Evaluation of Different Methods to Assess the Duration of Effectiveness of Permethrin-Treated Clothing against *Aedes albopictus* and *Aedes aegypti* Mosquitoes after Repeated Washing

by

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Mosquitoes can be vectors of pathogens that cause many diseases, such as malaria, West Nile encephalitis, dengue and Zika. Mosquito-borne diseases continue to be a global problem, affecting outdoor workers (e.g. forestry workers) and individuals participating in recreational activities, such as camping, hiking, hunting, or gardening. Measures to minimize the impact of arthropod exposure (e.g. mosquito and tick bites) are vital to protect public health. Personal protective measures, such as wearing insecticide-treated clothing, can provide some level of protection from arthropod exposure.

Consequently, the purpose of the current laboratory study is to assess the effects of repeated washing on the effectiveness of permethrin-treated clothing against mosquitoes. The specific aims are to: 1) quantify the amount of permethrin in two different fabric types after repeated washing, 2) investigate the knockdown/mortality of permethrin-susceptible and permethrin-resistant populations of *Aedes* mosquitoes after exposure to permethrin-treated clothing, and 3) examine the extent which mosquito exposure method impacts knockdown/mortality rate after exposure to permethrin-treated clothing. Permethrin-treated and untreated clothing of two fabric types (50% cotton/50% polyester and 100% cotton) were machine-washed and -dried for up to 15 cycles. Fabric swatch samples (n=2) were cut from each garment after 0, 5 and 15 washing/drying cycles. Two species of *Aedes* mosquitoes (*Ae. albopictus* and *Ae. aegypti*) known to be susceptible or resistant to permethrin (previously assessed by Centers for Disease Control and Prevention bottle bioassay) were used for this study. Subsets of mosquitoes were exposed to swatches for 2 min using 2 different exposure methods (Environmental Protection Agency petri dish method and World Health Organization cone method), and then observed for knockdown/mortality at 2 h and 24 h post-exposure.

Each fabric swatch was analyzed for permethrin content using gas chromatography. Analysis of variance was used to evaluate differences in permethrin content between treatment groups, while chi-square testing was used to evaluate proportions of mosquito knockdown and mortality against different variables. Results showed that permethrin-treated clothing was effective against the mosquito population that was susceptible to permethrin (*Ae. albopictus*) but not the resistant population (*Ae. aegypti*). Permethrin content decreased with increased number of washings. The petri dish and cone methods resulted in no significant differences in knockdown/mortality rate observed for either tested mosquito population. We expect the findings of this study to contribute to current research on permethrin-treated clothing as personal protection for reducing risk to mosquito-borne diseases. Understanding the extent to which fabrics retain permethrin may lead to the development of fabric blends that maximize permethrin retention. Moreover, understanding the efficacy of different mosquito exposure methods may help improve or standardize testing methods.

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I. INTRODUCTION

Arthropod bites and, in some cases, resulting infections impact public health. Arthropods can be vectors of pathogens that cause many diseases such as malaria (mosquito), West Nile encephalitis (mosquito), dengue (mosquito), and Lyme disease (tick). The number of cases of West Nile encephalitis (locally transmitted cases frequently detected in the United States [US]) and chikungunya (imported cases frequently detected in the US; minimal number of locally transmitted cases) is increasing (Moore *et al.*, 2012). Recent outbreaks of travel related cases and local transmission of dengue fever in Hawaii and Zika virus transmission in states such as Florida and Texas continue to show the spread of mosquito-borne viruses across the US. Actions to stop the impact of arthropod bites are vital to protect public health (Katz *et al.*, 2008).

Mosquito-borne diseases continue to be a global problem. Dengue fever cases are increasing and this disease impacts about 40% of the world's population (Centers for Disease Control and Prevention [CDC], 2014). There were 2,060 human cases of WNV infection reported to the CDC in the US from January - November 2016 and 66% of reported cases were neuroinvasive, affecting the brain and spinal cord (i.e., meningitis or encephalitis) (CDC, 2016). Malaria is one of the world's most common mosquito-borne diseases. Annually, 300 - 500 million malaria cases occur and at least 1 million people die of malaria each year worldwide (Kimani *et al.*, 2006). As of 2015, there has been a decrease in malaria cases due to better prevention and control measures such as early diagnosis and treatment, long-lasting insecticidal nets, and indoor residual spraying (World Health Organization [WHO], 2015). In 2015, there were approximately 214 million malaria cases worldwide, while the number of deaths related to malaria decreased from an estimated 584,000 deaths in 2014 to about 438,000 worldwide in 2015 (WHO, 2015).

In 2010, there were more than 34,000 cases of tick-borne diseases reported in the US (CDC, 2012). Estimates indicate that the incidence of Lyme diseases ranges from 240,000-440,000 new cases a year (CDC, 2015). Commonly reported tick-borne diseases are Lyme disease, spotted fever group rickettsiosis, ehrlichiosis, and anaplasmosis (CDC, 2016). Bartosik *et al.* (2008) stated that the reason for the high number of tick-borne disease cases in recent years is due to a lack of public knowledge of tick bite prevention methods. Host-seeking ticks are found in forested areas, meadows, and along walking trails. People at the greatest risk of contact with ticks involve outdoor workers and individuals participating in recreational activities such as camping, hiking, hunting, or gardening. Tick-borne diseases remain an occupational hazard for outdoor workers, especially forestry workers, park rangers, and military personnel. Personal protection methods, such as wearing insecticide-treated clothing, can be used to minimize arthropod bites and can be the first line of defense against vector-borne diseases (Moore *et al.*, 2012).

II. LITERATURE REVIEW

Occupational Hazard Exposure among Outdoor Workers

Although there are many vector-borne diseases, transmission of pathogens by ticks is considered a major occupational hazard for forestry workers and other outdoor workers such as military personnel. Since the nature of their job requires them to work outdoors for extended periods of time, outdoor workers are at a higher risk of contracting vector borne disease (Rossbach *et al.*, 2014; Covert & Langley, 2002). Personal protection measures for outdoor workers, such as foresters, are aimed at tick bite prevention. According to Cisak *et al*. (2012), *Borrelia burgdorferi* (commonly found in the US) and tick-borne encephalitis virus (commonly found outside the US) are, respectively, commonly associated with occupational Lyme borreliosis and tick-borne encephalitis in forestry and agricultural workers. A serosurvey done on US National Park Service employees revealed that 22% of employees were seropositive from past exposure to spotted fever group rickettsiae, 3% were seropositive from past exposure to *Ehrlichia chaffeensis*, and 8% of employees were seropositive from past exposure to *Anaplasma phagocytophylium* (Adjemian *et al.*, 2012). Most of the participants spent 26% of their time working outdoors. This same study also looked at mosquito-borne pathogens and found that 1.5% were seropositive from past exposure to West Nile virus, 12.6% were seropositive from past exposure to La Crosse virus, and 2.2% were seropositive from a flavivirus (type not specified), to name a few (Adjemian *et al.*, 2012). A participant who was infected with La Crosse virus was reported to have spent 38% of his time working outdoors (Adjemian *et al.*, 2012).

Military personnel are vulnerable to biting arthropods (e.g., mosquitoes, sand flies, ticks) due to the nature of their work in the field. Insecticide-treated tents, bed nets, and clothes have been used by military personnel to protect themselves from arthropod bites and vector-borne diseases. Using an Environmental Protection Agency (EPA) -approved amount of permethrin (0.125 mg/cm^2), treated clothing is widely used for military uniforms and can protect personnel from various arthropods (Khoobdel *et al*., 2005). Some countries, such as Australia and Iran, have adopted the use of permethrin-treated military clothing and most studies done on treated clothing have involved military personnel (Khoobdel *et al*., 2005; Pennetier *et al*., 2010; Frances *et al*., 2007; Faulde *et al.*, 2006; Frances *et al.*, 2014; Gopalakrishnan *et al.*, 2014; Faulde *et al*., 2003).

Workers may lack knowledge about measures to prevent arthropod exposure, how to use repellants, and other personal protection methods. A study on Polish forestry workers (Kurnatowski *et al.* 2010) found that forestry workers in Poland lacked knowledge of Lyme borreliosis and tick bite prevention measures. The same study showed a need for better disease surveillance and worker education.

Personal Protective Measures for Outdoor Workers and Non-Workers

Personal protection is essential for protecting public health from vector borne diseases. Wearing repellents, protective clothing, and limiting time outdoors are the best ways to prevent exposure. Arthropod repellants first started with the use of tar, smokes and plant oils (Katz *et al*., 2008). By 1946, Diethyltoluamide (DEET) was developed and proved to be the most effective type of repellent at the time (and still is a popular repellent) compared to commonly used insect repellants used such as, oil of citronella, dialkyl phthalates, indalone, and Rutgers 612 (Katz *et al.*, 2008). Formulations of insect repellents used today are aerosols, pump sprays, lotions, creams, suntan oils, powders, grease sticks, and cloth-impregnating laundry emulsions (Katz *et al*., 2008).

In a study on German forestry workers conducted by Rossbach *et al*. (2014), personal protection measures, particularly the use of long-sleeved shirts/trousers and daily application of repellants to exposed skin and/or clothing, were effective in reducing the risk of Lyme borreliosis. Remembering to tuck trousers into socks, checking one's body for ticks after potential exposure, and removing ticks right away once discovered are also other personal protection measures identified (Rossbach *et al*., 2014). The removal of ticks after outdoor activity is important because *ca*. 50% of infectious ticks can potentially transmit the infection after 48 hours of attachment. After 72 hours of attachment, most infectious ticks would likely be able to transmit a pathogen to the host subject (Buczek *et al.,* 2003; Mathers, 1993). An animal study on Lyme borreliosis by Cook (2014) stated that some transmission of Lyme disease spirochetes happened in less than 16 hours and transmission increased by 24 hours. There is difficulty in identifying attached ticks quickly but nevertheless, it is important to carry out tick checks regularly and also once one is done with outdoor activities.

Mosquito Diversity

There are > 41 genera of mosquitoes in the world that encompass approximately 3,500 species (CDC, 2015). Different mosquito species exhibit different blood feeding habits and not all mosquito species transmit pathogens that cause diseases. Others have shown differences in insecticide resistance between mosquito genera (Hemingway & Ranson, 2000). Hence, identifying and testing different mosquito populations against permethrin-treated clothing is important for worker protection.

Permethrin and Impregnation Methods

Synthetic pyrethroids, such as permethrin, can be applied to various fabrics (e.g., bed net, clothing, carpet) for protection against arthropods. This type of fabric treatment has proven to be safe, effective and durable, resulting in their widespread use (Faulde *et al*., 2003). Permethrin is the most commonly used synthetic pyrethroid used in treatment of fabric, but other pyrethroids including bifenthrin, deltamethrin, and cyfluthrin have been tested (Banks, Murray, Wilder-Smith, & Logan, 2014) for use on clothing. Another study showed that combining permethrin with other repellants containing DEET could also provide protection against mosquitoes in insecticide treated clothes (Pennetier *et al*., 2010). Cotton, jute, polyester, and nylon fibers can react differently to various insecticides. For example, deltamethrin had better success for treating cotton fibers than other pyrethroids, while cyfluthrin has better success on treating jute fibers than other insecticides tested in the study (Khoobdel *et al*., 2005).

Permethrin was first marketed in 1973. It is used as a repellant and an insecticide and is effective against ticks, mosquitoes and other arthropods (Katz *et al*., 2008). When susceptible arthropods contact a lethal dose of permethrin, paralysis occurs via nervous system excitation and blockage of sodium channels by inhibiting adenosine triphosphates, acetylcholinesterase, and aminobutyric acid receptors (Katz *et al.*, 2008).

Permethrin is commonly used for pest control in forestry, agriculture, residential, and public health settings, including for head lice and mosquito control (Katz *et al*., 2008). The repellant DEET was once used in clothing impregnation; however, it offers a brief protection time (the specific time was not mentioned in the study) and was soon replaced by permethrin that has a longer durability (Pennetier *et al*., 2010). Permethrin remains the first choice for fabric impregnation because of its effective repellency and low toxicity to humans. It also affects a wide range of arthropods, has a rapid reactivity rate, photostability, and is resistant to weathering (Gopalakrishnan *et al*., 2013).

Even though permethrin is considered safe and effective, there is concern that permethrin residue released via washing could contaminate water sources, hence endangering aquatic life. The loss of permethrin in fabric is due to various factors such as: 1) method of impregnation, 2) number of washes, and 3) exposure to light (France *et al.*, 2014; Richards *et al*., 2016). Some scientists believe that potential adverse health effects from long-term low dose exposure to permethrin through clothing could be potentially carcinogenic (Rossbach *et al.*, 2014). Permethrin is a class C carcinogen and small amounts of it can be absorbed by dermal contact. Because of this, contact with permethrin should be monitored and metabolism throughout the body should be measured (Banks *et al*., 2014). The EPA created regulations to estimate forms of absorption, ingestion, and uptake of permethrin into the body (EPA, 2011). The daily uptake of permethrin is estimated to be about 2.1 µg/kg/day (EPA, 1990). Evaluating daily uptake of permethrin is important, especially for those who wear treated clothing on a daily basis, such as military personnel or outdoor workers (Banks *et al*., 2014).

