MECHANISMS OF PYRETHRUM AND PYRETHROID REPELLENCY

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfilment of the requirements
for the degree of

Entomology-Doctor of Philosophy

2018

ABSTRACT

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Pyrethrum is a natural insecticide, extracted from the flower heads of *Chrysanthemum cinerariifolium*. Pyrethroids are synthetic compounds structurally derived from pyrethrins. Besides their insecticidal activity, it is well-documented from behavioral assays that pyrethrum and pyrethroids induce repellency. However, the molecular basis of pyrethrum/pyrethroid repellency is unknown. The aim of this study was to elucidate the mechanism of pyrethrum and pyrethroid repellency in mosquitoes and crop pests.

To investigate repellency against mosquitoes, a previously established Hand in cage assay was performed. We recorded mosquito landing rate on a hand in response to vapor from a mesh treated with pyrethrum or pyrethroids (transfluthrin, deltamethrin and permethrin).Three insecticide-susceptible *Aedes aegypti* strains, Waco, Orlando and Rock, two pyrethroid-resistant *Ae. aegypti* strains, Puerto Rico (PR) and Isokdr, an *Ae. aegypti* anosmic *Orco* (olfactory receptor co-receptor) mutant, and an insecticide-susceptible *Anopheles gambiae* strain, Kisumu were used. All Hand in cage experiments used serial dilutions of the test compounds to generate dose response curves. Different cohorts (50 per cohort) of nulliparous 6-8 day-old female mosquitoes were used per trial.

Throughout the investigation, significant levels of repellency by pyrethrum and pyrethroids were observed in the insecticide-susceptible mosquito strains although the magnitude varied with test compounds. The repellent effects by pyrethrum and pyrethroids were reduced in the two resistant strains, PR and Isokdr, compared to the susceptible strains, Waco and Rock. Pretreatment of mosquitoes with piperonyl butoxide (PBO), a P450 inhibitor, enhanced transfluthrin repellency in

both PR and Waco mosquitoes, but did not abolish the difference in repellency between them. Furthermore, repellency to DEET was not significantly different between susceptible and resistant mosquito strains.

Repellency against susceptible *Aedes* and *Anopheles* mosquitoes was also evaluated using two types of insecticide treated nets, PermaNet 2.0 and Olyset. Repellency of PermaNet2.0 on pyrethroid-susceptible *Ae. aegypti* Rock and Waco mosquitoes, and *An. gambiae* Kisumu mosquitoes was observed, but was reduced in pyrethroid-resistant Peurto Rico (PR) and Isokdr and anosmic *orco* mosquitoes. Repellency effect of the Olyset net on *An. gambiae* Kisumu mosquitoes was evident, but not on *Ae. aegypti* mosquitoes. Electroantennograph (EAG) recordings were conducted from antenna of adult *Ae. aegypti*. Pyrethrum and pyrethroids elicited EAG responses in Rock and Orlando mosquito strains. No EAG responses were recorded in Orco knockout mosquitoes.

Studies on the diamondback moth and maize grain borer using T-maze assays and feeding choice tests revealed that pyrethroids also evoke repellency in the maize grain borer and diamondback moth. The larvae of diamondback moth preferred eating a leaf in an untreated arena than the treated one. Adults of maize grain borer preferred the untreated control arm in the T-maze assay.

This study began to uncover the enigma of repellency of the one of the most important class of insecticides used globally to combat vector-borne human diseases. We confirmed repellency by pyrethrum/pyrethroids in both disease vectors and crop pests, and demonstrated that *Ae. aegypti* mosquitoes can sense pyrethrum/pyrethroids via an authentic Orco-dependent olfactory pathway. The repellency is likely mediated by dual activation of both sodium channels and/or olfactory receptors. Collectively, our study provides a conceptual framework for understanding of the modes of action of pyrethrum/pyrethroids as an important group of insect repellents.

ACKNOWLEDGEMENTS

My appreciation goes to my major professor Dr. Ke Dong who was so generous to dedicate her time and energy to supervise my work. She has been very supportive professionally and has helped me grow as a researcher. I am so thankful to my guidance committee: Dr. Edward Walker and Dr. Henry Chung at the Department of Entomology, Michigan State University (MSU) and Dr. Themba Mzilahowa at the Malaria Alert Center, College of Medicine (COM-UNIMA) for all their time and meaningful contributions to my research work. I also would like to thank late Dr Suzan Thiem for all her contributions, guidance and support. I would like to thank the Chair of Entomology department at MSU, Dr. Bill Ravlin for his support. Very special thanks to Heather Lenartson-Kluge for her assistance. I am thankful to all my lab mates for all kinds of assistance. I am grateful to Peng Xu, Qiang Wang and Feng Liu, postdoc associates in the Dong lab, for their help and collaboration to make this project work. My appreciation also goes to Dr Abel Sefasi (Bunda-LUANAR) for his support. Thanks to Dr Kingdom Kwapata (Bunda-LUANAR) for providing space in his lab. Thanks to the Borlaug Higher Education for Agricultural Research and Development (BHEARD) team at MSU for their support. This material is based upon work supported by United States Agency for International Development as part of the Feed the Future initiative, under CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013, and a grant from NIH (R01GM115475).

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CHAPTER 1

INTRODUCTION

Pyrethrins, pyrethroids and their use as insecticides

Pyrethrins were originally derived from extracts of the flower heads of *Chrysanthemum cinerariaefolium* and they comprise six insecticidal compounds namely pyrethrin I and II, cinerins I and II, jasmolins I and II (Anadón et al., 2009). In their natural state, they are potent insecticides but are unstable when exposed to light, air and heat (Ensley, 2007). Their use in form of crude extracts dates back to 400BC in persia (Ensley, 2007). In the past decades, three developments have helped establish their main uses including delivering pyrethrins by incorporating pounded flowers with other ingredients into mosquito coils that repel, expel, knock down or kill mosquitoes (Casida, 1980). The second way is through aerosol can or comb which produces droplets below 30um in diameter, the third one is the use of additive synergists the piperonyl butoxide (PBO) which by itself is not toxic but increases the potency (Casida, 1980).

Pyrethroids are synthetic analogs of pyrethrins with improved stability and greater insecticidal activity (Breckenridge et al., 2009; Davies et al., 2007; Ensley, 2007) With a few exceptions of more recently developed compounds, pyrethroids are typically esters of chrysanthemic acid (Fig. 1.1, Soderlund 2011). The first pyrethroid was allethrin which was identified in 1949 and has a basic cylopropane carboxylic ester structure as other type I pyrethroids, such as phenothrin and permethrin (Anadón et al., 2009). The insecticidal activity of pyrethroids was enhanced by adding a cyno group to give alpha-cyano at the phenocybenzyl alcohol moiety of type II pyrethroids, such as deltamethrin, cyfluthrin and lamda-cyhalothrin (Anadón et al., 2009). To date, pyrethroids remain important insecticides and have been used for more than thirty years to control insect vectors and crop pests.

In other parts of the world they are also used as an active ingredient in many household insecticidal products (Sugiura et al., 2008). Their use has extended to crop protection to minimize pre and postharvest losses. Cotton growing regions in the world , more especially in Africa , where transgenic cotton is rarely used, pyrethroids have been used in field sprays to control insect pests (Christian et al., 2011; Symington et al., 2011; Yang et al., 2004). Use of pyrethroids in vegetable crops such as tomatoes, have also been documented (Haddi et al., 2012). Dust formulated insecticides to control of storage pests in maize grains use a pyrethroid as an active ingredient (Kamanula, 2014).

Aside from agricultural and household uses, the use of pyrethroid insecticides has been largely documented in public health. To date, several pyrethroids, such as permethrin and deltamethrin (Fig. 1.1), are used in insecticide treated nets (ITN) (Denham et al., 2015; Enayati and Hemingway, 2006; Stevenson et al., 2011; Takken, 2002). To increase efficacy, some pyrethroid-bound nets are used together with piperoyl butoxide (PBO) as a synergist (Denham et al., 2015). They also have been used in indoor residual sprays (IRS) and they are incorporated in mosquito coils.

Recent research trends have documented the use of volatile pyrethroids such as transfluthrin (Fig. 1.1), inducing behavioral changes in mosquitoes due to sub-lethal exposures. A study by Ogoma et al., (2014) extensively evaluated the effect of airborne pyrethroids on entomological parameters of malaria and gave a detailed account of deterrence, toxicity and blood feeding inhibition in mosquitoes due to exposure to air borne pyrethroids. Mosquitoes that were captured in experimental huts did not feed or lay eggs. Details on several studies have revealed the use of pyrethroids in passive emanators, coils and transfluthrin impregnated hessian sacks to reduce outdoor mosquito bites (Govella et al., 2015; Ogoma et al., 2014, 2017; Ogoma, Ngonyani, et al., 2012; Ogoma et al., 2012).

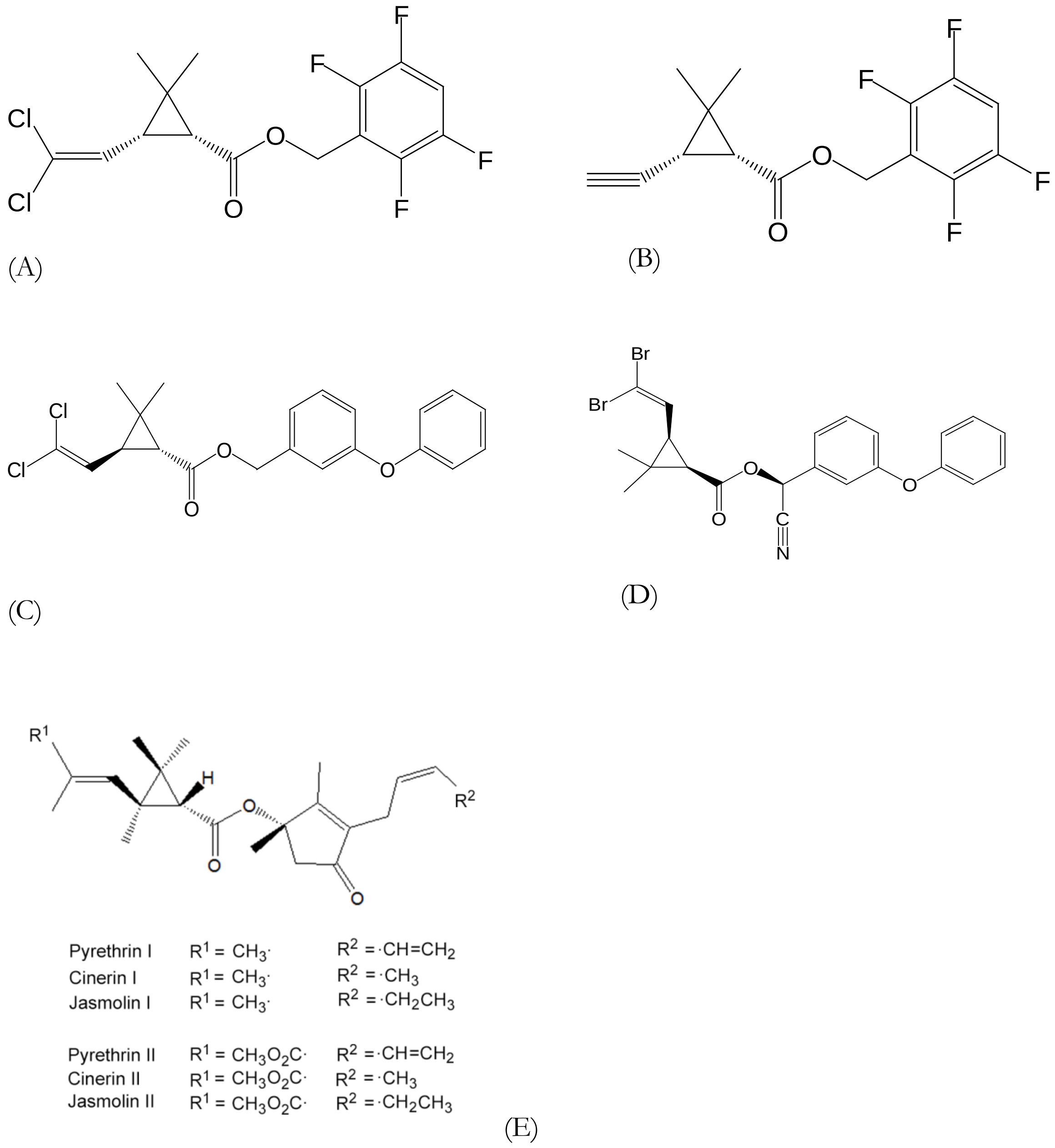

Figure1.1 Chemical structures of selected pyrethroids and pyrethrins. Indicated are: (A) Transfluthrin (B) Acetransfluthrin (ACTF), (C) deltamethrin and (D) permethrin. (E) Chemical structure of pyrethrin

Mode of action of pyrethroids

Voltage-gated sodium channels are integral transmembrane proteins that are critical for electrical signaling in most excitable cells. In response to membrane depolarization, sodium channels open (activate) and allow sodium ions to flow into the cell, causing depolarization of the membrane potential. A few milliseconds after channel opening, the channel is inactivated (closed), i.e., fast inactivation which plays an important role in the termination of action potentials and prevents excessive depolarization of the resting membrane potential. Thus, sodium channels are essential components of cellular excitability. Pyrethroids inhibit channel inactivation and stabilize the open state of sodium channels, causing prolonged channel opening (Narahashi, 2000, 2000; Narahashi et al., 1995). Type I pyrethroids cause repetitive discharges, whereas Type II pyrethroids cause membrane depolarization accompanied by suppression of cellular excitability (Narahashi 1986, Fig. 1.2).

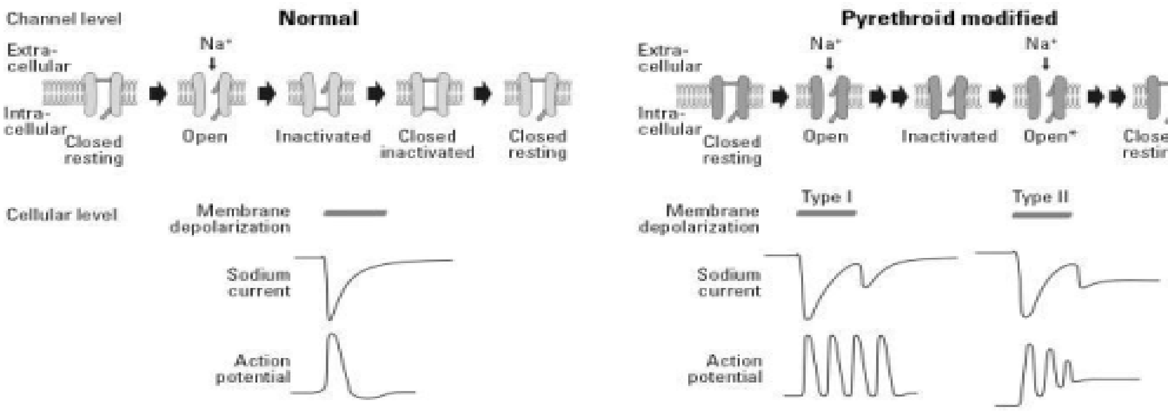

Figure 1.2 Schematic presentation of effect of pyrethroids on neuronal excitability. The diagram depicts the pyrethroid effects on individual channels on whole sodium currents and action potentials (Shafer et al., 2005).

Pyrethroid resistance

Although pyrethroids remain reliable in insect control because of their low mammalian toxicity, their potency on insects has been affected by development of resistance. Some of the mechanisms through which insects develop resistance to pyrethroids include increased metabolic detoxification, decreased sensitivity of the target site (sodium channels) to pyrethroids, reduced circular penetration or increased insecticide sequestration (Kasai et al., 2014; N. Liu, 2012; Nardini et al., 2012; Ranson et al., 2011; Toé et al., 2014). A growing body of literature has presented two most common mechanisms of pyrethroid resistance: enhanced metabolic detoxification (mainly P-450 mediated) and knockdown resistance (kdr) due to mutations in the sodium channel gene (Ffrench-Constant et al., 2004; Liu et al. 2015; Matowo et al. 2014; Dong et al., 2014).

Insect olfaction and pyrethroid repellency

Insects including *Drosophila* and mosquitoes rely on olfactory receptor neurons (ORNs) to sense odorants (Vosshal and Stocker, 2007; Carey and Carlson, 2011; Leal, 2013). ORNs are housed in olfactory sensilla on antennae and maxillary palps (Fig.3. Odorants bind to specific olfactory receptors (ORs) in ORNs, which confer odor-specificity. An obligate OR co-receptor (Orco) does not bind odorants by itself, but is necessary for the proper function of the OR/Orco complex as ligand-gated cation channels (Benton et al., 2006; Larsson et al., 2004; Sato et al., 2008; Vosshall and Hansson, 2011). Host seeking in mosquitoes is mediated by this sensory modality (Bohbot et al., 2010). Others have demonstrated the effect on body odor affecting flight and landing in mosquitoes (Webster et al., 2015). Detailed moment to moment flight maneuvers of the *Aedes* mosquitoes in response to human odor and carbon dioxide have been reported (Dekker and Carde, 2011). Several authors have emphasized that behavioral expressions in insects are mediated by olfaction (Takken et al., 2001; Zwiebel and Takken,2004; Wang et al., 2010, Takken and Verhulst, 2011) and olfactory receptors which are mainly found on the maxillary palpi and antenna of the insect are involved in the process (Zwiebel and Takken, 2004).

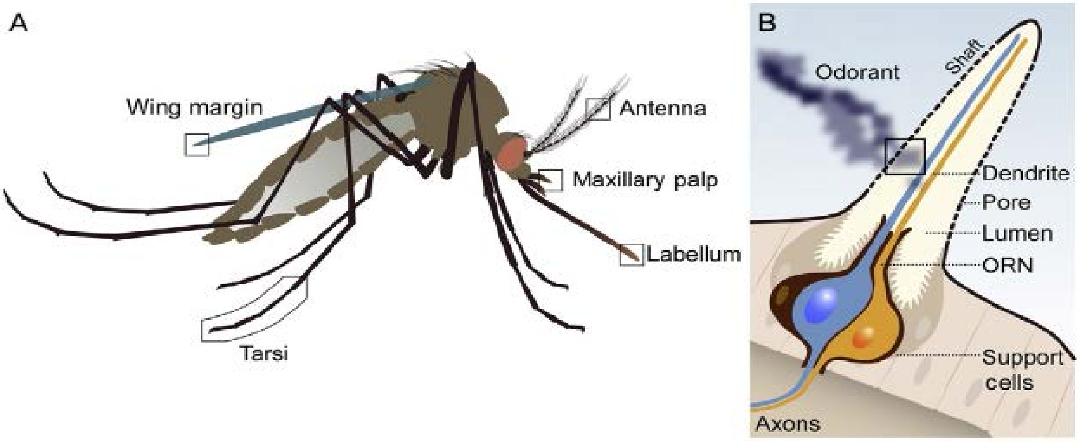

Figure 1.3 Peripheral olfactory system in mosquitoes. Different kinds of sensilla are located on different parts of the insect's body including, the antennae, maxillary palps, labellum, tarsi and wing margin. For olfaction mediated behaviors, the odorant passes through openings in the sensillum to sensilla lymph from the air as shown in (B). When in the sensilla lymph it binds to olfactory binding proteins which interact with olfactory receptors in the neurons. (Bennett and Chopra, 1993)

The olfactory sensilla can detect very low concentrations of air borne chemicals. For decades, DEET and other naturally derived insect repellents have been known to elicit behavior changes in mosquitoes through the olfactory pathway(Logan et al., 2010a; Masetti & Maini, 2006; McMahon et al., 2003; Syed and Leal, 2009).

Traditionally, most studies on pyrethroids have focused on contact toxicity and not the ability of the insects to detect very low concentrations which would elicit a change in their host seeking behavior. Details on the actual mechanisms underlying the resultant behavioral effects of exposure to pyrethroid sublethal effects are still elusive. Behavioral modifications due to insecticides with neurotoxic sub lethal effects such as pyrethroids, have been documented in a review by(Haynes, 1988). The review highlighted how permethrin and other insecticides affected mate locating behavior of males. Variations in the effect of the insecticides on the mate locating behavior were emphasized. A field study elucidated the impact of metofluthrin impregnated slow release plastic cylinder against mosquitoes under indoor conditions, citing over 6 weeks of activity and demonstrated significant reduction of mosquito indices after treatment (Kawada et al., 2006). Another study has pointed towards insensitivity of mosquitoes carrying sodium channel mutations to transfluthrin repellency (Wagman et al., 2015). They speculated that the repellency behavior evoked due to transfluthrin exposure in *Aedes* mosquitoes was mediated by the neuroexcitation that affected mosquito locomotor behavior.

Conflicting concepts have been presented in an attempt define a repellent when it comes to pyrethroids. Some studies have included the knockdown effect, mortality and deterrence in the term repellency (Adu-Acheampong et al., 2014, Ogoma et at., 2012) and others emphasize that repellents are not supposed to cause mortality in insects, but have to reduce vector host contact and affect insect behavior at very low detectable limits (Maia et al., 2013).

Attempts qualify or define chemicals in terms of the behaviors they elicit in the insects has attracted an evolution of technical terms. Repellency has been defined focusing on insect locomotor behavior; as when an insect steers its course away from the source of stimuli by (Debboun et al., 2006). Others have defined repellency depending on whether behavioral effects are observed after tarsal contact, and the resultant behavior has been referred to as "*contact repellency*" or the behavioral effects observed when an insect does not make a tarsal contact with a source of stimuli by steering its course away and this has been referred to as "*spatial repellency*" (Achee et al., 2009; Debboun et al., 2006; Dusfour et al., 2009; Sathantriphop et al., 2014). The terms spatial repellency and or contact repellency are still difficult to define as behavioral terms. A review by Miller et al.,(2009), emphasized on the terms to be desgnated to chemicals or insecticides in reference to the locomotor responses that they elicit in insects, updating terms by early behavioral scientists Diether and co investigators (1960). They emphasized the definition of a "*repellent* "as a chemical that causes a mover (an insect in this case) to make oriented movements away from the source of stimuli and an " *attractant* "as an oriented movement towards the source of stimuli. In the definition, Miller et al.,(2009) reinforced the assessment by Diether and coinvestigators, that these chemicals designated as attractants or repellents should act as odors. In the context of our study, we refer a repellent to a chemical that is causing a noncontact disengagement.

