Symbiont-Mediated Modification of Mosquitocide Toxicity in the Yellow Fever Mosquito, *Aedes aegypti* (Diptera: Culicidae)

Sara Stuart Scates

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Troy D. Anderson
Zachary N. Adelman
Carlyle C. Brewster

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ABSTRACT

The incidence of mosquito-borne human diseases is increasing worldwide, with effective chemical control limited due to widespread insecticide resistance in the insect. Recent evidence also suggests that bacterial symbionts of mosquitoes, known to be essential in nutritional homeostasis and pathogen defense, may play a significant role in facilitating mosquitocide resistance. Here, I examined the metabolic detoxification and toxicity of two mosquitocides, propoxur and naled, and the capacity of bacterial symbionts to modify the detoxification of the mosquitocides and, thus, alter their toxic action in the yellow fever mosquito, *Aedes aegypti*. The insecticide synergists piperonyl butoxide (PBO), triphenyl phosphate (TPP), and *S,S,S*-tributyl phosphorotrithioate (DEF) were used to examine the metabolic detoxification and toxic action of the two mosquitocides in mosquito larvae. A significant increase in the toxicity of propoxur was observed when applied in combination with PBO; however, there was no corresponding decrease in AChE activity. Naled applied in combination with PBO resulted in a decrease in anticholinesterase activity (higher residual AChE activity) and a subsequent decrease in toxicity of the insecticide. This suggests that esterases play a major role in the metabolic detoxification of both insecticides in mosquito larvae. The acute toxicities of naled and propoxur to *Ae. aegypti* larvae were also studied following a reduction of bacterial symbionts with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin. Antibiotic-treated mosquito larvae showed increased susceptibility and a reduction in cytochrome P450 monooxygenase and general esterase activities when treated with naled and propoxur. A reduction of bacteria in mosquito larvae treated with broad-spectrum antibiotics, therefore, appears to affect the metabolic detoxification of standard-use mosquitocides, such as propoxur and naled. The results also suggest that the bacteria themselves may contain metabolic detoxification enzymes that are functionally similar to

those in the mosquito larvae. Additional experiments, however, are needed to fully elucidate the contribution of bacterial symbionts in *Ae. aegypti* larvae in the metabolic detoxification of mosquitocides.

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Chapter 1

LITERATURE REVIEW

1.1 Public Health Impact

Mosquitoes affect millions of people worldwide as a result of their ability to vector a number of infectious microorganisms. Dengue fever, a disease that is suspected to be almost 1,000 years old, has reemerged in the past 35 years with devastating effects. The viruses themselves, as well as the principal mosquito vector, *Aedes aegypti*, has increased in geographic distribution due to demographic and societal changes such as population growth, urbanization, and advances in modern transportation (Gubler et al. 2002). Today, dengue is the most important mosquito-borne viral disease that affects humans (CDC 2010). It infects almost 400 million people each year and an estimated 3 billion people or 40% of the world's population are at risk for epidemic transmission (Bhatt et al. 2013). The dengue mosquito, *Ae. aegypti*, is found in almost 100 tropical countries with numbers increasing significantly each year. The increasing incidence of the more severe manifestation of dengue virus, dengue hemorrhagic fever, the simultaneous circulation of more than one viral serotype, and the absence of effective vaccines and treatment are factors that make dengue a serious public health concern (Gubler et al. 1998). The elimination of larval habitats and application of mosquitocides remains the primary approach for limiting virus transmission of *Ae. aegypti* (Morrison et al. 2008).

Dengue is an emerging disease. The four dengue viruses DEN 1,2,3, and 4 were first seen in monkeys and jumped to humans in Africa or Southeast Asia sometime

between 100 and 800 years ago. Until the mid-20th century, dengue remained a somewhat minor, geographically restricted disease but the disorder of World War II - mainly the unintentional transport of *Aedes* mosquitoes world-wide via cargo - are thought to have played a pivotal role in the distribution of the viruses (CDC 2010). Dengue hemorrhagic fever was first recognized only in the 1950s during epidemics in the Philippines and Thailand. However, It was not until 1981 that large numbers of DHF cases began to appear in the Caribbean and Latin America, where highly effective *Aedes* control programs had been in effect until the early 1970s (CDC 2010).

1.2 Vector Control

1.2.1 Environmental Management

The principal vector of dengue viruses, the *Aedes aegypti* mosquito, should be the primary target of surveillance and control activities. The most efficient way to control the mosquitoes that transmit dengue is through environmental management, mainly larval source reduction, i.e., elimination or cleaning of water-holding containers that serve as the larval habitats for *Ae. aegypti* in the domestic environment (Gubler et al. 1998). When container habitats are removed and water storage containers are covered to prevent mosquitoes from getting inside them, mosquitoes have fewer chances to lay eggs and cannot develop through their aquatic life stages. Source reduction can be especially effective when performed consistently, particularly when members of a community are mobilized and educated about the importance of vector control (WHO 2009). Other

environmental management initiatives focus on changes within the community- that is, improving water supply and storage so that mosquito-breeding habitats are eliminated. People can also reduce contact with mosquito populations by keeping their houses cool and closed up during the summer months, by wearing proper attire (e.g. long sleeves and long pants) when spending time outdoors, and wearing mosquito repellants that contain DEET, picaridin, lemon eucalyptus oil, or IR3535 as the active ingredient.

1.2.2 Biological Control & Genetic Manipulation

Biological approaches are also being considered as alternatives to control mosquito populations. Predatory crustaceans, fish, turtles, dragonflies, and other small insects have all been suggested to prey on insect larvae, thus effectively preventing mosquito development. Dengue is also a suitable target for genetic vector control strategies as it is specific to humans and has no significant animal reservoirs, has a single dominant vector, and widespread vector control programs have previously shown to be effective in controlling this disease, although those methods are now unacceptable or have not been sustainable (Alphey et al 2011).

Approaches utilizing a Wolbachia strain of bacteria have also been the focus of much research. Dr. Scott O'Neill and his colleagues at the University of Queensland, Australia showed that when *Wolbachia* are introduced into mosquitoes, they interfere with pathogen transmission and influence key life history traits such as lifespan (Hoffmann et al 2011). Their findings demonstrated that *Wolbachia*-based strategies can be deployed as

a practical approach to dengue suppression with the potential for area-wide implementation (Hoffmann et al 2011).

1.2.3 Chemical Control

Chemical control is another effective measure for controlling *Ae. aegypti* mosquitoes. The *Aedes* nervous system is a proven target site for high efficacy chemistries, including organophosphate-, carbamate- and pyrethroid-class mosquitocides. These chemistries work to kill mosquitoes by targeting acetylcholinesterase (AChE) and voltage-gated sodium channels (VGSC), respectively. The carbamate and organophosphate mosquitocides are AChE inhibitors that cause acetylcholine accumulation at the synaptic regions of cholinergic nerve endings resulting in excessive stimulation of cholinergic receptors leading to paralysis and death of the mosquito (Casida et al. 2004). The pyrethroid mosquitocides target the VGSC to prolong the course of sodium current depolarization thereby inducing a residual slow acting current that results in uncontrolled and persistent excitation of the neurons (Soderlund and Bloomquist 1990). Chemical control applications usually target specific life stages of mosquito development and are thus broken down into either larvicides (targeting larval life stage of development) or adulticides (targeting adult stage of development).

Larvicides should be used only to supplement environmental management and, except during emergency situations, should be limited to hard-to-manage containers (WHO, 2009). Currently there are several categories of larvicides available for use, these include: Insect Growth Regulators (IGRs), microbial larvicides, organophosphates (OPs), and

surface oils and films. Within each larvicide classification, there are a number of products and formulations. Method of application, application rates, and suggested treatment sites may differ from product to product as well (Connelly et al. 2009).

Adulticides target adult vectors and are intended to reduce mosquito densities, and effect mosquito longevity and factors relating to transmission. Adulticides can be applied as residual surface treatments or as space treatments in emergency situations. Insecticide treated materials (ITMs), most often seen in the form of insecticide treated bed-nets, have been shown to be highly effective in preventing diseases transmitted by nocturnally active mosquitoes. Therefore, current research initiatives are focusing on the efficacy of ITMs in controlling the diurnally active *Ae. aegypti* (WHO 2009). Ultra low volume (ULV) aerial spraying is often used in areas with known arboviral activity (Duprey et al. 2008). The organophosphate naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate) has been applied in large scale mosquito control programs via aircraft mounted sprayers using naphtha, an inert carrier. These ULV mosquitoide applications aerosolize into small droplets and remain aloft to kill mosquitoes upon contact (Duprey et al. 2008). Propoxur (2-isopropoxyphenyl methylcarbamate) is a carbamate mosquitocide that is most commonly applied via indoor residual spraying and is found to be effective in areas where mosquitoes were found to be resistant to organophosphate mosquitocides or as a DDT replacement in countries with high incidence of malaria (Sanil and Shetty 2010).

While highly effective, both metabolic and target-site resistance limits the use of these mosquitocides for the control *Aedes* mosquitoes (Vontas et al. 2012). A new public

health mosquitocide has not been produced for *Aedes*-borne disease control in over 30 years (Hemingway et al. 2006, Meyer et al. 2012) and, thus, there is an urgent need to better understand mosquitocide metabolism and resistance as a pre-requisite to enhancing the efficacy of these current-use chemistries for the control of vector mosquitoes. This resistance must be considered as a potentially serious threat to effective dengue control and routine monitoring of mosquitocide susceptibility should be integrated into control programs (WHO, 2009).

1.3 Mosquitocide Resistance

Insecticide resistance can be defined as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (IRAC). It is important to emphasize that insecticide use does not create resistance, but rather the misuse or overuse of insecticides can select for insects with resistant alleles. There are numerous biochemical and physiological barriers that affect the target site delivery of insecticides and, thus, contribute to insecticide resistance. These include cuticular penetration, metabolic detoxification, and target-site insensitivity.

1.3.1 Reduced Cuticular Penetration

Insecticide resistance due to reduced penetration is quite common among insect populations. Terriere (1982) proposed several possible mechanisms including a binding protein or lipid reservoir that is able to trap the insecticide in the cuticle, a degrading enzyme located within the cuticle or even a thicker more impermeable cuticle (Yu 2008).

These mechanisms have been implicated in organochlorine, organophosphate, carbamate and pyrethroid resistance in tobacco budworm, house flies, cotton bollworm and several other insect species (Vinson and Law 1971; Patil and Guthrie 1979; Ahmad et al. 2006).

1.3.2 Metabolic Detoxification

Every animal has detoxifying enzymes for components of the diet that may also be effective for xenobiotics. These enzymes recognize and transform functional groups to reduce reactivity and increase polarity (Phase I) or conjugate them to endogenous molecules for excretion (Phase II) (Casida et al. 2004) Three major enzyme groups are responsible for metabolic resistance to organophosphates, organochlorines, carbamates, and pyrethroids (Hemingway 2000). Glutathione *S*-transferase has been implicated in the development of mosquitocide resistance in both *Anopheles* and *Aedes* mosquitoes (Grant and Matsumura 1988; Prapanthadara et al. 1995). Esterases have been associated with organophosphate, carbamates, and less frequently, pyrethroid resistance. Cytochrome P450 monoxygenases have are involved in metabolic detoxification of pyrethroids, the detoxification as well as the activation of organophosphates, and less often carbamate resistance (Hemingway 2000).

1.3.2.1 Glutathione S-*Transferase-Based Resistance*

Gluthatione *S*-transferases (GSTs) are dimeric multifunctional enzymes that are involved in phase II biotransformation and detoxify a wide range of xenobiotics (Prapanthadara et al. 1996). These enzymes can generate resistance by conjugating reduced glutathione (GSH) to the insecticide itself or it's main toxic metabolite (Hemingway 2000). When

conjugated to GSH, the potentially toxic substrates become more water soluble and normally less toxic (Grant and Matsumura 1988). There are two classes of GSTs that have been classified according to their location within the cell: microsomal and cytosolic (Hemingway et al. 2004). These GSTs appear to have a role in pesticide resistance in arthropod pests, specifically organophosphate resistance in house flies, mosquitoes, and predaceous mites (Yu et al. 1996; Sun et al. 2001).

1.3.2.2 Esterase-Based Resistance

A wide range of insect pests have exhibited esterase-based resistance to organophosphate, carbamate, and pyrethroid insecticides (Field et al. 1988; Hemingway and Karunaratne 1998, Hemingway 2000). Most insecticides contain ester bonds and are therefore susceptible to hydrolysis by esterases during phase I biotransformation. These esterases work by rapidly binding to and slowly turning over the insecticide (i.e., sequestration, not hydrolysis) (Hemingway 2000). The overproduction of esterase genes via gene amplification has been implicated in enhanced degradation and sequestration of organophosphates, carbamates, and pyrethroids in green peach aphids (*M. persicae*), brown plant hoppers (*N. lugens*), and mosquitoes (*Culex* spp.) (Yu et al 2008). Esterases (Est) have been classified into two categories (A or B) on the basis of their inhibition by organophosphates. Currently, the terms Est-A and Est-B are used to distinguish between two groups of enzymes, such as those that hydrolyze organophosphates (Est-A) and those that are inhibited by organophosphates (Est-B) (Montella et al. 2012). The insecticide synergists *S,S,S*, tributyl phosphorotrithioate (DEF) and triphenyl phosphate (TPP) and have been routinely used in insecticide toxicity bioassays to provide a preliminary

assessment of the contribution of esterases in insecticide resistance (Koou et al 2014). The organophosphate esterase inhibitors TPP and DEF can inhibit several groups of insect hydrolases (Bernard and Philogene 1993). Both TPP and DEF can be competitive or non-competitive inhibitors of the general esterases that are involved in the metabolic detoxification of mosquitocides (Bernard and Philogene 1993). However, these synergists can affect the activity of the neurotransmission enyzme acetylcholinesterase in mosquitoes (Bernard and Philogene 1993).

1.3.2.3 Monooxygenase-Based Resistance

The cytochrome P450 monooxygenases (P450) are a highly intricate family of enzymes found in most organisms, including insects. These enzymes play a role in the metabolism of endogenous and exogenous compounds during phase I biotransformation. The P450s elicit *O*-, *S*-, and *N*-alkyl hydroxylation, aliphatic hydroxylation, ester oxidation, nitrogen oxidation, and thioether oxidation reactions in mosquito larvae. These P450-mediated reactions can either deactivate or activate the parent chemical structure of a mosquitocide in mosquito larvae. These proteins can affect the target-site activity of the mosquitocide and, in turn, alter its toxic action towards the mosquito larvae. There have been a number of reports establishing elevated P450 activities in mosquitocide-resistant mosquitoes (Vulule et al. 1999; Brogden et al. 1998; Hemingway et al. 1999). The methyldioxyphenyl synergists, such as piperonyl butoxide (PBO), are often used to characterize P450-mediated insecticide resistance. The synergist PBO can be a competitive or non-competitive inhibitor of the P450s that are involved in the metabolic detoxification or activation of mosquitocides (Omuro and Sato 1964). These synergists

form a complex with the P450s to prevent binding of the insecticide with the enzyme (Bernard and Philogene 1993).

1.3.3 Target Site Insensitivity

1.3.3.1 Insensitive Acetylcholinesterase

Carbamate and organophosphate mosquitocides both target acetylcholinesterase (AChE). Assays of several mosquito species have linked insensitive acetylcholinesterase to insecticide resistance. AChE hydrolyzes the excitatory neurotransmitter acetylcholine on the post-synaptic nerve membrane (Hemingway et al. 2000). Alteration of AChE in carbamate- and organophosphate-resistant insects leads to a decreased sensitivity to inhibition of the enzyme by these insecticides (Hemingway et al. 2000). In mosquitoes, only AChE1 seems to be involved in development of insecticide resistance (Hemingway et al. 2004). Insensitive AChE has been reported in *Culex* and *Anopheles* mosquitoes (Villani and Hemingway 1987; Ayad and Georghiou 1979; Hemingway and Georghiou 1983; Penilla et al. 1998; Bisset et al. 1990; Hemingway et al. 1985, 1986; Hemingway 1982).

