

EVALUATION OF PICARIDIN TREATED FABRICS FOR THEIR PROTECTION  
AGAINST MOSQUITO BITES IN COMPARISON WITH THE CURRENT US ARMY  
PERMETHRIN FACTORY-TREATED UNIFORM

by

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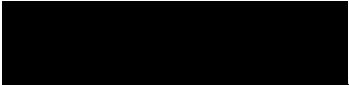

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## **DEDICATION**

To my wife, Silke, thank you for making all this possible. Your attention to detail, hard work and loving care throughout these last two years allowed me to focus on completing this thesis.

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May 18, 2018

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## ABSTRACT

Evaluation of picaridin treated fabrics for their protection against mosquito bites in comparison with the current US Army permethrin factory-treated uniform

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Development of next-generation repellent-treated uniforms is important in order to protect service members against bites of arthropod disease vectors. In this study, fabrics treated with two concentrations of microencapsulated picaridin repellent were evaluated for protection against the biting and landing activity of *Aedes aegypti* and *Anopheles gambiae* mosquitoes. The results were compared with the protection provided by the current permethrin treated uniform. Uniform fabrics tested included two concentrations of picaridin, permethrin treated and untreated uniform, and net. Twenty mosquitoes were added to each assay for ten minutes, landing counts observed and later examined for blood-feeding. The picaridin treated fabrics demonstrated the reduction of mosquito landing and biting activity. The fabrics provided 100% bite protection against *Ae. aegypti* and significantly reduced the landing counts. Landing counts and blood-feeding rates were significantly reduced by the picaridin treatment but it was not significantly different from the protection provided by the current permethrin treatment.

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## CHAPTER 1: Introduction

Throughout history, vector borne diseases have negatively affected military campaigns. A little over 75 years ago work on treating fabrics to repel mosquitoes and the other vectors that spread these diseases began when the U.S. Department of Agriculture was tasked to develop ways to protect soldiers from epidemic typhus, malaria and plague (6). Human arm-in-cage test are relied heavily on to conduct this research (13).

Increasing concern about the ethical aspects of testing human subjects, stricter regulations and the Institutional Review Board (IRB) requirements make this type of research a challenging endeavor. Conducting field tests outdoors in areas where wild mosquitoes may infect human test subjects with pathogens are very difficult to get an approval. A solution to these problems is to use an *in vitro* box bioassay that replaces humans with a membrane blood feeder. This box bioassay will be used to determine the efficacy of the next-generation military uniform fabric (an antimicrobial microencapsulated picaridin treatment) under development by the U.S. Army Research, Development and Engineering Command (RDECOM), Natick Soldier Research, Development, and Engineering Center (NSRDEC) against feeding mosquitoes.

### BACKGROUND

Vector-borne diseases continue to emerge or are resurging worldwide and few of them are vaccine-preventable. Malaria is the most important vector-borne disease and can be prevented, at least in part, using prophylactic drugs. However, drug resistance by the parasites (*Plasmodium* spp.) is on the rise throughout the world and other protective measures must be used to keep persons safe from infection (32). Personal protective

measures are important tools to use, especially in areas where the pathogens are present (10). These protective measures are skin repellent, permethrin-treated clothes and sleeping under a permethrin-treated bed net (1).

The treatment of military uniforms with repellents as the primary means to protect soldiers from exposure to insect bites had its beginnings with Philip Granett working at Rutgers University in 1942, when he found that a cheesecloth sleeve impregnated with butyl carbitol acetate protected an untreated arm against mosquitoes for 24 hours (26). Shortly thereafter, liquid repellents applied to clothing was successfully used to provide protection from mites that were responsible for the deadly pestilence of scrub typhus in the World War II's Pacific Theater. This treated clothing provided protection for up to 60 days in the field. The Army continued to develop clothing treatments and used one designated as M-1960 throughout the Korean and Viet Nam wars (7). M-1960 had a disagreeable odor, a tendency to irritate the skin and was not well accepted by soldiers and their commanders. It was not until 1991 that permethrin came into widespread use (1). Permethrin had the advantages of having no odor or greasy residue on the fabric, low toxicity and exceptional resistance to degradation by sunlight, heat and water. Permethrin is a contact repellent and tightly binds to cotton fabric which makes it resistant to washing out of the fabric (1). The repellent effect lasted for six weeks, even after weekly laundering (41).

After the 1990's, Army uniforms were individually treated by soldiers using spray cans, aerosol cans or by saturation in sealed bags (11). The uniforms were then hung out to dry after the treatment. Improvements to the treatment process continued. Two problems were identified. The first one was associated with the soldier's lack of skill or

attention while applying permethrin resulting in an inconsistent or uneven coverage of uniforms. The second issue was soldier exposure to concentrated chemical active ingredients during the mixing and application process (1). The effort to resolve the problems lead to the latest development of factory pretreatment. This method has the advantage of providing equal coverage throughout the garment, elimination of the need to mix chemicals by hand, and improved treatment technology increased the longevity of the repellent's activity (10). One issue that still holds true is that repellent effectiveness fades with an increased number of launderings (14; 15; 35; 41). Currently, the repellent activity of the Army factory-treated combat uniform lasts for up to 50 washes (launderings) but new impregnation techniques can extend that life up to 100 launderings (10). New impregnation techniques such as microencapsulation, polymer coating and plasma application process are being developed to prevent loss of effectiveness due to use and cleaning (4; 33).

Picaridin is a relatively new repellent and was registered for use in 2001 (45). It has been used mainly as a skin applied repellent and is part of the Department of Defense Insect Repellent System (1). It both repels and deters insects and is described as a spatial repellent that interferes with a mosquito's ability to smell a host (22).

#### **HEALTH SIGNIFICANCE TO MILITARY PERSONNEL**

Important arthropod vectors of disease include mosquitoes, ticks, sand flies and others. Diseases transmitted by these vectors include malaria, dengue fever, chikungunya fever, Zika, filariasis, Lyme disease, ehrlichiosis, rickettsioses, leishmaniasis and sand fly fever (21; 27; 31; 36; 40; 48; 49). Federal Drug Administration (FDA) approved vaccines do not exist for these diseases. Malaria can be prevented with the use of prophylaxis, but

with each passing year, drug resistance by the parasite *Plasmodium* spp. develops and it becomes more difficult to control this disease (30). Bed nets can be used only while you are sleeping and do not provide any protection from day biting mosquitoes like *Aedes aegypti* (16; 24). The Armed Forces Pest Management Board (AFPMB) recommends an integrated approach that incorporates multiple prevention methods to fully protect individuals. Treating clothing with a repellent to shield persons from being bitten by pathogen carrying vectors is an important part of the integrated approach (2). The treated clothing protects against vector's bites in two ways. First, it repels the vector and prevents it from biting the host and taking a bloodmeal (44). Second, if the treatment has insecticidal properties, it can kill the vector and prevent the vector from biting other people or it may negatively impact the insect fitness, such as its ability to lay eggs and create more vectors (9). However, the benefits of treating clothing with repellents may not last as long as the clothing does. Washing and even the normal wear of the insecticide treated clothes will reduce their effectiveness over time (14; 15; 35; 41). New repellents or an improved treatment process must be tested and researched in order to get the longest lasting benefits of protection against blood sucking vectors.

We also need to stay one step ahead of the insecticide resistance and tolerance issues that have developed from past pesticide misuse (19; 28; 31). Researching and testing new compounds on uniform fabrics is needed to accomplish this effort. Demonstrating the effectiveness of new products can be done by comparing them to the currently used factory-treated military fabric (43). Military medical personnel will be able to use this information to keep uniforms up-to-date and effective for protecting deployed

soldiers and soldiers conducting field exercises against bites of arthropod vectors and the diseases they transmit.

## **LITERATURE REVIEW**

### **Repellents and Treated Fabrics**

In the 1960's, researchers from the U.S. Army Medical Research Unit conducted a comparative efficacy test on the use of DEET (skin-application) and M-1960 (impregnated-clothing) against mosquito bites in the swamps of Malaya (43). Results from that study demonstrated that the combination of skin repellent and clothing treatment provided the best protection against mosquito bites. Use of skin repellent was effective at preventing bites to the skin but this resulted in an increase in mosquitoes landing on the untreated uniform and biting through it. Solely treating the uniform with repellent had a similar effect i.e., driving the mosquitoes to the unprotected skin areas. This study in Malaya required the use of volunteers to stand in the swamps and act as live bait for wild mosquitoes to land on them.

A group of researchers from the Letterman Army Institute of Research conducted a study of weathering effects on permethrin treated fabrics (14). The researchers explored contact toxicity and repellent effects that permethrin-impregnated fabrics had on mosquitoes. The fabric was weathered under tropical conditions in the lab for nine weeks. Results from this study demonstrated that the contact killing of mosquitoes stopped after one week, but the repellent continued to work for six weeks. Knock-down of mosquitoes was not needed to protect persons from mosquito bites. This experiment required the use of volunteers to expose their forearm to the bites of 15 laboratory reared mosquitoes.

In the late 1980's, arm-in-cage experiments continued to be important tools in repellent research. Two researchers from the U.S. Department of Agriculture (USDA) studied a variety of repellents and personal protection strategies against a newly invasive mosquito species, *Aedes albopictus* (38). Five chemicals were evaluated including a permethrin-impregnated clothing fabric. The researchers tested how well the chemicals protected subjects from mosquito bites. The skin repellents were measured for the longevity of bite protection and the permethrin-treated fabric was measured on bite protection through zero to five washings. This experiment required volunteers to expose their forearms to 100-500 laboratory-reared mosquitoes. Skin repellents and fabrics were tested separately and the results demonstrated the effectiveness of each individual product.

In another study, Royal Thai Army troops were used to evaluate permethrin-treated uniforms for protection against malaria in Thailand (8). Members of the Royal Thai Army were given malaria prophylaxis and bed nets, wore permethrin-treated uniforms but did not apply repellent on skin. This study demonstrated that relying solely on the use of permethrin-treated uniforms did not reduce the incidence of malaria in troops when in a malaria-endemic area where drug-resistant *Plasmodium* was present.

### **Fabric Treatments**

Researchers at the Central Institute of the Bundeswehr Medical Service (Germany) conducted a comparative study of a new polymer-coating treatment against the U.S. Army Individual Dynamic Absorption (IDA) Kit (10). The goal was to determine the knock-down effect and test how long the permethrin stayed active in the uniform. Mosquitoes (*Ae. aegypti*) and tick nymphs (*Ixodes ricinus*) were placed directly

on the fabrics to test its toxicity. The polymer-coated fabric remained effective for up to 100 washes whereas the IDA Kit treated fabrics lasted for 50 washes under identical conditions. This study did not measure repellency directly, but used toxicity/knock-down to determine if an effective amount of permethrin remained in the fabric. The time period for permethrin to knock-down the mosquitoes or ticks was measured and then correlated with the concentration of permethrin that remained in the fabric.

