COMPARISON OF A SILICONE MEMBRANE BLOOD-FEEDING SYSTEM TO HUMAN SKIN IN REPELLENCY BIOASSAYS WITH *AEDES AEGYPTI* MOSQUITOES

By

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To my husband and my family for their unending love and support

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COMPARISON OF A SILICONE MEMBRANE BLOOD-FEEDING SYSTEM TO HUMAN SKIN IN REPELLENCY BIOASSAYS WITH *AEDES AEGYPTI* MOSQUITOES

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It is expected that laboratory-based repellent bioassays should reliably evaluate the efficacy of compounds that deter mosquito feeding behavior. The variety of repellent bioassays available allows for flexibility in design, but makes it difficult to compare any two methods, including *in vitro* and *in vivo* comparisons. The most reliable data come from skin assays; however, this exposes volunteers to chemicals and mosquito bites. In this study, four repellent active ingredients were used: DEET, IR3535, picaridin and *para*-menthane-3,8-diol. Results from bioassays with a module-based method were operated *in vitro* with a membrane and *in vivo* on the skin of the leg and were then compared to a standard *in vitro* method where repellent-treated cloth is placed over an arm that is inserted into a cage of mosquitoes. Pooled data from six volunteers were used to create dose response curves and estimate effective doses for four repellents at the 50, 95, and 99% levels (ED_{50}, ED_{95}, ED_{99}) using dose response curves with a probit model from the module tests. The ED_{99} was estimated with repellent-treated cloth as the concentration that prevented 99% of the mosquitoes from feeding.

The baseline attraction did not differ statistically for the membrane and skin module tests. The membrane and skin module results also did not differ significantly as repellent treatments below 31 nmol/cm^2 dose level. However, since the chemical dose at which the skin and

membrane curves diverge is lower than standard thresholds used in screening these chemicals, the use of the membrane module system will require further alterations before it can substitute current screening methods using human volunteers. Interestingly, all four repellents tested on the membrane required much higher doses of repellent chemical to reach full protection. Based on the results of this study, cage-based tests appear to be a more reliable estimate of repellent activity on skin compared to module-based tests on membrane. However, with the knowledge of the effective dose ratios, module-based tests can be utilized for testing with infected vectors with previously evaluated repellents.

CHAPTER 1
INTRODUCTION

Mosquitoes and their Medical Relevance

There are over 3,500 described species of mosquitoes (Diptera: Culicidae), of which 156 species are of significant medical importance (Harbach 2012, Gaffigan et al. 2012, WRBU 2012). While both male and female mosquitoes feed on plant sugars, only adult females rely on haematophagy as a source of protein for the production of eggs. As humans have evolved with mosquitoes, our experience with these organisms has been less than pleasant, especially since humans are the preferred host for anthropophilic species, and serve as suitable alternatives for other opportunistic species. The severity of this experience ranges from a nuisance during the biting process and the allergic response that itches and irritates the skin, to the transmission of pathogens which cause debilitating disease and death.

Over the last few decades, global climate change, urbanization and increased human travel have contributed to the growing threat and spread of vector borne diseases (Weaver and Reisen 2010, Gubler et al. 2001). Countries that had previously eradicated or controlled mosquito-borne illnesses, such as yellow fever, dengue, and malaria are experiencing a marked resurgence in cases. In Brazil, where a malaria eradication program had previously controlled the local vector population, the incidence of malaria increased 76% in the Amazon region, primarily due to migratory workers and deforestation related to urbanization efforts (Gratz 1999, Sawyer 1986). Additionally, arthropod-borne viruses or arboviruses have emerged and spread into areas where they formerly could not have been sustained. Increased temperature and rainfall in areas with a previously temperate climate have provided new breeding grounds for tropical species. The vector for dengue and yellow fever, the *Aedes aegypti* (Linnaeus) mosquito originally from Northern Africa, has established itself in much of the western hemisphere including the United

States. However, unlike many Central and South American countries, dengue fever is not endemic to the U.S. (Gratz 1999). Some dengue transmission had previously been reported along the Texas-Mexico border since 1980, and more recently several locally-acquired cases of dengue in Key West, FL were confirmed in 2009-2010 (CDC 1999, CDC 2010). Knowledge of these cases and of juxtaposed *Ae. aegypti* mosquito populations highlight the need to explore preventative measures in anticipation of dengue outbreaks. The most recent major arbovirus outbreak of note was the West Nile virus epidemic, in which most cases in the United States occurred from 1999 to 2002 (Huhn et al. 2003). This event was particularly significant for developmental repellent research because it promoted the use of insect repellents to the public for the prevention of mosquito-borne diseases.

A Brief History of Repellent Research

Laboratory-based mosquito repellent testing methods can be classified broadly into those that involve application of compounds to skin (*in vivo*) and those that involve some surrogate, such as application of the compound to a membrane or cloth (*in vitro*). The *in vivo* methods simulate closely the actual use of insect repellents because the repellent is applied directly on the skin surface. *In vitro* methods for mosquito repellents refer to the testing in an indirect fashion, such as testing a candidate repellent soaked into a piece of cloth, followed by placement of the treated cloth over the skin surface. A common assumption in this area of research is that *in vitro* testing methods provide a fairly accurate means to screen, predict and extrapolate *in vivo* testing results and that these assays can be conducted in a convenient environment that exposes research staff and study volunteers to less overall risk.

While the use of plant-derived compounds to reduce the nuisance from insects has been documented since the time of the Egyptians by Herodotus and found across many native cultures, the extensive research and development effort to discover modern insect repellents

began during World War II with a goal to reduce the impact of disease to U.S. troops fighting overseas (Moore and Debboun 2007). An overwhelming breakthrough for the field of repellent research came in 1952 when *N*,*N*-diethyl-3-methylbenzamide (DEET) was invented by USDA in Beltsville, MD and tested by the USDA laboratory in Orlando, FL (Bernier and Tsikolia 2011). Since its discovery, DEET has been considered the gold standard for repellent testing against mosquitoes and many other arthropod species due to its effectiveness at low doses, broad-spectrum repellency and low toxicity to humans. Even DEET, however, is not an ideal repellent. DEET is not recommended for use on children or pregnant women, has an oily texture when applied on the skin, and is known to have plasticizing properties (Moore and Debboun 2007). Since the discovery of DEET, there are a number of effective repellents have been developed from two main areas, plant-derived extracts and synthetic compounds.

CHAPTER 2
A REVIEW OF MOSQUITO HOST LOCATION

Chemical Cues and Olfaction

Semiochemicals and their Role in Olfaction

Semiochemicals are chemicals that serve as messengers emitted by an organism that can modify the behavior emitting or receiving the chemical. Semiochemicals can be subdivided into three major classes, kairomones, allomones, and synomones. A kairomone is a chemical message that is released by one organism but which benefits the organism that receives the chemical message often to the detriment of the producer. Kairomones are contained within the skin emanations of humans and other animals. These kairomones have the undesired effect of providing the insect pest with olfactory cues to find its host.

Conversely, an allomone is a chemical message that benefits the organism producing it rather than the organism that receives the chemical message. Allomonal compounds frequently associated with mosquitoes are those that deter a mosquito from finding a host. A synomone is a chemical message that benefits both the producer and the receiver, such as a pheromone used to attract a sex of one species to the other sex. Of these, kairomones and allomones are the most significant in odor mediated host-vector relationships.

Carbon Dioxide as an Attractant

Several chemicals have been historically implicated as kairomones for the benefit of mosquito attraction to human hosts. Some of these chemicals include carbon dioxide, lactic acid, acetone, ammonia, 1-octen-3-ol, among others. The first of these, carbon dioxide is the oldest known attractant and is one highly prevalent both in the literature and in commercially available mosquito trapping equipment. Carbon dioxide has been debated as to whether it acts only as an activator to flight towards a potential host or whether it also has a synergistic response in

combination with host odors (Dekker et al. 2005, Roachell 1997, Gillies 1980). Some believe that carbon dioxide exhaled by a vertebrate is utilized by mosquitoes as well as other haematophagous arthropods as a cue for foraging behavior, helping them orient towards a host organism (Lehane 2005).

Khan et al. examined the role of carbon dioxide and its role at various concentrations in combination with heat at skin temperatures of 34˚C and moisture (1971). Addition of carbon dioxide did not increase blood-feeding behavior via probing, but was found to illicit an increase in flight activity towards the host although an increase in the amount of carbon dioxide did not increase flight activity further. Attraction to a human hand was found to be much preferred than a combination of heat, moisture and carbon dioxide, and the study was pessimistic that this sort of combination would not be sufficient to redirect mosquitoes from a host, stating that carbon dioxide was believed to act primarily as a flight stimulant in mosquitoes (Khan et al. 1967).

Skin Emanations and their Metabolites as Attractants

Hundreds of semiochemicals have been identified from compounds dissolved in sweat collected on human skin. Some of these are from sweat glands, from the breakdown of the foods we eat, and from bacteria that live on our skin. There were more than 270 chemicals identified on human skin (Bernier et al. 2000, 2002). Many volatile compounds found to be attractive to mosquitoes may occur as result of perspiration, which are produced by different types of sweat glands. Eccrine sudoriferous glands are most abundant on the hands and feet, but also on other parts of the body. Apocrine sudoriferous glands are more common in the armpits and the groin area. Sebaceous glands are found on the face and the scalp (Roachell 1997, Takken 1991). Studies using fresh vs. incubated sweat with *Anopheles gambiae s.s.* show that fresh sweat secretions are less attractive to mosquitoes than incubated sweat, indicating that bacterial decomposition of sweat by the microflora of the skin play a greater role in attracting mosquitoes

than do volatiles derived directly from human sweat glands and incubated sweat in the absence of bacteria diminished the attractiveness of the sample (Braks and Takken 1999, Braks et al. 2000). Khan et al. believed that convective heat, rather than simply being attractive in and of itself, aided in mosquito host location by means of odor transport of skin chemicals (1967), which has some merit as well because volatile chemicals are stimulated by heat and are able to stimulate mosquito at greater distances. Schreck et al. also found this to be true, noting that more *Ae. aegypti* mosquitoes were attracted to heated rather than unheated residues on the surfaces of glass that had been handled by human hands (1981).

Examination of skin exudates individually or in aggregates has increased the compounds known to attract mosquitoes. Brown suggested that the amino acids and estrogens have an additive effect to the attractiveness of a host to a mosquito, particularly when these are built upon the 'base attractiveness' of moisture, heat and carbon dioxide (1966). Other scientists surmised that chemicals other than carbon dioxide must be sufficiently attractive in its absence since only a negligible amount of carbon dioxide is respired via the skin (Frame et al. 1972). Ammonia was found to be an important attractant for *An. gambiae s.s.* mosquitoes, unlike lactic acid, and this was likely cause by the breakdown of urea and amino acids by bacterial microorganisms on the skin surface, increasing the pH of the incubated sweat (Braks et al. 2001) Incubated sweat was also found to be attractive to *Ae. aegypti*, thus ammonia may also be an attractant for this species (Geier et al. 1999). 1-octen-3-ol was originally identified as an attractant for tsetse flies from oxen was also found to be attractive to mosquitoes (Kline 1998). Human foot odor was attractive to *Ae. aegypti* with 66% of mosquitoes responding; however this was still less than the 80% which found the human hand to be most attractive in dual-port olfactometer tests (Kline 1998).

Some other potential attractants have been largely underexplored. Limburger cheese was identified as attractive to *Anopheles gambiae sensu stricto* (Knols and De Jong 1996), however despite its similarity to human foot odor, limburger cheese was not found to be attractive to *Ae. aegypti* (Kline 1998). Implications have been made about a correlation of attractiveness and human blood type but these have been largely refuted (Curtis 1986). Ahmadi and McClelland have suggested from experiments on *Aedes sierrensis* that an additional factor in host-seeking behavior might be an aggregation pheromone from mosquitoes; however, this theory appears to have garnered little support from a lack of subsequent testing or mention by other scientists (Ahmadi and McClelland 1985). Mehr et al. found that at concentrations of 10^{-6} to 10^{-4}, DEET was attractive to *Ae. aegypti* and *Aedes taeniorhynchus*, but not to *Anopheles albimanus* (Mehr et al. 1990), however this result was found to occur only over a short range of very low concentrations and the primary use of DEET currently is as a mosquito repellent.

L-Lactic Acid as an Attractant

L-lactic acid is another chemical that has been widely studied and debated for its attractive abilities (Reference, Acree et al. 1968 Science paper). Smith et al. reported that lactic acid was an attractant to *Aedes aegypti* at low concentrations, similar to those found on the skin surface or from breath, but repellent at much higher doses (1963). Chemical analyses of human skin emanations have shown that carboxylic acids and lactic acid are present in highest concentrations among many other volatile and moderately volatile skin compounds (Bernier et al. 2000, 2002). Bernier found evidence of attractant chemical synergism when he tested binary blends using L-lactic acid mixed with either dichloromethane, dimethyl disulfide or acetone and found that these blends produced a greater attraction of about 80% in *Ae. aegypti* mosquitoes than the mean attraction of about 50% for the two individual chemicals (Bernier et al. 2003). Later, a three compound mixture using lactic acid, acetone and dimethyl disulfide was found to provide even

greater attraction with almost 85% of mosquitoes responding to the blend (Bernier et al. 2007a). Conversely to the results found for *Ae. aegypti*, tests done with *Culex quinquefasciatus* found lactic acid to be attractive at concentrations that would normally be repellent to other species (Braks et al. 1999). Lactic acid was found to be attractive to *Ae. aegypti*, as well as surfaces touched by hands, but it has been contested that this is only true in the presence of carbon dioxide (Schreck et al. 1981).

Repellent Chemicals and Associated Mosquito Behavior

Repellents belong to a family of substances called allomones, *i.e.* substances that benefit the sender of chemical signals rather than receiver. Dethier et al. defined a 'repellent' as a chemical which causes insects to make oriented movement away from its source (1960). Usually, this is accomplished by a mosquito or other arthropod coming into contact with the repellent applied to the skin surface of a human or other animal. There is some disagreement with what is strictly considered a repellent *vs.* other classifications such as a pesticide or a deterrent (Cantrell et al. 2005). Although the US-EPA definition of pesticides is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest, the true mechanism of repellency does not necessarily involve insecticidal action (2012). While insecticides often irritate an insect and cause it to move away from the target source, sufficient exposure to the insecticide will ultimately kill the insect. There are many examples of repellents that are relatively non-toxic to target organisms. Dethier also noted that substances often classified as repellents may sometimes be more correctly defined as 'deterrents' in situations where the inhibition of feeding or oviposition is being measured rather than the movement away from a source. However, Dethier et al. also noted that the same compound may have multiple effects on behavior and be both a 'repellent' and a 'deterrent' (Dethier et al. 1960).

A second type of allomone involved in the vector-host relationship is an attraction-inhibitor. This is a compound produced by a host organism which induces anosmia or hyposmia in arthropods thereby masking host kairomones from the arthropod vector (Bernier et al. 2007b). This is the least understood class of compounds with the greatest potential for future research.

Mosquito Biology and Behavior Used in Host Location

Mosquito host location is controlled by both inherited behaviors and environmental factors whereby a mosquito is able to sense a potential host from a distance, locate a host in space at a short range, confirm the identity of a potential host, and initiate feeding behavior. While seemingly simple, these interactions rely on a number of cues that a mosquito must interpret in the central nervous system, weigh against internal cues and process to determine the appropriate action to be taken. Initiation of host location behavior can be passive or active. Some species actively seek out hosts once their internal physiological state has been assessed as adequate for the development of eggs. Other species feed opportunistically when a potential host wanders into a certain radius, which varies by species or by the strength of the odor stimulus, where a mosquito is resting (Day 2005). Once an organism has entered into this proximity, mosquitoes follow an odor plume produced by both skin chemicals and carbon dioxide towards a potential host organism (Dekker et al. 2001).

Mosquitoes usually fly upwind into a downwind plume. This anemotaxis towards human odors has been reported to occur from up to 30 ft away (Brown 1966). Mosquitoes may also orient upwind under continuous odor stimulation (Geier et al. 1999). Evidence of anemotaxis to host odors was provided by an experiment by Happold where individuals stood on a 20 ft platform above a forest (Happold 1965). The individuals were undisturbed by mosquitoes until a fan was used to direct a wind over the individual and into the forest, after which mosquitoes flew upwards 20 ft to the platform (Maibach 1969). In locating a host, a mosquito moves along a

route which provides an increasing concentration of stimulus and turns away from areas where the stimulus gradient declines (Brown 1966). This mechanism relating to the turning frequency, independent of orientation (which may also result), is known as klinokinesis. Environmental conditions also play an important role in long-range host location, particularly ambient temperature and humidity. Ambient temperature between 60˚F – 110˚F and relative humidity between 50-90% is necessary for a mosquito to adequately locate a host (Brown 1966).

The blood-seeking behavior in a female mosquito relies on receptors found on several anatomical structures to sense its external environment. Receptors vary in type and in number along the mosquito's body such as the ommatidia in the compound eyes, grooved pegs, small sensilla coeloconica and ampullacea, capitate palpal pegs, and for female *Ae. aegypti*, the blunt-tipped type I trichodea and short, pointed trichodea (McIver 1982). Receptors located in sensilla on a mosquito's antennae and the maxillary palps have been identified through olfactory signal transduction studies to be used in the detection of odors from potential host organisms (Zwiebel and Takken 2004). There are also receptors for odor, heat and humidity in a mosquito's palps, tarsi and proboscis, but these play a larger role in close range and tactile recognition, whereas the sensory receptors in the antennae are used in long range detection (McIver 1982). The exact function and binding abilities of many receptors are still an area of active research, particularly in relation to specific attractant or repellent chemical substances. Sensory receptors are used for the detection of heat (thermoreceptors), the detection of specific classes of chemicals (chemoreceptors), the detection of humidity (hygroreceptors) and the detection of movement (mechanoreceptors) (McIver 1982). Also, visual receptors in ommatidia are used for light/dark detection and shape recognition in long range host location (McIver 1982). Visual cues are also utilized in long-range detection of a host and *Ae. aegypti* has been found to be attracted to darker

colors in clothing (Brett 1938). Although the mosquito uses both visual and olfactory cues to orient toward a host, the olfactory system plays a prominent role in the response and its subsequent action (Bowen 1991). After landing, tactile and thermal stimuli play an important role in confirming the host and the decision making process to feed on the host (Maibach 1969).