Here, we hypothesize that olfactory receptor neurons are involved in the mechanisms of pyrethrum and pyrethroid repellency in mosquitoes and agricultural pests. We report different approaches to testing this hypothesis and these include; modified arm in cage assays (referred to as hand in cage assay hence forth)(Boyle et al., 2016; Kain et al., 2013; Logan et al., 2010; Masetti & Maini, 2006; Syed and Leal, 2009). To test involvement of the olfactory receptors, *orco*$^{5/16}$ mutant mosquitoes were used in the behavioral experiments. Aside hand in cage assay, other behavioral assays used to test the hypothesis include T-maze, two choice and feeding preference assays. We therefore report, repellency effect of pyrethrum and pyrethroids (transfluthrin and permethrin) in different *Aedes* and *Anopheles* mosquito strains. We also discuss the repellency effect of insecticide treated nets (PermaNet 2.0 and Olyset). Pyrethrum and transfluthrin repellency on two agricultural pests *Plutella xylostella* and *Sitophillus zeamais* has also been reported.

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CHAPTER 2

REPELLENCY OF PYRETHRUM AGAINST *Aedes aegypti* AND *Anopheles gambiae* MOSQUITOES

Abstract

Many vector-borne diseases, including malaria and dengue, are transmitted by insect vectors, which cause devastating impacts on global human health and sustainability. One of the most effective measures to reduce disease transmission is to reduce human contacts with disease vectors through the use of repellents in lotions, aerosols, coils, emanators and other repellent devices. Pyrethrum extracts from the flower heads of Chrysanthemum (*Tanacetum cinerariifolium*) have been used as insect repellent against various biting arthropods for thousands of years and since 1902, pyrethrum has been incorporated as a key ingredient in commercial mosquito coils (Moore and Debboun, 2007). However, the mechanism of pyrethrum-mediated repellency remains unknown. This study reports repellency effect of pyrethrum when tested in *Aedes aegypti Waco* mosquitoes and *Anopheles gambiae* tested alongside DEET as a positive control. The magnitude of repellency elicited by pyrethrum was not significantly different from the one elicited by DEET in susceptible *Aedes* and *Anopheles* mosquitoes. However, repellency of pyrethrum was reduced in pyrethroid resistant PR strain. Experiments using *orco*$^{5/16}$ mosquitoes in Hand in cage assays revealed reduced repellency of pyrethrum in the highest concentration used, and in the low concentrations, repellency was abolished. These behavioral results were consistent with electroantenogram (EAG) recordings of *Ae.aegypti* where pyrethrum elicited robust EAG responses in Orlando mosquitoes and little EAG response in *orco*$^{5/16}$ mosquitoes

Introduction

Pyrethrum is a botanical insecticide from the extracts of dry flowers of *Tanacetum cinerariifolium (*also known as *Chrysanthemum cinerariifolium). T. cinerariifolium* is grown commercially in many parts of the world, particularly in East Africa and Australia, for extraction of pyrethrum, which accumulates in the flower achenes (Crombie, 1995; Greenhill, 2007). Pyrethrum is non-persistent in the environment and possesses low mammalian toxicity. Pyrethrum extract is a mixture of multiple chemical components with the pyrethrin (I and II) as the major component which are responsible for its insecticidal property. In addition to pyrethrins, other components, like sequiterpenes, flavonoids, triperpenols and sterols, are also found in pyrethrum extract (Casida and Quistad, 1995).

Pyrethrum, and its synthetic analogs, the pyrethroids, are mostly known for their insecticidal activity (Anadón et al., 2009). However, pyrethrum is also an insect repellent against various biting arthropods for thousands of years and since 1902, it has been incorporated as a key ingredient in commercial mosquito coils (Moore and Debboun, 2007). For example, its use in the form of a coil against *Anopheles gambiae* mosquitoes was reported more than five decades ago (Smith and Opudho, 1967). The burning of pyrethrum coils reduced the biting activity and caused mosquitoes to leave the experimental huts (Smith and Opudho, 1967). This study by Smith and Opudho (1967) concluded that pyrethrum coils may greatly reduce the risk of malaria transmission and highlighted the mechanism of repellency was not known.

The use of pyrethrum oil spray on flies attacking cattle has been reported to have repellent effect with variations depending on species of flies (Howell and Fenton., 1944). Until recent decades, the use of pyrethrum as a repellent in micro doses is still evident (Glynne-Jones, 2001; Hoek et al., 2003). Whether pyrethrum repellency is olfaction-based remains unknown.

Perception of volatile chemicals by insects begins when the volatiles enter the lymph of olfactory sensilla (in antennae and also maxillary palps) via tiny pores and activate olfactory receptor neurons (ORNs) (Carey and Carlson et al., 2012; Leal, 2013). Odorant receptors (ORs) are located on the dendritic surface of olfactory receptor neurons (ORNs) that are housed in olfactory sensilla (Joseph and Carlson, 2015). Individual ORNs of basiconic and trichoid sensilla each expresses a single member of the OR family, which confers a characteristic odorant response profile of that neuron (Silbering & Benton, 2010). Each OR is co-expressed with an obligate olfactory receptor co-receptor (*ORCO*) (Vosshall and Hansson, 2011), which does not bind odorants by itself, but is essential for odorant perception (Benton et al., 2006; Larsson et al., 2004). ORs and *Orco* form a complex functioning as a ligand-gated ion channel (Sato et al., 2008; Wicher et al., 2008; Smart et al., 2008).

For decades, DEET has been a widely used repellent to minimize mosquito bites on humans, and it is known to evoke close range repellency through the olfactory system (Bohbot et al., 2011; Debboun et al., 2006; Naters and Carlson, 2006; Pickett et al., 2009; Stanczyk et al., 2013; Stanczyk et al., 2010; Syed and Leal, 2009). *Orco* and the OR pathway are necessary for the olfactory effects of DEET on mosquitoes (DeGennaro et al., 2013). This study assessed repellency effects of pyrethrum in comparison with DEET repellency on an *Ae. aegypti Orco* mutant, and several pyrethroid-susceptible and pyrethroid resistant *Aedes aegypti* and *Anopheles gambiae* strains to evaluate the involvement of sodium channels and olfactory receptors in pyrethrum repellency.

Materials and methods

Mosquitoes

In this study, four *Aedes aegypti* mosquito strains were used; Waco, Puerto Rico (PR), Orlando and an *Orco* mutant (*orco*$^{5/16}$). Waco is an insecticide-susceptible laboratory strain kindly provided by Dr. Zhiyong Xi at Michigan State University. Orlando is a wild-type *Ae. aegypti* strain kindly provided by Leslie Vosshall (Rockefeller University). PR and two *orco* mutant lines (*orco*^{5} and *orco*^{16}) are from BEI Resources, NIAID, NIH. PR is a pyrethroid resistant strain possessing P450-mediated pyrethroid resistance and three kdr mutations (Reid et al., 2014 ; unpublished data from the Dong lab). The two *orco* lines were crossed to generate *orco*$^{5/16}$ mosquitoes for this study.

The colonies of *Ae .aegypti* were reared at 27°C, at least 60% humidity, at12h:12h light: dark photoperiod in growth chambers. Larvae were fed with liver powder and adults with 10% sucrose solution throughout the rearing period. Fifty females (4-10 days old and mated) were used for behavioral experiments. Twenty four hours prior to the experiments, the mosquitoes were isolated into a clean cage and were given water only. Six hours before the experiments, the water was removed. Adults of *An. gambiae*, Kisumu strain were reared at Malaria Alert Center (MAC) insectary at University of Malawi, College of Medicine (COM). They were reared on 10% sucrose solution and larvae on fish food. Colonies were maintained in growth chambers at approximately 28^{0}C and 70% relative humidity.

Hand in cage behavior assay setup

i. Test arena

Behavioral assays were mostly carried out in summer. The temperature in the testing room ranged from 25-28^{0}C and humidity 40-70%. In colder weather, the room was conditioned by raising the temperature in the heating system and a humidifier was used to raise the humidity to at least 30%, making sure mosquitoes were active throughout the assay. The room was ventilated using a box fan to remove any background odors prior to the experiments. Test cages (30cmX30cmX30cm from Bio-quip) were cleaned in water with an odorless detergent and dried using a box fan. One to two hours prior to the experiment, the cages were cleaned again with 99% ethanol using cotton wool. The cages were left to dry for another 30 minutes and a paper towel fitting the bottom of the cage was lined and held in place using odorless tape.

ii. The glove

The study adopted Hand in cage assay from Boyle et al., (2016) with slight modifications. A 5.8 cm by 5 cm window was made on an Ansell^{Tm} sol-vex glove. To hold the window open, a magnet frame was glued to the glove. A piece of polyester mesh treated with test compounds was carefully cut and placed on top of the window (Fig 2.1A). On top of the treated mesh, five more magnet frames were stacked. An untreated mesh was place in between the fourth and the fifth magnet frames, which prevented the mosquitoes from getting in contact with the treated mesh (Fig 2.1 B-D).

iii. Test compounds

Pyrethrum from Sigma (Cat# N13151, 30.0% pyrethrin I and 19.9% pyrethrin II) was used. Pyrethrum was diluted volume by volume using acetone as a carrier solvent. A range of dilutions from 10^{-20} to 10^{-2} were tested. The range varied by the mosquito species to be tested. In this study, *Anopheles gambiae*, Kisumu strain was very sensitive to insecticides as such lower concentrations were used. Knockdown was not observed in *Aedes* strains, thereby; slightly higher concentrations were used to establish a dose dependent response.

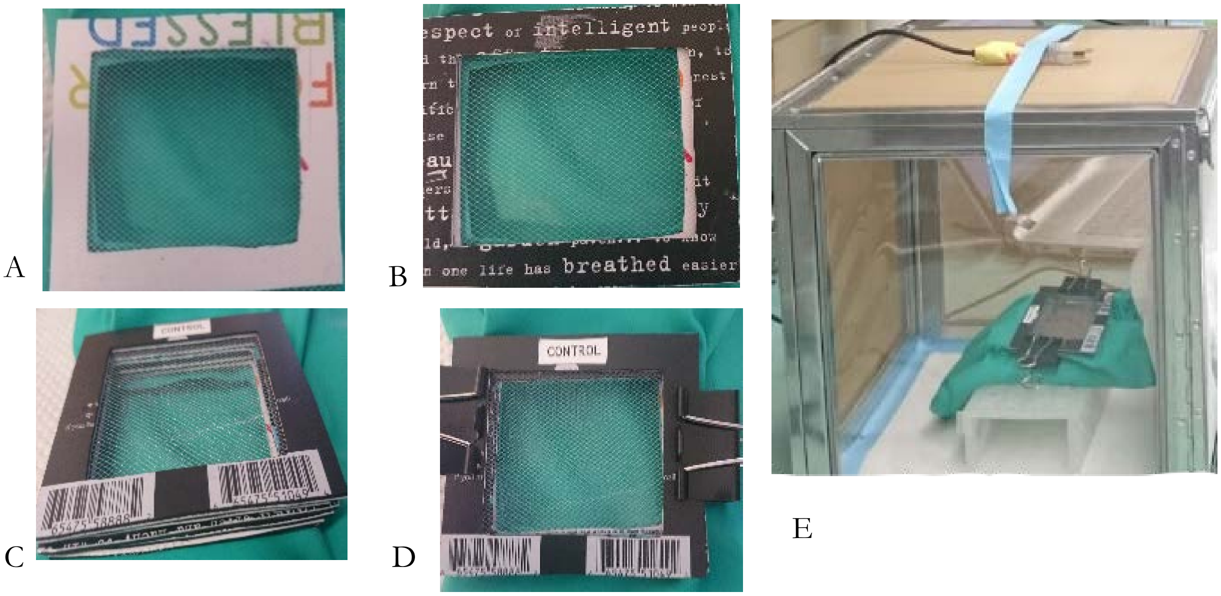

Figure 2.1 Hand in cage assay setup. (A-D) Assemblage of the glove to be used in the Hand in cage assay. (A) First layer of magnet frame that is glued to the glove, as a window frame and a first layer of mesh, which is usually treated with test compounds. It is placed on the top of the secured magnet frame. **(**B) Another magnet frame that holds the treated mesh in place (C) Extra stacks of four magnet frames and a second layer of mesh (untreated) between the fourth and the fifth magnet frames. (D) Complete set up of the glove, with clips that hold the stacked magnet frames and the treated and untreated mesh to the glove and (E) the complete setup of the Hand in cage assay. The camera on the top of the cage records landing activity of the mosquitoes during an arm visit.

Experimental design, data acquisition and analysis

i. Experimental design

Power analysis is important in determining the statistical power of an experiment. In this study, post-hoc power analysis was conducted using preliminary data in SAS 9.3 software and at least 3 replicates gave a power of more than 60%. To increase the power, most of the experiments were repeated at least 5 times, per compound per strain. Cohorts of 50 nulliparous mated and starved females were used as an experimental unit in one cage.

ii. Data acquisition

The assembled test glove worn was inserted into the cage (referred to as arm visit hence forth) for 5 minutes (Fig 2.1 E). For each arm visit, number of mosquitoes landing on the arm was counted starting from the second minute. The experiments recorded a cumulative number of mosquitoes that landed on the treatment window at time points 2, 3, 4, 5 minutes were recorded for each of the treatments for each replicate. Percent repellency for each treatment was calculated as;

$$Percent\ repellency = 1 - \frac{mean\ cumulative\ number\ of\ mosquitoes\ on\ a\ treated\ arm\ visit}{mean\ cumulative\ number\ of\ mosquitoes\ on\ untreated\ arm\ visit}\ X\ 100$$

Each test arena had 4 arm visits including the control arm. Mosquitoes were rested for at least 30 minutes before another concentration was tested. Each test was repeated for 5 times with different naïve mosquito cohorts. One cohort was used for each experiment and discarded after use. Experiments were completely randomized by day.

iii. Data analysis

Analysis of variance in SAS software (version 9.3) was used to compare mean percentage repellency. The repellency was compared within and between compounds and strains. A full model was used to establish the differences in the repellency of the mosquitoes on the treated window: ***model*** *y=u+compound+ dose +compound*dose+e* where ***y*** was the response: Percent repellency, ***u*** was overall mean, ***compound*** was an effect due to compound, dose was an effect due to ***dose*** and ***compound*dose***, was an interaction effect of dose and compound. Where necessary, separation of means was done using the Bonferroni posttests with alpha equal to 0.05.

Results

Repellency of pyrethrum and DEET on insecticide-susceptible Aedes and Anopheles mosquitoes

Hand in cage assay results revealed a significant repellent effect of pyrethrum when tested on *Aedes aegypti Waco* mosquitoes. The repellency increased with increasing concentrations of pyrethrum (Fig.2.2). Repellency was also observed from *Anopheles* mosquitoes, but at much low concentrations (Fig.2.2). For comparison, DEET was tested on Waco and *Anopheles* mosquitoes as a positive control. A comparison between pyrethrum and DEET repellency on *Aedes* mosquitoes revealed no significant differences (Fig. 2.2). Similar results were observed when repellency of pyrethrum and DEET was compared on *Anopheles* mosquitoes (Fig. 2.2). When concentration effects of pyrethrum and DEET repellency were compared on *Aedes* and *Anopheles* mosquitoes; at each of the dilutions used, no significant differences were observed. Statistical comparisons of repellency effect between the two compounds when tested on *Anopheles* and *Aedes* mosquitoes are shown in table 2.1.

Pyrethrum repellency is reduced in pyrethroid-resistant *Aedes* mosquitoes

Further investigation using resistant Puerto Rico (PR) strain revealed that DEET was a more potent repellent on the resistant mosquitoes than pyrethrum. Statistical results revealed compound effect on the Puerto Rico strain, with high mean repellency due to DEET ($p<0.0001$). Detailed comparisons of the test compounds by dose are shown in Table 2.1, Fig 2.3

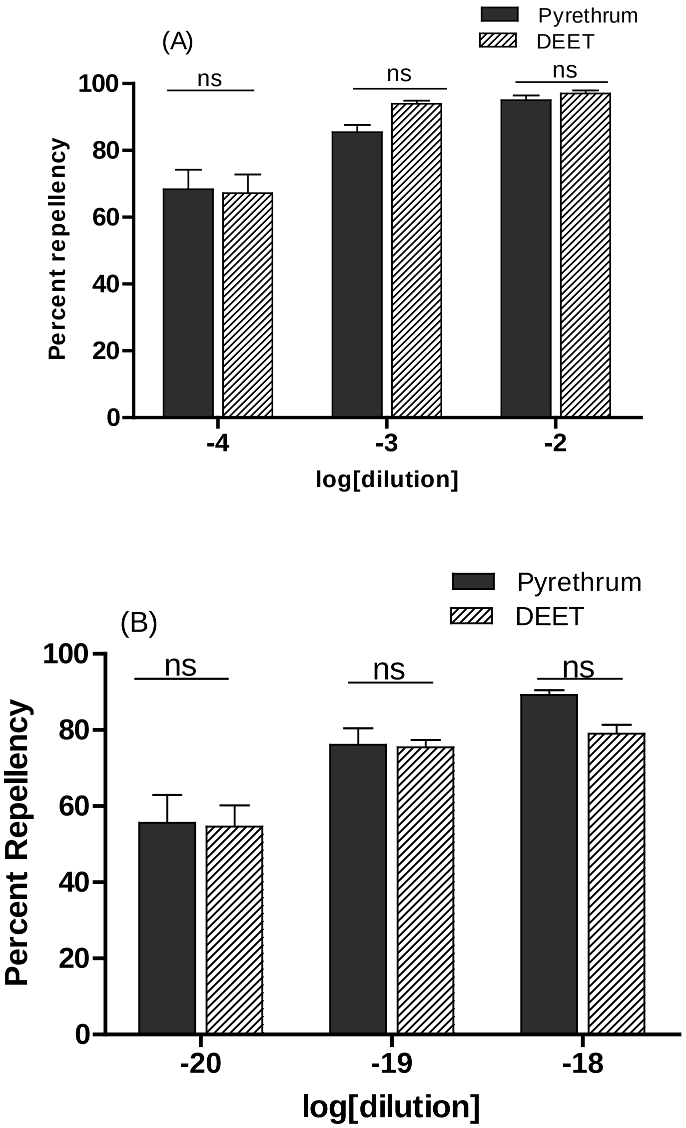

Figure 2.2 No difference in the repellency effects of pyrethrum and DEET. (A) Repellency of pyrethrum compared to DEET in *Ae.aegypti* Waco mosquitoes when tested at three different concentrations in Hand in cage assay. (B) Repellency of pyrethrum compared to DEET in *Anopheles gambiae* Kisumu strain tested using Hand in cage assay at three different concentrations (Note Kisumu was tested at very low concentrations because it was too sensitive to pyrethrum and pyrethroids. Higher concentrations caused a knockdown effect). Females were exclusively tested. Data analyzed using ANOVA. Bonferroni posttests (α=0.05); ns=not significantly different, * =P<0.05, ** = P<0.01, *** = P<0.001.

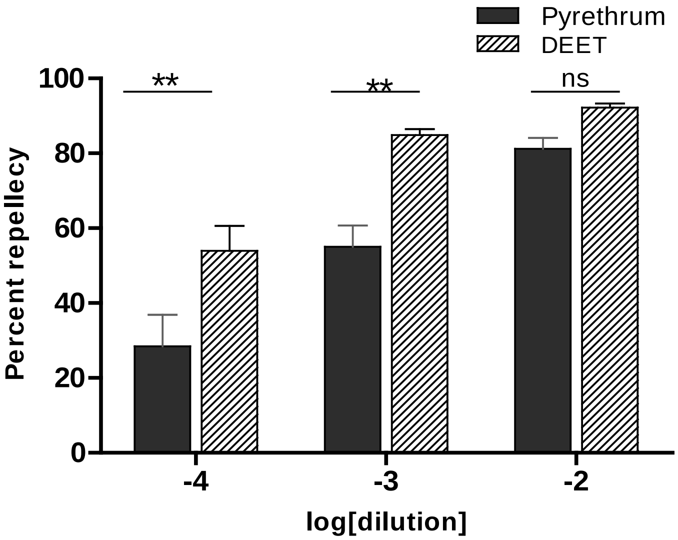

Figure 2.3 Difference in the repellency effects of pyrethrum and DEET on PuertoRico *Ae.aegypti* mosquitoes. The Peurtorico(PR) strain, has two pyrethroid resistance mechanism (P450-mediated and kdr mediated). Hand in cage assay was used to test the repellency effect at three different concentrations. Females were exclusively tested. Data analyzed using Two way ANOVA, Bonferroni posttests (α=0.05); ns=not significantly different, * =P<0.05, ** = P<0.01, *** = P<0.001.

Table 2.1 Repellency effect of pyrethrum compared to DEET between strains of *Aedes aegypti*[1] and *Anopheles gambiae* (Kisumu) [1].