Researchers at the Zhejiang University in China employed a combination of deltamethrin, a pyrethroid insecticide, and DEET, a repellent, to improve upon the protective qualities of permethrin-treated fabrics against mosquitoes (50). This study used both knock-down cone tests along with arm-in-cage repellency tests against *Ae. albopictus* mosquitoes. These combinations along with microencapsulated mixtures extended washing durability when compared to the conventional treatment method. Their results indicated promising methods for future impregnation techniques. In this study, the volunteers inserted a forearm treated with a test sample into a cage containing 400 laboratory reared mosquitoes to test for repellency.

Researchers at the USDA and the Academy of Health Sciences, U.S. Army Medical Department Center & School (22), tested the repellent and deterrent effects of picaridin and other repellents on mosquitoes and sand flies. This was the only study that tested the repellent effects of picaridin applied to a fabric. The fabric used was a 70% polyester and 30% nylon woven cloth. In this experiment it was demonstrated that picaridin applied to the skin repelled insects, even when the skin was covered with a cloth. This demonstrated that picaridin repelled even without contacting the insect. The

researchers also applied picaridin to the fabric and this too repelled the insects. The bioassay required volunteers to be exposed to six sets of five flies per test.

### ***In vitro* Bioassay System Development**

The development of *in vitro* bioassay systems has evolved over time as concern for human test subject safety has increased (13; 17). There continues to be variations in procedures with little consensus on the most appropriate system to use. Few studies have been dedicated to the testing of repellent in fabrics (29; 32; 44; 50), with the majority of the studies focused on skin-applied repellents.

Researchers at the Letterman Army Institute of Research developed a new method for testing the repellency of multiple chemical compounds and formulations (37). They developed a membrane blood feeding choice system that utilized 250 mosquitoes at a time, and a choice between 4-17 different feeding stations. Each station's membrane could be coated with a different repellent and the numbers of mosquitoes feeding was recorded in 2-minute intervals for a period of 20 minutes. It was suggested that such a system could be used in the preliminary evaluation and rapid screening of repellents prior to their testing on human subjects.

Later, in 2005, researchers at the USDA, concerned with human subject safety, developed an *in vitro* bioassay system that could test compounds of unknown toxicity without risk to test subjects (23). This system used small plastic modules, containing five mosquitoes each, placed on a repellent treated fabric that rested on a blood-filled membrane. Mosquitoes attracted to the warmed blood could bite through the fabric. With this system, it was possible to screen 100 repellent compounds per work week. Still,

the researchers determined that in vivo tests were best used to make final conclusions on a repellents usefulness once prospective compounds were found.

## **GOALS AND OBJECTIVES**

This study will be the first of its kind to examine the effectiveness of a microencapsulated picaridin fabric treatment in side-by-side comparisons with the U.S. Army currently utilized insect repellent (permethrin)-treated fabrics. The assays were focused on assessing the biting and repellent activity of two major mosquito vectors *Ae. aegypti* and *Anopheles gambiae*, and whether picaridin (at two different concentrations) conferred greater protection than the permethrin factory-treated uniform fabric.

The objectives of this research were:

- 1) Assess the effect that picaridin treated fabrics have at preventing *Ae. aegypti* and *An. gambiae* from blood-feeding.
  - a. Determine whether picaridin treated fabric conferred greater protection from *Ae. aegypti* and *An. gambiae* blood-feeding than a similar untreated military fabric.
  - b. Determine whether picaridin treated fabric conferred greater protection from *Ae. aegypti* and *An. gambiae* blood-feeding than the permethrin treated fabric.
- 2) Assess the effect that picaridin treated fabrics have at repelling *Ae. aegypti* and *An. gambiae*.
  - a. Determine whether picaridin treated fabric repel more *Ae. aegypti* and *An. gambiae* than untreated military fabric.

- b. Determine whether picaridin treated fabric repel more *Ae. aegypti* and *An. gambiae* than permethrin treated fabric.

Understanding the bite protection and repellency offered by new chemicals will allow medical material planners to determine if they show promising efficacy worth investing in a complete evaluation for future military use. This approach will save time and money during the testing phase by focusing on fabrics that demonstrate similar effectiveness to the current Army standard fabric. Ultimately, medical planners will be able to improve protection offered by current military uniforms against the threats of arthropod vectors and diseases they transmit.

#### **HYPOTHESES AND SPECIFIC AIMS**

**H<sub>0</sub> #1:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will = the mean blood-feeding rate of the untreated military fabric.

**H<sub>A</sub> #1:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will  $\neq$  the mean blood-feeding rate of the untreated military fabric. (i.e., bite through will significantly decrease).

**Specific Aim #1.** Compare the mean blood-feeding (bite-through) rate per mosquito of the two picaridin treated fabrics vs. untreated military fabric for *Ae. aegypti* and *An. gambiae*.

**H<sub>0</sub> #2:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will = the mean blood-feeding rate on permethrin treated military fabric (i.e., bite-through protection will not differ from the permethrin treated military fabric).

**H<sub>A</sub>#2:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will  $\neq$  the mean blood-feeding rate on permethrin treated military fabric.

**Specific Aim #2.** Compare differences between blood-feeding rates of all test fabrics for *Ae. aegypti* and *An. gambiae* using permethrin treated military fabric as the control.

**H<sub>0</sub> #3:** Mosquitoes' mean landing rate on picaridin treated fabric will = the mean landing rate of the untreated military fabric.

**H<sub>A</sub> #3:** Mosquitoes' mean landing rate on picaridin treated fabric will  $\neq$  the mean landing rate of the untreated military fabric (i.e., landing rate will significantly decrease).

**Specific Aim #3.** Compare the mean landing rate per mosquito of the two picaridin treated fabrics vs. untreated military fabric for *Ae. aegypti* and *An. gambiae*.

**H<sub>0</sub> #4:** Mosquitoes' mean landing rate on picaridin treated fabric will = the mean landing rate on permethrin treated military fabric (i.e., landing rate will not differ from the permethrin treated fabric).

**H<sub>A</sub>#4:** Mosquitoes' mean landing rate on picaridin treated fabric will  $\neq$  the mean landing rate on permethrin treated military fabric.

**Specific Aim #4.** Compare differences between landing rates of all test fabrics for *Ae. aegypti* and *An. gambiae* using permethrin treated military fabric as the control.

## CHAPTER 2: Materials and Methods

### MOSQUITOES

#### *Ae. aegypti*

Adult *Ae. aegypti* mosquitoes used in the studies were from the Uniformed Services University of the Health Sciences (USUHS) Insectary, Bethesda, MD maintained colony. The mosquitoes were reared by submerging filter paper containing eggs in 450 ml deionized water and stimulated to hatch by placing them under a vacuum for 30 min (T. Foggie & N. Achee, unpublished SOP) (18). Newly hatched larvae were fed with homogenized Tetra Betta® fish food pellets (Tetra®, Blacksburg, VA), until they pupated and later emerged into adults. Throughout the study, conditions of 28°C, 80% relative humidity (RH) and a photoperiod of 12:12 (L/D) was maintained. Adults were fed with 10% table sugar (sucrose) solution and only non-blood-fed female mosquitoes, 5-11 days old, were used in the testing.

#### *An. gambiae*

Adult *An. gambiae* mosquitoes used in the studies were from the USUHS Insectary, Bethesda, MD maintained colony. Eggs were placed in a plastic tray with 450 ml deionized water and allowed to hatch over a few days (5). Newly hatched larvae were fed with homogenized TetraMin® fish food flakes (Tetra®, Blacksburg, VA), until they pupated and later emerged into adults. Throughout the study, conditions of 28°C, 80% RH and a photoperiod of 12:12 (L/D) was maintained. Adults were fed with 10% table sugar (sucrose) solution and only non-blood-fed female mosquitoes, 5-11 days old, were used in the testing.

### TEST REPELLENTS

## **Picaridin**

Picaridin is registered for use on skin and clothes to repel mosquitoes, ticks, fleas and biting flies (12). It repels and inhibits mosquito feeding behavior by stimulating sensory hairs on the antennae and masking the host from detection (22). Exposure to picaridin does not kill *Aedes aegypti* mosquitoes or appear to alter their normal behavior (25). In the Schreck et al. (39) study, it was determined that the minimum effective dose for a 15 minute repellency test, defined as the lowest dose that resulted in 3 or fewer bites, to be 0.063 mg/cm<sup>2</sup> and 0.5 mg/cm<sup>2</sup> for the 24 hour test.

### ***Chemical Class and Type***

Picaridin belongs in the piperidine chemical family and its chemical name is 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester (45). It is a synthetic molecule developed by Bayer®. Picaridin is used in many countries throughout the world and is widely used in the U.S., Europe and Australia (20).

### ***Physical and Chemical Properties***

Technical grade picaridin is a colorless liquid with little odor (45). It has a vapor pressure of  $4.4 \times 10^{-4}$  mmHg at 25° C and a molecular weight of 229.3 g/mol (45). It is not soluble in water.

### ***Toxicity***

Picaridin is classified as slightly toxic if ingested (45). Picaridin is not considered a skin irritant and is not a sensitizer, but it can cause slight to moderate eye irritation (45). The U.S. EPA considered picaridin to be practically non-toxic for inhalation exposure (45).

## **Permethrin**

Permethrin is a versatile chemical and can be used in many ways. It has been registered for use on clothing and pets as a repellent (47). It can also be used as a pesticide in numerous residential sites, both indoor and outdoor or used on crops, nurseries and sod farms as a general use pesticide (42). It is a popular product used in public health mosquito abatement programs as well as used on food or feed crops and livestock (42). It has been formulated for the treatment of head lice and scabies on humans (47). Permethrin effects the nervous system of mosquitoes by interfering with the sodium channels to disrupt the function of neurons that can result in death (1).

### ***Chemical Class and Type***

Permethrin belongs in the pyrethroid chemical family and its chemical name is 3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate (47). It is considered a type I pyrethroid and is a blend of two stereoisomers (42).

### ***Physical and Chemical Properties***

Technical permethrin ranges from a colorless crystal to a yellow or brown viscous liquid (47). It has a vapor pressure of  $2.15 \times 10^{-8}$  mmHg and a molecular weight of 391.3 g/mol (47). Its solubility in water is  $5.5 \times 10^{-3}$  mg/L (47).

