EVALUATION OF NOVEL POTASSIUM CHANNEL-DIRECTED COMPOUNDS AS NEW MOSQUITOCIDES FOR CONTROL OF AEDES AEGYPTI AND ANOPHELES GAMBIAE

By

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To my family and friends who have always been there for me
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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

EVALUATION OF NOVEL POTASSIUM CHANNEL-DIRECTED COMPOUNDS AS NEW MOSQUITOCIDES FOR CONTROL OF *Aedes aegypti* AND *Anopheles gambiae*

By

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Vector control with insecticides is in a constant struggle to stay ahead of resistance development, so new insecticides with novel modes of action are needed. A novel class of target site for insecticide development would be potassium channels of nerve and muscle membranes. In this study, known and experimental potassium channel blockers were tested for toxicity against *Anopheles gambiae* and *Aedes aegypti* mosquitoes. 4-Aminopyridine was found to have the closest toxicity (10-fold less) to the industrial insecticide propoxur in a new headless larvae paralysis assay. Catechol PRC 725 was the most toxic experimental compound in all assays, but was at least 100-fold less toxic than propoxur in *Ae. aegypti* and 39-fold in *An. gambiae*. Potassium channel blockers increased tension in larval muscle sheets, along with increased duration of evoked contractions. For example, tetraethylammonium chloride (30 mM) increased baseline muscle tension and evoked contraction duration upwards of 200% and 500%, respectively. A novel symptom of melanization occurred after exposure to catechol compounds in the larval mortality assays, which may indicate their metabolism by phenoloxidase, followed by chemical polymerization. Compared to *An. gambiae* adults,
toxicity of the compounds decreased significantly when screened against *Ae. aegypti*.
Injection and synergist studies indicated that the decreased sensitivity was not primarily
due to the cuticle acting as a barrier, nor by metabolic degradation of the compounds.
While the toxicity of the experimental catechols was modest, the chemical structures of
these compounds may prove to be useful leads for the development of more powerful
compounds.
1.1 Vector-Borne Diseases of Anopheles and Aedes

There are 38 genera of mosquitoes, and three of them, *Anopheles*, *Aedes*, and *Culex* are considered to be of critical importance to pathogen transmission. Within these genera, the *Anopheles gambiae* (Giles) and *Culex pipiens* complexes, along with the *Aedes*, in the subgenus *Stegomyia*, are the mosquito species of greatest concern (Foster and Walker, 2009). A number of disease-causing pathogens are transmitted by these mosquitoes (Table 1-1). The resultant diseases, such as malaria, dengue fever, and yellow fever, have or have had major impacts on human society (Krauss, 2003). As seen in Table 1-1, *anopheline* mosquitoes transmit the *plasmodium* protozoan that causes human malaria. The World Health Organization (WHO) estimates that there were 216 million human cases and 655,000 deaths due to malaria in 2010, with most if these occurring in Africa (WHO, 2011). Reduction of disease incidence by control of the vector is primarily achieved through the use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) (WHO, 2011).

Similarly, the main vector associated with yellow fever virus transmission to humans is *Aedes aegypti* (L.) (Gubler, 1998). Besides vaccination, control of yellow fever, in the Americas, has centered on the control of the vector; which was achieved through the use of residual with insecticide treatments, public education, and source reduction from the 1940’s through the 1960’s (Gubler, 1998). Unfortunately, in the 1970’s the vector control programs suffered cutbacks which resulted in *Ae. aegypti* re-infestation into most of the tropical
areas in the Americas (Gubler, 1998). Because the yellow fever virus is maintained in the wild via sylvatic cycles involving monkeys and mosquitoes (Gubler, 1998), human populations living near these cycles are at risk of an outbreak. Currently the WHO estimates that there are around 200,000 yellow fever cases worldwide every year, with 30,000 of those resulting in deaths (WHO, 2013).

Dengue fever is caused by an eponymous virus, and the disease can manifest as a mild form or a severe form. The mild form is known as dengue fever, while the more severe form is dengue hemorrhagic fever (DHF) (Beran and Steele, 1994). There are four serotypes of the virus, which can be found circulating in most of the tropical regions of the world (Gubler, 2006; Halstead, 1980), and are vectored primarily by *Ae. aegypti* (Gubler, 2006). However, there are other members of the genus *Aedes* that transmit it as well, such as *Aedes albopictus* (Skuse). This mosquito is considered a secondary vector of dengue, and is responsible for much of the transmission of this disease in Asia (WHO, 2005). *Aedes polynesiensis* (Marks) and *Ae. scutellaris* (Walker) also have been thought to be responsible for virus transmission during dengue fever outbreaks (WHO, 2005).

With the effective control of *Ae. aegypti* through eradication efforts during the 1950’s and 1960’s, dengue was eliminated in Central and South American countries (Gubler, 1998). Globally, the number of cases of dengue and severe dengue reported to the WHO from 1955 to 1969 was 16,406 (WHO, 2012a). Over the next two decades the number of cases worldwide increased, with
295,554 cases being reported from 1980-89 (WHO, 2012a). Unfortunately, by 1998, following the re-expansion of *Ae. aegypti* into eradicated zones and the increased transmission of the dengue virus, dengue hemorrhagic fever reemerged in the American tropics and Pacific Islands (Gubler, 1998). The number of global cases reported in 2010 alone was 2,204,516 (WHO, 2012a). Worldwide, dengue is the most important viral disease transmitted by mosquitoes. Nearly half of the world’s population lives in countries where this virus is endemic, resulting in a WHO estimate of 50-100 million cases of dengue fever a year (WHO, 2012a).

### 1.2 Current Insecticides Available for Mosquito Control

Insecticides are an important component of vector control and disease risk reduction (Hemingway and Ranson, 2000), and are classified into groups based on their chemical structure (Yu, 2008). Insecticides currently used for the control of vectors fall into the carbamate, organophosphorus, pyrethroid, and organochlorine chemical classes (Guillet et al., 2001; WHO, 2011). Figure 1-1 shows the chemical structures of representatives from the four classes listed above.

Carbamates (Fig.1-1) are esters of carbamic acid, and they can be found in a number of different forms (Yu, 2008). Carbamates often contain an aryl ring as a part of their structure, such as in carbaryl and propoxur (Bloomquist, 1999). However, they also can be oximes, as represented by aldicarb and methomyl (Yu, 2008). Some of the carbamates are classified as proinsecticides, and contain a thioether group, such as aldicarb, which is activated metabolically to the sulfoxide or sulfone (Yu, 2008). Carbamate insecticides work by inhibiting
the enzyme acetylcholinesterase, which results in the buildup of the neurotransmitter acetylcholine. This causes a hyperexcitation of the nervous system, which can lead to death of the insect (Bloomquist, 1999). Carbamates are routinely used in indoor residual spraying (IRS) applications for the control of human malaria vectors in Africa (WHO, 2011).

Organophosphorus (OP) insecticides (Fig. 1-1) are also inhibitors of acetylcholinesterase. The chemistry of this class of insecticides can be varied; however, all the chemical structures are based around the phosphorus atom (Bloomquist, 1999). Many of the OPs exist as phosphates, phosphonates, phosphorothionates, phosphorodithioates, and phosphoramidothioates (Bloomquist, 1999). Some of these are proinsecticides that need to be activated by cytochrome P450 monooxygenases replacing P=S with P=O (Yu, 2008). Like the carbamates, these insecticides also are used in IRS applications for malaria vector control, and while both classes are effective in control of vectors, they also come with drawbacks (WHO, 2011). Both insecticide classes are more costly to use than pyrethroids and organochlorines, and there is also low selectivity for the target vectors, meaning humans and other animals could be detrimentally affected by their use (WHO, 2011).

The insecticides that are in the pyrethroid class (Fig. 1-1) typically consist of esters of chrysanthemic acid, and can be classified into two types; type 1, represented by permethrin, and type 2, represented by cypermethrin, based upon the chemical structure of the compound (Bloomquist, 1999). For pyrethroids containing a phenoxybenzyl alcohol, the difference between type 1
and type 2, is that type 1 pyrethroids do not possess a cyano group on the alpha
carbon in their structure (Bloomquist, 1999). It is this cyano group that gives the
type 2 pyrethroids superior activity when compared to the type 1 pyrethroids.
The effects that an insect displays, when poisoned by a pyrethroid, depends
again on the class. Type 1 pyrethroids will induce hyperexcitability and
convulsions in the insect, while type 2 pyrethroids induce lethargy and spastic
paralysis (Bloomquist, 1999). These effects are due to the pyrethroids acting
upon voltage-gated sodium channels in nerves. Both type 1 and type 2
pyrethroids alter sodium channel inactivation by either slowing it down (type 1) or
essentially preventing inactivation all together (type 2) (Bloomquist, 1999). When
a type 1 pyrethroid acts upon the channel, repetitive firing of action potentials in
the neuron will be recorded. In contrast, type 2 pyrethroids will cause a strong
depolarization in the cell, which will eventually lead to a block of excitability
(Bloomquist, 1999). Pyrethroids are used in IRS applications and are the only
insecticides that are used in insecticide treated nets/long-lasting insecticidal nets
(ITNs/LLINs), which in 2008-2010 helped protect 584 million people from malaria
infection (WHO, 2011). The pyrethroid insecticides are used preferentially due to
their low cost, persistence in the field after application, and their high selectivity
for the target vector which results in low toxicity to humans (WHO, 2011).

Organochlorines, also known as chlorinated hydrocarbons, consist of
several different insecticide types (Yu, 2008). However, many of the insecticides
are no longer used due to the potential for environmental harm (Yu, 2008). Of
the organochlorines, only dichlorodiphenyltrichloroethane (DDT, Fig. 1-1) is still
used against *Plasmodium* vectors (Yu, 2008). Many of the organochlorine compounds have been shown to persist within the environment for extended periods of time, which caused concern over their long-term toxic effects; thus, many of these compounds have restrictions on their usage (Yu, 2008; WHO, 2011). For instance, at the Stockholm Convention in 2001 DDT use was banned except for cases where it would be used for disease control (WHO, 2011). With its low cost and effectiveness against mosquitoes that transmit malaria protozoans, DDT is routinely used in IRS applications in Africa (WHO, 2011). The mode of action of DDT is similar to that of the pyrethroids, which could become problematic for malaria control because of mosquito cross-resistance developing against both types of compounds (WHO, 2011).

