EVALUATION OF IMPACT OF LONG-LASTING INSECTICIDAL HOUSE SCREENING (LLIS) ON PYRETHROID RESISTANT POPULATION OF THE DENGUE VECTOR *AEDES AEGYPTI* IN MEXICO

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Azael Che Mendoza

October 2016

Table of contents

Abstract

The public health importance of the endophilic mosquito *Aedes aegypti* has increased dramatically in the recent decade, because it is the vector of current outbreaks of dengue, chikungunya, Zika and Yellow fever viruses. The use of long-lasting insecticide nets fixed on doors and windows (LLIS) is one innovative approach recently proposed for *Aedes* control and under initial evaluation in different settings. Nevertheless, there is an urgent need to evaluate the efficacy of this intervention in endemic areas of dengue, where insecticide resistance in the mosquito populations has already developed after many years of selection pressure by the local vector control programmes. The aim of this PhD was to evaluate the efficacy of LLIS and assess the impact of insecticide resistance on this intervention.

In 2012, cluster randomised controlled trials were conducted in two Mexican cities -Acapulco (Guerrero state) and Merida (Yucatan state). The study compared ten control and ten intervention areas of 100 households each across both cities. Intervention clusters included LLIS (Acapulco and Merida during the first year), followed by targeted treatment (TT) in the productive water container types (in Acapulco during the second year). Cross-sectional entomologic surveys quantified mosquito infestations at baseline (pre-intervention) and in four post-intervention samples surveys approximately at 6-monthly intervals corresponding to dry/rainy seasons. Sequentially over two years from 2012-2014, WHO cone bioassays were performed in order to determine the insecticidal activity of LLIS under operational conditions, susceptibility tests using CDC bottles, biochemical assays and genotyping for kdr were performed on F1 adult mosquitoes emerged from eggs collected using ovitraps.

Overall, results showed significant reductions on adult vector densities in houses in the treated clusters with LLIS after two years at Merida and Acapulco: ca. 50% on the presence ($OR \leq 0.62$, $P<0.05$) and abundance ($IRR \leq 0.58$, $P<0.05$) of indoor-resting adults. In Acapulco, the combination of house screening with LLIS and TT of the most productive *Ae. aegypti* breeding sites had a significant impact on dengue vector populations and sustained that impact for up to 24 months. Based on the WHO efficacy criteria, the LLIS were efficient in killing susceptible *Ae. aegypti* (most of them achieving 80% of mortality) when first installed, but their activity rapidly declined. Much lower levels of mortality were achieved against the local and resistant *Aedes* population (less than 40%). The *Ae. aegypti* local populations demonstrated high levels of resistance to pyrethroids, mainly permethrin, some signs of decreased susceptibility for organophosphates but susceptibility to carbamates. Biochemical analysis showed a significant elevation of oxidases and GST enzyme activity and kdr-1016I and -1534C mutations were found at high frequencies in the two study sites. There was no clear effect attributable to the instalment

of LLIS on the mechanism of insecticide resistance (kdr frequencies and levels of enzyme activity), but in terms of intensity of resistance, resistance seemed to be higher within the study arms with LLIS after the intervention.

Although the efficacy of LLIS can be compromised by the degradation of the insecticide and/or the resistance of *Aedes* populations to pyrethroid-based insecticides, the physical barrier would still work as a preventive measure, as demonstrated in this study. The positive entomological impact observed in this study provides evidence of a sustained effect of LLIS on *Ae. aegypti*, and encourages the development of cluster randomised trials evaluating the epidemiological impact of this intervention.

Dedication

To my lovely wife Brenda and my daughter Alexia, they were always my inspiration and motivation, and my support during all this time.

To my mother Lilia, my sister (Lilian), all my brothers (Pedro and Joaquín) and my grandmother (Aurora) for love, endless support and encouragement

Acknowledgments

I would like to thanks the National Research Council of Mexico (CONACYT) to support me.

I am deeply indebted to Dr. Pablo Manrique Saide (UCBE/UADY) and Dra. Audrey Lenhart (Entomology Branch/CDC). Everything is impossible without their continuously support and kindness.

My deepest thanks to Dra Hilary Ranson and Dr. Phillip McCall, my LSTM supervisors, for all their efforts, patience, and support freely given during the development of this work.

Overall to all my supervisors, for their tremendous help and valuable instructions benefit me in all the time of my research and writing this thesis. Thanks again for all their inputs into my thesis.

To Dr. Gonzalo Vazquez Prokopec, who introduce me to basic principles of statistics and spatial analysis that helped me to analyse my data.

To Dra. Norma Pavia, for the support that she has given me in the last part of my Ph. D. work.

To all the students and friends from UCBE-UADY Mexico, that helped in different times during the fieldwork in Acapulco and Merida (Memo, Willy, Josue, Valentín, Anuar, Edgar, Emilio, Asael, Jedi, and Carolina) and labwork (Yamili, Valentin, and Vadillo). I have wonderful memories of all of you. Thank you so much guys!

To all the people from Laboratory of Entomology Branch of Centers for Disease Control and Prevention (CDC) Atlanta, USA (Kelly, Paula, Mariela, Nicole, Nsa and Lucy) who were very helpful and good company in the Lab. To my friend and lab partner Edgar, we did it!.

To Dr. Brogdon and Dr. Claudia for their advices and support. Many thanks for let me work in their Lab/CDC.

Special thanks to Lucrecia Vizcaino, for her patience and kind assistances during my stays in CDC. You was so nice to me. Many thanks Lucrecia and, go mafia Latina!

To MSc Felipe Dzul and all teamwork of health workers of Distrito de Renacimiento from Ministry of Health of Guerrero (Martini, May, Bibiano, Wilbert y los ovitramperos de Acapulco). I learned a lot from all of you.