Mosquitoes also react to their own physiological state when determining whether or not they require a blood meal. Adult female mosquitoes reproduce and sugar feed soon after emerging and then a few days later, they seek a host for a bloodmeal. Internal cues such as a mosquito's age, reproductive status, and whether or not the female is undergoing diapause, changes the tendency of a mosquito to seek out a host (Bowen 1991). In a 1984 study by Davis, *Ae. aegypti* mosquitoes were found to not display host-seeking behavior within two days of adult emergence, but after 5 days more than 90% of female mosquitoes display host-seeking behavior and this continued consistently up to 15 days post-emergence, showing that host-seeking behavior is age dependent (Roachell 1997). Other studies have also found that the frequency of bloodmeal seeking increases in mosquitoes as they age (Khan et al. 1971). Host seeking behavior studied in *Culex pipiens* stimulated with lactic acid has been shown to be related to sensitivity of sensilla type A3 receptors for females that have terminated diapause and resumed reproductive development *vs.* females that were still in reproductive diapause (Roachell 1997, Bowen 1991).

CHAPTER 3
A REVIEW OF MOSQUITO REPELLENT TESTING METHODS

Current Standards for Repellent Testing Methods

The major guidelines that govern the testing of mosquito repellents are contained in the three main documents that delineate the recommended materials and methods for testing. Two of these guidelines are produced by the American Society for Testing and Materials (ASTM) for the laboratory testing of mosquito repellent formulations on the skin (2000a) and for the field testing of topical repellent compounds (2000b). The third is the World Health Organization's (WHO) guidelines for efficacy testing of mosquito repellents for use on human skin (2009).

Although the methods are meant for application to a range of vector species, the WHO recommends the use of *Aedes aegypti* for use in laboratory tests. There are several reasons for selecting this particular species. Not only does *Ae. aegypti* respond well to laboratory rearing and can produce a large amount of insects for testing at regular intervals, but it is also an avid biter. Additionally, *Ae. aegypti* is a diurnal feeder which is preferred over other crepuscular mosquito species. This single species of mosquito is often used for large scale screening of potential repellent compounds, but other medically important genera should also be considered. Mosquito species of the *Anopheles* and *Culex* genera are frequently used in addition to *Ae. aegypti* mosquitoes in the most accurate evaluations (WHO 2009). The benefit of using all three genera in laboratory testing is to provide for a range of repellent responses. Data derived from experiments using all three genera can then more accurately predict the response of the mosquito population that the researcher is attempting to characterize, since several species inhabit the same regions.

The three previous guidelines cited stress safety and standardized processes which are aimed at providing a baseline for comparative analysis. This baseline is important as the

techniques used for repellent testing of mosquitoes can vary widely from region to region and across different target species of mosquitoes. While these methods allow for customization, the basic recommended procedures stated by the guidelines are noted below alongside examples of individual *in vitro* and *in vivo* methods. First, to provide some context for the individual examples, the three major systems of repellency measurement will be described to establish the basics involved in all testing methods.

Measurements of Repellent Efficacy

With the increased need for repellent research came the necessity to quantify the efficacy of potential repellent chemicals. The most widely used measure for determining effectiveness was one of the earliest developed, the time to first bite (Granett 1940) or complete protection time (CPT) which measures the time that the repellent offers 100% protection from bites. For this measurement, a test subject's arm or leg is exposed for a three minute period every thirty minutes to a biting insect until a bite occurs. The complete protection time is then recorded when a confirmatory bite occurs either during the same three minute period or during the subsequent three minute exposure period.

Another metric to assess repellency that is frequently used is percent protection, which calculates the percentage of biting mosquitoes prevented from taking a blood meal during a specified amount of time. This measurement is common for laboratory rather than field use as it requires an accurate count of the total female mosquitoes exposed to the test subject as well as the total number of blood-fed mosquitoes (Barnard et al. 2007). Females mosquitoes are almost exclusively used in the testing of mosquito repellents as they and not males are haematophagous or blood-feeding, requiring a blood meal in order to lay eggs. Blood feeding can be determined by direct observation of mosquito probing behavior on the test subject, by counting the number

of visibly engorged mosquitoes, or by sacrificing and examining the mosquitoes directly by individually crushing them on white paper and noting blood spots.

A third, more involved measure of repellency is the ED_{50} measurement or average effective dose. While the time to first bite measurement offers a practical and intuitive frame of reference for the typical repellent user, the ED_{50} or sometimes the ED_{90} or ED_{99} provide the researcher with a standardized measurement for comparative analysis over a range of testing methods. This measurement involved the exposure of a test subject's arm to approximately fifty mosquitoes for a thirty second period first without repellent, as a control exposure, and then with increasingly higher doses of the experimental repellent compound until complete repellency is attained. Mosquito biting is measured through observation of probing behavior during the exposure period. After the complete repellency dose is determined, the arm is re-introduced hourly until the number of bites received during the exposure period equals 50% of the bites received during the control exposure (Moore and Debboun 2007).

A fourth measurement conducted to measure repellent efficacy in the laboratory is the measurement of the minimum effective dosage (MED) (USDA 1977). The MED is an estimate of the surface concentration at which a repellent compound fails to prevent bites. Depending on the number of mosquitoes in the trial and the defined failure point, e.g. for 500 mosquitoes and a failure point of 5 bites, the MED is essentially an estimate of the ED_{99}. The MED also has a bearing on the measurement of CPT. As a compound evaporates from skin, the ability of the compound to repel will decrease and in theory, the surface concentration at which the repellent fails is the MED. However, the CPT measurement is influenced by many other factors such as evaporative loss of the compound, abrasive loss from volunteers usually by accidental contact with other surfaces, and dermal absorption and migration. Measurement of the MED removes all

these other sources of variability and focuses solely on the amount of repellent needed to deter mosquitoes (or other arthropods) from biting.

A Survey of *in vitro* Test Methods

The use of cloth sleeves as an *in vitro* test method is perhaps the simplest experimental design of this type. The idea behind the use of a cloth sleeve stems from the precautionary need to avoid direct contact with the skin. Direct contact with the skin is avoided in compounds where toxicological studies have not been performed and the safety of the chemical for use on skin is questionable. Cloth sleeves typically are fabricated of a well-ventilated stretchable material to allow for the emanations from human skin to pass through and illicit the host-seeking behavior in the mosquitoes. These sleeves are often constructed for testing on the forearms, spanning the area from the wrist to elbow. A sleeve can also be used for testing on the lower leg, spanning the area from the ankle to the knee. In order to prevent direct contact of the treated sleeve with the exposed skin of the participant, a second untreated sleeve is worn underneath the first to provide a protective barrier since the chemical treated in the sleeve may produce harmful effect to humans (WHO 2009).

Experimental repellent chemicals for use on cloth are diluted in 95% ethanol or acetone and the concentration applied is between 1.5 mg/cm^2 or 1 g of compound for a 650 cm^2 area. Ethanol and acetone are used as solvents in these experiments due to their high volatility. After the application of the chemical mixture to the cloth, the sleeve is allowed to dry for a specific amount of time. Typically fifteen minutes is sufficient time to allow for all of the volatile solvent to evaporate from the cloth sleeve, leaving only the desired test chemical. Once dry, the sleeve can be placed over the arm or leg of the volunteer. When testing the forearm, a latex glove is worn over the hand to protect the volunteer from painful bites on that sensitive region of the body. The arm or leg is then placed into a screened cage containing a minimum of 200 adult

female mosquitoes of a certain species, aged 5-10 d and preferably starved for one hour prior to testing (Barnard 2005).

A modification of the cloth sleeve experiment is the use of a cloth patch. In this experiment the area of skin on the arm or leg exposed to bites from mosquitoes is much smaller, and can vary according the needs of the experimenter. One example of this test uses a 50 cm^2 area of cloth soaked in the test chemical/solvent mixture as used for the cloth sleeve (Carroll et al. 2011, Katritzky et al. 2008, 2010, Rosa et al. 2012). The concentration of test chemical is also similar, often 1.5 mg/cm^2. For testing, however, the volunteer requires not only a latex glove to cover the hand, but also an impenetrable material to cover the rest of the arm as well. One model for this covering is the use of a Velcro-lined vinyl sleeve fashioned to the shape of the volunteer's arm with area cut out to allow for mosquitoes to bite through. The cloth patch can then be placed over this window during the testing procedure. The cloth patch modification is beneficial for large scale screening of chemicals because less cloth is used per test chemical/concentration and cloth patches can easily be replaced over the vinyl sleeve window for rapid trials (Bernier et al. 2005).

When human volunteers are scarce, the use of animals can provide a skin surface on which repellent chemicals can be tested. Rats, rabbits, gerbils, or guinea pigs are among the animals most often chosen for this type of assay. The desired area of skin surface is exposed by first shaving the animal and then the repellent chemical is applied to the skin surface. Sedation is recommended for the animals in these tests so that the chemical is not removed by rubbing and so that the mosquitoes may feed freely (Robert et al. 1991). Another benefit of this type of experiment is that it removes the potential risk to human volunteers with the use of wild-caught mosquitoes in disease-endemic areas.

The use of synthetic membranes that mimic the skin surface can be utilized as well. Several types of synthetic membranes have been explored as cost-effective means to avoid the use of human volunteers. Some of these membranes include collagen film, cow mesentery or baudruche film, and lamb skin membranes. These synthetic membranes can be stretched over receptacles of any size filled with a blood source. It is preferable that the blood source be maintained at a temperature of 28-40 ˚C so that the temperature cues that the mosquito is adapted to seeking out for their host blood meal will be consistent with the membrane/blood reservoir system. Although the concept of using an artificial membrane can be applied to most *in vitro* methods, this particular modification was developed in conjunction with the Klun & Debboun (K & D) *in vitro* module (Klun et al. 2005). The K & D module itself is a modification of an ASTM module for the testing multiple repellents on the skin of a single research participant at once (ASTM 2000b). The K & D module consists of a Plexiglas rectangle divided into six individual cells measuring 3 cm by 4 cm by 4 cm each. Each cell was designed with a small, pluggable hole at the top of the cell for the loading of 5 mosquitoes into each compartment. At the bottom of each cell is a Plexiglas sliding door to introduce the mosquitoes to the desired repellent testing surface, in this case the membrane/blood reservoir system. Cloth patches cut to the dimensions of the cell's bottom opening and treated with the desired concentration of repellent chemical are then placed in between the K & D module containing the mosquitoes and the blood reservoir system.

An uncommon and often criticized method for determining repellency involves the use of an olfactometer. Olfactometers are elongated chambers which are normally used for determining attraction in mosquitoes. Mosquitoes are placed at one end of the chamber and an odor stimulus is introduced at the other end of the chamber. The circulation of air uniformly through the

chamber allows an odor plume produced by the chemical stimulus to guide mosquitoes. Movement of the mosquitoes in the chamber toward the source of the odor stimulus provides a basis as to whether a chemical is attractant or not. If a chemical is not attractant, the mosquito will not be stimulated to fly and will remain in the area of the chamber where it settled before the test. Some researchers have modified this design to allow for the testing of attraction-inhibitors and other repellents in the olfactometer (Bernier et al. 2005, Kline et al. 2003).

One such modification involves the use of a three chamber olfactometer. In this design, mosquitoes are places in a median chamber prior to the test and allowed to acclimate for an hour. At the start of the test a repellent chemical is placed in one end, referred to as the proximal chamber. At the other end of the olfactometer is the distal chamber which has a mesh netting funnel which mosquitoes must pass through into a collection tube. Barriers separating the proximal and distal chambers from the median chamber are opened once the chemical stimulus has been placed in the proximal end. Movement of the mosquitoes away from the source of the chemical stimulus is indicative of a repellent and the repellency can be quantified by calculating the percentage of mosquitoes in each of the three chambers compared to the total number of mosquitoes (Dogan and Rossignol 1999). For this design male mosquitoes may be used as well as female mosquitoes since the focus is not on mosquitoes seeking a blood source, but simply being repelled from their position in the median chamber.

A Survey of *in vivo* Test Methods

In contrast to the great variation of *in vitro* methods, there are only a few *in vivo* methods. *In vivo* methods are often more rigid than *in vitro* methods since only a few chemicals that have previously passed toxicological safety tests can be administered and screened on a volunteer at a time. However, *in vivo* methods provide fairly consistent results, with high correlation to other *in vivo* tests and are realistically comparable to the end use of the repellent.

Topical skin repellent tests are very closely related to the cloth sleeve tests, and are very similar for both laboratory and field tests. Experimental repellent chemicals are diluted to the desired concentration, often in 25% ethanol or acetone. The concentration typically applied is 1.5 mL of compound for a 650 cm^2 area, the average skin surface area from the wrist to the elbow. Typically fifteen minutes is sufficient time to allow for all of the volatile solvent to evaporate from the skin surface, leaving only the desired test chemical. If the forearm is being tested, the volunteer should place a latex glove over the hand to protect from painful bites. The arm is then placed into a screened cage containing a minimum of 200 adult female mosquitoes of a certain species, aged 5-10 d and starved for one hour prior to testing (Barnard and Xue 2004). Human skin topical repellent tests in large outdoor cage or field tests follow the same basic procedure as the laboratory tests but are subject to the weather and environmental effects of the region in which the test is performed (Debboun et al. 2000, ASTM 2000a). Recommendations from the WHO guidelines indicate that repellent tests of this kind should be performed in the vicinity of human homes, have test subjects spaced 10 m apart and rotated in a randomized fashion to minimize positional errors (WHO 2009).

The K & D module for *in vivo* testing systems, like the module used for *in vitro* testing also consists of a Plexiglas rectangle divided into six individual cells measuring 3 cm x 4 cm x 4 cm each. Each cell was designed with a small, pluggable hole at the top of the cell for the loading of 5 mosquitoes into each compartment. At the bottom of each cell, which is curved for *in vivo* methods to lay closer to the skin surface of the rounded leg or arm, is a Plexiglas sliding door to introduce the mosquitoes to volunteer's chemically treated skin surface where corresponding rectangular treatments have been delineated and dried (Klun et al. 2006). This design is a marked improvement over the ASTM module, although is it also still used (Rutledge

and Gupta 2004). The ASTM module consists of a long, rectangular loading cell where 10 to 15 mosquitoes are loaded from a pluggable hole in one side. The bottom of this module is fashioned with five 29 mm diameter openings which are placed on the arm or leg skin surface of the volunteer where corresponding treatments have been delineated and dried (ASTM 2000b).

Previous Reviews of Mosquito Repellency Methods

While there is great diversity in the techniques employed in repellent testing methods, and several scientists have utilized these various methods to compare the efficacy of repellent compounds across mosquitoes species or even provide comparative analysis across several medically relevant insect orders, in the past forty years only two researchers have attempted to provide a direct comparative analysis and review of the many of the methods involved. Earlier reviews of this topic have had a broader focus, providing critiques of insect repellents in general, as in the 1977 article by Schreck. While mosquitoes provide the majority of the paper's focus, repellent testing measures for tick, fleas, and other biting flies are also mentioned in detail. While experimental designs can overlap to other insects from mosquito research, results from tick, flea, or other biting fly research are not usually applicable to mosquitoes and vice versa. One alteration Schreck advocates is the use of a large amount of mosquitoes for laboratory cage testing, suggesting that a higher biting pressure reduces the variability (Schreck 1977). While a thorough comparison of all relevant *in vivo* methods are presented in Barnard's review, Barnard largely disregards *in vitro* methods, citing that the comparability of *in vitro* methods is poor and should be avoided in favor of *in vivo* methods (Barnard 2005).

CHAPTER 4
COMPARISON OF DOSE-RESPONSE CURVES FOR MOSQUITO REPELLENTS TESTED *IN VIVO* AND *IN VITRO* USING A MODULE SYSTEM

Background

Though most *in vitro* methods for mosquito repellent tests provide a fast, safe, inexpensive way to test chemical compounds regardless of whether toxicity has been established or not, there are limitations that should be recognized when results from these methods are compared or even more importantly, used to estimate performance in the field. One limitation is the difficultly in correlation of results from *in vitro* studies to *in vivo* studies. Since by its nature, an *in vitro* test is not directly tested on the target host out of convenience or necessity, extrapolation of these results to *in vivo* systems without a basis for comparison limits their usefulness. Few studies have been conducted to compare these methods and validate that they provide results that are an accurate prediction of how repellents will perform on skin.

In vivo methods, however, are not without their disadvantages. These studies require human volunteers that are willing to subject themselves to the accumulation of bites and the potential for allergic reactions makes it difficult to perform statistically significant experiments with large sample sizes. Although field testing is most similar to the real use of repellent chemicals, the risk of exposure to pathogens carried by wild mosquitoes in disease endemic areas makes it impractical and unethical to perform these outdoor experiments in some locations.

Despite the drawbacks of current *in vitro* systems and their unpopularity with some researchers, *in vitro* systems are still valuable tools for the future of mosquito repellent research because they can serve to predict repellency when *in vivo* testing is not feasible. The desire to produce an accurate, rapid-screening method has produced a myriad of *in vitro* testing methods in comparison to the handful of *in vivo* methods. Modification to some of the more promising *in vitro* methods could result in a mechanism by which *in vivo* test results could be reliably

predicted without the need for human volunteers. With the ability to narrow down potential repellents through screening, the costly safety screening process for promising novel repellent compounds could also be minimized. Furthermore, the US EPA Human Studies Review Board (HSRB) has recently expressed concerns over use of humans in repellent studies, and these results would be of interest to US EPA for their new rules on repellent registrations. The first objective of this research project was to examine the dose-response curves of four mosquito repellent active ingredients tested *in vivo* on human skin and *in vitro* on a silicone membrane using a module system for significant differences.

Materials and Methods

Mosquito Rearing and Selection

The mosquitoes used in all bioassays were female *Ae. aegypti* (Orlando strain, 1952) from the colony maintained at the Center for Medical, Agricultural, and Veterinary Entomology location of the United States Department of Agriculture, Agricultural Research Service (USDA-ARS-CMAVE) in Gainesville, FL. Pupae were obtained from the colony and kept in laboratory cages where newly emerged mosquitoes were maintained *ad libitum* on a 10% sucrose solution at 25-28^{o}C ambient temperature, 60-80% relative humidity and a 14:10 (light:dark) photoperiod. Nulliparous female mosquitoes aged six to eleven days displaying host seeking behavior were pre-selected from stock cages using a hand-draw box and trapped in a collection trap (Posey et al. 1981). Female mosquitoes were then transferred to a smaller cage (28,316 cm^3) from which the mosquitoes were sorted into groups of 10 by mechanically aspirating them into acrylic holding tubes (15 cm long, 1.25 cm wide). Each tube contained approximately 10 mosquitoes, contained by screen gauze on one end and stoppered with a small cork (Fisher Scientific, Catalog No. 07781D Size 1) at the other end. Mosquitoes held in the tubes were allowed to acclimatize

for 15-20 min (Barnard et al. 2007) before being transferred into an empty chamber of the module for testing.