1.3 Rising Concerns with Resistance Development

Insecticide resistance development in insect vectors threatens the successes that have been seen with the use of insecticides for vector control (WHO, 2011). In 1908, the first sign of insecticide resistance was observed in a scale insect in the state of Washington. Since then, hundreds of insect species have been found to have some resistance to one or more insecticides (Yu, 2008). In terms of mosquito vectors, about 119 species have been documented as having resistance to insecticides; 63 of the species are in the genus *Anopheles*, 21 in *Aedes*, and 26 in *Culex* (Whalon, et al., 2012). In 2011, the World Health Organization reported that out of 87 countries that were monitoring insecticide resistance, 45 of the countries found a malaria vector mosquito that was resistant to at least one type of insecticide, and in 39 of those 45 countries resistance to pyrethroids was reported (WHO, 2011).
1.4 Target-Site Resistance

Resistance mechanisms can be grouped into two main types, target site resistance and metabolic resistance (Hemingway and Ranson, 2000). In target site resistance, non-silent point mutations usually cause alterations in the binding regions that insecticides use in their mode of action (Hemingway and Ranson, 2000). These mutations ultimately reduce the affinity of the sites to bind to the insecticide (Soderlund and Knipple, 2003). In contrast, metabolic resistance occurs when detoxication mechanisms are elevated or modified so that they are more efficient in sequestering or degrading xenobiotics (Hemingway and Ranson, 2000; Ranson et al., 2011).

An example of target site resistance was observed in Culex pipiens L. and An. gambiae where genes that encoded for acetylcholinesterase (AChE) were altered (Weill et al., 2003). There are two genes in mosquitoes that encode for AChE, ace-1 and ace-2, and mutations within both genes can confer resistance to insecticides that act upon AChE, such as carbamates and organophosphates (Weill et al., 2003; Nabeshima et al., 2004). In An. gambiae and two species of the Cx. pipiens complex (Cx. p. pipiens L. and Cx. p. quinquefasciatus Say), the specific alteration that occurs within the ace-1 gene is a substitution producing the G119S mutant (Weill et al., 2003). Under normal conditions propoxur was shown to inhibit 50% of the AChE activity at $10^{-6}$ M, however, in mosquitoes with the G119S mutation activity was 85% at $10^{-3}$ M (Weill et al., 2003). In the mosquito Cx. tritaeniorhynchus Giles, a phenylalanine to tryptophan substitution at codon 455 (F455W) within the ace-2 gene conveyed insecticide resistance (Nabeshima et al., 2004). Sometimes multiple mutations can occur in the genes
encoding AChE, such as with the mosquito *Ae. aegypti*. In this case, there are three different codon locations that mutated from the wild-type (Vaughan et al, 1997). These mutations are a F105S, G285A, and finally F350Y and each of these has been found singularly or in combination with the other mutations (Vaughan et al, 1997).

Target-site resistance has been shown to affect pyrethroid and DDT activity as well (Soderlund and Knipple, 2003). With DDT and the pyrethroid insecticides, target site resistance is commonly referred to as “knockdown resistance” (*kdr*) due to a reduction in rapid incapacitation (knockdown) in the resistant insect (Yu, 2008). *Kdr* was first discovered in a strain of house fly in 1951 after the flies were not incapacitated following exposure to DDT or pyrethrins (Busvine, 1951). Sodium channel gene analysis of *Musca domestica* (L.), revealed that the cause of *kdr* is due to a replacement of leucine by phenylalanine in the 6th transmembrane segment of domain 2 in the sodium channel, expressed as L1014F (Williamson et al., 1996). In 1998, the identical mutation was found within the primary malaria vector *An. gambiae* (Martinez-Torres et al., 1998). This finding is problematic, because malaria control is highly dependent upon the use of pyrethroids and *kdr* conveys resistance against virtually all pyrethroids and DDT (WHO, 2011; Soderlund and Knipple, 2003). The *kdr* mutation has been shown to reduce the toxicity of permethrin by 43-fold in a resistant strain of *An. gambiae* (Chandre et al., 2000).

### 1.5 Metabolic Resistance

The mechanisms chiefly involved with metabolic resistance are the monooxygenases, esterases, and the glutathione-S-transferases (Hemingway
and Ranson, 2000). The P450 monooxygenases are key enzymes involved in many functions within insects. The P450’s are a large family of genes involved in the growth, reproduction, and protection of the insect from chemical attack, including man-made insecticides (Feyereisen, 1999). P450 enzymes chemically alter insecticides through hydroxylation, epoxidation, dealkylation, desulfuration, and sulfoxidation reactions (Yu, 2008). There can be numerous P450 genes within an insect’s genome; however, it has been found that only a few are involved with insecticide metabolism (Ranson et al. 2011). In the case of An. gambiae, there are 111 P450 enzymes, but only three are overexpressed in resistant strains of the mosquito (Ranson et al., 2002 and 2011). These three genes fall into the CYP6 family of P450s and encode enzymes that bind well to insecticides, with two of the enzymes (CYP6P3 and CYP6M2) also having the ability to metabolize insecticides (Ranson et al., 2011).

Esterase-based resistance confers protection to insects from insecticides that contain ester linkages susceptible to hydrolysis; these include the organophosphates, carbamates, and pyrethroids (Yu, 2008). There are two types of esterases that are able to metabolize insecticides; the A-esterases (phosphatases) and the B-esterases (carboxylesterases) (Yu, 2008). The esterases work by hydrolyzing the ester bond through addition of water, which results in the dissociation of the insecticide into an acid and alcohol (Yu, 2008). Esterases also can work by sequestering insecticides; i.e., binding and then slowly releasing them (Karunaratne et al., 1993). Sequestration provides resistance by preventing the insecticide from reaching its target site. In order for
sequestration to be an effective means of resistance there needs to be an increased amount of the esterases within the insect, as one esterase enzyme will only bind one molecule of insecticide at a time (Hemingway and Karunaratne, 1998). In \textit{Cx. quinquefasciatus}, this resistance results from the elevation of the two esterases estα21 and estβ21 (Vaughan et al., 1997). Through immunological assays, the elevation in the esterases was estimated to be 70-fold for estα21 and 500-fold for estβ21, compared to the susceptible strains (Mouches et al., 1987).

Finally, the glutathione-S-transferases (GSTs) are able to degrade a number of xenobiotics via several types of reactions (Yu, 2008). In terms of vector resistance to insecticides, they play a large role in the metabolism of DDT, as well as a role in degradation of organophosphates (Hemingway and Ranson, 2000). As with the previous metabolic resistance mechanisms, GST-based resistance is presumably caused by the overexpression of GST enzymes (Hemingway and Ranson, 2000). This conclusion is evident from observations of a 3-fold increase of GST enzyme activity found in DDT-resistant \textit{An. gambiae} compared to a susceptible strain (Hemingway et al., 1985). An increase in GST enzyme activity has been documented for \textit{An. subpictus} (Grassi), and was thought to be caused by DDT selection pressure (Hemingway et al., 1991).

It should be noted that these resistance mechanisms do not necessarily occur in isolation. A combination of the different mechanisms can allow for strong resistance to insecticides. It was found that increased carboxylesterase and GST activity (two metabolic-type resistance mechanisms), coupled with
reduced AChE sensitivity, conferred an elevated resistance to both malathion and fenitrothion in oriental house flies, *Musca domestica vicina* (Macquart) (Yeoh et al., 1981). Accordingly, the LD$_{50}$ of the resistant strain was 238-fold higher for fenitrothion and 519-fold higher for malathion than corresponding LD$_{50}$ values for the susceptible strain (Yeoh et al., 1981). It was determined that the three resistance mechanisms were multiplicative in how they contributed to the resistance factor (Yeoh et al., 1981). For malathion resistance the carboxylesterase activity increased resistance by a factor of 5, GST activity by a factor of 10, and AChE by a factor of 10 (Yeoh et al., 1981). For fenitrothion, two factors increased the observed resistance, GST activity by a factor of 10 and AChE by a factor of 25-30 (Yeoh et al., 1981).

**1.6 Effects of Synergists on Metabolism and Resistance**

Synergists are compounds that have low toxicity by themselves; however, if an insecticide is degraded by a metabolic mechanism, combining it with a synergist will increase its toxicity through the inhibition of the metabolic enzymes that degrade xenobiotics (Casida, 1970; Raffa and Priester, 1985). Piperonyl butoxide is a well-known inhibitor of the P450 monooxygenases, DEF (S,S,S-tributyl phosphorotrithioate) is an esterase inhibitor, and DEM (diethylmaleate) is a GST inhibitor (B-Bernard and Philogene, 1993). Synergists can be used to detect the type of metabolic resistance mechanism that is being expressed in resistant populations (Raffa and Priester, 1985). For example, piperonyl butoxide has been used to determine if resistance is being caused by P450 monooxygenases (Hodgson and Levi, 1998). In these studies, the addition of the synergist to the insecticide resulted in increased toxicity over the insecticide
alone. Synergists added to insecticide formulations can assist in the control of P450-based resistant insect populations by returning insecticide efficacy to the level of the susceptible strain (Raffa and Priester, 1985).

Another beneficial use for synergists is the potential to delay resistance development in an insect population. This effect can occur when mortality in insects expressing a metabolic resistance phenotype is equivalent to the susceptible strain, so that resistant alleles are kept at a minimum, thus reducing the probability of resistance propagation (Raffa and Priester, 1985). Finally, synergists may keep beneficial insect populations from being as affected by an insecticide application through the use of a lower insecticide application rate. This is most probable if selectivity of the synergist is high for the pest and relatively low for beneficial insects (Raffa and Priester, 1985). Unfortunately, synergists can act only upon resistance mechanisms that are involved with enhanced metabolism; they have no effect on target-site mutations like \textit{kdr} (Soderlund and Knipple, 2003).

\textbf{1.7 Effects of Insecticides upon the Insect Immune System}

The insect immune system is comprised of three mechanisms. The first is the cuticle, which provides a physical and chemical barrier to foreign entities (James and Xu, 2012). The second and third mechanisms are within the insect body and are the humoral and cellular responses that are activated upon microbiotic or xenobiotic encroachment (James and Xu, 2012). The cuticular mechanism has been studied very little with regard to the interaction with insecticides and whether or not this interaction has an effect upon the immune system of insects (James and Xu, 2012).
In contrast, the humoral mechanism has been studied extensively in the fruit fly *Drosophila melanogaster* (Meigen), and is known to provide protection against bacterial and fungal invasion (James and Xu, 2012). However, there has been no evidence shown to correlate a relationship between the humoral response and chemical insecticides, although bacteria-based insecticides, such as *Bacillus thuringiensis*, do induce a humoral response (James and Xu, 2012).