This project was partially sponsored by Consejo Nacional de Ciencia y Tecnologia (Research projects GUE-2008-C01-91330 and GUE-2008-02-108686 FOMIX-CONACYT, and SALUD-2011-1-161551 Fondo Sectorial de Investigación en Salud y Seguridad Social (SSA/IMSS/ISSSTE-CONACYT)), and UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) (Research project "Innovative Community Based Ecosystem Management Interventions for Improved Dengue Prevention in Mexico").

To residents of Merida and Acapulco for giving access to their homes for the collection of field material.

Finally, I would like to thank my wife, Brenda, who always believes in me and comforts me. Her love and support mean everything to me.

List of abbreviations

ACh: Acetylcholine.

AChE: Acetylcholinesterase.

AIC: Akaike Information Criterion.

ANOVA: Analysis of variance

ATCH: Acetylthiocholine iodide.

ATPase: Adenosine triphosphatase.

BI: Breteau index.

Bti: *Bacillus thuringiensis* var. *israelensis*.

CAs: Carbamates.

CCEs: Carboxylesterases.

CDC: Center for Disease Control and Prevention, USA.

CDNB: 1-chloro-2,4'-dinitrobenzene.

CHIKV: Chikungunya viruses.

C. I.: Confidence intervals.

CNEP: National Campaign of Malaria Eradication.

DD: Diagnostic doses.

DDT: Dichloro-diphenyl-trichloroethane.

DENV: Dengue viruses.

DF: Dengue fever.

DGCP: General Direction of the Campaign against Malaria.

DGE. General Directorate of Epidemiology.

DHF: Dengue haemorrhagic fever.

DNA: Deoxyribonucleic acid:

DT: Diagnostic times.

DTNB: Dithiobis 2-nitrobenzoic acid.

EDTA: Ethylenediaminetetra-acetic acid.

ERC: Ethical review committee.

GAM: Generalized additive model.

GAMM: Generalized additive mixed model.

GSH: Reduced glutathione.

GSTs: Glutathione S-transferases.

HI: House index.

H2O2: Peroxide.

iAChE. Insensitivity to acethylcholinesterase.

IAS: Indoor adult survey.

IDRC: International development research centre.

IGRs: Insect growth regulators.

IRR: Incidence rate ratios.

IRS: Indoor residual spraying.

IS: Intra-domiciliary spraying.

ITCs: Insecticide treated curtains.

ITMs: Insecticide treated materials.

ITNs: Insecticide treated bednets.

IVCRI: Integrated vector control in response to risk indices.

KD: Knock-down.

kdr: Knockdown resistance.

KDT50: Half knock-down time.

KPO4: Phosphate.

LLINs: Long-lasting insecticidal mosquito nets.

LLIS: Insecticide-treated house screening.

LPS: larval&pupal survey.

MoH: Ministry of Health.

NaCl: Sodium chloride.

OCs: Organochlorines.

OPs: Organophosphates.

OR: Odds ratios.

PAHO: Pan American Health Organization.

PAIS: Program of Simultaneous and Intensive Actions.

PCR: Polymerase chain reaction.

PI: Post-intervention.

PPI: Pupae per person index.

PYs: Pyrethroids.

RR: Homozygous resistant.

RRKDT50: Knockdown resistance ratio.

SE: Error of mean.

SINAVE: Web-based epidemiological surveillance system.

SR: Heterozygotes.

SS: Homozygous susceptible.

TDR: Special programme for research and training in tropical diseases.

TEPP: Tetraethyl pyrophosphate.

TMBZ: 3,3',5,5'-tetramethyl benzidine dihydrochloride.

TT: Targeted treatment.

UCBE-UADY: Collaborative unit for entomological bioassays from university of Yucatan.

ULV: Ultra low volume spraying.

UV: Ultraviolet light.

WC: Water-holding containers.

WHO: World Health Organization.

WHOPES: World Health Organization Pesticide Evaluation Scheme.

ZIKV: Zika viruses.

PU= number of pupae.

% PU=contribution of pupae collected.

Chapter 1: General introduction

Dengue has re-emerged as a major international public health concern in the tropical and subtropical regions. Currently dengue viruses (DENV) are endemic in at least 100 countries with annual incidence ranging from 100-390 million of cases and an estimated 22,000 fatalities (Bhatt et al., 2013; Brady et al., 2012; WHO, 2012).

Contemporaneous routine vector control efforts targeting *Aedes* vectors (i.e. source reduction, hand-applied larvicides to containers and Ultra-Low Volume [ULV] adulticiding), have to date achieved only a limited and temporary impact in preventing disease because they are poorly efficacious or are limited in their coverage (Bowman et al., 2016; Tun-Lin et al., 2009). Improving urban *Aedes* control and achieving a measurable impact on DENV transmission will require a re-formulation of current strategies and a stronger focus on both lowering vector abundance and preventing human-vector contacts (Achee et al., 2015; Morrison et al., 2008; WHO, 2012). In the absence of effective treatment or vaccines for dengue (Sabchareon et al., 2012) and in the context of multiple co-circulating viruses transmitted by *Aedes* mosquitoes, the development of preventive and long-lasting methods for *Aedes* control has become a top global health priority.

Insecticide treated materials (ITMs), particularly bednets, are among the most effective approaches for controlling mosquito-borne infections and reducing the global burden of malaria (Lengeler 2004; Lindsay et al., 2002), lymphatic filariasis, and Japanese encephalitis and other arboviruses (Ogoma et al., 2010; Wilson et al., 2014). The recent adaptation of long-lasting insecticidal nets (LLIN) fitted as window curtains (ITC), has proven to reduce *Aedes aegypti* abundance densities and theoretically reduce dengue transmission risk (Kroeger et al., 2006; Lenhart et al., 2008 & 2013; Loroño-Pino et al., 2013; Rizzo et al., 2012; Vanlerberghe et al., 2011a). While screens can be easily introduced within DENV endemic areas, recent studies have shown that, as found with bednets, they require proper handling and use by local communities to be effective. An alternative innovation to curtains, is using LLINs fitted to windows and doors, i.e. insecticide-treated house screening (LLIS). LLIS on doors and windows are 'user-friendly', requiring little additional work or behavioral change by householders.