Chemical Treatments and Control

Chemical compounds used as repellent treatments included technical *N,N*-diethyl-3-methylbenzamide, 97% (Aldrich, CAS#134-62-3) hereafter referred to as 'DEET', technical 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester, 98% (CAS#119515-38-7) hereafter referred to as 'KBR3023', technical ethyl 3-[acetyl(butyl)amino]propanoate, 98% (CAS#52304-36-6) hereafter referred to as 'IR3535', and technical *para*-menthane-3,8-diol, 98% (Bedoukian, CAS# 42822-86-6) hereafter referred to as 'PMD'. The repellents used in this study are summarized in Table 4-1. Serial dilutions of these four chemicals were obtained by adding 1mL of completely denatured ethyl alcohol (Mallinckrodt) into 1mL of the previous concentration of chemical to produce a range of concentrations (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) when applied to the 14.19 cm^2 treatment area. The control treatment consisted of only completely denatured ethanol.

Alterations Made to Module from Previous Designs

Several modifications were made to the module and protocols from those used by Klun and Debboun (2000) and Weldon et al. (2003). Modifications made to the original Klun and Debboun (K & D) module by Weldon included an increase in the internal volume of each chamber from 100 cm^3 to 125 cm^3, an increase in the spacing between the chambers from 0.25 cm to 1.25 cm, and the opening under each chamber was reduced to a circular aperture with a diameter of 4.25 cm (Weldon et al. 2003). Modifications made the Weldon module system for this project include an increase in the number of mosquitoes placed in each chamber from 5 to 10, to allow for more precise feeding proportions to be recorded at each concentration level. A

glass plate with drilled holes in line with the blood wells and apertures of the feeding module was added. The glass plate was placed under the feeding module to act as an inert barrier between the feeding module and the loading module or between the feeding module and the skin of the volunteers (Fig. 4-1). This glass plate also prevented absorption of chemicals into the Plexiglass bottom of the module from which contract with the chemicals was most likely to occur. Elastic rubber bands were added to the sliding doors to prevent accidental opening of the chamber doors by the volunteers during testing.

A procedural modification to this experiment was the use of layering of chemical doses on the skin or membrane, so that each dose can be evaluated in all 6 chambers simultaneously. This differed from other protocols that used different doses in each of the 6 chambers. The testing of the same concentration across all chambers, in combination with the increased spacing between the chambers is an improvement over the original K & D design which assured that there was minimal effect of the repellent dose in one chamber affecting an adjacent chamber.

An early obstacle encountered during the trials was the lengthy amount of time needed to test volunteers by running each of the six chambers individually in a series. To overcome this, a multi-port connector was fashioned from nylon tubing and nylon barbed Tee connectors, arranged in parallel that allowed for CO_2 to be simultaneously pumped into several chambers (Fig. 4-2). This allowed three or six chambers to be tested concurrently and cut down testing time at least three fold from the previous testing scheme.

***in vivo* Module Bioassays on Skin**

For this part of the study, the proportion of *Ae. aegypti* mosquitoes blood feeding on six human volunteers (4 male, 2 female) was determined for a range of concentrations to the repellent chemicals DEET, IR3535, KBR3023, and PMD. Ten nulliparous female *Ae. aegypti* mosquitoes (6-11d) were loaded into each of the six testing chambers (5 cm x 5 cm x 5 cm) in

the previously described loading module (Klun and Debboun 2000, Klun et al. 2005, Weldon et al. 2003, Rutledge and Gupta 2004). Each of the six chambers in the loading module had two apertures, a 4.25 cm diameter circular aperture at the bottom of each chamber, covered by a sliding door, as well as a small 1.5 cm diameter circular aperture on the front of each chamber closed by a small cork.

For the *in vivo* test, the loading module was placed over the thighs of each human volunteer. The areas where test solution was to be delivered on the thighs were demarcated with 4.25 cm diameter circles corresponding to the six chambers. A 50 μL ethanol-diluted dose of DEET, IR3535, KBR3023, or PMD (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) was applied to the six circles in successive layered doses from the lowest concentration to the highest concentration. The first treatment applied in any set of tests was the ethanol control to establish the baseline for mosquito feeding behavior. The application was allowed to dry for 3-5 min to allow for the ethanol to evaporate. The loading module was then placed over the volunteer's thigh with a glass spacer placed between the module and the skin to prevent direct contact of the module with the chemicals. A sliding door was opened under three chambers at a time to expose mosquitoes in those chambers to the repellent for a 3 min period. At the end of the exposure period, the mosquitoes were knocked down with CO_2 gas via the corked hole, removed from the chamber with an aspirator and crushed to record the proportion of mosquitoes blood-feeding out of ten (Fig. 4-3). This process was repeated with the rest of the six chambers and with all concentrations of the repellent until all feeding in all chambers ceased. Volunteers for all repellent tests signed informed consent forms and were enrolled in an IRB study (Project # 636-2005).

***in vitro* Module Bioassays on Silicone Membranes Treated with Skin Odors**

In the second part of this study, the loading module was placed over the feeding module with six 4.25 cm diameter wells, corresponding to the six circular openings in the loading chamber (Klun and Debboun 2000, Klun et al. 2005, Weldon et al. 2003, Rutledge and Gupta 2004). Each of the wells in the feeding module was filled with 7 mL of citrated bovine blood, kept at approximately 37˚C by continuously pumping hot water through the feeding chamber with a Cole Parmer Polystat circulating water bath (Fig. 4-4). Prior to testing, strips of silicone membranes were worn against the upper thigh by the volunteers for 3-4 h, held in place with an Ace™ elastic bandage.

The worn silicone membranes were then placed across the six wells of the feeding module to come into contact with the blood, and the glass spacer was placed over the membranes leaving only the membrane-covered well area exposed. A 50 µL ethanol-diluted dose of DEET, IR3535, KBR3023, or PMD (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) was applied to the six circles on the silicone membrane above each well in successive layered doses from the lowest concentration to the highest. The first treatment applied in any set of tests was the ethanol control to establish the baseline for mosquito feeding behavior. The dose was allowed to dry for 3-5 min to allow for the ethanol to evaporate. The loading module was placed onto the feeding module and lined up to correspond to each well. A sliding door was opened under all six chambers to expose the mosquitoes to the repellent treatment for a 3 min period. At the end of the exposure period, the mosquitoes were knocked down with CO_2 gas via the corked hole, removed from the chamber with an aspirator and crushed to record the proportion of mosquitoes blood-feeding out of ten. This process was repeated with the rest of the six chambers and with all concentrations of the repellent until all feeding in all chambers ceased.

Statistical Analysis

Data from the six volunteers were pooled for each of the four repellent chemicals and at each of the concentration levels to reduce random effects that were the result of biological testing as well as to minimize person-to-person variability in attraction of the mosquitoes. The pooled data from the repellency bioassays were analyzed using a generalized linear mixed model using a binomial distribution with a probit link. The equation for the fitted model was:

probit(y/n) = mu + Nchem + Medium + Nchem*Medium + x + Nchem*x + Medium*x Nchem*Medium*x + e

where mu was the overall mean, Nchem was the fixed effect of the chemical, Medium was the fixed effect of the medium, Nchem*Medium was the interaction between Nchem and Medium, x was a fixed covariate corresponding to x = log(Dose+100), and the other terms correspond to interactions with this variate. In addition, e was the random error with e ~N(0, σ^2). Also, an overdispersion parameter was considered for this model. The above model was fitted using the procedure GLIMMIX as implemented in SAS 9.2 (SAS 2012). The significance of the model term effects were evaluated using an approximated F-test. PoloPlus v2.0 was also used to produce dose-response curves with the pooled data for visual comparison of treatments tested *in vivo* and *in vitro* (PoloPlus 2012).

Results

The results from the approximated F-test analysis, summarized in Table 4-2, indicate that there are significant differences for all of the effects accounted for in the fitted model. Hence, chemical and medium differ among its levels and there are significant interactions among them. Also, the continuous variate (logDose) is significant, and the slope associated with each of the factors Nchem and Medium and their interaction, are also highly significant.

A comparison of baseline attraction revealed that the mean blood-feeding on the control treatment was 59.16% for *in vivo* and 56.17% for *in vitro*, which is not a statistically significant difference for all volunteers pooled together (Fig. 4-5). Data from preliminary tests conducted with unworn membranes using both blood and a 10% sucrose solution treated with red food coloring failed to adequately attract mosquitoes, 38.3% and 12.8% bloodfeeding respectively, in the module tests at a level comparable to that of human skin. When examined individually, volunteer M5 had a significantly different proportion of mosquitoes blood-feeding on skin *vs.* membrane without repellent (Fig. 4-5), and because of the high variability in individual data only the pooled data was used for further analysis. Comparisons were made using a student's t-test for paired means at the p=0.05 level. Line graphs of the pooled dose-response data for DEET, KBR3023, IR3535, and PMD illustrated that the proportion of mosquitoes repelled on skin *vs.* membrane differed significantly from each other beginning at either the 31 nmol/cm^2 (Fig. 4-6), the 61 nmol/cm^2 (Fig. 4-7) or at the 123 nmol/cm^2 (Fig. 4-8, Fig. 4-9) dose level, according to a student's t-test of means with a confidence level of p=0.05. Probit-linked dose-response curves were produced in PoloPlus for visual comparison of the paired treatments for each of the chemicals tested (Fig. 4-10, Fig. 4-11, Fig. 4-12, Fig. 4-13). These illustrated the sigmoidal curve expected and provided visual cues as to the precision of the six data points at each dose level.

Discussion

Two sources of kairomones, human skin and silicone membranes treated with human odors, were used to evaluate the blood feeding behavior of female mosquitoes when exposed to four repellent chemicals in a laboratory setting. Since the baseline attraction was found to be statistically the same for both of these attractant sources, the attractiveness of the membrane to mosquitoes supports the hypothesis that this could be a good surrogate for humans in laboratory tests. The membranes were worn on the skin of the volunteers for 3-4 h prior to testing in an

effort to transfer some of the attractive skin chemicals on the membranes. This appears to have been successful, since the attractiveness of the membrane tested without human odors using both blood and a 10% sugar solution, did differ significantly from the control treatments on human skin.

The membrane served as a suitable alternative for the skin in the testing of all four repellents at lower concentrations, since the repellent results tested on skin and those tested on the membrane did not differ significantly below the 31 nmol dose level. Above this level, DEET differed significantly beginning at 31 nmol/cm^2 (Fig. 4-6), KBR3023 differed at 61 nmol/cm^2 (Fig. 4-7), and both IR3535 and PMD differed at 123 nmol/cm^2 (Fig. 4-8, Fig. 4-9). This indicates that near the point where half of the mosquitoes are deterred from feeding, the two models diverge and above these doses the membrane fails to provide a good estimate of the repellent dose required on the skin.

Interestingly, all four repellents tested on the membrane required much higher doses of repellent chemicals to reach full protection from mosquito blood-feeding, *i.e.* 100% of the mosquitoes deterred from feeding. The mean membrane dose level for full protection was 2-3 dose levels higher, or 4-6 times as much repellent than was needed for the skin. Given that the chemical doses were doubled at each interval, much more of the repellent chemical was necessary to achieve comparable feeding deterrence in the membrane *vs.* the skin. This is interesting because it is unclear whether the membrane became more desirable to the mosquitoes than the skin at these doses or if perhaps the skin became less desirable to the mosquitoes. Natural repellents as well as attraction-inhibitors have been documented in the exudate of humans (Bernier et al. 2002, 2005, 2007b). These natural allomones have also been found to vary from individual to individual. Perhaps the mixture of these naturally repellent or inhibiting

chemicals produced by the skin when mixed with the higher doses of repellents applied during the laboratory test, reduced the total amount of repellent needed to prevent blood-feeding by the female mosquitoes. Although, the membranes worn on the skin of the volunteers appears to have successfully transferred attractive skin chemicals onto the membranes, it is unclear whether any of these allomonal compounds were transferred or if they are produced at higher rates when humans undergo stress, *i.e.* being bitten by mosquitoes, similar to a plant's use of defensive chemicals for feeding deterrence by insects.

The human skin-chemical interactions with the repellent compounds were not explicitly explored in this study but could be further examined in additional studies with the membrane module system. By applying a standardized human-derived complement of chemicals to the membrane along with repellent treatments, the membrane system could be examined to see if it produces results more similar to the skin at dose applications higher than 31 nmol/cm^2.

Preliminary data on the individual volunteers showed evidence of an increase in feeding behavior that was inconsistent with the increased application of repellent chemical. This occurred mostly at the dose level just prior to the statistically significant divergence of the skin and membrane curves from each other. However, this effect was reduced by the pooling of the data from all volunteers, but is still evident on visual examination of the curves and line graphs. These unexpected results may be due to potentially attractive properties of repellent chemicals at very low doses, which have been documented with DEET (Mehr et al. 1990, Dogan and Rossignol 1999, Bernier et al. 2005). Further studies with lower doses of KBR3023, IR3535, and PMD should be conducted to test whether this phenomenon is occurring with these compounds as well.

There are several benefits of using an *in vitro* testing method in the laboratory instead of performing testing directly on human volunteers, which are not limited to exposing humans to less risk by not subjecting them to being bitten by mosquitoes, testing compounds of unknown toxicity, screening many successive compounds quickly, not needing to schedule human volunteers for testing or even needing to register volunteers for an IRB study. This last consideration encompasses significant savings in time and cost for scientists that wish to conduct repellent testing. However, since the chemical dose at which the skin and membrane curves diverge is lower than standard thresholds used in screening these chemicals, the use of the membrane module system would require some further modifications before it can be fully utilized as a replacement for screening methods using human volunteers.

Table 4-1. Chemical structures, names and other properties of the four repellent treatments.

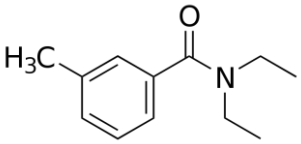
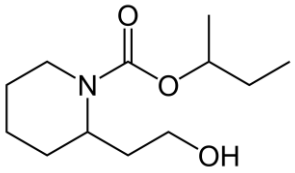
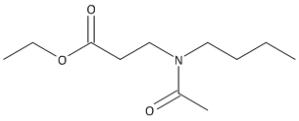
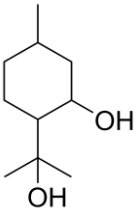
Repellent	Name	Structure	MW (g/mol)	CAS #
DEET	*N*,*N*-diethyl-3-methylbenzamide		191.27	134-62-3
KBR3023	2-(2-hydroxyethyl)-1-methylpropylester		229.32	119515-38-7
IR3535	ethyl-3-[acetyl(butyl)amino] propanoate		215.29	52304-36-6
PMD	*p*-menthane-3,8-diol		172.26	42822-86-6

Table 4-2. Results of F Tests in SAS 9.2 for the fitted model using pooled data from six subjects examining the effects of the chemicals, the testing media, the dose of repellent as well as their interactions.

Effect	Numerator DF	Denominator DF	F Value	Pr > F
Nchem	3	71	6.48	0.0006
Medium	1	71	83.23	<.0001
Nchem*Medium	3	71	5.30	0.0023
logDose	1	71	646.71	<.0001
logDose*Nchem	3	71	7.05	0.0003
logDose*Medium	1	71	101.93	<.0001
logDose*Nchem*Medium	3	71	5.91	0.0012

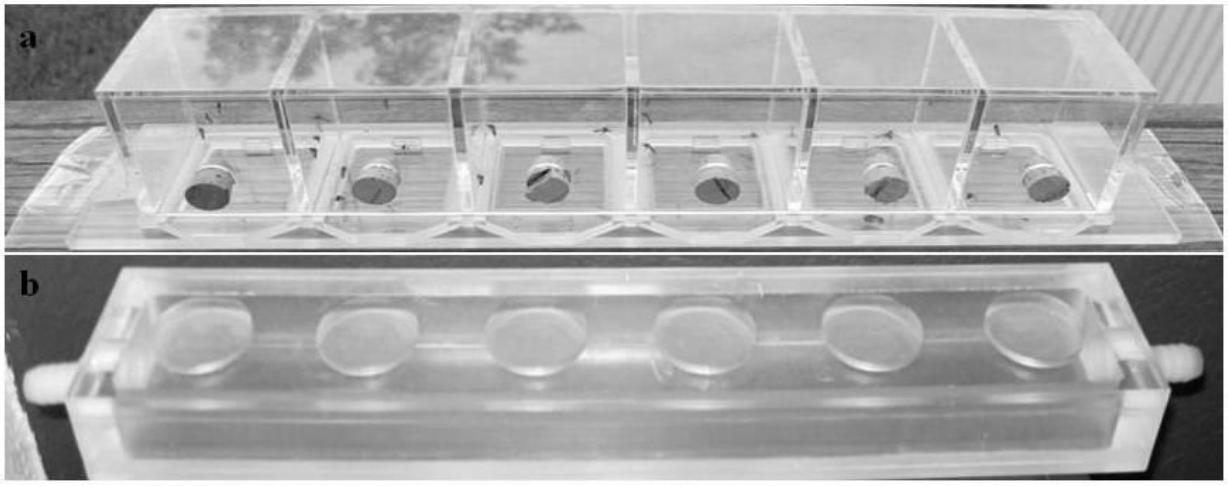


Figure 4-1. Pictures of *in vitro* loading and feeding modules. a) Mosquito loading module with six testing chambers corked and containing mosquitoes. b) Mosquito feeding module with 6 well receptacles for blood feeding. *Photos courtesy of Natasha M. Agramonte.*

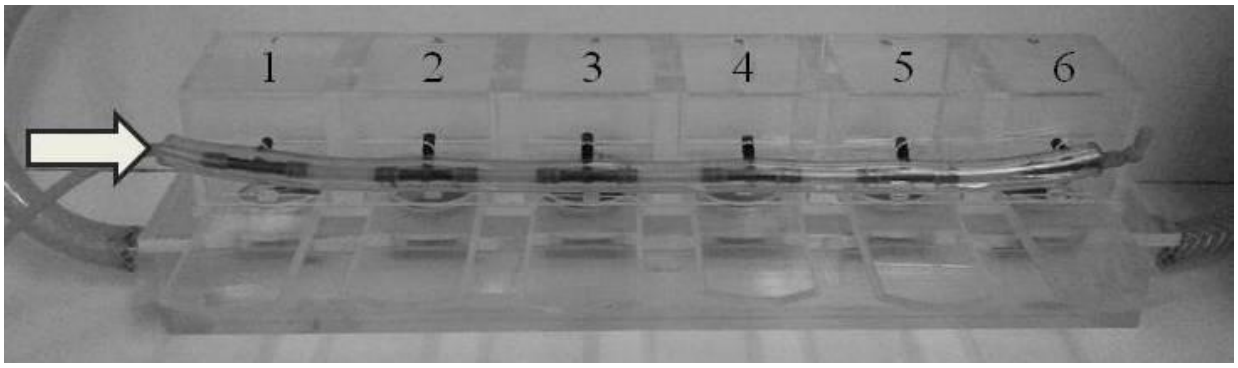


Figure 4-2. Assembled mosquito loading and feeding modules with arrow indicating the CO_2 tubing connected in parallel to the front of the six testing chambers via the cork hole. *Photo courtesy of Natasha M. Agramonte.*