Dose	**Pyrethrum Waco[2] (Mean repellency)**	**DEET Waco[3] (Mean repellency)**	**Difference**	**t-value**	**P value**
-4	68.35	67.22	-1.130	0.2290	P > 0.05
-3	85.49	93.99	8.500	1.723	P > 0.05
-2	95.05	97.05	2.004	0.4061	P > 0.05
Dose	**Pyrethrum PR[4] (Mean repellency)**	**DEET PR[5] (Mean repellency)**	**Difference**	**t-value**	**P value**
-4	44.19	54.00	9.802	1.559	P > 0.05
-3	56.71	84.88	28.17	4.479	P<0.001
-2	74.90	92.18	17.28	2.747	P < 0.05
Dose	**Pyrethrum Kisumu [6] (Mean repellency)**	**DEET Kisumu[7] (Mean repellency)**	**Difference**	**t-value**	**P value**
-20	54.60	55.64	1.038	0.1594	>0.05
-19	76.48	76.10	0.625	0.0960	>0.05
-18	79.00	89.20	10.20	1.567	>0.05

[1] 4-10 days old, nulliparous female's sugar starved

[2] Pyrethrum Waco = *Aedes aegypti*; Waco strain exposed to pyrethrum

[3] DEET Waco= *Aedes aegypti*; Waco strain exposed to DEET

[4] Pyrethrum PR= *Aedes aegypti*; Puerto-Rico strain exposed to Pyrethrum

[5] DEET PR= *Aedes aegypti*; Puerto-Rico strain exposed to DEET

[6] Pyrethrum Kisumu= *Anopheles gambiae*; Kisumu strain exposed to pyrethrum

[7] DEETKisumu= *Anopheles gambiae*; Kisumu strain exposed to pyrethrum

Repellency effect of pyrethrum is *Orco*-dependent

An earlier study has reported lack of DEET repellency in anosmic *orco*$^{5/16}$ mosquitoes where the *Orco* gene was mutated (Degennaro et al., 2013), confirming that DEET repellency is mediated by the OR pathway. To determine whether pyrethrum repellency is mediated by the OR pathway, we next examined the behavioral response of *orco*$^{5/16}$ mosquitoes to pyrethrum in the Hand in cage assay. At the low concentrations, pyrethrum repellency was abolished in *orco*$^{5/16}$ mosquitoes and a low level of repellency was detected at 10^{-2} (Fig. 2.5), whereas both compounds elicited robust repellency in wild-type Orlando mosquitoes, from which the *orco* mutants were generated (Fig. 2.5). These results indicate that pyrethrum repellency is *Orco*-dependent.

Dr. Feng Liu, a postdoc in the Dong lab, conducted electroantennograph (EAG) recording of antenna of *Ae. aegypti* in response to pyrethrum. Pyrethrum elicited robust EAG responses in Orlando mosquitoes indicating that mosquitoes can sense the pyrethrum vapor and also suggesting that pyrethrum effectively activate olfactory receptor neurons. Consistent with the behavioral results, little EAG response was detected in the antenna of *orco*$^{5/16}$ mosquitoes.

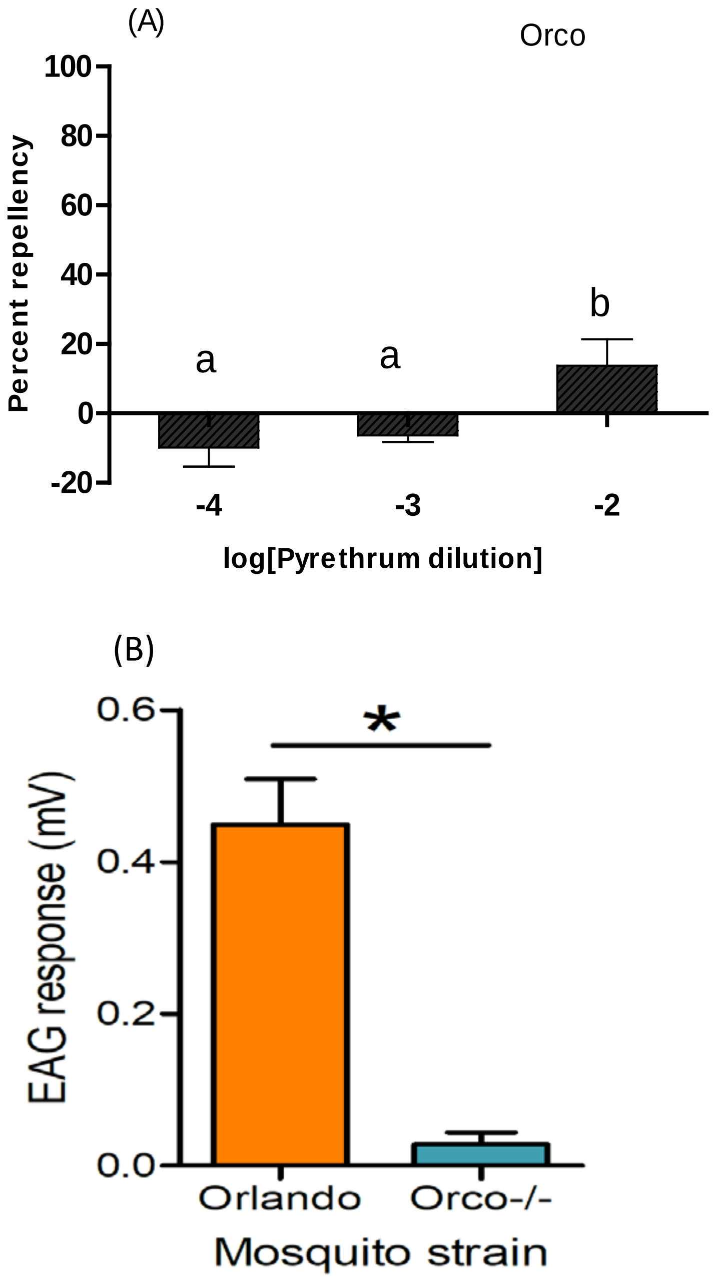

Figure 2.4 Reduced repellency effect of pyrethrum in *Aedes aegypti orco*$^{5/16}$ mosquitoes. (A) Showing repellency effect of pyrethrum in *orco*$^{5/16}$ mosquitoes tested at three different concentrations in Hand in cage assay. Repellency was abolished in the low concentrations slightly maintained in the high concentration. Data analyzed using ANOVA, Bonferroni posttests (α=0.05). Means followed by the same letter not significantly different from each other. (B) Showing Robust EAG response in Orlando strain and reduced response in *orco*$^{5/16}$ mosquitoes. * =P<0.05, Students T-tests (α=0.05) *(EAG recordings done by Feng Liu).*

Discussion

It is well-established that pyrethrum and pyrethroids target sodium channels for their insecticidal activity (Corbel et al., 2004a; Dong et al., 2014; Du et al., 2011; Du et al., 2006; Li et al., 2012). Pyrethrum and pyrethroids disrupt sodium channel function by prolonging sodium channel opening, increasing sodium ions influx resulting in overstimulation of the insect nervous system which eventually leads to death. Although it has been well documented that pyrethrum repels mosquitoes and other insect pests, the mechanism of pyrethrum repellency has not been well understood. The findings from this study established pyrethrum elicits repellency in *Ae. aegypti* and *An. gambiae* and indicated that pyrethrum repellency is olfaction-based.

DEET is a well-known repellent which has been used for more than five decades (Stanczyk et al., 2010; Vinauger et al., 2014) in many parts of the world. In this study, our results revealed that repellencies by DEET and pyrethrum are comparable denoting that pyrethrum is a potent repellent. Our study on pyrethroid susceptible *Anopheles gambiae* mosquitoes of the Kisumu strain confirmed repellency, indicating that pyrethrum as a repellent is not limited to one *Aedes* mosquito species. These results highlighted the need to explore the use of pyrethrum as a repellent to control mosquito bites. Pyrethrum has been cultivated in Africa, such as Kenya, and other parts of the world, implying access to crude extracts in the places where it is grown, can make a difference in reducing mosquito bites.

The instability of insecticidal activity of pyrethrum under heat and light conditions have been documented (Glynne-Jones, 2001). It would be interesting to discover the length of time at which its repellency can last if it is to be used outdoors as a repellent. In places such as Africa, where pyrethrum is grown and mosquito borne diseases are endemic, pyrethrum crude extracts may be easily accessible. Perhaps, most urgent studies should focus on how pyrethrum could be used as a repellent in semi field and field studies.

While results in this study point towards pyrethrum being an efficient repellent, some important questions on its residual efficacy as a repellent need to be answered. Although pyrethrum might be readily available as a crude extract, since it is grown in some parts of Africa such as Kenya(Wandahwa et al., 1996), how its long-term use as a repellent might impact entomological parameters in semi field and field trials needs to be investigated further. Decreased repellency of pyrethrum compared to DEET when tested on pyrethroid resistant PR, indicate that pyrethrum potency as a repellent, is more effective against pyrethroid-susceptible mosquitoes. Knowledge on the resistance status of mosquito populations in the areas where pyrethrum is to be used as a repellent is vital. It should also be emphasized that not all mosquito species may respond the same way to pyrethrum repellency. More studies on other mosquito species, to compare the minimum doses of pyrethrum as a repellent are vital.

The current study observed a difference in the repellency due to pyrethrum when compared between two vector species, *Anopheles gambiae* and *Aedes aegypti* mosquitoes. While the differences in the sensitivity of the two vector species may be attributed to other factors such as behavioral plasticity, significant differences between species responses to pyrethrin based repellents has also been reported. A study by Sathantriphop et al. (2014) reported differences in the repellency effect between vector species of *Cx.quinquefasciatus,* and *An.minimus* which exhibited a stronger behavioral response to pyrethroids as well as essential oils compared to *Ae.abopictus* and *Ae.aegypti* species.

Reduced repellency in the *orco* mutants highlighted the importance of olfactory receptors in pyrethrum repellency, like DEET repellency (Degennaro et al., 2013). However, detection of pyrethrum repellency in *orco* mutants at the high concentration suggested that the neurotoxic effects by pyrethrum on sodium channels could also evoke repellency. Reduced pyrethrum repellency in pyrethroid resistant mosquitoes further supports the involvement of sodium channels in pyrethrum repellency. Potential synergistic effects between these two mechanisms are worth further investigation in future studies and should benefit the development of novel strategies in mosquito control, which will help reduce the risk of disease transmission.

Conclusion

Pyrethrum targets voltage-gated sodium channels which are critical for electrical signaling in the nervous system by prolonging the opening of sodium channels resulting in over-excitation the insect central nervous system (Dong et al, 2014). Here we found that pyrethrum activates olfactory receptor neurons in mosquito antennae and evokes repellency in both *Aedes* and *Anopheles* mosquitoes. The comparable repellencies observed between pyrethrum and DEET in this study depicts pyrethrum as a potent repellent. Furthermore, we showed that pyrethrum repellency was reduced in *Orco* mutants and pyrethroid resistant mosquitoes, suggesting that pyrethrum repellency is olfaction-based and further enhanced by activation of sodium channels. Although pyrethrum might be readily available as a crude extract, since it is grown in some parts of Africa such as Kenya (Wandahwa et al., 1996), how its long-term use as a repellent might impact entomological parameters in semi field and field trials remains to be investigated.

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CHAPTER 3

TOXICODYNAMICS OF TRANSFLUTHRIN ON *Aedes aegypti* MOSQUITOES

Abstract

Pyrethroid insecticides act on voltage-gated sodium channels for their insecticidal activity, and have played a critical role for effective control of insect pests and disease vectors. Besides their insecticidal activities, pyrethroids possess repellency against mosquitoes and other insects and have been widely used in bed nets, coils, emanators and vaporizer mats to combat mosquitoes. More recently, volatile pyrethroids, such as transfluthrin (TF), received more attention and exhibit great potential for global mosquito control (Govella et al., 2015; Ogoma et al., 2012, 2017). However, details on the mechanism of action of transfluthrin are still elusive. This study reports olfaction-based repellency of transfluthrin in *Aedes aegypti* and *Anopheles gambiae* mosquitoes. The hand in cage assay was conducted to evaluate the action of TF on pyrethroid-susceptible and resistant mosquitoes. The main findings are 1) At lower concentrations, TF vapor elicited repellency in *Ae aegypti* and *An gambiae* ; 2) At higher concentrations, TF vapor induced knockdown (paralysis) of pyrethroid-susceptible mosquitoes, but not of pyrethroid-resistant mosquitoes carrying kdr mutations; 3) TF repellency was reduced in pyrethroid-resistant mosquitoes carrying kdr mutations; 4) Pretreatment of piperonyl butoxide (PBO), an inhibitor of P450s, enhanced TF repellency in both susceptible and resistant mosquitoes; 5) TF repellency was significantly reduced in anosmic Orco mosquitoes. These findings demonstrated the complex toxicodynamics of transfluthrin action on the mosquito nervous system: at high concentrations TF induces knockdown by targeting sodium channels, but induces repellency likely by activation of both olfactory receptors and sodium channels at sublethal concentrations.

Introduction

Pyrethroids have been highly featured in control of mosquito borne diseases, either as aerosol sprays, indoor residual prays (IRS) and mosquito coils as repellents. It is well-established that pyrethroid insecticides act on voltage-gated sodium channels for their insecticidal activity. The success of pyrethroids in insect control, however, has been greatly affected by the development of pyrethroid resistance. Different mechanisms of resistance development, including target site resistance, kdr, and P450-mediated resistance have been reported in several studies (Feyereisen, 1995; Hemingway et al., 2004; Liu, 2012; Reid et al., 2014; Salgado et al., 1983). A number of studies have reported the implications of pyrethroid resistance in control of insect vectors. (Matowo et al., 2014; Nardini et al., 2012; Strode et al., 2014)

In transmission dynamics, the biting rate of a mosquito is key to disease transmission among other important factors. For vectors, such as mosquitoes to locate a host for a blood meal, cues such as carbon dioxide and heat are important (Dekker and Carde,2011; Lacey and Cardé, 2012;Webster et al., 2015). Although pyrethroids have been traditionally understood as contact insecticides, in recent years, volatile pyrethroids, such as transfluthrin and metofluthrin, are main active ingredients of widely used insect repellent products including mosquito coils, emanators and vaporizer mats. The repellency of pyrethroids is now being evaluated for human disease vector control and has the potential to become an important component of future malaria control programs in Africa (Hill et al., 2014; Kawada et al., 2005; Ogoma et al., 2014; Ogoma et al., 2012;2017; Reddy et al., 2011; Sugano and Ishiwatari, 2011). For example, TF has been reported to have a 90% protective efficacy when impregnated in hessian sacks over a period of 6 months in a semifield trial (Ogoma et al., 2012). Reduction of outdoor bites with a 99% for Anopheles and 92% for Culex was evident when transfluthrin was used outdoors (Govella et al., 2015). A recent behavioral study reported reduced pyrethroid repellency in pyrethroid resistant *Aedes* mosquitoes carrying kdr mutations (Wagman et al., 2015). However, the mechanism(s) underlying the repellency elicited by these compounds is largely unknown.

In this study, we showed that transfluthrin evoked olfactory responses from the antennae of *Ae. aegypti* mosquitoes and elicited repellency. We further evaluated TF repellency using two types of mutant mosquitoes: orco mutants and kdr mosquitoes. Our study established unique dual actions of TF on ORs and sodium channels as the underlying mechanism of TF repellency. We also showed that pretreatment of a P450 inhibitor piperonyl butoxide (PBO) enhanced both repellency and vapor toxicity of TF. Our study established a new paradigm for the understanding of the modes of action of volatile pyrethroids in mosquito control.

Materials and Methods

Mosquitoes

In this study, six *Aedes aegypti* mosquito strains were used; Rockefeller (Rock), Isokdr, Waco, Puerto Rico (PR), Orlando and an *Orco* mutant (*orco*$^{5/16}$). Rock and Isokdr were provided by Jeff Scott's laboratory at Cornell University. Isokdr is highly resistant to pyrethroids possessing two kdr mutations in the sodium channel and Rock is pyrethroid-susceptible; and they are isogenic (Smith et al., 2018). Waco is an insecticide-susceptible laboratory strain kindly provided by Dr. Zhiyong Xi at Michigan State University. Orlando is a wild-type *Ae. aegypti* strain kindly provided by Leslie Vosshall (Rockefeller University). PR and two *orco* mutant lines (*orco*^{5} and *orco*^{16}) are from BEI Resources, NIAID, NIH. PR is a pyrethroid resistant strain possessing P450-mediated pyrethroid resistance and three kdr mutations (Reid et al., 2014 ; unpublished data from the Dong lab). The two *orco* lines were crossed to generate *orco*$^{5/16}$ mosquitoes for this study.

The colonies of *Ae .aegypti* were reared at 27°C, at least 60% humidity, at12h:12h light: dark photoperiod in growth chambers. Larvae were fed with liver powder and adults with 10% sucrose solution throughout the rearing period. Fifty females (4-10 days old and mated) were used for behavioral experiments. Twenty four hours prior to the experiments, the mosquitoes were isolated into a clean cage and were given water only. Six hours before the experiments, the water was removed. Adults of *An. gambiae* , Kisumu strain were reared at Malaria Alert Center (MAC) insectary at University of Malawi, College of Medicine (COM). They were reared on 10% sucrose solution and larvae on fish food. Colonies were maintained in growth chambers at approximately 28^0C and 70% relative humidity.

Test compound and arm visits

Transfluthrin (95% purity) kindly provided by Dr. Kamal Chauhan (USDA), was used in this study. The compound was prepared volume by volume (v/v) with acetone as a carrier solvent. In a glass petri dish, 450μl was applied to white rectangular polyester netting on the treatment mesh (see assembled glove design, Fig 2.1). Carrier solvent without the test compound was tested first, and the rest of the concentrations were tested from lowest to the highest concentration to avoid contamination. A range of concentrations from 10^{-8} to 10^{-2} was used for repellency assays using transfluthrin on PR and Waco. The assembled test glove worn was inserted into the arena (referred to as arm visit hence forth) for 5 minutes. Landing response of mosquitoes was recorded for 5 minutes. Each test arena had 4 arm visits including the control arm. Mosquitoes were rested for at least 30minutes before another concentration was tested. Each test was repeated for 5 times with different naïve mosquito cohorts. One cohort was used for each experiment and discarded after use. Experiments were completely randomized by day.

Hand in cage assay with cytochrome P450 inhibited mosquitoes using PBO

Following slightly modified methods by (Reid et al., 2014) a nonlethal dose of 1ug was applied on the dorsal side of the thorax of the mosquitoes. Very minimum chilling was used in the mosquito preparation to avoid altering the behavior of the mosquitoes before the behavioral assay was conducted. Very active, ready to bite female mosquitoes starved on water were gently aspirated from the holding cage and chilled for 1 minute and then transferred to a 4-degree Celsius glass petri dish for immobilization and 1ug of PBO was applied using a Hamilton syringe. Mosquitoes were treated in batches of 10 for efficient treatment and to reduce the cold treatment time. At least 50 PBO treated mosquitoes were transferred into a behavioral assay arena (a 30x30x30 bio quip metal cage) and the cage was transferred into a behavioral experiment room, with at least 30% relative humidity and temperatures of 28 degrees Celsius. The mosquitoes were left in the behavioral test room for an hour to let the PBO to take effect as well as for the mosquitoes to acclimatize. The mosquitoes were then tested in hand in cage assay using transfluthrin and landing rates which were later transformed to percent repellency as previously illustrated in (Equation 1, chapter 2) were recorded. The procedure was repeated 5 times with different cohorts of mosquitoes.

Hand in cage knockdown assay

Using hand in cage assay, mosquitoes were exposed to higher concentrations of transfluthrin to observe the knockdown effect. This assay used dilutions of 10^{-5} to 10^{-3}, and recorded the number of mosquitoes knocked down over a period of 60 minutes. After 60 minutes the mosquitoes were provided with 10% sucrose and mortality was recorded after 24 hours. A similar procedure was followed with mosquitoes that were pretreated with PBO.

Results

Transfluthrin (TF) repellency in susceptible mosquito strains

To evaluate whether mosquitoes perceive TF through the olfactory system, we first conducted electroantennagram (EAG) recordings from the antennae of female *Aedes* mosquitoes. EAG signals were detected in response to TF vapor (Fig.3.1A), indicating that *Aedes* mosquitoes can sense the vapor of TF. We then conducted the hand in cage assays using susceptible *Aedes* mosquitoes, which revealed transfluthrin (TF) elicited repellency effect in Waco and Rock mosquitoes (Fig. 3.1 A-B). For both mosquito strains, the repellency of TF increased with increasing concentration ($p<0.001$). To assess whether TF repellency is also in other mosquitoes, the experiments were repeated using *Anopheles gambiae* mosquitoes of the Kisumu strain. Repellency of TF in Kisumu mosquitoes was observed at the concentration as low as 10^{-20} (Fig.3.2). Unlike in *Aedes* mosquitoes where an overt dose response curve was observed, in *Anopheles* mosquitoes, the dose response curve plateaued at a low concentration (10^{-19}).

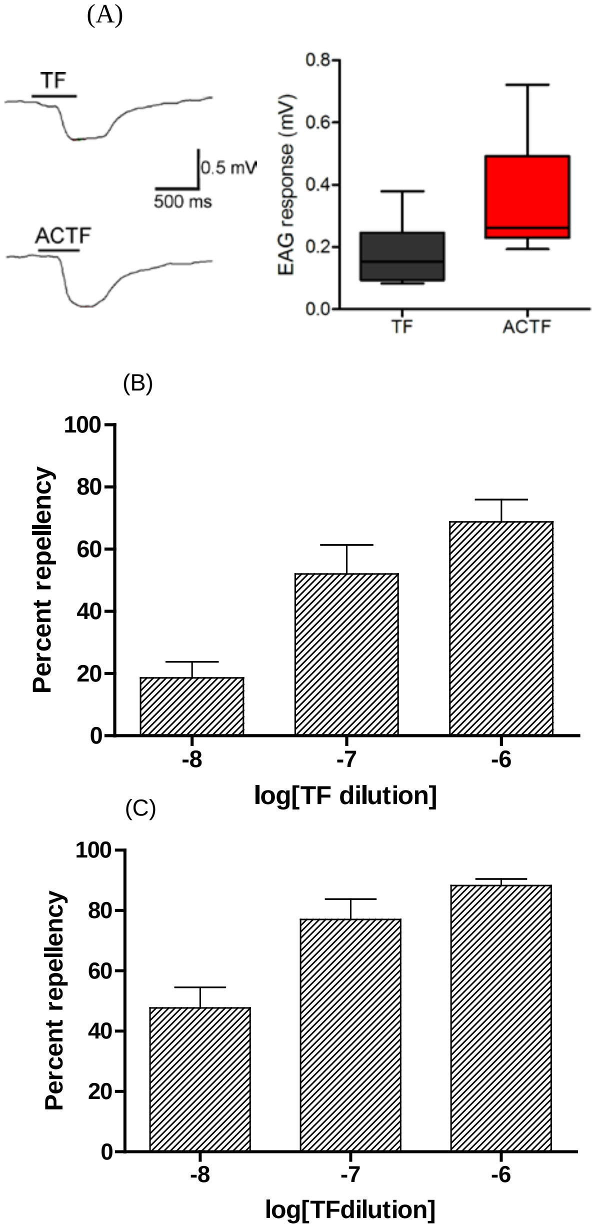

Figure 3.1 Transfluthrin repel susceptible *Aedes* mosquitoes. (A) Showing EAG signal from female mosquito antenna using TF and ACTF (Acetransflutrin), a TF-like structure shown in chapter 1 (Fig. 1.1). (B) Showing repellency of TF in Waco in tested in hand in cage assay at three different concentrations (C) Showing repellency of TF in Rock strain tested in hand in cage assay at three different concentrations. Female mosquitoes (4-8 days) tested exclusively *(EAG recordings done by Feng Liu)*.