1.3.3.2 GABA Receptors

Though resistance to dieldrin has been observed since the 1950s, the mechanism behind this resistance remained unknown until the 1990s. The GABA receptor in insects is a chloride-gated-ion channel and is suggested as a site of action for pyrethroids, avermectins, and cyclodienes (Hemingway et al. 2000). Binding of GABA to the receptor

leads to rapid gating of a fundamental chloride selective ion channel. A single substitution mutation from an alanine residue to a serine, or more rarely to a glycine, in the *Rdl* (resistance to dieldrin) gene has been recorded in all dieldrin-resistant insect species up to the present time (Hemingway et al 2004). This mutation appears to confer both insensitivity to the insecticide and decreased rate of desensitization.

1.3.3.3 Voltage-Gated Sodium Channels

Pyrethroid insecticides are known to have a rapid "knock-down" effect. The intense overuse of DDT and pyrethroids has led to the development of knock-down resistance *(kdr)* in many insect species (Liu et al. 2000, Soderland and Knipple 2003) The pharmacological effect of DDT and pyrethroids is development of persistent activation of sodium channels by modifying the normal voltage dependent gating mechanism of inactivation (Hemingway et al. 2000). *kdr* occurs because of a single or multiple substitutions in the sodium channel gate, which leads to a subsequent change in affinity between the insecticide and its binding site on the sodium channel (Hemingway et al. 2004). *kdr* resistance has been reported in both *Aedes* and *Anopheles* mosquitoes (Pinto et al. 2006; Brengues et al. 2003) .

1.3.4 Behavioral Resistance

The mechanisms of behavioral resistance are less clear than mechanisms of physiological resistance. Behavioral resistance is described as the ability to avoid a dose of insecticide that would otherwise prove lethal (Yu 2008). Behavioral resistance is primarily stimulus dependent and deals with hypersensitivity or hyperirritability (Yu 2008). It is suggested

that insects with behavioral resistance contain receptors that detect insecticides more efficiently than normal insects, and are therefore better able to sense to and avoid lower concentrations of insecticide than normal insects (Yu 2008). This has been documented in mosquitoes with DDT, horn flies with pyrethroids, fall armyworm with carbaryl, diamondback moths with fenvalerate, and more (Yu 2008; Sparkes et al. 1989; Young and McMillian 1979; Moore et al. 1989).

1.3.5 Bacterial Contribution to Insecticide Resistance

Recent evidence suggests that the bacterial symbionts of insects, known to be essential in nutritional homeostasis and pathogen defense, may also play a significant role in facilitating insecticide metabolism. Kikuchi et al. (2012) demonstrated that the bean bug, *Riportus pedestris*, harbor mutualistic gut symbionts of the genus *Burkholderia* that are capable of degrading organophosphate insecticides, and thus confer a unique insecticide resistance mechanism in this insect pest. The bacterial species exposed to these insecticides have evolved mechanisms to degrade insecticides so that they can use them as nutritional sources to increase their rates of reproduction and proliferation (Russell et al. 2011). Both Gram-negative and Gram-positive bacterial symbionts have been found to be active in insecticide degradation and, thus, may limit the toxic action of these compounds to insect pests. The involvement of bacterial symbionts in the detoxification of insecticides usually involves various enzyme systems that work to transform insecticides into carbon and energy sources for growth (Yu 2008). Some bacteria have the ability to metabolize organophosphates and use them as sources of carbon, phosphorous, or nitrogen, facilitating degradation of these compounds in the environment

(Werren et al 2012). These bacterial symbionts can perform all basic chemical reactions, including oxidation, hydrolysis, and reduction (Yu 2008). For example, bacterial strains that are able to degrade amide pesticides have been isolated, and their microbial metabolic pathways have also been described (Hashimoto et al. 2002; Li et al. 2010; Matsumura and Goush 1966; Konrad et al. 1969; Pandey et al. 2010; Sethunathan and Yoshida 1973; Tomasek et al. 2010, Zhang et al. 2011). Currently, three enzymes responsible for the first step in degradation pathways have been identified and characterized for bacterial strains degrading insecticidal carbamates (Russell et al. 2011). These enzymes include the carbofuran hydrolase MCD and the carbaryl hydrolases CehA and CahA (Derbyshire et al. 1987; Hayatsu et al. 2001; Hashimoto et al. 2002; Cheesman et al. 2007). These enzymes hydrolyze carbamate insecticides and, in turn, the reaction products are catabolized by other cellular pathways (Russell et al. 2011). Pyrethroid-degrading bacterial enzymes have also been characterized including the carboxylesterase PytH of *Sphingobium* sp. strain JZ-1 (Wang et al. 2009), the carboxylesterase permethrinase of *Bacillus cereus* SM3 (Maloney et al. 1993) and the pyrethroid-hydrolyzing esterase EspA of *Klebsiella* ZD 112 (Wu et al. 2006). These enzymes perform the initial catabolic pathways for bacteria, and the products formed during the hydrolysis reactions are likely catabolized further as each of these organisms mineralize the synthetic pyrethroids (Russell et al. 2011). Organophosphates such as phorate, methyl parathion, fenitrothion, dichlorvos, and fenthion have all been shown to degrade in the presence of bacteria (Ragnarsdottir 2000). Some of the bacteria participating in the degradation of OPs include: *Arthrobacter* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Trichoderma* sp., and *Streptomyces* sp. (Sethunathan and Yoshida 1973; Singh 2009).

Three enzymes have been implicated in these degradation activities: the Organophosphorous hydrolase (OPH) (Serdar et al. 1982; Siddavattam et al. 2003; Mulbry et al. 1989), Methyl parathion hydrolase (MPH) (Dong et al. 2005), and the Organophosphorous acid anhydrolase (OPAA) (Cheng et al. 1993, Cheng et al. 1995). OPHs have been purified from *B. diminuta* and *Flavobacterium* spp.. OPH is a member of the amidohydrolase superfamily, it has a broad substrate specificity and can hydrolyze P-O, P-F, and P-S bond, but with varying efficiency (Singh and Walker 2006). MPH has been identified in several phylogenetically unrelated bacteria, and is active against several OP compounds, but has a narrower substrate range than OPH (Singh et al. 2009). OPAA is another OP-degrading enzyme that has received substantial attention. This enzyme belongs to the family dipeptidase and has been isolated from *Alteromonas undina* and *Alteromonas haloplanktis* (Cheng et al. 1993; Cheng et al. 1999).

While many of these insecticide-degrading symbionts have been characterized for other insect pests, there is no information regarding mosquitocide-metabolizing bacterial species that could establish a symbiotic association and confer mosquitocide resistance in *Aedes* mosquitoes. In this study the capacity of bacterial symbionts to modify the metabolic detoxification of mosquitocidal chemistries and, thus, alter the toxic action of these chemistries towards the yellow fever mosquito, *Aedes aegypti* was examined.

Chapter 2

METABOLIC DETOXIFICATION FOR THE MOSQUITOCIDES, NALED AND PROPOXUR

2.1 INTRODUCTION

The mosquito *Aedes aegypti* is a highly anthropophilic, day-biting insect that is a major vector of viruses such as dengue, chikungunya, and yellow fever. It is found in almost 100 sub-tropical and tropical countries throughout the world and represents a significant public health challenge. Dengue is currently the most important mosquito-borne viral disease that affects humans (CDC 2010). It infects an estimated 390 million people each year and 40% of the world's population is at risk for epidemic transmission (Bhatt et al. 2013). Because there is no vaccine or specific treatment available, the elimination of mosquito larval habitats and application of mosquitocides remains the primary approach for limiting dengue virus transmission (Morrison et al. 2008). Unfortunately, the increasing incidence of insecticide resistance limits the use of many current-use insecticides for *Ae. aegypti* control. This problem is further exacerbated by the fact that no new insecticides have been developed and marketed for the chemical control of *Ae. aegypti* in over thirty years (Hemingway et al. 2006). Therefore, a thorough understanding of mosquitocide resistance mechanisms is essential for enhancing the efficacy of existing mosquito chemical control strategies and for the development of novel strategies.

Mosquito acetylcholinesterase (AChE) is a proven target site for two major insecticide families: the organophosphates and carbamates. AChE is a serine hydrolase responsible

for terminating synaptic transmission at cholinergic synapses in the central nervous system of mosquitoes by rapidly hydrolyzing the neurotransmitter acetylcholine (Weill et al. 2002). When AChE is inhibited by organophosphate and carbamate insecticides, paralysis and subsequent death of the insect occurs. Naled (Dibrom®) and propoxur (Baygon®) are examples of current-use insecticides used for the control of *Ae. aegypti.* Naled is an organophosphate insecticide most often applied through aerial spraying and targets adult and larval mosquitoes. Propoxur is a carbamate insecticide most often used in indoor residual spraying (IRS) in countries within Africa and targets adult mosquitoes. Naled mimics the structure of acetylcholine and, therefore, has a relatively high affinity for the catalytic center of AChE (Gupta 2011). The phosphate group of the compound is attracted to the esteratic site and the rest of the molecule is aligned by interaction with other side-groups of amino acid from the total catalytic area of the enzyme. While acetylcholine acetylates the enzyme, organophosphates will phosphorylate the enzyme. Following phosphorylation, the rate of hydrolysis and reactivation of the AChE will be significantly slower than for the hydrolysis of the acetylated enzyme. This is due to the fact that the phosphorous-oxygen bond is significantly stronger than the carbon-oxygen bond. Propoxur has a similar mechanism of action to that of naled; however, it will carbamylate AChE rather than phosphorylate the enzyme. Following AChE carbamylation, the rate of hydrolytic breakdown of AChE is intermediate to that of the acetylated and phosphorylated enzyme. To assess the inhibition potency of these compounds towards AChE, the residual AChE activity can be measured using *in vitro* and *in vivo* assays (Ellman et al 1961). While naled and propoxur are potent acetylcholinesterase inhibitors, the increasing incidence of resistance jeopardizes their

efficacy for the control of vector mosquitoes. Target site insensitivity, reduced cuticular penetration, and enhanced detoxification enzyme activity have been implicated in mosquitocide resistance. For this study, we focus primarily on the contribution of metabolic detoxification enzymes to insecticide resistance in *Ae. aegypti*.

Insecticide resistance has appeared in the primary insect vectors from every genus (Brogdon and McAllister 1998). Metabolic detoxification can be a major mechanism of resistance that involves enhanced levels or altered activities of general esterases, cytochrome P450 monooxygenases, or glutathione *S*-transferases (Brogdon and McAllister 1998). These enzymes recognize and transform functional groups to reduce reactivity and increase polarity (Phase I) and conjugate them to endogenous molecules for excretion (Phase II) (Casida et al. 2004) The general esterases and cytochrome P450 monooxygenases represent two major enzyme groups that are responsible for metabolic resistance to organophosphates, organochlorines, carbamates, and pyrethroids (Hemingway et al. 2000). The general esterases have been associated with organophosphate, carbamate, and less frequently, pyrethroid resistance. The carboxylesterases is a a group of enzymes, which hydrolyze carboxylic esters (Hemingway 1998). The general esterases act by sequestering the insecticide and rapidly bind or slowly release the insecticide metabolites (Karunaratne et al., 1993). The cytochrome P450 monooxygenases are a complex family of enzymes and are found in most organisms, including mosquitoes. The cytochrome P450 monooxygenases are involved in metabolic detoxification of pyrethroids, the activation and inactivation of organophosphates, and, less often, carbamate detoxification (Hemingway et al. 2000).

The cytochrome P450 monooxygenases metabolize insecticides through *O*-, *S*-, and *N*-alkyl hydroxylation, aliphatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation (Wilkinson 1976). Biochemical assays have been developed that can detect the activity of these various detoxification enzymes in individual insects. These biochemical assays can measure enhanced levels of detoxification enzymes responsible for insecticide resistance.

Synergists have been developed to counteract insecticide resistance conferred by metabolic detoxification enzymes. By combining synergists with insecticides in bioassays, the resistant mosquitoes will return to apparent susceptibility if the inhibited enzyme is responsible for resistance within that particular insect (Matowo et al 2010). Synergists are compounds that when applied alone are non-toxic to insects but, when applied in combination with insecticides, result in increased toxicity or effectiveness against the targeted pest (Yu 2008). The insecticide synergists piperonyl butoxide (PBO), *S,S,S*-tributyl phosphorotrithioate (DEF) and triphenyl phosphate (TPP) have been consistently used in insect toxicological bioassays to provide a preliminary assessment of the contribution of metabolic detoxification enzymes, such as cytochrome P450 monooxygenases and esterases in insecticide resistant insects (Koou et al 2014). Methyldioxyphenyl synergists, such as PBO, form a complex with cytochrome P450 monooxygenases and prevent binding of the insecticide with these enzymes, a prerequisite for detoxification (Bernard and Philogene 1993). The inhibition of cytochrome P450 monooxygenases by PBO can competitive or noncompetitive (Omuro and Sato 1964). Treatment with these synergists does not necessarily result in synergism.

For example, certain insecticides require activation by cytochrome P450 monooxygenases into metabolites more toxic than the parent compound. In these instances, a synergist will prevent this activation and act as an antagonist of this insecticide (Bernard and Philogene 1993). The organophosphate esterase inhibitors, TPP and DEF, can inhibit several groups of insect hydrolases (Bernard and Philogene 1993).

The hypothesis for this study is that the metabolic detoxification and toxic action of the mosquitocides naled and propoxur is regulated by the cytochrome P450 monooxygenase and general esterase activities of *Ae. aegypti* larvae. Therefore, the goal of this study was to examine the acute toxicities of naled and propoxur to *Ae. aegypti* larvae following the inhibition of cytochrome P450 monooxygenase and general esterase activities using the standard mosquitocide synergists PBO, TPP, and DEF. Here, I have examined the acute toxicities of naled and propoxur alone and in combination with PBO, TPP, and DEF for *Ae. aegypti* larvae. Next, I have assessed the cytochrome P450 monooxygenase, general esterase, and acetylcholinesterase activities in *Ae. aegypti* larvae following the exposure to PBO, TPP, and DEF. The data gathered in this study provide new information for the manner with which metabolic detoxification enzymes in *Ae. aegypti* larvae can alter the toxic action of standard-use chemistries for mosquito control.

2. 2 MATERIALS AND METHODS

2.2.1 Mosquito Colony

A laboratory colony of *Aedes aegypti* (Liverpool strain) was initially obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA), and has been cultured in the Insect Toxicology and Pharmacology Laboratory at Virginia Tech (Blacksburg, VA) since 2011. The mosquito colony was maintained according to the standard operating procedures described by Pridgeon et al. (2009) with slight modifications. The adult mosquitoes were held in a screened cage and provided 10% sucrose *ad libitum*. To encourage egg development in adult females, a membrane feeder containing defibrinated sheep blood (Colorado Serum Co., Denver, CO) was warmed to 37 °C and provided to the mosquitoes. Eggs were collected from the adult females and vacuum-hatched in a 1 L Erlenmeyer flask. The mosquito larvae were transferred to a plastic tray containing deionized water. A larval diet of flake fish food (Tetramin, Blacksburg, VA) was added to the plastic tray. Adults and larvae were reared in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod.

2.2.2 Acute Toxicity of Mosquitocides and Synergists

The acute toxicity bioassays were conducted for 24 h using third-instar mosquito larvae exposed to five concentrations of propoxur and naled providing a range of 0-100% mortality. The appropriate dilutions of each mosquitocide were prepared in acetone. The mosquitocides were delivered by adding 5 µl of mosquitocide solution to 5 ml deionized

water containing ten larvae in a 10 ml glass beaker. The same procedure was used to treat larvae with corresponding concentrations of acetone in deionized water as a control treatment. The bioassays were repeated three times for each mosquitocide concentration and control treatment. The treated larvae were maintained in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod. The endpoint for each bioassay was measured as a lethal concentration (LC) according to World Health Organization (WHO) guidelines. The larvae that were unable to perform an active movement upon gentle probing were considered dead.