On the other hand, insecticide resistance in mosquitoes, particularly to pyrethroid insecticides (PYs), the major component of LLIS, is becoming increasingly widespread and is a major concern for dengue-vector control programmes in many countries (WHO, 2012). The impact of PY resistance on LLIS efficiency to control mosquitos is currently unclear. In

Mexico there is strong evidence of resistance to PY in multiple *Ae. aegypti* populations, including in cities with high levels of DENV transmission. Data on the actual impact of resistance on current and future control activities, such as LLIS, and the development and implementation of effective evidence-based resistance management strategies are important priorities. Furthermore, there is an urgent need for evaluate LLIS efficacy in areas where insecticide resistance is already developed in the local dengue vector population.

The aim of this PhD is to evaluate the field-efficacy of LLIS on windows and doors, and assess the impact of insecticide resistance impact on this kind of intervention. The work was performed in dengue endemic areas of two Mexican cities and comprised of the following two main phases and objectives:

Phase 1. Studies on the susceptibility status and resistance mechanisms to insecticides in *Ae. aegypti* populations:

a. To determine the susceptibility levels and resistance intensity of *Ae. aegypti* local populations to the different insecticide chemical groups used by the Mexican vector control program with the CDC-bottle method: Pyrethroids (Permethrin, Alpha-cypermethrin), Carbamates (Propoxur), and Organophosphates (Chlorpyriphos).
b. To use biochemical assays to determine the mechanisms involved in the resistance of *Ae. aegypti* populations in the study sites.
c. To identify the principal polymorphisms associated with insecticide resistance in the voltage-gated sodium channel gene domain II and III of segment 6 (kdr mutations).

Phase 2. Impact of insecticide resistance on vector control strategies (e.g. LLIS) for *Ae. aegypti*.

a. To evaluate the field-efficacy of an intervention based on LLIS on *Ae. aegypti* populations at baseline and 6, 12, 18 and 24 months post implementation.
b. To determine the efficacy of LLIS in laboratory-based bioassays after 6, 12, 18, 24 months on both insecticide susceptible and local mosquito strains.
c. To monitor levels of insecticide susceptibility/resistance (after 6, 12, 18 and 24 months) of *Aedes* populations from areas with LLIS and areas with traditional control using the methods developed in Phase 1.

Chapter 2. Literature review

2.1 Classification and use of insecticides in vector control

2.1.1. Chemical groups and modes of action of insecticides.

Insecticides are chemicals used to control (via deterring or killing) insect populations. These chemicals can be inhibitors, activators, synergists, chemosterilants, hormonal agents or bacterial toxins. The most widely used insecticides are neuro-inhibitors. There are four main classes of insecticides used in public health for vector control: organochlorines (OCs), pyrethroids (PYs), organophosphates (OPs), and carbamates (CAs).

2.1.1.1. Insecticide compounds affecting voltage-gated sodium channels

An ion channel is a transmembrane protein complex that forms a water-filled pore across the lipid bilayer through which specific inorganic ions can diffuse down their electrochemical gradients. Controlled modulation of ion channels is critical for normal physiological processes in the cells. In neurons, ion channels play a crucial role in conduction of nerve impulses and release of neurotransmitters.

The voltage-gated sodium channel is the principal molecular target for action of the pyrethrins and PYs, dichlorodiphenyltrichloroethane (DDT) and its analogues, the synthetic analogues of naturally-occuring N-alkylamide insecticides and dihydropyrazole derivatives such as Indoxocarb (Davies et al., 2007; Zlotkin, 1999). The PYs, DDT and analogues strongly alter channel function by binding to specific receptor sites, causing excitatory paralysis of insects followed by death.

Organochlorines (OCs). Organochlorines are compounds that contain carbon, chlorine, and hydrogen. Since they resist metabolism and are readily stored in fatty tissue of any animal ingesting them, they accumulate in animals in higher trophic levels (Bloomquist, 1999).

Of the organochlorines only DDT is now used in vector control. The DDT affects mainly the peripheral nervous system (Figure 1). The DDT prolongs the inward sodium current and inhibits the increase in potassium permeability. Together these effects lead to a prolonged falling phase and an increased negative after-potential therefore resulting in repetitive activity. The treated insects rapidly become hypersensitive to external stimuli and develop tremors of the body and appendages leading to violent motion and eventually paralysis (Corbett et al., 1984). Although the primary target site of DDT is the sodium channel in the nervous system, it has also been shown to affect the activity of ATPase (Matsumura, 1985).

Pyrethroids (PYs). PYs are esters containing both alcohol and carboxylic acid moieties. Depending on the alcohol substituent PYs are classified as type I (have a descyano-3-phenoxybenzyl or other alcohols) and type II (have an α-cyano-3-phenoxybenzyl alcohol) (Bloomquist, 1999). Many of the older non-phenoxybenzyl Type I compounds (e.g. pyrethrins, allethrin, tetramethrin) are unstable under UV light and this characteristic prevents their use in indoor residual spraying. Introduction of the phenoxybenzyl pyrethroids (e.g. permethrin) or certain halogenated alcohols (e.g. tefluthrin) improved chemical stability and allowed the use of theses types of PYs in the field (Bloomquist, 1999).

PYs affect both the peripheral and central nervous systems of insects (Figure 1). They inhibit sodium channel deactivation and maintain them in an open configuration (O´Really et al., 2006). In addition, they shift the membrane potential, causing a new and relatively stable abnormal state of nerve cell hyperexcitability, leading to a sublethal incapacitating effect on insects (loss of normal posture and locomotion) known as 'knockdown' (reviewed by Suppiramaniam et al., 2010). Type I compounds are generally good knockdown agents because of their direct effects on peripheral sensory and motor nerves (as well as interneurons within the central nervous system). In contrast, type II pyrethroids provide a better kill by causing irreversible depolarization of the nerve axons and terminals and consequently a pronounced convulsive phase (reviewed in Suppiramaniam et al., 2010). Type II PYs hold the channels open for a longer time than type I (Suppiramaniam et al., 2010).