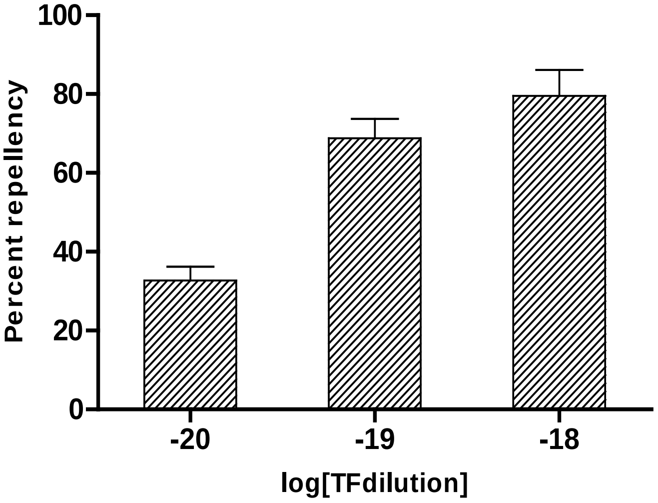

Figure 3.2 Transfluthrin repel susceptible *Anopheles gambiae,* Kisumu strain mosquitoes. Female mosquitoes (4-6 days old) tested exclusively in Hand in cage assay at three different concentrations. For *Anopheles gambiae*, TF was tested at very low concentrations because of its high sensitivity to pyrethroids.

Reduced transfluthrin repellency in resistant *Aedes* strains

In the hand in cage assay, we observed that the landing of pyrethroid resistant PR and Isokdr mosquitoes when the second mesh close to the hand was not treated with any chemicals, was not different from those of the two susceptible strains (Waco and Rock) indicating that the kdr mutations did not alter host-finding behavior. TF repellency was significantly reduced in both PR and Isokdr mosquitoes at all three concentration tested (Fig. 3.3). Hand in cage experiments using DEET, revealed no significant differences between the susceptible and resistance strains when compared at all the concentrations used (Fig. 3.4, Table 3.1).

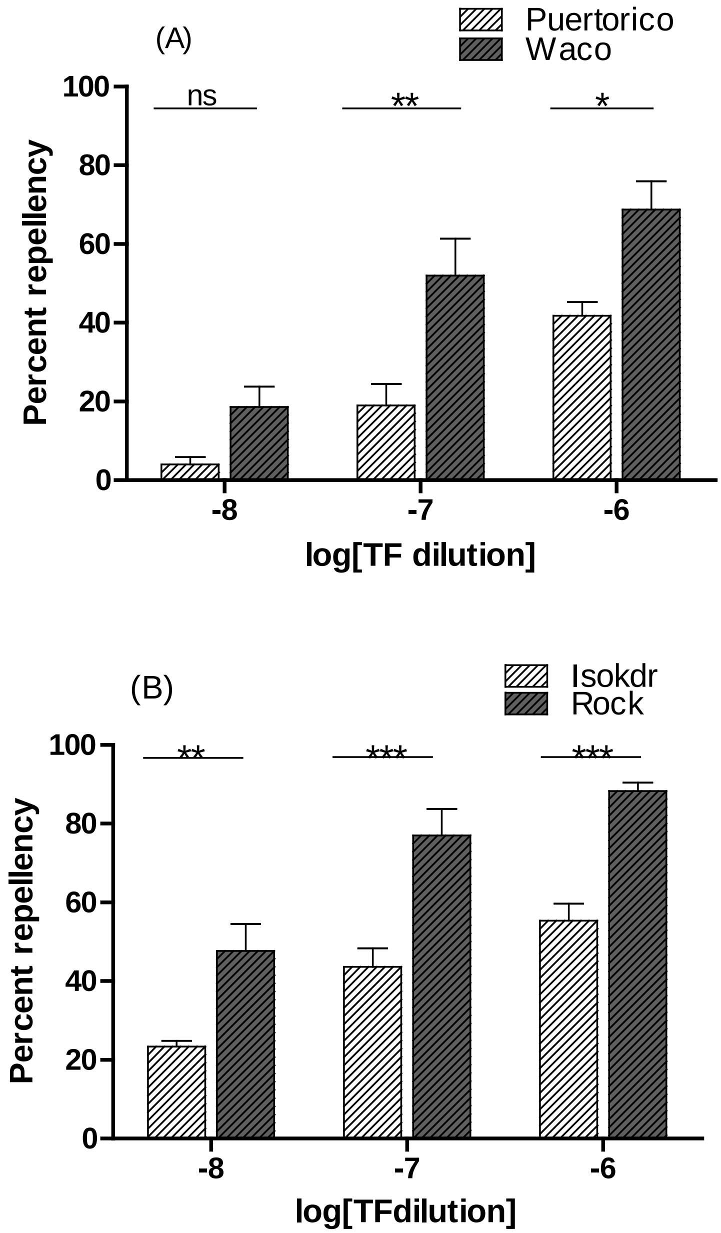

Figure 3.3 Transfluthrin repellency is reduced in resistant mosquito strains. (A) Showing repellency effect of TF in PeurtoRico (PR) and Waco tested in Hand in cage assay at three different concentrations (B) Showing repellency effect of TF, in Isokdr and Rock mosquitoes tested in Hand in cage assay. Data analyzed using two way ANOVA. Bonferroni posttests (α=0.05) ; ns=not significantly different, * =P<0.05, ** = P<0.01, *** = P<0.001.

Table 3.1 Repellency effect of TF compared between strains of *Aedes aegypti*[1] mosquitoes before and after PBO pre-treatment.

Dose	PRTF[2] (Mean repellency)	Waco TF[3] (Mean repellency)	Difference	t-value	P value[5]
-8	4.0	18.6	-14.6	-1.73	P>0.05
-7	19.0	52.0	-33.0	-3.92	P<0.001
-6	41.8	68.8	-27.0	-3.21	P<0.001
Dose	**Isokdr[4] (Mean repellency)**	**Rock[4] (Mean Repellency)**	**Difference**	**t-value**	**P value**
-8	23.40	47.68	24.28	3.554	P<0.01
-7	43.60	77.07	33.47	4.898	P<0.001
-6	55.40	88.32	32.92	4.819	P<0.001

[1] 4-10 days old, nulliparous female's sugar starved

[2] PRTF= PR strain exposed to transfluthrin, without PBO pretreatment

[3] WacoTF=Waco strain exposed to transfluthrin, without PBO pretreatment

[4] TF+PBO= Mosquitoes exposed to transfluthrin after PBO pretreatment

[5] Analysis of Variance(ANOVA) Bonferroni posttests;* =P<0.05,**= P<0.01, *** = P<0.001

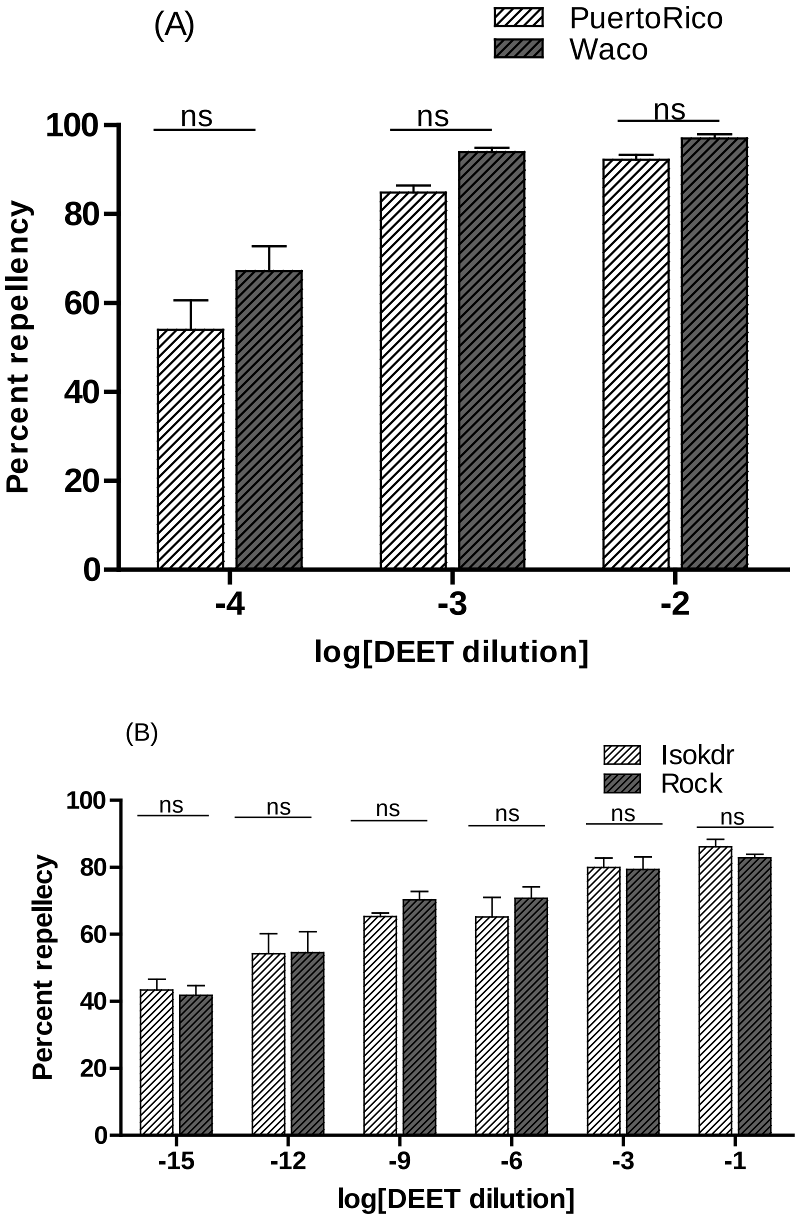

Figure 3.4 Repellency of DEET is not reduced in pyrethroid resistant mosquito strains. (A) Showing repellency effect of DEET on PeurtoRico and Waco tested in Hand in cage assay at three different concentrations (B) Showing repellency effect of DEET, in Isokdr and Rock mosquitoes tested in Hand in cage assay at six different concentrations. Data analyzed using ANOVA. Bonferroni posttests (α=0.05); ns=not significantly different, * =P<0.05, ** = P<0.01, *** = P<0.001.

Table 3.2 Repellency effect of DEET compared between strains of *Aedes aegypti*[1] mosquitoes before and after PBO pre-treatment

Dose	**Rock DEET [2] (Mean repellency)**	**Isokdr DEET[3] (Mean repellency)**	**Difference**	**t-value**	**P value[6]**
-15	43.39	41.79	-1.600	0.2947	P > 0.05
-12	54.18	54.58	0.4045	0.07451	P > 0.05
-9	65.31	70.27	4.961	0.9137	P > 0.05
-6	65.18	70.77	5.592	1.030	P > 0.05
-3	79.94	79.40	-0.5375	0.09901	P > 0.05
-1	86.13	82.89	-3.239	0.5966	P > 0.05

Dose	**Waco DEET [4] (Mean repellency)**	**PR DEET[5] (Mean repellency)**	**Difference**	**t-value**	**P value**
-4	54.00	67.22	13.22	2.568	P > 0.05
-3	84.88	93.99	9.112	1.770	P > 0.05
-2	92.18	97.05	4.874	0.9468	P > 0.05

[1] 4-10 days old, nulliparous female's sugar starved

[2] Rock DEET= Rock strain exposed to DEET

[3] Isokdr DEET= Isokdr strain exposed to DEET

[4] Waco DEET= Waco strain exposed to DEET

[5] PR DEET= PR strain exposed to DEET

[6] Analysis of Variance(ANOVA) Bonferroni posttests;* =P<0.05,**= P<0.01, *** = P<0.001

Repellency effect of transfluthrin is Orco-dependent

To determine whether the OR pathway is involved in the olfactory response to TF in *Ae. aegypti* mosquitoes, we examined the response of *orco*$^{5/16}$ mutant mosquitoes from DeGannaro et al. (2013) and a wildtype strain, Orlando, which is isogenic to *orco*$^{5/16}$ in the hand-in-cage assay. As shown in Fig. 3.5, like in Waco and Rock, significant levels of TF repellency were observed in Orlando mosquitoes, but TF repellency was significantly reduced in *orco*$^{5/16}$ mutants. About 30% of repellency was observed in *orco*$^{5/16}$ mosquitoes at the highest concentration of TF tested.

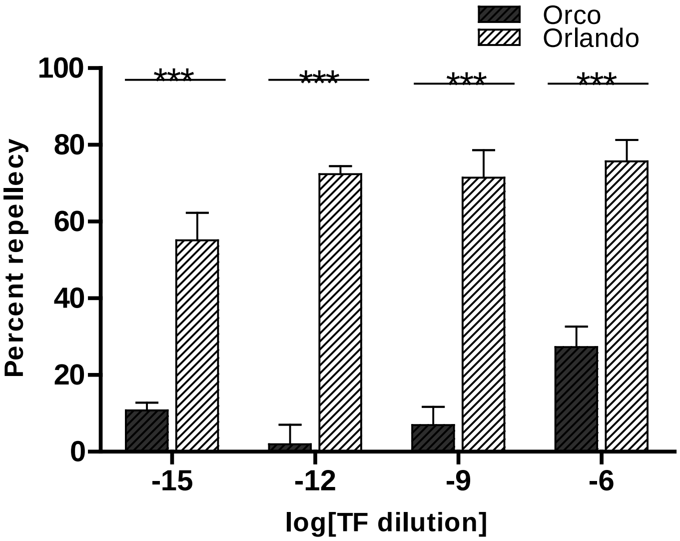

Figure 3.5 Transfluthrin repellency is reduced in *Aedes orco*$^{5/16}$ and it is significantly high in Orlando *Aedes aegypti* strain. Repellency was tested at four different concentrations in Hand in cage assay. Female mosquitoes exclusively tested. Data analyzed using ANOVA. Bonferroni posttest (α=0.05) were used to separate the means in both cases; ns=not significantly different, * =P<0.05, ** = P<0.01, *** = P<0.001

Pre-treatment of piperonyl butoxide (PBO) enhanced repellency elicited by transfluthrin

As shown earlier, both PR and Isokdr displayed a reduced repellency to transfluthrin compared to the pyrethroid-susceptible strains (Fig.3.3). The Isokdr strain possesses only the kdr-mediated resistance and lacks P450-mediated resistance mechanism. The reduced TF repellency in Isokdr is likely due to the kdr mutations in the sodium channel. However, the PR strain has both P450 mediated and kdr resistance mechanisms. To examine the role of P450-mediated resistance in TF repellency, a topical application of 1μg of PBO was used to pretreat the mosquitoes before they were used in the hand in cage assay to inhibit the activity of P450s. The PBO pretreatment resulted into enhanced repellency behavior in both Waco and PR mosquitoes. However, TF repellency maintained lower in PR mosquitoes pretreated with PBO compared to Waco mosquitoes (Fig 3.6, Table 3.3). These results suggest that both the P450-mediated and kdr mechanisms contributed to reduced repellency in PR mosquitoes. To confirm that PBO itself did not affect the landing response of mosquitoes during an arm visit, untreated control was compared in PBO and non-PBO treated mosquitoes and no statistical differences were observed (Fig 3.7)

Table 3.3 Repellency effect of TF compared within and between strains before and after PBO pre-treatment of *Aedes aegypti*[1] mosquitoes

Dose	PRTF[2] (Mean repellency)	PR (TF+PBO)[3] (Mean repellency)	Difference	t-value	P value[5]
-8	4.0	52.16	-19.5	-3.03	0.0058
-7	19.0	75.60	-14.5	-2.24	0.0343
-6	41.8	88.75	-29.8	-4.61	0.0001
Dose	**Waco TF[4] (Mean repellency)**	**Waco(TF+PBO)[4] (Mean Repellency)**	**Difference**	**t-value**	**P value**
-8	18.6	52.16	-33.6	-3.85	0.0008
-7	52.0	75.60	-23.6	-2.71	0.0123
-6	68.8	88.75	-19.9	-2.29	0.0312
Dose	**PR(TF+PBO)[4] (Mean repellency)**	**Waco(TF+PBO)[4] (Mean Repellency)**	**Difference**	**t-value**	**P value**
-8	23.55	52.16	-28.60	-4.18	0.0194
-7	33.50	75.60	42.10	-6.15	0.0001
-6	71.59	88.75	17.16	-2.50	0.0003

[1] 4-10 days old, nulliparous female's sugar starved

[2] PRTF= PR strain exposed to transfluthrin, without PBO pretreatment

[3] WacoTF=Waco strain exposed to transfluthrin, without PBO pretreatment

[4] TF+PBO= Mosquitoes exposed to transfluthrin after PBO pretreatment

[5] Analysis of Variance(ANOVA) Bonferroni posttests;* =P<0.05,**= P<0.01, *** = P<0.001

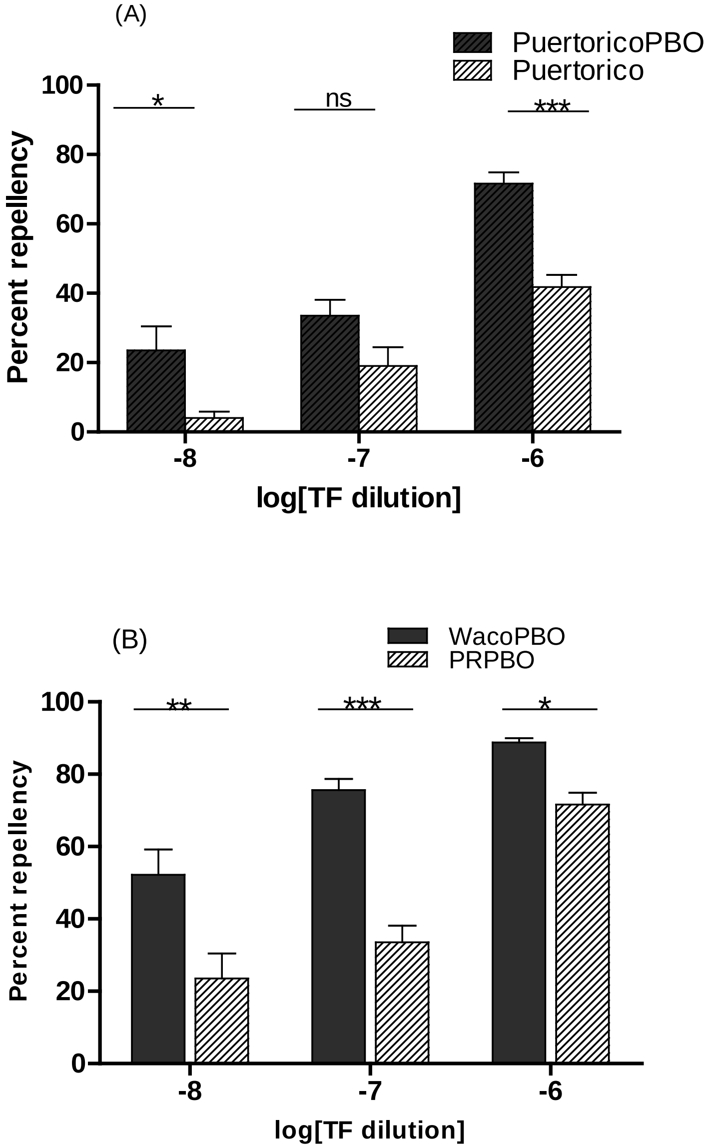

Figure 3.6 Transfluthrin repellency is enhanced when *Aedes* aegypti mosquitoes are pretreated with PBO. (A) Showing repellency effect of TF on PeurtoRico (PR) pretreated with PBO and without topical application of PBO tested in Hand in cage assay at 3 different concentrations (B) Repellency effect of TF, on Waco and PR mosquitoes when topically treated with PBO before the behavioral experiment. Least Significant Differences (LSD, α=0.05) were used to separate the means in both cases; ns=not significantly different, * =P<0.05, ** = P<0.01, *** = P<0.001.

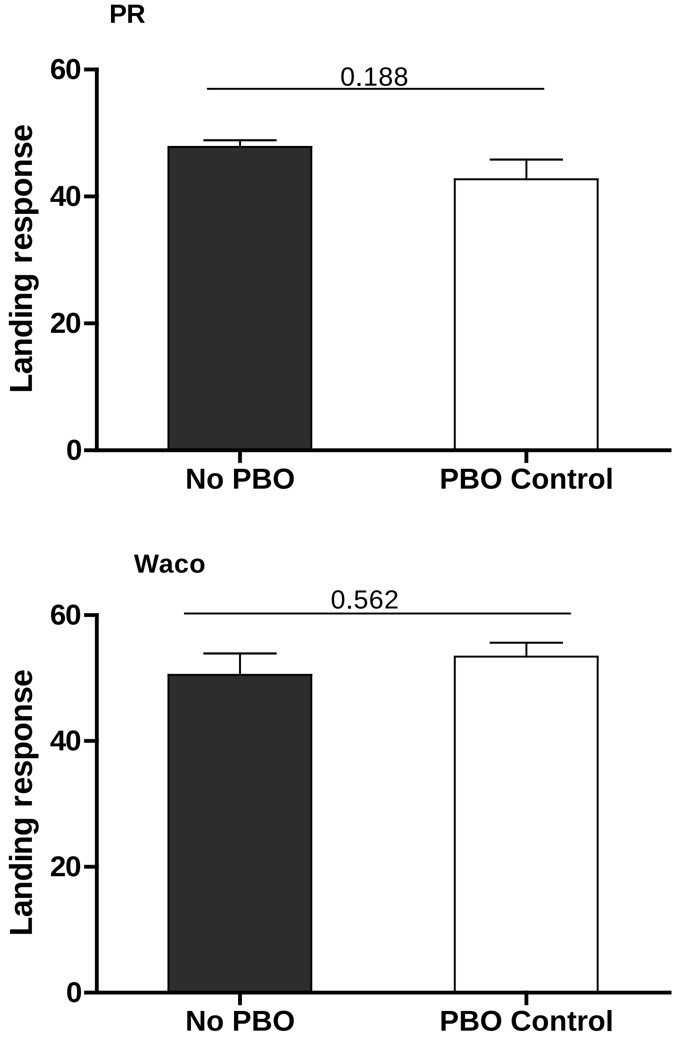

Figure 3.7 No difference in the landing response of PBO pretreated and untreated *Aedes aegypti* when exposed to untreated (control). Female mosquitoes exclusively tested; PBO-pretreatment conducted 60 minutes prior to experiment. Five cohorts of mosquitoes tested for each treatment, (unpaired two tailed t-tests, α =0.05)

Pre-treatment of PBO enhanced mosquito knockdown by transfluthrin.