The cellular mechanism of this response utilizes various hemocytes to induce encapsulation, nodulation, and phagocytosis (James and Xu, 2012). Organochlorines and organophosphates have been shown to alter the quantity of hemocytes within the insect (James and Xu, 2012). Hemocytes also activate a melanization pathway, arising from activation of the phenol oxidase (PO) (James and Xu, 2012). The primary role of PO in the melanization cascade is conversion of phenols into quinones, with the subsequent polymerization of the quinones resulting in melanin (González-Sontoyo and Córdoba-Aguilar, 2012). Synthesized melanin is then deposited onto foreign bodies for sequestration, and in the case of live invaders to encapsulate and ultimately kill by starvation of the target (González-Sontoyo and Córdoba-Aguilar, 2012). Finally, it has been shown that melanization can aid in preventing plasmodium infection in mosquitoes (Collins et al., 1986).

**1.8 Potassium Channels as Possible New targets for Insecticide Development**

Potassium channels perform multiple roles within organisms. They control the flow of potassium ions, having effects upon the resting potential of cells, neurosecretion, and excitability of nerves and muscles (Jan and Jan, 1997).
Alterations of potassium channels can have major effects on an organism (Jan and Jan, 1997), including neurological and cardiac dysfunction (Browne et al., 1994; Curran et al., 1995). There are a number of different types of potassium channels; the voltage-gated potassium channels, the calcium-activated potassium channels, the inward rectifying potassium channels, and the leak or pore channels, and their classification is discussed below (McCormack, 2003).

The voltage-gated potassium channels are comprised of four α subunits, each of which consists of six transmembrane helices (Jan and Jan, 2012; Coetzee et al., 1999). Similarly, calcium-activated potassium channels are comprised of six transmembrane helices, while the pore channels have four helices and the inward rectifying channels have only two (McCormack, 2003). The first gene complex to be described to encode for a voltage-gated potassium channel was the *Drosophila shaker* gene (Kamb et al., 1987). Soon thereafter, another three gene complexes that resembled *shaker* were discovered in *Drosophila* and named *shab*, *shaw*, and *shal* (Butler et al., 1989). Identification of the homologous vertebrate potassium channel genes showed the need for a simplified naming scheme and thus the Kv nomenclature was developed (Chandy, 1991). The “K” signifies that it is a potassium channel gene and the “v” signifies that it is voltage-gated (Chandy, 1991). The nomenclature continues with addition of numerals to indicate subfamily and in what order they were identified, so that Kv1.1 is the first member of the *shaker* subfamily (Chandy, 1991). Thus, the corresponding homologs to *shab*, *shaw*, and *shal* in vertebrates would be Kv2, Kv3, and Kv4 respectively (McCormack, 2003).
1.9 Kv2 (Shab) Channels in Insects and Mammals

Through the use of BLAST searches, the *An. gambiae* genome was queried using *D. melanogaster* Shaker, Shab, Shaw, and Shal to find the corresponding genes (McCormack, 2003). It was established that *An. gambiae* has eight voltage-gated potassium channels compared to *D. melanogaster* with only six (McCormack, 2003). The Kv2 gene in *D. melanogaster* is located on chromosome 3L:63A1, whereas in *An. gambiae* it is located on the chromosome 2L:23C (McCormack, 2003). Although the two orthologous genes are found on different chromosomes, it was discovered that the gene sequence for Kv2 is conserved in *An. gambiae*, suggesting that the function of the gene is also conserved (McCormack, 2003). *Shab* (Kv2) is the gene that encodes for the delayed rectifier current in *Drosophila* neurons and muscle (Tsunoda and Salkoff 1995).

1.10 Potassium Channel Inhibitors

There are a number of potassium channel blocking compounds that have been described (Fig. 1-2). Tetraethylammonium chloride (TEA) is a quaternary ammonium ion, and blocks the potassium channel pore, either intracellularly or extracellularly (Heginbotham and Mackinnon, 1992). When TEA is injected into giant squid axons and an action potential is generated, the action potential is prolonged instead of returning rapidly to the resting state (Tasaki and Hagiwara, 1957). This result occurs because potassium channels play a role in in the falling (repolarizing) phase of the action potential. 4-Aminopyridine is another known potassium channel blocker that prolongs action potential duration (Malhotra et al. 2012). It has been shown to help patients suffering from multiple sclerosis, and it
also is marketed as a bird poison under the name Avitrol (Malhotra et al. 2012). Salgado (1992a) showed that 4-aminopyridine blocks voltage gated potassium currents in *M. domestica* larvae.

Peptide toxins are another source of potassium channel inhibitors (Swartz and Mackinnon, 1995). The neurotoxin apamin (Fig. 1-2), derived from bee venom, has been shown to selectively block Ca\(^{2+}\)-dependent K\(^+\) channels (Hugues et al., 1981). Other peptide toxins include dendrotoxin from snakes, which target the *shaker* related subfamily of channels, and hanatoxin, isolated from tarantula venom that inhibits the Kv2.1 potassium channel (Swartz and Mackinnon, 1995).

Other compounds are known to modulate potassium channel currents (Fig. 1-2). Tacrine and 3-benzidino-6-(4-chlorophenyl)pyridazine, both of which are AChE inhibitors, have been found to reduce the amplitude of delayed rectifying potassium channel currents (Kraliz and Singh, 1996; Du et al., 2006). The compound quinine hemisulfate is a plant alkaloid used as an antimalarial medication (WHO, 2011), and it has been shown to block a number of potassium channels, including the delayed rectifier potassium channel and the Ca\(^{2+}\)-dependent potassium channel (Castle and Haylett, 1986). Quinidine is the stereoisomer of quinine and has been shown to block insect voltage gated potassium channels, as well (Salgado, 1992a). Through two electrode voltage clamping of muscle of third instar *D. melanogaster* larvae it was shown that quinidine is a more potent blocker of the delayed rectifier potassium current I\(k\), with an IC\(_{50}\) of 26.3 \(\mu\)M, than quinine with an IC\(_{50}\) of 38.6 \(\mu\)M (Kraliz et al., 1998).
In contrast to the inhibition on the potassium channel currents, there are also compounds that can prolong the open state of the channels, such as chlorzoxazone. In pituitary cells, it has been shown that chlorzoxazone increases the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} current (Liu et al, 2002). Finally, the substituted catechol 48f10 has been identified as a Kv2.1 potassium channel inhibitor as well (Zaks-Makhina et al., 2003). The chemical structure of a catechol is benzenediol; 48f10 has this moiety linked to a norbornane group (Zaks-Makhina et al., 2003). Through the continued study of the toxic action of these compounds, it could be possible to develop novel potassium channel inhibitors useful as novel insecticides.

1.11 Objective of this Study:

The primary objective of this study was to explore the viability of potassium channel directed compounds as potential insecticides against two species of mosquito; Ae. aegypti and An. gambiae. Insecticidal activity was determined through insect bioassays. Additionally, characterization of the effects seen from the introduction of the compounds was carried out through electrophysiological assays. The following thesis details the findings of these experiments.
Table 1-1. Mosquito genera and the human diseases that they vector (Beran and Steele, 1994; Krauss, 2003)

<table>
<thead>
<tr>
<th>Mosquito Genera</th>
<th>Human Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anopheles</strong></td>
<td>Sindbis Fever, O’Nyong-Nyong Fever, Lymphatic Filariasis, Malaria</td>
</tr>
<tr>
<td><strong>Aedes</strong></td>
<td>Chikungunya Fever, Yellow Fever, Dengue and Dengue Hemorrhagic Fever, Eastern Encephalitis</td>
</tr>
<tr>
<td><strong>Culex</strong></td>
<td>Western Equine Encephalitis, St. Louis Encephalitis, Venezuelan Equine Encephalitis, Rift Valley Fever, Chikungunya Fever West Nile Fever</td>
</tr>
</tbody>
</table>
Figure 1-1. Chemical structures of pyrethroids: permethrin and cypermethrin; carbamates: propoxur, aldicarb, and methomyl; Organochlorine: p,p-DDT; Organophosphorus: malathion. Compound structure source: Sigma-Aldrich.
Figure 1-2. Structures of potassium channel blockers and stimulator (Chlorzoxazone). Compound structure source: Sigma-Aldrich.
CHAPTER 2
TOXICITY OF POTASSIUM CHANNEL DIRECTED COMPOUNDS AND THE CHARACTERIZATION OF EFFECTS SEEN THROUGH INSECT AND ELECTROPHYSIOLOGICAL STUDIES

2.1 Introduction

The development of insecticide resistance in mosquitoes threatens our ability to control diseases, such as malaria, effectively (WHO, 2011). Resistance to insecticides in the pyrethroid class has been increasing, especially in Africa, due to heavy use in insecticide-treated nets (ITNs), which are used for protection of sleeping individuals (WHO, 2011). The other three major insecticide classes that are in use for vector control are the organochlorines (DDT), the carbamates, and the organophosphates (WHO, 2011). Each of these classes has had reports of insect resistance development (Hemingway et al., 1990; Karunaratne et al., 1993).

While resistance is growing, the supply of available and usable insecticides is diminishing due to a number of factors. The cost of developing new chemicals for use in the public health sector is high; most of the insecticides that are currently used are repurposed agricultural insecticides (Zaim and Guillet, 2002). Another issue is our increasing knowledge of the harmful effects that insecticides can have on the environment and humans; thus, older, inexpensive insecticides are being removed from use or, as in the case with DDT, severely restricted in applications (Zaim and Guillet, 2002; WHO, 2011). Development of new insecticides with novel modes of action is imperative to successfully combat resistance and keep diseases under control.
Choosing the path to follow in the development of an insecticide can be
difficult. By developing an insecticide with a similar mode of action as the
currently used insecticides, such as acetylcholinesterase inhibition or sodium
channel modification, there is the risk that vector populations having already
developed resistance against those insecticide types also would express
resistance against the new insecticide. In order to best circumvent the issue of
pre-existing resistance, one would want to develop a new class of insecticide that
works via a novel site and mode of action. It is with this thought that the present
study was undertaken. Potential targets of a new insecticide class are the
voltage-gated potassium channels. A number of compounds such as 4-
aminopyridine (4-AP), tetraethylammonium chloride (TEA), and a substituted
catechol (48f10) are known to block these channels (Malhotra et al., 2012;
Heginbotham and Mackinnon, 1992; Zaks-Makhina et al., 2003). Accordingly,
there is evidence that 4-AP causes convulsions in vertebrate and invertebrate
nervous systems (Pelhate and Pichon, 1974). Given these observations, this
study was undertaken to screen compounds that are known to be potassium
channel blockers and novel compounds that could potentially act on these
channels for toxicity to *Anopheles gambiae* (Giles) and *Aedes aegypti* (L.).
2.2 Materials and Methods

2.2.1 Chemicals

All of the industrial manufactured compounds including propoxur, 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA), 4-tert-octylcatechol (4TOC), eugenol, chlorzoxazone, β-thujaplicin, quinine, quinidine, and n,n-diethynicotinamide were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 2-1). The substituted catechols PRC 725 and 728 were obtained from the laboratory of professor Paul Carlier, Department of Chemistry, Virginia Polytechnic Institute and State University (Fig. 2-1). Substituted catechols were tested in insects due to the inhibition of human potassium channels by these compounds observed by Zaks-Makhina et al. (2003). The solvents dimethyl sulfoxide (DMSO, 99.9%), and ethanol (EtOH, 100%) were purchased from Sigma-Aldrich.

