AMERICAN UNIVERSITY OF BEIRUT

MONITORING PYRETHROID RESISTANCE IN THE HOUSE MOSQUITO, *CULEX PIPIENS*, IN LEBANON

by
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AN ABSTRACT OF THE THESIS OF

Reem Abed Masri for Master of Science
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Title: Monitoring Pyrethroid Resistance in the House Mosquito, *Culex pipiens*, in Lebanon

Pyrethroid insecticides have been extensively used against mosquitoes in Lebanon and worldwide for public health control. However, mosquitoes are known to develop resistance with time against chemicals. In this study, we evaluate percent resistance to pyrethroids in populations of the house mosquito, *Culex pipiens,* in selected cities in Lebanon and we determine the mechanisms of resistance, target site-resistance and/or metabolic resistance. Target site resistance consists of mutations in the sodium channel, which is the target of pyrethoids and DDT; while metabolic resistance involves elevated levels of detoxifying enzymes, which can be mixed function oxidases and non-specific esterases. For this study a sample of 672 mosquitoes was collected from six sites from May 2014-2016 and screened for resistance to pyrethroids, type I and type II using the WHO susceptibility test, then analyzed for the L1014F mutation in the sodium channel gene and for the over production of mixed function oxidases and non-specific esterases. Our results show that all sampled mosquito populations were resistant to pyrethroids, type I and type II. Only the population from Beirut showed high resistance to pyrethroids type II but borderline susceptibility to pyrethroids type I. This was because only in Beirut the municipality has been spraying type II pyrethroids. Moreover, resistance in the Lebanese *C.pipiens* populations to pyrethroids was caused by two mechanisms involving an over production in the detoxifying enzymes and the L1014F mutation. It was found that 66.96% of the sampled mosquitoes at the national level were homozygote for the mutation L1014F having the genotype [RR], 16.22% are heterozygote for the mutation exhibiting [RS] genotype and 16.82% were susceptible [SS]. High levels of oxidases and esterases were also detected in *C.pipiens* populations in most of the sites in Lebanon, mainly agricultural regions.

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ABBREVIATIONS

%	Percent
A.	*Aedes*
ace	acetylcholinesterase gene
Ach	Acetylcholine
AChE	Acetylcholinesterase
bp	basepair
Bt	*Bacillus thuringensis*
Bti	*Bacillus thuringensis israelenis*
C.	*Culex*
Ca	Calcium
cm	centimeter
Co.	county
CTAB	acetyl trimethyl ammonium bromide
CYP	Cytochrome P450
DDE	Dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEM	diethyl meleate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Est	Esterase
F	phenylalanine
g	gram
GPx	Glutathione peroxidase

GS	Thiolate anion
GSH	Glutathione
GST	Glutathione S-transferase
IRS	Indoor residual spraying
ITN	Insecticide-treated nets
Kdr	Knock-down resistance
L	Leucine
LPO	Lipid peroxidation
M	Molar
MFO	Mixed function oxidase
Mg	milligram
Mg	Magnesium
$MgCl_2$	Magnesium chloride
Min	Minute
ml	millileter
mm	millimeter
nm	nanometer
NSE	Non-specific esterase
OPs	Organophosphates
P450	Cytochrome P450-dependent monooxygenase
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
pmol	picomole
Rdl	resistance to dieldrin gene
ROS	Reactive oxygen species

RT	Room temperature
sec	Seconds
t	time
U	unite
ul	microliter
V	Valine
WHO	World Health Organization

CHAPTER I

INTRODUCTION

A. Introduction:

Mosquito are the most important vectors of disease. Besides acting as annoying biting insects, they are responsible for spreading malaria, dengue, yellow fever, and encephalitis, which are responsible for several million deaths and hundreds of millions of cases every year. Hence, mosquito control is an essential public health measure (WHO, 2014). Awareness on mosquito has been significantly increasing in the past decade because of the geographical spread of some medically important species such as *Aedes albopictus* and *Aedes aegypti* worldwide. Research studies implicated in understanding the physiology and behavior of mosquito is gaining great importance to help restrain vector-borne diseases through original strategies.

B. Mosquito Taxonomy and distribution

Mosquitoes are relatively small insects, measuring an average of 6mm long and weighing around 2.5mg (Service, 1996). The oldest known mosquito with an anatomy similar to modern species was found in a 79-million-year-old Canadian amber dating from the Cretaceous (G. O. Poinar; et al. 2000; Service, 1996). Mosquitoes belong to the family Culicidae, order Diptera, which comprises the true, two-winged flies (Beaty and Marquardt, 1996; Clements, 1992). One distinctive feature of the Diptera is the modification of the hind wings into small balancing organs called halteres (Walker, 1996; Clements, 1996). The Diptera is broadly divided into two suborders, Nematocera and Brachycera. The Nematocera consists of primitive flies with elongated bodies and many-segmented, often feathery antennae.

The Brachycera includes more advanced flies with proportional round body and much shorter antennae (Walker, 1996). The Culicidae, is a large family which belongs to the suborder Nematocera; therefore, mosquitoes are more closely related to midges and craneflies than higher flies such as houseflies and blowflies (Clements, 1992). The Culicidae family contains around 3,543 species belonging to 41 genera. It is divided into three subfamilies: Anophelinae, Toxorhynchitinae and Culicinae (Snow, 1990) (Clements, 1992; Service, 1996).

The subfamily Anophelinae contains the medically important genus *Anopheles*, which include 422 species, as well as two other rare genera, *Bironella* and *Chagasia*, (Service 1996; Walker, 1996).

The Toxorhynchitinae includes the single genus *Toxorhynchites* consisting of approximately 70 species. The world's largest mosquitoes belong to this subfamily. Toxorhynchites species show a curved proboscis adapted to feeding on nectar rather than blood (Service, 1996). Their larvae are predaceous on other mosquito larvae; therefore, they are used as mosquito control agents in some Gulf Coast states (Walker, 1996).

The Culicinae is the largest subfamily comprising 33 genera and approximately 3000 species (Service, 1996) which makes it difficult to generalize distinguishing features between them. It includes some of the most medically important genera: *Aedes*, *Culex*, *Mansonia*, *Haemagogus*, *Sabethes*, and *Psorophora* (Service, 1996).

Mosquitoes colonize a wide range of habitats with contrasting environments, which enable them to distribute worldwide. They can be found in tropical and

temperate regions as well as beyond the Arctic Circle; they are absent only from Antarctica and few islands (Service, 1996). The genera which are widely distributed and of medical importance are *Anopheles, Aedes, and Culex* (McGavin, 1992). The geographical distribution and development rate of insect vectors is strongly related to temperature, rainfall and humidity. A rise in temperature accelerates the insect metabolic rate, increases egg production and makes blood feeding more frequent. With the increase in temperature in the last years, mosquito's distribution worldwide increased dramatically (WHO, 2004).

C. Life Cycle

Mosquitoes go through four stages of development through their life cycle (Fig. 1); hence, they undergo complete metamorphosis. Mosquito life cycle starts by a female mosquito laying eggs on the water surface or on sites that will be flooded. Depending on the species a female mosquito can lay anywhere between 50 to 500 eggs at one time (Service, 1996). Embryonic development starts as soon as eggs are deposited in water and need days to few weeks, depending on temperature, to develop into a fully formed larva (Clements, 1996; Service, 1996). *Culex* mosquitoes lay their eggs attached to each other in several rows called rafts; on the contrary, *Aedes* and *Anopheles* lay their eggs singly (Service, 1996).

The immature stages comprise four active larval instars, between each instar the larva molts, shedding its skin to produce the following instar (Snow, 1990). Size, number, arrangement and complexity of certain hairs and other surface structures differentiate the larval stages (Snow, 1990).

Mosquito larva is legless with a well-formed head. Larval habitats are diverse in nature and size. Any selection of permanent or temporary water can serve

as a good habitat for mosquito larva such as: rice fields, marshes, shallow pools, man-made containers, leaf axils or animal footprints (Clements, 1996).

The respiratory organs in mosquito larva are the spiracles, situated at the posterior end of the abdomen (Clements, 1996; Service, 1996; Walker 1996). The siphon, found in *Aedes* and *Culex,* is an exterior extension of the spiracles that enable the larva to obtain oxygen from water surface by hanging down at an angle (Service, 1996). However, the spiracles of anopheline larvae are found on the dorsal surface of the last abdominal segment, and the larvae lie parallel to the water surface to obtain air (Clements, 1996).

Most larvae are saprophagous and feed on 'particulate matter', this includes aquatic microorganisms such as bacteria, diatoms, and algae (Clements, 1996); or animal carcasses and detritus (Service, 1996).

Mosquito larvae live a few days to a few weeks.

The final mosquito larva molts to become a comma-shaped pupa called tumbler. The pupa's head and thorax are fused into a large cephalothorax from which a pair of small, dorsal, trumpet-like structures, the respiratory siphons, emerge (Beaty and Marquardt, 1996). The pupa does not feed and is seen floating at the surface. The pupae gains it's buoyancy from the air contained in the cephalothorax (Service, 1996). The contraction of the abdominal muscles enables the pupa to move rapidly, (Beaty and Marquardt, 1996) tumbling below the water surface when disturbed. During pupation, larva tissues are broken down and adult tissues are generated. The pupal stage lasts between one to few days depending on the water temperature. The adult breaks down the pupal case and crawls onto the surface of the water (Snow, 1990). The adult mosquito first rests while its exoskeleton hardens then spreads it's

wings to dry preparing itself for life on land. The mosquito now is a flying land insect.

Adult mosquitoes are slender, delicate flies, measuring around 3-6 mm in length, but can range from 2 to 19 mm (Service, 1996). A reddish-brown exoskeleton protects the body, which is divided into three main parts: head, thorax, and abdomen (Becker *et al.*, 2003).

The head is spherical with a pair of antennae, a pair of compound eyes and external slim sucking mouthparts known as the proboscis or stylet. It also has a pair of palps that may be short or long depending on the species (Borror *et al.*, 1989). The thorax bears three pairs of jointed legs, one pair of functional wings and a pair of halteres. The abdomen is cylindrical and consists of ten segments, only eight are visible. The two terminal segments have been modified to carry out reproductive functions (Beaty and Marquardt, 1996).

Mating of most mosquitoes occurs when female enter swarms of flying males. Usually males form swarms over a marker (object that contrasts with the surrounding) at low light intensity (Becker *et al.*, 2003). When swarming, males move in many directions and patterns known as the " dancing flight". The sound produced by male's wing beating attracts the females to enter the swarm and in turn mating occurs (Becker *et al*., 2003;Clements, 1996).

Longevity of mosquitoes is dependent upon the type of environment. In general, adults in temperate and arctic regions live on average about four to five weeks. In hot tropical areas, the life span is about one to two weeks (Service, 1996). Female mosquitoes, especially those in temperate and arctic regions, may hibernate or aestivate and hence live much longer. For example, some species of *Culex pipiens* survive from August until May (Service, 1996; Becker *et al.*, 2003).