Knockdown of Waco mosquitoes by TF was observed at the concentration of as low as 10^{-5} in the hand in cage assay indicated TF vapor entered into mosquitoes interacting with sodium channels in the nervous system. At the highest concentration tested, 10^{-3}, most of the 50 mosquitoes were knocked down but recovered within sixty minutes of observation (Fig 3.8). Therefore, the study sought to evaluate the effect of pretreatment with PBO on knockdown effect of TF on Waco mosquitoes. As shown in Fig. 3.8, pretreatment of Waco mosquitoes with PBO an hour before the hand-in-cage assay increased the knockdown effect by TF at 10^{-4} and 10^{-3}. The knocked down mosquitoes did not recover at the end of the assay. The recovery from knockdown of the mosquitoes without PBO pretreatment (Fig. 3.8 A) is likely due to rapid metabolism of TF by P450s.

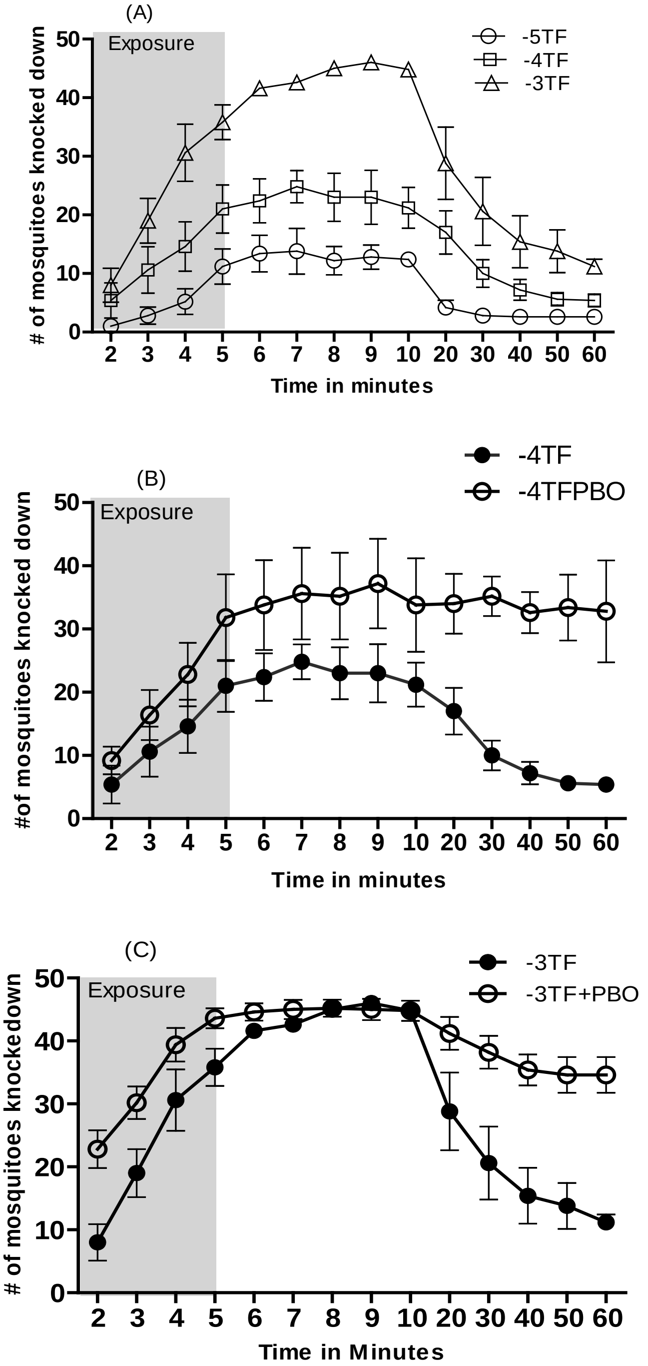

Figure 3.8 Transfluthrin knockdown effect is enhanced in mosquitoes pretreated with PBO (A) Showing knockdown effect of TF when cohorts of 50 female *Aedes aegypti* Waco strain was exposed to TF in hand in cage assay for five minutes (shaded portion) to TF dilutions of 10^{-5}, 10^{-4}, 10^{-3} (n=750). (B) Showing a comparison of Waco pretreated with PBO and without when exposed to 10^{-4} TF. (C) Showing a comparison of Waco pretreated with PBO and without when exposed to 10^{-3} TF.

Table 3.4 Knockdown effect on *Aedes aegypti*[1] pretreated with PBO when exposed to 10^{-3} TF dilution in hand in cage assay.

+	**TF[2]**	**TF+PBO[3]**	**Difference**	**95% CI of diff.**	**t**	**P value[4]**	**Summary**
2	8	22.8	14.8	2.509 to 27.09	3.584	P<0.01	**
3	19	30.2	11.2	-1.091 to 23.49	2.712	P > 0.05	ns
4	30.6	39.4	8.8	-3.491 to 21.09	2.131	P > 0.05	ns
5	35.8	43.6	7.8	-4.491 to 20.09	1.889	P > 0.05	ns
6	41.6	44.6	3	-9.291 to 15.29	0.7265	P > 0.05	ns
7	42.6	45	2.4	-9.891 to 14.69	0.5812	P > 0.05	ns
8	45	45.2	0.2	-12.09 to 12.49	0.04844	P > 0.05	ns
9	46	45	-1	-13.29 to 11.29	0.2422	P > 0.05	ns
10	44.8	44.8	0	-12.29 to 12.29	0	P > 0.05	ns
20	28.8	41.2	12.4	0.1089 to 24.69	3.003	P < 0.05	*
30	20.6	38.2	17.6	5.309 to 29.89	4.262	P<0.001	***
40	15.4	35.4	20	7.709 to 32.29	4.844	P<0.001	***
50	13.8	34.6	20.8	8.509 to 33.09	5.037	P<0.001	***
60	11.2	34.6	23.4	11.11 to 35.69	5.667	P<0.001	***

[1] 4-10 days old, nulliparous females sugar starved

[2] TF= Mosquitoes exposed to transfluthrin, without PBO pretreatment

[3] TF+PBO= Mosquitoes exposed to transfluthrin after PBO pretreatment

[4] Repeated Measures ANOVA (RMA), Bonferroni posttests;* =P<0.05,**= P<0.01, *** = P<0.001

Table 3.5 Knockdown effect on *Aedes aegypti*[1] pretreated with PBO when exposed to 10^{-4} TF dilution in hand in cage assay.

Treatment	**4TF**	**TF+PBO**	**Difference**	**95% CI of diff.**	**t**	**P value[4]**	**Summary**
2	5.4	9.2	3.8	-16.30 to 23.90	0.5628	P > 0.05	ns
3	10.6	16.4	5.8	-14.30 to 25.90	0.8591	P > 0.05	ns
4	14.6	22.8	8.2	-11.90 to 28.30	1.215	P > 0.05	ns
5	21	31.8	10.8	-9.297 to 30.90	1.6	P > 0.05	ns
6	22.4	33.8	11.4	-8.697 to 31.50	1.688	P > 0.05	ns
7	24.8	35.6	10.8	-9.297 to 30.90	1.6	P > 0.05	ns
8	23	35.2	12.2	-7.897 to 32.30	1.807	P > 0.05	ns
9	23	37.2	14.2	-5.897 to 34.30	2.103	P > 0.05	ns
10	21.2	33.8	12.6	-7.497 to 32.70	1.866	P > 0.05	ns
20	17	34	17	-3.097 to 37.10	2.518	P > 0.05	ns
30	10	35.2	25.2	5.103 to 45.30	3.732	P<0.01	**
40	7.2	32.6	25.4	5.303 to 45.50	3.762	P<0.01	**
50	5.6	33.4	27.8	7.703 to 47.90	4.118	P<0.01	**
60	5.4	32.8	27.4	7.303 to 47.50	4.058	P<0.01	**

[1] 4-10 days old, nulliparous females sugar starved

[2] TF= Mosquitoes exposed to transfluthrin, without PBO pretreatment

[3] TF+PBO= Mosquitoes exposed to transfluthrin after PBO pretreatment

[4] Repeated Measures ANOVA (RMA), Bonferroni posttests;* =P<0.05,**= P<0.01, *** = P<0.001

Discussion

Pyrethroids are known to disrupt sodium channel function by prolonging its opening, increasing sodium ions influx, which causes the knockdown effect, and may lead to death. Recently, studies (Xu et al., unpublished data) have confirmed the ability of pyrethroids to evoke olfactory response in Drosophila and mosquitoes. Evidence on transfluthrin's ability to interfere with mosquito biting behavior of different mosquito species in field and semi field trials have been reported (Govella et al., 2015; Ogoma et al., 2017; Ogoma, Lorenz, et al., 2014; Ogoma, Ngonyani, et al., 2014)

Despite pyrethroid success, resistance in arthropods is a common phenomenon. Mutations in different arthropod species associated with pyrethroid resistance, have been reviewed in Dong et al. (2014). Studies reporting P450 mediated pyrethroid resistance are not rare (Liu, 2012; Martin et al., 2003; Ranson et al., 2011). Insensitivity of insects to volatile repellent pyrethroid; transfluthrin, was recently reported in *Aedes aegypti* as a heritable trait (Wagman et al., 2015).

In this study we demonstrated the impact of both kdr and P450 mediated pyrethroid resistance on the toxicodynamics of transfluthrin action as a repellent and as a knokdown agent in *Aedes aegypti* mosquitoes. Higher repellency levels in Waco and reduced repellency in resistant PR denote pyrethroid repellency efficacy is dependent on susceptibility of the mosquitoes to pyrethroids in general and these results concur with the finding from Wagman et al. (2015).

Transfluthrin seem to have a duo action on sodium channels and olfactory receptors. It activates olfactory receptors to transduce action potentials, which are propagated and processed in the higher brain centers to evoke repellency behavior in the mosquitoes. At the same time, in its vapor state, it diffuses into an insect body and bind to the Na channels, through which action potentials may be propagated to the higher brain centers to induce knockdown effect. The duo action of a single compound, transfluthrin possibly leading to a synergistic propagation of action potentials through the olfactory pathway and sodium channel prolonged opening leading to enhanced repellency behavior, seem to be dependent on the susceptibility of mosquitoes to pyrethroids. Hence the observed low repellency levels in mosquitoes with kdr mutations when exposed to transfluthrin in the hand in cage assay could be attributed to the lack of synergy in between the signal transductions caused by the olfactory pathway and prolonged opening of sodium channels. Because of the mutations in the sodium channel, most likely, the signals that are propagated to the higher brain centers in the mosquito are only mediated through the olfactory pathway. Disruption of motor-neuron activity by transfluthrin has been reported (Wagman et al., 2015). This attests that resultant insect behavioral stimuli, termed as TF repellency is not exclusively due to the olfactory pathway.

An increased repellency in both strains after pretreatment with PBO, denote the important role that P450s may play in TF repellency. The P450s metabolize TF and therefore its availability is reduced for its action on sodium channels and olfactory receptors. The inhibition of P450 activity by PBO in the PR and Waco strains allowed us to elucidate the impact of P450 mediated pyrethroid resistance on transfluthrin repellency and toxicodynamics. Although contact bioassays using transfluthrin in the presence of PBO showed no enhanced toxicity in mosquitoes with P450 mediated mechanisms (Horstmann and Sonneck, 2016), here we report an enhanced vapor toxicity and repellency of TF on mosquitoes pretreated with PBO (see Fig 3.6). Enhanced repellency in PBO pretreated mosquitoes could be explained by the inhibition of P450s in the insects body to increase the availability of TF for its interaction with sodium channels and` olfactory receptors which together with the olfactory responses elicited repellency behavior. Besides as an inhibitor of P450s, PBO has been suggested to enhance penetration of insecticides (Kasai et al., 2014). Therefore, alternatively, enhanced TF repellency could be due to enhanced penetration of transfluthrin into the insect body (Kasai et al., 2014). While, detailed studies by (Zhu et al., 2010) have highlighted on a brain specific cytochrome P450 responsible for detoxification of deltamethrin in Tribolium insect species, others (Maïbèche-Coisne et al., 2004; Pottier et al., 2012) have demonstrated a key role of P450s as olfactory degrading enzymes.

Reported results attest the importance of P450s and kdr in TF toxicodynamics when used as a vapor and its implications in insect vector control. The increase in the knockdown effect of TF with increase in concentration, as well as repellency effect when used at sub lethal doses, denote the repel and kill effect that TF may have on mosquitoes. The repellency due to TF may decrease the mosquito biting rate. Biting rate is important as it somewhat relates to the mosquito densities which are key in vectoral capacity (Brady et al., 2016). Although it was beyond the scope of this study to assess the vectoral capacity parameters that may be affected by the use of transfluthrin, these results certainly present a possibility of TF significantly affecting the transmission parameters of vector borne diseases.

Conclusion

Collectively, this study reports repellency of TF against mosquitoes. TF repellency was almost abolished in Orco mutant mosquitoes, indicating olfaction-based TF repellency. TF repellency was also reduced in PR and Isokdr strains with both P450 and/or kdr mediated resistance. However, DEET repellency was not different between PR and Waco as well as Isokdr and Rock confirms that activation of sodium channels by TF may be important for TF repellency. Thus, the volatile TF may not work on olfactory receptors exclusively, it is likely that the resultant repellency stimuli, is due to a combined effect of TF being neuro-excitatory, interfering with Na channel opening and olfactory processing.

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CHAPTER 4

REPELLENCY EFFECT OF PermaNet 2.0 AND Olyset NET ON *Anopheles gambiae* AND *Aedes aegypti*

Abstract

The use of pyrethroid long lasting insecticidal nets (LLINs) remains one of the major ways in the control of mosquito borne diseases. The use of LLINs has registered tremendous success in reducing vector host contact although this has been limited due to development of resistance in some areas. Most studies on the mechanism of action of LLINs have reported excito-repellent effect and contact toxicity on mosquitoes. This present study reported olfactory based spatial repellency of deltamethrin treated net (PermaNet 2.0) and permethrin treated net (Olyset) against mosquitoes. Specifically, in the Hand in cage assay, we observed 1) repellency effect of PermaNet 2.0 on pyrethroid-susceptible *Ae. aegypti* Rock and Waco mosquitoes, and *An. gambiae* Kisumu mosquitoes; 2) reduced repellency of PermaNet 2.0 against *Ae. aegypti* pyrethroid-resistant Puerto Rico (PR) and Isokdr and *orco*$^{5/16}$ mosquitoes; and 3) repellency effect of the Olyset net on *An. gambiae* Kisumu mosquitoes, but not on *Ae. aegypti* mosquitoes even though repellency was observed from permethrin-treated mesh. Furthermore, we found that permethrin and deltamethrin elicited electroantennogram (EAG) responses from *Ae. aegypti* mosquitoes, but not from anosmic *orco*$^{5/16}$ mosquitoes, providing the olfactory basis of the repellency of LLINs.

Introduction

Among other known malaria vectors, *Anopheles gambiae* is considered one of most important (Riabinina et al., 2016; Takken et al., 2001; World Health Organization(WHO), 2017). It transmits a malaria parasite plasmodium *falciparium* which accounts for 99% of all the malaria cases in the world. In the year 2016, 216 million cases of malaria occurred worldwide, causing estimated deaths of around 455000 (WHO, 2017). Insecticide treated nets (LLINs) remain one of the most important tools in malaria control (Pennetier et al., 2013; Takken, 2002; Vantaux et al., 2014; World Health Organization, 2017). Pyrethroids are the only class of insecticides that have been approved to be used in LLINs (Ranson et al., 2011). Most commonly used pyrethroids in LLINs include; permethrin,α-cypermethrin and deltamethrin (Mosha et al., 2008). Commonly used LLINs include permethrin LLIN also referred to as Olyset Net and deltamethrin LLIN also referred to as PermaNet 2.0 (Guessan et al., 2001; Soleimani-Ahmadi et al., 2012). The use of LLINs is popular but not limited to *Anopheles* mosquito control. They have also been used in the control of *Aedes* mosquitoes. For example, the use of LLINs in the control of dengue vectors has been reported in Haiti (Lenhart et al., 2008)

Although LLINs remain important in mosquito control, reduced efficacy in resistant mosquito populations has been reported (Enayati and Hemingway, 2006; Guessan et al., 2001; Thiam et al., 2012; Toé et al., 2014). In the aforementioned studies, there was reduced toxicity in resistant mosquitoes compared to pyrethroid susceptible ones. In addition, the need to increase the concentration of pyrethroids in insecticide treated nets to effectively reduce the host-vector contact in mosquitoes with kdr has been reported (Corbel et al., 2004). Moreover, the impact of agricultural insecticide use in cotton and rice growing areas has been emphasized as one of the causes in reduced efficacy of the insecticide treated nets in agrarian societies (Bigoga et al., 2012; Diabate et al., 2002; Fane et al., 2012; Hien et al., 2017).

To curb the reduced efficacy due to resistance development in mosquitoes, some LLINs have been incorporated with a synergist PBO. A study assessing the efficacy of PermaNet 2.0 (without PBO) and PermaNet 3.0 (with PBO) revealed increased efficacy of PermaNet 3.0 on resistant mosquitoes (Koudou et al., 2011). Similar findings were reported when PermaNet 2.0 and 3.0 were compared in an experimental hut trial in an earlier study (N'Guessan et al., 2010). Similarly, reduced efficacy of Olyset nets in mosquitoes with pyrethroid resistance and evidence on increased efficacy of the Olyset plus, a permethrin treated net with PBO incorporated has been reported (Guessan et al., 2001; Pennetier et al., 2013).

Research on the mode of action of LLINs has mainly focused on contact toxicity of the nets (Ochomo et al., 2013). Analysis of differential behavioral responses of *Anopheles gambiae* mosquitoes revealed a reduced frequency of contact of mosquitoes with pyrethroid treated nets but recorded an increased flying and sitting behavior (Siegert et al., 2009). The observed behaviors were attributed to the neurotoxic effect due to contact with the pyrethroid treated nets. Similar results have been reported by Kawada et al., (2014). A study, examining the length of time a mosquito spends in physical contact with the insecticide treated net and untreated one using infrared tracking system, revealed that mosquitoes spent less time on LLINs compared to untreated control (Parker et al., 2015). In assessing the interaction of the mosquitoes with the bed net overtime, Parker et al., (2015) observed less contact of the mosquitoes with the LLINs compared to untreated control and concluded that the LLINs did not elicit repellency in the mosquitoes prior to physical contact, suggesting that LLINs mechanism of action is dependent on contact of the mosquito with the net.

Different studies have attributed exiting behavior, deterrence and or contact irritancy (also referred to as contact disengagement) to use of permethrin and deltamethrin treated nets and surfaces (Chareonviriyaphap et al., 2004; Ogoma, Lorenz, et al., 2014). And, investigations on distribution of *Anopheles gambiae* mosquitoes due to the effect of permethrin treated nets, revealed a decrease in the mosquito densities with decreasing distance from the intervention areas (Gimnig et al., 2003). Another study also reported a spill over insecticide treated net effect to houses that were not using the bed nets in Haiti (Lenhart et al., 2008). Although the study did not clearly evaluate the mechanism of the bednet action, the significant decrease in the dengue vectors was attributed to the deltamethrin treated net intervention. In addition to the reported spillover effect due to insecticide treated nets, exiting behavior of mosquitoes from houses containing insecticide treated nets has been reported (Miller et al., 1991, Mosha et al., 2008). A study conducted in India, concur with earlier mentioned studies; that evaluation of the efficacy of Olyset plus (a permethrin treated net with PBO incorporated) showed a significant reduction in the house entry, a decline in the blood feeding rates in experimental hut and the deterrence effect of permethrin treated nets on *Anophelesfluviatile* was evident (Gunasekaran et al., 2016). Moreover, the use of deltamethrin treated nets has been documented to significantly reduce host seeking by *Culex* mosquitoes when used around cattle enclosures (Maia et al., 2012). The present study, we hypothesized that LLINs repellency (non-contact disengagement) in different *Anopheles* and *Aedes* mosquito strains. In this study, we used the Hand in cage assay to examine potential repellency effect of Olyset and Permanet 2.0 on *An.gambiae* (the Kisumu strain) and *Aedes aegypti* (Waco, Rock, Isokdr and *orco* strains).

Materials and Methods

Mosquitoes

In this study, five *Aedes aegypti* mosquito strains were used; Rockefeller (Rock), Isokdr, Waco, Puerto Rico (PR), and an *Orco* mutant (*orco*$^{5/16}$). Rock and Isokdr were provided by Jeff Scott's laboratory at Cornell University. Isokdr is highly resistant to pyrethroids possessing two kdr mutations in the sodium channel and Rock is pyrethroid-susceptible; and they are isogenic (Smith et al., 2018). Waco is an insecticide-susceptible laboratory strain kindly provided by Dr. Zhiyong Xi at Michigan State University. Orlando is a wild-type *Ae. aegypti* strain kindly provided by Leslie Vosshall (Rockefeller University). PR and two *orco* mutant lines (*orco*^{5} and *orco*^{16}) are from BEI Resources, NIAID, NIH. PR is a pyrethroid resistant strain possessing P450-mediated pyrethroid resistance and three kdr mutations (Reid et al., 2014 ; unpublished data from the Dong lab). The two *orco* lines were crossed to generate *orco*$^{5/16}$ mosquitoes for this study.