2.2.2 Insects

For all larval assays, 4th instar Aedes aegypti larvae were obtained from a colony at the USDA, ARS, CMAVE insectary located in Gainesville, FL, USA and used prior to pupation. A brief description of how the larvae are reared is provided by Pridgeon et al (2008). Larvae were placed into a large plastic container that had been partly filled with tap water and were given food (ground liver and yeast mixture) provided by the CMAVE insectary. Pupae were collected and placed into insect rearing cages (BioQuip Products, Inc., Rancho Dominguez, CA, USA). Cotton balls soaked in 10% sugar water were placed on top of the cages to provide adult sustenance.
Anopheles gambiae larvae were raised from eggs obtained from a colony located at the CDC (Atlanta, GA, USA, FDACS permit: 10-33). Eggs were placed into large plastic containers with tap water. Emerged larvae were fed ground up beta-fish food (Tetra Holding, Blacksburg, VA, USA). Pupae were collected and newly emerged adults fed, as described for the Aedes mosquitoes. For all larval bioassays, 4\textsuperscript{th} instars served as experimental units.

2.2.3 Headless Larval Paralysis Bioassay

The headless larva bioassay was developed by Drs. Jeffrey Bloomquist and Rafique Islam (unpublished) to circumvent the problem of poor cuticle penetration, and provide a better estimate of the intrinsic paralytic activity of test compounds. Thus, the method allows compounds to cause toxicity to the larvae regardless of whether the chemical can pass through the cuticle. Larvae are placed into a Sylgard-filled 35mm dish (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the water level reduced to restrict larval movement. Decapitation of the larvae was performed by removal with forceps, and the larvae \((n = 10)\) were then placed into a chemical/solvent/larva saline suspension and observed for toxic effects after a period of 5 hours. Longer incubation times caused significant control paralysis (data not shown). The amount of solvent (ethanol) did not exceed 0.3\% (v/v). The mosquito larval saline (LS) was used in all electrophysiological and larval assays and was composed of 154 mM sodium chloride, 1.8 mM calcium chloride, 2.7 mM potassium chloride, and 1.2 mM of sodium bicarbonate with a pH 6.9, as described by Hayes (1953). All compounds were tested in Ae. aegypti while only 4-aminopyridine and tetraethylammonium chloride were tested in An. gambiae.
An active headless larva will show a strong bilateral contractile motion when probed. A headless larva that has been poisoned by a compound will respond with no movement or a sluggish, unilateral contractile motion when probed. These two observations were used to distinguish dead or paralyzed larvae from unaffected larvae. Completely paralyzed larvae may be spastic (strongly contorted and unable to move) or flaccid (normal relaxed position of the larval body but no movement when probed). Results were input into SAS 9.3 (SAS Institute, Cary, NC, USA) using a probit log10 procedure to determine a paralysis concentration (PC$_{50}$).

### 2.2.4 Intact Larval Toxicity Bioassays

In order to determine if compounds can penetrate the integument of *Ae. aegypti* and *An. gambiae* to cause toxicity, a 24 hr bioassay was utilized. Intact fourth instar larvae (*n*=10) were placed into a 35mm dish. The control dish contained larval saline plus the ethanol vehicle, while the experimental dishes contained the compound/solvent/larval saline mixture. Larvae were observed after 1 hour for any immediate toxic effects. The assay was allowed to run for an additional 23 hrs, where after larvae were evaluated. Larvae were probed to determine paralysis/death. An initial screen at a concentration of 100 ppm was run on all compounds. Results from those experiments were used to determine the concentrations for LC$_{50}$ determination. A minimum of three concentrations were included for each compound, with each concentration being evaluated a minimum of two times. Mortality data were pooled for each compound and evaluated using the SAS 9.3 statistical analysis package (SAS Institute, Cary, NC, USA).
2.2.5 Adult Topical Assay

Non-blood fed female *Ae. aegypti* (2-5 day old) were collected and anesthetized by placing the holding container on ice. Anesthetized adults were then placed onto a glass petri dish lined with a paper filter disk (Fisher Scientific, Pittsburgh, PA, USA) sitting on ice. Applications of compound (0.2 μl) were done by hand using a Hamilton repeating syringe (Hamilton, Reno, NV, USA). A 100% ethanol vehicle was used to dissolve and apply each compound. For the control treatment, females (n=10) were dosed with 100% ethanol. Dosed females were then placed into a paper cup with a mesh top, and a cotton ball soaked in 10% sugar water was placed upon the mesh top to provide a food source. Mortality was assessed 24 hours after treatment. To assess mortality, the cup was jostled to disturb the adults within, and if no movement or only slight leg movements were observed, that female was considered dead. Adult females were weighed after the completion of the assays, the average weight was taken and the compound concentrations were adjusted so that toxicity could be expressed on a per mg weight basis. Mortality data were pooled and evaluated using probit log10 analysis in the statistical analysis package SAS 9.3 (SAS Institute, Cary, NC, USA). A minimum of 3 concentrations were tested per compound with each concentration being tested a minimum of 3 times.

2.2.6 Adult Topical + Synergist Assay

Adult females were anesthetized in the same manner as in the adult topical assays. Ten females per concentration to be tested were topically dosed with the piperonyl butoxide (PBO) at 500 ng/mosquito. The adults were placed into containers and left for 1 hour. After that time period, the adults were
anesthetized again as before and then topically dosed with concentrations of propoxur or PRC 728. The mosquitoes were then returned to the containers and given a 10% sugar solution as in the adult topical assay. Mortality was accessed after 24 hours in the same manner as in the adult topical assay. After the completion of the experiments, the adults were weighed and the concentration dosages were adjusted so that they could be expressed in a per mg weight basis. The adjusted mortality data was then input into SAS 9.3 (SAS Institute, Cary, NC, USA) using a probit log10 analysis. Five concentrations were tested for each compound and each concentration was evaluated three times.

2.2.7 Adult Injection Assay

Using a model P-1000 micropipette puller (Sutter Instrument, Novato, California, USA) thin walled glass capillaries (WPI, Sarasota, FL, USA) were made into injection needles with a tip opening of ca. 10 microns (Heat = 500; Pull = 25; Velocity = 200; Time = 500; Pressure = 500). The pulled capillary was then stamped along the barrel with ink lines at 1 mm increments, and placed into a World Precision Instruments manual microsyringe pump (MMP, Sarasota, FL, USA). The dispensed volume of the injection system was calibrated as follows. MMP tubing was back-filled with mineral oil (ACROS Organics, NJ, USA), and any air bubbles were carefully removed. Water was then drawn up into the capillary, and the meniscus was used to calibrate delivery by positioning it at one of the stamped increments. The MMP’s digital readout was then zeroed so that it moved the meniscus to the next mark, and the change on the digital screen could be recorded. This result was averaged, and by using the internal diameter of the capillary glass, the volume expelled from the capillary was calculated. Using
these procedures, when the MMP’s digital screen showed a reading of -0.108, 200 nl had been dispensed from the capillary. Capillaries were filled by drawing toxicant solutions (5% ethanol in larval saline) into the pipette, backed by mineral oil.

Adult *Ae. aegypti* (2-5 days post eclosion) were collected and anesthetized by placing them on ice, as described previously. Working under a microscope, 200 nL of treatment suspension without insecticide was injected into the side of the thorax of 10 control treatment mosquitoes using the MMP. These mosquitoes were then held for 24 hr and assessed for mortality as described for the adult topical assay. For each compound, five concentrations were run a minimum of two times. Mortality data were pooled and then analyzed in SAS to determine an LD$_{50}$ value. As in the adult topical assays, females were weighed after the completion of the assays, the average weight was taken and the compound concentrations were adjusted so that toxicity could be expressed on a per mg weight basis. Adjusted mortality data were analyzed using probit log10 analysis in SAS 9.3 to calculate the adjusted LD$_{50}$ value.

### 2.2.8 Electrophysiology Hardware and Software

A Grass S9 stimulator (Grass Instruments, Quincy, MA, USA) was used to produce the electrical stimulus in the electrophysiology experiments. Muscle tension was recorded with a BAM21 optical force transducer amplifier (Sarasota, FL, USA) and ADInstruments PowerLab 4/30 (Colorado Springs, CO, USA) or World Precision Instruments’ LabTrax 4, data acquisition hardware (WPI, Sarasota, FL, USA). A World Precision Instruments’ (WPI) SP120P Syringe Pump (Sarasota, FL, USA) was used for application of compound onto the larval
body. Waveforms were then logged into Labscribe software (WPI, Sarasota, FL, USA). Concentration-response curves of electrophysiological data were created and analyzed using Prism™4.0c software (GraphPad Software, San Diego, CA, USA). The change in muscle tension seen with the addition of the compounds was compared to the baseline tension of the control solution that was applied.

2.2.9 Electrophysiology Muscle Tension Assay

For each assay, a 35mm dish with a wax-filled bottom was used. A depression in the wax was made to provide a well for the addition of compound. Compounds that were tested were PRC-728, 4TOC, and TEA. A minimum of 6 larvae were used for each compound with a minimum of 4 concentrations for each preparation. To immobilize each Ae. aegypti larva, a micro-insect pin was inserted into the head of the larva. The larva was then placed into the well dorsal side up. Forceps were used to stretch the larva’s body and another micro-pin was inserted into the siphon for full immobilization.

An incision was made, dorsally along the midline, starting at the thorax of the mosquito larva and was continued down to the anal region, and the gut of the larva was removed and discarded. The exoskeleton of the larva was then pinned flat with additional micro-pins positioned into the thorax and anal region so that a clear view of the ventral nerve cord (VNC) was obtained. The VNC was removed by pulling the cord out of the body starting from the caudal end working up to the thoracic ganglia. The anal region micro-pins were then removed along with the pin located in the siphon. The caudal end of the larva was then attached to an insect micro-pin hook on the force transducer. The connection between the larva and transducer was positioned so that there was little slack in the larval body.
Two electrodes were then inserted into the wax on either side of the thorax of the opened larva.