A human biomonitoring study was conducted in German armed forces that wore permethrin-treated battle dress uniforms (BDUs) (Rossbach *et al*., 2010). The purpose of the study was to compare permethrin exposure between volunteers wearing permethrin-treated BDUs and non-impregnated BDUs. The same study showed an increase in urinary metabolites in soldiers wearing permethrin-treated uniforms over time (up to 28 d) and there was a decrease in internal exposure after the 28-day period (after the uniforms were no longer worn) (Rossbach *et al*., 2010). However, during the 28 period after uniforms were no longer worn, permethrin metabolite concentrations were still about five times higher than the starting point values on the first day of the study before uniforms were worn, which shows that there may be a residual effect (Rossbach *et al.,* 2010). Urinary metabolite concentrations of permethrin measured on days 14 and 28 of wear were similar to exposure levels observed in pest control operators with occupational pyrethroid exposure (Rossbach *et al*., 2010). However, the biomonitoring results here are below the WHO acceptable daily intake (ADI) of permethrin (5-6 µg/kg). Nevertheless, the results were higher than uptake estimations (3 µg/kg) recommended by the US National Research Council (NRC) (Rossbach *et al*., 2010; NRC, 1994). The intake levels are different between the WHO and the NRC because of how the dermal absorption was studied. The WHO conducted their study using rabbits, whose skin is more permeable to chemicals than humans, while the NRC study was conducted on humans in various field studies (WHO, 2009; NRC, 1994).

Permethrin impregnation of fabric typically involves one or all the following processes: 1) absorption, 2) incorporation, 3) polymer-coating, and 4) microencapsulation (Faulde *et al*., 2015). The absorption method involves spraying or dipping the fabric (e.g. clothing) in insecticide to help with the binding process. Spraying/dipping fabrics can result in patchy distribution of the ingredient, loss of activity after laundering, and potential human exposure to treatment solution (Rossbach *et al.*, 2010). The incorporation method uses heat and salt gradients to bind permethrin into wool or silk fibers (Faulde *et al*., 2006) and is used on carpets (primarily to prevent flea infestations). Polymer coating involves a layer of polymer (where insecticide is bound) coated over a fabric surface. Microencapsulation is similar to polymer coating but the insecticide is put in a capsule and mixed into a binding solution (Faulde *et al.,* 2015). The material is washed with the insecticide solution, allowing a thin layer of polymer to bind to the fibers (Banks *et al*., 2014). The absorption method is commonly used for fabric impregnation (Faulde *et al*., 2003). The polymer-coating method provides longer-lasting protection (up to 100 washes) than the other methods, is the most expensive, and results in minimal skin absorption of insecticide (Faulde *et al*., 2003; Banks *et al*., 2014).

Permethrin-Treated Clothing and Mosquito Exposure

Mosquito- and tick-borne diseases are threats for travelers, troops, outdoor workers, and those that participate in outdoor recreational activities. Deployed troops and tourists coming from regions endemic for anthroponotic vector borne diseases such as dengue or malaria run the risk of importing pathogens to their homelands. Personal protection against biting arthropods and arthropod-borne diseases are a first line of defense (Abdel-Mohdy *et al.,* 2009) since vaccines do not exist for most vector-borne diseases.

The creation of repellants and insecticides that can be impregnated into clothing, tents, and bed nets have increased protection against arthropod exposure (Abdel-Mohdy *et al*., 2009). The purpose of using a chemical on clothes is to repel or irritate potential vectors that land and probe, even though there is already some type of barrier provided by the fabric (Wilder-*Smith et al*., 2012). Clothes to which permethrin is bound tightly to fabric fibers may provide effective and odorless protection against common mosquito species (*Anopheles, Culex*, and *Aedes*) and ticks (Wilder-Smith *et al*., 2012). Currently, there are products available to the public, such as permethrin-based sprays for clothes and permethrin impregnated clothing that are also widely used for military personnel who are deployed to areas endemic for vector borne diseases (Pennetier *et al*., 2010).

A study by Frances *et al*. (2014) tested permethrin-treated Disruptive Pattern Camouflage Uniforms (DPCU) used by Australian Army personnel against *Aedes aegypti* Linnaeus and *Anopheles farauti* Laveran. The same study aimed to determine protection from biting mosquitoes by measuring knockdown and mortality rate among the mosquitoes exposed to the

DPCU fabric. All uniforms were impregnated with permethrin using different methods: 1) Perigen Defense emulsion using dipping method, 2) Factory A treated by InsectShield (Greensboro, NC) using the polymerization method, and 3) Factory B treated by a factory in Belgium using the polymerization method (Frances *et al.*, 2014). The same study showed that Perigen defense fabric was washed up to 10 times, and the factory treated fabrics (Factory A and B) were washed up to 50 times. However, a reason was not given for why one fabric was washed more than the other (Frances *et al.* 2014). In the same study, after each wash, the fabric was cut, rolled into a tube shape and clipped into the WHO susceptibility test kit cylinder. Adult female mosquitoes were exposed to each fabric for 3 minutes after 1, 3, 5, and 10 washes for Perigen-treated fabric and 1, 3, 5, 10, 30, and 50 washes for the factory-treated fabrics. The knockdown of mosquitoes was recorded after 60 min, along with mortality rate that was measured after 24 h (Frances *et al.*, 2014). In the aforementioned study, *Ae. aegypti* was more susceptible to the permethrin-treated fabric than *An. farauti* against all treated fabrics, regardless of impregnation method. However, no indication of resistance/susceptibility status was reported for these mosquito populations. For example, in Factory A treated fabric, the knockdown rates (after 60 min) were: 96.3% (1 wash), 100% (3 washes), 90.1% (5 washes), 76.0% (10 washes), 25.0% (30 washes), and 0% (50 washes) (Frances *et al.*, 2014). The knockdown rate for *An. farauti* were: 93.3% (1 wash), 57.1% (3 washes), 66.7% (5 washes), 25.0% (10 washes), 0% (30 washes), and 4.0% (50 washes). The same study showed that the knockdown rates for *An. farauti* were lower as the number of washes increased, compared to *Ae. aegypti*. The mortality rate (after 24 h) for both species was low (range 0 - 47%). However, significantly more *Ae. aegypti* died than *An. farauti*.

Khoobdel *et al.* (2006) field tested permethrin-treated (dipping method) military uniforms in Iran against *An. stephensi* Liston and four species of *Culex (Cx. bitaeniohynchus* Giles, *Cx. tritaeniorhynchus* Giles, *Cx. perexiguus* Theobald, and *Cx. theileri* Theobald*).* In the same study, eight male participants wore permethrin-treated (N=6 participants) or untreated (N=2 participants) uniforms (8:00 pm to 1:00 am) in a revolving manner over eight nights to decrease subject-caused bias. Participants wearing treated clothing sat outside at 5-10 meters from each other, while the participants wearing untreated clothing sat 50 – 100 m away from the participants wearing treated clothing (Khoobdel *et al.* 2006). In the same study, mosquitoes that landed but did not bite were not captured but those that landed and bit were captured and transferred into a cup (replaced every 30 min for analysis of temporal abundance). Participants wearing permethrin-treated uniforms had the highest protection against *Cx. perexiguus* (89.8%), followed by *Cx. tritaeniorhynchus* (87.0%), *Cx. theileri* (84.3%), *An. stephensi* (78.7%), and *Cx. bitaeniorhynchus* (72.7%) (Khoobdel *et al.* 2006).

Similar studies done by Kimani *et al*. (2006) and Khoobdel *et al*. (2005) tested different mosquito populations against permethrin-treated clothing and found that biting rates decreased compared to untreated clothing. Kimani *et al*. (2006) conducted a community trial in a Dadaab refugee camp, located in Kenya, using active case detection in the community to determine malaria infection rate. Active case detection includes screening populations regardless of the presences of signs or symptoms of malaria to find infections and residual parasite carriers. The refugees' clothes were treated with permethrin by the dipping method (Kimani *et al*., 2006). The study continued for six months with clothes getting re-treated every three weeks during the study period. People wearing treated clothes experienced a lower malaria infection rate, reduced mosquito bites and reduced bites by other insects (bedbugs, body lice and head lice) (Kimani *et* *al*., 2006). Different age groups showed different results, wherein treated clothing was more beneficial for children 5-14 years of age, youth aged 15-24 years, and those > 50 years old. For adults aged 25-49 years and children less than five years old, the results were less beneficial (Kimani *et al*., 2006).

In the Khoobdel *et al*., (2005) study conducted in Tehran, Iran, three different fabric types (i.e., cotton, polyester, and nylon) were dipped in permethrin to investigate if fabric types would have any impact on the effectiveness of permethrin in protecting against biting *Cx. pipiens* Linnaeus mosquitoes. All fabrics were treated using the dipping method and the same amount of permethrin (0.125 mg/cm^2) was used. People wearing treated clothing experienced 89% less mosquito bites than those wearing untreated clothes (Khoobdel *et al*., 2005).

In a lab study done by the Army Malaria Institute (AMI), permethrin-treated DPC uniforms were washed in a commercial washing machine for 30 min using warm water and laundry detergent (Frances & Cooper, 2007). However, neither the treatment method of the DPC uniforms nor mosquito exposure methods were mentioned in the study. The results of the aforementioned study showed that 100% of *Ae. aegypti* mosquitoes were dead 60 min after a 1 min exposure to unwashed fabrics. After two washes of the fabric, mosquito mortality (after 1 min exposure) was less than 5% at 60 min post-exposure (Frances & Cooper, 2007). To measure the effect of multiple washes, DPC uniforms were washed up to five times (Frances & Cooper 2007). The types of fabric of all DPC uniforms were not given in this study. The untreated DPC uniform shirts had a 25% biting protection rate, while the treated shirts and treated trousers had a range of biting protection from 84%-99% and 86%-100%, respectively (Frances & Cooper 2007). After one wash, the knockdown effect for both DPC shirts and trousers was reduced from 93% to less than 20%. A chemical assay that was done showed that the first wash removed 64%

of the permethrin, and 80% of the initial permethrin had been removed after three washes (Frances & Cooper, 2007).

Permethrin Residual Activity and Impregnation Studies

According to Frances *et al*. (2014), permethrin concentrations are higher in clothes treated using the polymer coating method compared to fabrics treated with permethrin through dipping methods. However, biting protection rate, knockdown rate, and mortality rate of mosquitoes still decrease after repeated washings even in clothing that are treated using the polymer coating method (Frances *et al.,* 2014).

A German study on contact toxicity and residual activity of impregnation methods (polymer coating compared to dipping) demonstrated that higher residual quantities of permethrin are detected when military uniforms were impregnated with the polymer coating methods (Faulde *et al*., 2003). The same study compared factory-treated permethrin clothes using the polymer method (The UTEBXEL company used 1300 mg/m² permethrin) against two commercially available dipping methods (Peripel 10: 650 mg/m² permethrin; The Insect/Arthropod Repellent Fabric Treatment (IARFT: 1250 mg/m² permethrin). Both treated and untreated fabrics were laundered up to 100 times and air dried (Faulde *et al.* 2003). After the fabrics were washed, exposed to adult *Ae. aegypti* female mosquitoes, and tested for permethrin using the Scanning Electron Microscopy Analysis, they found the polymer coated fabric had a higher residual content of 280 mg/m² after 100 launderings. The washing-specific results were not given for the two dipping methods but Faulde *et al*., (2003) stated that the amount of 280 mg /m² of permethrin was present after three washings for the Peripel 10 dipping method and after six washings for the IARFT dipping method.