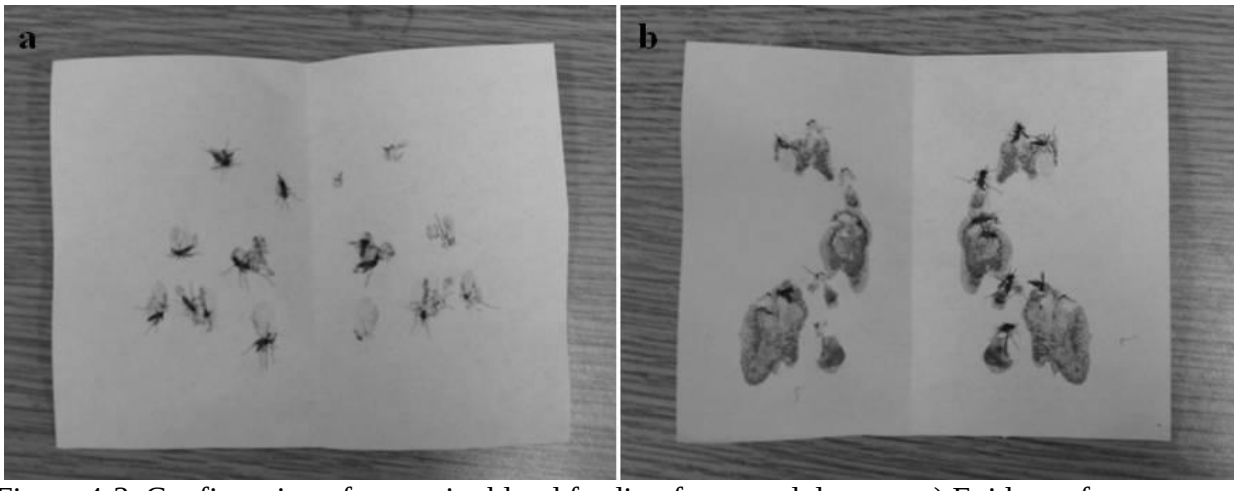


Figure 4-3. Confirmation of mosquito blood feeding from module tests. a) Evidence from module test indicating 0 mosquitoes feeding. b) Evidence from module test indicating 10 mosquitoes feeding. *Photos courtesy of Natasha M. Agramonte.*

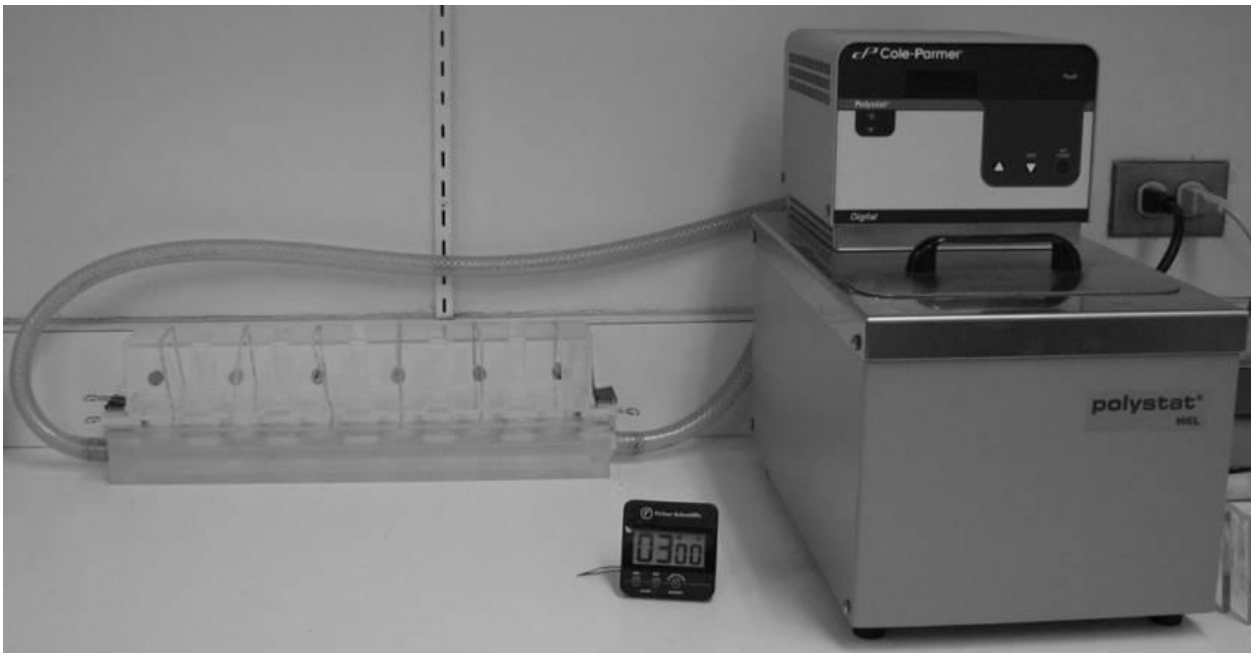


Figure 4-4. Assembled mosquito loading and feeding modules connected to heated water circulator via tubing for *in vitro* bioassay, showing rubber elastic bands placed around each of the six testing chambers. *Photo courtesy of Natasha M. Agramonte.*

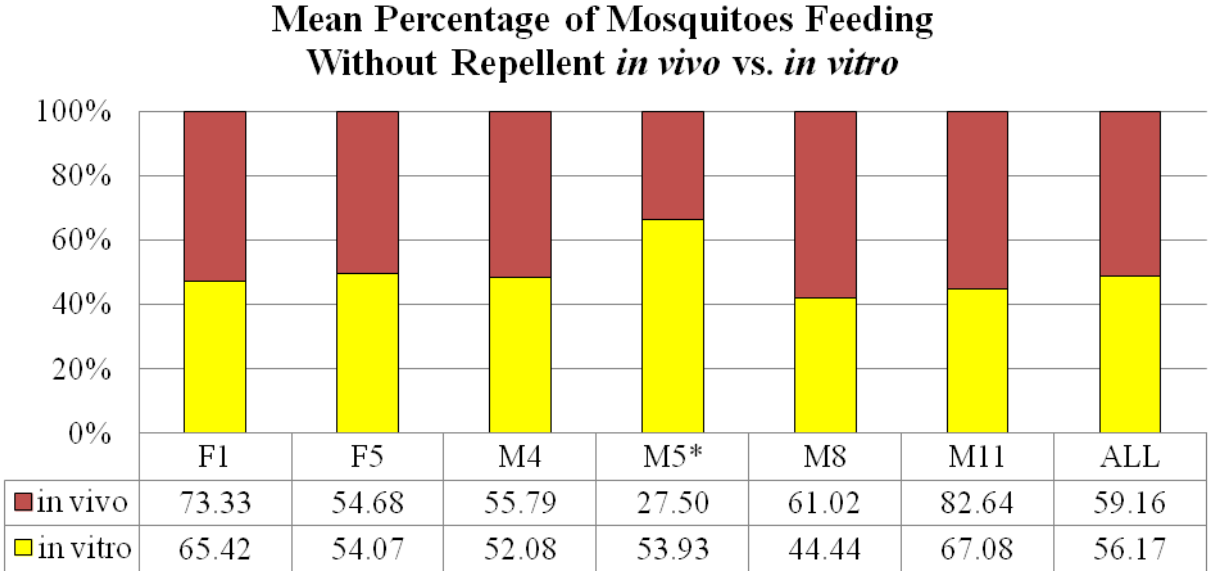


Figure 4-5. Stacked column chart comparing the mean attraction for the volunteers individually and pooled comparing *in vivo* vs. *in vitro* mosquito feeding on the control with asterisk indicating where controls differed from each other significantly ($p \leq 0.05$).

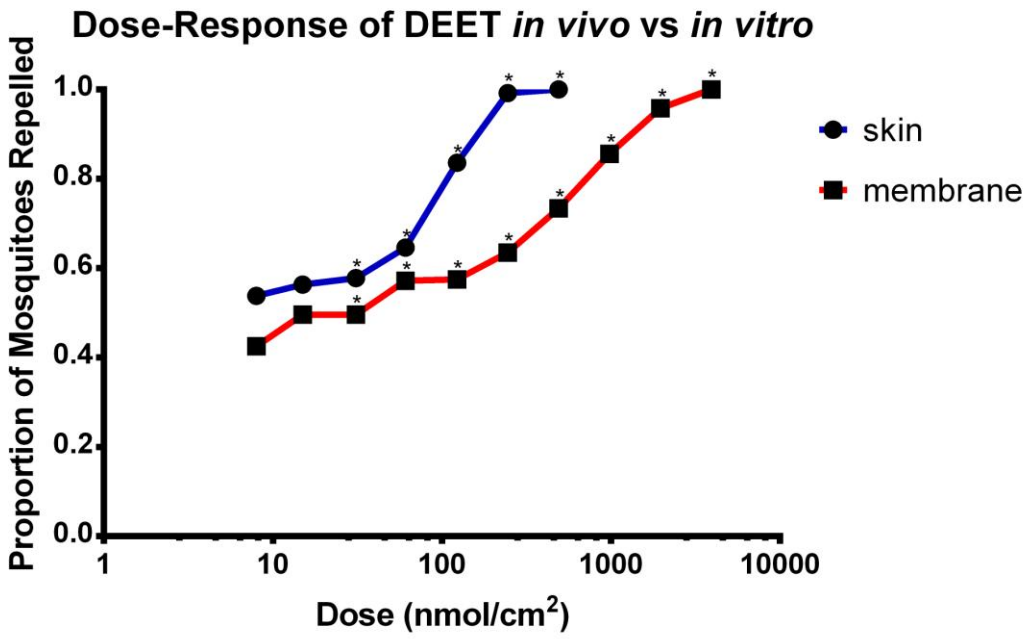


Figure 4-6. Line graph of dose-response data indicating the proportion of mosquitoes repelled by DEET on skin (*in vivo*) and on membrane (*in vitro*) with asterisk indicating where the two media differed from each other significantly ($p \leq 0.05$).

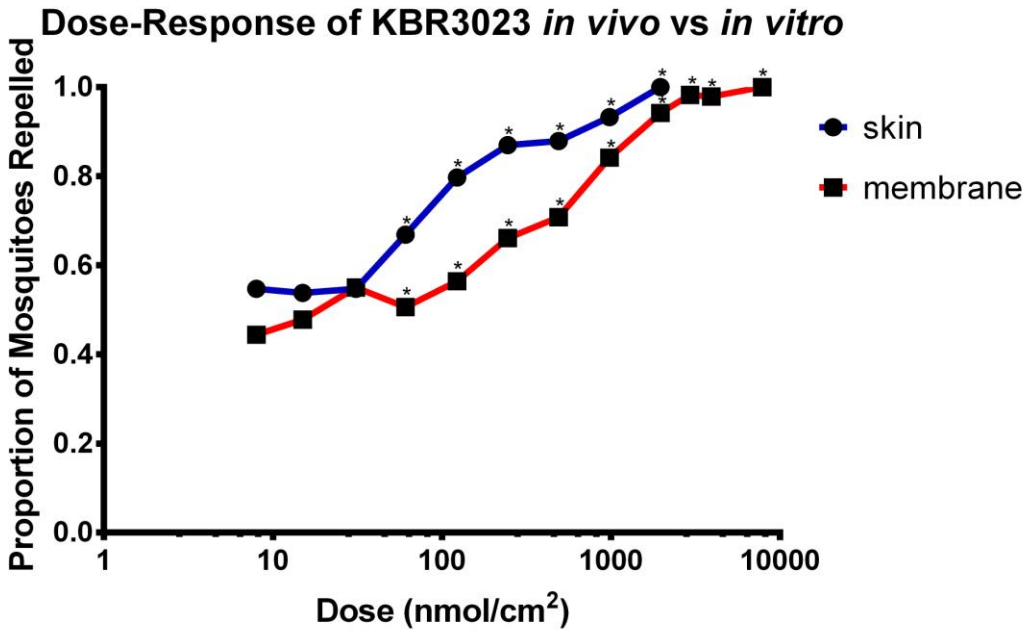


Figure 4-7. Line graph of dose-response data indicating the proportion of mosquitoes repelled by KBR3023 on skin (*in vivo*) and on membrane (*in vitro*) with asterisk indicating where the two media differed from each other significantly ($p \leq 0.05$).

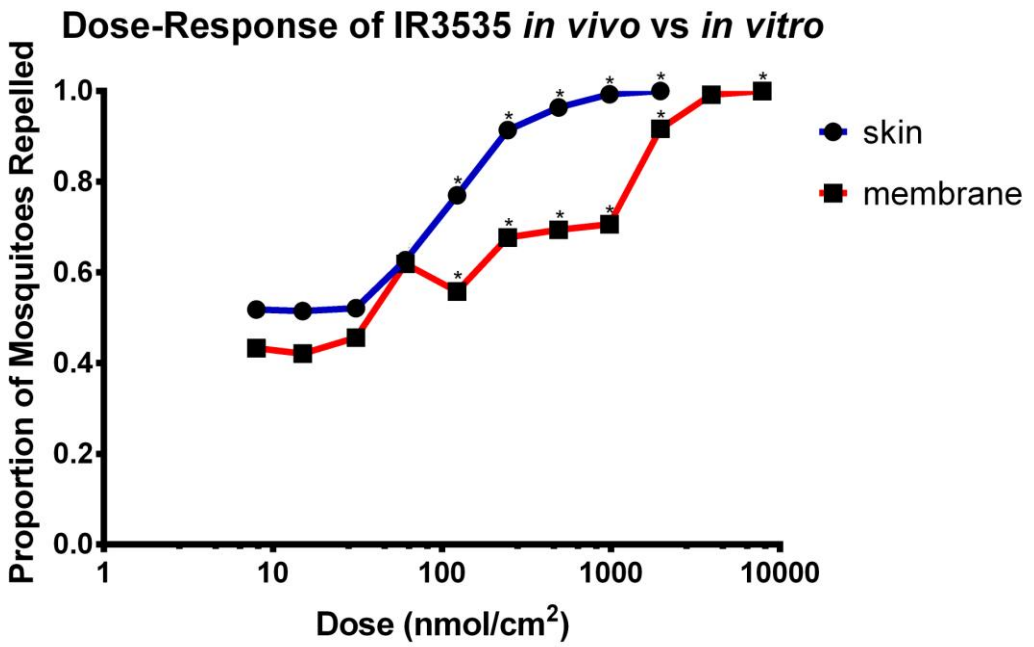


Figure 4-8. Line graph of dose-response data indicating the proportion of mosquitoes repelled by IR3535 on skin (*in vivo*) and on membrane (*in vitro*) with asterisk indicating where the two media differed from each other significantly ($p \leq 0.05$).

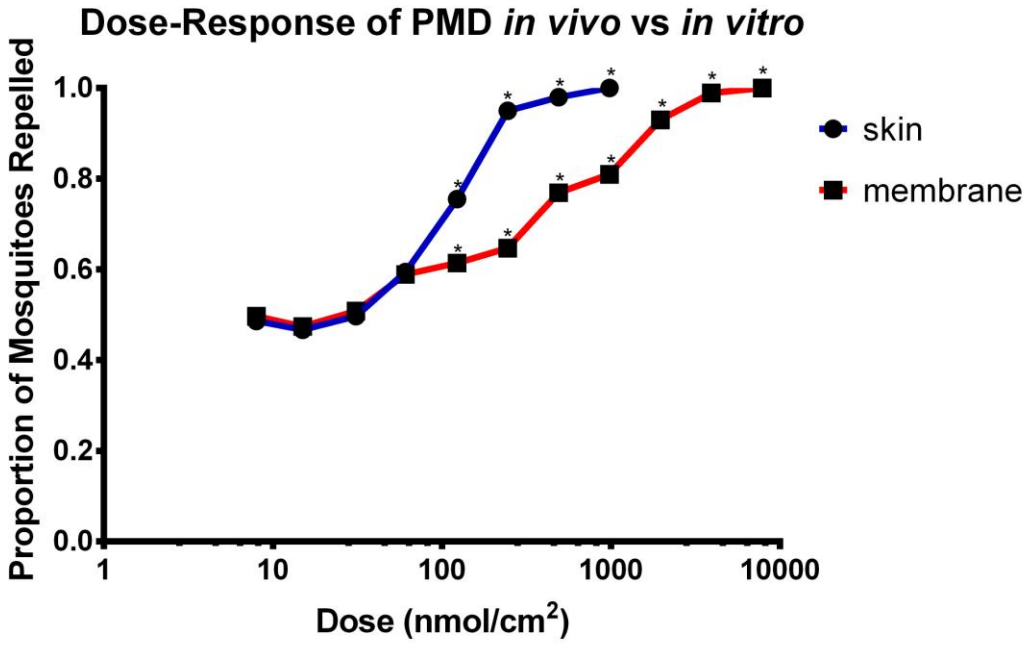


Figure 4-9. Line graph of dose-response data indicating the proportion of mosquitoes repelled by PMD on skin (*in vivo*) and on membrane (*in vitro*) with asterisk indicating where the two media differed from each other significantly ($p \leq 0.05$).

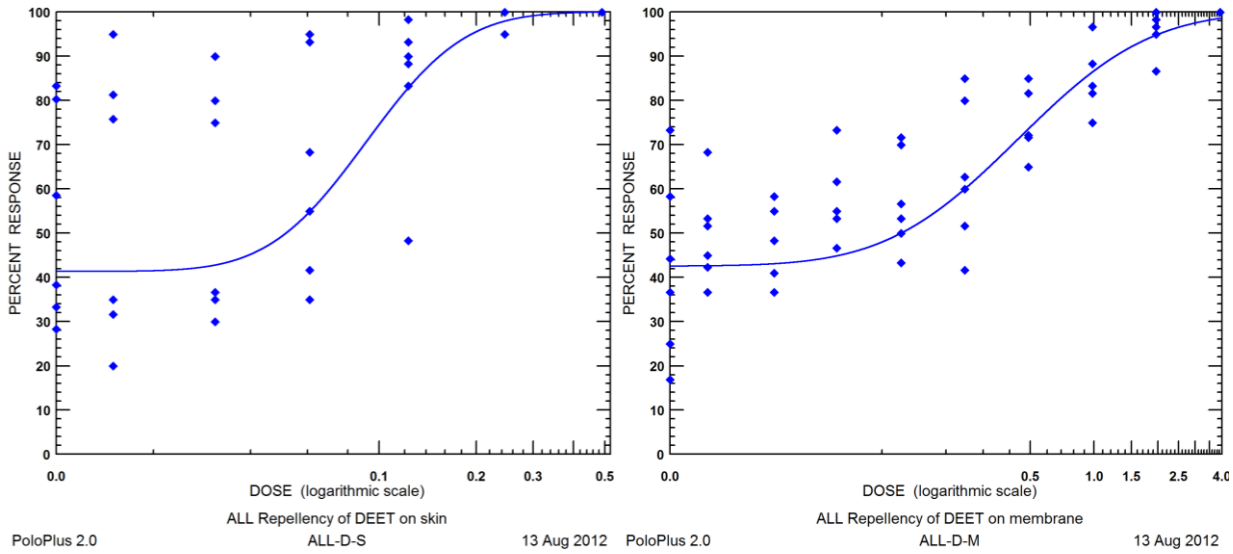


Figure 4-10. Dose-response curves produced by PoloPlus displaying the percentage of mosquitoes repelled by DEET on skin (left) and on the membrane (right) over a range of chemical doses on a log scale.