To assess the combined effect of propoxur and naled with the synergists TPP, DEF, and PBO, third-instar mosquito larvae were exposed to each mosquitocide at the same five concentrations determined above, individually and in combination with TPP (2,000 ppb), DEF (500 ppb) and PBO (2,500 ppb). Synergist exposures were optimized for time and concentration with the purpose of obtaining maximal inhibition of the desired enzymes without any larval mortality (see Appendix 1). The synergist stock solutions were prepared in acetone and 5 µl of each solution was dissolved in 5 ml of deionized water containing 10 larvae. The mosquitocide exposure methods are the same as described above in the acute toxicity bioassays; however, the larvae were exposed to each synergist for 12 h prior to the mosquitocide exposure.

2.2.3 In vivo Inhibition of Detoxification Enzyme Activity

Third-instar mosquito larvae were exposed to propoxur or naled at the LC_{25} individually, and in combination with triphenyl phosphate (TPP) at 2,000 µg/L (parts per billion or

ppb), *S*,*S*,*S*-tributyl phosphorotrithioate (DEF) at 500 ppb, or piperonyl butoxide (PBO) at 2,500 ppb for 12 h prior to mosquitocide treatment. The synergist stock solutions were prepared in acetone and 5 µl of each solution was dissolved in 5 ml of deionized water containing 10 larvae. The treated larvae were maintained in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod. Larval mortality was assessed for each treatment and all surviving mosquitoes were collected from each treatment for the metabolic detoxification enzyme activity bioassays. The procedure was replicated three times for the control (synergist untreated), synergist alone treatment, and synergist-mosquitocide treatment.

The general esterase activity of larvae treated with propoxur or naled alone (LC_{25}) and in combination with the synergists TPP and DEF was measured according to the method described by Rakotondravelo et al. (2006). Each mosquito sample was homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% (v/v) Triton X-100 (Sigma Aldrich, St. Louis, MO) at the rate of one mosquito larva per 100 µl sodium phosphate. The individual homogenates were centrifuged at 10,000 x *g* for 10 min at 4 ˚C. The supernatant was used as the enzyme source for measuring general esterase activity using 0.3 mM α-naphthyl acetate (α-NA) (Sigma Aldrich) as a substrate. The hydrolysis of α-NA to the product α-naphthol was measured using a microplate absorbance reader (Molecular Devices, Sunnyvale, CA) at 600 nm and 560 nm for α-NA. The total protein in each sample preparation was determined using a bicinchoninic acid assay as described by Smith et al. (1985) with bovine serum albumin (Sigma Aldrich) as a standard. The

amount of protein in each sample was measured using a microplate absorbance reader at 560 nm.

The cytochrome P450 monooxygenase activity of larvae treated with propoxur or naled alone (LC_{25}), or in combination with the synergist PBO was measured according to the method described by Anderson and Zhu (2004) with some modifications. Following the PBO treatments, the individual live larvae were transferred to the wells of a 96-well microplate containing 50 mM sodium phosphate (pH 7.2) and 0.4 mM 7-ethoxycoumarin. The larvae were incubated at 37 ˚C for 4 h followed by the addition of 50% (v/v) acetonitrile and Trizma base (pH 10). The reaction buffer was then removed from the microplate wells and transferred into wells of a new microplate, being careful not to aspirate any larvae. The deethylation of 7-ethoxycoumarin to the product 7-hydroxycoumarin was measured using a microplate fluorescence reader (Molecular Devices) at 480 nm while exciting at 380 nm.

2.2.4 In vivo Inhibition of Acetylcholinesterase Activity

The acetylcholinesterase (AChE) activity of larvae treated with propoxur or naled alone (LC_{10}), and in combination with the mosquitocide synergists TPP, DEF, and PBO was determined according to the method of Ellman et al. (1961) as modified by Anderson et al. (2009). Each mosquito sample was homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% (v/v) Triton X-100 at the rate of one mosquito larva per 100 µl sodium phosphate. The individual homogenates were centrifuged at 10,000 x *g*

for 10 min at 4 ˚C and the supernatant was used as the enzyme source for measuring AChE activity with acetylthiocholine iodide (ATCh) and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB). The hydrolysis of ATCh and the formation of thionitrobenzoic acid was measured using a microplate absorbance reader at 405 nm.

2.2.5 Statistical Analysis

Log-probit analysis was used to estimate the $LC_{10,}$ $LC_{25,}$ LC_{50} values for each mosquitocide, alone and in combination, with the synergists. Synergistic ratios (SR) were calculated by dividing the LC_{50} of the mosquitocide-only treatments by the LC_{50} of the mosquitocide + synergist treatments (e.g., $SR = LC_{50\ \text{mosquitocide-only}} \div LC_{50\ \text{mosquitocide + synergist}}$). The significant differences between the LC_{50} for each mosquitocide, alone and in combination, with the synergist treatments was based on the non-overlapping 95% confidence intervals estimated for each bioassay. The percentage of mosquitoes affected by the different treatments was statistically compared using a one-way ANOVA and a Tukey's multiple comparison post-test.

2. 3. RESULTS

2.3.1 Acute Toxicities of Mosquitocides and Synergists

The synergists TPP, DEF, and PBO at 2,000, 500, and 2,500 ppb, respectively, were not acutely toxic to third-instar *Ae. aegypti* larvae under the bioassay conditions. The LC_{50} estimates for propoxur or naled alone and in combination with TPP, DEF, and PBO were

used to calculate synergism ratios (Table 2.3.4-1). The toxicity of propoxur was significantly increased by 1.31-, 2.37-, and 4.47-fold when applied in combination with a fixed concentration of TPP, DEF, or PBO, respectively (Table 2.3.4-1). In addition, the toxicity of naled was significantly increased by 3.39- and 5.61-fold when in combination with a fixed concentration of TPP or DEF, respectively (Table 2.3.4-1). The toxicity of naled was slightly decreased when in combination with a fixed concentration of PBO; however, this alteration of toxicity was not significantly different due to the overlapping 95% confidence intervals of their LC_{50} estimates (Table 2.3.4-1).

2.3.2 In vivo Inhibition of Detoxification Enzyme Activity in Combined Treatments

The *in vivo* inhibition of general esterases (α-naphthyl acetate hydrolysis) in third-instar *Ae. aegypti* larvae exposed to the LC_{25} of propoxur or naled alone and in combination with the synergists TPP and DEF at 2,000 and 500 ppb respectively, is shown in Fig. 2.3.4-1, 2.3.4-2, and 2.3.4-3, respectively. The TPP and DEF treatments alone resulted in a 89.6% and 99.2% decrease in esterase specific activity, respectively, compared to the untreated larvae (Fig. 2.3.4-1).

The propoxur treatment alone resulted in a 25.7% reduction in esterase specific activity compared to the untreated larvae (Fig. 2.3.4-2). A significant reduction of esterase specific activity did occur in the larvae exposed to propoxur in combination with TPP or DEF. The TPP and DEF treatments resulted in a 82.9% and 98.5% decrease in the

esterase specific activity of propoxur, respectively, compared to the larvae treated with propoxur alone (Fig. 2.3.4-2).

The naled treatment alone resulted in a 55.3% reduction in esterase specific activity compared to the untreated larvae (Fig. 2.3.4-3). A significant reduction of esterase specific activity did occur in the larvae exposed to naled in combination with TPP or DEF. The TPP and DEF treatments resulted in a 100% and 100% decrease in the esterase specific activity of naled, respectively, compared to the larvae treated with naled alone (Fig. 2.3.4-3).

The *in vivo* inhibition of cytochrome monooxygenases (*O*-deethylation) in third-instar *Ae. aegypti* larvae exposed to the LC_{25} of propoxur or naled alone and in combination with the synergist PBO at 2,500 ppb is shown in 2.3.4-1, 2.3.4-2, and 2.3.4-3. The PBO treatment alone resulted in a 93.9% decrease in cytochrome P450 monooxygenase *O*-deethylation activity, respectively, compared to the untreated larvae (Fig. 2.3.4-1).

The propoxur treatment alone did not result in a significant reduction in cytochrome P450 monooxygenase *O*-deethylation activity compared to the untreated larvae (Fig. 2.3.4-2). A significant reduction of cytochrome P450 monooxygenase *O*-deethylation activity did occur in the larvae exposed to propoxur in combination with PBO. The PBO treatment resulted in a 94.0% decrease in the cytochrome P450 monooxygenase *O*-deethylation activity of propoxur compared to the larvae treated with propoxur alone (Fig. 2.3.4-2).

The naled treatment alone did not result in a significant reduction in cytochrome P450 monooxygenase *O*-deethylation activity compared to the untreated larvae (Fig. 2.3.4-3). A significant reduction of cytochrome P450 monooxygenase *O*-deethylation activity did occur in the larvae exposed to naled in combination with PBO. The PBO treatment resulted in a 78.7% decrease in the cytochrome P450 monooxygenase *O*-deethylation activity of naled compared to the larvae treated with naled alone (Fig. 2.3.4-3).

2.3.3 In vivo Inhibition of Acetylcholinesterase Activity

The residual acetylcholinesterase (AChE) activity of third-instar *Ae. aegypti* larvae exposed to the LC_{10} of propoxur or naled alone and in combination with the synergists PBO, TPP, and DEF at 2,500, 2,000, and 500 ppb respectively, is shown in Fig. 2.3.4-6, 2.3.4-7, and 2.3.4-8. The propoxur or naled treatments alone resulted in a 48.7% and 40.1% reduction in AChE activity, respectively, compared to the untreated larvae (Fig. 2.3.4-4, 2.3.4-5, and 2.3.4-6). The PBO, TPP, and DEF treatments alone resulted in a 25.1%, 11.0%, and 35.0% decrease in AChE activity, respectively, compared to the untreated larvae (Fig. 2.3.4-4, 2.3.4-5, and 2.3.4-6), however the decrease observed in the TPP treatment was not significant.

Increases in residual AChE activity did occur in the larvae exposed to propoxur or naled in combination with PBO. The PBO treatment resulted in a 7.3% (not significant) and 48.3% (significant) decrease in the anticholinesterase activity of propoxur or naled, respectively, compared to the larvae treated with propoxur or naled alone (Fig. 2.3.4-4).

Significant decreases in residual AChE activity did occur in the larvae exposed to propoxur or naled in combination with TPP. The TPP treatment resulted in a 35.8% and 39.8% increase in the anticholinesterase activity of propoxur or naled, respectively, compared to the larvae treated with propoxur or naled alone (Fig. 2.3.4-5).

Significant decreases in residual AChE activity did occur in the larvae exposed to propoxur or naled in combination with DEF. The DEF treatment resulted in a 59.0% and 69.7% increase in the anticholinesterase activity of propoxur or naled, respectively, compared to the larvae treated with propoxur or naled alone (Fig. 2.3.4-6).

2.3.4 Tables and Figures

Table 2.3.4-1. Effects of triphenyl phosphate (TPP, 2,000 µg/L), *S*,*S*,*S*-tributyl phosphorotrithioate (DEF, 500 µg/L), and piperonyl butoxide (PBO, 2,500 µg/L) on the acute toxicity of propoxur or naled to third-instar *Ae. aegypti* larvae.

Treatment	N	LC_{10} (95% CI)	LC_{25} (95% CI)	LC_{50} (95% CI)	Slope ± SE	X^2 (prob)	SR
Propoxur	**540**	**339 (266-398)**	**453 (383-512)**	**626 (559-694)**	**4.80 ± 0.35**	**2.43**	
+ TPP	**150**	**264 (194-320)**	**349 (280-406)**	**477 (411-541)**	**4.99 ± 0.69**	**0.57**	**1.31***
+ DEF	**150**	**123 (60-176)**	**177 (104-233)**	**264 (189-323)**	**3.87 ± 0.73**	**0.40**	**2.37***
+ PBO	**390**	**32 (21-44)**	**62 (45-79)**	**126 (101-157)**	**2.70 ± 0.28**	**1.47**	**4.97***
Naled	**900**	**17 (15-20)**	**25 (22-28)**	**38 (35-43)**	**3.69 ± 0.21**	**1.84**	
+ TPP	**150**	**5.9 (4.1-7.3)**	**8.0 (6.2-9.5)**	**11 (10-13)**	**4.47 ± 0.73**	**0.63**	**3.39***
+ DEF	**150**	**3.2 (1.3-4.6)**	**4.6 (2.5-6.0)**	**6.8 (4.9-8.4)**	**3.86 ± 0.87**	**0.21**	**5.61***
+ PBO	**150**	**23 (10-32)**	**31 (18-40)**	**43 (31-60)**	**4.95 ± 0.79**	**2.46**	**0.90**

[a]Propoxur and naled acute toxicity data are presented as LC_{10}, LC_{25}, and LC_{50} and their 95% confidence intervals (95% CI) in micrograms per liter (µg/L), the concentrations at which 10, 25, and 50% of the tested larvae were dead, respectively, in a 24-h bioassay. Log-probit analysis was used to estimate the endpoint concentrations for each mosquitocide.

[b]Pearson's chi-square and the probability of χ^2. The probability of > 0.05 indicates that the observed regression model is not significantly different from the expected model (i.e., a significant fit between the observed and expected regression models).

[c]Synergism ratio (SR) was calculated by SR = LC_{50} mosquitocide only / LC_{50} mosquitocide-synergist mixture. The asterisk next to the ratio indicates a significant difference between the LC_{50} of the mosquitocide and the binary combination with a synergist based on the non-overlapping 95% confidence intervals of the LC_{50} values.