Mosquito mouthparts are used to pierce and draw plant juices as a source of carbohydrates using their proboscis. Moreover, female mosquito of most species will require a blood meal for egg maturation and deposition (Service, 1996). Therefore, mosquitoes have developed a complex host-seeking behavior to locate the host. Location of potential host is usually dependent on olfactory, visual, or thermal stimuli. The main olfactory stimuli are carbon dioxide, phenolic compounds, and lactic acid (Becker *et al.,* 2003).

When a female lands on the host, it may poke the skin several times with the labellae searching for a capillary for blood intake (Davis and Sokolove, 1975). To be able to successfully complete a blood meal, the female injects saliva into the wound that contains anesthetics and anticoagulants that help remain blood in a liquid form. The introduction of these foreign proteins with saliva into the host stimulates an immune response, causing itching and inflammation (Becker *et al*., 2003).

It is good to note that only few autogenous species such *as Culex p.pipiens* biotype *molestus* are able to produce their first egg batch without a blood meal (Clements, 1996; Becker *et al*., 2003).

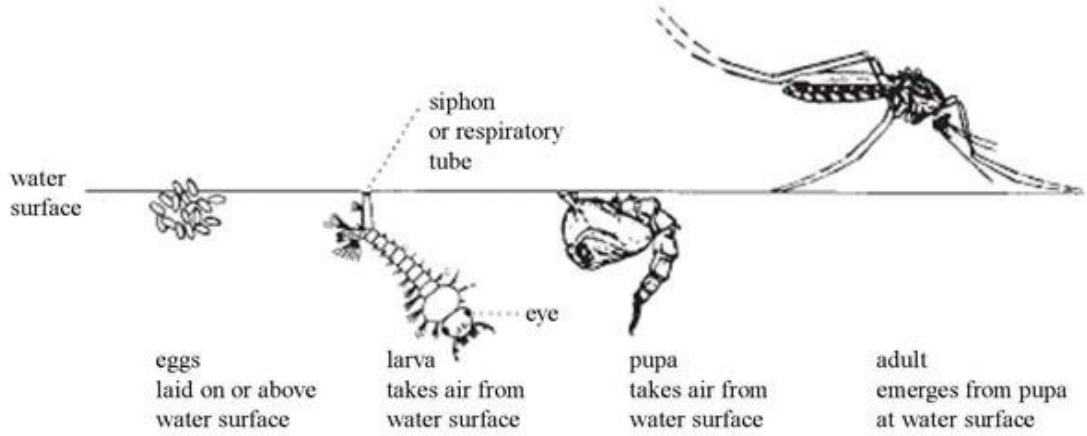

Figure 1: Stages of mosquito life cycle

Source:

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D. Medical importance

Since female mosquitoes are blood sucking and salivate into bloodstreams, mosquitoes are able to acquire pathogens and parasites from one vertebrate and transmit it to another; the transmitted pathogens cause vector borne diseases (Clements, 1996; Becker *et al*., 2003). But even so, the mosquito's ecology and physiology must be appropriate for it to acquire, foster, and transmit a particular microorganism. Vector-borne diseases account for more than 17% of all infectious diseases, infect more than one billion people each year and around one million people die from these diseases that include: malaria, dengue, schistosomiasis, leishmaniasis, Chagas disease, yellow fever, lymphatic filariasis and onchocerciasis (WHO, 2014).

Malaria cases and deaths has decreased by 18% from year 2000 until 2015 due to the drastic and reform measures applied by WHO especially in Africa to limit exposure to mosquito bites. These include the use of the insecticide-treated mosquito nets (ITNs) or indoor residual spraying (IRS) that reduced malaria mortality rates by an estimated 55% in children aged less than 5 years in sub-Saharan Africa (WHO, 2015). For this reason, monitoring mosquito resistance to current insecticides used is crucial.

Despite that, 218 million cases of malaria and 438,000 deaths were reported in 2015 indicating the heavy burden mosquito can inflict on public health (WHO, 2016).

Some of the diseases transmitted by medically important mosquito species are summarized in Table 1.

Table 1: List of diseases transmitted by mosquitoes.

Mosquito	Agent	Disease transmitted
Anopheles	*Plasmodium falciparum* *Plasmodium vivax* *Plasmodium ovale* *Plasmodium malariae*	Human malaria
Aedes	Dengue virus Zika virus Yellow fever virus } Flavivirus virus	Dengue fever Zika Yellow fever
Culex	*Wuchereria* *Brugia* (nematode) West Nile Virus	Filariasis Filariasis West Nile Disease

E. Mosquito Control

1. Biological control

Reducing mosquito population can be performed biologically, that is, by the use of predators, parasites, competitors, pathogens, or toxins from microorganisms. Biological control or the use of beneficial organisms for the control of mosquitoes was first introduced in the 19th century when dragonflies were established to decrease mosquito number (Lamborn, 1890; Becker 2003). The major advantage of biological control is that predators are conserved in the environment and will restore natural balance (Becker, 2003). An important step before introducing any predator is a

precise knowledge on the biology of the antagonist and its interaction with the ecosystem. For instance, the introduction of fish species to diminish mosquito larvae may affect the numbers of crustaceans and small fish found. Since, mosquitoes inhabit different environments, a specific antagonist for each habitat must be introduced (Becker, 2003). In general, predators of the immatures are more effective than predators of the adult stage because eggs, larvae and pupae are concentrated at their breading sites. Examples of predators that target the immature stages are: mosquito fish (*Gambusia affinis*), caudata, hydras, amphibians, dragonflies, and crustaceans (Clements, 1996; Becker, 2003).

Biological control by using pathogens, such as *Bacillus thuringensis*, is another method of reducing mosquito population. *Bacillus thuringensis* (Bt) is a facultative anaerobic, Gram-positive bacterium that forms characteristic protein inclusions near the endospore. These inclusions are toxic to the insect larvae in the order Coleoptera, Diptera and Lepidoptera (WHO, 2009). The subspecies *israelensis* (Bti) is a natural biological enemy that is specific for the diptera, such as mosquito larvae. It is safe for humans and all creatures except for fungus gnats and black flies. Bti spores release crystals that are toxic when mosquito larvae ingest them. The presence of an alkaline environment with enzymes in the midgut trigger toxin crystals to dissolve activating the toxin, which cause cells in the larvae's gut to rupture, killing it within 24 hours. However, mosquitoes have been recently accumulating resistance mechanisms against Bti (Tetreau *et al*., 2012).

Recently, the use of transgenic bacteria and fungi as a strategy for mosquito control, also known as paratransgenesis, is being extensively studied. Symbiotic

bacteria or fungi in mosquito are designed to express effector molecules that aim in targeting the pathogen inside the mosquito (Wilke and Marrelli, 2015).

2. Physical Control

Physical control of mosquitoes focuses on the reduction of mosquito breeding sites and mosquito-human contact. The elimination of unwanted water sources or sanitation is an important aspect of mosquito breeding site reduction. Many actions can be made to achieve this step. Public information programs that aim at educating society on the importance of reducing unwanted water sources is considered one of the main steps. Moreover, essential water containers such as bird baths and outdoor pet dishes should be emptied at least once a week and re-filled with fresh water. When not in use, swimming pools must be fully drained and kept free of water. In addition to education, city authorities have a direct role in decreasing mosquito-breeding sites. For instance, sewage systems and street waste containers should always be monitored to ensure they do not become water-holding containers for mosquito breeding (Becker *et al.*, 2003).

Physical control methods also focus on the human-mosquito contact. For example, wearing long trousers and sleeves outdoors when mosquitoes are most active. Installing nets on windows and doors also reduce the number of mosquitoes indoors. Moreover, when accommodations are not adequately screened or air conditioned, bed nets are important in providing protection to humans. Furthermore, natural repellent products are becoming commercially available. Examples of these products are castor oil, lemon eucalyptus oil, lavender oil, thyme oil and catnip oil. Other physical control such as mosquito coils, spatial repellents and vapourising mats all contain one or more of the insecticides classes (Becker *et al.,* 2003).

3. Chemical Control

Mosquito control is crucial for reducing the impact of mosquito transmitted diseases on human health (Becker *et al.,* 2003). Chemical control by the use of insecticides plays an essential part in reducing adult mosquito populations. The first chemical to be used against mosquitoes was a powder extracted from the heads of pyrethrum flowers by Boccone in 1697 and Buxbaum in 1728 (Ruigt, 1985; Becker, 2003). A series of dramatic discoveries started from 1939 when the "wonder" insecticide, best known as DDT, was introduced (Becker, 2003). Since then, a totally new concept of insect control began.

Insecticides used in mosquito control belong to four major chemical groups, chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids. Chlorinated hydrocarbons such as DDT are extremely stable in the environment and hence contaminate the ecosystem and are toxic to animals and humans. For these reasons they have been banned by most countries (Becker, 2003). DDT distorts the balance of sodium and potassium ions within sensory neurons thereby causing spontaneous firing and hyperexcitability (Vijverberg, H. P, 1982).

Organophosphates (OPs), chemically less stable, replaced chlorinated hydrocarbons. All OPs are derived from phosphoric acid and have a lower chemical stability; however, they are much more toxic to vertebrates (Becker, 2003). OPs target the synapses of sensory neurons by inhibiting acetylcholinesterase and hence increase the concentration of acetylcholine. The accumulation of acetylcholine causes rapid twitching of muscles and paralysis of the insect (Becker, 2003).

Carbamates were first introduced in 1951 in Switzerland and the first natural discovery was in grapes in the mid-19th century. The carbamates' mode of action is

the same as that of organophosphates targeting acetylcholinesterase. They have been effectively used against vectors that have developed resistance to organochlorines and organophosphates (Becker, 2003).

Pyrethroids are a new generation of synthetic insecticides. They emerged from natural pyrethrins, which are extracted from the flower heads of *Chrysanthemum* spp. Nowadays pyrethroids are replacing the three previous groups of insecticides because of their low mammalian toxicity and low persistence in nature (WHO, 2013, Becker *et al*., 2003).

Pyrethroids are neurotoxic to insects and are divided into class I and class II pyrethroids. When the chemical is applied, insects show uncoordinated movements followed by paralysis. Usually, pyrethroids bind to the sodium channel altering its gating properties and keeping it open. The nerve becomes depolarized for a longer time, keeps on firing and the insect dies of exhaustion (Martinez-Torres, 1998; Nannan, 2015). One interesting factor is that pyrethroids become more toxic to insects as the temperature is lowered (Becker, 2003) probably because higher temperatures reduce the residual life of the insecticides (Mansoor *et al.*, 2015).