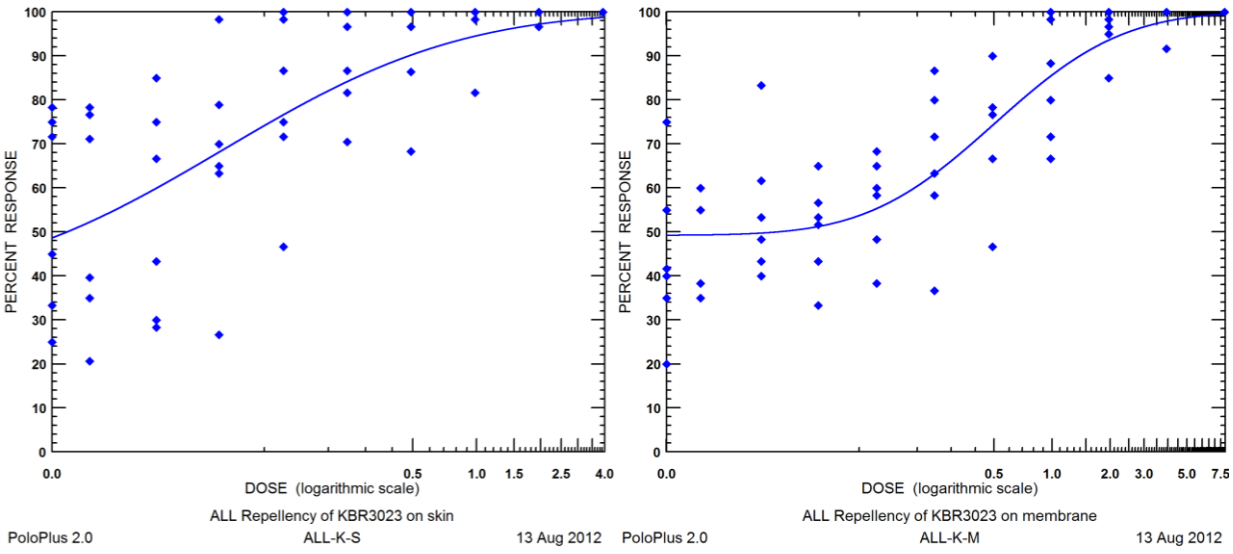


Figure 4-11. Dose-response curves produced by PoloPlus displaying the percentage of mosquitoes repelled by KBR3023 on skin (left) and on the membrane (right) over a range of chemical doses on a log scale.

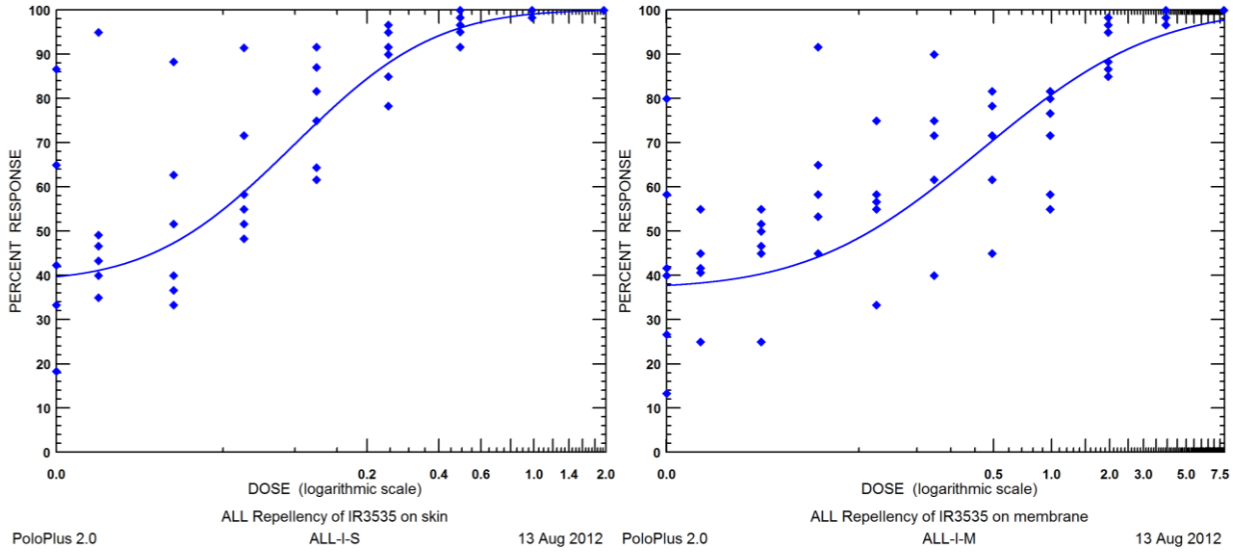


Figure 4-12. Dose-response curves produced by PoloPlus displaying the percentage of mosquitoes repelled by IR3535 on skin (left) and on the membrane (right) over a range of chemical doses on a log scale.

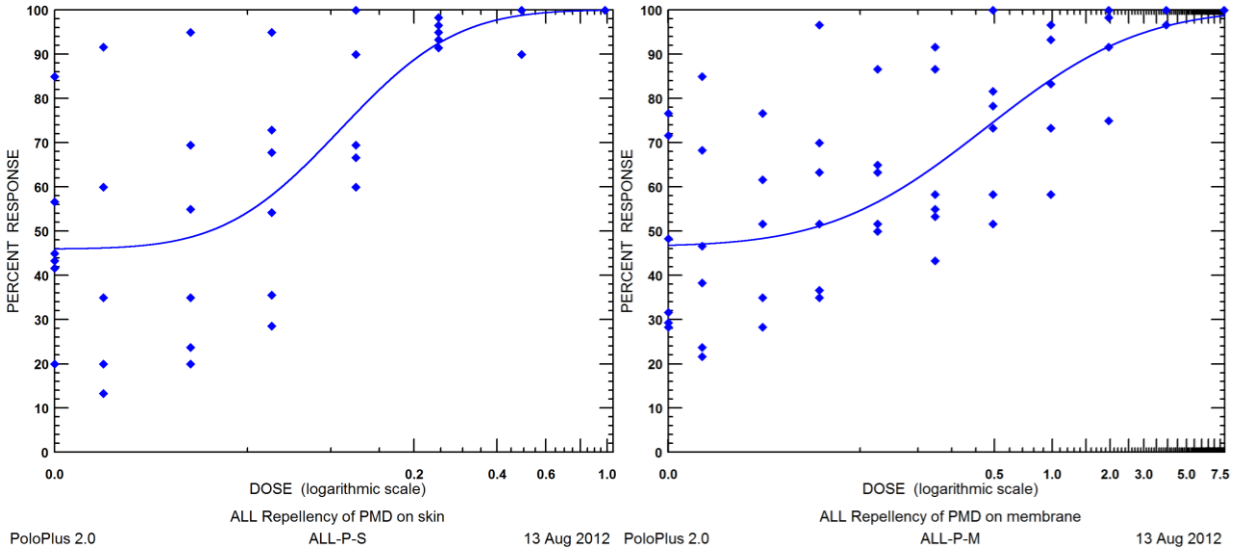


Figure 4-13. Dose-response curves produced by PoloPlus displaying the percentage of mosquitoes repelled by PMD on skin (left) and on the membrane (right) over a range of chemical doses on a log scale.

CHAPTER 5
ASSESSMENT OF THREE MEDIA FOR THE ESTIMATION OF EFFECTIVE DOSE IN MOSQUITO REPELLENT BIOASSAYS

Background

An ideal repellent screening test method should provide a fast, safe, inexpensive way to test chemical compounds, whether toxicity has been established or not, and without requiring the use of human volunteers. Currently, no such testing method exists but advances in mosquito repellent testing have allowed for indirect methods of testing mosquito repellents. Alternative testing strategies have included testing on cloth, membranes, animals of other species, as well as the use of complex human surrogate systems. While many clever testing methods have been fashioned, many of these novel methods have not undergone comparative analysis with skin or with other standardized methods of repellent screening to examine how well the results using the alternative methods accurately predict how well the repellent will perform on the skin.

One method used for the rapid screening of repellent compounds of unknown toxicity is the cloth patch bioassay (Carroll et al. 2011, Katritzky et al. 2008, 2010, Rosa et al. 2012). Although the cloth patch bioassay allows for the presence of human skin volatiles to affect the behavior of the mosquitoes in combination with repellent treatments, complex chemical interactions are not fully explored due to the protective barriers used in these tests. Because of this, potentially synergistic interactions of repellent chemicals with kairomonal skin emanations such as CO_2, lactic acid, acetone, and dimethyl disulfide cannot be explored until the chemicals are deemed safe.

Several areas of arboviral research would benefit from a well-defined correlation of indirect and direct methodologies, particularly in the testing of virus-infected mosquitoes. Since it is not ethical to perform assays with virus-infected mosquitoes on human volunteers using direct testing methods, a well developed indirect method would be beneficial, especially one that

precludes the use of human volunteers. Testing methods that preclude the use of human volunteers would also expand the ability of scientists to test mosquito repellents in their laboratories without the hassle of first obtaining approval from a human-use review board.

Materials and Methods

Mosquito Rearing and Selection

The mosquitoes used in all bioassays were female *Ae. aegypti* (Orlando strain, 1952) from the colony maintained at the Center for Medical, Agricultural, and Veterinary Entomology location of the United States Department of Agriculture, Agricultural Research Service (USDA-ARS-CMAVE) in Gainesville, FL. Pupae were obtained from the colony and kept in laboratory cages where newly emerged mosquitoes were maintained *ad libitum* on a 10% sucrose solution at 25-28^{o}C ambient temperature, 60-80% relative humidity and a 14:10 (light:dark) photoperiod. Nulliparous female mosquitoes aged six to eleven days displaying host seeking behavior were pre-selected from stock cages using a hand-draw box and trapped in a collection trap (Posey et al. 1981).

For use in the module bioassays on skin and membrane, female mosquitoes were then transferred to a smaller cage (28,316 cm^3) from which the mosquitoes were sorted into groups of 10 by mechanically aspirating them into acrylic holding tubes (15 cm long, 1.25 cm wide). Each tube contained approximately 10 mosquitoes, contained by screen gauze on one end and stoppered with a small cork (Fisher Scientific, Catalog No. 07781D Size 1) at the other end. Mosquitoes held in the tubes were allowed to acclimatize for 15-20 min (Barnard et al. 2007) before being transferred into an empty chamber of the module for testing.

For use in the cage tests on cloth, 500 (± 10%) females were preselected and collected in the trap and transferred to a test cage (approximately 59,000 cm^3 with dimensions 45 x 37.5 x 35 cm) and allowed to acclimatize for 15-20 min prior to testing (Barnard et al. 2007).

Repellent Chemical Treatments and Control

Chemical compounds used as repellent treatments included technical *N,N*-diethyl-3-methylbenzamide, 97% (Aldrich, CAS#134-62-3) hereafter referred to as 'DEET', technical 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester, 98% (CAS#119515-38-7) hereafter referred to as 'KBR3023', technical ethyl 3-[acetyl(butyl)amino]propanoate, 98% (CAS#52304-36-6) hereafter referred to as 'IR3535', and technical para-menthane-3,8-diol, 98% (Bedoukian, CAS# 42822-86-6) hereafter referred to as 'PMD'. Serial dilutions of these four chemicals were obtained by adding 1mL of completely denatured ethyl alcohol (Mallinckrodt) into 1mL of the previous concentration of chemical to produce a range of concentrations (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) when applied to the 14.19 cm^2 treatment area. The control treatment consisted of only ethanol.

Skin Bioassays for the Estimation of Effective Dose

In the first portion of this study, the proportion of *Ae. aegypti* mosquitoes blood feeding on the skin of six human volunteers (4 male, 2 female) was evaluated for a range of concentrations to the repellent chemicals DEET, IR3535, KBR3023, and PMD. Ten nulliparous female *Ae. aegypti* mosquitoes (aged 6-11d) were loaded into each of the six testing chambers (5 cm x 5 cm x 5 cm) in the previously described loading module (Klun and Debboun 2000, Klun et al. 2005, Weldon et al. 2003, Rutledge and Gupta 2004). Each of the six chambers in the loading module had two apertures, a 4.25 cm diameter circular aperture at the bottom of each chamber, covered by a sliding door, as well as a small 1.5 cm diameter circular aperture on the front of each chamber closed by a small cork (Fig. 5-1a).

For the skin test, the loading module was placed over the thighs of each human volunteer. On the thighs, areas for treatment were demarcated with 4.25 cm diameter circles corresponding

to the six chambers. A 50 μL ethanol-diluted dose of DEET, IR3535, KBR3023, or PMD (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) was then applied to the six circles in successive layered doses from the lowest concentration to the highest concentration. The first treatment applied in any set of tests was the ethanol control to establish the baseline for mosquito feeding behavior. The dose was allowed to dry for 3-5 min to allow for the ethanol to evaporate. The loading module was placed over the volunteer's thigh with a glass spacer placed between the module and the skin to prevent direct contact of the module with the chemicals. A sliding door was opened under three chambers at a time to expose mosquitoes in those chambers to the repellent for a 3 min period. At the end of the exposure period, the mosquitoes were knocked down with CO_2 gas via the corked hole (Fig. 5-2), removed from the chamber with an aspirator and crushed to record the proportion of mosquitoes blood-feeding out of ten (Fig. 5-3). This process was repeated with the rest of the six chambers and with all concentrations of the repellent until all feeding in all chambers ceased. Volunteers for all repellent tests signed informed consent forms and were enrolled in an IRB study (Project # 636-2005).

Membrane Bioassays for Estimation of Effective Dose

In the second portion of this study which examined the membrane medium, the loading module was placed over the feeding module with six 4.25 cm diameter wells, corresponding to the six circular openings in the loading chamber (Klun and Debboun 2000, Klun et al. 2005, Weldon et al. 2003, Rutledge and Gupta 2004). Each of the wells in the feeding module were filled with 7 mL of citrated bovine blood (Fig. 5-1b), kept at approximately 37˚C by continuously pumping hot water through the feeding chamber with a Cole Parmer Polystat circulating water bath (Fig. 5-4). Prior to testing, strips of silicone membranes were worn against the upper thigh by the volunteers for 3-4 h, held in place with an Ace™ elastic bandage.

The worn silicone membranes were placed across the six wells of the feeding module to come into contact with the blood, and the glass spacer was placed over the membranes leaving only the membrane-covered well area exposed. A 50 μL ethanol-diluted dose of DEET, IR3535, KBR3023, or PMD (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) was then applied to the six circles on the silicone membrane above each well in successive layered doses from the lowest concentration to the highest. The first treatment applied in any set of tests was the ethanol control to establish the baseline for mosquito feeding behavior. The dose was allowed to dry for 3-5 min to allow for the ethanol to evaporate. The loading module was placed onto the feeding module and lined up to correspond to each well. A sliding door was opened under all six chambers to expose the mosquitoes to the repellent treatment for a 3 min period. At the end of the exposure period, the mosquitoes were knocked down with CO_2 gas via the corked hole, removed from the chamber with an aspirator and crushed to record the proportion of mosquitoes blood-feeding out of ten. This process was repeated with the rest of the six chambers and with all concentrations of the repellent until all feeding in all chambers ceased.

Cloth Bioassays for Estimation of Minimum Effective Dose

The third portion of this study examined the estimation of minimum effective dose for repellency on cloth. In preparation for these tests, 2 dram screw -top glass vials containing the 1mL ethanol-diluted doses of DEET, IR3535, KBR3023, or PMD (7840 nmol/cm^2, 3920 nmol/cm^2, 1960 nmol/cm^2, 980 nmol/cm^2, 490 nmol/cm^2, 245 nmol/cm^2, 123 nmol/cm^2, 61 nmol/cm^2, 31 nmol/cm^2, 15 nmol/cm^2, 8 nmol/cm^2) were arranged and a 50 cm^2 (5 cm x 10 cm) piece of muslin cloth was rolled up and placed inside each vial to soak up the chemical treatment. Just prior to the experiment, the pieces of treated cloth were removed from the vials and card stock tabs (5 cm x 3 cm) were stapled onto them lengthwise. These cloth patches were

hung on a drying rack using masking tape for 5 min to allow the ethanol to volatilize off, leaving only the chemical treatment on the dry cloth.

Volunteers in the study used latex gloves to pull a nylon stocking over their arm and a Velcro™-sealed vinyl sleeve was then placed over their forearm (Fig. 5-5). The sleeve had a 32-cm^2 (4 cm x 8cm) window to allow attractive skin odors to draw mosquitoes to that open area. The purpose of the nylon stocking was to produce a barrier between the dried cloth and the skin, thereby avoiding direct contact of chemical to skin. Gloves were worn to protect the sensitive hands of the volunteers which would be bitten by the mosquitoes if not covered (Fig. 5-6). The dried cloth patch was then attached with masking tape over the opening in the sleeve. Participants then inserted their arm with the sleeve and patch into a screened cage that contained 500 female *Ae. aegypti* mosquitoes (Fig. 5-7).

Tests were conducted on each control or treated patch for 1 min periods. A control patch treated only with ethanol was tested prior to the start of experiments (Fig. 5-8a). When testing a treated patch, if approximately 1% or 5 mosquito bites were received within 1 min, the chemical dose was considered to have failed, i.e. was not repellent at that concentration (Fig. 5-8b). If a treated cloth patch received 0-4 bites within a minute, then it was considered as passed, i.e., repellent at that concentration. A median concentration treated patch was tested in the first round and treated patches were then tested successively at higher or lower concentrations depending upon whether the previous patch failed or passed, respectively. The estimate of the minimum effective dose, or ED_{99} was the lowest concentration that passed for each repellent chemical. This process of estimating an ED_{99} was replicated five times for each volunteer in the study.