The colonies of *Ae. aegypti* were reared at 27^{o}C, at least 60% humidity, at12h:12h light: dark photoperiod in growth chambers. Larvae were fed with liver powder and adults with 10% sucrose solution throughout the rearing period. Fifty females (4-10 days old and mated) were used for behavioral experiments. Twenty four hours prior to the experiments, the mosquitoes were isolated into a clean cage and were given water only. Six hours before the experiments, the water was removed. Adults of *An. gambiae*, Kisumu strain were reared at Malaria Alert Center (MAC) insectary at University of Malawi, College of Medicine (COM). They were reared on 10% sucrose solution and larvae on fish food. Colonies were maintained in growth chambers at approximately 28^{0}C and 70% relative humidity.

Behavioral assays

i. Repellency effect of permethrin on *Aedes aegypti*

This study was conducted to test the repellency of permethrin itself as a compound before testing the permethrin treated insecticide treated nets. Unlike other pyrethroids, permethrin is less volatile yet it is used in insecticide treated nets as a contact irritant. This study was conducted to establish non-contact repellency of permethrin. The permethrin was of 99% purity, from Sigma Aldrich. Fifty milligrams of the compound was weighed and diluted in acetone as a carrier solvent to be used in Hand in cage assay. Hand in cage assays were carried out following methods explained in Fig 2.1. The tests were conducted on both resistant (PR) and susceptible (Waco) mosquito strains. Females were tested exclusively.

ii. Repellency effect of Olyset and PermaNet 2.0 on *Ae.aegypti* and *An.gambiae*.

The test nets were provided by Malaria Alert Centre in Malawi. Olyset net is impregnated with 1000mg per meter squared of the treated net(Siegert et al., 2009). For the 5cmx5.8cm net pieces used in this study, they contained approximately 3mg. PermaNet 2.0 mosquito nets have been reported to have 55mg of deltamethrin per meter square(Koudou et al., 2011), converting to 0.159mg on the Hand in cage net piece used. To assess the repellency effect of insecticide treated nets, Hand in cage assays were carried out following methods explained in Fig 2.1 except that, an untreated insecticide treated net was used as control. For the other treatments; 5cmx5.8cm net pieces were cut and used in the behavioral assays. At least 5 different cohorts of mosquitoes were tested on each of the treatments. Untreated net was tested first.

Results

Permethrin elicited repellency on pyrethroid-susceptible *Ae. aegypti* mosquitoes

We first examined the potential permethrin repellency in the Hand in cage assay using permethrin-treated mesh. Repellency by permethrin was detected on Waco mosquitoes and there was an increase in the repellency effect with an increase in the concentration of the compound used (Fig. 4.1). There were no significant differences in the repellency effect of permethrin between the two lowest concentrations used (10^{-4} and 10^{-3} in Fig 4.1). A significant difference in the repellency effect of permethrin due to concentration was only observed between the lowest (10^{-4}) and the highest concentration (10^{-2} equivalent to 5mg of permethrin). However, the repellency on the PR strain was abolished (Fig. 4.4). Table 4.1 is an excerpt of statistical multiple comparisons comparing the PR and Waco strain at specific doses.

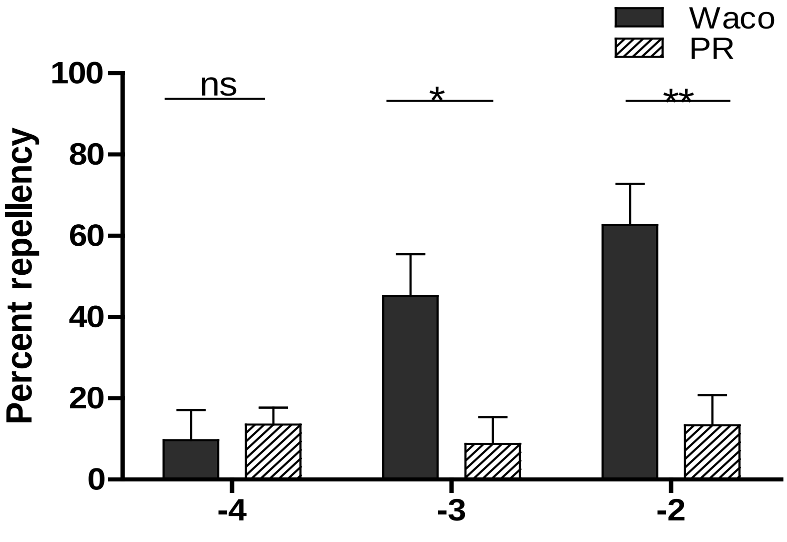

Figure 4.1 High permethrin repellency in pyrethroid susceptible *Aedes aegypti* Waco strain than pyrethroid resistant PR strain tested at three different concentrations in Hand in cage assay. Repellency was abolished in pyrethroid resistant PR strain and was maintained in susceptible Waco strain. Data analyzed using ANOVA, Bonferroni posttests ($\alpha=0.05$); ns=not significantly different, * =$P<0.05$, ** = $P<0.01$, *** = $P<0.001$

Table 4.1 Repellency effect of permethrin on Waco and PR *Aedes aegypti*[1] strains

Dose	**Waco[2] (Mean repellency)**	**PR[3] (Mean repellency)**	**Difference**	**t-value**	**P value[4]**
-4	9.708	13.54	3.830	0.3195	$P > 0.05$
-3	45.18	8.793	-36.38	3.035	$P < 0.05$
-2	62.59	13.35	-49.24	4.107	$P<0.01$

[1] 4-10 days old, nulliparous female's sugar starved

[2] Waco=Waco strain exposed to permethrin in Hand in cage assay

[3] PR= PuertoRico strain exposed to permethrin in Hand in cage assay

[4] Analysis of Variance(ANOVA) Bonferroni posttests;* =P<0.05,**= P<0.01, *** = P<0.001

PermaNet 2.0 repelled susceptible *Aedes aegypti* strains (Waco and Rock) and *Anopheles gambiae* (Kisumu)

We later carried out the non–contact Hand in cage assay to assess potential repellency of the deltamethrin treated net (PermaNet 2.0) and permethrin treated net (Olyset). Indeed, significant levels of repellency of PermaNet 2.0 was found in the pyrethroid susceptible *Ae. aegypti* mosquitoes: Waco, Rock, and the *An. gambiae* Kisumu strain. However, repellency of the Olyset net was observed in the *An. gambiae* mosquitoes, surprisingly not in the *Aedes* mosquitoes (Fig. 4.3). In all the tests, repellency effect on Kisumu was significantly higher than that on *Aedes aegypti* strains (Waco and Rock). However, repellency of PermaNet 2.0 was abolished in Isokdr and PR strains and *orco* mosquitoes. Detailed multiple comparisons of repellency effect on mosquito strains due to insecticide treated net effect are presented in table 4.2.

Table 4.2 Repellency effect of PermaNet 2.0 and Olyset compared when tested on *An. gambiae*[1] and different *Ae.aegypti*[1] strains

Strain/species	**PermaNet 2.0**[2]	**Olyset** [3]	**Difference**	**t-value**	**P-value** [9]
Waco[5]	54.01	12.39	-41.62	5.450	$P<0.001$
Rock[5]	66.97	18.31	-48.66	6.373	$P<0.001$
Isokdr[6]	4.486	-3.064	-7.551	0.7659	$P > 0.05$
PR[7]	-1.780	-6.354	-4.574	0.5990	$P > 0.05$
Orco[8]	-6.150	-3.768	2.382	0.3119	$P > 0.05$
Kisumu[4]	85.96	60.54	-25.42	2.978	$P < 0.05$

[1] 4-10 days old, nulliparous female's sugar starved
[2] PermaNet 2.0= Deltamethrin treated net, without PBO, manufactured by Sumitomo company
[3] Olyset= Permethrin treated net, without PBO. Manufactured by Sumitomo company
[4] Kisumu= *Anopheles gambiae* mosquito of Kisumu strain
[5] Waco and Rock = *Aedes aegypti*; pyrethroid susceptible laboratory strains
[6] Isokdr= *Aedes aegypti* strain with Kdr mediated resistance
[7] PR=*Aedes aegypti* strain with both Kdr and P450 mediated resistance
[8] *Orco*=*Aedes aegypti* strain, with *Orco*-coreceptor knocked out
[9] Analysis of Variance(ANOVA) Bonferroni posttests;* =$P<0.05$,**= $P<0.01$, *** = $P<0.001$

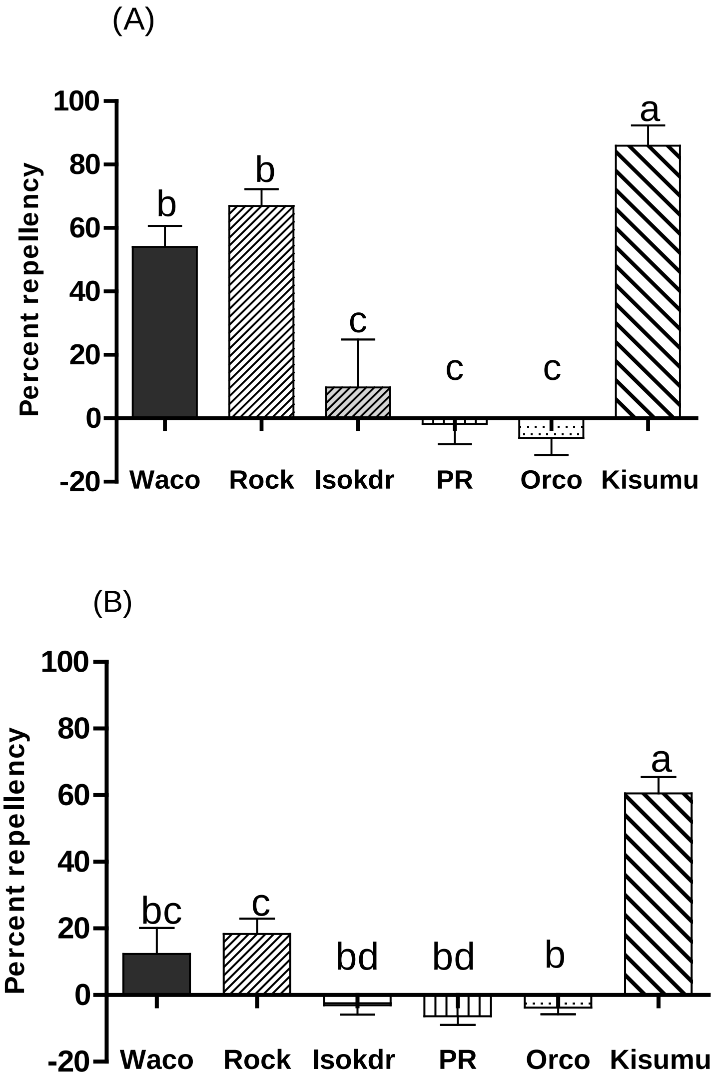

Figure4.2 PermaNet2.0 repel pyrethroid susceptible *Aedes aegypti* strains and *Anopheles gambiae*. (A) Showing repellency effect of PermaNet2.0 tested using net fabric in Hand in cage assay. (B) Showing reduced repellency effect of olyset net fabric on susceptible Aedes aegypti mosquito strains and increased repellency in *Anopheles gambiae*. Both nets are manufactured by Sumitomo Company; PermaNet 2.0 has 50mg/m^2 of deltamethrin and Olyset 1000mg/ m^2 of the net.

Permethrin and deltamethrin elicited electroantennogram (EAG) signals

In EAG recording experiments conducted by Feng Liu a postdoc associate in Dong Lab, deltamethrin and permethrin evoked olfactory responses from the mosquito antenna. Both deltamethrin and permethrin, when heated up, following methods by Slone et al., (2017), evoked EAG signals from the antenna of Rock mosquitoes in a dose dependent manner (Fig 4.3A and 4.3 A). The EAG recording experiments were repeated using Orlando and *Orco* mosquitoes. Similarly, EAG signal was detected from mosquitoes of another susceptible strain, Orlando, in response to both deltamethrin and permethrin (Fig 4.3A and 4.4 A). However, no EAG response was observed from *Orco* mosquitoes (Fig 4.3B and 4.4B), indicating EAG responses by deltamethrin and permethrin are *Orco*-dependent.

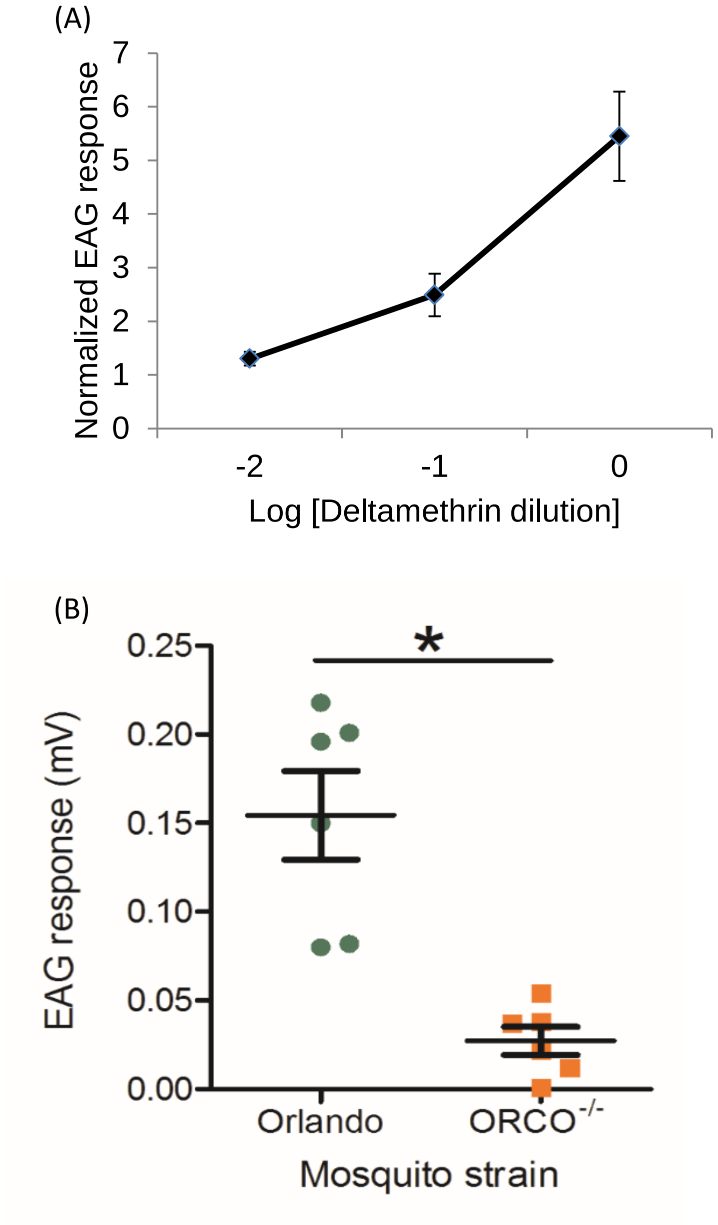

Figure 4.3 Normalized EAG responses of *Aedes aegypti* to deltamethrin. (A) Showing EAG responses in females *Aedes aegypti* Rock strain. (B) Showing a comparison of EAG responses in Orlando and *Orco Aedes aegypti* mosquitoes. Female mosquitoes tested exclusively. * =P<0.05 (*EAG recording done by Feng Liu)*

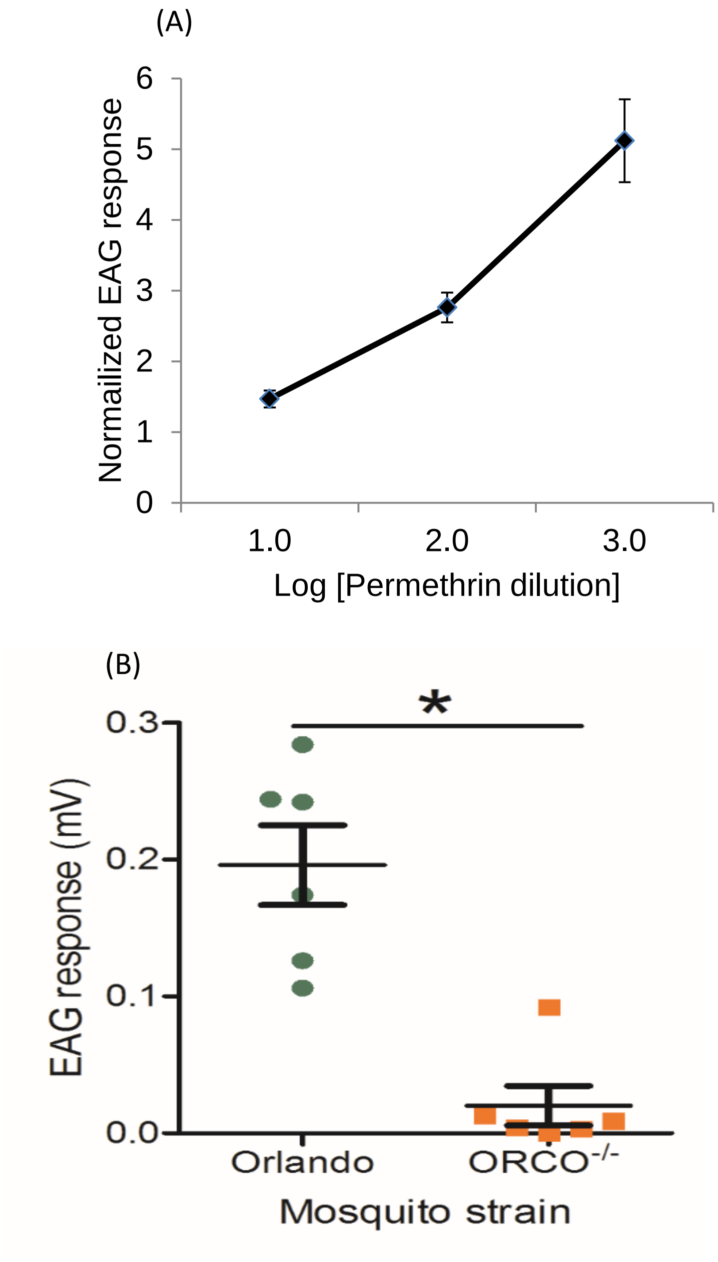

Figure 4.4 Normalized EAG responses of *Aedes aegypti* to permethrin. (A) Showing EAG responses recorded in *Aedes aegypti* Rock strain. (B) Showing a comparison of EAG responses in Orlando and *Orco Aedes aegypti* mosquitoes. Female mosquitoes tested exclusively. * =P<0.05 (*EAG recording done by Feng Liu)*

Discussion

The use of pyrethroid treated insecticide bed nets remains important in the control of mosquito borne diseases, more especially malaria. The bed nets act as a barrier from mosquito bites for people sleeping inside them. Research has demonstrated excito-repellency effects, others mortality in mosquitoes due to contact with the pyrethroid insecticide treated nets and surfaces (Corbel et al., 2004; Denham et al., 2015; Ritthison et al., 2014). The current study has demonstrated an olfactory basis of action of the pyrethroids, deltamethrin and permethrin, which are used in LLINs. Results on behavioral assays using permethrin compound on *Aedes aegypti* mosquitoes demonstrated a dose dependent noncontact repellency in the pyrethroid susceptible strain Waco and not in the resistant strain PR, depicting that pyrethroid repellency is not very much restricted to very volatile pyrethroids, such as transfluthrin and metofluthrin.

The positive impact of insecticide treated nets on houses that are close to intervention areas compared to those that were far, supporting the hypothesis that bed nets may have a community effects in controlling mosquito vectors has been documented (Gimnig et al., 2003). The actual mechanism underlying the community effects of pyrethroid treated nets is still debatable. The current study has demonstrated the ability of permethrin and deltamethrin to evoke olfactory response in EAG recordings in the antenna of the mosquito. The lack of EAG responses in mosquitoes with *Orco* co-receptor knocked out, support the olfactory basis of action of these test compounds, which somewhat might point towards the underlying mechanism of the community effects of the nets among other factors. Behavioral experiments evaluating the repellency effect of PermaNet 2.0 and Olyset net adopted the Hand in cage assay, importantly because the assay was designed in such a way that insects do not come into contact with the treated net, avoiding the excito repellent effects of insecticide treated nets that have been reported by a growing body of literature including (Kawada et al., 2014; Killeen and Smith, 2007).

The lack of LLIN repellency against pyrethroid-resistant mosquitoes (PR) observed in this study echoes a challenge of the use of deltamethrin treated bednets in areas with mosquito populations that are resistant. The efficacy of pyrethroid treated bednets when mosquitoes that have both kdr-type and metabolic resistance mechanisms was questioned (Enayati and Hemingway, 2006b). In their study, they highlighted significant differences between entry rates of pyrethroid and susceptible and resistant *Anopheles* mosquitoes in an exposure arena containing permethrin treated bed net. Results in the current study, support their findings. Similar findings have been reported (Ochomo et al., 2013, Toé et al., 2014). This study has presented evidence on the repellency effect of net bound permethrin and deltamethrin on different strains of *Ae.aegypti*. Different mosquito populations may develop resistance against the insecticides or change their behavior by avoiding contact with treated surfaces (Takken and Verhulst, 2011). In this study, the noncontact repellency effect of the deltamethrin treated net, PermaNet 2.0 evident in susceptible *Ae.aegypti* mosquito strains; Waco and Rock and the reduced repellency in the resistant strain, Isokdr, signify the impact of resistance development on the efficacy of the insecticide treated nets in areas with and without resistant mosquito populations.

A study by Kawada et al., (2014) revealed that L1014S ^{KDR} *Anopheles* mosquito field populations with reduced frequency takeoffs from a permethrin treated nets and those lacking kdr mutations maintaining high levels of contact repellency irrespective of their metabolic factors to pyrethroids. Our study has demonstrated that non-contact repellency due to Olyset and PermaNet 2.0 exposure may be affected by mosquito metabolic factors. Repellency effect of both nets was abolished in PR *Aedes* strain which has both P450 and kdr mediated resistance, unlike in Isokdr which only has kdr mediated resistance. While this study does not offset the excito repellency effect of pyrethroid treated nets, the abolished repellency in *Orco* due to non-contact exposure of the mosquitoes through Hand in cage assay confirm the role that olfactory receptors play in the repellency effect of the Olyset and PermaNet 2.0 insecticide treated nets.