In insects, the effects of PYs (especially type I) can develop within 1-2 minutes of treatment (Matsumura, 1985). The toxic potency of PY increases greatly with lowering of temperature (Zlotkin, 1999). This is probably because low temperatures prolong channel opening (O´Really et al., 2006), thus increasing the affinity of these insecticides to sodium channels.

2.1.1.2. Acetylcholinesterase inhibitors

The neurotransmitter operating in the autonomic nervous system, neuromuscular junctions and parts of the central nervous system is acetylcholine (ACh), which is released by cholinergic neurons. The synaptic action of ACh is terminated by the enzyme acetylcholinesterase (AChE), which rapidly hydrolyzes the ester group in ACh (Bloomquist, 1999). OPs and CAs work by inhibiting AChE in an irreversible bond[1]. Consequently, the enzyme AChE is unable to function and an accumulation of ACh occurs at the

[1] The time of reactivation of the enzyme is very long (from hours to days, depending of the molecule substituents) compared to the half-life of the enzyme, which is about 50 hr, and they are usually considered irreversible inhibitors.

neuromuscular junction, which causes over-stimulation and leads ultimately to the death of the insect (Figure 1).

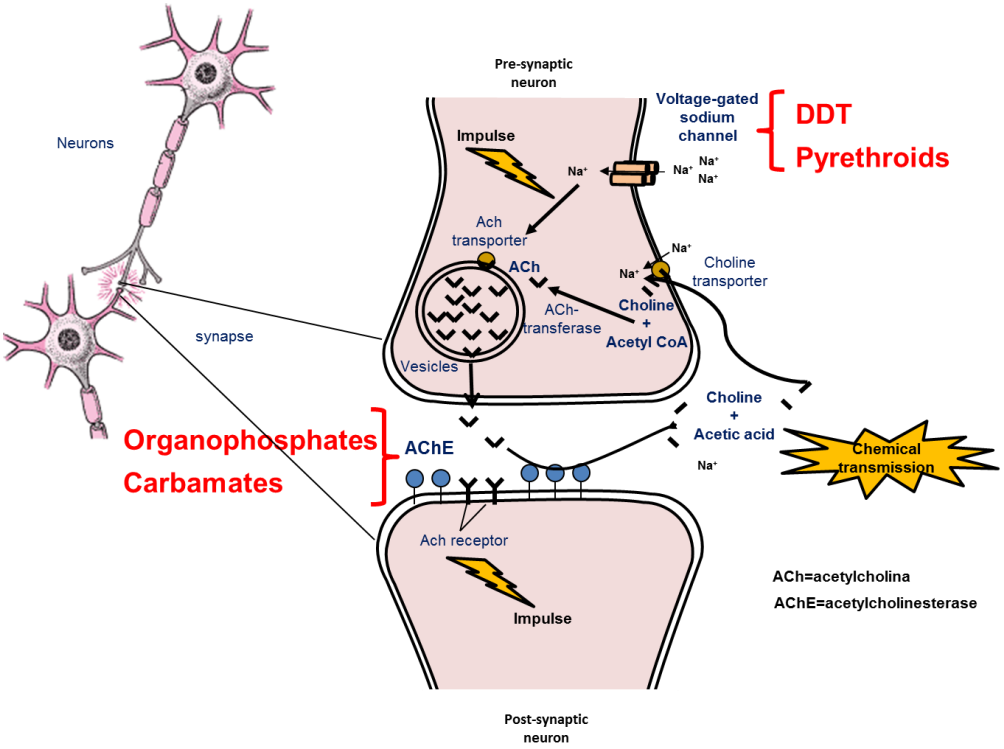


Figure 1. Biochemical target sites of synthetic insecticides. Modified from David et al., 2013.

Organophosphate (OPs). Most OP insecticides are usually applied in the non-insecticidal phosphorothionate form and are bio-activated to the insecticidal phosphate form (oxon analogue) by the oxidative action of cytochrome P450s within the insect. These oxons are more neurotoxic (potent AChE inhibitors) than their thionate analogues. The phosphorylation of AChE is persistent; reactivation of the enzyme can take many hours or even days (Matsumura, 1985). The signs of intoxication include restlessness, hyperexcitability, tremors, convulsions, and paralysis. In insects, the effects of OPs are confined to the central nervous system (Figure 1), where virtually all of the cholinergic synapses are located. Because they often require bioactivation and must penetrate into the central nervous system, the OPs do not have as rapid an action as the PYs (Casida and Quistad, 2004; Bloomquist, 1999).

Carbamates (CAs). These compounds are generally stable and most soluble in organic solvents (Bloomquist, 1999). The majority of the CAs in use are N-monomethyl carbamates, frequently referred to as N-methyl-carbamates or just methylcarbamates (Ecobichon, 2001). CAs are direct inhibitor of AChE and the signs of intoxication are similar to those of OPs

(Figure 1). Compared to phosphorylated AChE, the carbamylated enzyme complex is relatively less stable, so they have a shorter duration of action compared to OPs (Bloomquist, 1999).

2.1.2. Use of insecticides in vector control

Although the use of natural insecticides has been reported in ancient civilizations such as Greece in 1000 BC and China in AD 900 to control pests (Casida and Quistad 1998), it was not until the mid 19th century that insect pests were controlled with any degree of success using chemicals following the discovery of synthetic organic insecticides.

The genesis of the modern era for pesticide use began with the development of DDT in 1939 and its first use during World War II. The widespread use of DDT greatly contributed to the increased control (and in some areas, near elimination) of many vector-borne diseases such as typhus, yellow fever, malaria, onchocerciasis and schistosomiasis from the 1940s-1960s (Williams, 1964). Despite its low acute toxicity, the possible long-term toxicity from widespread use of DDT, led to it being banned in the 1970s, first for agricultural use together with several other OCs (e.g. aldrin and dieldrin), and eventually for public health use. However, its limited reintroduction in 2000 for public health use was only justified for high malaria transmission areas, such as parts of sub-Saharan Africa, where DDT has demonstrated to be effective because of its repellent and irritant (exito-repellency) propiertes (Sadasivaiah et al., 2007; WHO, 1960; WHO, 2007a).