The larva was stimulated with electrical pulses (frequency = 0.5 Hz; duration = 0.8 ms; Volts = 40), and the resulting contractile force recorded through the use of the LabChart or Labscribe software. To establish baseline contraction amplitude, the preparation was allowed to run for 3-5 minutes before any treatment was added. Addition of 0.2 mL of control solution (ethanol + larval saline) was added to the preparation via a 1 mL syringe after the 3-5-minute normalization period and effects were observed. The preparation was allowed to run for 3-5 minutes so that any effect from the solvent was determined, and then experimental solutions were added in the same quantity and manner. Experimental compounds were added to the same larva beginning with the lowest concentration and progressing up to the highest concentration evaluated. Preparations were not rinsed between concentrations, as treatment solutions completely displaced the saline volume within the larval body cavity. Observations occurred over a 3-5 minute interval between each addition. Concentration-response curves were constructed from the data, such as peak amplitude and twitch duration effects of evoked responses, or area under the curve if there was an effect on baseline tension.

2.2.10 Electrophysiology Analysis

Analysis of compound effect on baseline tension (area under the curve) was done using ImageJ (National Institutes of Health, USA), with a protocol developed by Reinking (2007). Grab software (Apple Inc., Cupertino, CA, USA) was used to take three-minute interval screenshots of each application treatment
in a recording. The screenshots were then opened in Paint 6.1 (Microsoft Corporation, Redmond, WA, USA) and a reference line was drawn in if there was an observed effect that changed the baseline tension. Graticule lines or any extraneous markings from the recording were removed. The area under the curve was calculated for the control application of a recording and subtracted from the areas of the applications that had compound effects. This result was then divided by the control area and multiplied by 100 to determine the percentage increase from control muscle tension. The resulting percentages were then input into Prism™4.0c software (GraphPad Software, San Diego, CA, USA) to produce a concentration response curve for baseline tension change. A sigmoidal concentration-response curve was created in Prism™ by inputting the log values of the concentrations and the corresponding percentage changes of muscle tension.

An analysis of evoked contraction duration was performed using the aforementioned procedure. The last 10 evoked responses of the control and final compound concentration from each recording were analyzed as representative of the compound effect. The time between the point at which the evoked contraction started and then returned to baseline was recorded. These values were then input into Prism™4.0c software (GraphPad Software, San Diego, CA, USA) and analyzed using an unpaired t-test to produce a bar graph comparing the control evoked contraction duration to the duration of evoked contractions from the final concentration used.
An analysis of evoked contraction amplitude was also performed. The same last 10 evoked responses of the control and final compound concentration that were analyzed for width change, were also analyzed for height change. Peak amplitude was calculated by taking the voltage output at the start of the evoked contraction and then subtracting that value from the top point of the contraction. The data points were then input into Prism™4.0c software (GraphPad Software, San Diego, CA, USA) and analyzed using an unpaired t-test to produce a bar graph comparing the control evoked contraction height to the height of evoked contractions from the final concentration used.

2.3 Results

2.3.1 Headless Larval Bioassays

The industrial insecticide standard propoxur had the highest toxicity in the headless assay to 4th instar Ae. aegypti (Table 2-1). For the potassium channel compounds, 4-AP was the most toxic, but was 11-fold less toxic than propoxur. 4-AP was significantly more toxic than TEA by about 56-fold. Quinidine and quinine are stereoisomers of 6-methoxy-α-(5-vinyl-2-quinuclidinyl)-4-quinolinemethanol, and were among the weakest compounds tested, with each being more than 1,000-fold less active than propoxur. Chlorzoxazone was more toxic than β-thujaplicin by about 1.4-fold, with both being ca. 500-fold weaker than propoxur. For the experimental synthetic compounds, PRC 725 was 9-fold less toxic than 4-AP, but ca. 3-fold more toxic than PRC 728 (Table 2-1). Of all the catechols, PRC-725 was the most toxic followed by 4-TOC; however, they were not significantly different. Eugenol was slightly (1.2-fold) more toxic than
PRC-728. *N*,*N*-diethylnicotinamide was the weakest compound evaluated, with no mortality at 400 ppm. For comparison, a limited series of experiments were done within headless *Anopheles gambiae*. The *An. gambiae* PC₅₀ for 4-AP and TEA was respectively in ppm: 4 (2 – 6) and 61 (44 – 81). The PC₅₀ of 4-AP was similar to that of headless *Aedes*; however, the value for TEA was 3-fold less than in *Aedes* larvae, indicating slightly greater toxicity potency.

### 2.3.2 Intact Larval Bioassays

There were three compounds whose LC₅₀ values could not be calculated under the conditions of this assay. 4-AP and TEA caused no mortality at 200 ppm, while quinidine showed only 5% mortality at 400 ppm. Quinidine’s solubility limit prevented higher concentration testing. Quinine was the only compound to have an LC₅₀ that was 3 times greater than that of its PC₅₀ (Table 2-1). The remaining compounds had calculated LC₅₀ values that were increased by a factor of two or less from the corresponding PC₅₀ values.

Within intact larvae, PRC-725 was shown to be 2-fold more toxic than 4-TOC and again about 3 times more toxic than the weakest catechol, PRC-728. PRC-728, eugenol, and β-thujaplicin were not significantly different from each other. Interestingly, the LC₅₀ for β-thujaplicin was slightly less than the PC₅₀ value; however, they were not significantly different. Chlorzoxazone was 6-fold weaker than PRC-725 (Table 2-1). For intact *An. gambiae*, PRC-725 was again the most toxic catechol being 7-fold more toxic than PRC-728 (Table 2-1), which again turned out to be the least active catechol. 4-AP was one of the weakest compounds in the intact assay, being 12-fold less active than PRC-725. As
anticipated, TEA showed no mortality at 200 ppm (Table 2-1). Propoxur was again the most toxic compound tested, being 39-fold more toxic than PRC-725.

A melanization effect in *An. gambiae* (Fig. 2-2) was observed in larvae exposed to the experimental catechols and 4-tert-octylcatechol. This effect was observed to be dose dependent, as can be seen in Figure 2-3. The effect also was observed in *Aedes*; however, it was limited to the anal gills and was less extensive than that observed for *An. gambiae* larvae, as can be seen in Figure 2-4.

Spastic paralysis was evident at the end of the 24-hr assays of intact larvae when exposed to all of the catechols and quinine. The remainder of the effective compounds did not show spastic paralysis at the end of the 24-hr assay. However, there may have been spastic-type paralysis earlier in the assays as they were not checked systematically for this effect. Object 2-1 shows larvae uncontrollably twitching after being exposed to a high concentration of propoxur (10 ppm) for four hours; a sign expected from carbamate poisoning. Object 2-2 shows larvae exposed to the catechol PRC-725 (100 ppm), which express impaired movement and spastic paralysis.

Object 2-1. Fourth instar *Aedes aegypti* larvae exposed to 10 ppm propoxur (.avi file 98.2MB)

Object 2-2. Fourth instar *Aedes aegypti* larvae exposed to 100 ppm PRC-725 (.mov file 63.3MB)

### 2.3.3 Adult Topical, Topical + Synergist, and Injection Assays

Propoxur was by far the most toxic compound evaluated against adult mosquitoes (Table 2-2). In *Ae. aegypti*, PRC 725 was 669-fold less toxic than propoxur. However, PRC 725 was the most toxic catechol with a LD$_{50}$ that was
1.6-fold more toxic than the next catechol, PRC 728. In comparison to the anopheline data, the LD$_{50}$ values for all of the compounds were increased by a minimum of 4-fold; with the exception of propoxur, which kept nearly the same LD$_{50}$ in both species. The LD$_{50}$ values of PRC-728 and β-thujaplicin increased in *Aedes* by a factor of 6 and 5 respectively. PRC-728 was more toxic than β-thujaplicin, though the LD$_{50}$ values are not significantly different. 4-TOC had the largest toxicity difference across species of 20-fold, making it the weakest compound affecting adult *Ae. aegypti*. Within *An. gambiae*, propoxur was 150-fold more toxic than the next most active compound, PRC-725, the most toxic catechol in *Anopheles*. However, the LD$_{50}$ of PRC 725 was not significantly different from β-thujaplicin, the other catechols, but was different from eugenol. Eugenol was the least toxic compound with a LD$_{50}$ that was 1,000-fold less toxic than propoxur. Toxicities for PRC-728, 4-TOC, and β-thujaplicin were not significantly different from each other.

The potassium channel-directed compounds induced signs of intoxication different from propoxur. Females from both species were observed to recover from chilling anesthesia at a slower rate than untreated controls when topically dosed with the lower concentrations of propoxur. When dosed with high concentrations of propoxur, it appeared as though the females did not recover from anesthesia; and progressed directly into poisoning, because they remained at the bottom of the container with no movement. Both species exhibited uncontrolled movements including leg twitching and wings fluttering while the mosquito was laying ventral side up on the container bottom.
Experiments with the catechol compounds found that the mosquitoes were, like propoxur, slow to recover from anesthesia at low doses and no recovery was seen at high doses. Control mosquitoes dosed with ethanol immediately responded with coordinated flight when disturbed and returned to the walls of the container. This behavior can be seen in Object 2-3. In contrast, mosquitoes dosed with 4-tert-octylcatechol (7 ug/mg) walked more than controls and were observed to be less coordinated. The mosquitoes had splayed legs when at rest on bottom of the containers, and were sluggish when disturbed. When disturbed, they fell to the bottom of the container and were less likely to respond with directed flight, as can be seen in Object 2-4. Object 2-5 shows mosquitoes dosed with propoxur at 5 ng/mg. Many of the mosquitoes remained at the bottom of the container with twitching legs and uncontrolled flight.

Object 2-3. Adult Aedes aegypti topically dosed with 100% ethanol control (.mov file 24.5MB)
Object 2-4. Adult Aedes aegypti topically dosed with 7 ug/mg 4-tert-octylcatechol (.mov file 57.9MB)
Object 2-5. Adult Aedes aegypti topically dosed with 5 ng/mg propoxur (.mov file 3.2MB)

Two compounds were tested with piperonyl butoxide as a synergist applied before the compound to be tested. These two compounds were propoxur and the catechol PRC-725. As can be seen in Table 2-2 the LC$_{50}$ of propoxur with PBO is significantly lower than the LC$_{50}$ of PRC-725, indicating that with a synergist propoxur is about 18 thousand times more toxic than PRC-725.