Similar studies done by Faulde *et al*. (2015), Frances *et al*. (2014) and Banks *et al*. (2015) also found that higher starting permethrin concentration in insecticide-treated clothes results in higher residual permethrin content after repeated washing cycles, compared to clothing that started with lower permethrin content. Faulde *et al*. (2015) studied different fabric types obtained from different vendors (Insect Shield 100% cotton t-shirts, ExOfficio 15% cotton/85% polyester t-shirts, Sol's Monarch 100% cotton-shirts, and Labonal socks), and found that the Labonal socks had the highest initial permethrin concentration of 4300 mg/m², followed by Sol's Monarch t-shirts with 1310 mg/m², Insect Shield t-shirts with 1300 mg/m², and ExOfficio t-shirts with 870 mg/m². After 100 launderings, Labonal socks' residual content went down by 58.1% (1800 mg/m²). The lower the initial permethrin concentration on the fabric, the lower the residual permethrin content after washing (Faulde *et al*., 2015). Insect Shield t-shirts had an initial concentration of 1300 mg/m² and, after 100 launderings, the residual content was 20 mg/m² (98.5% reduction) (Faulde *et al*., 2015). The study done by France *et al*. (2014) tested Australian military DPCU (washed up to 50 times) against two different impregnation methods: factory treatment (polymer coated) and dipping emulsion. After 1, 3, 5, 10, 30, and 50 wash cycles, swatches were cut and unfed female *Ae. aegypti* L. and *An. farauti* L. mosquitoes were exposed to swatches of the uniforms that were placed into a cylinder provided by the WHO susceptibility test kit. Ten adult female mosquitoes were exposed for three minutes and after each exposure transferred to a holding cylinder and placed in a polystyrene container to observe knockdown of mosquitoes for up to 60 min. Compared to observations made from studies mentioned above, they found higher concentrations of residual permethrin in the factory treated clothing after washing compared to the fabric that was dipped (Frances *et al*., 2014).

Banks *et al*. (2015) tested four different types of permethrin impregnation methods to see how effective it would be on school uniforms to protect children from dengue fever in Thailand. The four samples used were: 1) factory dipped clothing (polymer coated), 2) home dipped clothing, 3) microencapsulated clothing, and 4) factory dipped school uniforms (polymer coated). Clothes were washed up to 30 times and exposed to previously sugar fed *Ae. aegypti* female mosquitoes using the WHO cone test. The factory treated clothing not only produced higher knockdown rates compared to the other two fabrics but also retained more permethrin after 30 washes (Banks *et al*., 2015).

Permethrin-Treated Clothing and Tick Exposure

Insecticide treated clothing aids in reducing tick bites and tick-borne diseases. Two studies done by Vaughn *et al*. (2014) and Miller *et al*. (2011) observed the effectiveness of permethrin impregnated clothing against tick bites for outdoor workers and for those who engage in recreational activities outside in the summer. Vaughn et al. (2014) conducted a two-year trial evaluating the protective effectiveness of long lasting permethrin impregnated uniforms among outdoor workers in the North Carolina Division of Water Quality, and revealed that workers wearing treated clothing were better protected in their first year of wear (82% reduction in tick bites) compared to their second year (34% reduction in tick bites), hence clothing should be replaced each year if worn/washed regularly (Vaughn *et al*., 2014). In the same study, clothes were treated using the polymer coating method and 93% of workers wearing treated clothing had fewer tick bites than those who had used standard tick bite prevention measures and wore untreated clothing. Standard tick bite prevention measures include bathing or showering after being outside, examining the body for ticks after being outside, or using repellent containing 20% to 30% DEET (CDC, 2016). Miller *et al*. (2011) found that individuals wearing permethrin-treated summer clothes (e.g., cotton shorts, cotton t-shirts, cotton socks, and sneakers) (polymer coating method by Insect Shield) were 3.36 times less likely to have ticks attached to their bodies than those who wore untreated clothes. In the same study, some participants used "do-at-home" treatment kits and these results were compare to the commercial polymer coating method. Both methods showed protective benefits from ticks but people wearing commercially treated clothes reported fewer tick attachments (19.33%) than the do-at-home treated clothes (24.67%) (Miller *et al*., 2011). It was also noted that subjects who wore treated socks and shoes had fewer tick attachments (0.5%) than those who wore untreated shoes and socks (27%) (Miller *et al*., 2011).

Washing Permethrin-Treated Clothing

One important issue in using permethrin-treated fabric is the persistence of the insecticide in the fabric once it has been washed and worn several times. In the Thailand study done by Banks *et al*. (2015), two washing methods were used: the WHO washing technique (simulating hand washing) and machine washing. The WHO washing technique entailed submerging fabric swatches in one liter bottles of water/soap, placing in a 30ºC shaking water bath for 10 min, and drying at 30ºC for 45 minutes (Banks *et al.*, 2015). In the aforementioned study, machine washing consisted of washing fabrics for 30 min at a water temperature of 30º C, followed by air drying. In the same study, the WHO washing technique decreased permethrin content by 41.3% after five washes and then by 97.2% after 30 washes. Conversely, machine washing decreased permethrin content by 28% after five washes and then by 81% after 30 washes. Ironing and/or exposure to ultraviolet (UV) light had no significant effect in the concentration of permethrin (Banks *et al*., 2015).

Petri Dish Method vs. Cone Method for Insecticide Susceptibility Testing

Methods for testing the insecticide susceptibility of mosquitoes include the EPA petri dish method and the WHO cone method. In the petri dish method adapted from the EPA Mosquito Knock Down Protocol (EPA, 2009), the fabric swatches are placed inside the petri dish and covered with a lid that has a small opening on top. Ten to 20 mosquitoes are aspirated from a cage and transferred to the petri dish through the lid hole, which is then covered with transparent tape. The mosquitoes are left in the petri dish touching the fabric for the length of exposure time, and the fabric is then pulled out from the dish at the end of the exposure time (EPA, 2009). Mosquitoes are left in the dish and knockdown is recorded at 15 and 60 min post-exposure. In this method, mosquitoes are not generally assessed for mortality.

In the cone method (WHOPES, 2013), a polyvinyl chloride (PVC) cone is placed over the fabric. Mosquitoes are introduced into the cone through a hole at the top of the cone and then closed with a polyethylene plug. At the end of the exposure time, the female mosquitoes are placed in plastic cups and given a sugar solution. The knockdown rate is measured after 60 minutes; then they are put into an incubator for 24 hours to test for mortality rate (WHOPES, 2013).

Published research studies that compare the petri dish and cone methods are lacking, hence our study represents the first known attempt to compare these methods. Using the petri dish method, mosquitoes are confined to a smaller space and are forced to come into contact with the treated fabric. On the other hand, using the cone method, mosquitoes are able to fly and, in theory, may be able to avoid constant contact with the treated fabric. If we observe differences in mosquito knockdown/mortality rates between the petri dish and cone methods, further assessment of these methods may be warranted.

III. PURPOSE AND SPECIFIC AIMS OF STUDY

Specific Aims of Study

The main purpose of this study was to evaluate biological and chemical aspects of permethrin-treated fabrics. We aimed to:

1) determine the amount of permethrin in two fabric types (50% cotton/50% polyester and 100% cotton) after repeated washing;
2) investigate the knockdown/mortality of permethrin-susceptible and permethrin-resistant populations of *Aedes* mosquitoes after exposure to permethrin-treated clothing, and
3) examine the extent to which mosquito exposure method (petri dish and cone) impacts knockdown/mortality rate after exposure to permethrin-treated clothing .

Research Questions

To reach these aims, we answered these research questions:

1) What type of treated fabric retains more permethrin after 0, 5, or 15 washes?
2) Which population of mosquito (*Ae. aegypti* or *Ae. albopictus*) is more susceptible/resistant to permethrin-treated clothing?
3) What type of treated fabric results in higher/lower mosquito knockdown/mortality rates?
4) Do the petri dish or cone exposure methods yield differences in mosquito knockdown/mortality rates?

Study Objectives

The first objective was to determine the extent to which two different fabric types show a difference in retention of permethrin after they have been washed 0, 5, and 15 times. This objective was accomplished by washing each shirt and quantifying permethrin content via gas chromatograph. Gas chromatography testing was conducted after mosquitoes had been exposed to swatches of fabric. The second objective was to determine the extent to which fabric type (100% cotton or 50% cotton/50% polyester) impacted knockdown/mortality rate of different mosquito populations. The final objective was to conclude if any differences are observed in knockdown/mortality in mosquitoes exposed to treated fabrics via the WHO cone method or EPA petri dish method.

Hypotheses

The following hypotheses were tested:

First Hypothesis: *The 50% cotton/50% polyester fabric has a higher retention rate for permethrin than the 100% cotton fabric.* Polyester is generally more durable than cotton and less prone to fading or wrinkling. The durability of polyester allows for the clothing to be washed more frequently and withstand harsher detergents as opposed to cotton. Polyester and cotton blends provide softness and durability which could prove to be better in retaining permethrin because you have both qualities of polyester and cotton fabric.

Second Hypothesis: *Mosquitoes exposed to 50% cotton/50% polyester fabric have a higher knockdown/mortality rate than mosquitoes exposed to 100% cotton fabric.* If polyester retains permethrin at a higher rate than 100% cotton, higher knockdown/mortality rates are expected.

Third Hypothesis: *The EPA petri dish method provides higher knockdown/mortality rate compared to the WHO cone method.* Using the petri dish method, the mosquitoes will have no choice but to stay on the fabric since the petri dish is a smaller area. They can move up and to the sides but the area is much smaller, so encountering the fabric will be inevitable. Using the cone method, the fabric swatches are placed under a cone and the mosquitoes are exposed to the swatch. However, the cone provides a larger area which could allow the mosquitoes to not stick on the fabric as much, especially if there is a repellant effect of the permethrin.

IV. SIGNIFICANCE OF THE STUDY

The findings of this study will contribute to current research on mosquito knockdown/mortality by permethrin-treated clothing. Personal protection is one of the first steps taken that can reduce the risk of exposure to arthropods. Understanding the extent to which fabrics retain permethrin is important as this could lead to development of fabric blends that maximize permethrin retention.

Understanding the efficacy of different mosquito exposure methods is important as this could help improve/standardize testing methods. It is not a matter of assessing which method is better than the other but more about which method can provide a more realistic picture of mosquito knockdown/mortality. If the petri dish method or the cone method provides a more realistic picture of mosquito knockdown/mortality, then that is something that can be taken into consideration and possibly be implemented in more mosquito exposure experiments.

V. MATERIALS AND METHODS

Test Mosquitoes

The *Ae. albopictus* eggs (F_7) originated from Savannah, Georgia and the *Ae. aegypti* eggs (F_3) were obtained from Anna Marie Island, Florida. All eggs were reared to adults in the laboratory using standard procedures. Briefly, eggs were hatched in plastic rearing pans with 1.0 L of tap water and 200 mg of larval food (1:2 mixture of brewer's yeast and liver powder). Larvae were fed every other day for approximately four days until they became pupae. The pupae were picked from the pans and put into a cup that was placed in their respective cages until adult emergence. Adults were fed with 20% sucrose solution, and only nonblood fed female mosquitoes (6 d post-emergence for *Ae. albopictus* and 8 d post-emergence for *Ae. aegypti*) were used in the study. A CDC bottle bioassay (Brogdon & Chan, 2009) was conducted on a subset of each mosquito population to gather insecticide resistance/susceptibility data for permethrin before starting the permethrin-treated clothing exposure experiment.

Treatment of Fabrics

Permethrin-impregnated and untreated clothing were supplied by Insect Shield: 1) Men's High Vis SS Tee containing no SPF, short sleeved, 50% cotton/50% polyester, and 2) Men's Chambray Work Shirt containing no SPF, long sleeved, 100% cotton. Both shirts were treated by Insect Shield following the polymerization method, using a proprietary formulation of permethrin and tightly bound to the fabric fibers of each garment. All fabrics used in the study were treated with a permethrin concentration of 125 $\mu g/cm^2$. The untreated clothing used consisted of the same two types of shirts, however, they did not contain any insecticide. Swatches (2 swatches used for the experiment and 2 duplicate swatches) were cut from both treated and untreated fabrics and used for experiments for a total of 96 swatches (Figure 1).

Laundering of Fabric

The washing machine model used was General Electric (GE) washer model WCSR2090DBWW and the dryer model used was GE dryer model DJXR433EC3WW. The washing machine was adjusted for delicate textiles at a warm temperature of 27ºC for a washing time of 39 min/wash. A commercially available fragrance-free Tide detergent was used for laundering with 2 oz. of detergent used for each wash cycle. After washing, the garments were dried in the drying machine for 30 min at a setting of optimum dry with a temperature of about 50ºC. Fabric swatches were cut from each garment after 0, 5, and 15 launderings and drying cycles and stored in a Ziploc bag in a closed drawer away from light until the mosquitoes were ready for exposure. To avoid any cross contamination from potential permethrin residue, the control group (untreated) fabrics were washed first, followed by the permethrin-treated group. Each fabric swatch was used for the mosquito knockdown/ mortality experiment and analyzed for permethrin using gas chromatography.

Mosquito Knockdown/ Mortality Experiment

The research design of this study follows a quantitative research approach. The variables in this study were treatment (permethrin-treated or untreated), fabric type (100% cotton and 50% cotton/50% polyester), mosquito population (*Ae. albopictus* [susceptible to permethrin], *Ae. aegypti* [resistant to permethrin]), and number of washes (0, 5, 15). Fabric swatches (60 mm x 15 mm) were sampled and cut out from different locations on the garment.

