end goal of this work is to assay the functionality of these olfactory receptors through knockdown expression of terpene olfactory receptors following injections of dsRNA, and evaluate behavior in a choice-test system.

Introduction

The majority of knowledge on insect olfaction is derived from studies with attractants. Molecules enter insect sensory structures (i.e. antennae and maxillary palpi) and are bound, then transported through the aqueous sensilla environment to olfactory receptor neurons (ORNs) by odorant-binding proteins. Odorants then interact with olfactory receptors
(ORs) located on the dendrites of specific ORNs, and cause conformational changes that activate downstream messengers (cyclicAMP and IP3+ DAG) capable of opening Na+ or Ca++ channels for ion influx and depolarization of the neuron. ONs converge on the antennal lobe for sensory processing. Information conveyed by ONs can include chemical identity, concentration, and chemical dynamics (Zwiebel and Takken 2004; Hallem et al. 2006).

Insect ORs are classified in a putative family of G-protein-coupled receptors (seven transmembrane domains). Studies of sequence similarities between insect OR genes show that these are highly divergent within and across species, excluding a group of single gene orthologs that have been identified in multiple species (Drosophila melanogaster, Heliothis virescens, Anopheles gambiae, and Ae. aegypti) (Melo et al. 2004). This sequence is expressed in the majority of olfactory neurons and is thought to act in the localization of other proteins and might also assist in odor binding and signal transduction (dimerization) (Larsson et al. 2004).

In recent years, there is an increased emphasis on studies of mosquito olfaction, including the identification of 79 ORs in An. gambiae. In vivo characterization studies on specific Anopheles OR functionality show that some receptors respond to specific components of human sweat (4-methylphenol and 2-methylphenol) (Hallem et al. 2004). There is currently a large effort to analyze Anopheles receptors that respond to odors emitted by humans and identify approaches to alter olfactory-related behaviors. The significance of this work is apparent as Anopheles is the primary vector of malaria parasites that cause more than one million deaths each year (86% of these deaths occur in sub-Saharan Africa), according to the World Health Organization.
The genome of another important disease vector, *Ae. aegypti*, was sequenced and assembled by the Institute of Genomic Research (TIGR) and the Broad Institute, and is currently an accessible web-based resource (Lawson et al. 2006). Each year, there are millions of cases of dengue fever and thousands of dengue hemorrhagic fever reported. Research on *Aedes* olfactory processing, including the identification of additional olfactory receptors, would not only improve our understanding of olfaction and interspecific variation, but potentially contribute to studies that alter mosquito olfaction.

The neural mechanisms that mediate mosquito behavioral responses (attraction and repellency) remain poorly understood and basic studies are needed. Recent work in model systems has started to connect specific olfactory receptors with repellency effects. More specifically, one study showed that DEET was able to block *Drosophila* sensitivity to certain food odors by inhibiting the DOR +DOR83b (co-receptor) complex, in select neurons located in the antennae (Ditzen et al. 2008). The same study also showed inhibition of ON response to 1-octen-3-ol, a component of human breath in *An. gambiae*, following applications of DEET. These data suggest that DEET acts on the mosquito gustatory receptors GPOR8+GPOR7 (co-receptor) complex.

The work presented here serves as a starting point for studies on terpene olfactory receptors in *Ae. aegypti*, and includes the AaOR7 (in this summary, presented as odorant receptor 83b, putative, VectorBase) (Melo et al. 2004), an ortholog to *An. gambiae* (AgOR7) (Hill et al. 2002); *D. melanogaster* (DOR83b) (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999); *H. virescens* (HvirR2) (Krieger et al. 2003), and *Apis mellifera* (AmelR2) (Krieger et al. 2003). Anticipated future studies will include further exploration of
how these receptors affect mosquito responses and behaviors to a variety of terpenoid compounds.