The first OP insecticides were developed in 1937, tetraethyl pyrophosphate (TEPP) and parathion. Because of their powerful insecticidal propierties, rapid detoxification in mammals and useful propiertes for pest control, OPs quickly replaced OCs (Casida and Quistad 1998). For vector control OPs has been historically used for larviciding (e.g. use of temephos), but after mid-90s the use of OPs was extended to residual spraying and space spraying, increasing its use, while the OCs experimented a declining trends (WHO, 2002).

The use of CAs began in the 1950s, when there was a search for insecticides having anticholinesterase activity, greater selectivity, and less mammalian toxicity than some of the organophosphorus esters then in use. This led to the synthesis of several potent aryl esters of methyl carbamic acid, these agents becoming insecticides of choice for pest control in the 1960s and 1970s (Casida and Quistad 1998; Ecobichon, 2001). Before 2000 CAs were rarely used for vector control, this type of insecticide represented less than 1% of global coverage with insecticides used for house-spraying. The use of carbamates in vector control is relatively rare compared with other classes of insecticides (Berg et al., 2012).

The PY original compounds in this series were the natural pyrethrins, which were isolated from the flowers of the chrysanthemum. Pyrethrums (dried chrysanthemum flowers based infusion) are one of the oldest and most widely used botanical insecticides but they have a high rate of photodegradation. Synthetic PYs are much more photostable. They are highly lipophilic, have a short half-life in the environment, have low toxicity to terrestrial vertebrates and do not biomagnify like older chemical classes, such as OCs (Casida and Quistad 1998; Schleier and Peterson, 2011). The first photostable PYs was permethrin, followed by cypermethrin and deltamethrin (Schleier and Peterson, 2011). Synthetic pyrethroids are commonly used in crop protection, animal health and in public health (reviewed by Davies et al., 2007).

The OPs and PYs are the most widely used groups of insecticides for dengue control in the Americas region. Use of carbamates has been limited to residual spraying in the region, due to their recent introduction for dengue control (Berg et al., 2012; WHO, 2011).

2.1.3. Overview of dengue chemical control in Mexico.

DDT and dieldrin in Mexican anti-malaria campaign. The history of mosquito chemical control in Mexico started with an anti-malaria campaign initiated in the mid-1940s, when The Rockefeller Foundation supported the world´s first field trials for DDT residual spraying against *Anopheles* mosquitoes in the central states of Morelos and Michoacan (Gómez-Dantés and Birn, 2000; Stapleton 1998). With the creation of the General Directorate of the Campaign against Malaria (DGCP-SSA) in 1947 (which preceded the National Campaign to Eradicate Malaria, CNEP), vector control was mostly based on DDT or dieldrin use in residual spraying and oil application on water bodies (Gómez-Dantés and Birn, 2000).

Initially, dieldrin was the insecticide of choice, due to its long lasting residual effect that allowed a single application per annum (Gómes-Dantés & Birn, 2000). Dieldrin was replaced by DDT in 1960, because of concerns regarding dieldrin toxicity levels and evidence of resistance to this insecticide (Martínez-Palacios, 1965; Gómes-Dantés & Birn, 2000). During the CNEP programme, more than 81,900,000 houses were sprayed with DDT throughout Mexico from 1956-1983 (Figure 2).

In 1984, the CNEP was dismantled and the malaria program was integrated into the Ministry of Health (MoH) in the General Directorate of Preventive Medicine within a division responsible for all vector-borne diseases. During the 1980s, a new version of the eradication scheme called "Program of Simultaneous and Intensive Actions" (PAIS) was implemented in 955 specific foci of malaria transmission identified as the main sources of

infection. The features of PAIS included environmental sanitation, use of protective measures like screening and bed nets and the improvement of the quality of walls in houses enabling better impregnation with the insecticide (Gómes-Dantés & Birn, 2000).

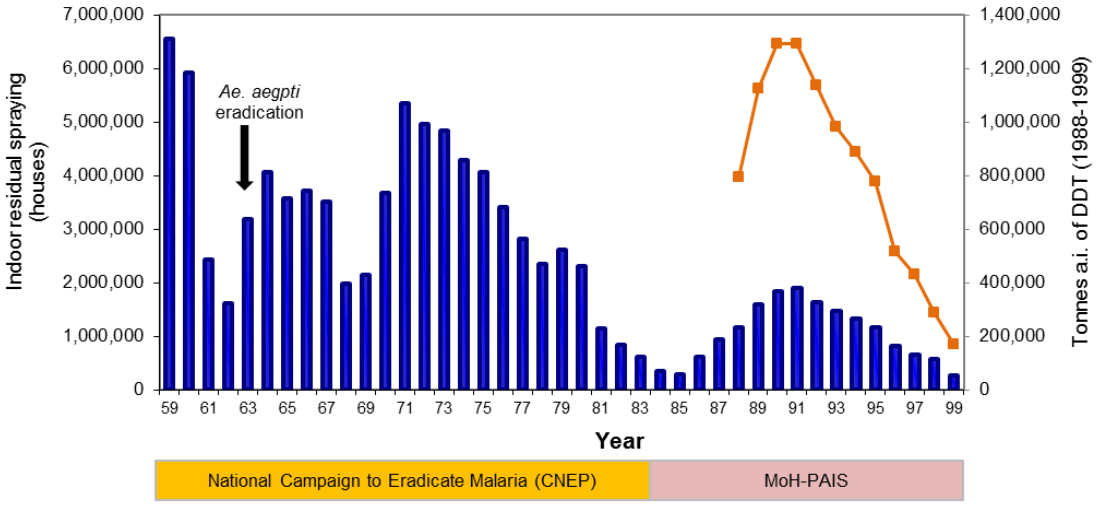


Figure 2. DDT and malaria control in Mexico. Blue bars represent number of houses sprayed with DDT from 1959-1999. Orange line represent tonnes of active ingredient (a.i.) used of DDT from 1988-1999. Source: SISPA data bases (2013).