The petri dish exposure method was adapted from the EPA bioassay for insecticidal activity of treated fabric samples against adult mosquitoes (EPA, 2009). Each swatch was placed securely into a petri dish, wherein 3-10 adult female mosquitoes were chilled in ice for 45 s before being transferred to the fabric. The petri dish was covered and the mosquitoes were observed for 2 min. After the two-minute period, the petri dish containing mosquitoes was chilled in a -20°C freezer for 45 s and mosquitoes were transferred to 0.25 L cardboard cages with mesh screening, provided 20% sucrose solution, and transferred to an incubator at 28°C with approximately 80% humidity and 14:10 h day: night cycle. At 2 hr. and 24 hr. post-exposure to clothing swatches, knockdown rate (i.e. lying on back or side and unable to fly) and mortality rate (i.e. the number of dead mosquitoes) were recorded. The same procedure was done using the WHO cone method adapted from WHOPES (WHO, 2013). Once the mosquitoes had been chilled, they were placed onto the fabric, covered with the clear plastic cone (8.5 cm in diameter at the base and 5.5 cm high), and observed for 2 minutes before being chilled and transferred to cardboard cages. After each mosquito experiment, fabric swatches used were analyzed for permethrin residue by gas chromatography. Figure 1 is a flow chart showing the various parameters of the study, including the number of washes, exposure methods, mosquito species, fabric types, and number of swatches tested.

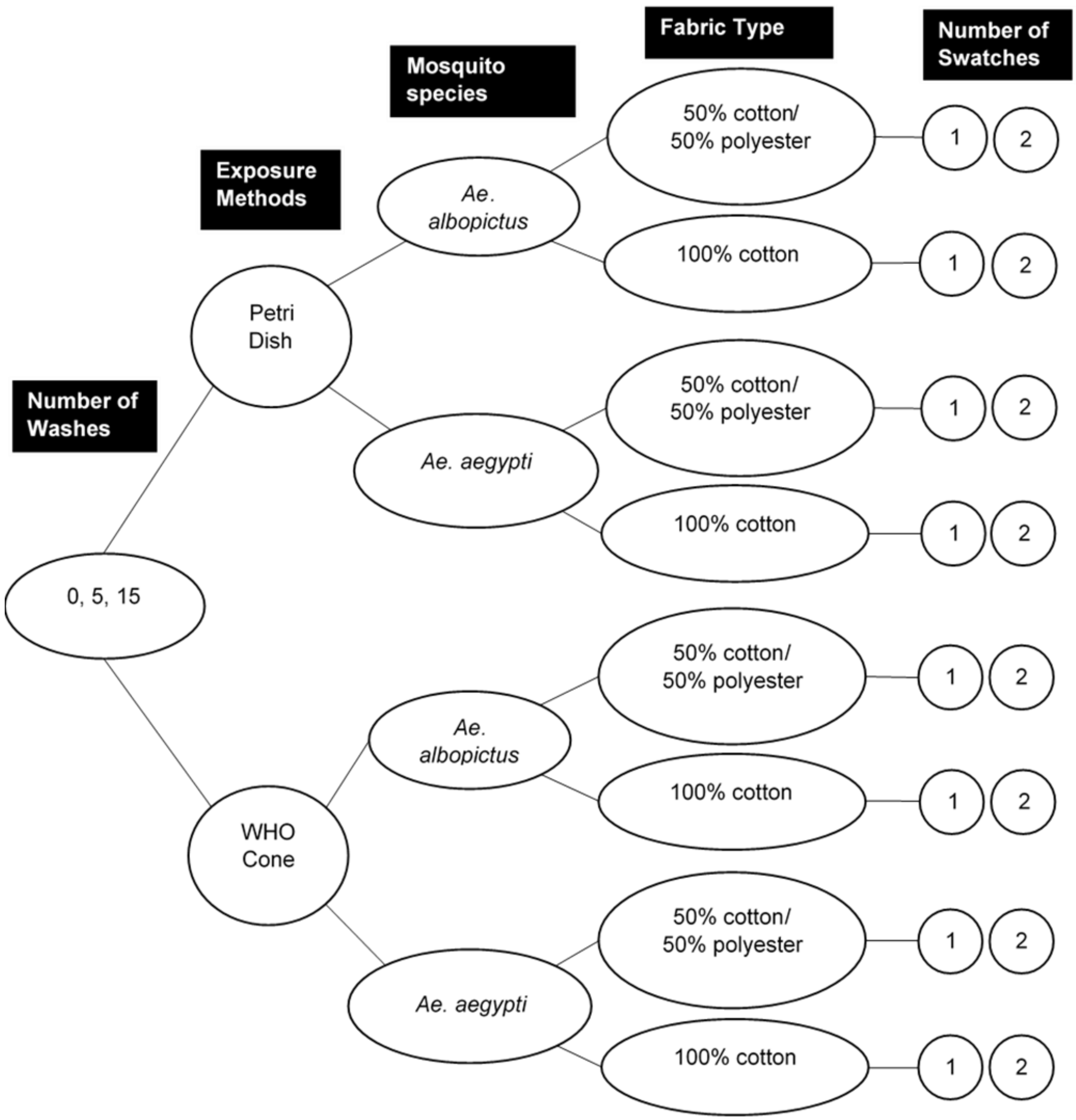

Figure 1. Flowchart of Study Parameters for both untreated and treated fabrics

Permethrin Content Analysis in Fabrics

After the designated number of wash cycles and each mosquito experiment, the treated swatches were tested for permethrin residue using a gas chromatograph (GC). For the controls, 3 swatches from each fabric type and each number of washes were tested, and the same method was followed for the treated samples. The method used to analyze permethrin content was adapted from Gupta *et al.* (1989). After mosquito knockdown/ mortality experiments were completed, the fabric swatches were transferred to separate 60 mL amber glass vials containing 40 mL acetone and soaked for one hour to elute permethrin in a water-filled Sonicator (Fisher Scientific Ultrasonic Bath, 2.8L) with settings at a starting temperature of 75ºF (23.8ºC) and timer for 60 min. Eluent samples (1.5 ml) from three swatches were transferred to 1.5 mL amber GC vials. A portion of the eluent (1 µL) was analyzed directly by capillary GC with flame ionization detector (GC-FID) using an Agilent GC 6850 (Agilent Technologies, Alpharette, GA). The capillary column used was DB-5MS (5% phenyl-methylpolysiloxane), 15 mm x 0.25 (i.d.) mm, 0.25 µm (film thickness) (Agilent Technologies, Alpharette, GA). The injector and detector temperatures were set at 250°C and 260°C, respectively. The oven temperature was programmed from 200°C to 250°C (adapted from Hengel et al. 1997) at 10°C/min and held for 7 min, with a total run time of 12 min. Nitrogen was used as both carrier (32.6 mL/min) and make-up (10 mL/min) gas, and hydrogen were used as the detector gas (30 mL/min). A permethrin stock solution was prepared by dissolving 0.01 g permethrin standard in 40 mL acetone, and was used to prepare the calibration standards. Five-point calibration curves were used to generate the calibration curve for quantification. The linearity of the detector response was checked before conducting analysis by using these calibration curves. The average permethrin content (n=3) for each fabric type and number of washes was determined. To confirm that there were no erroneous peaks, a blank vial filled with 1.5 mL of acetone was run in between sample runs.

Statistical Analysis

Cross tabulation and chi square tests were performed to analyze the proportions of mosquitoes knocked down and dead at 2 h post-exposure and 24 h post-exposure against several independent variables (i.e., fabric type, number of washes, mosquito population, and exposure method) (SPSS Institute, Chicago, IL). *P* values for treated clothing were determined by using Pearson chi-square values under asymptotic significance (2 sided). *P*-values < 0.05 were considered significant. Analysis of variance (ANOVA) was used to evaluate differences in permethrin content between fabric types and number of washes. Permethrin quantities were log-transformed $(x + 1)$ prior to using ANOVA to improve normality and ensure normal distribution among all values. A Duncan test was used to determine differences in means and to find significance between permethrin concentrations between fabric types and/or number of washes.

VI. RESULTS

Permethrin Content by Fabric Type and Number of Washes

The treated 100% cotton fabric showed higher initial permethrin content at 0 washes (29.2 ± 2.9 µg/cm²) compared to the 50% polyester/50% cotton fabric (6.3 ± 1.0 µg/cm²), and consequently has a higher permethrin content after 15 washes (Table 1). Regardless of the fabric type, the permethrin content decreased as the number of washes increased, such that 0 washes had the highest permethrin concentration while 15 washes had the lowest. For the treated clothes, 50% polyester/50% cotton started at 6.3 ± 1.0 µg/cm² and decreased to 2.3 ± 0.3 µg/cm² (for 63.4% reduction) for 15 washes, while 100% cotton started at 29.2 ± 2.9 µg/cm² and decreased to 8.9 ± 0.9 µg/cm² (69.5% reduction) for 15 washes (Table 1, Figure 2). A small amount of permethrin in the controls was detected, which may be attributed to contamination during packaging and shipping (Table 1). Fabric type (P=0.001, df=1, F=698.3) (highest for 100% cotton) and the number of washes (P=0.007, df=2, F=137.2) (highest in 0 wash group) significantly impacted permethrin content.

Table 1. Average Permethrin Content by Fabric Type & Number of Washes (n=3)

Sample Type	Fabric Type	No. of Washes	Permethrin Concentration (µg/cm^2) Mean ± Standard Deviation
Treated	50% Polyester/50% Cotton	0	6.3 ± 1.0
		5	4.2 ± 0.3
		15	2.3 ± 0.3
	100% Cotton	0	29.2 ± 2.9
		5	16.7 ± 1.4
		15	8.9 ± 0.9

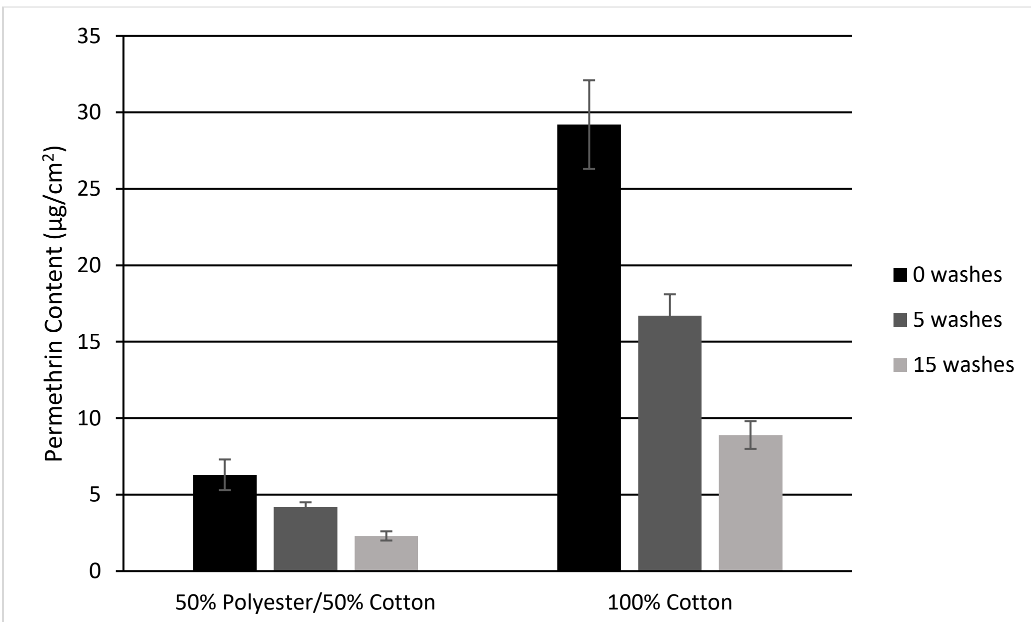

Figure 2. Permethrin Content by Fabric Type and Number of Washes (mean ± standard deviation)

Knockdown and Mortality Rates of *Ae. albopictus* Population

Exposure Method

The *Ae. albopictus* population used here were known to be susceptible to permethrin. When conducting a CDC bottle bioassay, within 15 minutes of exposure to the diagnostic dose of permethrin, 86% of the mosquitoes in the bottle were knocked down and by 30 minutes (the diagnostic time), 100% of the mosquitoes were knocked down. For *Ae. albopictus*, when comparing the two fabric exposure methods, the petri dish method resulted in a higher knockdown (29.9%) and mortality rate (39.0%) compared to the cone method (17.3% and 25.3%, respectively), but the differences were not statistically significant between exposure methods for either knockdown (P=0.069; X^2=3.304; df=1) or mortality (P=0.072; X^2=3.230; df=1) rates (Table 2; Figure 3 & 4).