### ***Toxicity***

Permethrin is highly toxic to honeybees, fish, and aquatic invertebrates because of the way it disrupts the sodium channels (46). Mammals are less susceptible to permethrin compared to insects because their sodium channels are less effected by pyrethroids and

recover faster (42). The U.S. EPA considered permethrin to be low in toxicity for acute oral exposure, low in toxicity for potential eye irritation and very low in toxicity for potential skin irritation and is not considered a skin sensitizer (47).

## **TEST FABRICS**

The following test fabrics were used, see Figure 1: 1) untreated net with 100 holes / cm<sup>2</sup>, to test the willingness of the mosquitoes to feed while providing minimal resistance to feeding (Figure 1a); 2) military uniform fabrics (referred to as OCP, Operational Camouflage Pattern), supplied by the U.S. Army Research, Development and Engineering Command (RDECOM), Natick Soldier Research, Development, and Engineering Center (NSRDEC), Natick, MA. The military fabric provided (OCP) consisted of untreated (Figure 1b) and treated Army Combat Uniform (ACU) with the OCP composed of 50:50 nylon/cotton (NyCo) ripstop weave. Each swatch was cut into 12.7 cm by 12.7 cm squares. The permethrin treated (Perm-OCP) fabric was factory-treated and came from the manufacturer unwashed and without any further treatments, see Figure 1c. The picaridin treated fabrics were prepared by NSRDEC using a microencapsulated formulation of picaridin applied to an untreated sample of ACU 50:50 NyCo fabric as described in the Place et al. (33) article. Two aliquots of the picaridin microcapsules equivalent to 18 ml and 36 ml were applied to their respective swatch, resulting in an average loading of approximately 0.9 mg/cm<sup>2</sup> (0.9 Pic-OCP) and 1.8 mg/cm<sup>2</sup> (1.8 Pic-OCP) of picaridin, see Figures 1d & 1e. A white residue is visible on the picaridin treated fabrics and is the result of the microencapsulated treatment process. Table 1 lists the fabrics used.

Table 1. Types of fabrics tested

Fabric ID	Use	Treatment	Composition	Weave
Net	Commercial	None - mesh 100 holes / cm <sup>2</sup>	Polyester	Plain
Untreated OCP	Military- ACU	None	50:50 nylon/cotton	Ripstop
Perm-OCP	Military- ACU	Polymer-coated with permethrin	50:50 nylon/cotton	Ripstop
0.9 Pic-OCP	Military- ACU	Micro-encapsulation 0.9 mg/cm <sup>2</sup> picaridin	50:50 nylon/cotton	Ripstop
1.8 Pic-OCP	Military- ACU	Micro-encapsulation 1.8 mg/cm <sup>2</sup> picaridin	50:50 nylon/cotton	Ripstop

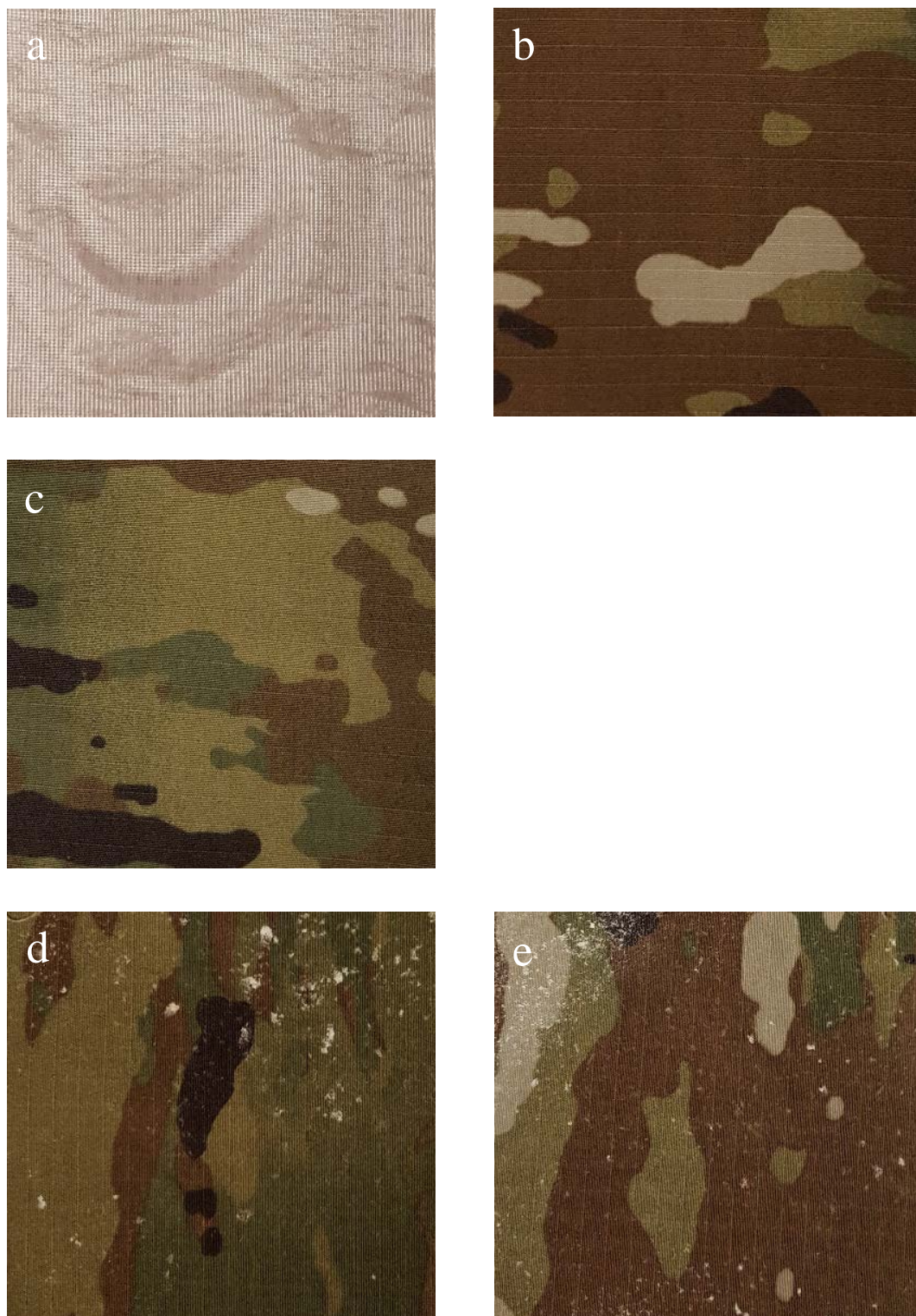


Figure 1. Test fabrics

(a) untreated net mesh 100 holes / cm<sup>2</sup>, (b) untreated OCP, (c) Perm-OCP polymer-coated with permethrin, (d) 0.9 Pic-OCP micro-encapsulation with 0.9 mg/cm<sup>2</sup> picaridin and (e) 1.8 Pic-OCP micro-encapsulation with 1.8 mg/cm<sup>2</sup> picaridin.

Repellent impregnated fabrics were stored separately and at room temperature (20°C) in sealed plastic bags, inside brown mail envelopes until the first mosquito landing and biting trials occurred under the conditions described. The picaridin samples were kept separate from the permethrin and untreated samples.

#### **MEMBRANE BLOOD-FEEDING SETUP**

Outdated human blood plasma was provided by Interstate Companies, Memphis, TN. The blood used in the bioassays was prepared by mixing 10 ml of blood (per glass bell feeder) with adenosine triphosphate (RPI, Mount Prospect, IL) (ATP) at a concentration of 0.025g ATP / 10 ml blood (37). Blood was pre-warmed in a circulating water bath (PolyScience, Niles, IL) to 36°C. A 7.5 x 7.5 cm square piece of synthetic Baudruche (Joseph Long Inc., NJ) membrane was secured, with the textured side of the membrane facing outwards, across the bottom of each glass feeder (Chemglass Life Sciences, Vineland, NJ), with a rubber band. Water at 36°C was constantly circulated around the glass feeders prior to the addition of the ATP supplemented blood (10 ml per feeder).

#### **REPELLENCY TESTING PROCEDURES**

In order to simulate mosquito host finding behavior, landing and biting avoidance (or protection) were measured following the protocols previously described by Schreck & McGovern (38) and Rutledge et al. (37) for fabric repellent and knock-down testing with a modification of the arm-in-cage test. Instead of using human subjects as the blood source, membrane blood-feeders were used. Box bioassay tests were carried out in Plexiglass cages sized 30.5 x 30.5 x 30.5 cm designated as Test box 1 and Test box 2, see Figure 2. On the front side, a 15.2 x 15.2 cm opening allowed access through a cotton

socket sleeve. The back side of the cage was lined with a white absorbent bench pad. On the top of the cage, a 50 mm diameter hole allowed mosquito access to the membrane blood-feeder. Test fabric samples sized 12.7 x 12.7 cm are affixed over the hole in the top of the box, with the outside surface facing into the cage, and the membrane blood-feeder