Pyrethroids today are approved by the WHO and are highly recommended because of their low toxicity to mammals. Nevertheless, improper use of these insecticides affects other insects such as honeybees and predators of common pests (Becker, 2003). Moreover, pyrethroids used to control mosquito larvae can endanger fish and aquatic plants. Selective control for mosquito larvae can be established by choosing the right concentration of pyrethroids (Becker, 2003).

F. Resistance Mechanisms in Mosquitoes

Although insecticide spraying proved to be one of the most efficient ways to control adult mosquitoes, it subjects the population to Darwinian selection and survival of the fittest (Casida *et al*., 1998). This results in killing susceptible individuals in the population and increasing the number of resistant ones.

With continuous use of insecticides, mosquitoes developed ways to overcome the toxicity of the chemical control. In fact, resistance has been defined as " the inherited ability of the strain of some organisms to survive doses of a toxicant that would kill the majority of the individuals in a normal population of the same species" (WHO, 1957). Resistance mechanisms are usually determined by a single genetic factor were species under continued selective pressure of insecticides will accumulate resistance genes and corresponding resistance mechanisms that may lead to cross or multiple resistances (Becker *et al*., 2003). It has been suggested that some rare individuals carry one or more resistance genes before being subjected to any stress (insecticide), this event is termed as the preadaptive phenomenon (Xu, 2012; Nannan, 2015). This proportion of individuals should increase in the population following insecticide selection. Resistance may develop to more complicated cases such as "cross resistance" or "multiple resistance". In cross-resistance, the mosquito population resists many classes of a particular insecticide even on primary encounter. In extreme cases of multiple resistance, mosquitoes build up mechanisms that resist unrelated insecticide classes, thus controlling such population with insecticide becomes extremely difficult (Becker, 2003).

The development of resistance to the widespread use of insecticides led the World Health Organization (WHO) to encourage companies to search for new compounds (Minton, 1988; Becker, 2003).

There are several resistance mechanisms in mosquitoes, namely, behavioral, physiological, and target site. When an insecticide is sprayed, it has first to penetrate the integument of the insect before reaching a specific target site. Studies on *Culex pipiens* have shown that insecticide resistance can occur at any of these points (integuments or target site) or by even changing the insect behavior.

Physiological resistance is a mechanism whereby the cuticle thickens; therefore, the penetration of the insecticide is reduced (Breaud, 1993).

Behavioral resistance evolves in response to selective pressures exerted by the toxicant. These actions strengthen the ability of a population of insects to escape and avoid the lethal effects of that toxicant (Lockwood *et al.*, 1984). This type of response can be further divided into stimulus-dependent or stimulus-independent. The first involves an insect leaving an insecticide treated area only after making physical contact with the chemical, whereas stimulus-independent or sometimes called "special repellency" occurs when insects move away from the insecticide-treated area without making a direct contact (Chareonviriyaphap, 2013). As an example, it is documented that DDT and permethrin can reduce rate of mosquitoes entry to houses, increase rate of early exit, and induce a shift in biting times (Hemingway *et al*., 2004). Behavioral changes in mosquitoes have been seen extensively in agriculture; however, behavioral adaption in vectors has always lagged behind (Gatton *et al*., 2013).

1. Target Site Resistance

Target site resistance occurs when the protein receptor that the insecticide is designed to attach to is altered by a mutation (WHO, 2013). Upon exposure to high doses of organophosphates or carbamates, point mutations in the acetylcholinesterase

active site occur. Point mutations also occur in the ion channel portion of a ϒ-Aminobutyric acid (GABA) receptor subunit in species resistant to cyclodiene insecticides. Moreover, extensive use of DDT and pyrethroids will cause a mutation in the sodium channel gene that alters the population resistant (Hemingway *et al*., 2000).

a. Mutation in the Voltage-Gated Sodium Channel

Voltage gated sodium channels are integral transmembrane proteins essential for the generation and propagation of action potential in excitable cells. Each channel comprises four domains (I- IV), each domain consists of six transmembrane helices (S1-S6). The selective conductance of sodium ions across the plasma membrane by this channel underlies the propagation of these potentials. During an action potential the sodium channel undergoes changes between closed-resting, activated, and inactivated states. Any molecule that binds to specific sites on the channel either alters the equilibrium between these states or blocks the channel (O'Reilly *et al*., 2006). There are at least ten specific binding sites for ligands on the sodium channel, the binding site for lipid soluble DDT and prethroid insecticides has been shown to be at site "7" (Wang *et al*., 2003). Moreover, DDT and pyrethroids prefer to target the open state of the channel, hence their binding appears to stabilize the open state inhibiting the transition to the deactivated state (O'Reilly *et al*., 2006). In other words, mutations at this position reduce the affinity of the "open channels" for pyrethroids by 10–30-folds due to enhanced closed-state inactivation, hence narrowing the number of high-affinity binding sites available for pyrethroids (O'Reilly *et al.*, 2006). The persistent depolarization of the plasma membrane, inward flux of sodium ions, induces repetitive nerve firing and hyperexcitability, leading to paralysis and death.

Since DDT was proven to be harmful to mammals and the environment, many countries switched to the low persistent and less toxic pyrethroid. Unfortunately, resistance mechanisms selected for the continuous use of DDT also confer cross-resistance to some classes of pyrethroids (Brengues *et al.,* 2003).

An important mechanism of resistance to DDT and pyrethroids is referred to as knockdown resistance or "kdr" because the insect can resist exposure to insecticides without being "knocked down" (Torres *et al.,* 1999). The most common mutation is a replacement of the amino acid leucine to phenylalanine found at codon 1014 in the sixth segment of domain II of the sodium channel (Leu^{1014} in IIS6) which was first detected in pyrethroid-resistant house fly *Musca domestica* (Torres et al, 1999). This alteration prevents the pyrethroids from binding to the sodium channel. An alternative substitution showing a replacement of the amino acid leucine to serine or histidine in pyrethroid-resistant *C. pipiens* was reported by a group in 1999 (Martinez-Torres *et al.,* 1999). Mutations at the domain II S4-S5 linker and IIS5 residues are generally correlated with higher levels of resistance (up to 1000-fold) and are often reffered to "super-kdr" (O'Reilly *et al.,* 2006). It has been noted that a super-kdr is always associated with a kdr mutation. These observations led scientists to agree that if *kdr* mutation is very important in pyrethroid resistance, than its frequency, heterozygosity, and/or homozygosity should be correlated with different levels of resistance (Liu *et al.,* 2006).

b. Mutation in Acetylcholinesterase

Organophosphates and carbamates target the synaptic enzyme acetylcholinesterase (AChE) that hydrolyzes the neurotransmitter acetylcholine to terminate nerve impulse. AChE is encoded by an *ace* gene. Most insects, including

mosquitoes possess two ace genes, *ace*-1 and *ace*-2, except in true flies were *ace*-1 is not found. In mosquito resistant species, the mutation is associated with *ace*-1 gene while *ace*-2 in not involved (Humingway *et al.*, 2004). The most common mutation is a single amino acid substitution from glycine to serine (G119S). Thus, a mutation in the enzyme will lead to accumulation of ACh in the synaptic cleft, which causes excessive nerve firing, paralysis and eventually death.

c. Mutation in GABA Receptor

Type A receptor for the neurotransmitter GABA is the target site for cyclodiene (organochlorine compound) insecticides such as dieldrin. The $GABA_A$ receptor is a chloride channel that consists of five subunits; each subunit is composed of an extracellular cysteine loop and four transmembrane domains (M1-M4). Resistance to cyclodiene is present in several species including mosquitoes. The mutation is a substitution of a conserved amino acid alanine to a serine or more rarely to glycine in the *Rdl* gene (Humingway *et al.*, 2004; Nannan, 2015). The mutation causes a prolonged opening of the channel once the ligand binds.

2. Metabolic Resistance

Metabolic resistance arises when increased enzymatic activity results in a more rapid detoxification of the insecticide preventing it from reaching the target site (WHO, 2013). Increased enzymatic activity is due to gene amplification or overexpression. The detoxification of insecticides in mosquitoes involves three major groups: cytochrome P450s (P450s), esterases, and glutathione S-transferases (GSTs).

a. Cytochrome P450s Monooxygenases

Cytochrome P450s-dependent monooxygenases are an extremely important and diverse family of hydrophobic, heme-containing enzymes. They are found in almost all aerobic organisms from bacteria to mammals. They are critical for the detoxification of endogenous and exogenous compounds such as drugs, pesticides and plant toxins (Scott, 1999; Liu, 2015). Monooxygenases are unusual because they can metabolize diverse substrates and are capable of producing different reactions. P450 is a hemoprotein, which acts as the terminal oxidase in monooxygenase systems. In eukaryotes, P450s are found in the endoplasmic reticulum and mitochondria. The monooxygenases of insects have several functions, including growth, feeding, resistance to pesticides and tolerance to plant toxins. Insect monooxygenase can be detected in a wide range of tissues. Highest levels of monooxygenase activities are in the midgut, fat bodies and Malpighian tubules (Scott, 1999). Moreover, levels of monooxygenase activities and P450 concentrations can differ dramatically between individuals and throughout development. In general, total concentrations of P450 are unnoticeable in eggs, fluctuate during each larval instar, decrease tremendously during pupae and boost up again in adults. Among metabolism based insecticide resistance monooxygenase-mediated resistance is the most frequent type although increase in esterases are also very common (Scott, 1999). It was noted that levels of monooxygenase-mediated detoxification in susceptible strains of certain species limits the toxicity of some insecticides such as pyrethrins. Moreover, it is frequently found in major mechanisms of resistance and has the potential to confer cross-resistance to many classes of insecticide (Scott, 1999). Cytochrome P450-Dependent Mixed Function Oxidases (MFO) System is one of the systems in P450s that is known for the detoxification of pyrethroids (Vulule *et al.*, 1999). Microarray studies have

shown that several P450s in *An. gambiae* associated with pyrethroid resistance. Some examples include CYP6Z1, CYP6Z2, and CYP6P6 (David *et al.*, 2013).