Statistical Analysis

Pooled data from repellency analysis was used to estimate the various effective doses for each treatment for the three media examined. Effective doses at 50, 95, and 99 were estimated

for the skin and membrane bioassays using a dose response curve model with a probit link. The equation used for the model is:

probit(y/n) = ln (Dose_nmol +100)

where y is the pooled number of mosquitoes repelled at each treatment group across all replicates and all volunteers, n is the pooled total number of mosquitoes exposed at each treatment group across all replicates and all volunteers (Table 5-1), and Dose_nmol is the concentration of repellent chemical to which mosquitoes were exposed at each concentration interval. Estimations of the effective dose at 50, 95, and 99 (ED_{50}, ED_{95}, and ED_{99}) were made using SAS 9.2 (SAS 2012). Estimation of 95% confidence intervals (CI 95) for each treatment and effective dose from the skin and membrane bioassays were calculated using trial and error in SAS 9.2. The significance at a confidence of p=0.05 for the model term effects, as well as their interactions were evaluated using an approximated F-test. PoloPlus v2.0 was also used to produce dose-response curves for secondary confirmation of effective dose estimations at 50, 95, and 99 levels for the skin and membrane media (PoloPlus 2012).

Pooled data was used to estimate the MED on the cloth medium for each repellent compound tested. Effective doses at 50 and 95 could not be determined for this test since only the point estimate at ED_{99} was estimated. The ED_{99} values for each repellent chemical were then averaged for the five replicates and then across the six study volunteers, and reported as the mean ED_{99}. The 95% confidence intervals were estimated using twice the value of the standard deviation ± the mean ED_{99}.

Results

Mean values for the ED_{50}'s and ED_{95}'s ($nmol/cm^2$) for the four repellent chemicals tested were estimated for the skin and membrane media using data pooled from all six volunteers (Table 5-2, 5-3). Ratios comparing the ED_{50} and ED_{95} results on membrane to the skin for the

four repellent chemicals were also included (Table 5-2, 5-3). Mean values for the ED_{99}'s ($nmol/cm^2$) for the four repellent chemicals tested were estimated for the skin, membrane and cloth media using pooled data as well (Table 5-4). Ratios comparing the ED_{50} and ED_{95} results from membrane and cloth to the skin as well as from membrane and skin to the cloth for the four repellent chemicals were also included (Table 5-4). Secondary confirmation of the estimated ED_{50}, ED_{95}, and ED_{99} values on skin and membrane were supported by estimates provided by PoloPlus using the dose-response curves data described in the previous chapter. Bar graphs were used to illustrate the differences in the mean ED_{99}'s estimated for each of the repellent treatments on skin, membrane and cloth (Figure 5-9). The ED_{99} results for the skin and cloth were more similar than either skin or cloth to the membrane, and these fine differences were highlighted in a second bar graph which excluded the membrane values (Figure 5-10).

Discussion

The objective of this portion of the study was to compare the skin and membrane bioassay results with the results of a standard method to assess the usefulness of this alternative method in estimating repellent dose. This study allowed for a comparison of three methods using different media to estimate minimum effective dose (ED_{99}). Only two media, the skin and the membrane could be compared at the median effective dose (ED_{50}) and the 95% effective dose (ED_{95}). Effective doses at 50 and 95 could not be determined for the cloth patch bioassay since this type of assay is used only to estimate the ED_{99} dose. These three dose levels are those that are most commonly reported in repellency and toxicity studies, although toxicity studies report lethal dose levels (LD).

A comparison of the ED_{99}'s indicates that the minimum effective doses estimated on membrane were much higher than those of the same chemical repellents applied to skin or cloth.

There are potentially for two reasons for this discrepancy. One reason that the results of the membrane ED_{99} are not comparable to the cloth results, as was alluded to in the previous chapter previously since the membrane results were statistically different from the results on skin possibly above at least 31 nmol/cm^2. It is possible that allomonal chemicals produced by the skin adversely effected the concentration of repellent needed to achieve full protection, both in the skin as well as in the cloth bioassays. While the bioassays using cloth did not have repellent directly applied to the skin surface, the skin of the volunteers was available to the mosquitoes to bite, as well as to provide odors for attraction or possibly repulsion via allomonal chemicals. The presence of these human skin volatiles could have affected the behavior of the mosquitoes in combination with repellent treatments.

A second reason for the discrepancy may lie in the validity of the ED_{99} estimation. The ED_{99} results for the membrane contain a high level of inaccuracy due to estimation of a point in an area of the dose response curve where small changes in response (repellency) occur over a relatively larger range of concentrations. According to Robertson et al., the sample size necessary for an accurate estimation of the ED_{95} from a dose-response curve requires a sample size of 600-1000 to determine significance (1984, 2007). This means that for the estimation of an ED_{99}, more than 1000 samples, likely between 2000-5000 samples would be necessary for accurate estimation of an ED_{99} using a dose-response curve. This sacrifice in accuracy is accepted since the number of mosquitoes and replicates required to produce an estimate with a high level of confidence was beyond the time and mosquito resources available for this study.

For an accurate estimation of ED_{50}, however, a sample size of only 300-500 is necessary to determine significance (Robertson et al. 1984, 2007), which was easily achieved in all the bioassay runs since the average number of mosquitoes tested per volunteer per treatment was

between 400-700 (Table 5-1). For this reason, the ED_{50} estimates on the skin and membrane are more likely to predict the median effective dose and the ED_{99} is likely a less precise indicator since these were estimated from dose-response curves, which is further supported by the wide confidence intervals calculated for both skin and membrane bioassays at the ED_{95} and ED_{99} levels (Table 5-3, 5-4).

While the estimated ED_{99}'s for the skin and cloth were more similar than either of these to the membrane results, the wider confidence intervals for the skin indicate a lower degree of precision when tested by this method. Thus, the cloth module bioassay appears to produce the best estimation of ED_{99} of these three bioassays for mosquito repellent screening. Interestingly, the ED_{99} values estimated on cloth in this study were roughly two times higher than estimates for these same four repellent chemicals tested by the same method, but using acetone as a solvent (Agramonte and Bernier, unpublished data).

The density of mosquitoes within each testing chamber for all three bioassays was 0.08 mosquitoes per cm^3 or 1 mosquito per 12.5 cm^3 for the modules tests using skin and membrane, and 0.008 mosquitoes per cm^3 or 1 mosquito per 118 cm^3 for the cage tests using cloth (Table 5-5). This means that the mosquito density for module bioassays is approximately 10 times greater than the density of mosquitoes used in the cage tests with cloth. Further testing should examine ED_{99}'s on skin in larger test cages, such as those in the cloth patch bioassay, to rule out the effect of higher mosquito density or limited ventilation in the module.

Table 5-1. Total number of mosquitoes bioassayed per treatment for six human volunteers.

Treatment[1]	Total Mosquitoes Tested[2] (N)
DEET-skin	2570
KBR3023-skin	2574
IR3535-skin	3298
PMD-skin	2800
DEET-membrane	3900
KBR3023-membrane	3840
IR3535-membrane	4018
PMD-membrane	3714
DEET-cloth	2500[3]
KBR3023-cloth	2500[3]
IR3535-cloth	2500[3]
PMD-cloth	2500[3]

[1] Treatment defined as repellent chemical applied and medium on which it was applied
[2] Average number of mosquitoes tested per volunteer per treatment is ~400-700
[3] Number of mosquitoes per test cage in cloth patch bioassays was 500 (±25)

Table 5-2. Mean ED_{50} (95% CI) and ED_{50} ratios for four chemicals estimated using repellency bioassay data (nmol/cm^2) pooled from six human volunteers on membrane and skin.

Medium	DEET	KBR3023	IR3535	PMD
Skin	11.55 (1.45-20.28)	4.05 (-14.20-20.46)	17.04 (4.41-28.74)	20.28 (8.53-31.14)
Membrane	43.05 (17.68-69.02)	35.07 (7.39-63.37)	60.72 (30.26-92.87)	23.73 (-3.91-51.41)
Membrane: Skin Ratio	3.73	8.66	3.56	1.17

Table 5-3. Mean ED_{95} (95% CI) and ED_{95} ratios for four chemicals estimated using repellency bioassay data (nmol/cm^2) pooled from six human volunteers on membrane and skin.

Medium	DEET	KBR3023	IR3535	PMD
Skin	218.14 (169.89-307.89)	751.64 (519.55-1278.84)	385.25 (299.41-537.78)	341.19 (261.04-491.70)
Membrane	2480.87 (1681.12-4185.53)	3448.89 (2182.44-6520.99)	4190.35 (2721.43-7477.97)	3498.19 (2137.24-7050.95)
Membrane: Skin Ratio	11.37	4.59	10.88	10.25

Table 5-4. Mean ED_{99} (95% CI) and ED_{99} ratios to skin and cloth for four chemicals estimated using repellency bioassay data (nmol/cm^2) pooled from six human volunteers on membrane, skin and cloth.

Medium	DEET	KBR3023	IR3535	PMD
Skin	391.13 (287.61-606.27)	1934.65 (1174.11-4075.54)	774.74 (561.82-1202.45)	655.94 (467.93-1052.86)
Membrane	8456.34 (4914.05-17754.31)	13648.87 (7159.02-34100.65)	16629.62 (9082.00-38077.44)	14436.30 (7159.02-40034.84)
Cloth	239.00 (173.97-304.03)	258.67 (-211.24-441.28)	1494.67 (130.81-3865.76)	381.33 (38.13-567.88)
Membrane: Skin Ratio	21.63	7.06	21.48	22.04
Membrane: Cloth Ratio	35.38	52.90	11.13	37.89
Skin: Cloth Ratio	1.64	7.50	0.52	1.72

Table 5-5. Densities calculated for all three bioassay types using the internal volume of each test chamber or cage (cm^3) and the number of mosquitoes used for each test (N).

Bioassay Type	N (mosquitoes)	Volume (cm^3)	Density (N/cm^3)
Module Test on Skin	10	125	0.080
Module Test on Membrane	10	125	0.080
Cage Test on Cloth	500	59000	0.008

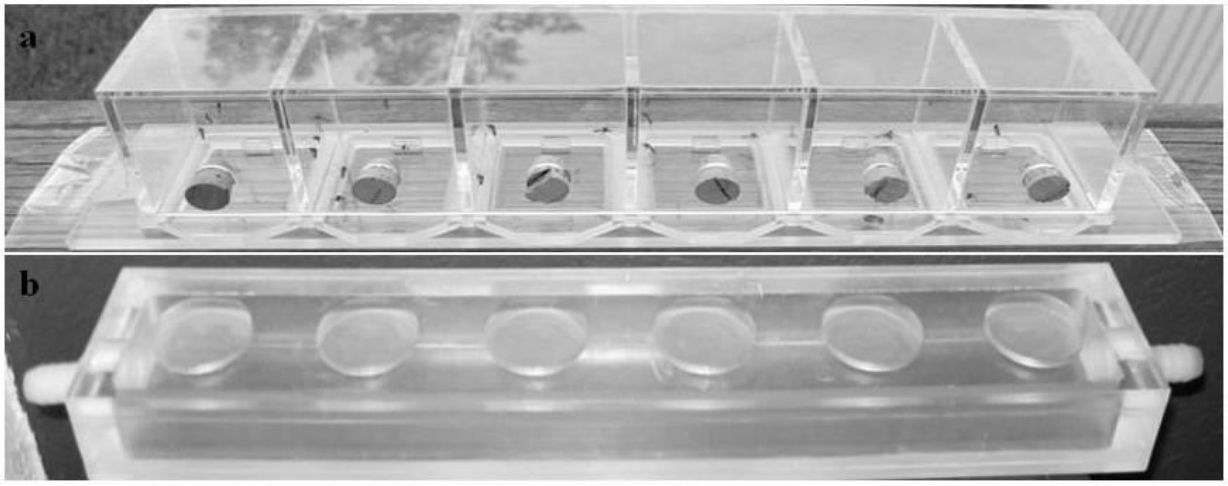


Figure 5-1. Pictures of *in vitro* loading and feeding modules. a) Mosquito loading module with six testing chambers corked and containing mosquitoes. b) Mosquito feeding module with 6 well receptacles for blood feeding. *Photos courtesy of Natasha M. Agramonte.*

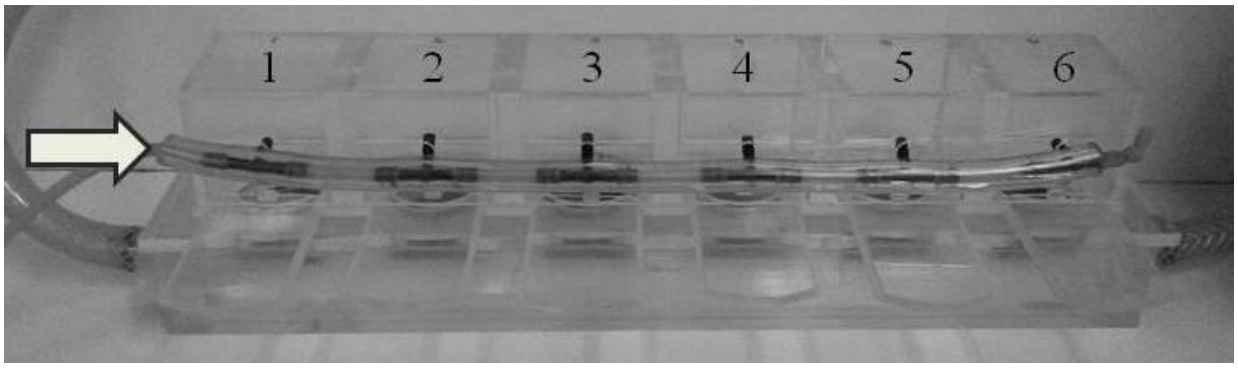


Figure 5-2. Assembled mosquito loading and feeding modules with arrow indicating the CO_2 tubing connected in parallel to the front of the six testing chambers via the cork hole. *Photo courtesy of Natasha M. Agramonte.*

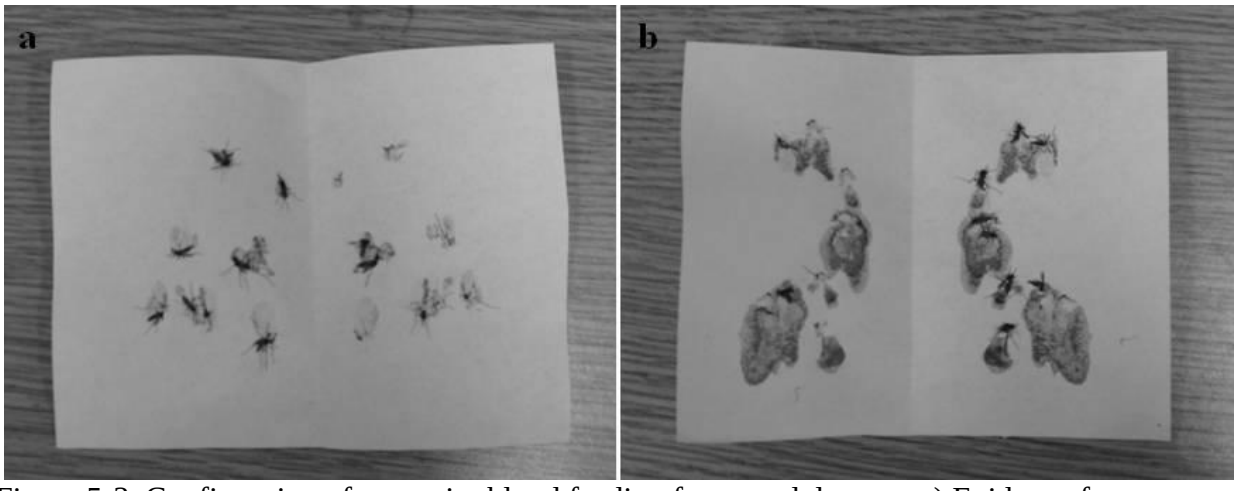


Figure 5-3. Confirmation of mosquito blood feeding from module tests. a) Evidence from module test indicating 0 mosquitoes feeding. b) Evidence from module test indicating 10 mosquitoes feeding. *Photos courtesy of Natasha M. Agramonte.*

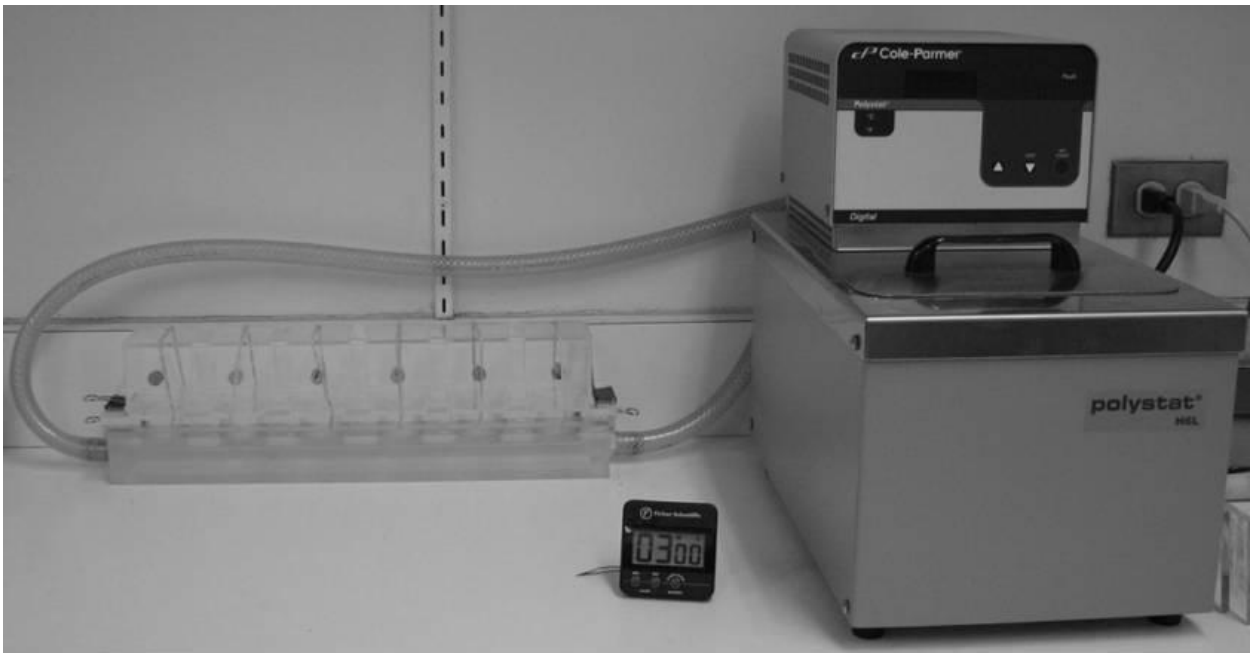


Figure 5-4. Assembled mosquito loading and feeding modules connected to heated water circulator via tubing for *in vitro* bioassay, showing rubber elastic bands placed around each of the six testing chambers. *Photo courtesy of Natasha M. Agramonte.*