Table 2. Knockdown (2-hr post-exposure) and Mortality (24-hr post-exposure) Rates by Exposure Method (*Ae. albopictus*)

Sample Type	Exposure Method	Knockdown						Mortality					
		n	Total	%	X^2	df	***P*-Value**	n	Total	%	X^2	df	***P*-Value**
Control	Cone	0	71	0.00%	---	--	**---**	0	71	0.00%	---	--	**---**
	Petri Dish	0	95	0.00%				0	95	0.00%			
Treated	Cone	13	75	17.3%	3.304	1	**0.069**	19	75	25.3%	3.230	1	**0.072**
	Petri Dish	23	77	29.9%				30	77	39.0%			

Number of Washes

Fabric having 0 washes resulted in the highest *Ae. albopictus* knockdown (37.5%) and mortality (46.4%) rates while the 15-wash group had the lowest knockdown and mortality rates (8.0% and 14.0%, respectively). The differences between the different wash groups were statistically significant for both knockdown (P=0.002, X^2=12.720; df=2) and mortality (P=0.002; X^2=12.912; df=2) rates (Table 3; Figure 3 & 4).

Table 3. Knockdown (2-hr post-exposure) and Mortality (24-hr post-exposure) Rates by Number of Washes (*Ae. albopictus*)

Sample Type	**No. of Washes**	**Knockdown**						**Mortality**					
		n	Total	%	X^2	df	***P*-Value**	n	Total	%	X^2	df	***P*-Value**
Control	0	0	57	0.0%	---	--	**---**	0	57	0.0%	---	--	**---**
	5	0	55	0.0%				0	55	0.0%			
	15	0	54	0.0%				0	54	0.0%			
Treated	0	21	56	37.5%	12.720	2	**0.002**	26	56	46.4%	12.912	2	**0.002**
	5	11	46	23.9%				16	46	34.8%			
	15	4	50	8.0%				7	50	14.0%			

Fabric Type

Ae. albopictus exposed to 100% cotton fabric had a slightly higher knockdown (23.5%) and mortality (30.9%) rate compared to fabric containing 50% cotton/50% polyester (23.9% and 33.8%, respectively), but the differences were not statistically significant for either knockdown (P=0.944; X^2=0.005; df=1) or mortality (P=0.699; X^2=0.150; df=1) rates (Table 4; Figure 3 & 4).

Table 4. Knockdown (2-hr post-exposure) and Mortality (24-hr post-exposure) Rates by Fabric Type (*Ae. albopictus*)

Sample Type	**Fabric Type**	**Knockdown**						**Mortality**					
		n	Total	%	X^2	df	***P*-Value**	n	Total	%	X^2	Df	***P*-Value**
Control	100% Cotton	0	79	0.0%	---	--	**---**	0	79	0.0%	---	--	**---**
	50% Polyester/50% Cotton	0	87	0.0%				0	87	0.0%			
Treated	100% Cotton	19	81	23.5%	0.005	1	**0.944**	25	81	30.9%	0.150	1	**0.699**
	50% Polyester/50% Cotton	17	71	23.9%				24	71	33.8%			

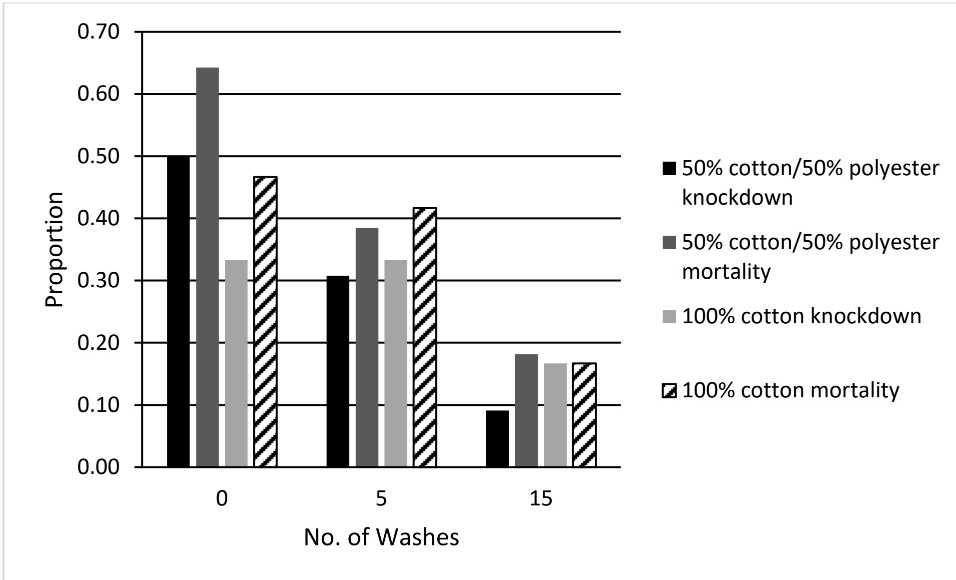

Figure 3. 2-hr Knockdown and 24-hr Mortality of *Ae. albopictus* using Petri Dish Exposure Method

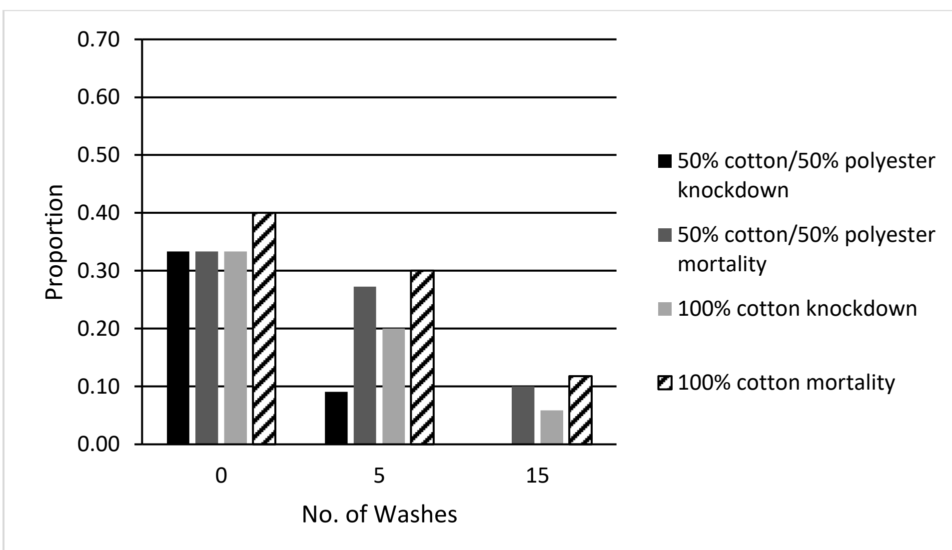

Figure 4. 2-hr Knockdown and 24-hr Mortality of *Ae. albopictus* using Cone Exposure Method

Knockdown and Mortality Rates for *Ae. aegypti* Population

The *Ae. aegypti* mosquitoes used in this experiment were resistant to permethrin. When conducting a CDC bottle bioassay, within 15 and 30 minutes (the diagnostic time) of being exposed to the diagnostic dose of permethrin, only 60% of the mosquitoes were considered dead. When looking at exposure methods, the cone method was not successful in knocking down or killing any mosquitoes, however the petri dish had a knockdown and mortality rate of 2.4% (P=0.178; X^2=1.811; df=1). Comparing the number of washes, 0 washes had a knockdown and mortality rate of 1.8%, 5 washes had a knockdown and mortality rate of 1.7%, and 15 washes did not produce any rates (P= 0.650; X^2=0.862; df=2) (Table 5, 6, & 7; Figure 5). Amongst fabric type, 100% cotton did not knockdown or kill any mosquitoes, while 50% cotton/50% polyester had a knockdown and mortality rate of 2.4% (P=0.168; X^2=1.903; df=1). Mosquitoes exposed to fabrics experiencing 15 washes did not show knockdown or mortality (Table 5, 6, & 7; Figure 5).

Table 5. Knockdown (2-hr post-exposure) and Mortality (24-hr post-exposure) Rates by Exposure Method (*Ae. aegypti*)

Sample Type	**Exposure Method**	**Knockdown**						**Mortality**					
		n	Total	%	X^2	df	***P*-Value**	n	Total	%	X^2	df	***P*-Value**
Control	Cone	0	69	0.00%	---	--	**---**	0	69	0.00%	---	--	**---**
	Petri Dish	0	98	0.00%				0	98	0.00%			
Treated	Cone	0	76	0.0%	1.811	1	**0.178**	0	76	0.0%	1.811	1	**0.178**
	Petri Dish	2	85	2.4%				2	85	2.4%			

Table 6. Knockdown (2-hr post-exposure) and Mortality (24-hr post-exposure) Rates by Number of Washes (*Ae. aegypti*)

Sample Type	**No. of Washes**	**Knockdown**						**Mortality**					
		n	Total	%	X^2	df	***P*-Value**	n	Total	%	X^2	df	***P*-Value**
Control	0	0	56	0.0%	---	--	**---**	0	56	0.0%	---	--	**---**
	5	0	56	0.0%				0	56	0.0%			
	15	0	55	0.0%				0	55	0.0%			
Treated	0	1	55	1.8%	0.862	2	**0.650**	1	55	1.8%	0.862	2	**0.650**
	5	1	58	1.7%				1	58	1.7%			
	15	0	48	0.0%				0	48	0.0%			

Table 7. Knockdown (2-hr post-exposure) and Mortality (24-hr post-exposure) Rates by Fabric Type (*Ae. aegypti*)

Sample Type	**Fabric Type**	**Knockdown**						**Mortality**					
		n	Total	%	X^2	df	***P*-Value**	n	Total	%	X^2	df	***P*-Value**
Control	100% Cotton	0	86	0.0%	---	--	**---**	0	86	0.0%	---	--	**---**
	50% Polyester/50% Cotton	0	81	0.0%				0	81	0.0%			
Treated	100% Cotton	0	78	0.0%	1.903	1	**0.168**	0	78	0.0%	1.903	1	**0.168**
	50% Polyester/50% Cotton	2	83	2.4%				2	83	2.4%			

Figure 5. 2-hr Knockdown and 24-hr Mortality of *Ae. aegypti* using Petri Dish Exposure Method

A more in-depth analysis was taken among mosquito populations to see if there was any significant relationship in mosquito knockdown or mortality between 1) fabric types under a different exposure method at a specific number of washes, 2) number of washes under a different fabric type using a specific exposure method, and 3) exposure methods under different fabric types among a specific number of washes. There was no significance found for any of the three different analyses (Tables 8, 9, 10). Even among the different number of washes (Table 9), one would expect to see statistical significance but, likely due to the small sample size here, no significance was found.

Table 8. Knockdown and Mortality Rates of Mosquito Populations by Fabric Type Under Different Exposure Methods and Number of Wash Cycles (100 = 100% Cotton; 50/50 = 50% polyester/50% Cotton)

Species	No. of Washes	Exposure Method	Fabric Type	Knockdown Rate				Mortality Rate			
				%	X^2	df	*p*-value	%	X^2	df	*p*-value
Ae. albopictus	0	Cone	100	33.3	0.000	1	1.000	40.0	0.127	1	0.722
			50/50	33.3				33.3			
		Petri dish	100	33.3	0.829	1	0.362	46.7	0.909	1	0.340
			50/50	50.0				64.3			
	5	Cone	100	20.0	0.509	1	0.476	30.0	0.019	1	0.890
			50/50	9.1				27.3			
		Petri dish	100	33.3	0.019	1	0.891	41.7	0.027	1	0.870
			50/50	30.8				38.5			
	15	Cone	100	5.9	0.611	1	0.434	11.8	0.020	1	0.888
			50/50	0.0				10.0			
		Petri dish	100	16.7	0.290	1	0.590	16.7	0.009	1	0.924
			50/50	9.1				18.2			
Ae. aegypti	0	Cone	100	0.0	---	---	---	0.0	---	---	---
			50/50	0.0				0.0			
		Petri dish	100	0.0	0.899	1	0.343	0.0	0.899	1	0.343
			50/50	6.7				6.7			
	5	Cone	100	0.0	---	---	---	0.0	---	---	---
			50/50	0.0				0.0			
		Petri dish	100	0.0	1.353	1	0.245	0.0	1.353	1	0.245
			50/50	7.7				7.7			
	15	Cone	100	0.0	---	---	---	0.0	---	---	---
			50/50	0.0				0.0			
		Petri dish	100	0.0	---	---	---	0.0	---	---	---
			50/50	0.0				0.0			

Table 9. Knockdown and Mortality Rates of Mosquito Populations by Number of Washes Under Different Fabric Types and Exposure Methods (100 = 100% Cotton, 50/50 = 50% Polyester/50% Cotton)