Summary of Current Research

Through collaborative efforts with Dr. Kimber’s Laboratory, two putative *Ae. aegypti* olfactory receptors (OR43b and OR83b) were selected from VectorBase that closely align with *D. melanogaster* olfactory receptors (DOR43a, DOR19a, DOR92a, DOR42b). These olfactory receptors were of interest as they have previously been shown to respond to terpene molecules in the literature (Fishilevich and Vosshall 2005). Transcript production of OR43b and OR83b sequences was investigated with *Ae. aegypti* (Liverpool strain) ORs from different RNA tissue samples. Whole body (N ≥ 10), body-only (N ≥ 10), and head-only (N ≥ 100), tissues were homogenized in Trizol (Invitrogen, Carlsbad, California), and total RNA was extracted via phenol/chloroform partition. Tissue-specific RNA extracts were used to synthesize cDNA from 2 µg of RNA, M-MLV reverse transcriptase (Promega, Madison, Wisconsin), Oligo(dT)15 primer (Promega, Madison, Wisconsin), and Recombinant RNasin ribonuclease inhibitor (Promega, Madison, Wisconsin). Final PCR products were examined on a 1% agarose gel containing ethidium bromide. Results showed a different pattern in expression of the two olfactory receptors in that OR43b was expressed in the body, but not the head tissues, whereas the OR83b was seen in both the body and head tissues (see Figure 1).

Multiple primer sets were designed from the two *Aedes* olfactory receptor sequences to produce OR43b and OR83b PCR templates containing a T7 RNA polymerase promoter sequence using RT-PCR, as specified by the MEGAscript RNAi Kit (Ambion, Austin,
The best performing primer was T7-OR83b-4F, T7-5’CCAAACTCGGCTGCCCTGTT-3’ and T7-OR83b-4R, T7-5’-AGTAGGTGACGACCGCTCCCAGAAC-3’, which produced a T7-template (approx. 700 bp in length) (Figure 2) under the following conditions: 1.5 mM Mg++, 10µm of each primer, 5 U/µl Taq DNA polymerase, annealing temperature of 59°C for 40 cycles. Attempts were made to improve the performance of other primers by adjusting the Mg++ concentration and adjustment of the thermocycler program (increased annealing temperature and varying the number of cycles), but proved unsuccessful. Additional primers were successful at producing a T7 template, but were unsuccessful in dsRNA synthesis with the MEGAscript kit. The resulting T7-OR83b-4 template yielded a dsRNA sequence 661 bp in length (Figure 3) from the MEGAscript Kit. A six hour incubation period yielded good quantities of dsRNA product. Final dsRNA products, following nuclease digestion and purification procedures as specified by the manufacture, were examined on 1% agarose gel containing ethidium bromide using a 6X non-denaturing gel loading buffer (Ambion, Austin, Texas).

The next component of this study is to complete injections using ds-OR83b-4 in Ae. aegypti adults. This will allow examination of knockdown expression of the odorant receptor 83b at different concentrations (3 levels, ranging from 100 ng to 2000 ng per injection) and time periods after injection (24 and 72 hour). Initial injections with Aedes saline have shown good levels of adult survival ranging from 84-92%. An anticipated concentration range of dsRNA for these injection studies with the OR83b target is based upon levels used and the duration of effects reported by Boisson et al. (2006) in An. gambiae. In their study, silencing in Anopheles midgut cells required approx. 140 ng of dsRNA, whereas much larger amounts (1600 ng) were required for a target gene in the salivary
glands. Silencing effects were shown for 4 and 7 days following injections, and reported to last at least 13 days. Once data become available on knockdown expression of the 83b odorant receptor, mosquitoes will be assayed for behavioral effects related to terpeneolfaction using a standardized choice-test system that has been developed in our laboratory (Schultz et al. 2006; Paluch et al. 2009).

**Acknowledgements**

Funding for this work was provided by the 2008 Henry and Sylvia Richardson Research Incentive grant.

**References**


Figure 1. RT-PCR analysis of *Aedes aegypti* OR43b (Vectorbase predicted size is 1191 bp) and OR83b (Vectorbase predicted size is 1437 bp) (AaOR7) putative olfactory receptor genes (VectorBase), in whole body, headless, and head-only preparations.
**Figure 2.** Production of a 700 bp dsRNA containing T7-RNA polymerase promoter sequence with OR 83b-4 primer set for silencing of *Aedes aegypti* OR 83b putative (AaOR07) (Vectorbase predicted size is 1437 bp). Positive and negative controls shown (+/-).
**Figure 3.** Undigested and unpurified dsRNA from different incubation times using a MEGAscript Kit from Ambion (Austin, TX) with *Aedes aegypti* OR83b putative (AaOR07) T7 primers for target dsRNA synthesis. Results from two different primer sets are shown, T7-Or83b-1 and T7-OR83b-4.