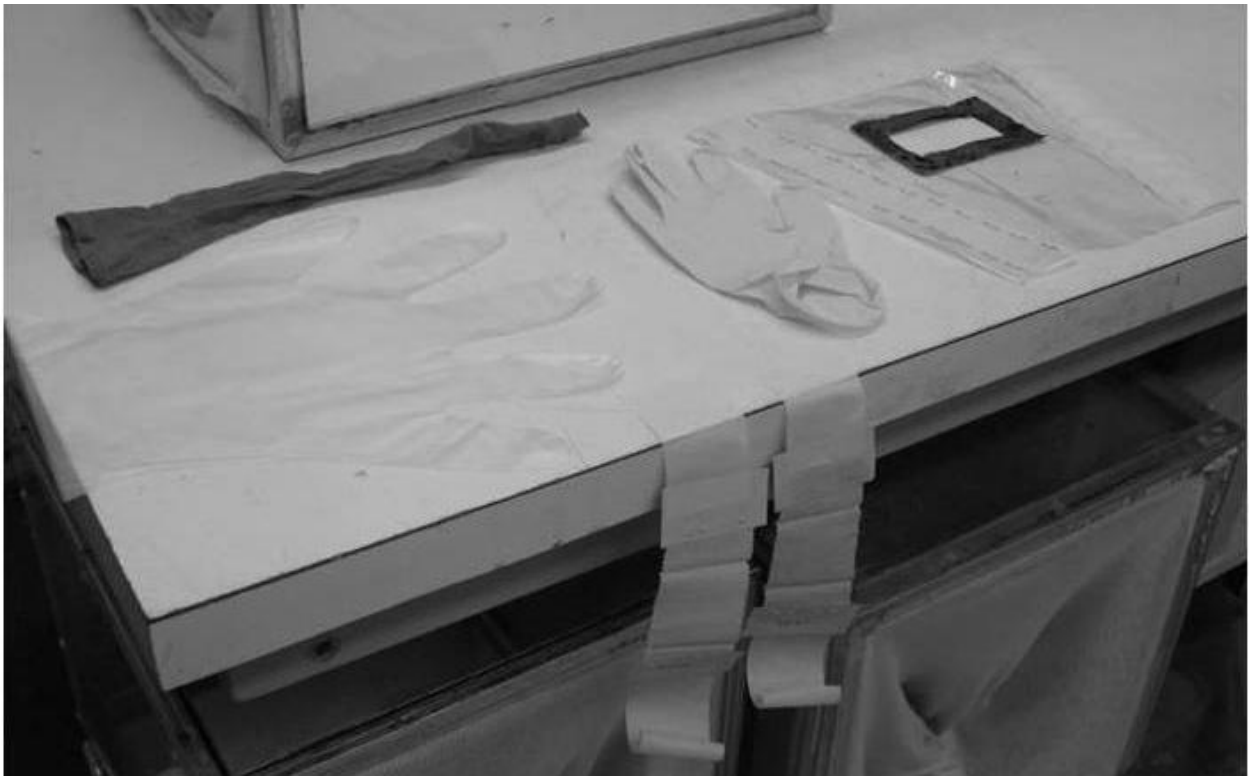


Figure 5-5. Materials used for the cloth patch bioassay. *Photo courtesy of Greg Allen.*

Figure 5-6. *Ae. aegypti* feeding behavior on hands without (left) and with (right) repellent DEET. *Photo courtesy of Greg Allen.*

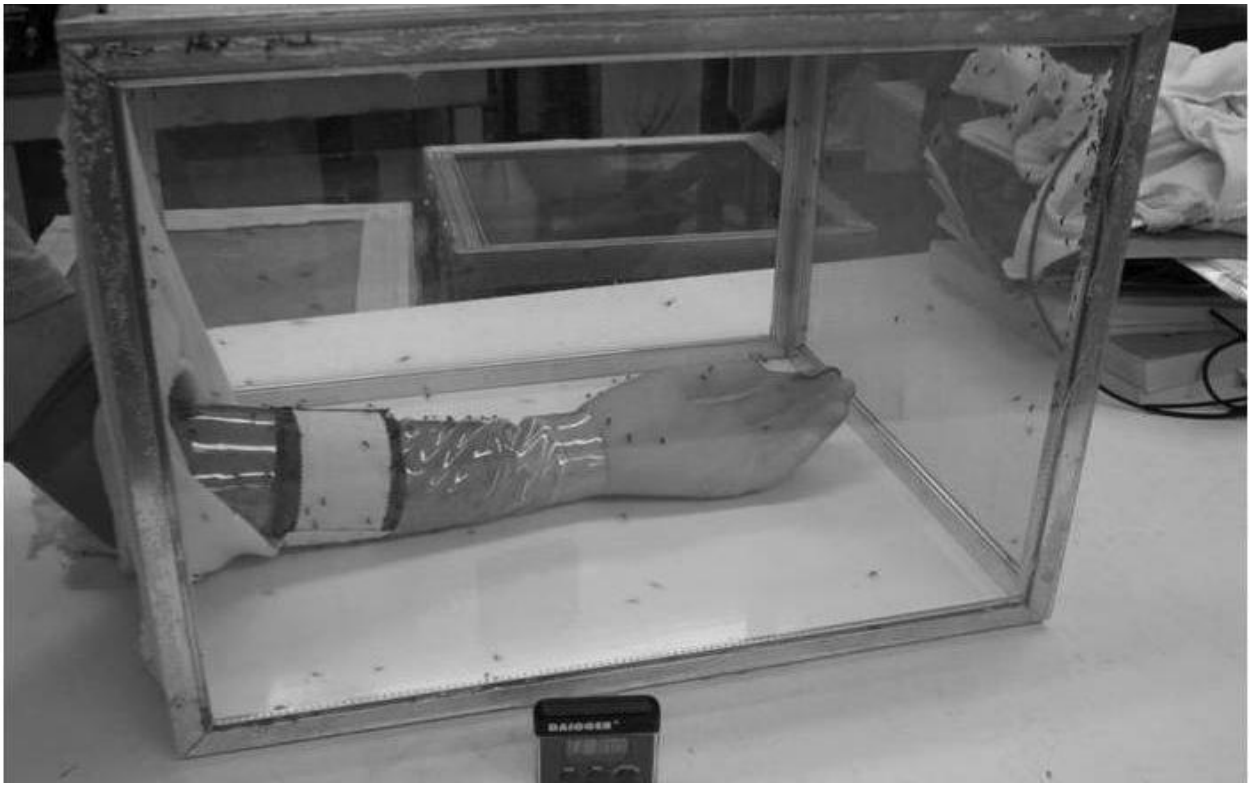


Figure 5-7. Example of cloth patch bioassay in progress. *Photo courtesy of Greg Allen.*

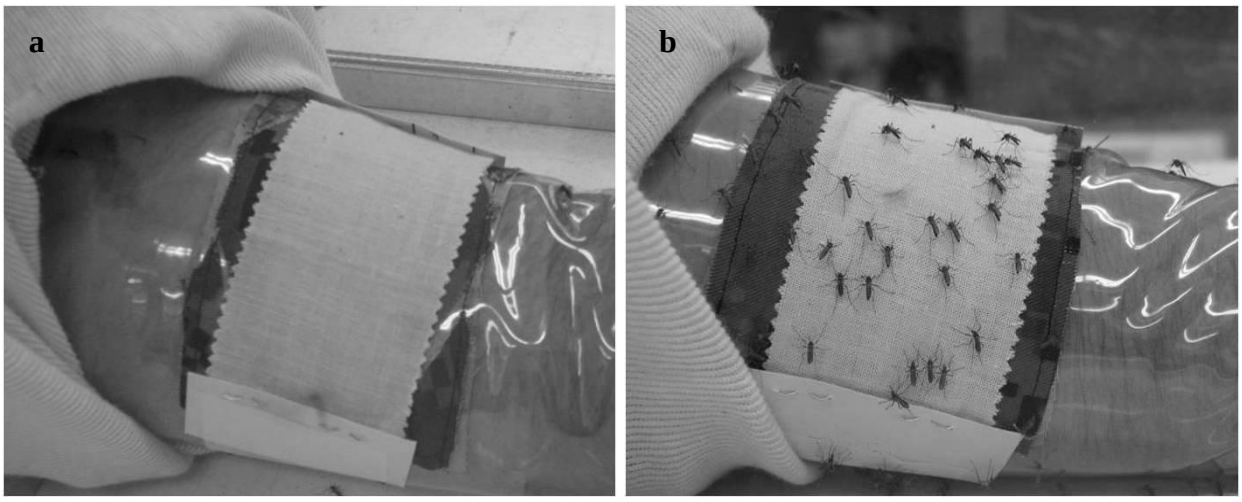


Figure 5-8. Cloth patch test showing confirmation of mosquito blood feeding. a) Cloth patch with 0 mosquitoes blood-feeding. b) Cloth patch with > 5 mosquitoes blood-feeding. *Photos courtesy of Greg Allen.*

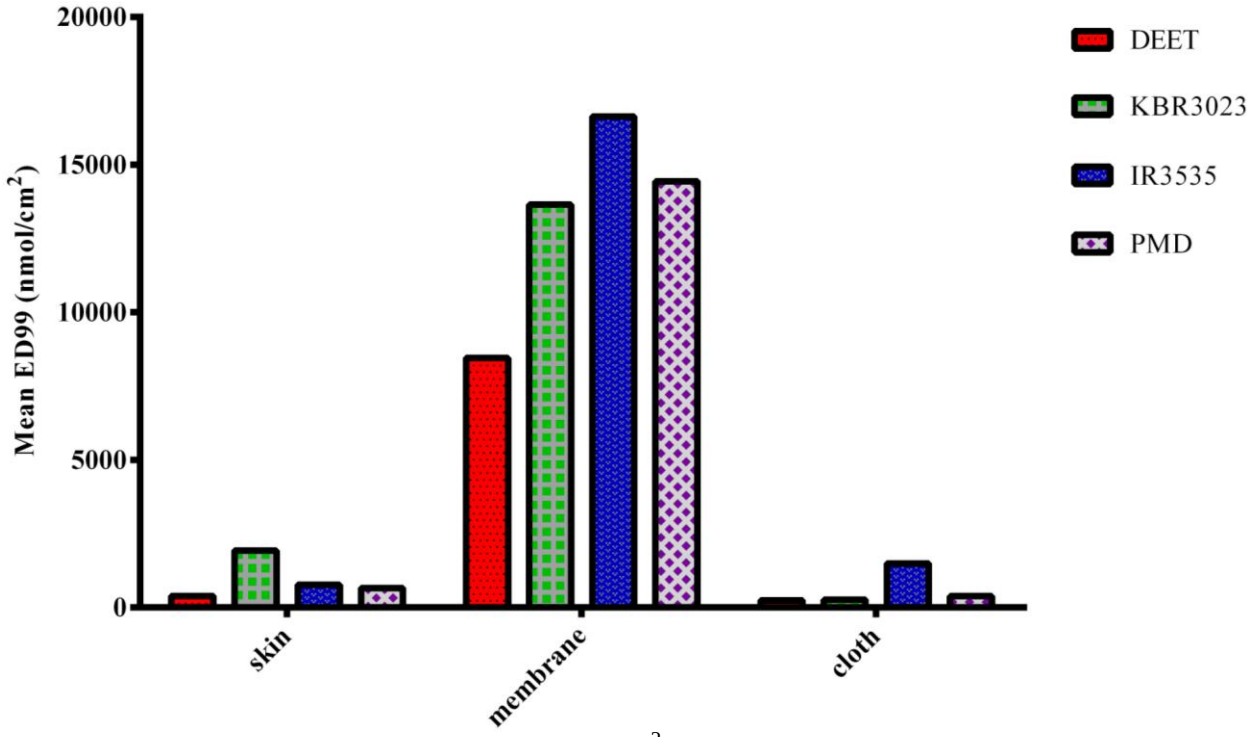


Figure 5-9. Bar graph of the mean ED_{99} (nmol/cm^2) estimated for each of the four repellent treatments evaluated on three media.

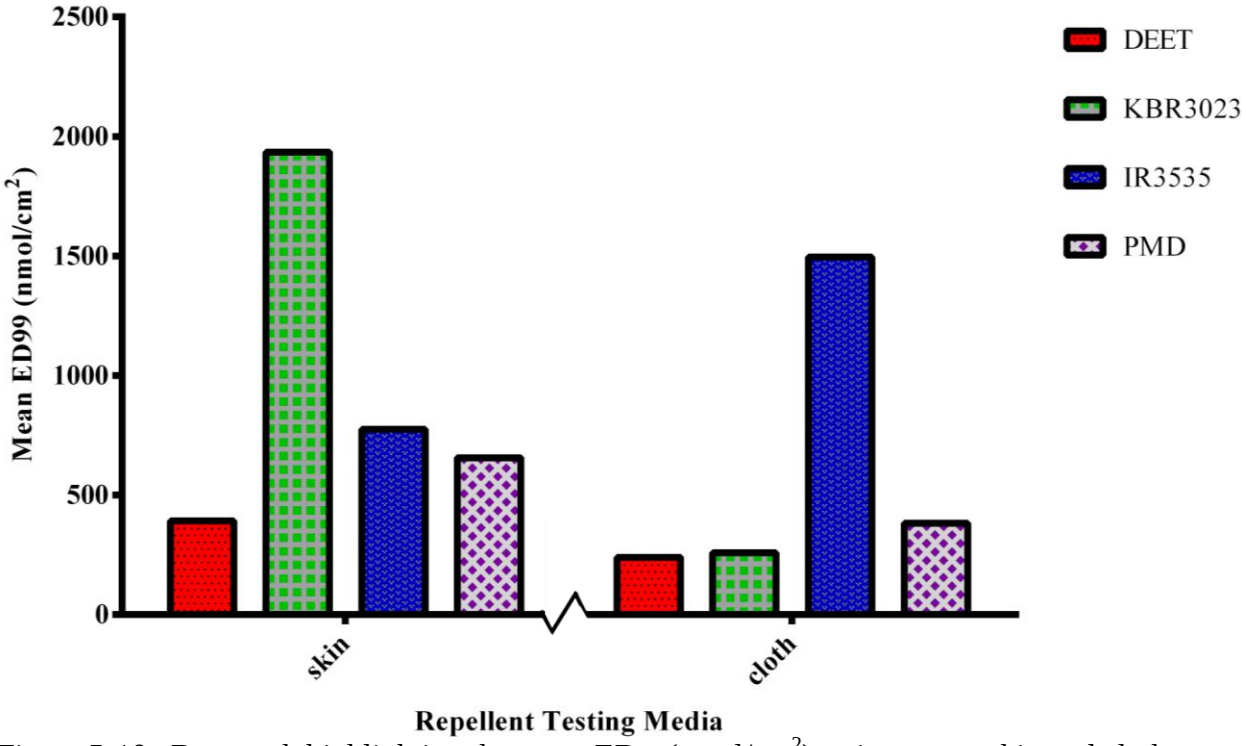


Figure 5-10. Bar graph highlighting the mean ED_{99} (nmol/cm^2) estimates on skin and cloth media for the four repellent treatments rescaled for direct comparison.

CHAPTER 6
SUMMARY OF FINDINGS AND IMPLICATIONS FOR FUTURE RESEARCH

The purpose of this research was to examine a silicone membrane blood-feeding system and its effectiveness as an alternative to application of repellents directly on human skin. A few standardized *in vivo* repellency assay methods exist which have directly utilized humans in testing repellents on the skin; however, these had not been shown to be sufficiently comparable to *in vitro* repellency assay methods. The goal of this project was to examine the results obtained from an *in vitro* assay method to determine the level at which results from this method compare to *in vivo* testing results in an effort to preclude the use of human volunteers in these kinds of studies.

Based on literature on mosquito host seeking behavior, it was logical to hypothesize that use of a silicone membrane treated with human odors and placed over a warmed blood source would produce similar repellent responses in the *Aedes aegypti* mosquito when compared to human skin. The efforts to transfer some of the attractive skin chemicals onto the membranes appeared to have been successful, since the attractiveness of the membrane tested without human odors using both blood and a 10% sugar solution, did differ significantly from the tests on human skin and from membranes treated with attractive skin chemicals. Additionally, the baseline attraction for the membranes worn by the volunteers was found not to differ statistically from the attractiveness for tests performed on the skin of these same volunteers.

This project also sought to create dose-response curves for four major repellent chemicals and examine if these could simulate the results of testing on skin. A comparison of the ED_{99}'s indicated that the minimum effective doses estimated on membrane were much higher than those of the same chemical applied to skin or cloth. Based on the comparative assessment of three repellent screening media, namely cloth, membrane and skin, the cloth bioassay appears to

produce the best estimation of the results on skin as well as the most accurate method for estimation of ED_{99} values from these bioassays for mosquito repellent screening.

All four repellents tested on the membrane required much higher doses of repellent chemicals to reach full protection from mosquito blood-feeding, *i.e.* 100% of the mosquitoes deterred from feeding. This is interesting because it is unclear whether the membrane became more desirable to the mosquitoes than the skin at these doses or if perhaps the skin became less desirable to the mosquitoes. If these are the result of allomonal compounds produced from human skin it was unclear whether any of these allomonal compounds were transferred on the membranes or if they are produced at higher rates when humans undergo stress, *i.e.* being bitten by mosquitoes, similar to a plant's use of defensive chemicals for feeding deterrence by insects. Also, early data from the individual volunteers showed evidence of an increase in feeding behavior that was inconsistent with the increased application of repellent chemical. This occurred mostly at the dose level just prior to the statistically significant divergence of the skin and membrane curves from each other. These unexpected results may be due to potentially attractive properties of repellent chemicals at very low doses. A third question of interest was whether the solvent the repellent in dissolved in plays a role in the effect of a repellent, since the ED_{99} values estimated on cloth in this study were roughly two times higher than estimates for these same four repellent chemicals tested by the same method, but using acetone as a solvent.