Species	Exposure Method	Fabric Type	No. of Washes	Knockdown				Mortality			
				%	X^2	df	*p*-value	%	X^2	df	*p*-value
Ae. albopictus	Cone	100	0	33.3	3.902	2	0.142	40.0	3.385	2	0.184
			5	20.0				30.0			
			15	5.9				11.8			
		50/50	0	33.3	5.186	2	0.075	33.3	1.699	2	0.428
			5	9.1				27.3			
			15	0.0				10.0			
	Petri Dish	100	0	33.3	1.140	2	0.566	46.7	2.858	2	0.240
			5	33.3				41.7			
			15	16.7				16.7			
		50/50	0	50.0	4.777	2	0.092	64.3	5.479	2	0.065
			5	30.8				38.5			
			15	9.1				18.2			
Ae. aegypti	Cone	100	0	0.0	---	---	---	0.0	---	---	---
			5	0.0				0.0			
			15	0.0				0.0			
		50/50	0	0.0	---	---	---	0.0	---	---	---
			5	0.0				0.0			
			15	0.0				0.0			
	Petri Dish	100	0	0.0	---	---	---	0.0	---	---	---
			5	0.0				0.0			
			15	0.0				0.0			
		50/50	0	6.7	0.918	2	0.632	6.7	0.918	2	0.632
			5	7.7				7.7			
			15	9.9				0.0			

Table 10. Knockdown and Mortality Rates of Mosquito Populations by Exposure Methods under Different Fabric Types and Number of Wash Cycles (100 = 100% Cotton, 50/50 = 50% Polyester/50% Cotton)

Species	No. of Washes	Fabric Type	Exposure Method	Knockdown				Mortality			
				%	X^2	df	p-value	%	X^2	df	p-value
Ae. albopictus	0	100	Cone	33.3	0.000	1	1.000	40.0	0.136	1	0.713
			Petri Dish	33.3				46.7			
		50/50	Cone	33.3	0.735	1	0.391	33.3	2.476	1	0.116
			Petri Dish	50.0				64.3			
	5	100	Cone	20.0	0.489	1	0.484	30.0	0.321	1	0.571
			Petri Dish	33.3				41.7			
		50/50	Cone	9.1	1.698	1	0.193	27.3	0.336	1	0.562
			Petri Dish	30.8				38.5			
	15	100	Cone	5.9	0.882	1	0.348	11.8	0.142	1	0.706
			Petri Dish	16.7				16.7			
		50/50	Cone	0.0	0.955	1	0.329	10.0	0.286	1	0.593
			Petri Dish	9.1				18.2			
Ae. aegypti	0	100	Cone	0.0	---	---	---	0.0	---	---	---
			Petri Dish	0.0				0.0			
		50/50	Cone	0.0	1.102	1	0.294	0.0	1.102	1	0.294
			Petri Dish	6.7				6.7			
	5	100	Cone	0.0	---	---	---	0.0	---	---	---
			Petri Dish	0.0				0.0			
		50/50	Cone	0.0	1.197	1	0.274	0.0	1.197	1	0.274
			Petri Dish	7.7				7.7			
	15	100	Cone	0.0	---	---	---	0.0	---	---	---
			Petri Dish	0.0				0.0			
		50/50	Cone	0.0	---	---	---	0.0	---	---	---

VII. DISCUSSION

Long-lasting permethrin-treated clothing has been proven to be an effective defense against arthropod exposure. However, lab and field studies indicate a reduction in effectiveness of permethrin-treated clothing against mosquito exposure after repeated washings. In the current laboratory study, both permethrin content and mosquito knockdown/mortality rate decreased with repeated washing and the extent to which this occurred varied when testing against two different mosquito exposure methods and two different mosquito populations.

Mosquito Species Knockdown/Mortality

Ae. aegypti mosquitoes used in this study were resistant to permethrin-treated clothing, while the *Ae. albopictus* were susceptible (determined by CDC bottle bioassay). According to CDC bottle bioassay guidelines, less than 80% mortality at the recommended diagnostic time suggests resistance (CDC, 2009). For these populations of *Aedes* mosquitoes, 30 minutes is the diagnostic time for permethrin (CDC, 2009). During the bottle bioassay conducted in our lab, within 30 minutes of exposure to permethrin, only 60% of Ae. *aegypti* mosquitoes were knocked down (Richards *et al*., unpublished). The bottle bioassay had a longer exposure time compared to the current study (i.e., two minutes). Results of the current study showed a difference in *Ae. aegypti* resistance to insecticide treated clothing compared to studies done by Frances *et al.* (2014), Faulde *et al.* (2003), and Banks *et al.* (2015) in which the *Ae. aegypti* used in their experiments were more sensitive permethrin treated clothing exposure. In the Frances *et al.* (2014) study, *Ae. aegypti* mosquitoes used were from Australia, were about 5-8 days old, and were exposed to treated swatches for about 10 minutes. At 5 washes, the knockdown rate was already up to 50% for one fabric and the mortality rate at 5 washes was 18.7% as opposed to our study in which only about 1.7% of *Ae. aegypti* mosquitoes had died. In the Faulde *et al.* (2003)

study, *Ae aegypti* mosquitoes were 5-8 days old and achieved 100% knockdown rate within 30-40 minutes after 15 washes. At 15 washes in our experiment, no *Ae. aegypti* mosquitoes had been knocked down or killed. In the Banks *et al*., (2015) study, *Ae. aegypti* mosquitoes were 3-5 days old and exposed for 3 minutes to tested swatches. In the same study, one hour post-exposure, the knockdown rates were up to 96%. Knockdown was maintained up to 80% for 15 washes and mortality was sustained above 80% for up to 5 washes (Banks et al. 2015). Mortality and knockdown at 5 washes for our study occurred at only 1.7% and no knockdown or mortality rate was observed in the 15 wash group. Such difference between this study and other studies could be the result of how the mosquitoes were reared in the lab, the location from which the mosquitoes were collected (degree of resistance/susceptibility), and/or how long the mosquitoes were exposed to the tested fabric swatches.

Effect of Washing on Mosquito Knockdown and Mortality Rates

Mosquito mortality and knockdown rates from exposure to permethrin-treated clothes are dependent on the number of washings and duration of exposure. In our study, the number of washings had significant effect on the *Ae. albopictus* population but not on the *Ae. aegypti* population. A knockdown rate of 37.5% for *Ae. albopictus* at 0 wash was reduced to 8.0% at 15 washes, while the mortality rate of 46.4% for *Ae. albopictus* at 0 wash was reduced to 14.0% at 15 washes. For both knockdown and mortality, the *Ae. aegypti* population was at 1.8% for 0 wash and fell to 0% at 15 washes. Gopalakrishnan *et al.* (2014) tested *Ae. albopictus* against permethrin treated clothing for 5 minutes and showed high knockdown rates of 77.6%, 15.9%, 9.5% and 3.5% for the first four washes and 100% mortality rates after 24 hours for the same first four washes. However, there was a significant decrease in these rate at 5 washes, producing no knockdown after 15 minutes and an 88% mortality rate after 24 hours. In our study, *Ae.*

albopictus produced a 23.9% knockdown rate after 2 hours and a 34.8% mortality rate after 24 hours. France *et al.* (2014) found that their polymer coated treated clothing was effective for up to 30 washes but saw a decrease in knockdown/mortality between 30-50 washes.

The washing technique and the impregnation method of permethrin can impact the duration of effectiveness of permethrin. Banks *et al*. (2015) observed a variation in *Ae. aegypti* mosquito knockdown rate when comparing two washing methods. In the same study, for *Ae. aegypti*, the 100% cotton treated shirts undergoing the WHO bottle washing technique achieved 90% mosquito knockdown for up to 6 washes, while the 100% cotton treated shirts undergoing the machine-washing technique maintained 90% knockdown for up to 10 washes. From 10-20 washes, the 100% cotton treated shirts experiencing the WHO washing technique, knockdown significantly decreased from 81.7% to 38.3% and mortality decreased from 77.5% to 32.4%. For machine washing, after 20 fabric washes, knockdown and mortality were 78.21% and 61.42%, while for 30 washes, it decreased to 57.9% and 40.9% (Banks *et al.*, 2015). Faulde *et al.* (2006) studied three types of treated clothing. The first two fabrics (Peripel 10 and IARFT) were treated using the dipping method. Both could achieve 100% knockdown rate in 1 hour between 20-40 washes, reaching 100% knockdown rate after 40 washes that took up to 200-300 minutes (Faulde et al. 2006). The same study suggested that Peripel 10 be retreated after 15 washes and IARFT be retreated after 50 washes. The third type of clothing was treated using the polymer coating method. There was no suggestion for retreatment with this method because it could achieve 100% knockdown in an hour for up to 100 washes (Faulde *et al*., 2006). The polymer coated method has shown to be much stronger in retaining permethrin after several washes compared to the dipping or absorption methods. Vaughn *et al.* (2014) found that long-lasting permethrin impregnation clothes could retain tick-repellent activity for over 70 washes. Their study followed outdoor workers for 2 years and found that 1 year of wear reduced tick bites by >80% but effectiveness significantly decreased in the second year. They suggested that after 1 year of wear, clothing should be retreated. It was also suggested that clothes that are treated using self-applied spray or dipping method lose effectiveness unless reapplied every 3-5 washes (Vaughn *et al.*, 2014). Polymer coating lasts longer against washing, aging, rinsing, wearing, and weathering. Individual impregnation methods (i.e., dipping or spraying) can also expose one to high concentrations of permethrin through inhalation or skin contact (Rossbach *et al.*, 2010). Thus, the polymer coated method also proves to be much safer.

Fabric Type and Mosquito Knockdown/Mortality

Here, results showed that the knockdown and mortality rates between the 2 tested fabrics were not statistically different. For the *Ae. aegypti* population, 100% cotton did not knockdown or kill any mosquitoes, while 50% cotton/50% polyester produced a knockdown and mortality rate of 2.4%. For the *Ae. albopictus* population, 50% polyester/50% cotton produced a slightly higher knockdown and mortality rate of 23.9% and 33.8% against 100% cotton that produced knockdown and mortality rates of 23.5% and 30.9%. There were no statistically significant differences for knockdown/mortality rates between the two fabrics among both mosquito populations.

This study demonstrated a difference in permethrin concentration between different fabrics, which could mean that they were not initially treated with the same amount of permethrin. The amount of permethrin impregnated into clothing is done by weight. So, the heavier cotton fabric was likely treated with more permethrin than the lighter weight blended fabric (50% polyester/50% cotton). The lack of significant difference in knockdown/ mortality rates between the two fabrics types, despite the higher permethrin content in 100% cotton, may be attributed to the short exposure time or because mosquitoes were sticking more to the 50% polyester/50% cotton fabric due to electrostatic attraction compared to 100% cotton. With a longer exposure time, the higher permethrin concentration in 100% cotton may result to a greater effect on the mosquitoes against 50% cotton/50% polyester fabric.

Petri dish method vs. Cone method

We observed no statistically significant difference in mosquito knockdown or mortality between the petri dish and cone exposure method among both mosquito populations. For the *Ae. aegypti* population, the cone method did not produce any knockdown or mortality rates while the petri dish method only produced a knockdown and mortality rate of 2.4%. For the *Ae. albopictus* population, the cone method resulted in knockdown and mortality rates of 17.3% and 25.3%, while the petri dish method showed knockdown and mortality rates of 29.9% and 39.0%. Even though, the petri dish method did have higher results than the cone method among *Ae. albopictus*, no significance was found between the two methods. Further research should be done using larger sample sizes or different exposure times for both exposure methods.

Strengths and Limitations of the Study

Based on the literature, a limited number of studies have tested the petri dish method against the WHO cone method for mosquito exposure to permethrin-treated clothing, and this study explored the differences between the two methods. Many studies use either a WHO plastic tube method (e.g. Faulde *et al*., 2003), the cone method (e.g. Gopalakrishnan *et al*., 2014), or a field exposure study (e.g. Kimani *et al*., 2006). Comparing the two methods is important because we can understand how to better monitor effectiveness of permethrin-treated clothing against mosquito exposure. This study also tested one genus but two different species of mosquitoes (*Ae. albopictus* and *Ae. aegypti*) with different resistant statuses.

The mosquito exposure time for this study was only set at 2 minutes. Against *Ae. albopictus*, the time of exposure seemed to make a difference in knockdown/mortality rate. However, against the *Ae. aegypti*, there was no change in knockdown or mortality rate. For further studies, it is recommended to increase exposure time to determine if there is a significant change in knockdown and mortality rates among *Ae. aegypti*. Another limitation of the study involves the transfer technique used in the experiment. After the mosquitoes were exposed to the fabrics, they were placed in the freezer to subdue them and successfully transfer them in cardboard cages for further incubation without flying away. Unfortunately, electrostatic attraction would make the mosquitoes stick to the fabric and often, use of forceps was necessary to take them off the fabric, wherein they get squished accidentally. This resulted in having less mosquitoes to observe than the initial number. For future studies, it would be best either to aspirate the mosquitoes from the cone and into the cardboard cage when using the cone method, or carefully remove the fabric from the petri dish when using the petri dish method. Moreover, while testing the untreated swatches using gas chromatography, small amounts of permethrin were detected, indicating a slight contamination on the controls. The detected amount was significantly less than the treated swatches but is still worth noting. When the clothes were packaged, treated and untreated clothes were placed next to each other in the plastic bags which could have led to some cross contamination. For further studies, it would be best to package treated and untreated clothing separately to avoid cross contamination.