At the end of the 1980s, the use of DDT began to decrease. DDT use continued sporadically in some areas of the country until 1998, when it was banned as part of the agreements established by the Commission for Environmental Cooperation of North American Free Trade Agreement (Environews Forum, 1997; SSA, 2011). DDT use was fully discontinued in 2000 (Méndez-Galván et al., 2004).

Mexican anti-*Aedes* campaign. The early years of *Aedes* eradication encompasses the anti-yellow fever campaign in the 19th century. Yellow fever control was the first priority overall in Mexican coastal cities (including Veracruz, Acapulco, Campeche and Merida). In 1921, the Special Commission for the Campaign against Yellow Fever was created in Mexico and in conjunction with agents and considerable funding from the Rockefeller Foundation International Health Board, waged a successful campaign to eliminate yellow fever in the state of Veracruz, Mexico through the elimination of *Aedes* breeding sites. The yellow fever campaign was also assisted by the use of DDT indoor residual spraying implemented by anti-malaria campaign (Torres, 1995; Novo, 1995). The eradication of yellow fever in Veracruz was achieved in 1923 (Gómes-Dantés & Birn, 2000; Novo 1995) and by 1925, urban yellow fever was declared eradicated from Mexico (Novo, 1995). Since 1923 and to date the yellow fever is considered eradicated in Mexico.

In the 1940s, the first evidence of dengue transmission in Mexico was reported (Narro-Robles & Gómez-Dantés, 1995). Although there was no explicit dengue control programme at that time, anti-mosquito activities against malaria and yellow fever were ongoing (Torres, 1995).

The history of dengue vector control in Mexico started with a vigorous campaign to eradicate *Ae. aegypti*, initiated by the Pan American Health Organization (PAHO) in all Latin American countries in 1957. All houses in positive localities were treated with intra-domiciliary DDT spraying (applying two treatments every six months). By 1960, no dengue cases were reported in the country and in 1963, PAHO certified *Ae. aegpti* eradication in Mexico. However, in 1978 the country experienced the re-emergence of dengue (Torres, 1995). It appears that while the Mexican health authorities were looking for *Ae. aegypti* on the borders, a gradual re-infestation by vector populations to levels of epidemiological importance occurred within the Mexican territory.

In 1980, and in view of these events, Mexico had to establish a National Contingency Programme for the Prevention, Surveillance, and Control of DENV. This programme aimed to control and prevent outbreaks, based on elimination of mosquito breeding sites through massive source reduction ("descacharrización") and larvicide application (1 % temephos), part of the PAHO recommended activities (Nelson, 1986). In addition, a new element was integrated: adult control with ULV application of malathion.

Organophosphate insecticides such as malathion were used from 1981 to 1999 (SSA, 2001). Initially malathion was used sporadically and gradually replaced DDT in 1984, mainly for malaria vector control (Gómes-Dantés & Birn, 2000) and later for ULV space spraying for *Aedes* control (SSA, 2001). Deltamethrin was sporadically used for intra-domiciliary spraying for a brief period between 1999-2001. In 2000, dengue control programs in Mexico switched to permethrin-based pyrethroid insecticides for ULV intra-domiciliary and space spraying for adult mosquito control, and permethrin was exclusively used for almost ten years (Figure 3). In 2009, the use of permethrin was banned because of evidence of resistance to this insecticide in several *Ae. aegypti* populations in Mexico (Saavedra-Rodríguez et al., 2007; Ponce-García et al., 2009). Since then, a broad group of insecticides have been approved to be used for dengue control in Mexico, including other pyrethroids and the recently approved CAs propoxur and bendiocarb (SSA, 2008; SSA, 2014). See figure 4.

Temephos 1% has been applied to bodies of water and domestic containers for immature *Aedes* control since 1980. Although other larvicides are available (based on Spinosad, Bti and insect growth regulators (IGRs)), temephos continues to be the larvicide of choice in Mexico

due to its low price. An overview of historical insecticide use in Mexico is shown in Figure 4.

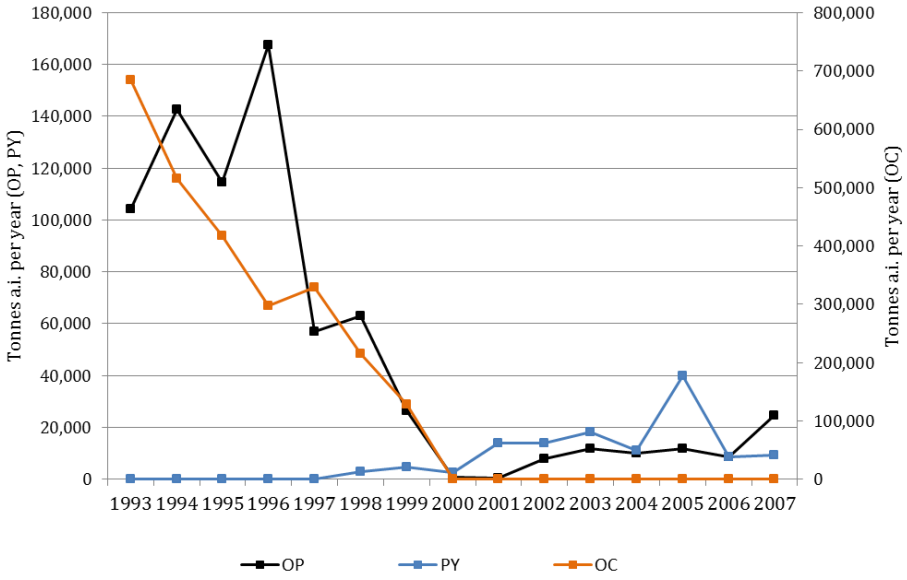


Figure 3. Use of insecticides for mosquito control in Mexico averaged during the period 1993–2007. PY, pyrethroids; OP, organophosphates; OC, organochlorines. Source: data from WHO, 2004, 2007b, 2009b and 2011