Injection of both propoxur and PRC-725 in Aedes adults resulted in a significant drop in LD$_{50}$ values (Table 2-2). The LD$_{50}$ of propoxur dropped by a
factor of 9, and PRC-725’s value dropped by a factor of 11. Propoxur was 426-fold more potent than PRC-725. Uncontrolled wing fluttering and leg twitching were observed with injections of propoxur, similar to those observed after topical treatment. Additionally, these convulsions were seen almost instantaneously; even occurring while the female was still impaled with the capillary. Injections of PRC-725 produced no immediate effects upon the injected female. Again, as was seen with the topical treatments, low doses of these compounds slowed the recovery of the adults from anesthesia, while higher doses prevented recovery, and led straight to mortality.

2.3.4 Electrophysiology

Figure 2-5A shows typical effects of TEA upon larval muscle sheets. The drop in tension at the beginning of this recording was a slip in the larval body on the hook attached to the force transducer after the larval saline was added. The addition of 1 mM TEA onto the preparation increased the overall baseline tension slightly and also increased the individual evoked contractions (Fig. 2-5A). The tension continued to increase as higher concentrations were applied. At the top concentration of 30 mM, the baseline showed a large jump in baseline tension (3 mg of force). Quantitation of these effects showed that baseline tension of the larval body increased upwards of 200%. The amount of force generated by the larval body was calculated to be around 1 mg/50 mV of transducer electrical output. Thus, it can be seen in Figure 2-5A that prior to chemical administration, there was 5-6 mg of force generated by the larval body through the evoked contractions. At 30 mM TEA, the total force (baseline tension + twitch) increased to around 10 mg, and the maximal force reading was about 16 mg. It also was
observed that at 30 mM TEA there was an increased frequency of spontaneous contraction unrelated to electrical stimulation.

The concentration-response curve for TEA-dependent increase in muscle tension (baseline and electrically-evoked) is shown in Figure 2-5B. This effect had an EC$_{50}$ of 20.5 mM, assuming maximal effect at 30 mM. Higher concentrations were not attempted. The lack of saturation for tension increase prevented any meaningful calculation of fiducial limits.

Evoked responses were altered in appearance by TEA (Fig. 2-5C). Closer inspection of the recordings revealed that the evoked contractions were elongated in duration. The bar graph in Figure 2-5C displays a comparison of the average duration of the evoked contractions from the untreated control to the contractions observed following administration of 30 mM TEA. TEA increased the duration of contractions by about three-fold; the mean duration of the control treated evoked contractions was 176 msec, while the mean duration of the contractions from the 30 mM concentration was 584 msec. An unpaired t-test showed that these means were statistically different (p-value < 0.0001; t = 14.6, df = 118). Additionally, evoked contraction height was also altered with the addition of TEA. Figure 2-5D shows that TEA reduced the evoked contraction height. The average height of the control treated evoked contractions was 157 mV, while the average height of the 30mM TEA treated evoked contractions was 116 mV. An unpaired t-test showed that the means were significantly different (p-value = 0.0046; t = 2.89, df = 116). A total of six preparations were used for the analyses.
Figure 2-6A shows electrophysiological data for the experimental catechol PRC-728. Baseline muscle tension increased over the course of the experiment with the addition of higher concentrations of compound, similar to that seen with TEA. The amount of force generated by the muscle sheet increased significantly from the control at 0.8 mM PRC-728 (Fig. 2-6A). The baseline tension increased by 5 mg and the total amount of force being applied to the transducer (tension + top evoked contraction) was approximately 10 mg. The force generated from the control-evoked contractions was consistently around 4 mg, but peaked at 6 mg. The PRC-728 (Fig. 2-6B) concentration response curve showed a total tension increase of 125% with an EC$_{50}$ of 1.17 mM. An increase in evoked contraction duration was also seen with increase in compound concentration (Fig. 2-6C). The mean duration of the control contractions was 126 msec, while the mean width of the contractions at the final concentration of 0.8 mM was 189 msec, making this a 50% increase in duration. An unpaired t-test showed that these means were statistically different (p-value < 0.0001; t = 10.41; df = 118). An increase in spontaneous spiking was not observed at any concentration. Figure 2-6D shows that PRC-728 also had a significant effect upon evoked contraction height. Control contractions averaged 165 mV, while 0.8 mM PRC-728 treated contractions averaged 61 mV. An unpaired t-test showed that these means were significantly different (p-value < 0.0001; t = 11.99, df = 118). A total of 6 preparations were used for the analyses.

The compound 4-tert-octylcatechol (Fig. 2-7A) increased the baseline tension of the larval preparation by around 250% (Fig. 2-7B). This particular
preparation experienced a fair amount of drift, and the decrease in the evoked contraction height after the 0.1 mM concentration was not observed in other replicates. Note that contraction height, along with baseline tension, is recovered later in the recording. At 1 mM, the baseline tension in the muscle sheet increased by about 6.5 mg of force. This effect, with the evoked contractions, put a total amount of force generated by the larval body around 7-8 mg, with a peak of 13 mg. The overall concentration-response relationship had an estimated \( EC_{50} \) value of 0.77 mM. As with TEA and PRC-728, the duration of the individual evoked contraction was increased (Fig. 2-7C). The mean duration of the control contractions was 170 msec, and at 1 mM was 246 msec, a 45% increase. The unpaired t-test showed that these means were statistically different (p-value < 0.0001; \( t = 4.9; \ df = 98 \)). Similar to PRC 728, there was not an increase in spontaneous contractions at any of the concentrations. Figure 2-7D shows that 4TOC had little effect upon the evoked contraction height, and an unpaired t-test showed that the mean height of the contractions from both the control treated and the 1 mM 4TOC treated were not statistically different from each other. A total of 5 preparations were used in the analyses.

The compounds effects were also video recorded on the dissected larval muscle sheets without electrical stimulation. Object 2-6 shows a muscle sheet treated with 1mM 4-AP. At 1 minute 22 seconds in the video, control larval saline was applied by hand pipetting over the dissected muscle sheet an Aedes aegypti larva. There was no effect observed from the addition of the saline. At the 2-minute mark, 1 mM of 4-AP was similarly applied. The muscle sheet reacted
immediately with uncontrollable twitching at the lateral margins of the muscle sheet. Object 2-7 is a similar video showing the effects of 4TOC. Control (0.2% ethanol in larval saline) was added to muscle sheet 6 seconds into the video with no resulting effects. Then at the 30 second time mark, 1 mM of 4TOC was pipetted onto the body, and an immediate and strong contraction of the muscles in the center of the muscle sheet was observed. Additionally, the muscle strands appear to turn a milky white color after the 4TOC addition.

Object 2-6. Fourth instar *Aedes aegypti* larvae dissected and pinned open dosed with 1mM 4-Aminopyridine (.avi file 564MB)

Object 2-7. Fourth instar *Aedes aegypti* larvae dissected and pinned open dosed with 1mM 4-tert-octylcatechol (.avi file 104MB)

Objects 2-8 and 2-9 are videos of the experimental catechols PRC-725 and 728. Object 2-8 shows 0.1% ethanol being applied at approximately 10 seconds in (no effect), and then at 36 seconds 1 mM PRC-725 was added, which caused a slow strong contraction of the medial muscles in the preparation. Object 2-9 shows 0.1% ethanol being applied at 7 seconds in (no effect), and at 30 seconds into the video 1 mM PRC-728 was added and caused an immediate strong medial contraction. In both object 2-6 and 2-7, the muscles affected by the compounds turned a milky white, same as observed with muscles exposed to 4TOC (Object 2-7). It should be noted that control preparations were run, which included multiple applications of the ethanol in larval saline control solutions and showed no effect on the larval muscle sheets.

Object 2-8. Fourth instar *Aedes aegypti* larvae dissected and pinned open dosed with 1mM PRC-725 (.avi file 152MB)

Object 2-9. Fourth instar *Aedes aegypti* larvae dissected and pinned open dosed with 1mM PRC-728 (.avi file 138MB)
2.4 Discussion

Headless larval assays were conducted for the sole purpose of determining whether or not known potassium channel blockers in solution could cause mortality within a mosquito without having to cross the cuticular barrier. It was shown that the potassium channel compounds did indeed cause mortality within headless mosquitoes. The next step was to see if the compounds could pass the cuticle barrier and still cause mortality. A compound that cannot pass the cuticle would have little utility as a contact insecticide, but could be useful as a stomach poison if it could pass through the midgut lining to its intended target site. To test whether or not these compounds could penetrate the larval cuticle, responses in headless and intact larvae were compared. It was shown that there was little difference between the headless *Ae. aegypti* PC$_{50}$ results and the intact *Ae. aegypti* LC$_{50}$ results for PRC compounds and the standard insecticide, propoxur. For PRC-725, and β-thujaplicin, the 95% confidence intervals of the PC$_{50}$ and the LC$_{50}$ values overlapped, indicating that the cuticle had no significant effect on penetration. For propoxur, 4TOC, eugenol, and chlorzoxazone the PC$_{50}$ and LC$_{50}$ values were significantly different, but still always <2-fold. The compounds that were most affected by the presence of the cuticle were 4-AP, TEA, quinine, and quinidine, indicating that the cuticle barrier plays a limiting role in the toxicity of these compounds, but also that there was a useful intrinsic potency on the channels, *in vivo*. 4-AP and TEA also were shown to have decreased toxicity when tested on intact *An. gambiae* larvae.

There are a number of reasons as to why the cuticle decreases the toxicity of these compounds. The cuticle itself is made up of many different parts, but it
is mainly a two-phase barrier (Yu, 2008). The outermost layer is a waxy lipophilic layer, which would prevent any polar compound from penetrating (Yu, 2008). The next phase is a hydrophilic layer, which would slow the movement of any non-polar, hydrophobic, compounds (Yu, 2008). Thus, to penetrate the cuticle successfully a compound needs to be able to interact with both phases (Yu, 92008). In the case of 4-AP, TEA, and quinidine, which readily dissolve in water, the outermost layer of the cuticle likely prevented penetration as they are hydrophilic and possess quaternary (TEA) or basic amine moieties (4-AP, quinine, and quinidine). Quinine’s solubility in water is 3.6-fold greater than quinidine’s solubility, thus higher concentrations of quinine were able to be tested. Other explanations for the decrease in toxicity could be cuticular thickening, or the penetration of a compound may be slowed by active removal via epithelial transporters within the cuticle itself. Cuticle thickening has been documented in a resistant strain of An. funestus (Giles), a mosquito that transmits Plasmodium in southern Africa (Wood et al., 2010). Similarly, P-glycoproteins are transporters expel xenobiotics from insects (Porretta et al., 2008). These transporters have been shown to reduce toxicity of both temephos and diflubenzuron within the mosquito Aedes caspius (Pallas) (Porretta et al., 2008).