b. Esterase-Based Resistance

Insecticide resistance genes have developed in a wide variety of insects in response to heavy chemical application. In mosquitoes, esterases are a major part of this resistant mechanism. Most esterases belong to the carboxylesterase gene family. Esterases are enzymes that hydrolyze carboxylic esters or esters into acids and alcohols. There is a wide range of esterases that differ in their substrate specificity, their protein structure, and their biological function. Several patterns have been adopted to classify these enzymes. Esterases are divided into A and B esterases according to their preference for hydrolyzing the synthetic α- and β- napthyl acetate respectively. Also, esterases are distinguished by the "Aldridge" classification; which proposes a system based on the interaction of esterases with OP molecules. According to this classification system, esterases A (Est-A) are capable of hydrolysing OP compounds, esterases B (Est-B) are inhibited by them and esterases C (Est-C) do not interact with OPs (Montella *et al.*, 2012). Proteins of this family do not share a high degree of similarity in their primary protein structure and have widely differing substrate specificities. The increased interest in this enzyme is due to their wide range of roles. Similar to oxidases, esterases are ubiquitous and important in the metabolism of several classes of exogenous and endogenous compounds. They perform a number of crucial functions in insect development and behavior, such as odorant degradation as well as in reproduction and digestion (Montella *et al.*, 2012). Esterases are targets of OPs and carbamates. Oxons of these insecticides bind quickly to esterases and esterify the serine residue in the active site. This is accompanied by a

slow hydrolysis of the newly formed ester bond. Thus, the OPs and carbamates are considered inhibitors of esterases since they bind the enzyme with high affinity and hydrolyze it very slowly. To counteract this effect, mosquitoes developed ways to overproduce esterases. The first method involves gene amplification and the second one consists of gene overexpression (Raymond *et al.,* 1998). As a result, overproduced esterases act as high affinity "sponges" and sequester more insecticide. Esterases protect acetylcholinesterases, the major target of OPs and carbamates, by interactiong with the oxons with high affinity. In resistant *Culex* species, esterase overproduction rates are estimated to range between 70 to 500 folds depending on the species (Hemingway *et al.,* 1998). The amplified esterases are found in all life stages of *Culex* and are responsible for cross-resistance (Karunaratne *et al.,* 1993). Although esterases studies show to be specific for organophosphates and carbamates, non-specific esterases are shown to be involved in pyrethroid detoxification (Vulule *et al.,* 1999).

c. Glutathione S-Transferase

Glutathione S-transferases (GSTs) comprise a family of eukaryotic and prokaryotic multifunctional intracellular enzymes known to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. Elevated levels of GSTs have always been implicated in all classes of insecticide resistance. GSTs can also serve as non-enzymatic binding proteins (known as ligandins) participating in the intracellular transport and signaling processes. In mosquitoes, the metabolic resistance based on GST is the major mechanism of DDT-resistance (Che-Mendoza *et al*., 2009). There exists three unrelated families of GSTs in eukaryotes: microsomal, cytosolic and mitochondrial. They are classified according

to their location in the cell. The mitochondrial kappa family is found in mammalian mitochondria and peroxisome and is not related to the other two classes. The third class seems to be also distinct from the order Diptera. Little is known about the insect microsomal GSTs. They are membrane bound protein, distinct from cytosolic GSTs in structure and origin, yet they catalyze similar reactions (Che-Mendoza *et al*., 2009). Cytosolic GSTs on the other hand, have been implicated in insecticide detoxification. The cytosolic GSTs are homo or heterodimer proteins, each subunit folds into two domains the N-terminal and C-terminal domain. The N- terminal contain the G-site or the active site, which is the binding site of endogenous tripeptide glutathione (GSH). The larger C-terminal consists of a variable number of helices that makes the hydrophobic H-site were the substrates usually bind (Che-Mendoza *et al*., 2009). The high level of diversity in this region allows GSTs to react with a broad range of electrophilic substrates.

Mechanisms of GST detoxification will differ depending on the type of insecticide being detoxified. Four mechanisms of detoxification can occur by GSTs: glutathione conjugation, dehydrochlorination, passive binding, or glutathione peroxidase (GPx). In a reactive conjugation, the active site interacts with GST to yield an active thiolate anion (GS^-). This nucleophilic anion is then capable of attacking the center of any lipophilic compound to form the corresponding GS-conjugate. This conjugation neutralizes the electrophilic sites of the substrates rendering the product more water-soluble and hence can move out of the cell. These conjugates are then eliminated via the glutathione S-conjugate (Che-Mendoza *et al*., 2009).

Detoxification of organophosphates occurs via the conjugation of GSH with OP insecticide via two pathways: O-dealkylation or O-dearylation. The GSTs are found to be secondary resistance mechanisms to P450s or esterases.

GSTs can detoxify organochlorines by two mechanisms, conjugation and dehydrochlorination. The latter being the most predominant mechanism in eliminating the toxicity of this insecticide. In dehydrochlorination of DDT, GSH acts a cofactor rather than a conjugate; hence, the levels of GSH at the end of the reaction are the same. GS^- acts as a general base in this mechanism and removes hydrogen from DDT, which renders the removal of chlorine generating non-toxic DDE (Che-Mendoza *et al*., 2009).

GSTs act as an antioxidant defense against pyrethroid insecticides. Usually pyrethroid oxidation in the cell generates reactive oxygen species (ROS) that causes damages in cell components and activates the generation of lipid peroxidation (LPO). GSTs have the ability to decrease peroxidase damage induced by pyrethroids by detoxifying lipid peroxidase products. GSTs can also passively sequester pyrethroid compounds and inhibit their action (Che-Mendoza *et al*., 2009). The use of GST inhibitors (e.g diethyl maleate) in pyrethroid resistant *Culex* strains suggests that GST-mediated metabolism has a relative contribution in pyrethroid resistance (Xu et al., 2005).

Studies have shown that resistance to pyrethroids decreased when adding piperonyl butoxide (PBO) and diethyl maleate (DEM), which are known to be cytochrome P450 and glutathione transferase inhibitors (Scott & Georghiou, 1986). The mechanism by which pyrethroid sequestration occurs requires the presence of

oxidases and esterases in large amounts, which can be accomplished by the amplification of their corresponding genes (Hemingway *et al.*, 1998).

G. Status in Lebanon

Because of the great importance of mosquitoes as vectors diseases many species have been studied in great detail (Snow, 1990). In contrast, our knowledge of the Lebanese species is very patchy. Preliminary studies about the types and distribution of mosquito species in Lebanon were made in the 1940's and the 1970's (Matossian & Ibrahim, 1974; Parr, 1943). In 1943, Parr identified nine species of mosquitoes in Lebanon. Then, in 1974, Matossian *et al* mentioned that an eradication program for malaria was done in 1956; however, the fact that malaria was not completely eradicated in Syria and Israel continues to pose a threat to Lebanon. Moreover, they mentioned that antibodies against dengue virus were present but the disease was not spreading because the vector, *A. aegypti*, was absent. It is important to note that *A. aegypti* was later documented to be present at a very low density in Lebanon by a survey done by Knio *et al* (2005), but this is controversial.

A survey done between 1999 and 2001 showed that twelve species of mosquitoes are found in Lebanon. *Culex pipiens* was the most abundant species and was found throughout the year both indoors and outdoors with diverse breeding sites (Knio *et al.*, 2005). *Anopheles claviger*, the vector of malaria, was found at low density in few breeding sites with cool and clean water. This species was responsible for a low occurrence of indigenous cases of malaria in the country (Knio et al. 2005). In 2007, *Aedes albopictus* was detected in Lebanon by (Haddad *et al.*, 2007) and has been established since then.

According to a previous study done on mosquito resistance to organophosphates in Lebanon, it was found that the frequency of *Culex pipiens* resistant to organophosphates dramatically decreased in 2008-2009 compared to that in 2005 (Alout *et al*., 2009)(Osta *et al.*, 2012). This reduction was due to the shift from the use of organophosphates to pyrethroids. In 2005, the frequency of G119S resistant AChE1 was high; 41.4% of the sample population were heterozygote resistance and no susceptible mosquitoes were documented (Alout *et al*., 2009). However, these percentages decreased in 2008-2009 upon the introduction of pyrethroids that target other resistance mechanisms in mosquitoes (Osta *et al.*, 2012). Moreover, the F290V substitution that was previously detected at low frequency in Lebanon was not detected in the study.

An explanation for the disappearance of the F290V substitution from natural populations was the increase resort to pyrethroids, which do not select for this mutation. In fact, in the past five years the municipalities in Lebanon have been using pyrethroids extensively. Therefore, an update on the frequency of mosquito resistance to pyrethroids in Lebanon is needed.

This work aims at assessing the level of pyrethroid resistance in *Culex pipiens* populations in Lebanon. More particularly, resistance to permethrin and deltamethrin of *C. pipiens* collected in eight sites of the country will be evaluated and the mechanisms of resistance will be determined. The levels of target-resistance will be estimated by determining the frequency of knockdown resistance "*kdr*" and the levels of metabolic resistance will be determined by measuring the levels of mixed function oxidases (MFO) and non-specific esterases (NSE).

CHAPTER II

MATERIALS AND METHODS

A. Mosquito Collection

House mosquitoes, *Culex pipiens*, were collected from eight regions in Lebanon: Tripoli (North Governorate), Koura (North Governorate), Horish Tabet (Maten Co., Mount Lebanon), Beirut, Hadath (Baabda Co., Mount Lebanon), Rmaileh (Mount Lebanon), Sour (South Governorate) and West Beqaa. The collections were made from May 2014 until May 2016. Adult mosquitoes were collected indoors using a plastic vial by placing it slowly over the mosquito and capping it.

B. Processing Mosquitoes

Mosquitoes collected in fall and winter, between September 2014 and March 2015, were placed in 1.5 ml eppendorf tube and given a number code starting with 1 for the first collected mosquito. The tubes were then stored in boxes at -70°C.The mosquitos' serial numbers, location of collection, and date of collection were kept in records. Mosquitoes collected in spring and summer, between April 2015 and August 2015, were placed in a cage with a glass bowl to lay eggs in and a cotton pad soaked with water and sugar to feed. Mosquito eggs were reared in mosquito plastic trays (30cmx20cm) covered with fine mesh and fed ground fish food. They were kept at room temperature. Upon emergence, adults were collected and used in the WHO susceptibility test.

C. Control Mosquitoes

Culex pipiens reference strains were obtained from the laboratory of Dr.

Zakaria Kambris (American University of Beirut). These stains were susceptible, never exposed to an insecticide for more than five years, fed only sugar.

D. WHO Susceptibility Test

1. Choice of specimens tested

Susceptibility testing were conducted using only female mosquitoes of age 3-5 days, which were not gravid. This is because the age, physiological status and gender of mosquitoes are factors that can affect the results of the susceptibility tests. It is generally not recommended to use males for resistance monitoring, being smaller and more fragile than females; therefore, resulting in higher control mortalities. Also older female mosquitoes are sometimes less resistant to insecticides, especially when resistance is conferred by the presence of a detoxifying enzyme, the activity of which decreases with age. It is therefore recommended that susceptibility tests be performed on females of age's 3-5-post emergence and preferably using non-blood fed females as blood contains oxidases that enhances the detoxification system and allows the mosquito to become more resistant (WHO, 2013).

2. Testing Technique

Whenever 20 adult mosquitoes were collected from the reared mosquitoes in the lab of ages 3-5 post emergence and belonging to the same locality, the WHO susceptibility test was conducted.