Recommendations for Future Studies

Field studies seem to be the most effective way in evaluating the effectiveness of permethrin treated clothing against mosquito exposure. Environmental exposures may be difficult to simulate in laboratory studies, such as continuous sun exposure, varying weather conditions (rainfall or snow) and wearing of the fabric due to heavy usage. However, more field studies need to be conducted to accurately determine sustainability and cost effectiveness, especially in poorer areas. There are few published studies on the effect of permethrin-treated clothes against different genera and species of mosquitoes. *Aedes* mosquitoes showed the highest sensitivity to permethrin compared to *Culex* or *Anopheles* mosquitoes in the Faulde *et al.* (2015) study and *Ae. aegypti* mosquitoes showed higher knockdown and mortality rates against *An. farauti* in Frances *et al.* (2014) study. However, resistance/susceptibility status was not assessed in the aforementioned studies.

Some arthropods can develop resistance against pyrethroids. The combination of permethrin with other repellants (DEET) or other pyrethroids (e.g., bioallethrin) have shown to be effective in controlling the transmission of vector-borne diseases (Chareonviriyaphap *et al.*, 2013). However, more studies need to be conducted on the effects of such combinations on mosquito resistance to pyrethroids.

The dermal exposure of permethrin continues to be an issue, and could potentially be a risk associated with individuals wearing permethrin-treated clothes. Although, Rossbach *et al*., 2010 has shown that the risk involved with dermal exposure is low, it is still an issue that needs to be continuously monitored and studied in order to understand and ensure the safe use of permethrin-treated clothes for adults and children.

VIII. CONCLUSION

Permethrin-treated clothing has demonstrated effectiveness in reducing exposure to blood feeding arthropods. They can be used universally against most common arthropods and have the potential to reduce the transmission risk of pathogens that cause diseases such as malaria, dengue, or Lyme disease. Studies have shown that permethrin-treated clothing can protect against both day-biting and night-time mosquitoes and is useful in protecting travelers, military personnel and people who work outdoors. However, even with this assurance, there are still some caveats. The effect of the permethrin-treated clothing against potential vectors relies on many factors, such as how often they are washed, the type of fabrics used, and the susceptibility of mosquitoes to permethrin, which can influence the knockdown activity over time. Protection provided by permethrin-treated clothes against mosquito bites may be reduced over time due to repeated washings and thus, there may be a need to re-treat or replace these protective clothing after certain period of use. The need to re-treat or replace clothing may depend on the number of washes, how often the clothing is worn, and environmental conditions (e.g., rain, snow, long-term sun exposure). More studies are needed to evaluate the extent to which these factors affect the length of effectiveness of permethrin-treated clothing.

Results from the current study showed that the efficacy of permethrin-treated clothing can vary between mosquito populations. *Aedes aegypti* knockdown/mortality rates were much lower than *Ae. albopictus*. The *Ae. aegypti* population was already a known highly resistant species from the CDC bottle bioassay previously performed. There may still be a need to evaluate different species against insecticide-treated clothing to understand the best way to offer more protection for outdoor workers and those who engage in recreational activities. When comparing the two different mosquito exposure methods, the petri dish method appeared to be much more effective at knocking down and killing mosquitoes than the cone method, and there may be a need to evaluate which method is more appropriate for mosquito knockdown and mortality experiments. Permethrin-treated clothing has demonstrated potential for significant protection against known vector species and can help support one's decision to regularly use permethrin-treated clothing whether for occupational purposes or recreational activity. Mosquito and tick borne diseases should be recognized as occupational health risks and the use of permethrin-treated clothing should be included in standard prevention practices.

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X. APPENDICES

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Appendix A

<u>Petri dish 2-hr knockdown results for *Aedes albopictus*</u>

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/10	0/8	0/7
50/50 B	0/6	0/9	0/9
Total	**0/16**	**0/17**	**0/16**
100 A	0/8	0/8	0/7
100 B	0/9	0/7	0/7
Total	**0/17**	**0/15**	**0/14**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	4/7	2/7	0/5
50/50 B	3/7	2/6	1/6
Total	**7/14**	**4/13**	**1/11**
100 A	2/6	2/6	1/6
100 B	3/9	2/6	1/6
Total	**5/15**	**4/12**	**2/12**

Appendix B

Petri dish 24-hr mortality results for *Aedes albopictus*

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/10	0/8	0/7
50/50 B	0/6	0/9	0/9
Total	**0/16**	**0/17**	**0/16**
100 A	0/8	0/8	0/7
100 B	0/9	0/7	0/7
Total	**0/17**	**0/15**	**0/14**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	5/7	3/7	1/5
50/50 B	4/7	2/6	1/6
Total	**9/14**	**5/13**	**2/11**
100 A	3/6	3/6	1/6
100 B	4/9	2/6	1/6
Total	**7/15**	**5/12**	**2/12**

Appendix C

Cone method 2-hr knockdown results for *Aedes albopictus*

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/6	0/6	0/6
50/50 B	0/8	0/5	0/7
Total	**0/14**	**0/11**	**0/13**
100 A	0/5	0/6	0/6
100 B	0/5	0/6	0/5
Total	**0/10**	**0/12**	**0/11**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	2/5	0/5	0/4
50/50 B	2/7	1/6	0/6
Total	**4/12**	**1/11**	**0/10**
100 A	3/7	1/5	1/8
100 B	2/8	1/5	0/9
Total	**5/15**	**2/10**	**1/17**

Appendix D

Cone method 24-hr mortality results for *Aedes albopictus*

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/6	0/6	0/6
50/50 B	0/8	0/5	0/7
Total	**0/14**	**0/11**	**0/13**
100 A	0/5	0/6	0/6
100 B	0/5	0/6	0/5
Total	**0/10**	**0/12**	**0/11**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	2/5	1/5	1/4
50/50 B	2/7	2/6	0/6
Total	**4/12**	**3/11**	**1/10**
100 A	3/7	2/5	1/8
100 B	3/8	1/5	1/9
Total	**6/15**	**3/10**	**2/17**

Appendix E

Petri dish 2-hr knockdown results for *Aedes aegypti*

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/8	0/6	0/7
50/50 B	0/9	0/8	0/8
Total	**0/17**	**0/14**	**0/15**
100 A	0/9	0/7	0/7
100 B	0/8	0/10	0/10
Total	**0/17**	**0/17**	**0/17**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/9	1/7	0/5
50/50 B	1/6	0/6	0/7
Total	**1/15**	**1/13**	**0/12**
100 A	0/8	0/10	0/8
100 B	0/5	0/7	0/7
Total	**0/13**	**0/17**	**0/15**

Appendix F

Petri dish 24-hr mortality results for *Aedes aegypti*

Control

Fabric Types	**0 washes**	**5 washes**	**15 washes**
50/50 A	0/8	0/6	0/7
50/50 B	0/9	0/8	0/8
Total	**0/17**	**0/14**	**0/15**
100 A	0/9	0/7	0/7
100 B	0/8	0/10	0/10
Total	**0/17**	**0/17**	**0/17**

Treated

Fabric Types	**0 washes**	**5 washes**	**15 washes**
50/50 A	0/9	1/7	0/5
50/50 B	1/6	0/6	0/7
Total	**1/15**	**1/13**	**0/12**
100 A	1/8	0/10	0/8
100 B	1/5	0/7	0/7
Total	**2/13**	**0/17**	**0/15**

Appendix G

Cone method 2-hr knockdown results for *Aedes aegypti*

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/6	0/7	0/6
50/50 B	0/3	0/5	0/7
Total	**0/9**	**0/12**	**0/13**
100 A	0/5	0/3	0/6
100 B	0/7	0/10	0/4
Total	**0/12**	**0/13**	**0/10**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/9	0/6	0/6
50/50 B	0/7	0/9	0/6
Total	**0/16**	**0/15**	**0/12**
100 A	0/5	0/7	0/3
100 B	0/6	0/6	0/6
Total	**0/11**	**0/13**	**0/9**

Appendix H

Cone method 24-hr mortality results for *Aedes aegypti*

Control

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/6	0/7	0/6
50/50 B	0/3	0/5	0/7
Total	**0/9**	**0/12**	**0/13**
100 A	0/5	0/3	0/6
100 B	0/7	0/10	0/4
Total	**0/12**	**0/13**	**0/10**

Treated

Fabric Types	0 washes	5 washes	15 washes
50/50 A	0/9	0/6	0/6
50/50 B	0/7	0/9	0/6
Total	**0/16**	**0/15**	**0/12**
100 A	0/5	0/7	0/3
100 B	0/6	0/6	0/6
Total	**0/11**	**0/13**	**0/9**

APPENDIX I

Permethrin Content Analysis (raw data)

TREATED				
Vial	**Sample (Fabric Type, no. of washes)**	**Area**	**Retention Time**	**Concentration (µg/cm²)**
1	Blank	0	0	1.1
2	Blank	0	0	1.1
3	50/50, 0 washes	71.7	9.853	5.6
4	50/50, 0 washes	99.2	9.854	7.4
5	50/50, 0 washes	73.7	9.858	5.8
6	Blank	0	0	1.1
7	100, 0 washes	484.6	9.844	31.5
8	100, 0 washes	395.2	9.845	25.9
9	100, 0 washes	461.6	9.844	30.1
10	Blank	0	0	1.1
11	50/50, 5 washes	53.8	9.853	4.5
12	50/50, 5 washes	46	9.825	4.0
13	50/50, 5 washes	45.5	9.815	4.0
14	Blank	0	0	1.1
15	100, 5 washes	260	9.846	17.5
16	100, 5 washes	222	9.835	15.1
17	100, 5 washes	261	9.843	17.5
18	Blank	0	0	1.1
19	50/50, 15 washes	22.3	9.825	2.5
20	50/50, 15 washes	14.5	9.819	2.0
21	50/50, 15 washes	16.3	9.815	2.2
22	Blank	0	0	1.1
23	100, 15 washes	124.8	9.825	8.9
24	100, 15 washes	110.7	9.841	8.1
25	100, 15 washes	139	9.831	9.9
26	Blank	0	0	1.1

APPENDIX I

Permethrin Content Analysis (raw data), cont'd

CONTROL				
Via l	**Sample (Fabric Type, no. of washes)**	**Area**	**Retention Time**	**Concentration ($\mu g/cm^2$)**
1	Blank	0	0	1.1
2	Blank	0	0	1.1
3	50/50, 0 washes	5.1	9.704	1.5
4	50/50, 0 washes	4.9	9.679	1.5
5	50/50, 0 washes	5.7	9.71	1.5
6	Blank	0	0	1.1
7	100, 0 washes	10	9.7	1.8
8	100, 0 washes	8.5	9.72	1.7
9	100, 0 washes	11.5	9.757	1.9
10	Blank	0	0	1.1
11	50/50, 5 washes	18	9.696	2.2
12	50/50, 5 washes	8.1	9.695	1.7
13	50/50, 5 washes	4.4	9.692	1.4
14	Blank	0	0	1.1
15	100, 5 washes	7.3	9.681	1.6
16	100, 5 washes	5.8	9.697	1.5
17	100, 5 washes	9.3	9.629	1.8
18	Blank	0	0	1.1
19	50/50, 15 washes	6.3	9.691	1.5
20	50/50, 15 washes	3.3	9.697	1.3
21	50/50, 15 washes	3.6	9.696	1.4
22	Blank	0	0	1.1
23	100, 15 washes	4.4	9.694	1.4
24	100, 15 washes	3.1	9.697	1.3
25	100, 15 washes	5	9.694	1.5
26	Blank	0	0	1.1

Appendix J

SPSS Knockdown/Mortality Table by Fabric Type (*Ae. aegypti* and *Ae. albopictus*)

Knockdown Table

Crosstab

Mosquito Species				Knockdown No	Knockdown Yes	Total
Ae. aegypti	Fabric	100	Count	78	0	78
			% within Fabric	100.0%	0.0%	100.0%
		50/50	Count	81	2	83
			% within Fabric	97.6%	2.4%	100.0%
	Total		Count	159	2	161
			% within Fabric	98.8%	1.2%	100.0%
Ae. albopictus	Fabric	100	Count	62	19	81
			% within Fabric	76.5%	23.5%	100.0%
		50/50	Count	54	17	71
			% within Fabric	76.1%	23.9%	100.0%
	Total		Count	116	36	152
			% within Fabric	76.3%	23.7%	100.0%
Total	Fabric	100	Count	140	19	159
			% within Fabric	88.1%	11.9%	100.0%
		50/50	Count	135	19	154
			% within Fabric	87.7%	12.3%	100.0%
	Total		Count	275	38	313
			% within Fabric	87.9%	12.1%	100.0%

Chi-Square Tests

Mosquito Species		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Ae. aegypti	Pearson Chi-Square	1.903[c]	1	.168		
	Continuity Correction[b]	.446	1	.504		
	Likelihood Ratio	2.674	1	.102		
	Fisher's Exact Test				.497	.264
	N of Valid Cases	161				
Ae. albopictus	Pearson Chi-Square	.005[d]	1	.944		
	Continuity Correction[b]	.000	1	1.000		
	Likelihood Ratio	.005	1	.944		
	Fisher's Exact Test				1.000	.547
	N of Valid Cases	152				
Total	Pearson Chi-Square	.011[a]	1	.916		
	Continuity Correction[b]	.000	1	1.000		
	Likelihood Ratio	.011	1	.916		
	Fisher's Exact Test				1.000	.527
	N of Valid Cases	313				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 18.70.

b. Computed only for a 2x2 table

c. 2 cells (50.0%) have expected count less than 5. The minimum expected count is .97.

d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.82.