Further, increased repellency effect of the nets on *An.gambiae* compared to *Ae.aegypti* mosquito strains, signify the differences in the sensitivity of different mosquito species to pyrethroids as repellents. Repellency effect observed in this study could be a resultant behavior of olfactory processing of odors. To dissect the differences in the sensitivity of the mosquito species to repellency effect of the pyrethroids, studies exploring in the actual signal propagation and neural circuits in the nervous system of the mosquito species resulting into the repellency behavior could help in elucidating the underlying cause of the sensitivity differences

In addition, this study has reported greater repellency effect of Permanent 2.0 compared to Olyset net in all the mosquito strains of *Aedes* and *Anopheles* tested. Other studies have also reported varying results on insecticide treated nets (Mosha et al., 2008; Spitzen et al., 2014). While Mosha et al. (2008) reported high protective efficacy of permethrin treated net over deltamethrin insecticide treated net, Spitzen et al., (2014) reported absence of close range repellency of deltamethrin treated net against *Anopheles* mosquitoes. In the present study, non-contact hand in cage assays using permethrin showed repellency in susceptible Waco mosquitoes yet there was no repellency of the permethrin treated net (Olyset). The differences in the repellency effect of the nets used in this study could be attributed to the fabric properties of the nets and the concentrations used.

Conclusion

In summary, pyrethroid repellency may not be limited to very volatile pyrethroids. Permethrin and deltamethrin also elicit repellency behavior in mosquitoes through the olfactory pathway as observed in the EAG recordings and behavioral assays. Lack of EAG response in *Orco* mosquitoes when exposed to permethrin and deltamethrin compounds, yet overt responses in mosquitoes with their *Orco* co-receptor intact, confirm the involvement of the olfactory receptors in the action of permethrin and deltamethrin compounds. As regards to PermaNet 2.0 and Olyset, the findings of this study point towards a non-contact repellency mechanism of action of the nets apart from the well-known contact repellency. Abolished repellency observed in behavioral assays in resistant mosquitoes indicate that the nets may be more efficient tools in reducing mosquito bites in areas with susceptible mosquito populations compared to areas with resistant mosquito populations. It is important to note that the conclusions presented here are from the evaluation of PermaNet 2.0 and Olyset. Further studies may consider using other nets that are available on the market from different manufacturers to evaluate the potency of their repellent activities on different mosquito species and mosquitoes with different resistance status.

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CHAPTER 5

TRANSFLUTHRIN AND PYRETHRUM REPELLENCY ON *Plutella xylostela* AND *Sitophilus zeamais*

Abstract

Pyrethroids are also important in the control of different agricultural pests. In their use in agricultural field pest control, they are sprayed on the crops to reduce damage by insects. In grain storage, they are mainly incorporated as active ingredients in pesticide dusts. The current use of pyrethroids in the control of agricultural insects attracts repeated sprays and pesticide applications which may be very costly for small holder farmers. The use of pyrethroids as repellents in agriculture is very rare yet it has potential to reduce pesticide load on food and the environment. This study reports the repellency effect of transfluthrin and pyrethrum on *Plutella xylostela* and *Sitophilus zeamais*. It illustrates 1) avoidance of pyrethrum and transfluthrin treated arenas in adult *Plutella xylostela* and *Sitophilus zeamais* insects 2) feeding preference of *Plutella xylostela* larvae in pyrethrum and transfluthrin treated arenas 3) reduced feeding in *Plutella xylostella* larvae in pyrethrum and transfluthrin treated arenas

Introduction

Insect pests rely on chemosensation to locate their plant hosts, to find oviposition sites, suitable mates and food. Reviews (Naters and Carlson, 2006; Tricoire-Leignel et al., 2012) emphasized that insect olfaction cues as crucial in agricultural production. Olfaction measures long range attraction (Naters and Carlson, 2006) or repellency and some studies have proved its importance in diamondback moth in distinguishing suitable plant hosts from unsuitable ones (Henniges-Janssen et al., 2011; Liu et al., 2005). Diamondback moth has been reported to avoid its natural enemies using olfactory cues (Reddy et al., 2002). Similar results have also been reported in *Drosophila*; to avoid natural enemies and harmful chemical substances in oviposition sites (Ebrahim et al., 2015; Stensmyr et al., 2012). These studies confirm the key role that olfaction plays for insects in crop production. In fact, the economically important damage that insect pests cause on crops is largely driven by olfactory cues which they use to find oviposition sites; which are mostly the plant host where the larvae hatch and cause direct damage.

Insects such as diamondback moth (*Plutella xylostella*) are a leading cause of damage to cruciferous crops and lesser grain borer (*Sitophilus zeamais*) cause significant post-harvest loss in maize crop in Africa and other parts of the world. Like other insect pests, effective control still relies on insecticides. Earlier, before restrictions were placed on some major insecticides, control of most agricultural pests in Africa relied on the inexpensive organochlorides and methyl carbamates which are not only toxic to insects but also humans (Casida, 1980). Currently, even with restrictions, use of less expensive yet toxic insecticides such as carbamates is still common for profit oriented small holder farmers. In some cases organophosphates and pyrethroids such as cypermethrin are used to reduce preharvest losses, more especially in Malawi. On the other hand, pesticide dusts with deltamethrin incorporated are used in reducing postharvest losses in maize yield to control maize weevils and other beetles such as larger grain borer. In some cases, multiple applications of these pesticides are required, to maintain yield quality. This may pose a health risk. Studies have confirmed health effects of overuse of insecticides in some parts of Africa (Naidoo etal., 2013). Unfortunately, the use of insecticide sprays also select for resistance in *Anopheles gambiae* mosquitoes (Hien et al., 2017) complicating the control of malaria.

Unlike carbamates and organochlorides, pyrethroids are less toxic to humans and other mammals (Casida, 1980; Dong et al., 2014; Ensley, 2007; Soderlund et al., 2002). Despite their favorable chemical properties, the use of pyrethroids in agriculture and public health has been affected by development of insecticide resistance requiring frequent reapplications to protect crops exceeding required recommendations, risking food toxicities and making agricultural production for small holder farmers in Africa, very costly.

Recent progress in insect chemosensation studies, offers opportunities for developing novel ways of insect pest control. Olfaction, in conjunction with the existing insect control strategies may enhance crop protection in Agriculture. Olfaction in agricultural insect pest control, is essential in trap designs (Dendy et al., 1989). Although the potential in using repellents to push insects away from crops has not been largely tapped, Arnold et al., (2015) emphasized on how in-depth knowledge on insects orientation to stimuli may play a vital role in designing mass trapping of insects for control. Their study demonstrated the importance of odor and color on the ability of lesser grain borer (LGB) in locating the maize grain. What these studies lacked was an exploration of chemical cues that would push the insects away from the storage facility and pulled into a trap, to enhance mass trapping. Majority of studies taking advantage of the insect olfaction cues for mass trapping to reduce crop damage, have maximized on the host odor and color cues. Yet, recent studies by have presented evidence on the ability of the pyrethroid; transfluthrin to prevent outdoor mosquito bites through repellency when used and reused in low cost impregnated hessian sacks (Govella et al., 2015; Ogoma et al., 2012, 2017). Although the studies mainly focused on repellency in mosquitoes, their results warranted further exploration of similar mechanisms in other insects, more especially agricultural pests. Pyrethroids feature highly in the control of insect pests of crops like cotton, horticultural crops and are also incorporated in pesticide dusts for control of storage pests such as LGB in Malawi. They are also important in the control of livestock pests such as tsetse fly. Currently, pyrethroid blanket sprays are common in the control of insect pests in the field. Establishing repellency effect of pyrethroids might be helpful in control of insect pests in the field as a push strategy at minimal insecticide amounts. This study sought to establish repellent effect of pyrethrum and transfluthrin on two agricultural insect pests; *Plutella xylostella* (diamondback moth) and *Sitophilus zeamais* (lesser grain borer) in Malawi.

Materials and Methods

Insects

Sitophilus zeamais

A laboratory colony of *Sitophilus zeamais* (lesser grain borer), also referred to as LGB was obtained from Lilongwe University of Agriculture and Natural Resources (LUANAR), Bunda campus and used for the experiments. The lesser grain borer was reared on untreated maize grain to avoid pre-exposure to pesticides before the insects were used in a behavioral assay. Both males and females were used in the assay.

Plutella xylostella

A colony of *Plutella xylostella* (Diamondback moth) was established by collecting larvae from Dedza, Bembeke area central Malawi. Dedza is an area where cabbage is commonly grown and the diamondback moth problems are not scarce. The larvae were collected from a farmer's field and reared at Bunda College campus biotechnology laboratory on fresh cabbage plants until pupation. Insects were collected from Dedza bi weekly to sustain the colony. Adults were fed 10% sugar solution and were reared in 30x30x30 bioquip plastic cages.

Test compounds

Technical grade transfluthrin and pyrethrum compounds with at least 95% purity were used for the study. The test compounds were diluted in acetone as a carrier solvent. In preliminary behavioral studies, transfluthrin caused knockdown in the behavioral assays, as such it was used at a slightly lower concentration (volume/volume) 10^{-3} as opposed to pyrethrum which was diluted at a higher concentration 10^{-2}. These concentrations were maintained throughout the whole study.

Behavioral assays

i. T-Maze trap assay

T-Maze assays for *Drosophila melanogaster* (Stensmyr et al., 2012) and (Ebrahim et al., 2015) were modified to examine repellency of pyrethrum and transfluthrin on lesser grain borer (Fig 5.1). Insects were gently released into the set up through the delivery tube (A) into the decision tube (B). Once all the insects were released into the decision tube they were left for 60 minutes to make a choice. The assay was designed in such a way that once the insect makes a choice and goes into either of the holding cups (C) it has limited opportunity to walk back into the decision tube. The holding cups contained filter papers (D1 or D2) which were treated with either 100 µl of acetone or a test compound. Twenty beetles were used per assay. Each assay was repeated atleast five times with different cohorts of insects. The experiments were conducted in a room with temperature of 25°C and 60% relative humidity.

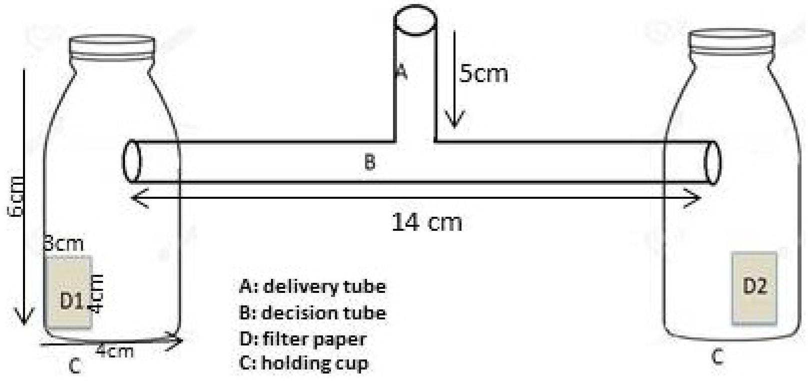

Figure 5.1 T-Maze trap assay set up for *Sitophilus zeamais* (LGB). (A) Showing a delivery tube, both males and females were used in the assay. (B) Showing a decision tube; insects walk freely into the decision chamber to make a choice, insects are left to make a choice for at least 60 minutes, after which any insects remaining in the decision tube are scored as undecided. (C) Showing a holding cup and (D1-D2) showing filter papers (3x4cm) loaded with either 100 µl of acetone or test compound.

ii. Two-choice repellency assay

The two choice repellency assay was set using three world health organization (WHO) bioassay test tubes. The three tubes were connected together to make; a decision tube (A in Fig. 5.2), and choice tubes on each side (B1 and B2 in Fig 5.2). Diamondback moth adults (5males and 5 females) were released into the decision tube and left in the behavioral test room (25^{0}C; 60% relative humidity) for 30 minutes to acclimatize. Later the choice tubes with filter papers loaded with acetone (for control) or test compound as treatment were connected. Soon after the choice tubes were connected, doors (C in Fig. 5.2) on both sides of the decision tube were opened to allow the insects move and make their choice. After 30 minutes, once the choices were made, the doors were closed and insects that remained in the decision tube were considered undecided.

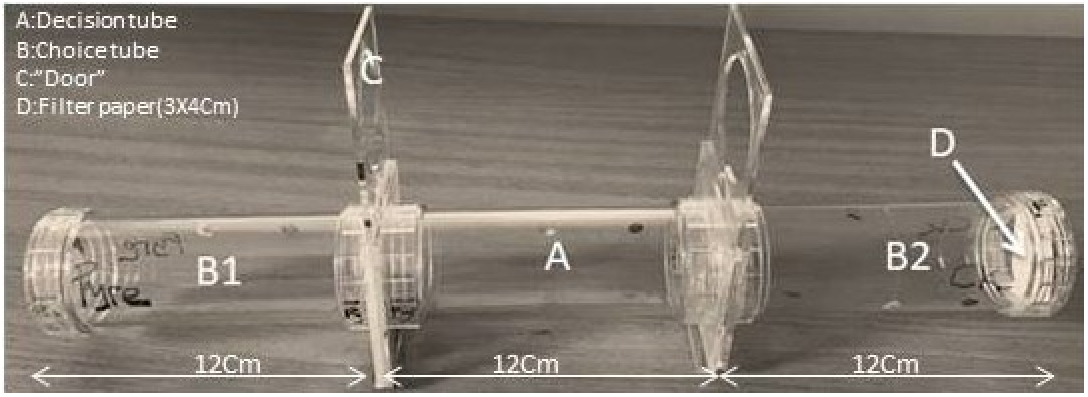

Figure 5.2 Two choice assay setup for adult diamondback moth insects. (A) Showing a decision tube which holds both male and female diamondback moths. (B1-B2) Showing choice tubes containing filter papers either loaded with acetone (as control) or test compound (pyrethrum or transfluthrin) diluted in acetone. (C) Showing decision tube doors and (D) side view of a filter paper loaded with acetone on B2, the decision tube B1 also contains a filter paper loaded with a test compound pyrethrum.

iii. Larvae feeding preference assay

Third instar larvae of diamondback moths, starved for 12 hours, were used in the assay. Two plastic sample bottles, 6cm high and 4cm in diameter on the base were used in the assay as feeding arenas (Fig. 5.3) The bottles (used as a feeding arena) into which the leaves were placed were cleaned to certainty first with water then with 95% alcohol and left to dry. A filter paper, once loaded with either acetone or a test compound, was placed inside the cover of the bottle to hold it and the bottle (the feeding arena) with the leaf and filter paper were placed in a feeding chamber (Fig. 5.3). Discs (4cm in diameter) were cut from a freshly harvested leaves and kept in distilled water to maintain freshness until they were used for experiments. Then, 12 hour starved, 3rd instar larvae were carefully released into the middle of the feeding chamber using a camel brush. The feeding chamber was placed in an experimental room with at least 25^0C and 60% relative humidity for 24 hours. Results were observed the next day. Number of larvae in each of the feeding arena was counted; those that remained in the feeding chamber were considered as undecided.

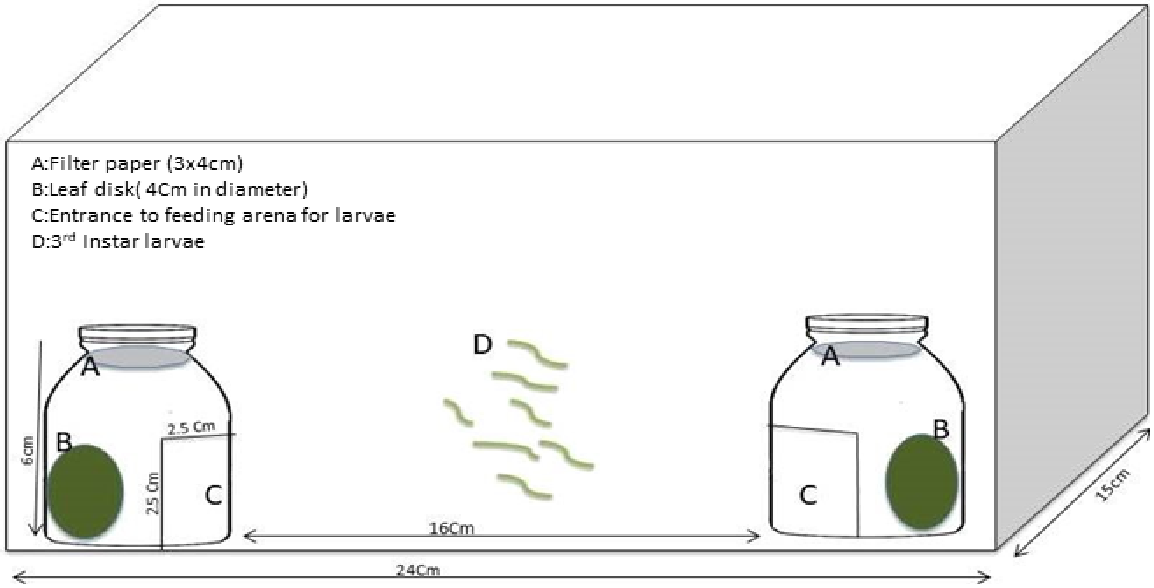

Figure 5.3 Feeding preference assay set up for diamondback moth larvae. The assay was carried out in a feeding chamber (30cmx15cmx10cm). (A) Showing a filter paper treated (3x4cm) with either acetone (for control) or test compound (transfluthrin or pyrethrum) as treatment. (B) Showing a leaf disc of a cabbage plant (4cm in diameter). The leaf was cleaned with clean water and had no contact with the test compounds. (C) Showing an arena window for entrance of larvae that has made a choice and (D) Diamondback moth larvae; 3rd instar, n=10, released in the middle of the feeding chamber.

Data analysis

The response index/repellency index (RI) from all the behavioral assays was calculated following modified methods by (Stensmyr et al., 2012). In brief the indices were calculated as (Ta-Tb)/T, where Ta is the number of insects in the treatment and Tb number of insects in the control(acetone), and T is the number of insects that participated in the trial (Ta+Tb). The index ranged from -1 to +1, where the latter signified complete avoidance (repellency) and the former, attraction. The RI were analyzed using Students't-test, with α=0.05. For larvae feeding preference assay, further analysis of the images of the leaf discs remaining after 24 hours of feeding in the feeding arenas, were captured and processed in Image J software to calculate the leaf area left. The area of the leaf discs remaining either in control arena or treated arena were calculated and subjected to Student's t-tests with α=0.05.

Results

Repellency effect of pyrethrum and transfluthrin on *Sitophilus zeamais* (Lesser gain borer)

To assay the behavioral effects of exposing lesser grain borer (LGB) to pyrethrum (10^{-2} dilution), a T-Maze trap assay was used (Fig 5.1). Adult LGB, were used in cohorts of 20 released into the decision tube. Notably, when the beetles were released, they oriented towards the untreated arena of the maze, steering their course away from the treated one. Similar experiments were repeated using a 10^{-3} transfluthrin dilution. Exposed LGB, displayed a similar pattern orienting towards the untreated arena, depicting a negative response from transfluthrin. Although transfluthrin was used at a lower concentration than pyrethrum, results still revealed significantly high percent response towards the control arena denoting that in both cases, pyrethrum and transfluthrin are effectively repelling the LGB.

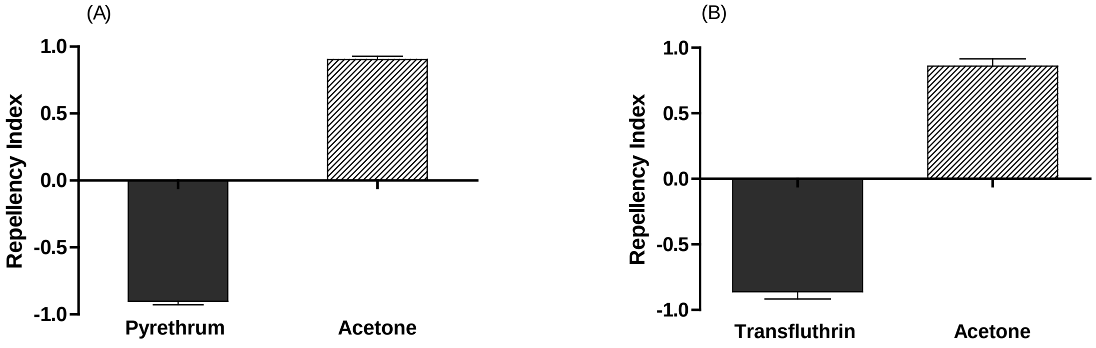

Figure 5.4 Pyrethrum and transfluthrin repel *Sitophilus zeamais* (lesser grain borer) in a T-maze assay. (A) Showing repellency index due to pyrethrum in lesser grain borer (LGB). Negative repellency index denote avoidance/ or an orientation away from the arena and positive index denote attraction to the arena. (B) Showing repellency of transfluthrin against LGB. Both males and females tested in the T-maze assay, 20 insects released in the decision tube (A in Fig 5.2). Experiments replicated 5 times, with 4 experimental units a day, total insects used in the experiment; n=400. Treatments significantly different from each other (P<0.0001), Student's T-tests (α=0.05).

Table 5.1 Percent response[2] of *Sitophilus zeamaise*[1] when pyrethrum and transfluthrin were used in a T-maze choice trap assay

Treatment	Difference[3]	t-value	95% CI of diff	P value	Summary[3]
Acetone vs pyrethrum	87.50	41.23	82.26 to 92.74	P<0.0001	***
Acetone vs undecided	89.25	42.05	84.01 to94.49	P<0.0001	***
pyrethrum vs undecided	1.750	0.8246	-3.485 to 6.985	P>0.05	ns
Treatment	**Difference[2]**	**t-value**	**95% CI of diff**	**P value**	
Acetone vs transfluthrin	85.25	26.15	77.21 to 93.29	P<0.0001	***
Acetone vs undecided	91.50	28.07	83.46 to 99.54	P<0.0001	***
transfluthrin vs undecided	6.250	1.917	-1.791 to 14.29	P>0.05	ns

[1] *Sitophilus zeamais* (Lesser grain borer) adults

[2] Percent response denotes a proportion of insects steering a course towards an arena.

[3]Difference (mean percent response) compares the number of insects that oriented to each of the arenas. The control attracted more insects than the treatment, hence the positive differences.