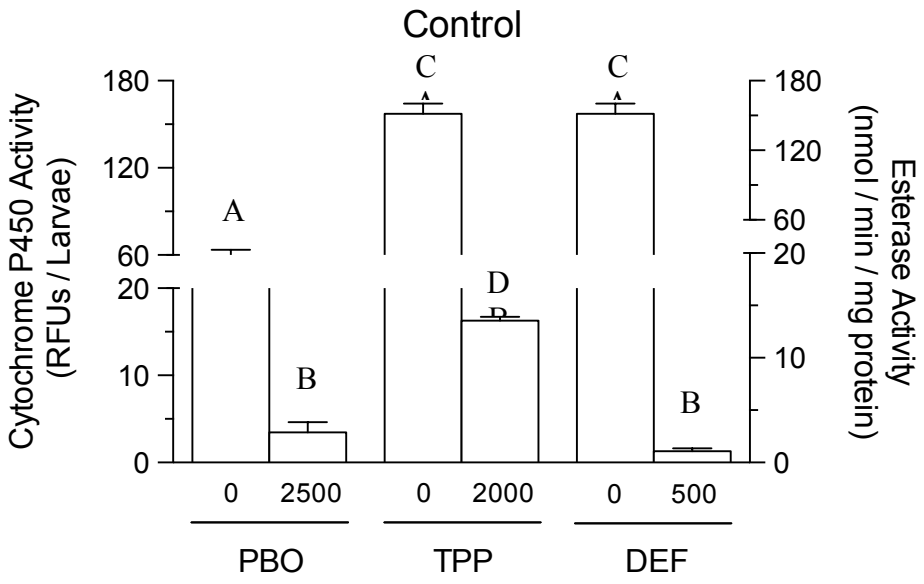

Figure 2.3.4-1. *In vivo* inhibition of cytochrome P450 monooxygenase and general esterase activities in third-instar *Ae. aegypti* larvae treated with the synergists piperonyl butoxide (PBO), triphenyl phosphate (TPP), or *S,S,S*-tributyl phosphorotrithioate (DEF) . Larvae were treated with PBO (2,500 µg/L), TPP (2,000 µg/L), or DEF (500 µg/L) for 12 h. Enzyme activities were assessed for syergist treated larvae compared to untreated control larvae. Enzyme activities are presented as the mean $\pm$ standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

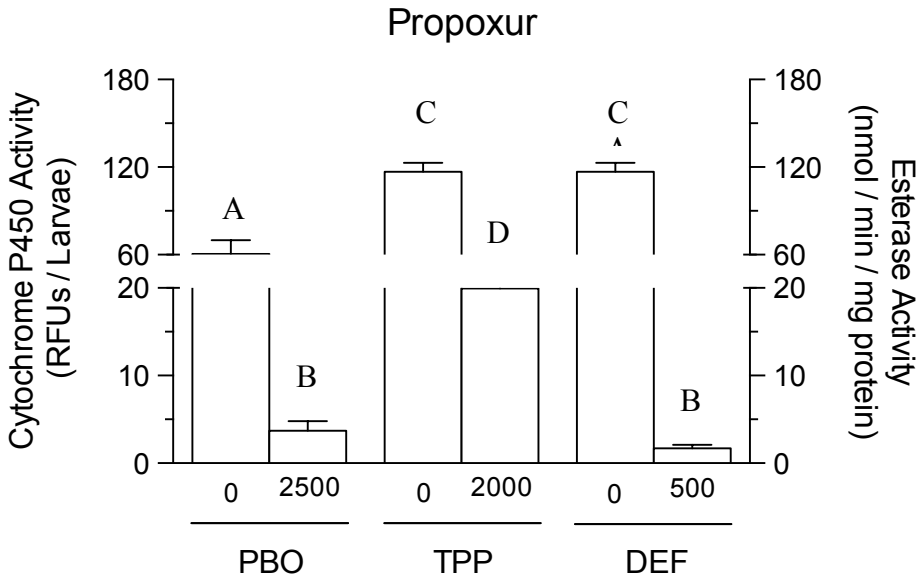

Figure 2.3.4-2. *In vivo* inhibition of cytochrome P450 monooxygenase and general esterase activities in third-instar *Ae. aegypti* larvae treated with propoxur alone and in combination with synergists piperonyl butoxide (PBO), triphenyl phosphate (TPP), or *S,S,S*-tributyl phosphorotrithioate (DEF). Larvae were treated with PBO (2,500 µg/L), TPP (2,000 µg/L), or DEF (500 µg/L) for 12 h followed by a treatment of propoxur at 453 µg/L (LC_{25}) for 24 h. Enzyme activities are presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

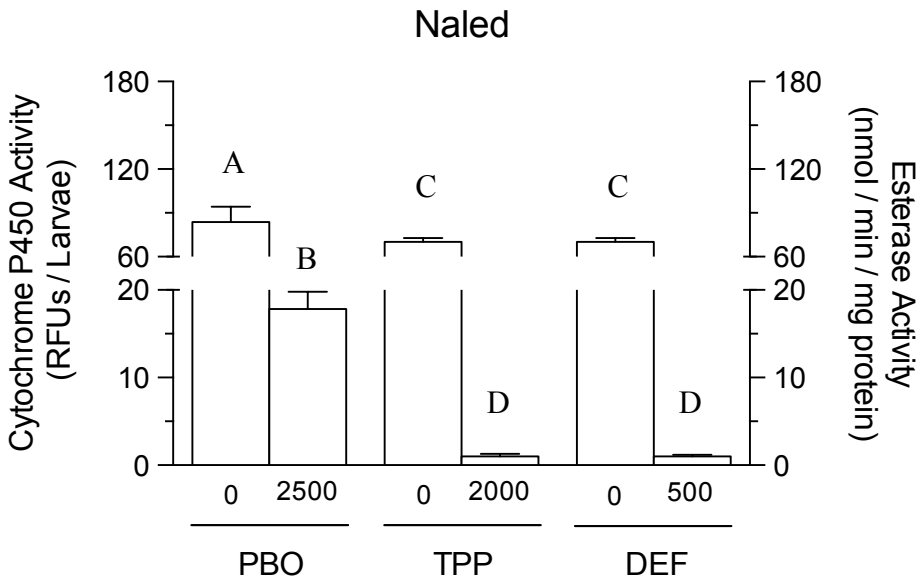

Figure 2.3.4-3. *In vivo* inhibition of cytochrome P450 monooxygenase and general esterase activities in third-instar *Ae. aegypti* larvae treated with naled alone and in combination with synergists piperonyl butoxide (PBO), triphenyl phosphate (TPP), or *S,S,S*-tributyl phosphorotrithioate (DEF). Larvae were treated with PBO (2,500 µg/L), TPP (2,000 µg/L), or DEF (500 µg/L) for 12 h followed by a treatment of naled at 25 µg/L (LC_{25}) for 24 h. Enzyme activities are presented as the mean $\pm$ standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

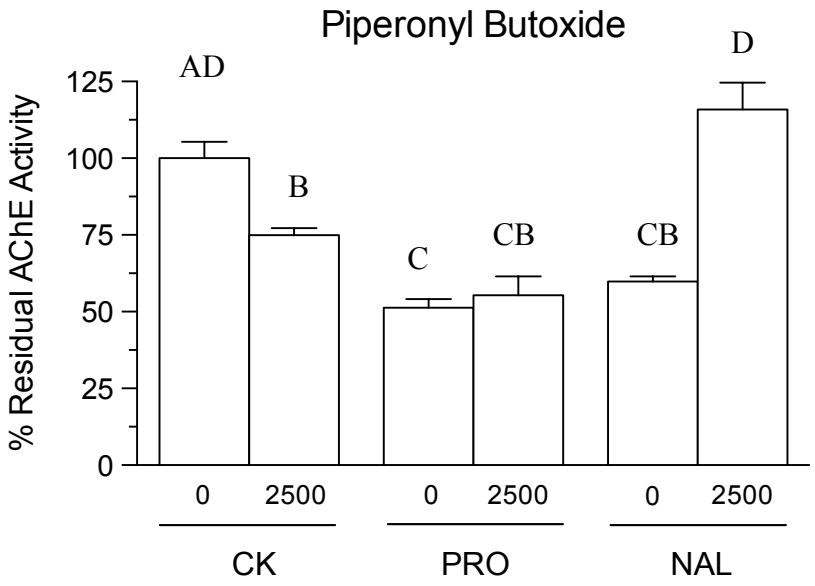

Figure 2.3.4-4. *In vivo* inhibition of acetylcholinestease (AChE) activity in third-instar *Ae. aegypti* larvae treated with propoxur (PRO) or naled (NAL) alone or in combination with the synergist piperonyl butoxide (PBO), Larvae were treated with PBO (2,500 µg/L) for 12 h followed by a treatment of propoxur at 339 µg/L (LC_{10}) or naled at 17 µg/L (LC_{10}) for 24 h. AChE activity is presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

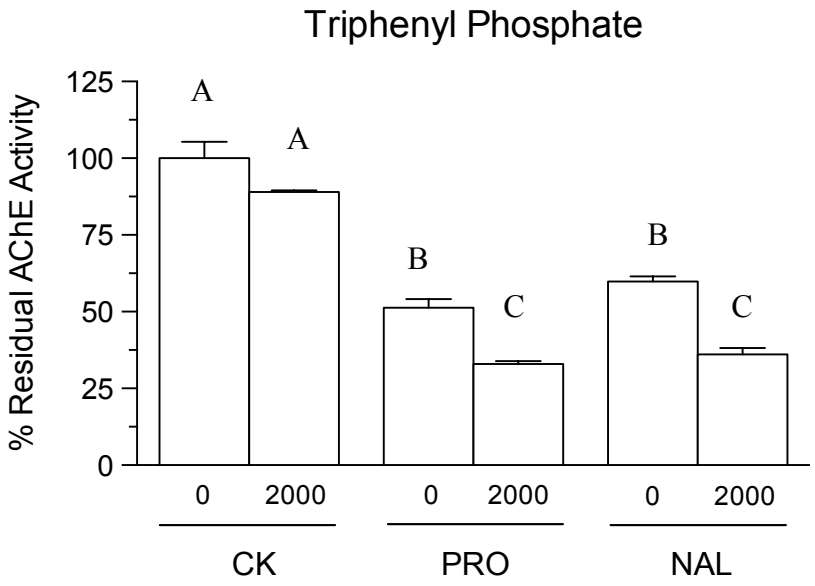

Figure 2.3.4-5. *In vivo* inhibition of acetylcholinestease (AChE) activity in third-instar *Ae. aegypti* larvae treated with propoxur (PRO) or naled (NAL) alone or in combination with the synergist triphenyl phosphate (TPP). Larvae were treated with TPP (2,000 µg/L) for 12 h followed by a treatment of propoxur at 339 µg/L (LC_{10}) or naled at 17 µg/L (LC_{10}) for 24 h. AChE activity is presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

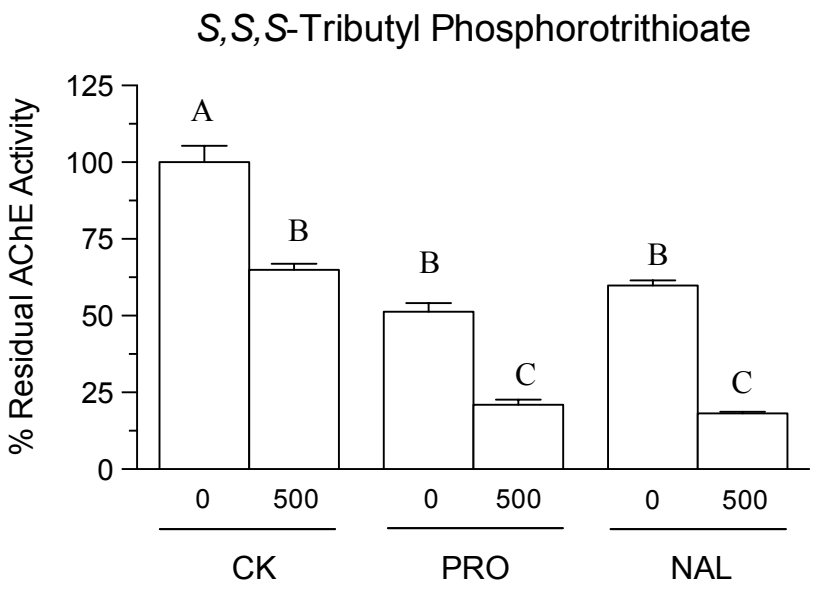

Figure 2.3.4-6. *In vivo* inhibition of acetylcholinestease (AChE) activity in third-instar *Ae. aegypti* larvae treated with propoxur (PRO) or naled (NAL) alone or in combination with the synergist S,S,S- tributyl phosphorotrithiote (DEF). Larvae were treated with DEF (500 µg/L) for 12 h followed by a treatment of propoxur at 339 µg/L (LC_{10}) or naled at 17 µg/L (LC_{10}) for 24 h. AChE activity is presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

2.4 DISCUSSION OF RESULTS

The goal of this study was to examine the acute toxicities of naled and propoxur to *Ae. aegypti* larvae following the inhibition of cytochrome P450 monooxygenase and general esterase activities using the standard mosquitocide synergists PBO, TPP, and DEF. The data gathered in this study provides new information for the manner with which metabolic detoxification enzymes in *Ae. aegypti* larvae can alter the toxic action of standard-use chemistries for mosquito control.

Mosquito acetylcholinesterase (AChE) is a proven target site for two major mosquitocide families: the organophosphates and carbamates. AChE is a serine hydrolase responsible for terminating synaptic transmission at cholinergic synapses in the central nervous system of mosquitoes by rapidly hydrolyzing the neurotransmitter acetylcholine (Weill et al. 2002). When AChE is inhibited by organophosphate and carbamate mosquitocides, paralysis and subsequent death of the insect occurs. While naled and propoxur are effective acetylcholinesterase inhibitors, the increasing incidence of resistance jeopardizes their efficacy for the control of *Ae. aegypti* mosquitoes. Target site insensitivity, reduced cuticular penetration, and enhanced detoxification enzyme activity have been implicated in mosquitocide resistance. For this study, I focused primarily on the contribution of metabolic detoxification enzymes to mosquitocide resistance in *Ae. aegypti*.

The synergists PBO, TPP, and DEF are standard-use chemistries for assessing the metabolic detoxification activities of cytochrome P450 monooxygenases and general

esterases towards current-use mosquitocides in *Ae. aegypti* (Koou et al 2014). The hypothesis for this study was that the metabolic detoxification and toxic action of the mosquitocides naled and propoxur is regulated by the cytochrome P450 monooxygenase and general esterase activities of *Ae. aegypti* larvae. This study demonstrates that the acute toxicity of propoxur was significantly increased for the mosquito larvae exposed to PBO whereas this synergist had no effect on the acute toxicity of naled to the mosquito larvae. In addition, the acute toxicities of propoxur and naled were significantly increased for mosquito larvae exposed to TPP and DEF. This information expands our knowledge of cytochrome P450 monooxygenase- and general esterase-mediated metabolic detoxification and toxic action of standard-use mosquitocides, such as naled and propoxur, in *Ae. aegypti* larvae, which appears to be influenced by the chemical structure of the mosquitocide. Hemingway et al. (2000) report that general esterases are often associated with the reduced toxic action of carbamate and organophosphate mosquitocides. The cytochrome P450 monooxygenases are more frequently associated with the reduced toxic action of organophosphate mosquitocides compared to the carbamate mosquitocides (Hemingway et al. 2000). However, I have observed that cytochrome P450 monooxygenases are an important metabolic detoxification mechanism for *Ae. aegypti* larvae exposed to propoxur, but not for those larvae exposed to naled. Lykken and Casida (1969) report that cytochrome P450 monooxygenases can facilitate the debromination of naled to the more toxic metabolite dichlorvos in insects. The acute toxicity of naled to the *Ae. aegypti* larvae exposed to PBO suggests that the cytochrome P450 monooxygenase-mediated debromination of naled to dichlorvos may not be an important factor in the toxic action of the mosquitocide. To date, there is no evidence of

cytochrome P450 monooxygenase-mediated activation or deactivation of naled reported for *Ae. aegypti* larvae and, thus, a further examination of this mechanism is warranted for these mosquitoes.

The anticholinesterase activities of propoxur and naled were significantly increased in *Ae. aegypti* larvae exposed to the mosquitocide synergists TPP and DEF. These data suggest that general esterases are important metabolic detoxification mechanisms for *Ae. aegypti* larvae exposed to propoxur and naled and, in turn, can alter the toxic action and target-site activity (i.e., reduced acetylcholinesterase inhibition) of these mosquitocides. However, TPP and DEF appear to reduce the metabolic detoxification of propoxur and naled and, thereby, sustain the toxic action and target-site activity (i.e., increased acetylcholinesterase inhibition) of these mosquitocides. Interestingly, the anticholinesterase activities of propoxur and naled were reduced in mosquito larvae exposed to the mosquitocide synergist PBO. These data suggest that cytochrome P450 monooxygenases may not be an important factor in the toxic action and target-site activity (i.e., reduced acetylcholinesterase inhibition) of naled to *Ae. aegypti* larvae. However, there is a discrepancy with regard to the cytochrome P450 monooxygenase-mediated toxic action and target-site activity (i.e., reduced acetylcholinesterase inhibition) of propoxur to mosquito larvae, which might suggest a different mechanism of toxicity. Sanchez-Arroyo et al. (2001) report that PBO can reduce the cuticular penetration of propoxur in insects and, thereby, limit the delivery of propoxur across the blood-brain barrier to the target site acetylcholinesterase. Khot et al. (2008) reveal that PBO can create a blockade between insect anticholinesterases and other esterases to

reduce the target-site activity of these compounds. This observation might explain the reduced acetylcholinesterase inhibition of propoxur and naled in the PBO-treated *Ae. aegypti* larvae, although, a further examination of this mechanism is warranted for these mosquitoes.