The intact An. gambiae results showed that the toxicity of the compounds differed significantly between the two mosquito species. There was a difference in size between the two species, with Ae. aegypti adults being 2-2.5-fold larger than An. gambiae adults. The species ratio (SR) in Table 2-1 show that for the
catechols there was ca. 6-fold difference for each of the compounds; with the exception of PRC-728, which was approximately a 3-fold difference. Had all of the SR values turned out to be 3, then it could have been postulated that the difference in toxicity is only due to a size difference. However, as most of the remaining SR values were around 6 it would premature to conclude that size was the only reason for the toxicity differences. Even though the LD$_{50}$s were corrected for weight, there is a possibility that some physiological difference related to size could affect the toxicity of the compounds.

The increase in toxicity of injected PRC-725 puts the LD$_{50}$ value for *Ae. aegypti* well within the range of the LD$_{50}$ for *An. gambiae*. This result shows that the cuticle in *Ae. aegypti* plays some role in creating the difference in toxicity seen between the species. However, due to the fact that the toxicity of both PRC 725 and propoxur both increase by about the same amount, the cuticle is not the primary mechanism creating the disparity of toxicity for the catechols between the species. Increased metabolism within *Ae. aegypti* was also eliminated due to the results found from the topical with synergist assay. The synergist PBO increased the toxicity of propoxur by about 22-fold while having no effect upon the toxicity of PRC-725, at least in a 1 hr synergist pretreatment.

Different signs of intoxication suggest different modes of action between carbamates and potassium channel blockers. Typical symptoms of carbamate poisoning include restlessness, hyper excitability, tremors, convulsions, and paralysis (Yu, 2008), similar to what was observed in mosquitoes here. These symptoms were not seen with the potassium channel compounds. Instead,
spastic paralysis was seen when larvae were dosed with the catechols PRC-725, 728, eugenol, and 4TOC. Blocking the delayed rectifier (Kv2) potassium channel should result in action potential prolongation, as these channels are responsible for returning cells to their resting potential. It has been shown that the Kv2 channel is blocked when 4-AP is applied to voltage-clamped house fly larval muscle (Salgado, 1992a), and a prolongation of action potentials was observed after application of TEA to giant squid axons by Tasaki and Hagiwara (1957).

Galvan et al. (1980) observed, that with the introduction of 4-aminopyridine to rat sympathetic ganglia, spontaneous action potentials and sub-threshold excitatory postsynaptic potentials. They suggested these results were due to 4-AP causing presynaptic membrane potential changes resulting in the release of acetylcholine (Galvan et al., 1980).

In this study, electrophysiology recordings have shown that potassium channel compounds affect the larval body by causing muscles within the body to contract, increasing the tension in the body of the mosquito. It is at the top concentrations that the tension increase is the most apparent and contraction can be seen in the videos included in this study. The medial muscles within the larva undergo the most significant contraction at single doses of top concentrations of both 4-TOC and PRC-728. The compounds also had an effect upon the individual evoked contractions in the form of lengthening the duration of the measured twitch. The evoked contractions affected by 30 mM TEA expressed the largest increase in duration. This is most likely due to the fact that the top concentration of TEA was 30-fold greater than both the concentrations of
PRC-728 and 4-TOC. Unfortunately higher concentrations of catechols could not be used due to the amount of ethanol that would have been needed to maintain solubility in the applications. This broadening of evoked contractions is not a surprising observation, as TEA has been shown to lengthen spikes from action potentials in mouse cortical neurons by prolonging the repolarization phase at 1 mM (Rudy et al., 1999). Though this affect is shown upon a different channel (Kv3), it can be postulated that TEA would have a similar effect on the delayed rectifier channel (Kv2), which could then affect the relaxation of muscles within a body.

A novel melanization was observed within the bodies of the An. gambiae larvae, and the anal gills of the Ae. aegypti larvae, was melanization. Compounds causing this effect were the catechols PRC-725 and 728, and 4TOC, but not eugenol. The anopheline larvae appeared uniformly black at the completion of the assays, while the anal gills of the Aedes were browned and spotted. The melanization process in insects is controlled by the phenol oxidase cascade (James and Xu, 2012). Phenol oxidase converts catechols into quinones that then polymerize together to form melanin (James and Xu, 2012). It is hypothesized that this cascade is what is causing the blackening of the larval bodies and browning of the anal gills. The effect is dose dependent and does not correlate with mortality, as there were live Ae. aegypti larvae with melanization in the anal gills at the end of multiple bioassays. While An. gambiae larvae were completely melanized, this effect only occurred in the anal gills of Aedes aegypti, and may have been due to a difference in the amount of phenol oxidase found
within the two species. Further experiments are required to determine whether this is indeed the mechanism underlying the difference in melanization between the two species.

Larval mosquito control is usually performed through habitat manipulation, habitat modification, chemical larviciding, and biological larviciding (WHO, 2012b), while the greatest need for new compounds is for adult control through indoor residual spraying and insecticide treated bed nets (WHO, 2012b). Propoxur was used as a standard in larval and adult mosquito toxicity comparisons for the tested potassium channel compounds. The results show that of the tested compounds, 4-AP was the most toxic, but was 10-fold lower in comparison to propoxur (Table 2-1). There was a little difference between species within the headless larvae data for 4-AP. However, 4-AP was significantly less toxic in intact larvae than propoxur, regardless of species. The compound with the next closest activity to propoxur was the experimental catechol PRC-725, closely followed by 4TOC (Table 2-1). Unfortunately the difference between the most toxic experimental catechol and the commercial carbamate is 100-fold, and thus would not prove useful as a practical larvicide. Propoxur was also by far the most potent of the compounds against adults, having an LD$_{50}$ ca. 1 ng/mg in each of the species (Table 2-2). Unfortunately, none of the potassium channel compound’s toxicities came anywhere near the potency of propoxur. PRC-725 was the most potent compound from the potassium channel compounds tested in both species. That said, LD$_{50}$ values of PRC-725 are still 150- and 670-fold weaker than propoxur in An. gambiae and
Ae. Aegypti respectively (Table 2-2). It is doubtful that, even through the use of a synergist, or commercial formulation chemistry, the LD\textsubscript{50} values of the catechols could be brought down to commercial levels.

Overall, the data generated in the present study confirms that potassium channels are viable targets for insecticides, especially the Kv2 subtypes. Compounds that have been previously discovered to block potassium channels do cause effects consistent with Kv2 channel block. Salgado (1992b) observed that RH-5849, a diacylhydrazine observed to block potassium channels, caused acute neurotoxic effects within *Periplaneta americana* (L.) and *Musca domestica* (L.). The toxicity of the experimental catechols though is poor when compared to a commercialized insecticide. However, that does not necessarily mean that these compounds are without utility. Because they show some toxicity, it may be possible to change the structure of the experimental compounds to create more potent materials that attack the same target site.
Figure 2-1. Structures of compounds screened in assays.
<table>
<thead>
<tr>
<th>Compound</th>
<th>5 hr PC&lt;sub&gt;50&lt;/sub&gt; ppm (CI) Ae. aegypti</th>
<th>n</th>
<th>24 hr LC&lt;sub&gt;50&lt;/sub&gt; ppm (CI) Ae. aegypti</th>
<th>n</th>
<th>24 hr LC&lt;sub&gt;50&lt;/sub&gt; ppm (CI) An. gambiae</th>
<th>n</th>
<th>Species Ratio Ae./An.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4- Aminopyridine</td>
<td>3.1 (0.52 – 7.35)</td>
<td>240</td>
<td>&gt;200</td>
<td>20</td>
<td>76 (66 – 88)</td>
<td>140</td>
<td>&gt;2.6</td>
</tr>
<tr>
<td>Tetraethylammonium chloride</td>
<td>173 (139 – 222)</td>
<td>160</td>
<td>&gt;200</td>
<td>20</td>
<td>&gt;200</td>
<td>20</td>
<td>--</td>
</tr>
<tr>
<td>Propoxur</td>
<td>0.28 (0.21 – 0.36)</td>
<td>190</td>
<td>0.6 (0.5 – 0.7)</td>
<td>140</td>
<td>0.17 (0.11 – 0.25)</td>
<td>330</td>
<td>3.5</td>
</tr>
<tr>
<td>4-tert-octylcatechol</td>
<td>38 (20 – 52)</td>
<td>151</td>
<td>0.6 (0.5 – 0.7)</td>
<td>14</td>
<td>14 (12 – 15)</td>
<td>196</td>
<td>6.4</td>
</tr>
<tr>
<td>PRC 725</td>
<td>28 (18 – 54)</td>
<td>120</td>
<td>40 (36 – 45)</td>
<td>45</td>
<td>6.6 (1 – 15)</td>
<td>221</td>
<td>6.1</td>
</tr>
<tr>
<td>PRC 728</td>
<td>98 (66 – 180)</td>
<td>100</td>
<td>141 (121 – 164)</td>
<td>24</td>
<td>45 (11 – 113)</td>
<td>221</td>
<td>3.1</td>
</tr>
<tr>
<td>Eugenol</td>
<td>81 (62 – 99)</td>
<td>199</td>
<td>132 (113 – 159)</td>
<td>24</td>
<td>24 (22 – 26)</td>
<td>250</td>
<td>5.5</td>
</tr>
<tr>
<td>Chlorzoxazone</td>
<td>139 (93 – 170)</td>
<td>163</td>
<td>226 (198 – 261)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-thujaplicin</td>
<td>188 (173 – 208)</td>
<td>154</td>
<td>154 (134 – 174)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinine</td>
<td>349 (121 – 647)</td>
<td>170</td>
<td>1051 (885 – 1251)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinidine</td>
<td>299 (161 – 346)</td>
<td>20</td>
<td>&gt;400</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N,N-Diethylnicotinamide</td>
<td>&gt;400</td>
<td>20</td>
<td>&gt;400</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All PC<sub>50</sub> and LC<sub>50</sub> values are given in ppm (95% Confidence Intervals). Chemicals were considered significantly different when 95% confidence intervals did not overlap. *Anopheles* 5-hr PC<sub>50</sub> data 4-AP and TEA respectively in ppm: 4 (2 – 6) and 61 (44 – 81)
Table 2-2. Toxicity (LD$_{50}$ values (95% Confidence Intervals)) of propoxur and five experimental compounds to adult *Aedes aegypti* and *Anopheles gambiae*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Adult Topical LD$_{50}$ An. gambiae ng/mg</th>
<th>n</th>
<th>Adult Topical LD$_{50}$ Ae. aegypti ng/mg</th>
<th>n</th>
<th>Species LD$_{50}$ Ratio Ae./An.</th>
<th>Adult Injected LD$_{50}$ Ae. aegypti ng/mg</th>
<th>n</th>
<th>Treatment LD$_{50}$ Ratio Topical/Injected</th>
<th>Adult Topical w/PBO Ae. aegypti ng/mg</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>1.08 (0.58 – 1.48)</td>
<td>139</td>
<td>1.3 (0.72 - 1.8)</td>
<td>150</td>
<td>1</td>
<td>0.14 (0.1 – 0.19)</td>
<td>190</td>
<td>9</td>
<td>0.06 (0.05 – 0.07)</td>
<td>269</td>
</tr>
<tr>
<td>PRC 725</td>
<td>163 (96 – 350)</td>
<td>140</td>
<td>870 (720 – 1000)</td>
<td>150</td>
<td>5</td>
<td>81 (18 – 140)</td>
<td>120</td>
<td>11</td>
<td>1073 (784 – 1602)</td>
<td>169</td>
</tr>
<tr>
<td>PRC 728</td>
<td>252 (177 – 341)</td>
<td></td>
<td>1400 (1000 – 1900)</td>
<td>110</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-tert-octylcatechol</td>
<td>263 (310 – 511)</td>
<td></td>
<td>5200 (4600 – 5900)</td>
<td>230</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-thujaplicin</td>
<td>393 (313 – 471)</td>
<td></td>
<td>1800 (1400 – 4200)</td>
<td>120</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eugenol</td>
<td>1023 (948 - 1136)</td>
<td></td>
<td>3600 (3000 – 4200)</td>
<td>120</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mortality determined 24 hr after exposure and dose was adjusted for weight, and the average weight of the *Aedes* female was 2.12 mg while the average weight for the *Anopheles* females was 0.83 mg. Compounds were injected through the thorax.
Figure 2-2. *Anopheles gambiae* 24-hr intact larval assay. A) Control group exposed to tap water + ethanol. B) 100 ppm PRC-725 exposure. C) Close up view of a control larva after 24-hrs. D) Close up view of larvae exposed to PRC-725.