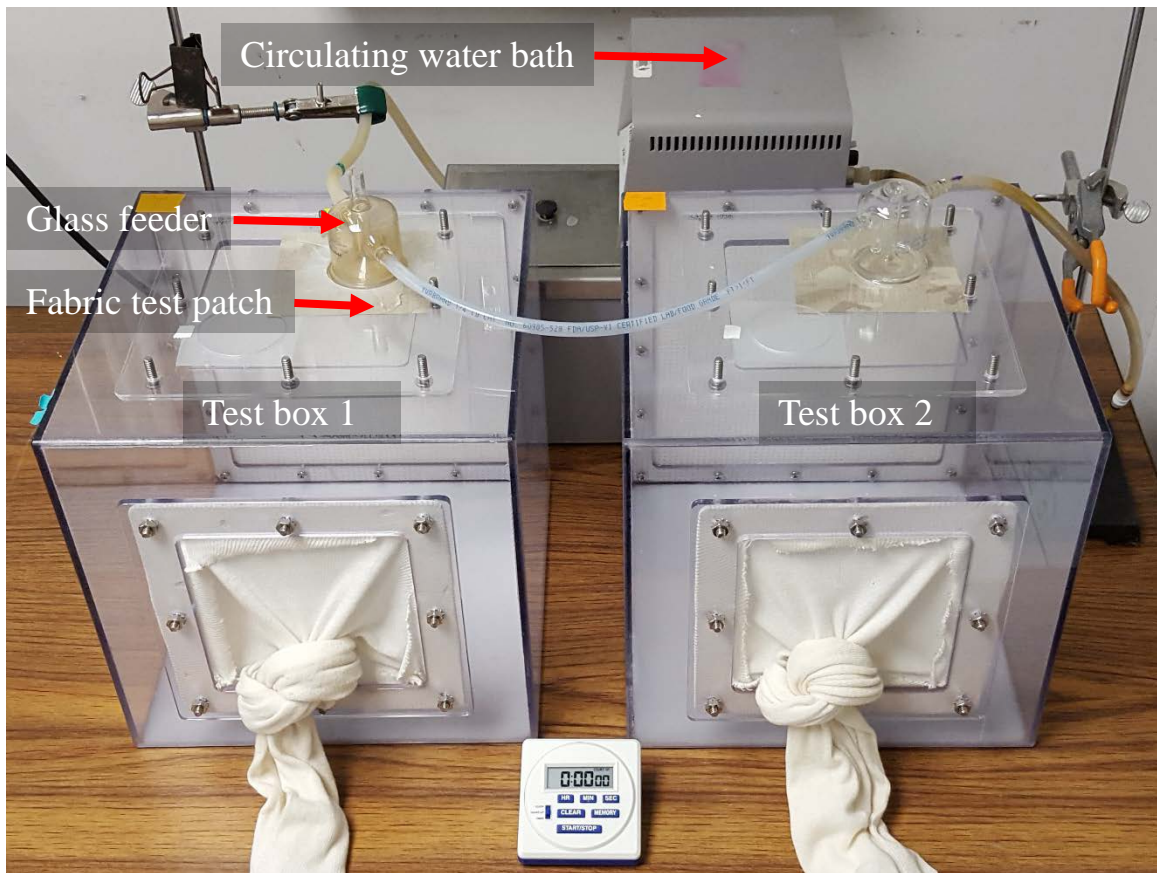


Figure 2. Box bioassay setup

resting on the fabric, see Figure 3. Twenty female mosquitoes aged 5-11 days, fed constantly on a 10% sugar solution on cotton pads and then sugar starved for 24 hours before the test, were placed into the cage at 27°C, 70% RH at time zero. Mosquito behavior, including landing, probing and feeding, was observed every 30 seconds for 10 minutes. The data was recorded on the worksheet, see Appendix A. Mosquitoes were aspirated, by mouth, out of the test box into a 50 mm plastic cup with a mesh lid and

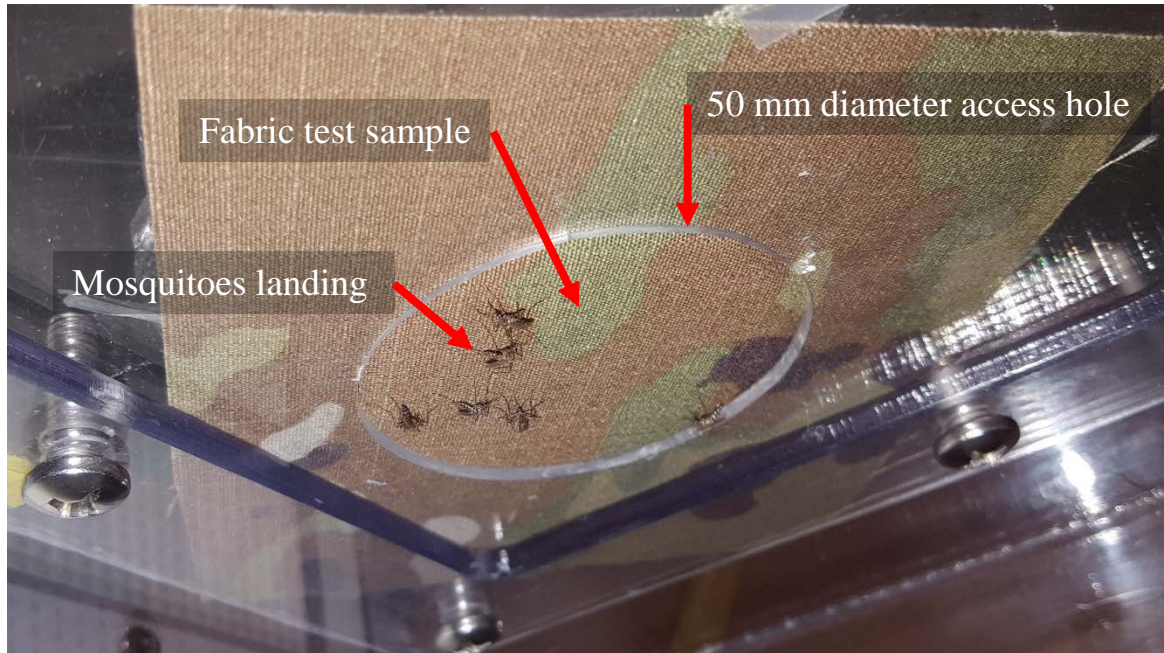


Figure 3. View of test fabric from inside the test box

placed in a  $-20^{\circ}\text{C}$  freezer to immobilize them. They were later observed using a stereo microscope (Leica Microsystems Inc., Buffalo Grove, IL) to determine the number of individuals that had blood-fed. For this test, blood-fed is defined as a mosquito that, upon completion of the replicate is confirmed to have blood in its abdomen. Two test cages were experimented side-by-side. Each test was repeated four times for each species of mosquito, each time using a new batch of twenty 5-11 day-old mosquitoes. The fabric testing schedule is shown in Table 2. In order to offset any mosquito mortality during the 24 hour sugar starvation period, 21 female mosquitoes were added to the plastic holding cups.

Table 2. Fabric testing schedule

Test #	Box Bioassays			
	Box 1		Box 2	
	Rep.	Fabric	Rep.	Fabric
1 <sup>st</sup>	1	Net	1	Untreated OCP
2 <sup>nd</sup>	2	Net	1	Perm-OCP
3 <sup>rd</sup>	3	Net	1	0.9 Pic-OCP
4 <sup>th</sup>	4	Net	1	1.8 Pic-OCP
5 <sup>th</sup>	2	Untreated OCP	2	0.9 Pic-OCP
6 <sup>th</sup>	3	Untreated OCP	2	1.8 Pic-OCP
7 <sup>th</sup>	4	Untreated OCP	2	Perm-OCP
8 <sup>th</sup>	3	Perm-OCP	3	0.9 Pic-OCP
9 <sup>th</sup>	4	Perm-OCP	3	1.8 Pic-OCP
10 <sup>th</sup>	4	0.9 Pic-OCP	4	1.8 Pic-OCP

## DATA ANALYSIS

### Mosquito Bite-through and Blood-feeding

The number of blood-fed mosquitoes in each trial was recorded and the biting rate per mosquito was calculated by dividing the total number of blood-fed mosquitoes in a given feeding assay by the number of mosquitoes used in that assay. Assays were repeated 12 times. Data were analyzed with one-way analysis of variance (ANOVA) and nonparametric Kruskal-Wallis test, comparing the mean rank of each fabric with the mean rank of the control fabric (Perm-OCP) and correcting for multiple comparisons using Dunn's test performed by GraphPad Prism version 7.03 for Windows, (GraphPad Software, La Jolla, CA). A *p*-value of < 0.05 was used to determine statistical significance.

Blood-feeding rates per mosquito of two fabrics were also analyzed with a two-tailed t-test using the Mann-Whitney test performed by GraphPad Prism version 7.03. A *p*-value of < 0.05 was used to determine statistical significance.

Standard error (SE) values for the percentage mosquitoes biting through the fabric and blood-feeding were calculated:  $SE = \sqrt{p(1-p)/n} \times 100$ , where  $p$  is the proportion of insects biting through the fabric and  $n$  is number of insects observed to calculate  $p$ .

The percent fabric bite protection is calculated by the following formula:

$$\left[ 1 - \left( \frac{\text{treatment rate}}{\text{control rate}} \right) \right] \times 100$$

Treatment rate = (# blood-fed female mosquitoes after test interval) / (total # of female mosquitoes in test cage). For the treated OCP fabric.

Control rate = (# blood-fed female mosquitoes after test interval) / (total # of female mosquitoes in test cage). For the untreated OCP fabric.

### **Mosquito Landing Counts**

The total number of landings in each trial is recorded and the landing rate per mosquito was calculated by dividing the total number of landings on the fabric for each 10 minute repetition by the number of mosquitoes in the repetition. Data were analyzed with one-way analysis of variance (ANOVA) and nonparametric Kruskal-Wallis test, comparing the mean rank of each fabric with the mean rank of the positive control fabric (Perm-OCP) and correcting for multiple comparisons using Dunn's test performed using GraphPad Prism version 7.03 for Windows. A  $p$ -value of  $< 0.05$  was used to determine statistical significance.

Landing rates per mosquito of two fabrics were analyzed with a two-tailed t-test using the Mann-Whitney test performed by GraphPad Prism version 7.03. A  $p$ -value of  $< 0.05$  was considered to indicate statistical significance. A landing rate comparison on the two picaridin treated fabrics were made to compare differences between the two different concentrations of repellent. The landing rates per mosquito were compared using a two-

tailed t-test using the Mann-Whitney test performed by GraphPad Prism version 7.03 for Windows. A  $p$ -value of  $< 0.05$  was considered to indicate statistical significance.

## CHAPTER 3: Results

### BITE-THROUGH AND BLOOD-FEEDING PREVENTION

The results of 12 *Ae. aegypti* assay replicates and eight *An. gambiae* assay replicates utilizing the membrane feeding box bioassay device to evaluate the bite through and blood-feeding prevention of the two treated picaridin fabric samples at 0.9 mg/cm<sup>2</sup> and 1.8 mg/cm<sup>2</sup> are presented in Table 3. The following fabrics were tested: 1) untreated net, 2) untreated OCP, 3) permethrin-treated OCP (Perm-OCP), 4) 0.9 mg/cm<sup>2</sup> picaridin-treated OCP (0.9 Pic-OCP) and 5) 1.8 mg/cm<sup>2</sup> picaridin-treated OCP (1.8 Pic-OCP).

Table 3. Bite through and blood-feeding of *Ae. aegypti* and *An. gambiae* on treated and untreated fabrics

Item	Fabrics				
	Untreated Net	Untreated OCP	Permethrin OCP	0.9 mg/cm <sup>2</sup> Picaridin OCP	1.8 mg/cm <sup>2</sup> Picaridin OCP
	<i>Ae. aegypti</i>				
Replicates	12	12	12	12	12
Mean # mosquitoes/rep	20.5	20.7	21	21	21
Total # mosquitoes	246	248	252	252	252
Total # bite-through	113	30	0	0	0
Mean bites through	9.4	2.5	0	0	0
Percent bite through (SE)	46.0 (3.2)	12.1 (2.1)	0	0	0
	<i>An. gambiae</i>				
Replicates	8	8	8	8	8
Mean # mosquitoes/rep	20.3	20.3	20.4	20	20
Total # mosquitoes	162	162	163	160	160
Total # bite-through	11	0	0	0	0
Mean bites through	1.4	0	0	0	0
Percent bite through (SE)	6.8 (2.0)	0	0	0	0

During the *Ae. aegypti* assays, both the untreated net and the untreated OCP fabric had mosquitoes successfully bite through the fabric and blood-fed. The mean of the successful bites through were 9.42 for the untreated net and 2.50 for the untreated OCP fabric per assay. There were zero successful bites through for the treated fabrics. The overall percent of *Ae. aegypti* that bit through the test fabrics and blood-fed were as follows: untreated net 45.5%, untreated OCP 12.1%, Perm-OCP 0%, 0.9 Pic-OCP 0% and 1.8 Pic-OCP 0%.