The knowledge of metabolic detoxification activities in insects to standard-use insecticides is essential for better understanding the mechanisms of toxicity, resistance, and synergism (Shrivastava et al 1970). There are numerous mosquitocides that are limited for use to control *Ae. aegypti* as a result of mosquitocide resistance. This problem is further exacerbated by the fact that no new insecticides have been developed and marketed for the chemical control of *Ae. aegypti* in over thirty years (Hemingway et al. 2006). Therefore, a thorough understanding of mosquitocide resistance mechanisms is warranted for enhancing the efficacy of existing mosquito chemical control strategies and for the development of novel strategies. One possible factor contributing to increased mosquitocide resistance, may be the presence of naturally-acquired microbes within the mosquito. Recent evidence suggests that the bacterial symbionts of insects, may also play a significant role in facilitating insecticide metabolism. Kikuchi et al. (2012) demonstrated that the bean bug, *Riportus pedestris*, harbor mutualistic gut symbionts of the genus *Burkholderia* that are capable of degrading organophosphate insecticides, and thus confer a unique insecticide resistance mechanism in this insect pest. The bacteria exposed to these insecticides have evolved mechanisms to degrade them, so that they can be used as nutritional sources to increase the bacterial rate of reproduction and proliferation (Russell et al. 2011). The involvement of bacterial symbionts in the

detoxification of insecticides usually involves various enzyme systems that work to transform insecticides into carbon and energy sources for growth (Yu 2008). So the same esterases and P450s that are present within the mosquitoes themselves, are also present within the bacteria colonizing them. A primary component of objective two focuses on the removal of naturally occurring bacteria within the mosquito using antibiotics and the resulting changes in enzyme degradation activities within the mosquito. Therefore, a primary aim of the first objective is to understand the extent with which these metabolic detoxification enzymes are working within the mosquitoes prior to antibiotic treatment, so that the contribution of the bacteria to these degradation activities can be more clearly understood. Thus, a substantial biochemical profile of these metabolic enzyme activities is provided here.

Chapter 3

BACTERIAL CONTRIBUTION TO METABOLIC DETOXIFICATION OF THE MOSQUITOCIDES, NALED AND PROPOXUR

3.1 INTRODUCTION

Mosquitocide resistance is increasing at an alarming rate, threatening the efficacy of mosquito control programs, worldwide. Areas where vector-borne diseases, such as dengue, are prevalent are of particular concern. The lack of effective vaccines and therapeutics as well as the absence of novel mosquitocidal chemistries underscores the need for successful resistance management strategies; however, these require a comprehensive understanding of the various adaptive mechanisms underlying resistance.

Symbiosis was first defined by Anton de Bary in 1869 and is defined as "the living together of parasite and host" (de Bary 1893). Symbiotic bacteria are those bacteria living in symbiosis with another organism, or each other. Many insects harbor large communities of diverse microorganisms that probably exceed the number of cells in the insects themselves (Gusmão 2000). Extensive research has focused on the contribution of microbial endosymbionts to the host's reproduction mode (Haine 2008), nutritional homeostasis (Dillon 2004), mate selection (Werren 2008), as well as pathogen defense (Moran 2007, Dong et al 2009, O'Neill et al 2011, Hoffmann et al 2011). Recently, however, certain bacterial endosymbionts have also been implicated in the development of insecticide resistance in their insect hosts (Kikuchi et al. 2012). Kikuchi et al. (2012) demonstrated that the bean bug, *Riportus pedestris*, harbor mutualistic gut symbionts of the genus *Burkholderia* that are capable of degrading the organophosphate insecticides

and, thus, confer a unique insecticide resistance mechanism in this insect pest. The bacterial species exposed to these insecticides have evolved mechanisms to degrade insecticides so that they can use them as nutritional sources to increase their rates of reproduction and proliferation (Russell et al. 2011). Both Gram-negative and Gram-positive bacterial symbionts have been found to be active in insecticide degradation and, thus, may limit the toxic action of these compounds towards insect pests. The involvement of bacterial symbionts in the detoxification of insecticides usually involves various enzyme systems that work to transform insecticides into carbon and energy sources for growth (Yu 2008). Russell et al (2011) described a few of these enzymes found in carbamate-degrading bacteria and organophosphate-degrading bacteria. These include: MCD and CehA for the carbamates and phosphotriesterases (PTEs), methyl parathion hydrolases (MPHs), and organophosphorus acid anhydrolases (OPAA) for the organophosphates. MCD isolated from *Achromobacter* W111 (Derbyshire et al. 1987) and CehA isolated from *Rhizobium sp* AC100 (Hashimoto et al. 2002) are esterases that are able to hydrolyze the carboxylester bond of 1-naphthyl acetate. All three of the enzymes associated with organophosphate metabolism function via hydrolysis of the R3 phosphoester bond (Russell et al 2011). It has also been suggested that there may exist an uncharacterized oxidative route for degradation of some organophosphates, as well (Munnecke and Hsieh 1976). Some bacteria have the ability to metabolize organophosphates and use them as sources of carbon, phosphorous, or nitrogen, facilitating degradation of these compounds in the environment (Werren et al 2012). These bacterial symbionts can perform all basic chemical reactions, including oxidation, hydrolysis, and reduction (Yu 2008). While many of these insecticide-degrading

symbionts have been characterized for other insect pests, there is currently no information regarding mosquitocide-metabolizing bacterial species that could establish a symbiotic association and confer mosquitocide resistance in *Aedes* mosquitoes. However, several primary midgut-associated bacteria genera have been identified in *Aedes aegypti* mosquitoes, these include: *Serratia, Klebsiella, Asaia, Bacillus, Enterococcus, Enterobacter, Kluyvera,* and *Pantoea.*

The hypothesis for this study is that the metabolic detoxification and toxic action of the mosquitocides naled and propoxur is regulated by the bacteria in *Ae. aegypti* larvae. Therefore, the goal of this study was to examine the acute toxicities of naled and propoxur to *Ae. aegypti* larvae as well as the cytochrome P450 monooxygenase and general esterase activities of the larvae following the reduction of bacteria with broad-spectrum antibiotics. Here, I have examined the acute toxicities of naled and propoxur alone and in combination with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin for *Ae. aegypti* larvae. Next, I have assessed the cytochrome P450 monooxygenase, general esterase, and acetylcholinesterase activities in *Ae. aegypti* larvae following the exposure to gentamycin, penicillin, and streptomycin. The data gathered in this study provides information for the manner with which the metabolic detoxification enzymes in bacteria of *Ae. aegypti* larvae may alter the toxic action of standard-use chemistries for mosquito control.

3.2. MATERIALS AND METHODS

3.2.1 Mosquito Colony

A laboratory colony of *Aedes aegypti* (Liverpool strain) was initially obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA), and has been cultured in the Insect Toxicology and Pharmacology Laboratory at Virginia Tech (Blacksburg, VA) since 2011. The mosquito colony was maintained according to the standard operating procedures described by Pridgeon et al. (2009) with slight modifications. The adult mosquitoes were held in a screened cage and provided 10% sucrose *ad libitum*. To encourage egg development in adult females, a membrane feeder containing defibrinated sheep blood (Colorado Serum Co., Denver, CO) was warmed to 37 °C and provided to the mosquitoes. The mosquito eggs were collected from the adult females and vacuum-hatched in a 1 L Erlenmeyer flask. The mosquito larvae were transferred to a plastic tray containing deionized water. A larval diet of unsterilized flake fish food (Tetramin, Blacksburg, VA) was added to the plastic tray; this was the primary source of microbiota. The mosquito adults and larvae were reared in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod.

3.2.2 Antibiotic Optimization

Third-instar mosquito larvae were exposed to a mixture of antibiotics containing gentamycin sulfate (1500 μg/ 1 ml), penicillin (1000 units/ 1 ml), and streptomycin (1 mg/ml) for 12 h. The antibiotic stock solutions were prepared in sterilized water and each solution was dissolved in 5 ml of deionized water containing 10 larvae. The treated larvae were maintained in an environmental chamber at 28 °C and 75% relative humidity

with a 16 h:8 h (light:dark) photoperiod. Larval mortality was assessed for each treatment. The procedure was replicated three times for the control (antibiotic untreated) and antibiotic treatments. After these exposures, mosquitoes were homogenized in a PBS solution (pH 7.4) and centrifuged at 10,000 x g. The supernatants were then serially diluted up to 10^6 and plated onto Luria Bertani (LB) agar plates. The plates were incubated at 28 °C for 48 hours after which time colony forming units (CFU) were determined.

3.2.3 Acute Toxicity of Mosquitocides and Antibiotics

The acute toxicity bioassays were conducted for 24 h using third-instar mosquito larvae exposed to five concentrations of naled or propoxur providing a range of 0-100% mortality. The appropriate dilutions of each mosquitocide were prepared in acetone. The mosquitocides were delivered by adding 5 µl of mosquitocide solution to 5 ml deionized water containing ten larvae in a 10 ml glass beaker. The same procedure was used to treat larvae with corresponding concentrations of acetone in deionized water as a control treatment. The bioassays were repeated three times for each mosquitocide concentration and control treatment. The treated larvae were maintained in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod. The endpoint for each bioassay was measured as a lethal concentration (LC) according to World Health Organization (WHO) guidelines. The larvae that were unable to perform an active movement upon gentle probing were considered dead.

To assess the combined effect of naled or propoxur with the antibiotics, third-instar mosquito larvae were exposed to each mosquitocide at the same ranges above, alone, and in combination with the antibiotics (gentamycin sulfate (1500 μg/ 1 ml), penicillin (1000 units/ 1 ml), and streptomycin (1 mg/ml)). The mosquitocide exposure methods are the same as described above in the acute toxicity bioassays; however, the larvae were exposed to antibiotics, alone, for 12 h prior to the mosquitocide exposure.

Mosquito larvae were also exposed to the mosquitocides at the LC_{25} alone and in combination with the antibiotic mixture. Larval mortality was assessed for each treatment and all surviving mosquitoes were collected from each treatment for the metabolic detoxification enzyme activity bioassays. The procedure was replicated three times for the control (antibiotic untreated) and antibiotic treatments.

The general esterase activity of larvae treated with the antibiotics, alone, and in combination with naled or propoxur at the LC_{25} was measured according to the method described by Rakotondravelo et al. (2006). Each mosquito sample was homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% (v/v) Triton X-100 (Sigma Aldrich, St. Louis, MO) at the rate of one mosquito larva per 100 μl sodium phosphate. The individual homogenates were centrifuged at 10,000 x *g* for 10 min at 4 ˚C and the supernatant was used as the enzyme source for measuring general esterase activity with 0.3 mM α-naphthyl acetate (α-NA) (Sigma Aldrich) as a substrate. The hydrolysis of α-NA to the product α-naphthol was measured using a microplate absorbance reader (Molecular Devices, Sunnyvale, CA) at 600 nm and 560 nm for α-NA.. The total protein

in each sample preparation was determined using a bicinchoninic acid assay as described by Smith et al. (1985) with bovine serum albumin (Sigma Aldrich) as a standard. The amount of protein in each sample was measured using a microplate absorbance reader at 560 nm.

The cytochrome P450 monooxygenase activity of larvae treated with the antibiotics, alone, and in combination with naled or propoxur at the LC_{25} was measured according to the method described by Anderson and Zhu (2004) with some modifications. Following the treatments, the individual live larvae were transferred to the wells of a 96-well microplate containing 50 mM sodium phosphate (pH 7.2) and 0.4 mM 7-ethoxycoumarin. The larvae were incubated at 37 ˚C for 4 h followed by the addition of 50% (v/v) acetonitrile and Trizma base (pH 10). The larvae were removed from the microplate wells and the deethylation of 7-ethoxycoumarin to the product 7-hydroxycoumarin was measured using a microplate fluorescence reader (Molecular Devices) at 480 nm while exciting at 380 nm.

3.2.4 In vivo Inhibition of Acetylcholinesterase Activity

The acetylcholinesterase (AChE) activity of larvae treated with antibiotics, alone, and in combination with naled or propoxur was determined according to the method of Ellman et al. (1961) as modified by Carlier et al. (2008). Each mosquito sample was homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3% (v/v) Triton X-100 at the rate of one mosquito larva per 100 µl sodium phosphate. The individual homogenates were centrifuged at 10,000 x *g* for 10 min at 4 ˚C and the

supernatant was used as the enzyme source for measuring AChE activity with acetylthiocholine iodide (ATCh) and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB). The hydrolysis of ATCh and formation of thionitrobenzoic acid was measured using a microplate absorbance reader at 405 nm.

3.2.5 Mosquitocide Degradation Bioassays

Davis minimal broth medium supplemented with casein (DMMC) was prepared. Propoxur or naled were prepared and added separately to flasks containing 5 mL of DMMC. The flasks were inoculated with a homogenate of *Ae. aegypti* larvae. The flasks were prepared with either DMMC media and DMMC plus naled or propoxur as a control treatment. After 12-, 24-, and 48-h incubation, 100 µl of growth medium (LC_{50} of propoxur and naled) was transferred to 4.9 mL of water containing 10 mosquito larvae. Percent mortality was assessed 24 h post treatment.

2.6 Statistical Analysis

Log-probit analysis was used to estimate the LC_{50} values for each mosquitocide, alone and in combination, with the synergists. Synergistic ratios (SR) were calculated by dividing the LC_{50} of the mosquitocide-only treatments by the LC_{50} of the mosquitocide + synergist or antibiotic treatments (e.g., $SR = LC_{50\ \text{mosquitocide-only}} \div LC_{50\ \text{mosquitocide + synergist}}$). The significant differences between the LC_{50} for each mosquitocide, alone and in combination, with the synergist treatments was based on the non-overlapping 95% confidence intervals estimated for each bioassay. The percentage of mosquitoes affected

by the different treatments was statistically compared using a one-way ANOVA and a Tukey's multiple comparison post-test.

3.3. RESULTS

3.3.1 Antibiotic Optimization

The antibiotics gentamycin sulfate (1,500 µg/mL), penicillin (1,000 units/ml), and streptomycin (1,000 µg/mL) resulted in a 99.92% reduction in the culturable bacteria isolated from *Ae. aegypti* mosquito larvae (Fig. 3.3.5-1)

3.3.2 Acute Toxicities of Mosquitocides and Antibiotics

The antibiotics gentamycin sulfate (1,500 µg/mL), penicillin (1,000 units/ml), and streptomycin (1,000 µg/mL), were not acutely toxic to third-instar *Ae. aegypti* larvae under the bioassay conditions. The LC_{50} estimates for propoxur or naled alone or in combination antibiotics were used to calculate synergism ratios (Table 3.3.5-1). The toxicity of propoxur was significantly increased by 2.62-fold when in combination with a fixed concentration of antibiotics (Table 3.3.5-1). In addition, the toxicity of naled was significantly increased by 1.58-fold when in combination with a fixed concentration of antibiotics (Table 3.3.5-1).

3.3.3 In vivo Inhibition of Detoxification Enzyme Activity in Combined Treatments

The *in vivo* inhibition of esterases (α-naphthyl acetate hydrolysis) in third-instar *Ae. aegypti* larvae exposed to the LC_{25} of propoxur or naled alone or in combination with antibiotics, is shown in Fig. 3.3.5-2. The propoxur or naled treatments alone resulted in a 25.7% and 55.3% reduction in esterase specific activity, respectively, compared to the untreated larvae (Fig. 3.3.5-2). The antibiotic treatment alone resulted in a 21.9%

decrease in esterase specific activity, respectively, compared to the untreated larvae (Fig. 3.3.5-2).