For this, a clean white paper (12x 15 cm) rolled into a cylinder shape, was inserted inside the holding tube (green dot) and closed tightly. Twenty female mosquitoes were aspirated from the cage into the holding tube through the hole in the slide. Once the mosquitoes were transferred, the slide was closed and the holding tube

was held upright for one hour. At the end of the recovery time, any damaged or dead mosquito was removed. An exposure tube (red dot) was prepared in the same way but was lined now with either insecticide impregnated paper or oil impregnated paper as control. The empty exposure tubes were attached to the complementary side on the holding tube, the slide was opened and mosquitoes were gently blown from the holding tube to the exposure tube. The sliding unit was then closed and the exposure tube was held upright for one hour (Fig.3).

At the end of the exposure time, mosquitoes were transferred to the holding tube and kept for 24 hours; the exposure tube was now detached. The tube was held upright with a cotton pad soaked with water and sugar so that mosquitoes could feed. At the end of the recovery period the number of dead mosquitoes was counted and recorded.

The complete test consisted of six exposure tubes that were divided into two categories, two tubes that were lined with oil-impregnated papers and four tubes lined with pyrethroid-impregnated papers. The pyrethroids used in this test were permethrin (type I pyrethroid) and deltamethrin (type II pyrethroid). For this reason the test was repeated twice.

An adult mosquito was considered to be alive if it was able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they had lost legs or wings, were considered immobile and counted as dead. Alive adult mosquitoes were then transferred into single eppendorf tubes that were labeled with a number and the collection site, and placed at -70°C in separate boxes labeled as "WHO susceptibility test".

3. Sample size

A WHO test tube was run every time 20 mosquitoes of age 3-5 days were collected from the reared mosquitoes in the lab of a specific region. A total of four tubes (n=80) and two control tubes (n=40) were run for each region to calculate percent mortality per site.

4. Reporting Susceptibility Test Results

The mortality of the test sample was calculated by summing the number of dead mosquitoes across all four exposure replicates and expressed as a percentage of the total number of exposed mosquitoes:

$$\text{Observed mortality} = \frac{\text{total number of dead mosquitoes}}{\text{total sample size}} \times 100$$

Another similar calculation was made for the control mortality.

- If the control mortality was above 20%, the tests must be discarded.
- If the control mortality was greater than 5% but less than 20%, then the observed mortality had to be corrected using Abbots formula, as follows:

$$\frac{(\%\ \text{observed mortality} - \%\ \text{control mortality})}{(100 - \%\ \text{control mortality})} \times 100$$

- If the control mortality was below 5%, it could be ignored and no correction was necessary.

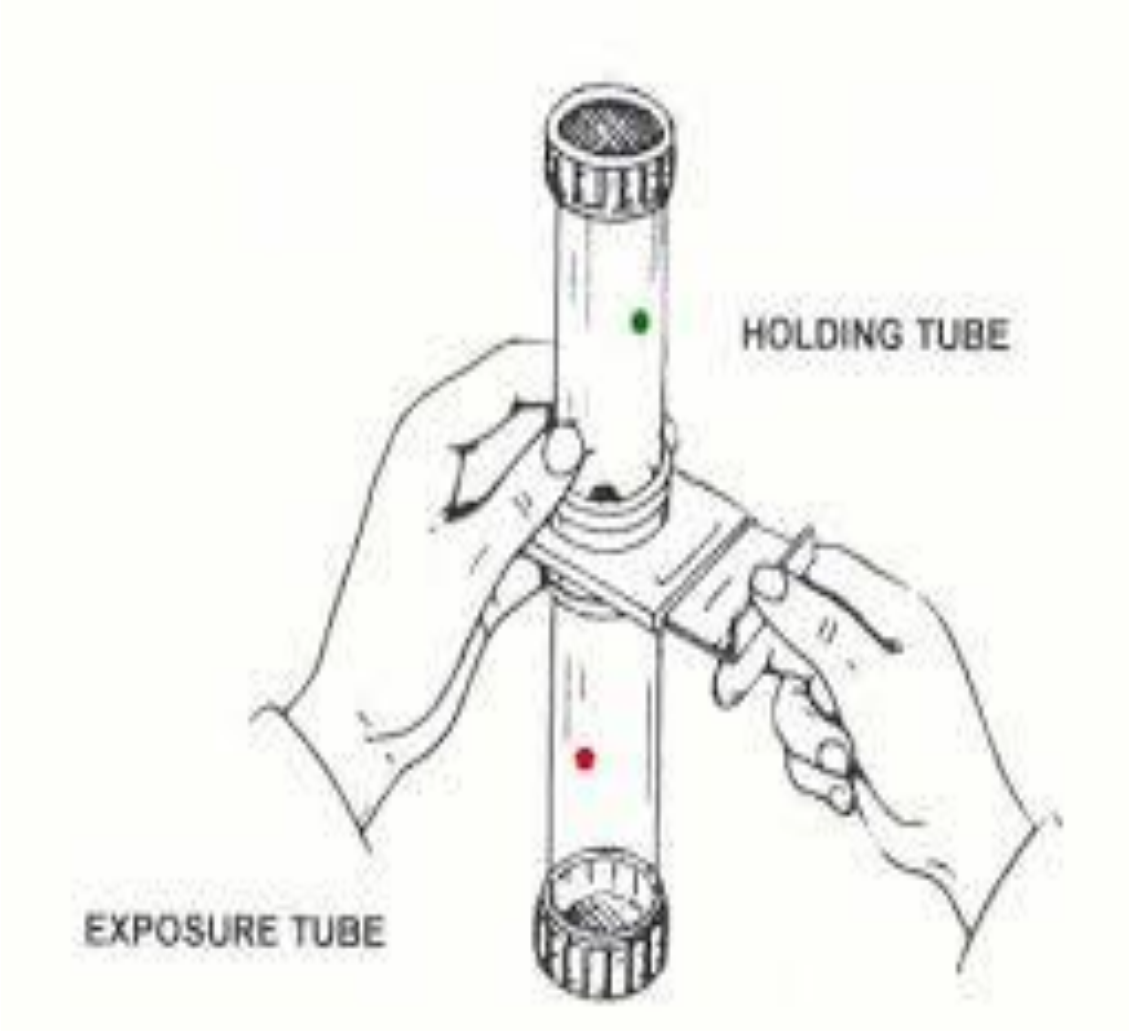

Figure 2: Picture for exposure and holding tubes of WHO susceptibility Test.

Source: http://www.who.int/whopes/resistance/en/WHO_CDS_CPE_PVC_2001.2.pd

E. Enzymatic Assay

Culex pipiens female, non-blood fed adults that survived the WHO susceptibility test, were used to test the enzymatic activity. The eppendorfs containing the mosquitoes were placed on ice to minimize the enzymatic degradation activity. Single mosquitoes were then homogenized in 100µl of cold 1 × PBS without Ca and Mg (Lonza). The homogenate was then diluted with an additional 400µl of this buffer.

For each homogenized mosquito two aliquots of 100µl each of the diluted homogenate were transferred to two wells of a 96 well flat-bottomed microtitration plate. Microtiter plates should be disposed of after use.

For the MFO assay, 200µl of ready-to-use cold TMB single solution (Invitrogen stored at 4 °C) was added to the 100µl of mosquito homogenate in each well, followed by 25µl of 3.0% hydrogen peroxide (Eau Oxygene). The plate was read immediately at T_0 on a microplate reader of wavelength 620nm. It was then read a second time 5 minutes later (T_5). The results were saved on an excel sheet for later analysis.

For the NSE assay, 100µl of a 3.0-mmol/liter solution of β-napthyl acetate (Sigma) was added to the 100µl aliquot of mosquito homogenate in each well and incubated for 10 minutes. The β-napthyl acetate solution was freshly prepared before each experiment as follows: 5.6mg of β-napthyl acetate powder (stored at -20°C) was dissolved in 2ml of acetone then diluted with 8ml of pH 7.2 potassium phosphate buffer. After the 10-min incubation period, 100µl of 3,3'-Dimethoxybenzidine dihydrochloride (#191248) that is synonym to *o*-Dianisidine dihydrochloride (Sigma) was added to each well. After 2 minutes, the absorbance of each well was read at λ=540nm. The results were saved on an excel sheet for later analysis.

In each plate, two replicates of blank were prepared for each assay. The blank contained all the above solutions in order of use minus the 100µl mosquito homogenate that was replaced with 100µl of 1 × PBS.

Mean absorbance values for replicate wells, rounded to the nearest 0.1, were used to indicate the values of MFO and NSE in each test mosquito.

F. Molecular Analysis of Mosquitoes Resistance status

1. Genomic DNA Extraction

Genomic DNA was isolated using acetyl trimethyl ammonium bromide solution (CTAB) protocol (Rotgers and Bendich 1988) from individual mosquito legs stored at -70°C. Mosquito legs were placed in 1.5ml labeled eppendorf tubes and homogenized for 1 minute in 200µl 2% CTAB using pestle tips. Tubes were then incubated for 5 minutes at 65°C. 200µl of chloroform (Sigma) was added and tubes were shaken well. The tubes were then centrifuged for 5 minutes at 19,810 g at room temperature (RT). Following centrifugation, two phases were formed; the upper aqueous phase was transferred into a new labeled tube and 1µl of glycogen (20mg/ml) was added. For DNA precipitation 100% ethanol (Sigma) was added (2.5 times the volume of the aqueous phase). After mixing well, the eppendorfs were put for 20 minutes at -20°C followed by a 20 minute centrifugation at -4°C at 26,960 g. After centrifugation, the supernatant was discarded, and the DNA pellet was washed with 100µl of 70% ethanol. Tubes were then centrifuged again for 5 minutes at 26,960 g at RT. Ethanol was then removed and the pellet was kept to dry for 5 to 10 minutes at RT. Finally, the pellet was dissolved in 20µl nuclease free water.

The concentration of DNA in each sample was measured and recorded using the NanoDrop machine.

The DNA samples were then stored at -20°C.