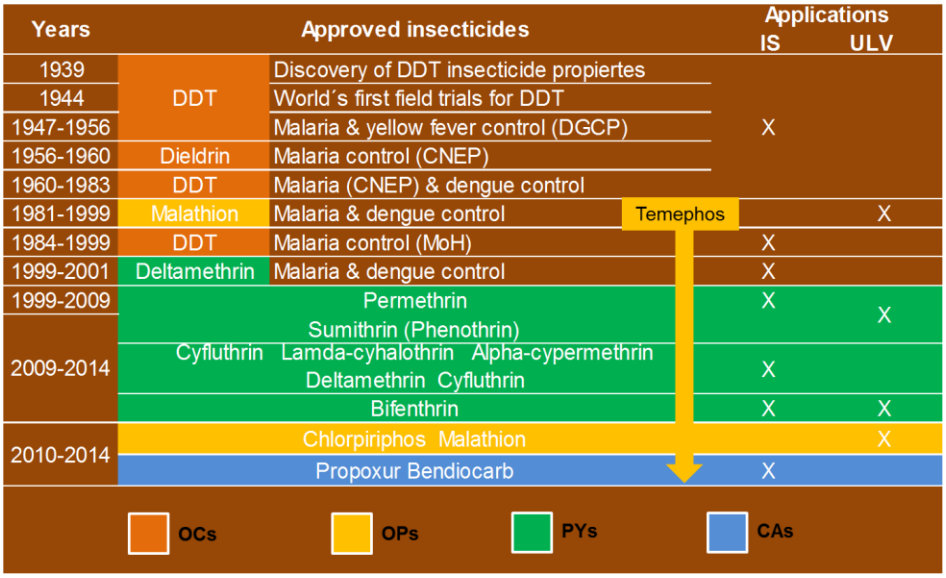


Figure 4. History of approved insecticide for mosquito control in Mexico. Since 1947 the vector program of the Ministry of Health in Mexico has used a series of insecticides for the control of dengue and malaria. DGCP, General Direction of the Campaign against Malaria; CNEP, National Campaign of Malaria Eradication; MoH, Ministry of Health. DDT, organochlorine; CA, carbamate; PY, pyrethroids; OP, organophosphate; IS, intra-domiciliary spraying; ULV, ultra low volume spraying.

2.2 Evolution of resistance

2.2.1. Importance of insecticide resistance.

The pressure exerted by the extensive and intensive use of insecticides has encouraged the development and evolution of resistance on more than five hundred species of arthropods of medical, agricultural and veterinary importance (Denholm et al., 2002; IRAC, 2013). The number of species resistant to any insecticide has had an increase of almost 50% in the last 40 years (Figure 5).

Resistance in insect pests results in increased insecticide application rates, decreased yields (crops and animal products), environmental damage, and outbreaks of human and animal diseases when vectors cannot be controlled. The World Health Organization (1976) has called insecticide resistance "the biggest single obstacle in the struggle against vector-borne disease". One estimate suggests that the cost of resistance may be $1.5 billion USD annually in the United States (Pimentel et al., 2005).

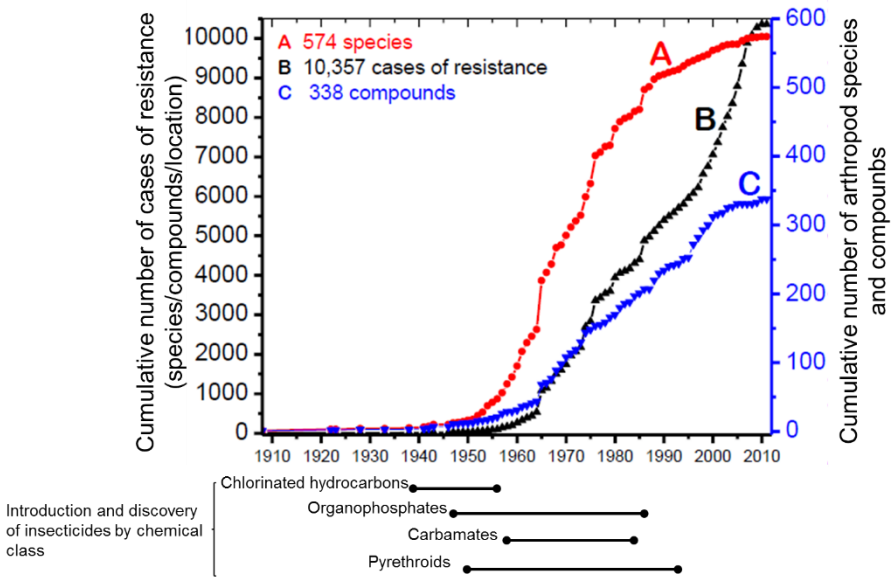


Figure 5. Evolution of arthropod insecticide resistance from 1908 to 2012. Source: http://www.pesticideresistance.org.

Development of insecticide resistance. Insecticide resistance is heritable and thus has a genetic basis (Hemingway et al, 2004). As insect populations are usually large in size and they have multiple generation in short periods of time, there is always a risk that insecticide resistance may evolve, especially when insecticides are misused or over-used. Natural selection by an insecticide allows some initially very rare, naturally occurring, pre-adapted

insects with resistance genes to survive and pass the resistance trait on to their offspring. Through continued application of the same type of insecticides (or with the same mechanism of action), selection for the resistant individuals continues so the proportion of resistant insects in the population increases, while susceptible individuals are eliminated by the insecticide. Under permanent selection pressure, resistant insects outnumber susceptible ones and the insecticide is no longer effective. Insect resistance generally is a local phenomenon and can vary greatly over space and time (Deming et al., 2016).