A significant reduction of esterase specific activity did occur when larvae were exposed to propoxur in combination with antibiotics, however when mosquito larvae were exposed to naled in combination with antibiotics there was only a slight decrease in esterase specific activity, though this decrease was not significant. The antibiotic treatments resulted in a 50.6% decrease in esterase specific activity when applied in combination with propoxur compared to the larvae treated with propoxur alone (Fig. 3.3.5-2). The antibiotic treatments resulted in a 12.5% increase in esterase specific activity when applied in combination with naled compared to the larvae treated with naled alone (Fig. 3.3.5-2).

The *in vivo* inhibition of cytochrome P450 monooxygenases (*O*-deethylation) in third-instar *Ae. aegypti* larvae exposed to the LC_{25} of propoxur or naled alone or in combination with antibiotics is shown in Fig. 3.3.5-3. The propoxur or naled treatments alone resulted in a 6.3% and 32.4% increases in cytochrome P450 monooxygenase *O*-deethylation activity, respectively, compared to the untreated larvae, however these increases were not significant (Fig. 3.3.5-3). The antibiotic treatment alone resulted in a 90.7% decrease in cytochrome P450 monooxygenase *O*-deethylation activity compared to the untreated larvae (Fig. 3.3.5-3).

A significant reduction of cytochrome P450 monooxygenase *O*-deethylation activity did occur in the larvae exposed to propoxur and naled in combination with antibiotics. The antibiotic treatment resulted in a 84.4% decrease in cytochrome P450 monooxygenase *O*-deethylation activity when applied in combination with propoxur compared to the larvae treated with propoxur alone (Fig. 3.3.5-3). The antibiotic treatment resulted in a 67.6% decrease in cytochrome P450 monooxygenase *O*-deethylation activity when applied in combination with naled , compared to the larvae treated with naled alone (Fig. 3.3.5-3).

3.3.4 In vivo Inhibition of Acetylcholinesterase Activity

The residual acetylcholinesterase (AChE) activity of third-instar *Ae. aegypti* larvae exposed to the LC_{25} of propoxur or naled alone or in combination with antibiotics, is shown in Fig. 3.3.5-4. The propoxur or naled treatments alone resulted in a 48.7% and 40.1% reduction in AChE activity, respectively, compared to the untreated larvae (Fig. 3.3.5-4). The antibiotic treatments alone resulted in a 19.7% increase in AChE activity, respectively, compared to the untreated larvae (Fig. 3.3.5-4). A significant reduction of AChE activity did occur in the larvae exposed to naled in combination with antibiotics compared to larvae treated with naled alone, however treatment with propoxur in combination with antibiotics only resulted in a slight reduction in AChE activity compared to the larvae exposed to propoxur alone. The antibiotic treatments resulted in a 29.7% decrease in the anticholinesterase activity of propoxur, respectively, compared to the larvae treated with propoxur alone (Fig. 3.3.5-4). The antibiotics treatments resulted in a 65.1% decrease in the anticholinesterase activity of naled, respectively, compared to the larvae treated with naled alone (Fig. 3.3.5-4).

3.3.5 Mosquitocide Degradation

Propoxur mortality decreased by *ca*. 20% and 25% in mosquitoes treated with the 24- and 48-h propoxur-bacteria inoculum, respectively, compared to the propoxur-only treatment (Fig. 3.3.5-5). Naled mortality decreased by *ca*. 10% and 15% in mosquitoes treated with the 24- and 48-h naled-bacteria inoculum, respectively, compared to the naled-only treatment (Fig. 3.3.5-6)

3.3.6 Figures and Tables

Table 3.3.5-1. Effects of gentamycin (1,500 µg/ml), penicillin (1,000 U/ml), and streptomycin (1,000 µg/ml) on the acute toxicity of propoxur and naled to third-instar *Ae. aegypti* larvae.

Treatment	N	LC_{10} (95% CI)	LC_{25} (95% CI)	LC_{50} (95% CI)	Slope ± SE	X^2 (prob)	SR
Propoxur	540	339 (266-398)	453 (383-512)	626 (559-694)	4.80 ± 0.35	2.43	
+ Antibiotics	150	134 (54-189)	349 (280-406)	477 (411-541)	4.99 ± 0.69	0.56	2.62
Naled	900	17 (15-20)	25 (22-28)	38 (35-43)	3.69 ± 0.21	1.84	
+ Antibiotics	150	9.2 (6.1-12)	14 (11-18)	24 (19-30)	3.13 ± 0.35	1.36	1.58

[a]Propoxur and naled acute toxicity data are presented as LC_{10}, LC_{25}, and LC_{50} and their 95% confidence intervals (95% CI) in micrograms per liter (µg/L), the concentrations at which 10, 25, and 50% of the tested larvae were dead, respectively, in a 24-h bioassay. Log-probit analysis was used to estimate the endpoint concentrations for each mosquitocide.

[b]Pearson's chi-square and the probability of χ^2. The probability of > 0.05 indicates that the observed regression model is not significantly different from the expected model (i.e., a significant fit between the observed and expected regression models).

[c]Synergism ratio (SR) was calculated by SR = LC_{50} mosquitocide only / LC_{50} mosquitocide-synergist mixture. The asterisk next to the ratio indicates a significant difference between the LC_{50} of the mosquitocide and the binary combination with a synergist based on the non-overlapping 95% confidence intervals of the LC_{50} values.

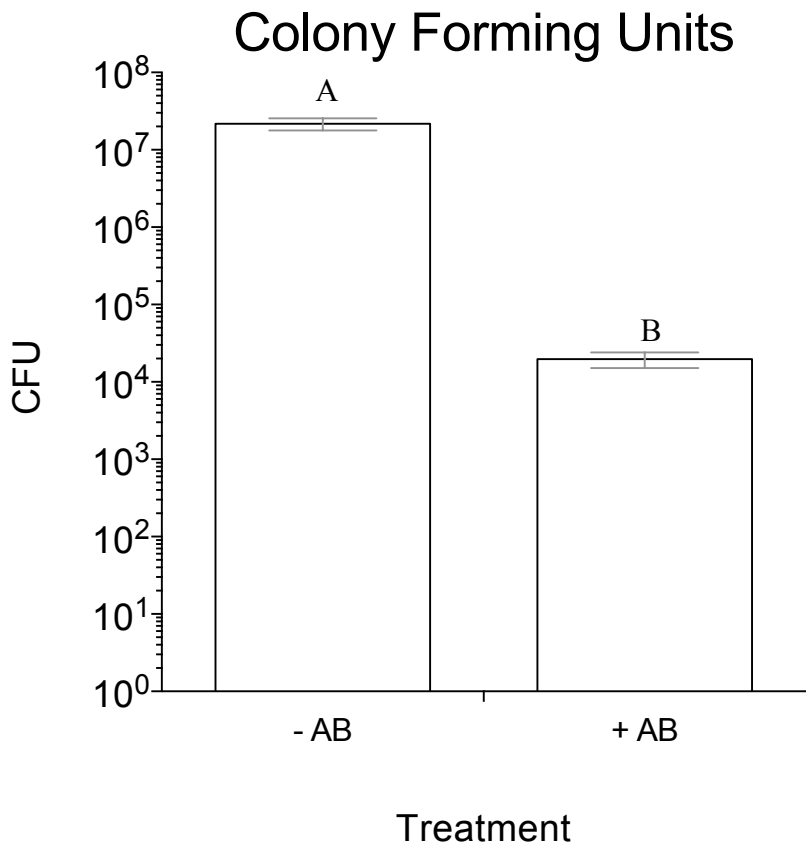

Figure 3.3.5-1 Colony forming units of untreated and antibiotic-treated mosquitoes. Larvae were treated with an antibiotic mixture containing 1,500 μg/mL gentamycin sulfate, 1,000 units/ml penicillin, and 1,000 μg/mL streptomycin for 12 h. Treated and untreated larvae were then homogenized with PBS and plated onto an LB agar plate. Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$).

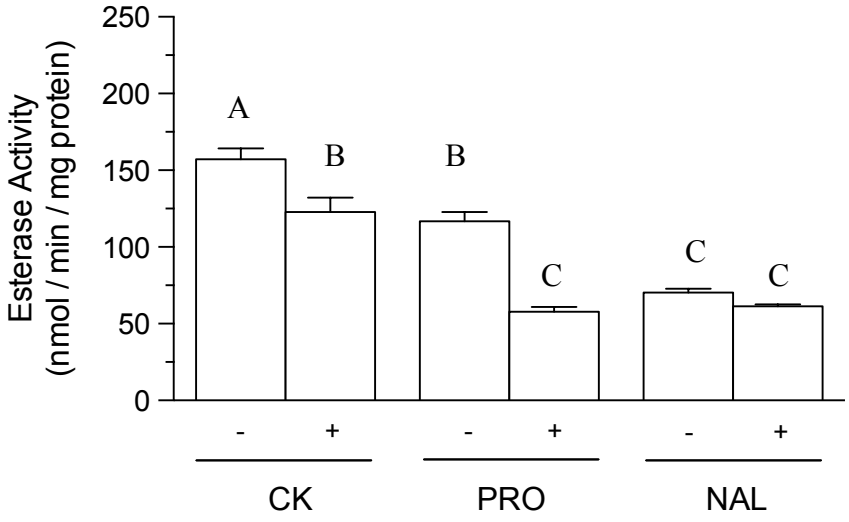

Figure 3.3.5-2 *In vivo* inhibition of esterase activities in third-instar Ae. aegypti larvae treated with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin alone and in combination with propoxur or naled. Larvae were treated with a combination of gentamycin (1,500 µg/ml), penicillin (1,000 U/ml), and streptomycin (1,000 µg/ml) for 12 h followed by a treatment of propoxur (PRO) at 453 µg/L (LC_{25}) or naled (NAL) at 25 µg/L (LC_{25}) for 24 h. CK is the control treatment. Enzyme activities are presented as the mean $\pm$ standard error ($n = 3$). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). Insecticide or control treatment alone, no antibiotics (-); insecticide or control treatment + antibiotics (+)

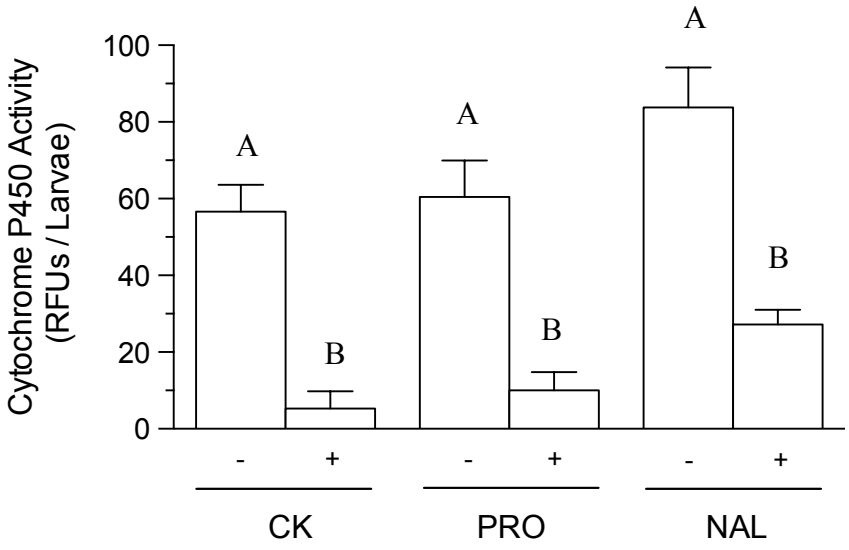

Figure 3.3.5-3 *In vivo* inhibition of cytochrome P450 monooxygenase activities in third-instar *Ae. aegypti* larvae treated with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin alone and in combination with propoxur or naled. Larvae were treated with a combination of gentamycin (1,500 µg/ml), penicillin (1,000 U/ml), and streptomycin (1,000 µg/ml) for 12 h followed by a treatment of propoxur (PRO) at 453 µg/L (LC_{25}) or naled (NAL) at 25 µg/L (LC_{25}) for 24 h. CK is the control treatment. Enzyme activities are presented as the mean $\pm$ standard error ($n = 3$). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). Insecticide or control treatment alone, no antibiotics (-); insecticide or control treatment + antibiotics (+)

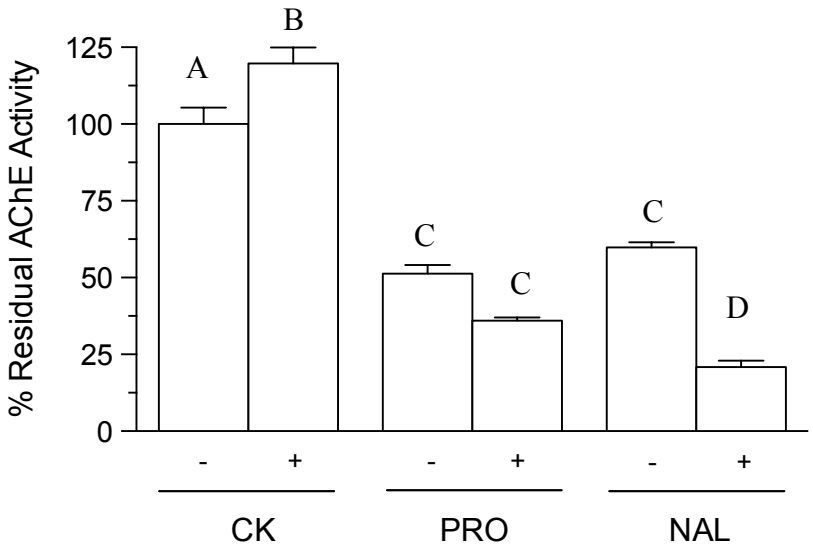

Figure 3.3.5-4 *In vivo* inhibition of acetylcholinesterse (AChE) activities in third-instar *Ae. aegypti* larvae treated with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin alone and in combination with propoxur or naled. Larvae were treated with a combination of gentamycin (1,500 μg/ml), penicillin (1,000 U/ml), and streptomycin (1,000 μg/ml) for 12 h followed by a treatment of propoxur (PRO) at 453 μg/L (LC_{25}) or naled (NAL) at 25 μg/L (LC_{25}) for 24 h. CK is the control treatment. Enzyme activities are presented as the mean $\pm$ standard error ($n = 3$). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). Insecticide or control treatment alone, no antibiotics (-); insecticide or control treatment + antibiotics (+)

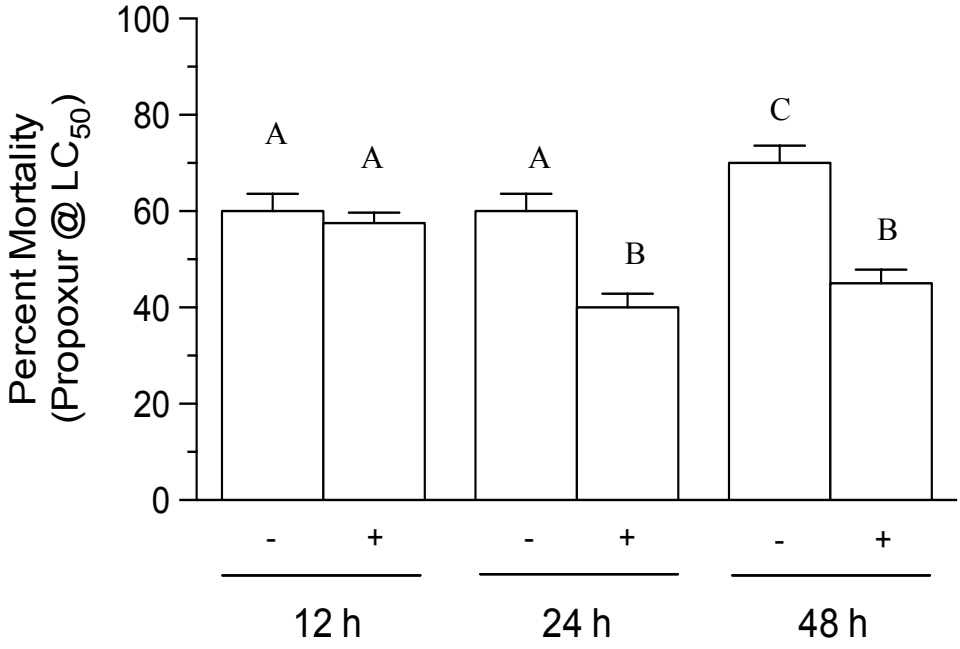

Figure 3.3.5-5. Percent mortality of third-instar *Ae. aegypti* larvae treated with bacteria-degraded propoxur. Bacteria culture was treated with propoxur at 626 µg/L and incubated for 12, 24, or 48 h. Larvae were treated with the propoxur-bacteria inoculum diluted to the LC_{50} (626 µg/L) of each mosquitocide for 24 h. Percent mortality is presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). DMMC + Bacteria + Mosquitocide (+); DMMC + Mosquitocide (-)