The human skin-chemical interactions with the repellent compounds could be further examined in additional studies with the membrane module system by applying a standard human-derived chemical treatment to the membrane along with repellents to examine if it produces results more similar to the skin at dose applications higher than 31 nmol/cm^2. Further studies with lower doses of KBR3023, IR3535, and PMD should be conducted to test whether

these compounds are also attractive to mosquitoes at low concentrations. Finally, further testing should examine ED_{99}'s on skin in larger test cages, such as those in the cloth patch bioassay, to rule out the effect of higher mosquito density or limited ventilation in the module and the effect of solvent on the minimum effective dose of a repellent chemical. A dose-response study could be undertaken with the cloth patch assay to better establish how well this method compares to *in vivo* module assays. Finally, a dose-response study could be conducted *in vivo* with direct topical application of these compounds since all four repellents are registered for use on human skin.

LIST OF REFERENCES

Ahmadi, A., and G. McClelland. 1985. Mosquito-mediated attraction of female mosquitoes to a host. Physiol. Entomol. 10: 251-255.

American Society for Testing and Materials [ASTM]. 2000a. ASTM E939 – 94, Standard test method of field testing topical applications of compounds as repellents for medically important and pest arthropods (including insects, ticks, and mites):1 mosquitoes. ASTM International, West Conshohocken, PA, 2003, DOI: 10.1520/E0939-94R12, www.astm.org.

American Society for Testing and Materials [ASTM]. 2000b. ASTM E951 – 94, Standard test methods for laboratory testing of non-commercial mosquito repellent formulations on the skin. ASTM International, West Conshohocken, PA, 2003, DOI: 10.1520/E0951-94R06, www.astm.org.

Barnard, D. R. 2005. Biological assay methods for mosquito repellents. J. Am. Mosq. Control Assoc. 21: S12-S16.

Barnard, D. R., U. R. Bernier, R. Xue, and M. Debboun. 2007. Standard methods for testing mosquito repellents, pp. 103-110. *In* M. Debboun, S. P. Frances and D. Strickman (eds.), Insect repellents: principles, methods, and uses. CRC Press, Boca Raton, FL.

Barnard, D. R., and R. Xue. 2004. Laboratory evaluation of mosquito repellents against *Aedes albopictus*, *Culex nigripalpus*, and *Ochlerotatus triseriatus* (Diptera: Culicidae). J. Med. Entomol. 41: 726-730.

Bernier, U. R., K. D. Furman, D. L. Kline, S. A. Allan, and D. R. Barnard. 2005. Comparison of contact and spatial repellency of catnip oil and *N*,*N*-diethyl-3-methylbenzamide (DEET) against mosquitoes. J. Med. Entomol. 42: 306-311.

Bernier, U., D. Kline, S. Allan, and D. Barnard. 2007a. Laboratory comparison of *Aedes aegypti* attraction to human odors and to synthetic human odor compounds and blends. J. Am. Mosquito Contr. 23: 288-293.

Bernier, U. R., D. L. Kline, D. R. Barnard, C. E. Schreck, and R. A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the Yellow Fever mosquito (*Aedes aegypti*). Anal. Chem. 72: 747–756.

Bernier, U. R., D. L. Kline, and K. H. Posey. 2007b. Human emanations and related natural compounds that inhibit mosquito host-finding abilities, pp. 77-102. *In* M. Debboun, S. P. Frances and D. Strickman (eds.), Insect repellents: principles, methods, and uses. CRC Press, Boca Raton, FL.

Bernier, U., D. Kline, K. Posey, M. Booth, R. Yost, and D. Barnard. 2003. Synergistic attraction of *Aedes aegypti* (L.) to binary blends of L-lactic acid and acetone, dichloromethane, or dimethyl disulfide. J. Med. Entomol. 40: 653-656.

Bernier, U., D. Kline, C. Schreck, R. Yost, and D. Barnard. 2002. Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). J. Am. Mosquito Contr. 18: 186-195.

Bernier, U. R., and M. Tsikolia. 2011. Development of novel repellents using structure-activity modeling of compounds in the USDA archival database, pp. 21-46. *In* J. R. Coats, and G. E. Paluch (eds.), Recent developments in invertebrate repellents. American Chemical Society: Washington, D.C.

Bowen, M. F. 1991. The sensory physiology of host-seeking behavior in mosquitoes. Annu. Rev. Entomol. 36: 139-158.

Braks, M., C. Koenraadt, O. Briet, and W. Takken. 1999. Behavioral responses of *Culex quinquefasciatus* (Diptera: Culicidae) to human odour, carbon dioxide and lactic Acid. Exp. Appl. Entomol. 10: 91-97.

Braks, M., J. Meijerink, and W. Takken. 2001. The response of the malaria mosquito, *Anopheles gambiae*, to two components of human sweat, ammonia and L-lactic Acid, in an olfactometer. Physiol. Entomol. 26: 142-148.

Braks, M., E. Scholte, W. Takken, and T. Dekker. 2000. Microbial growth enhances the attractiveness of human sweat for the malaria mosquito, *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Chemoecology. 10: 129-134.

Braks, M., and W. Takken. 1999. Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto*. J. Chem. Ecol. 25: 663-672.

Brett, G. A. 1938. On the relative attractiveness to *Aedes aegypti* of certain coloured cloths. T. Roy. Soc. Trop. Med. H. 32: 113-124.

Brown, A. 1966. The attraction of mosquitoes to hosts. J. Am. Med. Assoc. 196: 159-162.

Cantrell, C. L., J. A. Klun, C. T. Bryson, M. Kobaisy, and S. O. Duke. 2005. Isolation and identification of mosquito bite deterrent terpenoids from leaves of American (*Callicarpa americana*) and Japanese (*Callicarpa japonica*) beautyberry. J. Agric. Food Chem. 53: 5948-5953.

Carroll, J. F., N. Tabanca, M. H. Kramer, N. M. Elejalde, D. E. Wedge, U. R. Bernier, M. R. Coy, J. J. Becnel, B. Demirci, K. H. Can Baser, J. Zhang and S. Zhang. 2011. Activity of *Cupresus funebris*, *Juniperis communis*, and *J. chinensis* (Cupressaceae) Essential Oils as Repellents Against Ticks (Acari: Ixodidae) and as Repellents and Toxicants Against Mosquitoes (Diptera: Culicidae). J. Vector Ecol. 36: 258-268.

Centers for Disease Control and Prevention [CDC]. 1999. Imported Dengue – Florida, 1997-1998. Morbidity and Mortality Weekly Report. 48: 1150-1152.

Centers for Disease Control and Prevention [CDC]. 2010. Locally acquired Dengue – Key West, Florida, 2009-2010. Morbidity and Mortality Weekly Report. 59: 577-581.

Curtis, C. 1986. Fact and fiction in mosquito attraction and repulsion. Parasitol. Today. 2: 316-318.

Day, J. F. 2005. Host-seeking strategies of mosquito disease vectors. J. Am. Med. Assoc. 21: S17-S22.

Debboun, M., D. Strickman, V. B. Solberg, R. C. Wilkerson, K. R. McPherson, C. Golenda, L. Keep, R. A. Wirtz, R. Burge, and T. A. Klein. 2000. Field evaluation of DEET and a piperidine repellent against *Aedes communis* (Diptera: Culicidae) and *Simulium venustum* (Diptera: Simuliidae) in the Adirondack mountains of New York. J. Med. Entomol. 37: 919-923.

Dekker, T., M. Geier, and R. T. Carde. 2005. Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. J. Exp. Biol. 208: 2963-2972.

Dekker, T., W. Takken, and R. T. Carde. 2001. Structure of host-odour plumes influences catch of *Anopheles gambiae s.s.* and *Aedes aegypti* in a dual-choice olfactometer. Physiol. Entomol. 26: 124-134.

Dethier, V. G., L. B. Browne, and C. N. Smith. 1960. The designation of chemicals in terms of the responses they elicit from insects. J. Econ. Entomol. 53: 134-136.

Dogan, E. B., and P. A. Rossignol. 1999. An olfactometer for discriminating between attraction, inhibition, and repellency in mosquitoes (Diptera: Culicidae). J. Med. Entomol. 36: 788-793.

Frame, G. W., W. G. Strauss, and H. I. Maibach. 1972. Carbon dioxide emission of the human arm and hand. J. Invest. Dermatol. 59: 155-159.

Gaffigan, T. V., R. C. Wilkerson, J. E. Pecor, J. A. Stoffer, and T. Anderson. 2012. Systematic catalog of Culicidae. http://www.mosquitocatalog.org/.

Geier, M., O. Bosch, and J. Boeckh. 1999. Influence of odour plume structure on upwind flight of mosquitoes towards hosts. J. Exp. Biol. 202: 1639-1648.

Gillies, M. T. 1980. The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. Bull. Ent. Res. 70: 525-532.

Granett, P. 1940. Studies of mosquito repellents, I. test procedure and method of evaluating test data. J. Econ. Entomol. 33: 563-565.

Gratz, N. G. 1999. Emerging and resurging vector-borne diseases. Annu. Rev. Entomol. 44: 51-75.

Gubler, D. J., P. Reiter, K. L. Ebi, W. Yap, R. Nasci, and J. A. Patz. 2001. Climate variability and change in the United States: potential impacts on vector and rodent-borne diseases. Environ. Health Persp. 109: 223-233.

Harbach, R.E. 2012. Mosquito taxonomic inventory. http://mosquito-taxonomic-inventory.info/.

Happold, D. C. D. 1965. Mosquito ecology in Central Alberta, II. Adult population and activities. Canad. J. Zool. 43: 821-846.

Huhn, G. D., J. J. Sejvar, S. P. Montgomery, and M. S. Dworkin. 2003. West Nile virus in the United States: an update on an emerging infectious disease. Am. Fam. Physican 68: 653-660.

Katritzky, A. R., Z. Wang, S. Slavov, D. A. Dobchev, C. D. Hall, M. Tsikolia, U. R. Bernier, N. M. Elejalde, G. G. Clark, and K. J. Linthicum. 2010. Novel carboxamides as potential mosquito repellents. J. Med. Entomol. 47: 924-938.

Katritzky, A. R., Z. Wang, S. Slavov, M. Tsikolia, D. Dobchev, N. G. Akhmedov, C. D. Hall, U. R. Bernier, G. G. Clark, and K. J. Linthicum. 2008. Synthesis and bioassay of improvement mosquito repellents predicted from chemical structure. Proc. Natl. Acad. Sci. 105: 7359-7364.

Khan, A., H. Maibach, and W. Strauss. 1971. A quantitative study of variation in mosquito response and host attractiveness. J. Med. Entomol. 8: 41-43.

Khan, A., H. Maibach, W. Strauss, and J. Fisher. 1970. Differential attraction of the yellow fever mosquito to vertebrate hosts. Mosq. News. 30: 43-47.

Khan, A., W. Strauss, H. Maibach, and W. Fenley. 1967. Comparison of the attractiveness of the human palm and other stimuli to the yellow-fever mosquito. J. Econ. Entomol. 60: 318-320.

Kline, D. 1998. Olfactory responses and field attraction of mosquitoes to volatiles from limburger cheese and human foot odor. J. Vector Ecol. 23: 186-194.

Kline, D. L., Bernier, U. R., Posey, K. H., and D. R. Barnard. 2003. Olfactometric evaluation of spatial repellents for *Aedes aegypti*. J. Med. Entomol. 40: 463-467.

Klun, J., and M. Debboun. 2000. A new module for quantitative evaluation of repellent efficacy using human subjects. J. Med. Entomol. 37: 177-181.

Klun, J. A., A. Khrimian, and M. Debboun. 2006. Repellent and deterrent effects of SS220, Picaridin, and DEET suppress human blood feeding by *Aedes aegypti*, *Anopheles stephensi*, and *Phlebotomus papatasi*. J. Med. Entomol. 43: 34-39.

Klun, J. A., M. Kramer, and M. Debboun. 2005. A new *in vitro* bioassay system for discovery of novel human-use mosquito repellents. J. Am. Mosq. Control Assoc. 21: 64-70.

Knols, B. G. J., and R. De Jong. 1996. Limburger cheese as an attractant for the malaria mosquito *Anopheles gambiae s.s.* Parasitol. Today. 12: 159-161.

Lehane, M. J. 2005. Location of the host, pp. 27-55 *In* The biology of blood-sucking in insects, Cambridge University Press, Cambridge, UK.

Maibach, H., A. Khan, W. Strauss, and W. Skinner. 1969. Human skin in relationship to mosquito attraction and repulsion. Conn. Med. 33: 23-28.

McCabe, E. T., W. F. Barthel, S. I. Gertler, and S. A. Hall. 1954. Insect repellents. III. *N,N*-diethylamides. J. Org. Chem. 19: 493-498.

McIver, S. B. 1982. Sensilla of mosquitoes (Diptera: Culicidae). J. Med. Ent. 19: 489-535.

Mehr, Z., L. Rutledge, M. Buescher, R. Gupta, and M. Zakaria. 1990. Attraction of mosquitoes to diethyl methylbenzamide and ethyl hexanediol. J. Am. Mosquito Contr. 6: 469-476.

Moore, S. J. and M. Debboun. 2007. History of insect repellents, pp. 3-30. *In* M. Debboun, S. P. Frances and D. Strickman (eds.), Insect repellents: principles, methods, and uses. CRC Press, Boca Raton, FL.

PoloPlus. 2012. Probit and logit statistical analysis computer software program, version 2.0. LeOra Software, Petaluma, CA.

Posey K. H., and C. E. Schreck. 1981. An airflow apparatus for selecting female mosquitoes for use in repellent and attraction studies. Mosq. News 41: 566–568.

Roachell, W. 1997. Olfactory cues in mosquito host location. Insect Behavior Review Articles. http://www.colostate.edu/Depts/Entomology/courses/en507/papers_1997/roachell.html.

Robert, L. L., J. A. Hallam, D. C. Seeley, L. W. Roberts, and R. A. Wirtz. 1991. Comparative sensitivity of four Anopheles (Diptera: Culicidae) to five repellents. J. Med. Entomol. 28: 417-420.

Robertson, J. L., R. M. Russell, H. K. Preisler, and N. E. Savin. 2007. Binary quantal response: dose number, dose selection, and sample size. pp. 55-70. *In* Bioassays with arthropods. CRC Press, Boca Raton, FL.

Robertson, J. L., K. C. Smith, N. E. Savin, and R. J. Lavigne. 1984. Effects of dose selection and sample size on the precision of lethal dose estimates in dose-mortality regression. J. Econ. Entomol. 77: 833-837.

Rosa, L. H., N. Tabanca, N. Techen, D. E. Wedge, Z. Pan, U. R. Bernier, J. J. Becnel, N. M. Agramonte, L. A. Walker, and R. M. Moraes. 2012. Fungal diversity associated with micropropogated medicinal plant of *Echinacea purpurea* (L.) Moench. Amer. J. Plant Sci. 3: 1105-1164.

Rutledge, L. C. and R. K. Gupta. 2004. Evaluation of an *in vitro* bloodfeeding system for testing mosquito repellents. J. Am. Mosq. Control Assoc. 20: 150-154.

Sawyer, D. R. 1986. Malaria on the Amazon frontier: economic and social aspects of transmission and control. Southeast Asia J. Trop. Med. Public Health. 17(3): 342-345.

Statistical Analysis Software Institute [SAS]. 2012. Statistical analysis computer software program, version 9.2. SAS Institute, Cary, NC.

Schreck, C. E. 1977. Techniques for the evaluation of insect repellents: a critical review. Ann. Rev. Entomol. 22: 101-119.

Schreck, C., N. Smith, D. Carlson, G. Price, D. Haile, and D. Godwin. 1981. A material isolated from human hands that attracts female mosquitoes. J. Chem. Ecol. 8: 429-38.

Smith, C., I. Gilbert, H. Gouck, M. Bowman, F. Acree Jr., and C. Schmidt. 1963. Factors affecting the protection period of mosquito repellents. USDA Technical Bulletin 1285: 1-36.

Takken, W. 1991. The role of olfaction in host-seeking of mosquitoes: a review. Insect. Sci. Appl. 12: 287-295.

U.S. Department of Agriculture [USDA]. 1977. Repellent activity of compounds submitted by Walter Reed Army Institute of Research. I. Protection time and minimum effective dosage against *Aedes aegypti* mosquitoes. USDA Technical Bulletin No. 1549.

U.S. Environmental Protection Agency [US-EPA]. 2012. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), P.L. 112–177, effective Sept. 28, 2012, 7 U.S.C. § 136 et seq. http://www.ag.senate.gov/download/fifra.

Weaver, S. C. and W. K. Reisen. 2010. Present and future arboviral threats. Antiviral Research 85(2): 328-345.

Weldon, P., J. Aldrich, J. Klun, J. Oliver, and M. Debboun. 2003. Benzoquinones from millipedes deter mosquitoes and elicit self-anointing in capuchin monkeys (*Cebus* spp.). Naturwissenschaften 90: 301-304.

Walter Reed Biosystematics Unit [WRBU]. 2012. Medically important mosquito species. http://wrbu.si.edu/command_aors_MQ.html.

World Health Organization [WHO]. 2009. Guidelines for efficacy testing of mosquito repellents for human skin. Ref:WHO/CDS/NTD/WHOPES/2009.4, http://www.who.int/whopes/guidelines/en/.

Zwiebel, L. J., and W. Takken. 2004. Olfactory regulation of mosquito-host interactions. Insec. Biochem. Molec. 34: 645-652.

BIOGRAPHICAL SKETCH

Natasha Marie Elejalde was born in Miami, FL, the first of three daughters by Regina Maria Smit and Jose Antonio Elejalde, III. She grew up in Miami where she attended St. Theresa Catholic School for much of her primary education and Our Lady of Lourdes Academy for high school from 1998 to 2002. At 17, she enrolled at the University of Florida in Gainesville, FL and took courses in the basic sciences, psychology, genetics and entomology. Driven by her interest in molecular biology and genetics, she volunteered in Dr. Rebecca Kimball's evolutionary biology laboratory for two years of her undergraduate career and was selected as an undergraduate teaching assistant for Dr. Marta Wayne's genetics course. She received her Bachelor of Science in Zoology in 2006 from the University of Florida.

The semester prior to graduation, Natasha left Dr. Kimball's genetics laboratory to begin working as a student assistant for the USDA in Dr. Ulrich Bernier's mosquito repellents laboratory at the Center for Medical, Veterinary and Agricultural Entomology in Gainesville, FL. She worked up to a full time position as a research technician testing experimental chemicals as insect repellents as well as assisting with other projects testing military uniforms for efficacy in insecticide treatment. Her passion for public health issues, specifically those related to the transmission and spread of insect-borne diseases led her to enroll at the University of Florida again in 2009 to pursue a graduate education in medical entomology under Dr. Bernier. She maintained her full-time position at the USDA while pursuing her graduate degree part-time and developed a research project examining alternative methods for testing mosquito repellents which precluded direct testing on human volunteers. In 2012, she married her boyfriend of five years, Kent Joseph Agramonte, and took his name. She received her Master of Science in Entomology and Nematology in 2012 from the University of Florida.