Figure 2-3. *Anopheles gambiae* during a 24-hr larval exposure to PRC-728 where larvae show a concentration-dependent melanization effect. A) 48 ppm; black arrows indicate larvae that have melanization in the abdominal area. B) 96 ppm. C) 150 ppm.

Figure 2-4. Close up of anal gills of *Aedes aegypti* larvae exposed to synthesized catechols A) Control after 24 hrs exposure to control larval saline + ethanol. B) 100 ppm PRC-725. C) 24-hr exposure to 300 ppm PRC-728. Notice that the anal gills in the control solution are clear while the gills in the tested solutions are brown and black in color.
Figure 2-5. Electrophysiological recording and analysis of tetraethylammonium chloride. A) Muscle tension recording larval body treated with increasing concentrations of tetraethylammonium chloride. B) Concentration response curve showing increase in muscle tension of larval bodies as increasing concentrations of tetraethylammonium chloride are applied. C) Comparison of the duration of evoked contractions of muscle tension preparations when a control solution (larval saline + ethanol) is applied and then when the top concentration of 30 mM is applied. Inset. Plots of control waveforms (bottom) and those observed in 30 mM TEA (top). D) Comparison of the height of the evoked contractions when the control solution (LS = larval saline) is applied and then when the top concentration is applied.
Figure 2-6. Electrophysiological recording and analysis of PRC-728. A) Electrophysiology muscle tension recording; larval body treated with PRC-728. B) Concentration response curve showing increase in muscle tension of larval bodies as increasing concentrations of experimental catechol PRC-728 are applied. C) Comparison of the duration of evoked contractions of muscle tension preparations when a control solution (larval saline + ethanol) is applied and then when the top concentration of 0.8 mM is applied. Typical evoked waveforms for control and 0.8 mM PRC-728 are shown inside their respective bars. D) Comparison of the height of the evoked contractions when the control solution is applied and then when 0.8 mM PRC-728 is applied.
Figure 2-7. Electrophysiological recording and analysis of 4-tert-octylcatechol. A) Electrophysiology muscle tension recording; larval body treated with 4-tert-octylcatechol. B) Concentration response curve showing increase in muscle tension of larval bodies as increasing concentrations of 4-tert-octylcatechol were applied. C) Comparison of the duration of evoked contractions of muscle tension preparations when a control solution (larval saline + ethanol) is applied and then when the top concentration of 1mM is applied. Typical evoked waveforms for control and 1mM 4-tert-octylcatechol are shown inside their respective bars. D) Comparison of the height of the evoked contractions when the control solution is applied and then when the top concentration is applied.
CHAPTER 3
IMPLICATIONS AND FUTURE DIRECTIONS FOR THE DEVELOPMENT OF A POTASSIUM CHANNEL DIRECTED CLASS OF INSECTICIDES

3.1 Conclusion

Malaria control programs rely heavily upon the use of insecticide treated nets and indoor residual spraying to suppress vector populations to low levels (WHO, 2012b). Increasing resistance burdens the small pool of available insecticides that are employed. Presently there are four classes of insecticides that are utilized; organophosphates, carbamates, organochlorines, and pyrethroids, the last of which are used more than the previous three types (WHO, 2012b). A new class of insecticide is needed to ensure the continued successes of vector control, thus the primary goal of this study was to assess the toxicological value of designing compounds that target potassium channels.

We found that compounds already on the market that are known effectors of potassium channels do indeed cause mortality within the two medically-important mosquito species; *Aedes aegypti* (L.) and *Anopheles gambiae* (Giles). Unfortunately, only one of the compounds evaluated came within 10-fold of the toxicity of a standard insecticide used in vector control, and that was when the compound did not have to pass the mosquito cuticle. In terms of toxicity to intact insects, the commercially-available potassium channel compounds were extremely poor. There was one compound with toxicity that was within 39-fold of the commercial insecticide, propoxur, in intact *An. gambiae* larvae. This was an experimental catechol developed by Dr. Paul Carlier’s lab at Virginia Tech and was an important finding for two reasons. First, the toxicity of the experimental catechol PRC 725 was greater than any of the commercially-available potassium channel compounds that were tested. Secondly,
PRC 725 was synthesized based on the chemical structure of another substituted catechol (48f10), which had been determined to be an inhibitor of potassium channels (Zaks-Makhina et al., 2003). These results show experimental catechols, specifically (PRC 725) have the prospect of being a good lead compound for the development of target-specific potassium channel insecticides.

The significant differences seen in the toxicity of the compounds between the two mosquito species was an interesting observation that needs to be solved. Sequence alignment of the potassium channels of the two mosquito species revealed that there were no apparent differences (Max Totrov, personal communication); thus, there must be another mechanism affecting the toxicity. The cuticle was the next mechanism that was thought to be the cause of the toxicity disparity, and so injection studies were performed. This method of application brought the toxicity of the compounds, within Ae. aegypti, down to the toxicity values for topically dosed An. gambiae. This result demonstrated that the cuticle in Ae. aegypti differs from An. gambiae in some way that conveys increased protection against the compounds; however, it is not the primary reason for the toxicity difference. Synergist studies also concluded that metabolism within Ae. aegypti does not have a great role in the toxicity of catechols.

The electrophysiological experiments were conducted to compare effects of compounds known to block potassium channels to the experimental catechol compounds. Results showed that the effects of the compounds were similar in nature. The known potassium channel compound increased the body tension of the larval bodies by causing the continued contraction of muscles within the body lining. The compounds also elongated relaxation times of evoked contractions during these
experiments, which is consistent with other studies that have shown that blocking the potassium channel can prolong repolarization times of cells.

While these data show that steps towards a potassium channel directed insecticide are being taken, they are still far from completion. The toxicity of these compounds needs to be increased significantly to be of any practical use as vector control agents. However, with more extensive structural modification of these experimental compounds, it may be possible to bring their toxicity up to commercially viable levels.

### 3.2 Future Studies

The primary motivation behind the development of potassium channel directed insecticides has been to combat insecticide resistance within malaria endemic countries. However, as insecticide resistance is not solely found within disease vectors, it would be prudent to screen these same compounds within other pests as well. Agricultural pests, for example, express insecticide resistance and an additional class of insecticide would most likely prove useful in controlling those as well.

The cuticle within *Ae. aegypti* should be studied and compared to that of *An. gambiae*. Determining the thickness of the cuticle and its composition will allow for a more thorough explanation of how the cuticle decreases the toxicity of the compounds. This could aid in developing insecticides that target this insect.

The melanization effect is thought to be caused by the phenol oxidase cascade, however hard data needs to be acquired to confirm this hypothesis. Determining the quantities of phenol oxidase within each of the species would be important in explaining the differences in melanization between the species. It would also be important to run the compounds at maximal doses in *Aedes aegypti* to determine if increasing the
concentration of the catechols would create the whole body effect. Finally, an experiment that directly links phenol oxidase catalysis to the catechol compounds to melanization would need to be performed to be certain that this is what was causing the effect.

Additional electrophysiological assays should be performed upon the muscles directly. The assays performed in this study showed that muscles were indeed affected by compounds, resulting in contraction; however, having data showing depolarization of these muscles and block of Kv2 currents under voltage clamp would be beneficial. Additionally, there are no data on the nervous system effects for these compounds. Such studies would allow comparison of the relative sensitivity of these two tissues.
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BIOGRAPHICAL SKETCH

Nicholas Larson was born and raised in central Florida in a town by the name of Winter Haven. After graduating Winter Haven Senior High School in 2003 he attended the University of Central Florida. After a number of degree changes he graduated in 2008 with a Bachelor of Science degree in biology. For the next 3 years he worked for the Walt Disney World Company and for CVS Pharmacy. In the fall of 2010 he started volunteering with Polk County Mosquito Control working with Dr. Carl Boohene. During his time with the mosquito control his interest in insecticides and insects developed. He applied to the University of Florida to pursue a Master of Science degree in entomology and nematology. In the fall of 2011 he started his graduate career in the laboratory of Professor Jeffrey Bloomquist.

His career goals are to continue into a doctoral program and eventually make his way into government or industry working on insecticide/repellent toxicology and discovery.