2. Polymerase Chain Reaction Amplification for the Detection of L1014F Mutation

Polymerase chain reaction (PCR) amplifications followed by gel electrophoresis were used to screen for mutations in the sodium channel gene, mainly L1014F. The L1014F mutation creates a restriction site in the sodium channel gene of resistant individuals. The PCR/Gel electrophoresis test protocol was followed from Torres *et al.* (1999) to detect the presence of L1014F mutation in single mosquitoes. Two PCR reactions for each mosquito individual were done because of the presence of an intron, which is highly polymorphic in *Culex,* downstream from the mutation. The two reactions were totally the same except that one contained a sense-specific primer ending with the "susceptible codon" (TTA) Cgd3 5'CCACCGTAGTGATAGGAAATTTA 3'and the other contained a sense-specific primer ending with the "kdr codon" (TTT) Cgd4 5'CCACCGTAGTGATAGGAAATTTT 3'. Two additional common primers were added in each reaction. One primer is an anti-sense primer based on the sequence immediately downstream from the intron Cgd2 5' GCAAGGCTAAGAAAAGGTTAAG 3'and the other is a sense primer far upstream Cgd1 5' GTGGAACTTCACCGACTTC 3'. Each reaction yields a common band that differs in size depending on the length of intron 2. This band was used as an internal control for the PCR reaction. The presence of the additional band only occurred in the PCR reaction carrying the equivalent resistance-associated specific primer (kdr specific or susceptible specific). The presence of a second band in both PCR products indicates heterozygosity. The PCR amplification reactions were conducted in 25µl total volume. Reaction 1 contained: 2µl DNA template, 1µl (12.5 pmol) of each of primers Cgd1, Cgd2, and Cgd3, 10mM dNTPs, 2.5µl of 10× Taq polymerase buffer (Thermoscientific Dream Taq) that includes 20mM $MgCl_2$, 0.25µl of DNA

polymerase (Thermoscientific Dream Taq 5U/ µl) and the rest of the volume nuclease free water. The second reaction is identical except the substitution of primer Cgd3 with Cgd4. Amplification was conducted on a LifePro thermal cycler engine using the following program: denaturation at 95°C for 5 min, then 30 cycles of 95°C for 40sec, 48°C for 2 min, 72°C for 1 min 40 sec, followed by elongation at 72°C for 10 min.

The amplified fractions were analyzed by gel electrophoresis in a 1.5% agarose (Sigma) gel containing ethidium bromide (BIO-RAD) and electrophoresed using 1X Tris-EDTA (10x TBE: 108g of Tris Base, 55g of Boric acid, 20ml of 0.5 M EDTA, 980ml distilled water) as a running buffer at 85volts. A 100bp DNA ladder was used as a control for band size.

The number of genotypes [RR], [RS], and [SS] were counted for each population and compared to that of the susceptible lab-reared control using Fisher's exact test. Giving the P-value from this study, susceptibility in tested populations can be compared to that of the control.

It is good to note that in the diagnostic PCR test locations such as Horsh Tabet, Rmaileh, and Hadath were summed together as Mount Lebanon.

G. Statistics

For the molecular test conducted, the *p*-values were obtained from the Fisher's exact test using SPSS program. Moreover, enzymatic assay data were analyzed using Graphpad Prism software. Non-parametric (Mann-Whitney) test was chosen to obtain the p-values for each city compared to the control sample.

CHAPTER III

RESULTS

To assess pyrethroid resistance in *Culex pipiens* populations in Lebanon, a total sample of 672 mosquitoes were collected from various sites in a two-year period, from May 2014 until May 2016. Initially, WHO susceptibility assay was conducted to determine percent resistance in the different mosquito populations. Then, tests were performed to detect the mechanisms of resistance, whether target site and/or metabolic.

A.WHO susceptibility test

Mosquitoes from eight different regions in Lebanon were used to determine percent mortality against pyrethroid insecticides. Mosquitoes were exposed to known concentrations of an insecticide for a fixed period of time at the end of which the number of fatalities was recorded. This test was used to detect differences between baseline susceptibility and resistance to insecticides in adult mosquitoes. The known concentration used is referred to as a discriminating or diagnostic concentration that has been already established under standardized laboratory conditions for all insecticides currently used (WHO, 2013). Two subclasses of pyrethroids were used in this experiment, which were 0.75% permethrin (type I) and 0.05% deltamethrin (type II).

The results of the WHO susceptibility test to the pyrethroids, permethrin and deltamethrin, are summarized in Tables 2 and 3, respectively. Percent mortality was below 90% in most mosquito populations for both pyrethroids, indicating resistance.

In fact, according to the WHO test procedures report in 2013, mortality in the range of 98-100% indicates susceptibility, whereas values of If 90-98% indicates resistance. In the latter case, the presence of resistant genes in the population must be confirmed by performing additional bioassay tests with the same insecticide, or by conducting molecular assays for known resistance mechanisms. Furthermore, if the mortality obtained is less than 90%, than resistant is confirmed and additional bioassays are not necessary as long as a minimum of 80 mosquitoes is tested. A similar calculation is needed on the two control tubes to either accept or reject the test.

With respect to susceptibility tests against permethrin, four mosquito populations out of five showed mortality percentage below 90% (Table 2), which confirms the existence of resistance genes in the population studied. The only exception was the mosquito population from Beirut, which showed percent mortality of 93.75, which lies in the range of 90-97%. According to the WHO report any percentage in this range has to be confirmed by performing additional tests or molecular studies.

With respect to susceptibility tests against deltamethrin, all mosquito populations tested from three sites showed a mortality percentage below 90%, which confirms resistance (Table 3).

Table 2: WHO susceptibility test results for *Culex pipiens* population screened for permethrin resistance.

City	Number of dead mosquitoes in control tubes	Total control sample size	Number of dead mosquitoes in experimental tubes	Total experimental sample size	Control mortality %	Sample mortality %
Tripoli	1	38	17	80	2.6	21.25
Horsh Tabet	1	40	21	76	2.5	27.63
Beirut	0	40	75	80	0	93.75
Hadath	0	40	22	79	0	27.84
Sour	0	40	49	80	0	61.25

Table 3: WHO susceptibility test results for *Culex pipiens* population screened for deltamethrin resistance.

City	Number of dead mosquitoes in control tubes	Total control sample size	Number of dead mosquitoes in experimental tubes	Total experimental sample size	Control mortality %	Sample mortality %
Tripoli	0	40	28	76	0	36.84
Horsh Tabet	0	40	45	80	0	56.25
Beirut	0	40	39	76	0	51.31

Table 4 summarizes major types of pyrethroids used in the sampled cities.

Table 4: Type of insecticide used across Tripoli, Beirut, and Sour

Location	Type of insecticide
Tripoli	2013-2016 Tetramethrin (Type I pyrethroid)
Beirut	2013-2016 Cyhalothrin (Type II pyrethroid)
Sour	2013-2015 50% Permethrin (Type I pyrethroid) and 50% organophosphates 2015-2016 Permethrin

B. Enzymatic Assay

To determine whether the observed resistance is metabolic, biochemical tests were conducted to measure the level of enzymatic activities of esterases and oxidases. The test indirectly measures levels of oxidases and esterases using a colorimetric assay. Control lab-reared mosquitoes were used for comparison analysis.

A total of 633 mosquitoes from eight different cities in Lebanon were assayed for MFO and NSE levels. Control susceptible mosquitoes were also assayed in parallel to be able to compare each region with levels of oxidases and esterases in susceptible mosquitoes. The median for each sample was determined and compared to that of the control sample using Mann-Whitney statistical test. Six populations; HorshTabet, Sour, Basta(Beirut), Koura, West Beqaa, and Rmaileh were significantly different from the control sample for MFO at t=0 and t=5min (Fig 4A and 4B)

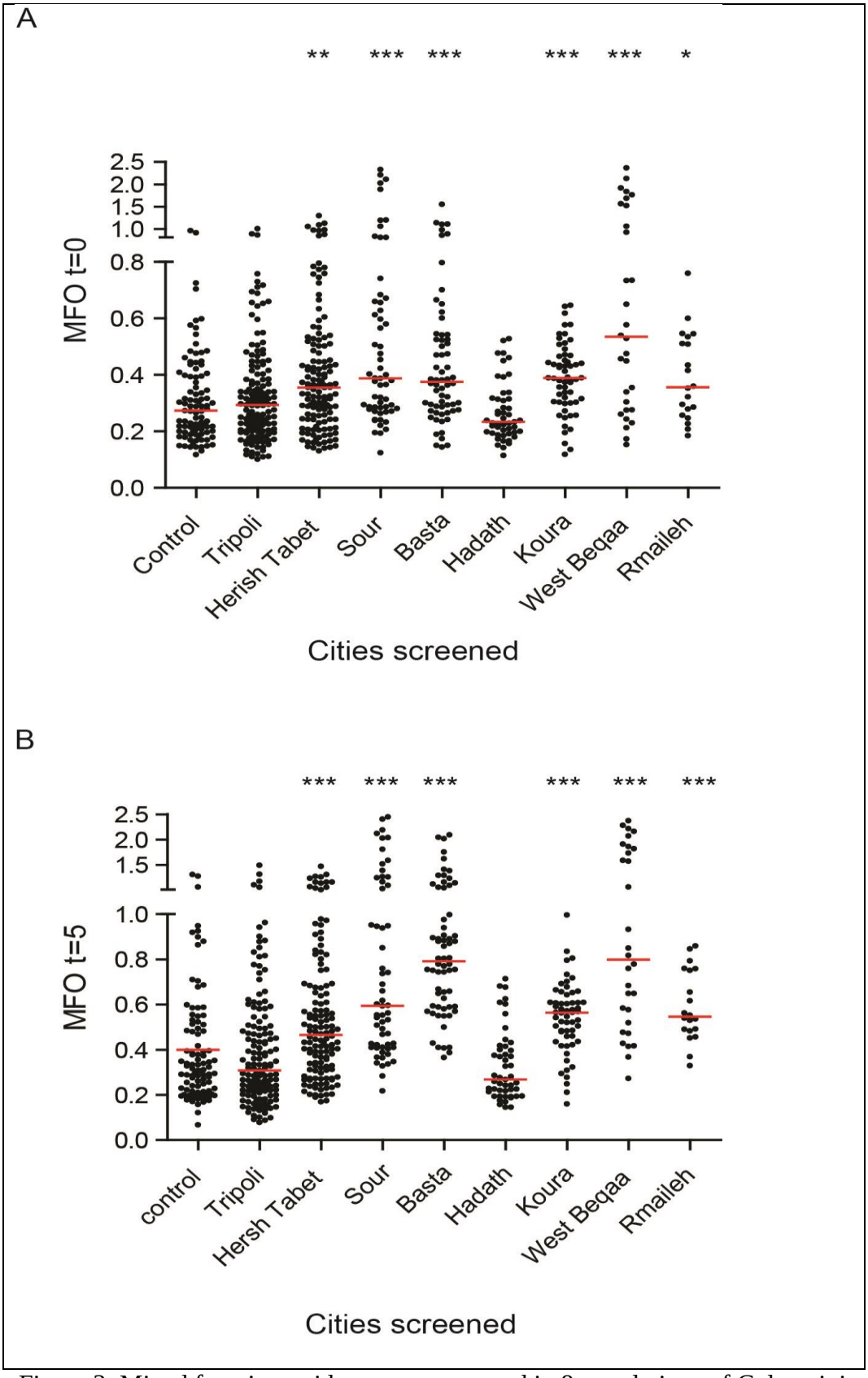

Figure 3: Mixed function oxidase assay screened in 8 populations of Culex pipiens mosquitoes in Lebanon. Measured at t=0min (A) and at t=5min (B). *** indicates $p<0.001$, ** $p<0.005$ and * $p<0.05$. The p-values are obtained from non-parametric Mann-Whitney test. Red bars are referred to the median of each population.