[4] ANOVA Bonferroni posttests (α=0.05); *** = P<0.001, ns= not significantly different

Repellency effect of pyrethrum and transfluthrin on *Plutella xylostella* (Diamondback moth)

Pyrethrum and transfluthrin elicited a repellency behavior when tested on diamondback moth two choice set up (Fig 5.2). Diamondback moths oriented towards the untreated arm than the treated arm containing a filter paper loaded with the test compound. For transfluthrin and pyrethrum, insects preferred the untreated side, hence the negative response index for both compounds (see table 5.3). In this experiment, transfluthrin was used at a lower dose (10^{-3}) compared to pyrethrum (10^{-2}) to keep the flying insects from knockdown effect. Insects that did not make a choice after 60 minutes of the experiment were considered undecided.

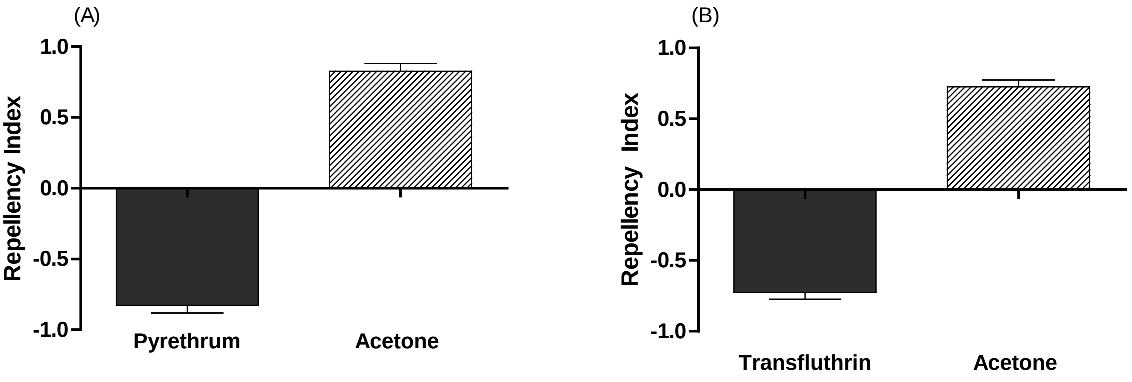

Figure 5.5 Pyrethrum and transfluthrin repel *Plutella xylostella* (diamondback moth) in a choice assay. (A) Showing repellency index in pyrethrum against diamondback moth (DBM) in a choice assay using WHO bioassay test tubes. Negative index denote avoidance in the insect, from the arena and positive index denote attraction to the specific arena. (B) Showing repellency index in transfluthrin against DBM. Five males and females tested in the choice assay, 10 insects released in the decision chamber. Experiments replicated 5 times, with 3 experimental units a day, total insects used in the experiment; n=300. Treatments significantly different from each other ($P<0.0001$), Student's T-tests ($\alpha=0.05$).

Table 5. 2 Percent response[2] of adult *Plutella xylostella*[1] when pyrethrum and transfluthrin were used in two choice test.

Treatment	**Difference[3]**	**t-value**	**95% CI of diff**	**P value**	**Summary[4]**
Control vs pyrethrum	66.67	10.35	49.32 to 84.02	P <0.0001	***
Control vs undecided	83.33	12.94	65.98 to 100.7	P <0.0001	***
pyrethrum vs undecided	16.67	2.588	-0.6828 to 34.02	P >0.05	ns

Treatment	**Difference[2]**	**t-value**	**95% CI of diff**	**P value**	Summary[3]
Control vs transfluthrin	70.00	11.74	53.94 to 86.06	P <0.0001	***
Control vs undecided	80.00	13.42	63.94 to 96.06	P <0.0001	***
transfluthrin vs undecided	10.00	1.677	-6.062 to 26.06	P >0.05	ns

[1] *Plutella xylostella* (Diamondback moth) adults (5 males and 5 females)

[2] Percent response denotes a proportion of insects steering a course towards an arena.

[3] Difference (mean percent response) compares the number of insects that oriented to each of the arenas. The control attracted more insects than the treatment, hence the positive differences.

[4] ANOVA Bonferroni posttests ($\alpha=0.05$); *** = $P<0.001$, ns= not significantly different

Pyrethrum and transfluthrin repel DBM larvae and reduce feeding on leaf discs in treated arena

Having assayed the repellency effect of pyrethrum and transfluthrin on adult DBM, the following studies sought to explore whether the test compounds would have repellent activity on the larvae and affect their feeding behavior. Results in this study confirmed significant differences in the percent response of the larvae, when released in the feeding chamber. Calculated response indices for both compounds revealed a negative response for the treated arenas compared to untreated ones (Fig 4.5 A-B). Calculated leaf areas using image J, revealed larger leaf areas remained in treated arenas compared to untreated ones(Fig 4.5 C-D and Fig 4.6)

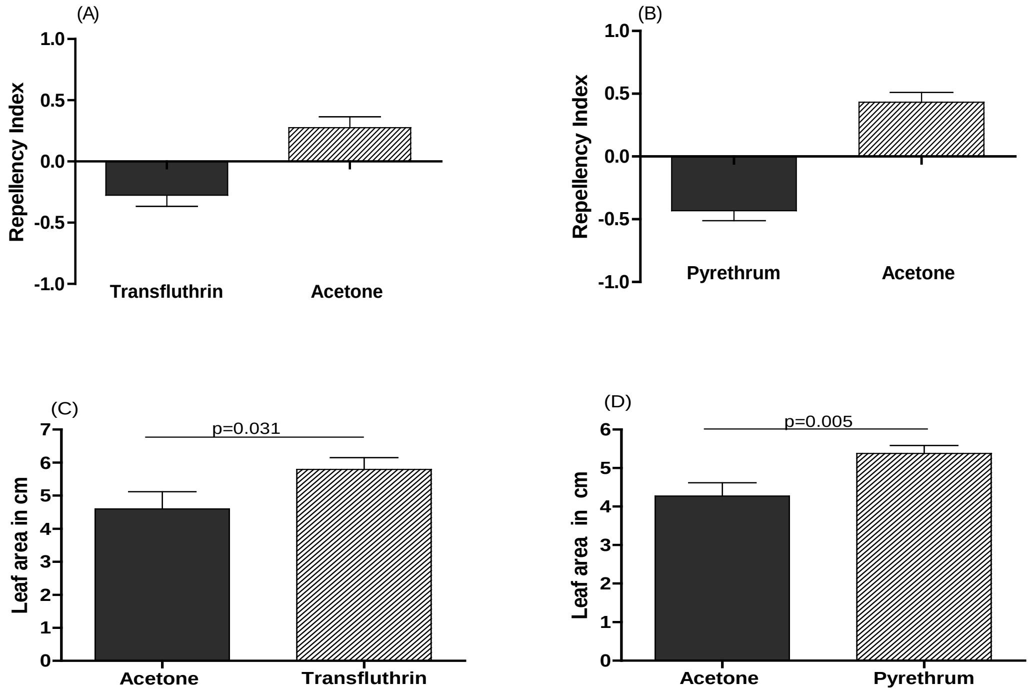

Figure5.6 Pyrethrum and transfluthrin repel and reduce feeding in *Plutella xylostella* (diamondback moth) larvae in a feeding choice assay. (A-B) Showing repellency index of transfluthrin and pyrethrum against diamondback moth larvae. (C-D) Showing the remaining leaf area in the feeding arenas, after 24hrs of the feeding trial. Experiments replicated 5 times, with 4 experimental units a day for each of the compounds. Six-twelve hour starved larvae, cohorts of 10, used per unit; n=400 for the whole experiment. Student's T-tests (α=0.05).

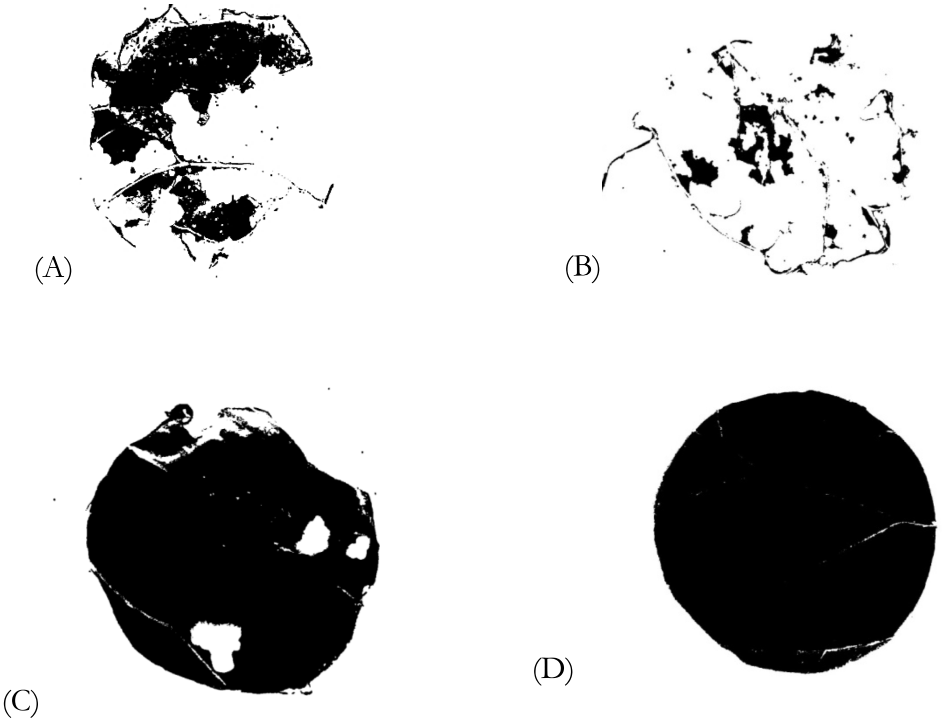

Figure5.7 Reduced feeding in pyrethrum and transfluthrin arenas in the choice feeding assay against DBM larvae. (A-B) Showing the leaf area left (dark shaded spots) in untreated arenas without pyrethrum and transfluthrin. (C-D) Showing the leaf area left in pyrethrum and transfluthrin treated arena .Ten 3rd instar larvae released into the feeding chamber. Faded regions of the leaf disc image depict the areas eaten by the larvae. Leaf image analyzed using ImageJ software.

Table 5.3 Percent response[2] of *Plutella xylostella*[1] larvae when pyrethrum and transfluthrin were used in a larvae feeding preference assay

Treatment	**Difference[3]**	**t-value**	**95% CI of diff**	**P value**	**Summary[4]**
Control vs pyrethrum	41.00	7.712	27.89 to 54.11	P <0.0001	***
Control vs undecided	61.50	11.57	48.39 to 74.61	P <0.0001	***
pyrethrum vs undecided	20.50	3.856	7.386 to 33.61	P <0.0001	***
Treatment	**Difference[2]**	**t-value**	**95% CI of diff**	**P value**	Summary[3]
Control vs transfluthrin	27.50	4.881	13.60 to 41.40	P <0.0001	***
Control vs undecided	54.50	9.674	40.60 to 68.40	P <0.0001	***
transfluthrin vs undecided	27.00	4.793	13.10 to 40.90	P <0.0001	***

[1] *Plutella xylostella* (Diamondback moth) larvae

[2] Percent response denotes a proportion of insects steering a course towards an arena.

[3] Difference (mean percent response) compares the number of insects that oriented to each of the arenas. The control attracted more insects than the treatment, hence the positive differences.

[4] ANOVA Bonferroni posttests (α=0.05); *** = P<0.001, ns= not significantly different

Discussion

In most parts of Africa, insecticide use remain important in pre- and post-harvest crop protection, although a number of studies have reported some occupational hazards due to overuse and misuse of pesticides in some parts of the continent (Naidoo et al., 2013). Repellency studies on insects of agricultural importance, due to sub lethal compound exposures are rare. This study was inspired by the efficiency of a low cost technology that uses transfluthrin hessian sacks to control outdoor biting mosquitoes (Govella, Ogoma, Paliga, Chaki, & Killeen, 2015; Ogoma et al., 2012, 2017). The observed behavioral responses of LGB when exposed to pyrethrum were consistent with responses to transfluthrin. More insects oriented towards the untreated arm than the treated one reinforcing the notion that pyrethrum and transfluthrin evoked repellency behavior in the LGB.

A closely related study focused on the use of permethrin treated nets to protect maize in storage(Anaclerio et al., 2015) targeting the contact toxicity of the permethrin treated net, to reduce the penetration effect of insects from one infected storage bag to uninfected one. Concurring with a recent study (Barbosa et al., 2017) which demonstrated the LGB avoided surfaces treated by deltamethrin more than spinosyns. They attributed contact irritancy to be the underlining cause of the avoidance behavior. Contrary to the findings of this study, our study has demonstrated the ability of pyrethrum and transfluthrin to repel LGB without getting in contact with treated arenas. Although our study did not look at the specific olfactory receptors responsible and did not repeat the experiments on LGB insects with their Orco co-receptors knocked out; earlier studies have demonstrated reduced repellency in Orco mosquitoes. Thus, in the current study, the possibility of olfaction mediated repellency behavior in LGB may not be overruled.

In addition to suggesting the olfactory mediated avoidance behavior, because of the volatile nature of pyrethrum and transfluthrin, neuro-physiological excitation of the LGB due to sodium channel activation when exposed to the sublethal vapors could also influence the resultant avoidance behavior of insects from the treated arm and orientation to the untreated arm. When extended to a field setup, this study presents an opportunity of decreasing pesticide load in maize storage by decreasing LGB infestations in storage facility not only through contact toxicity, but also the ability of the compounds to steer insects away from the storage facilities without contact. This may enhance push and trap of LGB in the storage facilities.

Apart from the LGB the current study further explored repellency due to pyrethrum and transfluthrin on diamondback moth adults in absence of attractant odors from a host plant. The increased response to the untreated arm of the test tube confirmed the ability of the transfluthrin and pyrethrum to elicit avoidance behavior in diamondback moth. Studies have investigated the roles of olfaction on diamondback moth host finding(Couty et al., 2006). Others have focused the role of chemo sensation on diamondback moth responses to natural enemies (Reddy et al., 2002) yet studies on olfactory mediated behaviors due to pyrethroid exposure are rare. Our laboratory trial on repellency and transfluthrin and pyrethrum, has demonstrated the ability of the test compounds to elicit repellency behavior on diamondback moth, suggesting the need to further explore the repellency effect in semi-field and field trials.

In addition to the adult insects, our observation on repellency effect of pyrethrum and transfluthrin on diamondback moth larvae in a feeding choice assay, confirm how repellency may not only be important to adult diamondback moth, but also in larval foraging behavior. The orientation of the larvae to the untreated arena more than the treated one in the feeding preference assay, depicts chemo sensation is not exclusively used in the adult diamondback moth insects. The effect of non-plant host odors such as deterrents on diamondback moth larvae using leaf discs that have been dipped in a compound of interest was demonstrated by (Guangli et al.,2011). Earlier review by (Ramaswamy, 1988), gave a detailed description of sensory modalities and behaviors in moths, diamondback moth included; highlighting findings by Diether (1982) that; host recognition and preference of moths could be complex. It may involve the complex neural and metabolic processes. Thus studies using feeding preference experiments with the substrate treated with a test compound, may be complex to interpret in such a way that it may be difficult to deduce the cause of the released behavior of the larvae since the larvae may have to come into contact with the treated substrate and the resultant behavior could either be acceptance, rejection and or deterrence not necessarily repellency. The results observed in the feeding preference experiments in this current study, were exclusively due to odor cues and not contact of the insects with the treated surface. The increased response to the leaf disc in the untreated arena than the one treated with either pyrethrum or transfluthrin; depict the importance of olfactory cues in the choices of food sources for the larvae diamondback moth.

The leaf area analysis revealing a small leaf area remaining in the untreated arena, compared to the treated ones for both compounds; pyrethrum and transfluthrin, depict that the larvae were not only repelled by the test compounds, but they also accepted the leaf in the untreated arena more than the one in the treated arena. These results confirmed, the repellency elicited by the test compounds steered the insects more into the untreated arena suggesting that repellency may have an important role in controlling crop damage, not just by steering the insects away but also reducing the crop damage itself. Although this claim warrants further exploration of the actual larvae olfactory receptors involved, it echoes a promising role repellents may play in the future control of crop pests to reduce pesticide overload in the environment.

Conclusion

These results have emphasized the importance of repellents in reducing food losses. Although repellents have mainly featured in public health, from this study, it seems they may be important in reducing insect-host contact, and reduce direct damage on crops. This study has shown that pyrethrum and transfluthrin can elicit repellency in *Plutella xylostella* and *Sitophilus zeamais*. Normally, prevention of food losses in most parts of the world, Africa specifically, involve repeated application of pesticides directly on the food products. These practice results into pesticide overload on food crops and may cause health hazards. The steering away of the test insects from treated arenas in this study presents a promising opportunity of reducing pesticides application on food. Additionally, the reduced feeding in diamond back moth larvae in arenas treated with pyrethrum and transfluthrin suggest that the repellency mechanism is not limited to adult insects only but also larvae. Further studies will test the extent to which these repellents can reduce food losses in semifield studies. Studies to test more pyrethroids and to identify olfactory receptors responsible for this repellency behavior in *Sitophilus* and *Plutella xylostella*, might be also be important.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Pyrethroids are compounds that are structurally derived from pyrethrins and they make up almost 17% of the insecticides that are sold on the market(Sparks, 2013). Pyrethrum and pyrethroids are well known for their insecticidal activity upon contact with insects through their action on the voltage gated sodium channel(Davies et al., 2007; Dong et al., 2014; Du et al., 2011). A growing body of literature has documented from behavioral assays that pyrethrum and pyrethroids induce repellency(Bibbs & Kaufman, 2018; Bowman et al., 2018; Chareonviriyaphap et al., 2004; Govella et al., 2015; Kawada et al., 2006; Ogoma et al., 2014; Sathantriphop et al., 2014). This study was conducted to elucidate the mechanism of pyrethrum and pyrethroid repellency in mosquitoes and agricultural pests. In an attempt to find out the mechanism of repellency, we utilized behavioral assays and electroantennogram recordings. We used different strains of *Aedes aegypti* mosquitoes that are pyrethroid susceptible and pyrethroid resistant (kdr and P450 mediated mechanisms). We also tested repellency on *Anopheles gambiae*, Kisumu strain. An *orco* mutant *Aedes aegypti* mosquito made from Orlando strain was used to verify the involvement of olfactory receptors. Our study used DEET as a positive control since it is a well-known mosquito repellent. We further explored repellency effect of pyrethrum and transfluthrin on agricultural insect pests, *Sitophilus zeamais* and *Plutella xylostella.*

The data in our study suggest that pyrethrum and pyrethroids evoke repellency behavior in mosquitoes. Our Hand in cage behavioral assay was designed in such a way that the mosquitoes do not get in contact with the treated surfaces so that we could exclusively test involvement of olfactory receptors in the resultant behavioral stimuli. Landing frequency transformed into repellency percentage was compared between compounds and mosquito strains. Unexpected, our study showed enhanced repellency due to pyrethrum and pyrethroids in pyrethroid-susceptible mosquito strains than in resistant ones. This difference between strains was not observed when DEET was tested. It was expected that if olfactory receptors were exclusively involved in the repellency effect of pyrethrum and pyrethroids, there would not be overt differences in the repellency levels between resistant and susceptible strains. This suggests that sodium channel activation also plays a role in the repellency. Interestingly the abolished and/or reduced repellency due to pyrethrum and pyrethroids in pyrethroid susceptible *orco* mutants when compared to a susceptible isogenic strain in the behavioral assays still signified the importance of olfactory receptors in the repellency mechanism. Our electroantennogram studies in the mosquito antenna showed that olfactory receptors were activated by pyrethrum and pyrethroids. These findings echo the importance of both olfactory receptors and sodium channel action in the repellency due to pyrethrum and pyrethroids. The details on the extent to which olfactory processing and sodium channel activation contribute to pyrethroid repellency in mosquitoes remain to be investigated.

In addition, our study has shown enhanced repellency and knock down effect of transfluthrin when mosquitoes are pretreated with piperonyl butixide (PBO). We have shown that inhibiting P450 activity in mosquitoes enhances transfluthrin repellency and toxicity. These studies have laid out a foundation for further investigation of the effect of P450 activity inhibition on transfluthrin repellency. It should be noted that these results cannot be generalized for all pyrethroids, they may vary. Future studies will consider testing this effect of P450 inhibition on repellency using different kinds of pyrethroids.

One of the ways through which pyrethroids have been used to combat vector borne diseases, is their incorporation in the insecticide treated nets. Their mechanism of action is still debatable. Our study has shown that the deltamethrin treated net (PermaNet2.0) and permethrin treated net (Olyset) repel mosquitoes and the magnitude of repellency may vary from one species to another. It should be emphasized that based on our findings, the repellency of the bed net may also vary from one net type to another. It would seem unlikely that the pyrethroid treated nets, especially the permethrin and deltamethrin treated nets would elicit repellency in mosquitoes because permethrin and deltamethrin are not very volatile. Our study has shown that the repellency effect is not limited to very volatile pyrethroids. Infact, our behavioral experiments showed repellency due to permethrin in corroboration with electroantennogram studies that showed a robust response. Why permethrin treated net showed reduced repellency than deltamethrin treated net, yet behavioral assay and electroantennogram recordings showed overt responses would depend on several factors, including; how the compound is incorporated in the nets as well as the material of the net used. Thus it should be noted that these results cannot be generalized on all bed nets and all mosquito species. Based on the observed variations in these results, further studies will test repellency of several pyrethroid treated net types that are being used.

Further, our study has revealed the potential of pyrethroid repellency in reducing food losses in laboratory experiments. Normally, insecticides such as pyrethroids are used in sprays and not as repellents in the control of insect crop pests. In our study, both pyrethrum and transfluthrin have shown the ability to steer insects away from the treated arenas without physical contact. Future studies on pyrethroid and pyrethrum repellency on agricultural insect pests, will attempt replicating the laboratory studies under field and semi field studies. The studies will also examine the impact of pyrethrum and pyrethroids on oviposition behavior of agricultural pests, more especially the diamond back moth.

Overall, our studies on mosquitoes and agricultural pests present a great step forward in exploring the use of pyrethrum and pyrethroids as repellents in the control of insect pests and vectors without overloading the environment with pesticides.

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