The *Ae. aegypti* assays were conducted on August 28, October 25 and October 27 in 2017. The blood-feeding rate by date of the *Ae. aegypti* was analyzed to check for variation between the different assay dates. A one-way analysis of variance, nonparametric Kruskal-Wallis test was conducted for the blood-feeding on the untreated net resulted in a  $p$ -value = 0.1141. Following correction for multiple comparisons (Dunn's test), our results indicated that 8/28 and 10/27 differed significantly with an adjusted  $p$ -value = 0.0179. The differences in blood-feeding rates between dates was not ascertained as this was beyond the scope of the study.

The number of assay replicates performed with *An. gambiae* was lower than the number obtained for *Ae. aegypti*, eight vs. 12 assay replicates. *An. gambiae* eggs were produced in sufficient quantities but successful larval development to adult was poor when compared to *Ae. aegypti*. Pothikasikorn et al. (34) indicated that *Ae. aegypti* and *Anopheles dirus* mosquitoes fed preserved human blood outdated by between five and 15 days did not significantly affect survivability to adult, but blood that was older did show lowered survivability. The difference in successful larval development to adult was not

ascertained as this was beyond the scope of this study. Only the untreated net had mosquitoes successfully bite through and blood-feed. The mean of the successful bites through was 1.375 for the untreated net. Overall biting activity for the *An. gambiae* was low. The remaining fabrics, untreated OCP, Perm-OCP, 0.9 Pic-OCP and 1.8 Pic-OCP all had zero successful bites through the fabrics. Only 6.8% successfully bit through the untreated net and blood-fed.

The *An. gambiae* tests were conducted on September 24 and November 1 in 2017. The blood-feeding rate by date of the *An. gambiae* was analyzed to check for variation between the different test dates. A two-tailed Mann-Whitney test to compare ranks was conducted for the blood-feeding on the untreated Net fabric. The resultant *p*-value was 0.3714 (CI of difference = 97.14%) indicates that the medians did not vary significantly.

Results of the tests to determine the blood-feeding rates of *Ae. aegypti* and *An. gambiae* on the respective test fabrics is shown in Table 4. The calculated percent fabric bite protection using the untreated net as the control for the *Ae. aegypti* tests were 73.67% bite protection from the untreated OCP fabric and 100% protection from the Perm-OCP, 0.9 Pic-OCP and 1.8 Pic-OCP fabrics. The *An. gambiae* tests results demonstrate that all the fabrics, both treated and untreated provided 100% bite protection when using the untreated net as the control.

Table 4. Rates of blood-feeding *Ae. aegypti* and *An. gambiae* tested against different test fabrics

Rep. No.	Fabrics				
	Untreated Net	Untreated OCP	Permethrin OCP	0.9 mg/cm <sup>2</sup> Picaridin OCP	1.8 mg/cm <sup>2</sup> Picaridin OCP
<b><i>Ae. aegypti</i></b>					
1	0.8571	0.4	0	0	0
2	0.3	0.4	0	0	0
3	0.7143	0.1905	0	0	0
4	0.7	0	0	0	0
5	0.5238	0.1905	0	0	0
6	0.5	0.0476	0	0	0
7	0.619	0.1429	0	0	0
8	0.5238	0.0476	0	0	0
9	0.2	0	0	0	0
10	0.0952	0.05	0	0	0
11	0.2105	0	0	0	0
12	0.2381	0	0	0	0
<b><i>An. gambiae</i></b>					
1	0.0952	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0.3684	0	0	0	0
6	0	0	0	0	0
7	0.05	0	0	0	0
8	0.0526	0	0	0	0

### Specific Aim 1

The Mann-Whitney test results comparing mosquito blood-feeding rate mean ranks of untreated OCP fabric with picaridin treated fabrics on *Ae. aegypti* are tabulated in Table 5. There is a significant difference between the biting rate mean ranks of 0.9 Pic-OCP ( $p = 0.0013$ ) and 1.8 Pic-OCP ( $p = 0.0013$ ) compared to the untreated OCP fabric. This represents a reduction in blood-feeding.

*An. gambiae* tests results to compare mean ranks indicated that there was no significant difference between the bite rate of the untreated OCP fabric and both picaridin OCP ( $p > 0.9999$ ). All means were equal to zero.

Table 5. *Ae. aegypti* biting rate mean ranks of untreated OCP fabric compared with picaridin treated fabrics

Item	<i>Ae. aegypti</i>		<i>Ae. aegypti</i>	
	Untreated OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	Untreated OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
Mann-Whitney test mean	0.1224	0	0.1224	0
Std. Deviation	0.1478	0	0.1478	0
Std. Error of Mean	0.04266	0	0.04266	0
Lower 95% CI	0.02854	0	0.02854	0
Upper 95% CI	0.2163	0	0.2163	0
Mann-Whitney test mean ranks	16.5	8.5	16.5	8.5
Mann-Whitney test mean $p$ -value	0.0013*		0.0013*	

Mann-Whitney test comparing mosquito biting rate mean ranks.

\*  $p$ -value of  $< 0.05$ .

An additional comparison was made between the net and untreated OCP. A two-tailed nonparametric  $t$  test, using the Mann-Whitney test to compare ranks was performed and the results demonstrate that for the *Ae. aegypti* there was a significant difference ( $p = 0.0002$ ) between the two fabrics, while *An. gambiae* did not demonstrate a significant difference ( $p = 0.0769$ ), see Table 6.

Table 6. Biting rate mean ranks of untreated net compared with untreated OCP fabric on *Ae. aegypti* and *An. gambiae*

Item	<i>Ae. aegypti</i>		<i>An. gambiae</i>	
	Untreated Net	Untreated OCP	Untreated Net	Untreated OCP
Mann-Whitney test mean	0.4568	0.1224	0.07078	0
Std. Deviation	0.2436	0.1478	0.1253	0
Std. Error of Mean	0.07031	0.04266	0.04432	0
Lower 95% CI	0.3021	0.02854	-0.03401	0
Upper 95% CI	0.6116	0.2163	0.1756	0
Mann-Whitney test mean ranks	17.42	7.583	10.5	6.5
Mann-Whitney test mean <i>p</i> -value	0.0002*		0.0769	

Mann-Whitney test comparing mosquito biting rate mean ranks.

\* *p*-value of < 0.05.

## Specific Aim 2

The one-way analysis of variance, nonparametric Kruskal-Wallis test (Table 7) indicated that there was a significant result. Following correction for multiple comparisons (Dunn's test), our results clearly demonstrate that the *Ae. aegypti* bite rate mean ranks of the untreated net ( $p < 0.0001$ ) and untreated OCP ( $p = 0.0171$ ) fabrics were significantly different from the positive control fabric Perm-OCP. The bite rate mean rank of the 0.9 Pic-OCP and 1.8 Pic-OCP were not significantly different from the positive control fabric Perm-OCP having a *p*-value greater than 0.9999.

For the *An. gambiae* assays, our results indicated a significant difference only for the untreated net ( $p = 0.0041$ ) (Table 7). For the remaining fabrics, when correcting for multiple comparisons (Dunn's test), between the bite rate mean rank of each fabric with the bite rate mean rank of the Perm-OCP fabric as the positive control, the untreated OCP

and both picaridin fabrics were not significantly different than the control fabric Perm-OCP, with a  $p$ -value  $>0.9999$ .

Table 7. Mosquito bite rates

Item	Fabrics				
	Untreated Net	Untreated OCP	Permethrin OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
<i>Ae. aegypti</i>					
Mean biting rate	0.4568	0.1224	0	0	0
Dunn's mean rank	53.42	37.58	20.5	20.5	20.5
Dunn's adjusted $p$ -value	$p<0.0001^*$	$0.0171^*$	Control	$p>0.9999$	$p>0.9999$
<i>An. gambiae</i>					
Mean biting rate	0.0679	0	0	0	0
Dunn's mean rank	28.5	18.5	18.5	18.5	18.5
Dunn's adjusted $p$ -value <sup>1</sup>	$0.0041^*$	$p>0.9999$	Control	$p>0.9999$	$p>0.9999$

<sup>1</sup>One-Way ANOVA (and Nonparametric) Kruskal-Wallis test using Dunn's test to correct for multiple comparisons, control fabric permethrin OCP.

\*  $p$ -value of  $< 0.05$ .

## LANDING COUNT PREVENTION

The results of 12 *Ae. aegypti* tests and eight *An. gambiae* tests utilizing the membrane feeding box bioassay device to evaluate the landing count prevention of the two treated picaridin fabric samples at 0.9 mg/cm<sup>2</sup> and 1.8 mg/cm<sup>2</sup> are presented in Table 8. During the *Ae. aegypti* tests, all test fabrics had mosquitoes successfully land and attempt to probe. The mean mosquito landing counts per test were 162.6 for the untreated net, 155.4 for the untreated OCP fabric, 10.0 for the Perm-OCP fabric, 15.8 for the 0.9 Pic-OCP fabric and 11.1 for the 1.8 Pic-OCP fabric.

Table 8. Landing counts of *Ae. aegypti* and *An. gambiae* on treated and untreated fabrics

Item	Fabrics				
	Untreated Net	Untreated OCP	Permethrin OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
<b><i>Ae. aegypti</i></b>					
Replicates	12	12	12	12	12
Mean # mosquitoes/rep	20.5	20.7	21	21	21
Total # mosquitoes	246	248	252	252	252
Landing Count	1951	1865	120	189	133
Mean landing count	162.6	155.4	10.00	15.8	11.1
<b><i>An. gambiae</i></b>					
Replicates	8	8	8	8	8
Mean # mosquitoes/rep	20.3	20.3	20.4	20	20
Total # mosquitoes	162	162	163	160	160
Landing Count	228	88	6	0	4
Mean landing count	28.5	11	0.8	0	0.5

During the *An. gambiae* tests, nearly all test fabrics had mosquitoes successfully land and attempt to probe. The mean mosquito landing counts per test were 28.5 for the untreated net, 11 for the untreated OCP fabric, 0.8 for the Perm-OCP fabric, 0.0 for the 0.9 Pic-OCP fabric and 0.5 for the 1.8 Pic-OCP fabric. Results of the tests to determine the landing rates of *Ae. aegypti* and *An. gambiae* on the respective test fabrics is shown in Table 9.