On the other hand, five populations of mosquitoes (Tripoli, Horsh Tabet, Hadath, Koura, and Rmaileh) were significantly different from the control sample when tested for non-specific esterases at t=2min (Fig. 5).

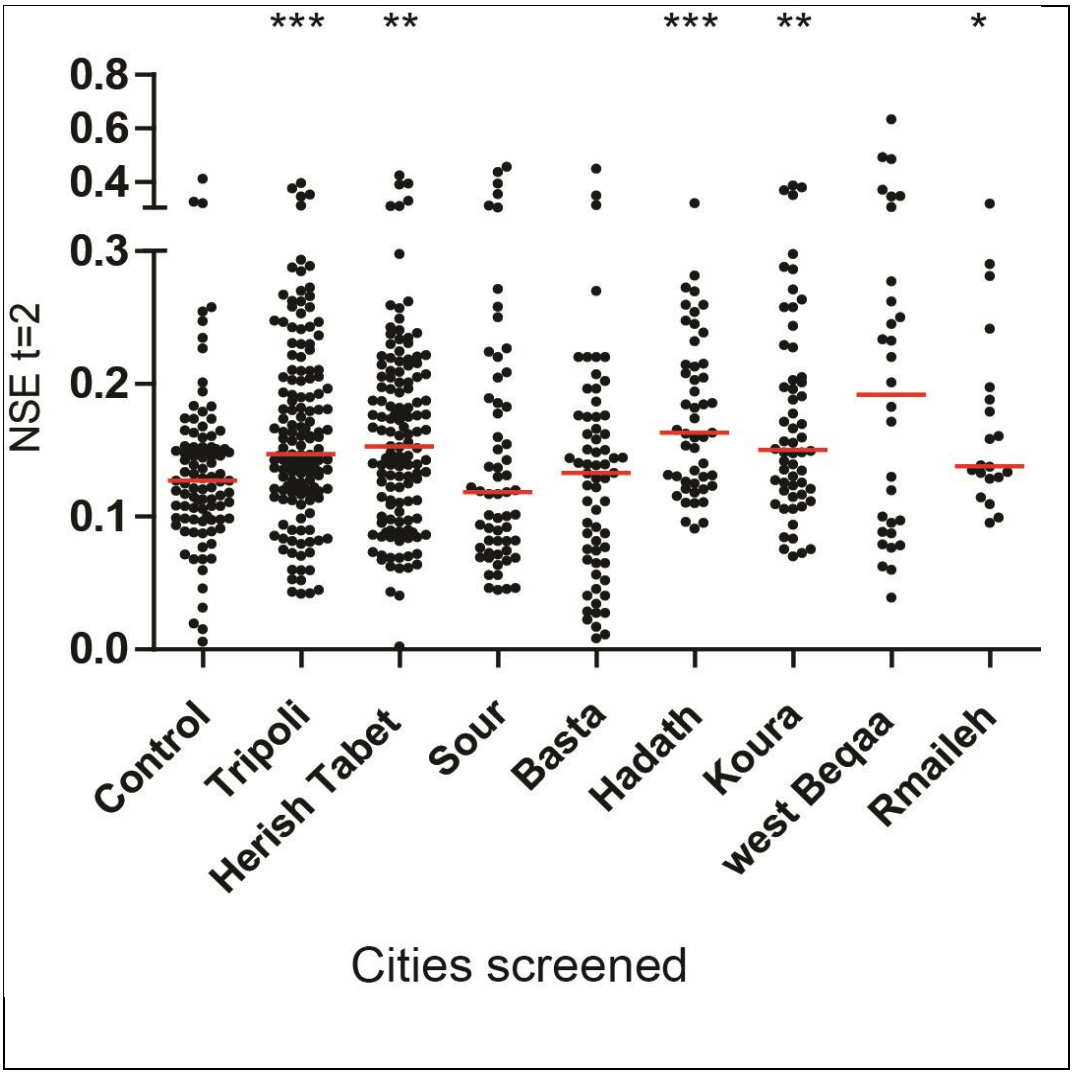

Figure 4: Non-specific esterase assay screened in 8 populations *Culex pipiens* mosquitoes in Lebanon. *** indicates p<0.001, ** p<0.005 and * p<0.05. The p-values are obtained from non-parametric Mann-Whitney test. Red bars are referred to the median of each population.

C. Molecular diagnostic test to detect L1019F mutation in Kdr gene

Target site resistance in the Lebanese *C. pipiens* was evaluated by screening

for the most common mutation in the sodium channel gene; the L1019F (leucine to phenylalanine substitution). Mosquitoes were given the genotype RR if homozygote for L1019F, RS if heterozygote for L1019F, and SS if the mosquito lacks any mutation at this site and is said to be susceptible (wild-type).

A total of 672 mosquitoes were screened for mutation in the sodium channel gene (L1019F). Genomic DNA was isolated from individual mosquito legs. PCR amplification involved two reactions, each using three different primers. Each reaction yielded a common band from 481 to 510bp that varied in size in different individuals as a result of the discrepancy of the length of intron 2. This band was used as an internal control for the PCR reaction. The presence of one additional band (≈ 354-383bp) only occurred in the PCR reaction that corresponds to the susceptible specific primer (Cgd3) or resistance-associated primer (Cgd4). The presence of a second band in both PCR products indicates a heterozygote resistance state (Fig. 6).

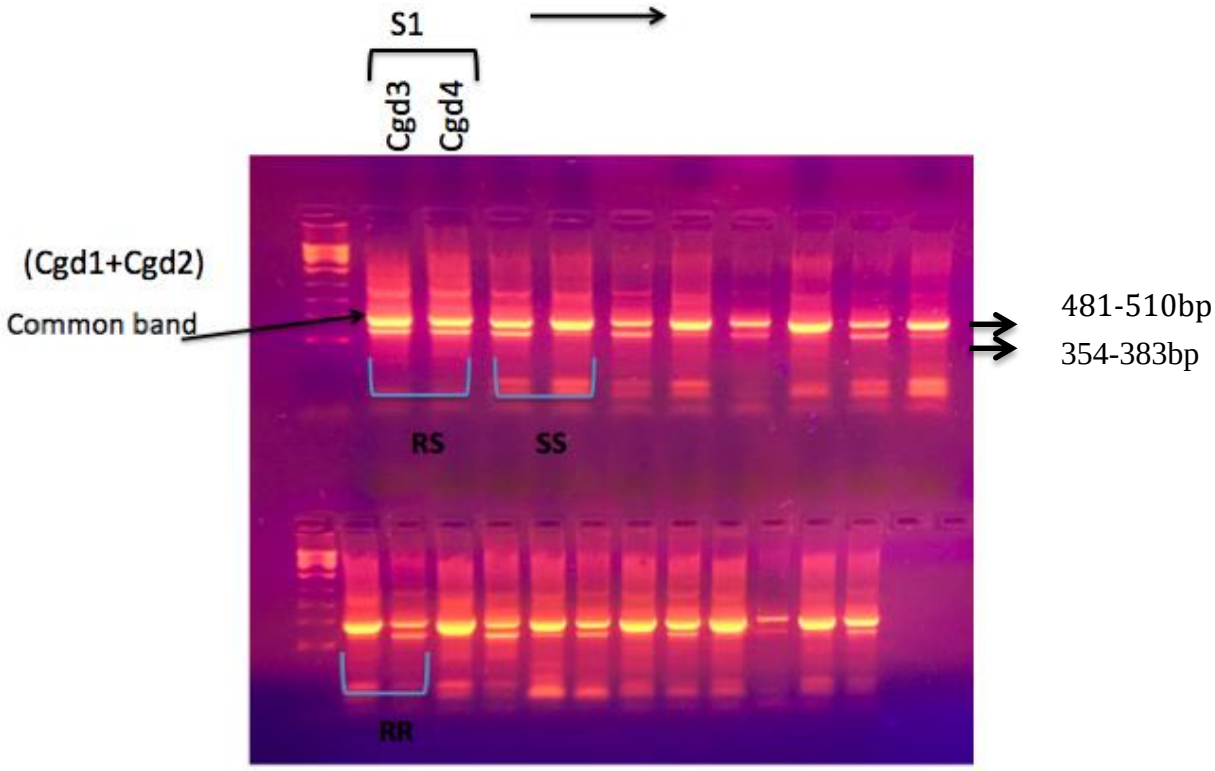

Figure 5: Diagnostic PCR test to detect L1014F mutation in *C.pipiens* individual mosquitoes. Genomic DNA amplification with the primers Cgd1 and Cgd2 produced a common band of 481 to 510bp. A second band of 354-383bp only occurred in the PCR reaction that corresponds to the susceptible specific primer (Cgd3) or resistance-associated primer (Cgd4). The presence of a second band in both PCR products indicates a heterozygote resistance state.

Allele frequency for each city was determined (table 5) and for the L1014F mutation, allele frequency showed a significant departure from the Hardy-Weinberg equilibrium. *P*-values of all populations except that of Beirut were significantly different from the control. Beirut population had a P-value of 0.717 suggesting that this population is not significantly different from the susceptible control. With a large *Fis* value, the results indicated an excess of resistance in the mosquito populations studied except that in Beirut.

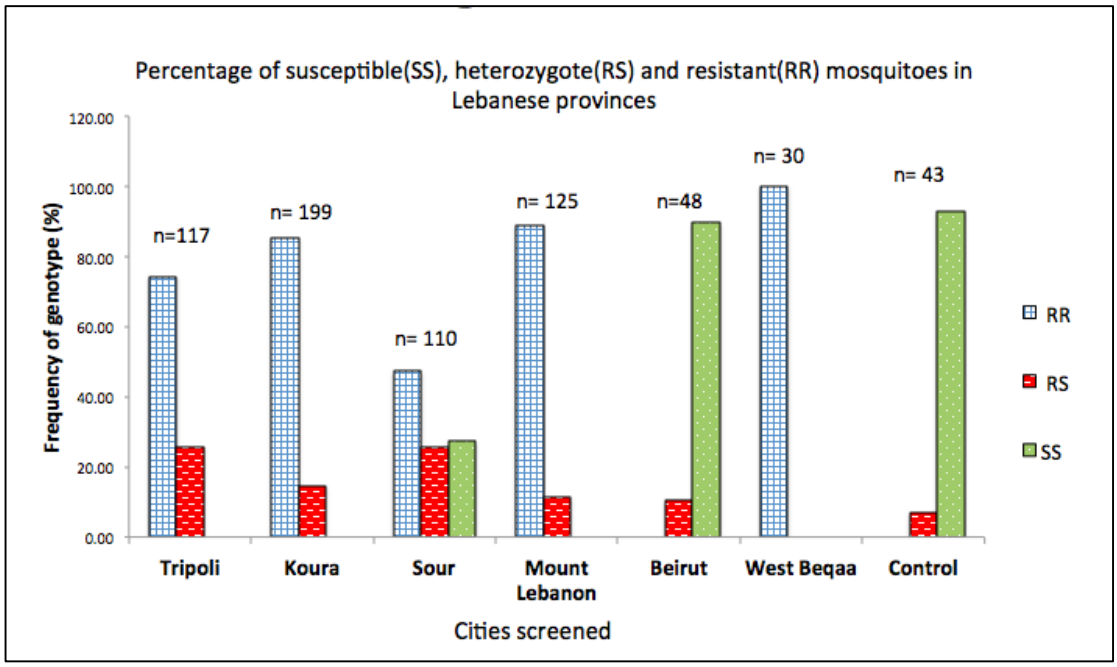

Figure 6: Percentage of susceptible (SS), heterozygote (RS) and resistant (RR) mosquitoes in Lebanese provinces with n= total sample size.