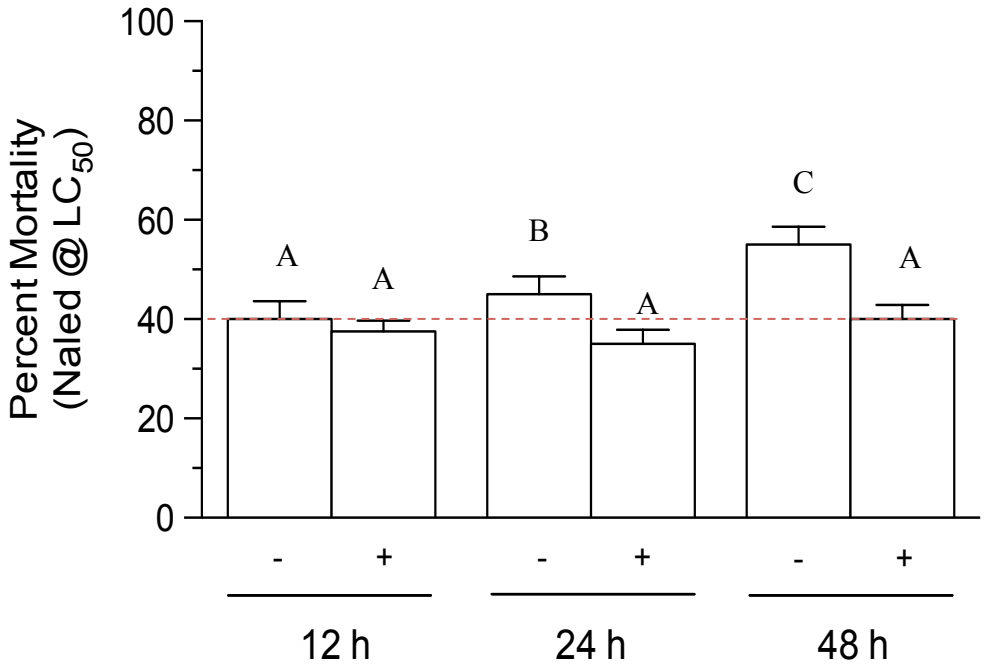

Figure 3.3.5-6. Percent mortality of third-instar *Ae. aegypti* larvae treated with bacteria-degraded naled. Bacteria culture was treated with naled and incubated for 12, 24, or 48 h. Larvae were treated with the naled-bacteria inoculum diluted to the LC_{50} (38 µg/L) of each mosquitocide for 24 h. Percent mortality is presented as the mean ± standard error (n = 3). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance with a Tukey's multiple comparison test ($p < 0.05$). DMMC + Bacteria + Mosquitocide (+); DMMC + Mosquitocide (-)

3.4 DISCUSSION OF RESULTS

The goal of this study was to examine the acute toxicities of naled and propoxur to *Ae. aegypti* larvae as well as the cytochrome P450 monooxygenase and general esterase activities of the larvae following the reduction of bacteria with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin. To my knowledge, the data gathered in this study provides new information for the manner with which the metabolic detoxification enzymes of bacteria in *Ae. aegypti* larvae may alter the toxic action of standard-use chemistries for mosquito control. The hypothesis for this study is that the metabolic detoxification and toxic action of the mosquitocides naled and propoxur is regulated by the bacteria in *Ae. aegypti* larvae. This study demonstrates that the reduced cytochrome P450 monooxygenase and general esterase activities in *Ae. aegypti* larvae treated with broad-spectrum antibiotics can decrease the acute toxicities of naled and propoxur. These data suggest that gentamycin, penicillin, and streptomycin decrease the number of bacteria colony-forming units in the mosquito larvae and, in turn, reduce metabolic detoxification activities of the mosquito larvae to naled and propoxur.

Kikuchi et al. (2012) report a previously unknown mechanism of insecticide resistance via the acquisition of mutualistic symbiotic bacteria in an insect and the metabolic detoxification of insecticides. It was observed that observed that an immature insect can acquire the bacteria *Burkholderia* sp. from the soil and these bacteria can increased the organophosphate insecticide tolerance of the host insect (Kikuchi et al. 2012). Here, I have observed a reduction in cytochrome P450 monooxygenase and general esterase activities in *Ae. aegypti* larvae treated with the broad-spectrum antibiotics gentamycin,

penicillin, and streptomycin. These results suggest that a broad-spectrum antibiotic-mediated reduction of bacteria in the mosquito larvae can decrease the metabolic detoxification of the standard-use mosquitocides propoxur and naled. In addition, these results suggest that the bacteria themselves may contain metabolic detoxification enzymes that are functionally similar to those of *Ae. aegypti* larvae. Russell et al. (2011) report the metabolic detoxification enzymes in both carbamate- and organophosphate-degrading bacteria. These enzymes include MCD and CehA for the carbamate insecticides and phosphotriesterases (PTEs), methyl parathion hydrolases (MPHs), and organophosphorus acid anhydrolases (OPAA) for organophosphate insecticides. The esterases MCD isolated from *Achromobacter* W111 (Derbyshire et al. 1987) and CehA isolated from *Rhizobium sp* AC100 (Hashimoto et al. 2002) are reported to hydrolyze carboxylester bonds of 1-naphthyl acetate. These enzymes are associated with organophosphate insecticide metabolism via hydrolysis of phosphoester bonds (Russell et al. 2011). It has also been suggested that there may exist an uncharacterized oxidative route for degradation of some organophosphate insecticides (Munnecke and Hsieh 1976). In this study, I have observed an increase in acetylcholinesterase activity in *Ae. aegypti* larvae treated with the antibiotics compared to the untreated mosquito larvae. However, the anticholinesterase activities of propoxur and naled was reduced in the antibiotic-treated *Ae. aegypti* larvae compared to those mosquito larvae only treated with propoxur and naled. These results suggest that the bacteria in mosquito larvae may modify the toxic action of standard-use mosquitocides.

In addition, this study examined the metabolic detoxification of propoxur and naled using culturable bacteria isolated from the *Ae. aegypti* larvae. Following the incubation of propoxur and naled with the culturable bacteria, the acute toxicities of these mosquitocides were reduced to mosquito larvae. Bacteria are reported to facilitate the metabolic detoxification of insecticides using enzyme systems that transform these compounds to carbon and energy sources for growth (Yu 2008). Werren et al. (2012) observed that bacteria are capable of metabolizing insecticides and utilizing the carbon-, phosphorous, and nitrogen degradation products. The degradation products of propoxur and naled were not analyzed in the bacteria cultures for this study and, thus, I cannot conclude that the culturable bacteria is in fact responsible the metabolic detoxification of these mosquitocides in *Ae. aegypti* larvae.

There are many insects that harbor large communities of diverse microorganisms, which that likely exceeds the number of cells in the insects themselves (Gusmão 2000). Research studies have focused on the contribution of microbial endosymbionts to the host's reproduction mode (Haine 2008), nutritional homeostasis (Dillon 2004), mate selection (Werren 2008), and pathogen defense (Moran 2007, Dong et al. 2009, O'Neill et al. 2011, Hoffmann et al. 2011). Kikuchi et al. (2012) and Patil et al. (2013) suggest that bacteria may actually modulate mosquito susceptibility to insecticides, which was the basis for this study to examine the metabolic detoxification and toxic action of standard-use mosquitocides that can be regulated by the bacteria in *Ae. aegypti* larvae. However, the need for additional experiments is warranted to fully elucidate the contribution bacteria in *Ae. aegypti* larvae to the metabolic detoxification of mosquitocides.

The use of insecticides in agriculture and public health in addition to the presence of anthropogenic xenobiotics (e.g., antibiotics) may affect the microbial biodiversity of the environment. The overall responses of mosquito larvae to standard-use mosquitocides may be altered and the selection of mosquitocide resistance mechanisms may occur as a consequence. In this context, future studies should be performed that focus on better understanding mosquitocide resistance mechanisms in *Ae. aegypti* larvae, with regard to the mosquito microbiome, and compiling data on the impact of the environment on mosquito symbiont biodiversity in an effort to explain how these factors might influence mosquitocide resistance. Because these studies were conducted using laboratory-reared *Ae. aegypti* larvae, it is possible that the results observed in this study might be even more or less pronounced in natural mosquito populations as a result of the different exposure that the natural populations would have to microbe-rich habitats. Therefore, it would be valuable to collect mosquitoes from various larval habitats, screen these populations of mosquitoes for differences in microbial diversity, and determine the mosquitocides resistance status. It would then be pertinent to identify various environmental factors (i.e. mosquitocide application, chemical leaching, etc.) that may be contributing to these differences and establish relevant trends. The information gained from these additional studies might be used to 1) improve current pharmacodynamic and pharmacokinetic models for established and experimental mosquitocidal chemistries; 2) develop more effective, sustainable, and targeted vector control interventions; and 3) highlight the potential environmental impacts of mosquito usage in public health and agriculture.

Chapter 4

SUMMARY

The specific aim of this study were to evaluate the bacterial contribution to the metabolic detoxification of standard-use mosquitocides in the yellow fever mosquito larvae, *Aedes aegypti*. The mosquitocides propoxur (carbamate class) and naled (organophosphate class) were used in this study. First, the mosquitocide synergists piperonyl butoxide (PBO), triphenyl phosphate (TPP), and *S,S,S*-tributyl phosphorotrithioate (DEF) were used to examine the cytochrome P450 monooxygenase- and general esterase-mediated metabolic detoxification and toxic action of propoxur and naled in the mosquito larvae. Second, the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin were used to examine the bacteria-mediated metabolic detoxification and toxic action of propoxur and naled in the mosquito larvae. Third, the metabolic degradation of propoxur and naled was examined using the culturable bacteria from the mosquito larvae.

General esterases are enzymes that are able to hydrolyze mosquitocides from the parent compound to less toxic metabolites. DEF and TPP are known general esterase inhibitors. The literature suggests that esterases present within the mosquito should degrade insecticides such as propoxur and naled which should subsequently lead to less of the compound getting to it's intended target site (lower antichlinesterase activity) and a corresponding increase in residual AChE activity. As a result of this, toxicity should also decrease. When combined with compounds such as naled and propoxur, insecticide synergists such as DEF and TPP will inhibit esterases, less insecticide will be degraded and will therefore have higher anticholinesterase activity (lower residual AChE activity)

which leads to an increase in toxicity. Our results confirm and suggest that esterases do play a major role in the metabolic detoxification of both propoxur and naled.

Cytochrome P450 monooxygenases are enzymes that either activate or inactivate mosquitocides by introducing or exposing functional groups to the compound. For the carbamates, such as propoxur, the literature suggests that cytochrome P450 monooxygenases will inactivate them to less toxic metabolites which should result in less of the compound getting to it's intended target site (lower antichlinesterase activity) and a corresponding decrease in toxicity. When PBO was applied in combination with propoxur, a significant increase in toxicity was observed, however there was not a corresponding decrease in AChE activity, as expected. It is likely that this can be explained by PBO's unknown mode of action. While PBO is a known cytochrome P450 monooxygenase inhibitor, it also has a number of other known functions including the ability to act as a blocker of esterases (including AChE), the ability to alter cuticular penetration, and the ability to alter blood brain barrier permeability. Even though a significant increase in propoxur toxicity was observed when applied in combination with PBO, this may not necessarily be a result of AChE inhibition but rather the result of some unknown function that PBO may be contribiting.

For the organophosphates, such as naled, the literature suggests that cytochrome P450 monooxygenases will actually act to metabolically activate these compounds into more toxic metabolites. So when they are inhibited by PBO we should see less insecticide being activated and a corresponding decrease in the anticholinesterase activity of the

compound (higher residual AChE activity) and a subsequent decrease in toxicity. Our results confirmed this.

The hypothesis for this study was that the metabolic detoxification and toxic action of the mosquitocides naled and propoxur is regulated by the cytochrome P450 monooxygenase and general esterase activities of *Ae. aegypti* larvae. To my knowledge, the data gathered in this study provides new information for the manner with which metabolic detoxification enzymes in *Ae. aegypti* larvae can alter the toxic action of standard-use chemistries for mosquito control.

The second objective of this study was to examine the acute toxicities of naled and propoxur to *Ae. aegypti* larvae as well as the cytochrome P450 monooxygenase and general esterase activities of the larvae following the reduction of bacteria with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin. An increased susceptibility of antibiotic-treated larvae to naled and propoxur toxicity was observed, along with a reduction in cytochrome P450 monooxygenase and general esterase activities in mosquito larvae treated with the broad-spectrum antibiotics. These results suggest that a reduction of bacteria in mosquito larvae treated with broad-spectrum antibiotics can decrease the metabolic detoxification of the standard-use mosquitocides propoxur and naled. In addition, these results suggest that the bacteria themselves may contain metabolic detoxification enzymes that are functionally similar to those of the mosquito larvae. An increase in acetylcholinesterase activity was also observed in the antibiotic-treated mosquitoes compared to the untreated individuals; however, the

anticholinesterase activities of propoxur and naled was reduced in the antibiotic-treated mosquitoes compared to those individuals only treated with propoxur and naled. These results suggest that the bacteria in mosquito larvae may modify the toxic action of standard-use mosquitocides.

In addition, this study examined the metabolic detoxification of propoxur and naled using culturable bacteria isolated from the *Ae. aegypti* larvae. Following the incubation of propoxur and naled with the culturable bacteria, the acute toxicities of these mosquitocides were reduced to mosquito larvae. Previous studies suggest that bacteria facilitate the metabolic detoxification of insecticides using enzyme systems that transform these compounds to carbon and energy sources for growth (Yu 2008). Werren et al. (2012) observed that bacteria are capable of metabolizing insecticides and utilizing the carbon-, phosphorous, and nitrogen degradation products. The degradation products of propoxur and naled were not analyzed in the bacteria cultures for this study and, thus, I cannot conclude that the culturable bacteria is in fact responsible the metabolic detoxification of these mosquitocides in *Ae. aegypti* larvae.

The goal of this study was to examine the acute toxicities of naled and propoxur to *Ae. aegypti* larvae as well as the cytochrome P450 monooxygenase and general esterase activities of the larvae following the reduction of bacteria with the broad-spectrum antibiotics gentamycin, penicillin, and streptomycin. To my knowledge, the data gathered in this study provides new information for the manner with which the metabolic detoxification enzymes of bacteria in *Ae. aegypti* larvae may alter the toxic action of

standard-use chemistries for mosquito control. The hypothesis for this study is that the metabolic detoxification and toxic action of the mosquitocides naled and propoxur is regulated by the bacteria in *Ae. aegypti* larvae. This study demonstrates that the reduced cytochrome P450 monooxygenase and general esterase activities in *Ae. aegypti* larvae treated with broad-spectrum antibiotics can decrease the acute toxicities of naled and propoxur. These data suggest that gentamycin, penicillin, and streptomycin decrease the number of bacteria colony-forming units in the mosquito larvae and, in turn, reduce metabolic detoxification activities of the mosquito larvae to naled and propoxur. Kikuchi et al. (2012) and Patil et al. (2013) suggest that bacteria may actually modulate mosquito susceptibility to insecticides, which was the basis for this study to examine the metabolic detoxification and toxic action of standard-use mosquitocides that can be regulated by the bacteria in *Ae. aegypti* larvae. However, the need for additional experiments is warranted to fully elucidate the contribution bacteria in *Ae. aegypti* larvae to the metabolic detoxification of mosquitocides.