Table 9. Landing rates of *Ae. aegypti* and *An. gambiae* on test fabrics

Rep. No.	Fabrics				
	Untreated Net	Untreated OCP	Permethrin OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
<b><i>Ae. aegypti</i></b>					
1	10.7619	10.8	0.9048	1.9524	0.7619
2	10.9	9.25	0.9048	0.9524	2
3	13	14.7143	0.5	1	0.8571
4	10.6	9.3333	1	2.0952	1.1429
5	7.2381	5.8095	0.2857	0.4762	0.3333
6	9.1	6.9524	0.619	0.4286	0.4286
7	11.381	10	0.3333	0.1905	0.1905
8	10.7619	13.2857	0.619	1	0.4762
9	2.4	1.0952	0.1905	0	0
10	1.0952	3.45	0.0952	0	0
11	3.5789	1.95	0.1905	0.5714	0
12	4	3.381	0.0476	0.3333	0.1429
<b><i>An. gambiae</i></b>					
1	1.4762	0.381	0	0	0
2	0	0.5714	0.05	0	0
3	0	0	0	0	0
4	0	0.5714	0	0	0
5	4.5263	1.3889	0.1	0	0
6	0.05	0.8947	0.1	0	0.1053
7	3.05	0.7	0.05	0	0
8	2.5789	0	0	0	0.1053

### Specific Aim 3

The Mann-Whitney test results comparing mosquito landing rate mean ranks of untreated OCP fabric with picaridin treated fabrics on *Ae. aegypti* are tabulated in Table 10. Our results demonstrate there is a significant difference between the landing rate mean ranks of the untreated OCP fabric with both picaridin fabrics ( $p < 0.0001$  for both fabrics). The *An. gambiae* landing rate comparisons are shown in Table 11. Our test

Table 10. Landing rate mean ranks of untreated OCP fabric compared with picaridin treated fabrics on *Ae. aegypti*

Item	<i>Ae. aegypti</i>		<i>Ae. aegypti</i>	
	Untreated OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	Untreated OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
Mann-Whitney test mean	7.901	0.75	7.901	0.5278
Std. Deviation	4.08	0.6904	4.08	0.5921
Std. Error of Mean	1.178	0.1993	1.178	0.1709
Lower 95% CI	5.309	0.3113	5.309	0.1516
Upper 95% CI	10.49	1.189	10.49	0.904
Mann-Whitney test mean ranks	18.33	6.667	18.33	6.667
Mann-Whitney test mean <i>p</i> -value	< 0.0001*		< 0.0001*	

Mann-Whitney test comparing mosquito landing rate mean ranks.

\* *p*-value of < 0.05.

Table 11. Landing rate mean ranks of untreated OCP fabric compared with picaridin treated fabrics on *An. gambiae*

Item	<i>An. gambiae</i>		<i>An. gambiae</i>	
	Untreated OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	Untreated OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
Mann-Whitney test mean	0.5634	0	0.5634	0.02633
Std. Deviation	0.4595	0	0.4595	0.04874
Std. Error of Mean	0.1625	0	0.1625	0.01723
Lower 95% CI	0.1793	0	0.1793	-0.01443
Upper 95% CI	0.9476	0	0.9476	0.06708
Mann-Whitney test mean ranks	11.5	5.5	11.25	5.75
Mann-Whitney test mean <i>p</i> -value	0.0070*		0.0120*	

Mann-Whitney test comparing mosquito landing rate mean ranks.

\* *p*-value of < 0.05.

results clearly indicate a significant difference between the untreated OCP fabric and both picaridin treated fabrics, 0.9 Pic-OCP ( $p = 0.0070$ ) and 1.8 Pic-OCP ( $p = 0.0120$ ).

Picaridin reduced the landing rates for *Ae. aegypti* and *An. gambiae*.

Additional results for the comparison between the untreated net and the untreated OCP fabric landing rates are shown in Table 12. The results demonstrate that there was no significant difference between the two fabrics for either species, *Ae. aegypti* ( $p = 0.6808$ ) and *An. gambiae* ( $p = 0.6185$ ). The landing rates between the two untreated fabrics did not differ.

Table 12. Landing rate mean ranks of untreated net compared with untreated OCP fabric on *Ae. aegypti* and *An. gambiae*

Item	<i>Ae. aegypti</i>		<i>An. gambiae</i>	
	Untreated Net	Untreated OCP	Untreated Net	Untreated OCP
Mann-Whitney test mean	7.901	7.502	1.46	0.5634
Std. Deviation	4.08	4.446	1.755	0.4595
Std. Error of Mean	1.178	1.283	0.6206	0.1625
Lower 95% CI	5.309	4.677	-0.007219	0.1793
Upper 95% CI	10.49	10.33	2.928	0.9476
Mann-Whitney test mean ranks	13.3	11.88	9.125	7.875
Mann-Whitney test mean $p$ -value	0.6808		0.6185	

Mann-Whitney test comparing mosquito landing rate mean ranks.  
 $p$ -value of  $< 0.05$ .

#### Specific Aim 4

The one-way analysis of variance, nonparametric Kruskal-Wallis test (Table 13) on the *Ae. aegypti* indicated that there was a significant result. Following correction for multiple comparisons (Dunn's test), our results demonstrate that the *Ae. aegypti* landing

rate mean rank of the untreated net ( $p < 0.0001$ ) and untreated OCP ( $p = 0.0002$ ) fabrics were significantly different from the positive control fabric Perm-OCP. The landing rate of the 0.9 Pic-OCP and 1.8 Pic-OCP were not significantly different from the positive control fabric Perm-OCP having a  $p$ -value greater than 0.9999.

Table 13. Mosquito landing rates

Item	Fabrics				
	Untreated Net	Untreated OCP	Permethrin OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
<i>Ae. aegypti</i>					
Mean landing rate	7.901	7.502	0.4742	0.75	0.5278
Dunn's mean rank	48.79	47.29	18	21.46	16.96
Dunn's adjusted $p$ -value	$p < 0.0001^*$	0.0002*	Control	$p > 0.9999$	$p > 0.9999$
<i>An. gambiae</i>					
Mean landing rate	1.46	0.5634	0.0375	0	0.02633
Dunn's mean rank	26.88	28.13	19.13	12	16.38
Dunn's adjusted $p$ -value <sup>1</sup>	0.5623	0.3481	Control	0.7019	$p > 0.9999$

<sup>1</sup>One-Way ANOVA (and Nonparametric) Kruskal-Wallis test using Dunn's test to correct for multiple comparisons, control fabric permethrin OCP.

\*  $p$ -value of  $< 0.05$ .

For the *An. gambiae* tests, our results indicated no significant differences (Table 13). Correcting for multiple comparisons (Dunn's test), the landing rate mean rank  $p$ -values of the untreated and picaridin OCP fabrics with Perm-OCP as the control were: untreated net  $p = 0.5623$ , untreated OCP  $p = 0.3481$ , 0.9 Pic-OCP  $p = 0.7019$  and 1.8 Pic-OCP  $p > 0.9999$ .

The two-tailed nonparametric  $t$  test, using the Mann-Whitney test results comparing mean ranks of the two different concentrations of picaridin are tabulated in Table 14. Our results indicated that there was no significant differences between the two

fabrics for both the *Ae. aegypti* ( $p = 0.3668$ ) and *An. gambiae* ( $p = 0.4667$ ) mosquitoes. Both concentrations of picaridin demonstrated similar effectiveness at reducing landing rates.

Table 14. Landing rate mean ranks of 0.9 Pic-OCP fabric compared with 1.8 Pic-OCP fabric on *Ae. aegypti* and *An. gambiae*

Item	<i>Ae. aegypti</i>		<i>An. gambiae</i>	
	0.9 mg/cm <sup>2</sup> picaridin OCP	1.8 mg/cm <sup>2</sup> picaridin OCP	0.9 mg/cm <sup>2</sup> picaridin OCP	1.8 mg/cm <sup>2</sup> picaridin OCP
Mann-Whitney test mean	0.75	0.5278	0	0.02633
Std. Deviation	0.6904	0.5921	0	0.04874
Std. Error of Mean	0.1993	0.1709	0	0.01723
Lower 95% CI	0.3113	0.1516	0	-0.01443
Upper 95% CI	1.189	0.904	0	0.06708
Mann-Whitney test mean ranks	13.83	11.17	7.5	9.5
Mann-Whitney test mean $p$ -value	0.3668		0.4667	

Mann-Whitney test comparing mosquito landing rate mean ranks.  
 $p$ -value of  $< 0.05$ .

## CHAPTER 4: Discussion

The use of repellents on clothing is an important part of the insect repellent system used to protect military personnel from mosquitoes and other arthropod vectors (6). This system is called the Department of Defense (DoD) Insect Repellent System, which consists of a skin treatment containing DEET (diethyltoluamide), picaridin or IR3535 and a uniform treatment that consists of permethrin. Permethrin is a contact repellent and tightly binds to cotton fabric which makes it resistant to washing out of the fabric (1). Picaridin is more of a spatial repellent, disrupting the mosquitoes odorant receptors making it difficult to find the host (3) and can be washed off the skin with soap and water (1). Currently, permethrin is used to treat clothing as well as Long-Lasting Insecticidal Nets to protect people worldwide from insect vectored disease. How long permethrin will continue to be an effective repellent remains to be seen as resistance to this compound increases throughout the world and can lead to a failure to protect (19; 28; 31). It is important to develop new compounds that can replace permethrin as a fabric treatment.

Picaridin is one of the newer compounds to be used as a repellent. It is mostly used as a direct application to the skin and is not widely used as a fabric treatment. During their investigation of methods for the preparation of antimicrobial microcapsule treatment of military uniform fabric, NSRDEC incorporated picaridin into the microcapsules and tested the treatment for its overall functionality and stability (33). If this treatment performs as well as permethrin treated fabrics, it may be used as a possible replacement fabric treatment in areas that have permethrin resistant vectors of disease.