SPSS Knockdown/Mortality Table by Fabric Type (*Ae. aegypti* and *Ae. albopictus*) cont'd.

Mortality Table

Crosstab

Mosquito Species				Mortality No	Mortality Yes	Total
Ae. aegypti	Fabric	100	Count	78	0	78
			% within Fabric	100.0%	0.0%	100.0%
		50/50	Count	81	2	83
			% within Fabric	97.6%	2.4%	100.0%
	Total		Count	159	2	161
			% within Fabric	98.8%	1.2%	100.0%
Ae. albopictus	Fabric	100	Count	56	25	81
			% within Fabric	69.1%	30.9%	100.0%
		50/50	Count	47	24	71
			% within Fabric	66.2%	33.8%	100.0%
	Total		Count	103	49	152
			% within Fabric	67.8%	32.2%	100.0%
Total	Fabric	100	Count	134	25	159
			% within Fabric	84.3%	15.7%	100.0%
		50/50	Count	128	26	154
			% within Fabric	83.1%	16.9%	100.0%
	Total		Count	262	51	313
			% within Fabric	83.7%	16.3%	100.0%

Chi-Square Tests

Mosquito Species		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Ae. aegypti	Pearson Chi-Square	1.903[c]	1	.168		
	Continuity Correction[b]	.446	1	.504		
	Likelihood Ratio	2.674	1	.102		
	Fisher's Exact Test				.497	.264
	N of Valid Cases	161				
Ae. albopictus	Pearson Chi-Square	.150[d]	1	.699		
	Continuity Correction[b]	.045	1	.831		
	Likelihood Ratio	.149	1	.699		
	Fisher's Exact Test				.730	.415
	N of Valid Cases	152				
Total	Pearson Chi-Square	.077[a]	1	.781		
	Continuity Correction[b]	.016	1	.901		
	Likelihood Ratio	.077	1	.781		
	Fisher's Exact Test				.879	.450
	N of Valid Cases	313				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 25.09.

b. Computed only for a 2x2 table

c. 2 cells (50.0%) have expected count less than 5. The minimum expected count is .97.

d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 22.89.

Appendix K

SPSS Knockdown/Mortality Table by Number of Washes (*Ae. aegypti* and *Ae. albopictus*)

Knockdown Table

Crosstab

Mosquito Species				Knockdown No	Knockdown Yes	Total
Ae. aegypti	Washes	0	Count	54	1	55
			% within Washes	98.2%	1.8%	100.0%
		5	Count	57	1	58
			% within Washes	98.3%	1.7%	100.0%
		15	Count	48	0	48
			% within Washes	100.0%	0.0%	100.0%
	Total		Count	159	2	161
			% within Washes	98.8%	1.2%	100.0%
Ae. albopictus	Washes	0	Count	35	21	56
			% within Washes	62.5%	37.5%	100.0%
		5	Count	35	11	46
			% within Washes	76.1%	23.9%	100.0%
		15	Count	46	4	50
			% within Washes	92.0%	8.0%	100.0%
	Total		Count	116	36	152
			% within Washes	76.3%	23.7%	100.0%
Total	Washes	0	Count	89	22	111
			% within Washes	80.2%	19.8%	100.0%
		5	Count	92	12	104
			% within Washes	88.5%	11.5%	100.0%
		15	Count	94	4	98
			% within Washes	95.9%	4.1%	100.0%
	Total		Count	275	38	313
			% within Washes	87.9%	12.1%	100.0%

Chi-Square Tests

Mosquito Species		Value	df	Asymptotic Significance (2-sided)
Ae. aegypti	Pearson Chi-Square	.862[b]	2	.650
	Likelihood Ratio	1.428	2	.490
	Linear-by-Linear Association	.780	1	.377
	N of Valid Cases	161		
Ae. albopictus	Pearson Chi-Square	12.720[c]	2	.002
	Likelihood Ratio	13.834	2	.001
	Linear-by-Linear Association	12.394	1	.000
	N of Valid Cases	152		
Total	Pearson Chi-Square	12.139[a]	2	.002
	Likelihood Ratio	13.100	2	.001
	Linear-by-Linear Association	11.517	1	.001
	N of Valid Cases	313		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.90.

b. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .60.

c. 0 cells (.0%) have expected count less than 5. The minimum expected count is 10.89.

SPSS Knockdown/Mortality Table by Number of Washes (*Ae. aegypti* and *Ae. albopictus*) cont'd.

Mortality Table

Crosstab

Mosquito Species				Mortality No	Mortality Yes	Total
Ae. aegypti	Washes	0	Count	54	1	55
			% within Washes	98.2%	1.8%	100.0%
		5	Count	57	1	58
			% within Washes	98.3%	1.7%	100.0%
		15	Count	48	0	48
			% within Washes	100.0%	0.0%	100.0%
	Total		Count	159	2	161
			% within Washes	98.8%	1.2%	100.0%
Ae. albopictus	Washes	0	Count	30	26	56
			% within Washes	53.6%	46.4%	100.0%
		5	Count	30	16	46
			% within Washes	65.2%	34.8%	100.0%
		15	Count	43	7	50
			% within Washes	86.0%	14.0%	100.0%
	Total		Count	103	49	152
			% within Washes	67.8%	32.2%	100.0%
Total	Washes	0	Count	84	27	111
			% within Washes	75.7%	24.3%	100.0%
		5	Count	87	17	104
			% within Washes	83.7%	16.3%	100.0%
		15	Count	91	7	98
			% within Washes	92.9%	7.1%	100.0%
	Total		Count	262	51	313
			% within Washes	83.7%	16.3%	100.0%

Chi-Square Tests

Mosquito Species		Value	df	Asymptotic Significance (2-sided)
Ae. aegypti	Pearson Chi-Square	.862[b]	2	.650
	Likelihood Ratio	1.428	2	.490
	Linear-by-Linear Association	.780	1	.377
	N of Valid Cases	161		
Ae. albopictus	Pearson Chi-Square	12.912[c]	2	.002
	Likelihood Ratio	13.824	2	.001
	Linear-by-Linear Association	12.817	1	.000
	N of Valid Cases	152		
Total	Pearson Chi-Square	11.266[a]	2	.004
	Likelihood Ratio	12.031	2	.002
	Linear-by-Linear Association	10.979	1	.001
	N of Valid Cases	313		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 15.97.

b. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .60.

c. 0 cells (.0%) have expected count less than 5. The minimum expected count is 14.83.

Appendix L

SPSS Knockdown/Mortality Table by Exposure Method (*Ae. aegypti* and *Ae. albopictus*)

Knockdown Table

Crosstab

Mosquito Species				Knockdown No	Knockdown Yes	Total
Ae. aegypti	Method	Cone	Count	76	0	76
			% within Method	100.0%	0.0%	100.0%
		Petri Dish	Count	83	2	85
			% within Method	97.6%	2.4%	100.0%
	Total		Count	159	2	161
			% within Method	98.8%	1.2%	100.0%
Ae. albopictus	Method	Cone	Count	62	13	75
			% within Method	82.7%	17.3%	100.0%
		Petri Dish	Count	54	23	77
			% within Method	70.1%	29.9%	100.0%
	Total		Count	116	36	152
			% within Method	76.3%	23.7%	100.0%
Total	Method	Cone	Count	138	13	151
			% within Method	91.4%	8.6%	100.0%
		Petri Dish	Count	137	25	162
			% within Method	84.6%	15.4%	100.0%
	Total		Count	275	38	313
			% within Method	87.9%	12.1%	100.0%

Chi-Square Tests

Mosquito Species		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Ae. aegypti	Pearson Chi-Square	1.811[c]	1	.178		
	Continuity Correction[b]	.401	1	.527		
	Likelihood Ratio	2.577	1	.108		
	Fisher's Exact Test				.498	.277
	N of Valid Cases	161				
Ae. albopictus	Pearson Chi-Square	3.304[d]	1	.069		
	Continuity Correction[b]	2.647	1	.104		
	Likelihood Ratio	3.340	1	.068		
	Fisher's Exact Test				.086	.051
	N of Valid Cases	152				
Total	Pearson Chi-Square	3.411[a]	1	.065		
	Continuity Correction[b]	2.801	1	.094		
	Likelihood Ratio	3.472	1	.062		
	Fisher's Exact Test				.083	.046
	N of Valid Cases	313				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 18.33.

b. Computed only for a 2x2 table

c. 2 cells (50.0%) have expected count less than 5. The minimum expected count is .94.

d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 17.76.

SPSS Knockdown/Mortality Table by Exposure Method (*Ae. aegypti* and *Ae. albopictus*) cont'd.

Mortality Table

Crosstab

Mosquito Species				Mortality No	Mortality Yes	Total
Ae. aegypti	Method	Cone	Count	76	0	76
			% within Method	100.0%	0.0%	100.0%
		Petri Dish	Count	83	2	85
			% within Method	97.6%	2.4%	100.0%
	Total		Count	159	2	161
			% within Method	98.8%	1.2%	100.0%
Ae. albopictus	Method	Cone	Count	56	19	75
			% within Method	74.7%	25.3%	100.0%
		Petri Dish	Count	47	30	77
			% within Method	61.0%	39.0%	100.0%
	Total		Count	103	49	152
			% within Method	67.8%	32.2%	100.0%
Total	Method	Cone	Count	132	19	151
			% within Method	87.4%	12.6%	100.0%
		Petri Dish	Count	130	32	162
			% within Method	80.2%	19.8%	100.0%
	Total		Count	262	51	313
			% within Method	83.7%	16.3%	100.0%

Chi-Square Tests

Mosquito Species		Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Ae. aegypti	Pearson Chi-Square	1.811[c]	1	.178		
	Continuity Correction[b]	.401	1	.527		
	Likelihood Ratio	2.577	1	.108		
	Fisher's Exact Test				.498	.277
	N of Valid Cases	161				
Ae. albopictus	Pearson Chi-Square	3.230[d]	1	.072		
	Continuity Correction[b]	2.636	1	.104		
	Likelihood Ratio	3.252	1	.071		
	Fisher's Exact Test				.084	.052
	N of Valid Cases	152				
Total	Pearson Chi-Square	2.946[a]	1	.086		
	Continuity Correction[b]	2.444	1	.118		
	Likelihood Ratio	2.979	1	.084		
	Fisher's Exact Test				.094	.058
	N of Valid Cases	313				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 24.60.

b. Computed only for a 2x2 table

c. 2 cells (50.0%) have expected count less than 5. The minimum expected count is .94.

d. 0 cells (.0%) have expected count less than 5. The minimum expected count is 24.18.

Appendix M

SPSS Permethrin Content Tables for Fabric Type and Number of Washes

Between-Subjects Factors

		N
Fabric Type	100	3
	50/50	3
No. of Washes	.0	2
	5.0	2
	15.0	2

Tests of Between-Subjects Effects

Dependent Variable: Lnconcentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.249[a]	3	1.416	324.243	.003
Intercept	26.022	1	26.022	5957.104	.000
FabricType	3.051	1	3.051	698.349	.001
No.ofWashes	1.199	2	.599	137.190	.007
Error	.009	2	.004		
Total	30.280	6			
Corrected Total	4.258	5			

a. R Squared = .998 (Adjusted R Squared = .995)

Lnconcentration

Duncan[a,b]

No. of Washes	N	Subset		
		1	2	3
15.0	2	1.5151		
5.0	2		2.1252	
.0	2			2.6074
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .004.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = .05.