Table 5: Frequencies for each genotype of *kdr* gene together with the corresponding number of individuals in brackets. N is the number of mosquitoes analyzed. SS, susceptible mosquitoes; RS, mosquitoes heterozygous for L1014F; RR, mosquitoes homozygous for L1014F. The Fis value indicates whether there is an excess of resistance. The p-value (SPSS, Fisher's exact test) is also indicated.

Location	N	[RR]	[RS]	[SS]	*Fis*	*p*-value
Tripoli	117	0.74(87)	0.25(30)	0(0)	169.903	0.000
Koura	199	0.85(170)	0.14(29)	0(0)	197.391	0.000
Sour	110	0.47(52)	0.25(28)	0.27(30)	61.649	0.000
Mount Lebanon	125	0.88(111)	0.11(14)	0(0)	444.570	0.000
Beirut	48	0(0)	0.10(5)	0.89(43)	-	0.717
West Beqaa	30	1(30)	0(0)	0(0)	370.553	0.000

CHAPTER IV

DISCUSSION

Little is known about mosquito resistance to insecticide in Lebanon. A previous study investigated the resistance *C. pipiens* mosquitoes collected across Lebanon during the years 2009-2010 against organophosphate insecticides (Osta *et al*., 2012). A dramatic reduction in resistance against organophosphates was seen in 2010 in Lebanon in comparison to previous data collected in 2005 (Alout *et al.,* 2009). This was explained by the decreased dependence on organophosphates and increased use of pyrethroids (Osta *et al*., 2012).

According to the information obtained from several municipalities in Lebanon including (Tripoli, Beirut, and Sour), pyrethroids are extensively used nowadays because of their low toxicity, and all types of these insecticides are sprayed all over the country for insect control. Thus monitoring resistance to pyrethroids became legitimate and valuable to assess the efficacy of mosquito control programs. This study was conducted to examine the status and levels of resistance of *C.pipiens* populations against pyrethroids, and the mechanisms underlying this resistance.

Mosquitoes were sampled from May 2014 till May 2016, from eight major cities in the country and screened for pyrethroid resistance using the WHO susceptibility assay. Percent sample mortalities against two major pyrethroids, permethrin and deltamethrin, were calculated and found to vary from one site to another. The lowest percent mortality against permethrin was 21.25% in mosquito samples from Tripoli (Tripoli Co.), followed by samples from Horsh Tabet (Maten Co.) and Hadath (Baabda Co.) with mortality rates of 27.63% and 27.84%,

respectively. Hence, mosquito populations from these three regions showed high resistance to permethrin. Mosquito samples from Sour (Sour Co.) showed a 61.25 % mortality which confirms resistance, but with a lower level than the previously mentioned regions. As for mosquito populations from Beirut, they showed the highest mortality rate (93.75%) against permethrin or the lowest resistance. Such level of mortality is considered as a borderline resistance and needs further testing to confirm resistance or susceptibility.

The same WHO susceptibility assay was conducted on mosquito populations from Tripoli, Horsh Tabet, and Beirut using deltamethrin as test insecticide. Resistance to deltamethrin was shown in mosquito populations from the three cities. Percent mortality against deltamethrin were much below 90% in populations from Tripoli and Horsh Tabet (36.84% and 56.25% respectively), which indicates resistance. However, these values from these two sites were higher for deltamethrin compared to permethrin. This indicates that mosquito populations from Tripoli and Horsh Tabet showed resistance to both pyrethroids. These results confirm that Type II pyrethroids are more effective than type I in mosquito control. In general, type II pyrethroids delay the inactivation of voltage-gated sodium channel longer than type I compounds and their effects are less reversible (O'Reilly et al., 2006). The municipality of Tripoli reported the use of teteramethrin (type I pyrethroids), during the last four years (Table 4), which confirms the increased resistance in Tripoli against permethrin compared to deltamethrin. The excessive use of some classes of insecticides for many years is likely driving the resistance observed.

One the other hand, percent mortality against deltamethrin in mosquito populations from Beirut was 51.31% compared to 93.75% for permethrin. This means that this population is more resistant to deltamethrin. This may be explained by the

selective usage of type II pyrethroids in the capital Beirut compared to other regions in Lebanon. Indeed, when contacted officials in Beirut municipality confirmed that lambda cyhalothrin, a type II pyrethroid has been used for the last four years as spraying or fogging in the city (Table 4).

The results of WHO susceptibility test provided an overview of the frequency of resistance in the country against pyrethroids. To determine the mechanisms responsible for the observed resistance enzymatic and molecular tests were conducted.

A total sample of 672 mosquitoes collected from different sites in Lebanon showed the presence of the three genotypes [SS], [RS], and [RR] in the kdr gene. Interestingly, homozygous resistant mosquitoes [RR] showed the highest frequency reaching 66.96% at the national level. The homozygous susceptible and heterozygous genotypes gave an approximate equal distribution in the population with 16.82% and 16.22% respectively, nationwide. These percentages confirm the results of low percent mortality using the WHO assay. The highest resistant percentages belonged to four sites in Lebanon which are: West Beqaa, Mount Lebanon, Koura and Tripoli with 100%, 88.8%, 85.43% and 74.36% respectively (Fig.7). According to table 3 all the cities gave a P-value smaller than 0.05 except Beirut population. Therefore; Tripoli, Koura, Sour, Mount Lebanon and West Beqaa are significantly different from the susceptible control. Only Beirut population is considered similar to the susceptible control population having high numbers of susceptible mosquitoes.

The absence of susceptible mosquitoes in the sample collected from Beqaa is likely due to excessive, uncontrolled use of pyrethroid to control insect pest in the wide agricultural plains in the Beqaa valley. Altogether, these results indicate that the

continuous use of pyrethroids in the country without rigorous control may drive resistant alleles to fixation in the mosquito population.

Development of pyrethroid resistance has been reported in many neighboring countries such as Tunisia (Daaboub et al., 2008), Egypt (Zayed et al., 2008) and Greece (Kioulos et al., 2014).

For instance, the frequency of *kdr* resistance mutation was significantly higher than organophosphates in Greece with 63.0% in one of the cities (Kioulos et al., 2014).

A biochemical enzymatic assay was also used to measure the levels of mixed function oxidases and non-specific esterases in sampled mosquitoes compared to the control. Mosquito populations from eight cities were screened in addition to the control mosquitoes and the median was calculated for each sample (Figures 4 and 5). The MFO levels at t=0 and t=5 for Horsh Tabet, Sour, Basta, Koura, West Beqaa, and Rmaileh were significantly different from the control susceptible sample with a P-value smaller than 0.05. This indicates increase in the oxidases activity to detoxify the insecticides. Whether this indicates overexpression of certain P450 genes or gene amplification remains to be determined.

Comparing diagnostic PCR tests with MFO enzymatic assay shows that five mosquito populations out of six which exhibited increase MFO activity also showed a high percentage of [RR] genotype indicating the presence of mixed resistance mechanisms in most cities. Moreover, Beirut diagnostic PCR revealed that most of the mosquito sampled are susceptible [SS], yet WHO revealed resistance to deltamethrin and borderline resistance to permethrin suggesting that the main mechanism of resistance in the capital is mainly metabolic. Indeed, mosquitoes from Beirut showed significantly increased MFO activities to control lab colony. On the

other hand, mosquito Tripoli population with 74.36% [RR] genotype, showed no increased activity in mixed function oxidase suggesting that the main resistance to pyrethroids is the target-site modification.

Regarding NSE levels, populations from five cities showed significantly higher esterase activities compared to controls, since esterases are known to be more involved in the detoxification of organophosphates and carbamates (Raymond *et al.*, 1998), the presence of increased esterases in some populations in Lebanon may be due to the continuous usage of these chemicals today in some localities.

It can be concluded from these results that most mosquito populations sampled exhibit both mechanisms, L1014F mutation and over-produced enzymes, to neutralize insecticides. Elevated levels of detoxifying enzymes such as mixed function oxidases can target several insecticide types and classes such as pyrethroids, DDT and organophosphates that are also used in agriculture. Thus, it is noticed that agricultural areas have the highest oxidases and esterases levels, such as Koura and West Beqaa (Figues 4 and 5).

An important observation was made during the study. Few of the mosquitoes that survived the WHO assay did not show the leucine to phenylalanine substitution in the kdr gene; moreover, they had low oxidase and esterase activity. This shows that another mechanism or mutation is taking place and allowing mosquitoes to resist insecticide exposure. This could be a different mutation in the sodium channel or an enhancement of an enzyme that was not tested in this study, such as GST.

The strong dependence on a confined number of active ingredients registered and used in public health, together with the use of analogous chemicals in agriculture, is likely to select for insecticide resistance.

One of the major reasons for developing resistance is the lack of routine

studies that aim in testing insecticide resistance. For instance the WHO malaria report 2015 showed that among 97 countries that reported adopting policies for vector control only 52 reported resistance data for 2014. Of these, 32 had reported data every 2 years. Moreover, three quarters of the countries monitoring this insecticide class in 2014 reported resistance (WHO, 2015).

To manage resistance adequately, the WHO recommends the use of rotations, insecticide mixture or novel insecticide classes that have different mode of action. Moreover, information about the status of resistance to insecticides is an important step for the rational selection of insecticides for use against disease vectors (WHO, 1995).

Finally, our study can be strongly improved by increasing the sample size as to have more statistically representative results especially in the Beqaa region. Also, sequencing will help in the detection of rare genotypes such as L1014H, L1014S, and M918T. For instance, mosquitoes that show no L1014F mutation in our study may be subject to other mutations. Moreover, assessing the levels of other metabolic resistant enzymes such as glutathione-S transferase and cytochrome P450 monoxygenase is important.

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