This paper investigates the effectiveness of two different concentrations of microencapsulated picaridin treatments on military-relevant fabrics and compares it to the current U.S. Army permethrin treated army combat uniform. All levels of significance were calculated for of  $p$ -value of  $< 0.05$

Bite protection is an important part in preventing the spread of mosquito borne disease. If a treated fabric prevents a mosquito from biting through it and blood-feeding, there can be no transmission of disease. In the first set of assays, five fabrics were tested to see how effective *Ae. aegypti* and *An. gambiae* mosquitoes were at biting through the fabric and blood-feeding. The first fabric was an untreated net with 100 holes per cm<sup>2</sup>. It was used to represent a situation that minimized any barriers to feeding and demonstrate the mosquitoes' willingness to bite. The second fabric was an untreated swatch of the U.S. Army combat uniform and contained no repellents. It was used to demonstrate how much of the bite protection was due to the physical barrier that the fabric itself confer. The remaining three fabrics were all the same type of fabric as the second fabric, the U.S. Army combat uniform with the OCP camouflage pattern, the only differences were the repellent treatments to the fabric. The third fabric was the standard U.S. Army combat uniform factory-treated with permethrin and represents the bite protection levels that are currently accepted by the military. This fabric was considered the positive control and used to compare the effectiveness of the treated picaridin fabrics. The remaining two fabric types were treated with the microencapsulated picaridin, containing an approximate concentration of 0.9 mg/cm<sup>2</sup> and 1.8 mg/cm<sup>2</sup> of picaridin.

## ***Ae. AEGYPTI* BITE PROTECTION**

The *Ae. aegypti* bite tests had the following mean bite-through and successful blood-feeding results of 9.4 for the untreated net, 2.5 for the untreated OCP fabric and zero for the permethrin and picaridin treated fabrics. This equated to only 46% of the mosquitoes successfully biting through the untreated net, 12.1% biting through the untreated OCP fabric and no successful bites through the permethrin and picaridin treated fabrics. The calculated percent fabric bite protection using the untreated net as the control were 73.67% bite protection from the untreated OCP fabric and 100% protection from the Perm-OCP and picaridin treated fabrics.

The comparison between the picaridin treated fabrics with the untreated OCP demonstrated that the picaridin treatment significantly reduced the amount of blood-feeding by the *Ae. aegypti* mosquito. Our test results clearly indicated that there was a significant difference between the biting rate mean ranks of the untreated OCP fabric with both picaridin fabrics ( $p = 0.0013$  for both fabrics). The picaridin treated fabrics significantly reduced the number of bites on top of what the untreated fabrics alone would reduce.

During the testing, not every repetition contained the same number of mosquitoes in the sample. An extra mosquito was added to each test cup in anticipation of any mosquito mortality leading up to the day of the experiment. Counting errors also resulted in a variation of the sample numbers. Because the sample size varied by one or two mosquitoes between repetitions, it was determined to use a bite rate when conducting the analysis of variance comparisons. Our test demonstrated that the untreated net ( $p < 0.0001$ ) and untreated OCP ( $p = 0.0171$ ) fabrics mean bite rate ranks were significantly different from the Perm-OCP fabric while the two picaridin treated fabrics were not. The

two picaridin treated fabrics (both  $p > 0.9999$ ) had a similar bite rate reduction as the Perm-OCP control. All treated fabrics had a significantly reduced *Ae. aegypti* bite rate compared to the untreated fabrics.

Our test results indicated that the barrier to biting created by the untreated OCP fabric significantly reduces the bite rate. This would be expected because uniform fabrics are designed to reduce mosquito bites (1; 14). The untreated OCP fabric did not prevent all bites which is why repellent treated fabrics are used. An effective fabric repellent treatment would be expected to add an additional amount of protection. Our test results indicate that the repellent fabrics significantly reduced *Ae. aegypti* biting beyond what would be expected from the untreated fabric by itself.

#### ***AN. GAMBIAE* BITE PROTECTION**

The *An. gambiae* blood-feeding tests had the following mean bite-through and successful blood-feeding results of 1.4 for the untreated net, zero for the untreated OCP fabric and zero for the permethrin and picaridin treated fabrics. This equated to only 6.8% of the mosquitoes successfully biting through the untreated net and no successful bites through untreated OCP fabric or the permethrin and picaridin treated fabrics. The calculated percent fabric bite protection using the untreated net as the control were 100% bite protection from the untreated OCP fabric and 100% protection from the permethrin and picaridin treated fabrics.

Difficulties in rearing *An. gambiae* mosquitoes in sufficient quantities resulted in only eight of the planned 12 tests to be completed. Eggs could be reared in sufficient quantities but successful larval development to adult was poor. The testing was also conducted during the same time of the day for both species. *An. gambiae* feeding activity

occurs in the evenings whereas *Ae. aegypti* feed during the day. These differences in feeding times along with the strength of the *An. gambiae* colony could account for some of the reduced bite rate numbers.

Our test results indicated that there was a significant result only for the untreated net ( $p = 0.0041$ ). Following correction for multiple comparisons (Dunn's test), the untreated OCP and both picaridin fabrics were not significantly different than the control fabric Perm-OCP, with a  $p$ -value  $>0.9999$ .

#### ***AE. AEGYPTI* LANDING COUNTS**

During the *Ae. aegypti* tests, all test fabrics had mosquitoes successfully land and attempt to probe. The mean mosquito landing counts per test were 162.6 for the untreated net, 155.4 for the untreated OCP fabric, 10.0 for the Perm-OCP fabric, 15.8 for the 0.9 Pic-OCP fabric and 11.1 for the 1.8 Pic-OCP fabric.

The comparison of the mean landing rate per mosquito between the picaridin treated fabrics vs. the untreated OCP demonstrated that the picaridin treatment significantly reduced the landing count by the *Ae. aegypti* mosquito. Our results clearly indicated that there was a significant difference between the landing rate mean ranks of the untreated OCP fabric with both picaridin fabrics ( $p < 0.0001$  for both fabrics).

The comparison of the landing rate mean rank of each fabric to the landing rate mean rank of the control Perm-OCP fabric indicated that the untreated net ( $p < 0.0001$ ) and untreated OCP ( $p = 0.0002$ ) fabrics were significantly different from the positive control fabric Perm-OCP. The landing rates of the two picaridin fabrics were not significantly different from the positive control fabric Perm-OCP having a  $p$ -value greater than 0.9999.

Our results indicated that there was no significant difference between the net and the untreated fabrics. The *Ae. aegypti* were just as likely to land on the untreated net as they were likely to land on the untreated OCP fabric. This demonstrates that the military fabric itself does not have a repellent effect. Any reduction in landing count rates can be attributed to the repellency effects of the different repellent treatments on the fabric.

#### ***AN. GAMBIAE* LANDING COUNTS**

During the *An. gambiae* tests, all test fabrics except the 0.9 Pic-OCP had mosquitoes successfully land and attempt to probe. Unlike the *Ae. aegypti* test, many of the *An. gambiae* repetitions had zero landings during the 10 minute test. The mean mosquito landing counts per test were 28.5 for the untreated net, 11 for the untreated OCP fabric, 0.8 for the Perm-OCP fabric, 0.0 for the 0.9 Pic-OCP fabric and 0.5 for the 1.8 Pic-OCP fabric.

The comparison of the mean landing rate per mosquito between the picaridin treated fabrics vs. the untreated OCP demonstrated that the picaridin treatment significantly reduced the landing count by the *An. gambiae* mosquito. The Mann-Whitney test results indicated that there was a significant difference between the landing rate mean ranks of the untreated OCP fabric with both picaridin fabrics ( $p < 0.0001$  for both fabrics).

Our results indicated that there was no significant differences between the landing rates mean rank of the untreated fabrics or of the landing rate mean rank of the picaridin OCP fabrics. Following correction for multiple comparisons (Dunn's test), the adjusted  $p$ -value for each fabric was as follows: untreated net  $p = 0.5623$ , untreated OCP  $p = 0.3481$ , 0.9 Pic-OCP  $p = 0.7019$  and 1.8 Pic-OCP  $p > 0.9999$ . This is not to say that the

*An. gambiae* were just as likely to land on any of the fabrics and that the repellents had no effect but is more indicative of the low activity of the mosquitoes and their lack of desire to feed.

## LIMITATIONS

Time was a limiting factor in conducting these tests. Only four repetitions of each fabric per day, for a total of 20 total tests, was possible. In between tests a new batch of eggs was used to prepare for the next test. Small variations between the populations could account for small differences between the test days.

The blood used for the different tests was not always from the same batch. The freshest batch of blood was used whenever possible and this resulted in some tests having blood that was a different age and possibly a different donor used. These variations could affect the mosquitoes' willingness to feed and fully engorge (34).

The blood-feeding rate by date of the *Ae. aegypti* was analyzed to check for variation between the different test dates. Our results indicate there was a significant difference between the dates ( $p = 0.0040$ ). Further analysis with Dunn's multiple comparison test indicated that 8/28 and 10/27 differed significantly with an adjusted  $p$ -value = 0.0179. Some of the differences between these dates were the eggs used and the age of the blood. The 28 August blood was four days old while the 27 October blood was 21 days old. Pothikasikorn et al. (34) demonstrated that out-of-date blood used on day 25 resulted in a reduction in blood-feeding compared to blood used on day five. This could account for the reduced numbers in blood-feeding for the latter date.

## CHAPTER 5: Conclusion

This was the first study to examine microencapsulated picaridin treatments on military uniform fabric. The goal of this study was to evaluate two picaridin treated fabrics and test their bite protection and landing count properties. The study successfully met its objectives and obtained data allowing decision-making on the study hypotheses.

### STUDY GOALS AND OBJECTIVES

The goal of this research was to evaluate the effectiveness of microencapsulated picaridin fabric treatments at repelling mosquitoes and preventing them from blood-feeding.

The objectives of this research were:

- 1) Assess the effect that picaridin treated fabrics have at preventing *Ae. aegypti* and *An. gambiae* from blood-feeding.
  - a. Determine whether picaridin treated fabric conferred greater protection from *Ae. aegypti* and *An. gambiae* blood-feeding than a similar untreated fabric.
  - b. Determine whether picaridin treated fabric conferred greater protection from *Ae. aegypti* and *An. gambiae* blood-feeding than the permethrin treated fabric.

In this study, picaridin treated fabric reduced the number of successful blood-feeds by *Ae. aegypti* mosquitoes. The fabric alone was able to reduce mosquito blood-feeding, but with the addition of a picaridin treatment to the fabric, an additional significant number of mosquitoes were prevented from blood-feeding. No mosquitoes were able to blood-feed through any of the treated fabrics. When the picaridin treated

fabrics were compared to the permethrin treated fabric, no significant difference was detected.

The blood-feeding activity of the *An. gambiae* was not as expected. Only 6.8% of the mosquitoes blood-fed, and of those none were able to bite through any of the military fabrics. Statistically there was no difference between the untreated OCP fabric and the net.