References Cited

Ahmed, S., and Ahmad, M. S., 2006. Effect of insecticides on the total number of soil bacteria under laboratory and field conditions. *Pak. Entomol.* 28 (2); 63-68.

Alphey, N., Alphey, L., & Bonsall, M. B., 2011. A model framework to estimate impact and cost of genetics-based sterile insect methods for dengue vector control. *PloS One*, *6*(10):e25384.

Anderson, T. D. and Zhu, K. Y., 2004. Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). *Pestic. Biochem. Physiol.* 80: 54-64.

Anderson. T. D., Paulson, S. L., Wong, D. M., Carlier, P. R., Bloomquist, J. R. 2009. Pharmacological mapping of the acetylcholinesterase active site gorge in mosquitoes with *bis*(n)-tacrines. In: Advanced in Human Vector Control. *ACS Sympos Ser*. 1014:143-151.

Bernard, C. B., and Philogène, B. J., 1993. Insecticide synergists: role, importance, and perspectives. *J of Toxicol. and Env. Health*, *38*(2):199–223.

Bhatt, Samir; Gething, Peter W; Brady, Oliver J; Messina, Jane P; Farlow, Andrew W et al., 2013. *Nature* vol. 496 (7446): 504-7

Benedict, M. Q., 1997. Care and maintenance of anopheline mosquito colonies. In: Crampton J. M., Beard, C. B., Louis, C. (Eds.) The Molecular Biology of Insect Disease Vectors. Chapman and Hall, London, pp. 3-13

Brogdon, W. G., & McAllister, J. C., 1998. Insecticide resistance and vector control. *Emerging Infectious Diseases*, *4*(4), 605–13.

Carlier P., Anderson T., Wong D., Hsu D., Hartsel J., Ma M., Wong E., Choudhury R., Lam P., Totrov M., Bloomquist J., 2008. Towards a Species-Selective Acetylcholinesterase Inhibitor to Control the Mosquito Vector of Malaria, Anopheles gambiae. *Chemico-Biol. Inter.,* 175: 368-75.

Casida, J. E., and Quistad, G. B., 2004. Why insecticides are more toxic to insects than people: The unique toxicology of insects. *J Pestic. Sci.* 29(2); 81 - 86.

Cheesman, M. J., Horne, I., Weir, K., Pandey, G., Williams, M., Scott, C., Russell, R. J., 2007. Carbamate pesticides and their biological degradation: prospects for enzymatic bioremediation. In: Kennedy, I. R., Solomon, K. R., Gee, S. J., Crossan, A. N., Wang, S., Sanchez-Bayo, F., et al., (Eds.). Rational Environmental Management of Agrochemicals; Risk Assessment, Monitoring and Remedial Action. Washington: *Amer. Chem. Soc.* pp. 288–305.

Cheng, T.C., Harvey, S.P., Stroup, A. N., 1993. Purification and properties of a highly active organophosphorous acid anhydrolase from Alteromonas undina. *Appl. Environ. Microbiol.* 59:3138-3140.

Connelly, C.R., and Carlson, D.B., (Eds.). 2009. Florida Coordinating Council on Mosquito Control. Florida Mosquito Control: The state of the mission as defined by mosquito controllers, regulators, and environmental managers. Vero Beach, FL: University of Florida, Institute of Food and Agricultural Sciences, Florida Medical Entomology Laboratory.

de Bary, A. 1879. *The Phenomenon of Symbiosis.* (Verlag Von Karl J. Trubner, Strasbourg).

Derbyshire, M. K., Karns, J. S., Kearney, P. C., Nelson, J. O., 1987. Purification and characterization of an *N*-methylcarbamate pesticide hydrolyzing enzyme. *J Agric. Food Chem.* 35:871–877.

Dong, Y., Manfredini, F., Dimopoulos, G., 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens* 5(5):e1000423.

Duprey, Z., Rivers, S., Luber, G., Becker, A., Blackmore, C., Barr, D., … Rubin, C., 2008. Community aerial mosquito control and naled exposure. *J Am Mosq Control Assoc.*, *24*(1): 42–6.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, *7*(2): 88–95.

Field, L.M., Devonshire, A.L., Forde, B.G., 1988. Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem. J.* 251, 309–312.

Grant, D. F., and Matsumura F., 1988. Glutathione S-transferase 1 and 2 in susceptible and insecticide resistant *Aedes aegypti. Pestic. Biochem. Physiol.* 33:132-143.

Gubler, D. J., 1998. Dengue and Dengue Hemorrhagic Fever. Clin. Microbiol. Rev. 11: 480–496.

Gubler, D. J., 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends. Microbiol.* 10: 100–103.

Gupta, R. (Ed.)., 2011. *Toxicology of Organophosphate & Carbamate Compounds.* p. 768. Academic Press.

Hashimoto, M., Fukui, M., Hayano, K., Hayatsu, M., 2002. Nucleotide sequence and genetic structure of a novel carbaryl hydrolase gene (*ceh*A) from *Rhizobium* sp strain AC100. *Appl. Environ. Microbiol.* 68:1220–1227.

Hemingway, J., 1982. Genetics of organophosphate and carbamate resistance in*Anopheles atroparvus* (Diptera: Culicidae) J Econ. Ent . 75:1055–8.

Hemingway, J., and Karunaratne, S. H., 1998. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med. and Vet. Ent.*, *12*(1), 1–12.

Hemingway, J., 2000. The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochemistry and Molecular Biology*, *30*(11); 1009–15.

Hemingway, J., Field, L., & Vontas, J., 2002. An overview of insecticide resistance. *Science*, *298*(5591), 96–97.

Hemingway, J., Beaty, B. J., Rowland, M., Scott, T. W., Sharp, B. L., 2006. The Innovative Vector Control Consortium: improved control of mosquito-borne diseases. *Trends Parasitol.* 22:308–312.

Hayatsu, M., Mizutani, A., Hashimoto, M., Sato, K., Hayano, K., 2001. Purification and characterization of carbaryl hydrolase from *Arthrobacter* sp RC100. FEMS Microbiol. Letters 201:99–103.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., … O'Neill, S. L., 2011. Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature*, *476*(7361):454–7.

Karunaratne, S. H, Jayawardena K. G., Hemingway J., and Ketterman A. J., 1993. Characterization of a B-type esterase involved in insecticide resistance from the mosquito Culex quinquefasciatus. *Biochem. J,* 294: 575–579

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., Fukatsu, T., 2012. Symbiont-mediated insecticide resistance. *PNAS* 109:8618-8622.

Konrad J. G., Chesters G., Armstrong D. E., 1969. Soil degradation of malathion, a phosphorodithioate insecticide. *Soil Sci Soc Am Proc.* 33:259-262.

Li, R., et al., 2010. Biochemical degradation pathway of dimethoate by *Paracoccus* sp. Lgjj-3 isolated from treatment wastewater. *Int. Biodeter. Biodegr.* 64: 51– 57.

Maloney, S. E., Maule, A., Smith, A. R. W., 1993. Purification and preliminary characterization of permethrinase from a pyrethroid-transforming strain of *Bacillus cereus*. *Appl. Environ Microbiol.* 59:2007–2013.

Matsumura F., Ward C. T., 1966. Degradation of insecticides by the human and the rat liver. *Arch Environ Health* 13:257-261.

Meyer, J. M., Ejendal, K. F. K., Avramova, L. V., Garland-Kuntz, E. E., Giraldo-Calderón, G. I., et al., 2012. A "Genome-to-Lead" Approach for Insecticide Discovery: Pharmacological Characterization and Screening of *Aedes aegypti* D1-like Dopamine Receptors. *PLoS Negl. Trop. Dis.* 6(1): e1478.

Morrison, A. C., Zielinski-Gutierrez, E., Scott, T. W., Rosenberg, R., 2008. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. *PLoS Med* 5: e68.

Matowo, J., Kulkarni, M. A., Mosha, F. W., Oxborough, R. M., Kitau, J. A., Tenu, F., & Rowland, M., 2010. Biochemical basis of permethrin resistance in Anopheles arabiensis from Lower Moshi, north-eastern Tanzania. *Malaria J*, *9*:193.

Mulbry, W. W., and Karns, J. S. 1989. Parathion hydrolase specified by the Flavobacterium opd gene-relationship between the gene and protein. *J. Bacteriol.* 171: 6740–6746

Okumu, F. O., and Moore, S. J., 2011. Combining indoor residual spraying and insecticide-treated nets for malaria control in Africa: a review of possible outcomes and an outline of suggestions for the future. *Malar. J.* 10: 208.

Pandey, G., et al., 2010. Cloning and biochemical characterization of a novel carbendazim (methyl-1H-benzimidazol-2-ylcarbamate)-hydrolyzing esterase from the newly isolated *Nocardioides* sp. strain SG-4G and its potential for use in enzymatic bioremediation. *Appl. Environ. Microbiol.* 76: 2940– 2945.

Patil, V. L., and Guthrie, F. E., 1979. Cuticular lipids of two resistant and a susceptible strain of houseflies. *Pestic. Sci.*, *10*(5): 399–406.

Prapanthadara, L., Hemingway, J., Ketterman, A. J., 1995. DDT resistance in *Anopheles gambiae* Giles from Zanzibar, Tanzania, based on increased DDT-dehydrochlorinase activity of glutathione S-transferases. *Bull. Entomol. Res.* 85:267–274.

Prapanthadara, L.,Koottathep, S., Promlet, N., Hemingway, J., Ketterman A.J., 1996. Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (species B). *Insect Biochem. Mol. Biol.* 26:277-285.

Ragnarsdottir, K., 2000. Environmental fate and toxicology of organophosphate pesticides. *J Geol. Soc. London* 157:859–876.

Rakotondravelo et al., 2006. Sublethal effects of three pesticides on activities of selected target and detoxification enzymes in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). *Arch. Environ. Contam. Toxicol.* 51:360-366.

Pridgeon et al., 2009. Toxicity comparison of eight repellents against four species of female mosquitoes. *J. Am. Mosq. Cont. Assoc.* 25(2):168-173.

Rani, A., Sharma, A., Rajagopal, R., Adak, T., Bhatnagar, R. K., 2009. Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*- an Asian malarial vector. *BMC Microbiol.* 9: 96.

Russell, R. J., Scott, C., Jackson, C. J., Pandey, R., Pandey, G., Taylor, M. C., Coppin, C. W., Liu, J.-W. and Oakeshott, J. G., 2011. The evolution of new enzyme function: lessons from xenobiotic metabolizing bacteria versus insecticide-resistant insects. *Evol. Appl.* 4: 225–248.

Sanil, D., and Shetty, N. J., 2010. Genetic study of propoxur resistance-a carbamate insecticide in the malaria mosquito, *Anopheles* stephensi Liston. *Malar. Res. Treat.*, *2010*, 502824.

Serdar, C. M., Gibson, D. T., Munnecke, D. M., and Lancaster, J.H.. 1982. Plasmid involvement in parathion hydrolysis by Pseudomonas diminuta. *Appl. Environ. Microbiol.,* 44: 246-249.

Sethunathan, N., and Yoshida, T., 1973. A Flavobacterium sp. that degrades diazinon and parathion. *Can. J Microbiol.*, *19*(7): 873–5.

Siddavattam, D., Khajamohiddin, S., Manavathi, B., Pakala, S. B., and Merrick, M. 2003. Transposon-like organization of the plasmid-borne organophosphate degradation

(opd) gene cluster found in Flavobacterium sp. *Appl. Environ. Microbiol.* 69: 2533–2539

Singh, B. and Walker, A., 2006. Microbial degradation of organophosphorus compounds. *FEMS Microbiol. Rev.* 30:428–471.

Singh, B. K. 2009. Organophosphorus-degrading bacteria: ecology and industrial applications. *Nat. Rev. Microbiol.* 7, 156–164.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C., 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem*. 150, 76-85

Soderlund, D. M., Bloomquist, J. R., 1990. Molecular mechanisms of insecticide resistance. In: Roush, R. T., Tabashnik, B. E., (Eds.), Pesticide Resistance in Arthropods. Chapman and Hall, London, pp. 58–96.

Srigiriraju, L., Semtner, P. J., Anderson, T. D., and J. R. Bloomquist., 2009. Esterase-based resistance in the tobacco-adapted form of the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Arch. Insect. Biochem. Physiol.* 72:105-123.

Sun, C. A., Wang, L. Y., Chen, C. J., Lu, S.L., You, S. L., Wang, L. W., Wang, Q., Wu, D. M., Santella, R. M., 2001. Genetic polymorphisms of glutathione S-transferase M1 and T1 associated with susceptibility to aflatoxin-related hepatocarcinogenesis among chronic hepatitis B carriers: a nested case-control study in Taiwan. Carcinogenesis. 22:1289–1294.

Terriere, L. C., 1982. The biochemistry and toxicology of insecticides. Oregon State University, Corvalis.

Tomasek, P. H., Karns, J. S., 1989. Cloning of a carbofuran hydrolase gene from *Achromobacter* sp. strain WM111 and its expression in gram-negative bacteria. *J. Bacteriol.* 171: 4038– 4044.

Vinson, S. B. and Law, P. K., 1971. Cuticular composition and DDT resistance in the tobacco budworm. *J. Econ. Entomol.* 64: 1387-1390.

Vontas, J., Kioulos, E., Pavlidi, N., Morou, E., della Torre, A., and Ranson, H., 2012. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. *Pestic. Biochem. Physiol*. 104:126-131.

Wang, B. Z., Guo, P., Hang, B. J., Li, L., He, J., Li, S.P., 2009. Cloning of a novel pyrethroid-hydrolyzing carboxylesterase gene from *Sphingobium* sp. Strain JZ-1 and characterization of the gene product. *Appl. Environ. Microbiol.* 75:5496–5500.

Weill, M., Fort, P., Berthomieu, A., Dubois, M. P., Pasteur, N., & Raymond, M., 2002. A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the ace gene in Drosophila. *Proc. Biolog. Sci. / The Royal Society*, *269*(1504):2007–16.

Werren, J. H. (2012). Symbionts provide pesticide detoxification. *Proc. Nat. Acad. Sci. USA.*, *109*(22): 8364–5.

Wilkinson, C. F., 1976. *Insecticide biochemistry and physiology*, p. 768. New York: Plenum Press.

Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. Geneva: World Health Organization; 2009. Available from: http://www.ncbi.nlm.nih.gov/books/NBK143157/

World Malaria Report 2011, The World Health Organization. Available: http://www.who.int/malaria/world_malaria_report_2011/9789241564403_eng.pdf.]

Wu, P. C., Liu, Y. H., Wang, Z. Y., Zhang, X. Y., Li, H., Liang, W. Q., Luo, N., et al., 2006. Molecular cloning, purification, and biochemical characterization of a novel pyrethroid-hydrolyzing esterase from *Klebsiella* sp strain ZD112. *J Agric. Food Chem.* 54:836–842.

Yu, S. J., 1996. Insect glutathione S-transferases. *Zool. Stud*. 35(1): 9-19.

Yu, S. J., 1982. Host plant induction of glutathione *S*-transferase in the fall armyworm. *Pestic. Biochem. Physiol.* 18:101-106.

Yu, S. J., 2008. The Toxicology and Biochemistry of Insecticides. Boca Raton, FL: CRC Press, pp. 235-240.

van Asperen, K., 1962. A study of house fly esterases by means of a sensitive colormetric method. *J. Insect Physiol.* 8:401:416.

Zhang, M. L., Scott, J. G., 1996. Cytochrome b(5) is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pestic. Biochem. Physiol.* 55:150–156.