- 2) Assess the effect that picaridin treated fabrics have at repelling *Ae. aegypti* and *An. gambiae*.
  - a. Determine whether picaridin treated fabric repel more *Ae. aegypti* and *An. gambiae* than untreated military fabric.
  - b. Determine whether picaridin treated fabric repel more *Ae. aegypti* and *An. gambiae* than permethrin treated fabric.

In this study, picaridin treated fabric reduced the number of *Ae. aegypti* mosquitoes landing on the fabric. Landing behavior did not differ between the net and the untreated OCP as expected. There was a significant reduction in landings between picaridin and the untreated OCP fabric. When the picaridin treated fabrics were compared to the permethrin treated fabric, no significant difference was detected.

The landing results of the *An. gambiae* was not much different than the *Ae. aegypti*. Landing behavior did not differ between the net and the untreated OCP as expected. There was a significant reduction in landings between picaridin and the untreated OCP fabric. When the picaridin treated fabrics were compared to the permethrin treated fabric, no significant difference was detected.

## HYPOTHESES DECISIONS

**H<sub>0</sub> #1:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will = the mean blood-feeding rate of untreated OCP fabric.

**H<sub>A</sub> #1:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will  $\neq$  the mean blood-feeding rate of untreated OCP fabric. (i.e., blood-feeding will significantly decrease).

**Specific Aim #1.** Compare the mean blood-feeding (bite-through) rate per mosquito of the two picaridin treated fabrics vs. untreated OCP fabric for each of the two mosquito species.

**Results:**     *Ae. aegypti*

- a) Mean blood-feeding rate of untreated OCP (0.1244) is significantly different ( $p = 0.0013$ ) than 0.9 Pic-OCP (0.0). The small  $p$ -value indicates that there is strong evidence against H<sub>0</sub>.
- b) Mean blood-feeding rate of untreated OCP (0.1244) is significantly different ( $p = 0.0013$ ) than 1.8 Pic-OCP (0.0). The small  $p$ -value indicates that there is strong evidence against H<sub>0</sub>.

*An. gambiae*

- a) Mean blood-feeding rate of untreated OCP (0.0) is not significantly different ( $p > 0.9999$ ) than 0.9 Pic-OCP (0.0). The large  $p$ -value indicates that there is little or no evidence against H<sub>0</sub>.
- b) Mean blood-feeding rate of untreated OCP (0.0) is not significantly different ( $p > 0.9999$ ) than 1.8 Pic-OCP (0.0). The

large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

**Decision:** *Ae. aegypti* reject  $H_0$  #1 and accept  $H_A$

*An. gambiae* fail to reject  $H_0$  #1 and reject  $H_A$

**$H_0$  #2:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will = the mean blood-feeding rate on Perm-OCP fabric (i.e., bite-through protection will not differ from the permethrin treated fabric).

**$H_A$  #2:** Mosquitoes' mean blood-feeding rate on picaridin treated fabric will  $\neq$  the mean blood-feeding rate on Perm-OCP fabric.

**Specific Aim #2.** Compare any difference between blood-feeding rates of all test fabrics for each of the two mosquito species.

**Results:** *Ae. aegypti*

- a) Mean blood-feeding rate of Perm-OCP (0.0) is not significantly different ( $p > 0.9999$ ) than 0.9 Pic-OCP (0.0). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .
- b) Mean blood-feeding rate of Perm-OCP (0.0) is not significantly different ( $p > 0.9999$ ) than 1.8 Pic-OCP (0.0). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

*An. gambiae*

- a) Mean blood-feeding rate of Perm-OCP (0.0) is not significantly different ( $p > 0.9999$ ) than 0.9 Pic-OCP (0.0). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

- b) Mean blood-feeding rate of Perm-OCP (0.0) is not significantly different ( $p > 0.9999$ ) than 1.8 Pic-OCP (0.0). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

**Decision:** *Ae. aegypti* fail to reject  $H_0$  #2 and reject  $H_A$

*An. gambiae* fail to reject  $H_0$  #2 and reject  $H_A$

**$H_0$  #3:** Mosquitoes' mean landing rate on picaridin treated fabric will = the mean landing rate of untreated OCP fabric.

**$H_A$  #3:** Mosquitoes' mean landing rate on picaridin treated fabric will  $\neq$  the mean landing rate of untreated OCP fabric (i.e., landing rate will significantly decrease).

**Specific Aim #3.** Compare the mean landing rate per mosquito of the two picaridin treated fabrics vs. untreated OCP fabric for each of the two mosquito species.

**Results:** *Ae. aegypti*

- a) Mean landing rate of untreated OCP (7.901) is significantly different ( $p < 0.0001$ ) than 0.9 Pic-OCP (0.75). The small  $p$ -value indicates that there is strong evidence against  $H_0$ .
- b) Mean landing rate of untreated OCP (7.901) is significantly different ( $p < 0.0001$ ) than 1.8 Pic-OCP (0.5278). The small  $p$ -value indicates that there is strong evidence against  $H_0$ .

*An. gambiae*

- a) Mean landing rate of untreated OCP (0.5634) is significantly different ( $p = 0.0070$ ) than 0.9 Pic-OCP (0.0). The small  $p$ -value indicates that there is strong evidence against  $H_0$ .

- b) Mean landing rate of untreated OCP (7.901) is significantly different fabrics ( $p = 0.0120$ ) than 1.8 Pic-OCP (0.02633). The small  $p$ -value indicates that there is strong evidence against  $H_0$ .

**Decision:** *Ae. aegypti* reject  $H_0$  #3 and accept  $H_A$

*An. gambiae* reject  $H_0$  #3 and accept  $H_A$ .

**$H_0$  #4:** Mosquitoes' mean landing rate on picaridin treated fabric will = the mean landing rate on Perm-OCP fabric (i.e., landing rate will not differ from the permethrin treated fabric).

**$H_A$  #4:** Mosquitoes' mean landing rate on picaridin treated fabric will  $\neq$  the mean landing rate on Perm-OCP fabric.

**Specific Aim #4.** Compare any difference between landing rates of all test fabrics for each of the two mosquito species.

**Results:** *Ae. aegypti*

- a) Mean landing rate of Perm-OCP (0.4742) is not significantly different ( $p > 0.9999$ ) than 0.9 Pic-OCP (0.75). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .
- b) Mean landing rate of Perm-OCP (0.4742) is not significantly different ( $p > 0.9999$ ) than 1.8 Pic-OCP (0.5278). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

*An. gambiae*

- a) Mean landing rate of Perm-OCP (0.0375) is not significantly different ( $p = 0.7019$ ) than 0.9 Pic-OCP (0.0). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

b) Mean landing rate of Perm-OCP (0.0375) is not significantly different ( $p > 0.9999$ ) than 1.8 Pic-OCP (0.02633). The large  $p$ -value indicates that there is little or no evidence against  $H_0$ .

**Decision:** *Ae. aegypti* fail to reject  $H_0$  #4 and reject  $H_A$ .

*An. gambiae* fail to reject  $H_0$  #4 and reject  $H_A$ .

#### **FUTURE DIRECTIONS FOR RESEARCH**

Although these microencapsulated picaridin treated fabrics demonstrated success in repelling *Ae. aegypti* mosquitoes and preventing biting, durability tests need to be conducted to determine how long the picaridin will remain active in the fabric. Permethrin can withstand up to 50 washings (1). If picaridin treated uniforms are to be considered as a replacement for permethrin treated uniforms, they will have to demonstrate similar results. Further testing by washing the treated fabrics for differing lengths of time will help to fill in the remaining gaps in knowledge.

#### **DISCLAIMER**

Testing was carried out at the USUHS entomology laboratory under the guidance of LTC Jittawadee Murphy and assistance of Ms. Thoai Dotrang. Laboratory best practices training and safety measures were strictly adhered to, according to the biosafety level (BSL) of the mosquitoes tested.

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Army, Department of Defense, or the US Government. LTC Jittawadee Murphy is a U.S. Army entomologist and Ms. Thoai Dotrang is an employee of USUHS.

# APPENDIX A

## Data Recording Sheet

Date
Time
Temp °C <span style="float: right;">Room      Incubator</span>
r.h. <span style="float: right;">Room      Incubator</span>
Waterbath
Blood <span style="float: right;">Serum      ATP</span>

Date
Time
Temp <span style="float: right;">Room      Incubator</span>
r.h. <span style="float: right;">Room      Incubator</span>
Waterbath
Blood <span style="float: right;">Serum      ATP</span>

Date
Time
Temp <span style="float: right;">Room      Incubator</span>
r.h. <span style="float: right;">Room      Incubator</span>
Waterbath
Blood <span style="float: right;">Serum      ATP</span>

Date
Time
Temp <span style="float: right;">Room      Incubator</span>
r.h. <span style="float: right;">Room      Incubator</span>
Waterbath
Blood <span style="float: right;">Serum      ATP</span>

Species
Age (days)
Starved

Species
Age
Starved

Species
Age
Starved

Species
Age
Starved

Rep #	Cage # 1	
Fabric:		
Time (min.)	Landing / Probing	Observed Blood Feeding
0.5		
1		
1.5		
2		
2.5		
3		
3.5		
4		
4.5		
5		
5.5		
6		
6.5		
7		
7.5		
8		
8.5		
9		
9.5		
10		

Rep #	Cage # 2	
Fabric:		
Time (min.)	Landing / Probing	Observed Blood Feeding
0.5		
1		
1.5		
2		
2.5		
3		
3.5		
4		
4.5		
5		
5.5		
6		
6.5		
7		
7.5		
8		
8.5		
9		
9.5		
10		

Rep #	Cage # 1	
Fabric:		
Time (min.)	Landing / Probing	Observed Blood Feeding
0.5		
1		
1.5		
2		
2.5		
3		
3.5		
4		
4.5		
5		
5.5		
6		
6.5		
7		
7.5		
8		
8.5		
9		
9.5		
10		

Rep #	Cage # 2	
Fabric:		
Time (min.)	Landing / Probing	Observed Blood Feeding
0.5		
1		
1.5		
2		
2.5		
3		
3.5		
4		
4.5		
5		
5.5		
6		
6.5		
7		
7.5		
8		
8.5		
9		
9.5		
10		

# Dead at start of test
Knockdown
Engorgement Status
Partial-      Full-
Total Blood Fed
N =

# Dead at start of test
Knockdown
Engorgement Status
Partial-      Full-
Total Blood Fed
N =

# Dead at start of test
Knockdown
Engorgement Status
Partial-      Full-
Total Blood Fed
N =

# Dead at start of test
Knockdown
Engorgement Status
Partial-      Full-
Total Blood Fed
N =

Observer
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Observer
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Observer
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Observer
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