Insects can become resistant to more than one class of insecticide in two ways. Resistance selected by one insecticide can confer resistance to another insecticide, even where the insect has not been exposed to the latter product, i.e. cross-resistance. This is because of insecticides have the same or very similar modes of action (e.g. CAs and OPs; PYs and DDT). If the resistance is conferred by two or more different resistance mechanisms in an individual insect (a resistant insect may have both target site and metabolic resistance), it is termed multiple resistance or multi-resistance (Brengues et al., 2003; Georghiou, 1965). The main mechanisms by which mosquitoes confer resistance are reviewed below.

2.2.2. Mechanism of insecticide resistance.

Resistance can be acquired through behavioural or physiological actions. Behavioural resistance occurs when insects are able to evade contact with pesticides through avoidance (deterrence). This type of response can be further divided into direct (tarsal) contact excitation (irritancy) and non-contact spatial repellency when insects move away from the insecticide-treated area without making direct contact (Roberts et al., 1997). In laboratory and field experimental trials, the repellent and irritant actions of DDT and some pyrethroids on *Ae. aegypti* mosquitoes have been desmostrated (Achee et al., 2009; Grieco et al., 2007). Behavioral resistance could have significant impacts on the effectiveness of mosquito chemical control; however is the less studied and it is more difficult to monitor in field populations compared to physiological resistance (Gatton et al., 2013).

Physiological resistance is the dominant type of insecticide response and the most extensively studied. It is defined as the ability of an insect population to survive exposure to a concentration of insecticide that would normally result in complete kill (Roberts and Andre, 1994). Physiological resistance may be achieved by various mechanisms, such as increased excretion, cuticular resistance, metabolic resistance or resistance due to the alteration of target sites.

In cuticular resistance changes in the chemical composition of the insect's cuticle would result in delaying the rate of insecticide penetration into the body, which would in turn

provide time for detoxification mechanisms to take effect, avoiding insecticides reach their binding site in a lethal dose. Some *Culex* mosquitoes have evolved thicker or altered cuticles, which reduces the penetration of insecticide (Apperson and Georghiou, 1975; Stone and Brown, 1969). In addition some genes have been identified encoding for cuticular protein over-expressed in PY resistance strains of *Anopheles* (Kwiatkowska et al., 2013) and *Ae. aegypti* (Lertkiatmongkol et al., 2010).

2.2.3. Insecticide resistance in mosquitoes.

2.2.3.1. Metabolic resistance

Metabolic resistance occurs when enhanced levels (quantitative changes) or modified activities (qualitative changes) of detoxification enzymes prevent the insecticide from reaching its site of action. When increased quantities occur, sequestration is the primary mechanism; qualitatively changes can hydrolyse insecticides at a faster rate than their counterparts in susceptible insects. Quantitative changes are usually mediated via upregulation through mutations in trans- and/or cis-acting regulatory loci (increasing the transcription) or through amplification of the structural gene encoding the enzyme; and qualitative changes result from mutations in the enzyme coding sequence (Bass and Field, 2011). See figure 6.

There are three major groups of enzymes families involved in metabolic resistance: the carboxylesterases, cytochrome P450s and glutathione S-transferases.

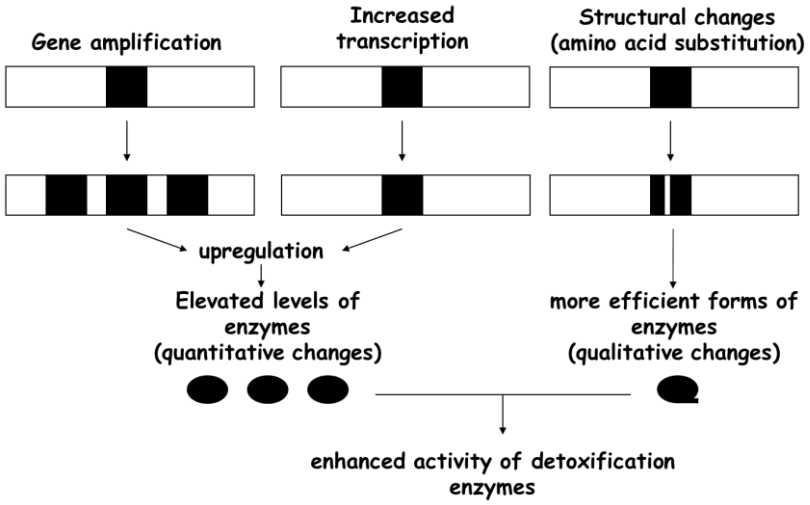


Figure 6. Molecular mechanism of metabolic resistance.

Carboxylesterases (CCEs). CCEs comprise a gene family within the α/β-hydrolase fold protein superfamily (Montela et al., 2012). Increased production of CCE causes

enhanced degradation and sequestration of insecticidal esters, such as OPs. This mechanism can be considered as the primary mechanism against OPs (Karunaratne, 1998). 49 CCE-encoding genes have been identified in *Ae. aegypti* (Strode et al., 2008).

The overproduction of esterase enzymes is a common mechanism of OPs resistance observed in *Culex* mosquitoes as result of gene amplification (e.g. *estα2^{1}*, *estβ1^{1}*, *estβ2^{1}*) (Gullemaud et al., 1997; Hemingway, 2000; Hemingway and Karunaratne, 1998; Peiris and Hemingway, 1993; Raymond et al., 2001; Vaughan and Hemingway, 1995). The amplified esterase form binding insecticides more effectivelly than their non-amplified counterparts (Karunaratne et al., 1995). The level of amplification is highly correlated with the resistance phenotype (Bass and Field, 2011).

Transcriptional or translational regulation may also play a role in esterase gene expression. In *Culex* mosquitoes, esterase genes (*estα2^{1}* and *estβ2^{1}*) are co-amplified in a 1:1 ratio within the genome (Hemingway and Karunaratne, 1998), but they are differentially expressed in a 2:1 (*esta11- estβ11*) to 30:1 (*estβ2^{1}- estα2^{1}*) ratio (Cui et al., 2007b; Paton et al., 2000).

Increased OP-CCE activity, by amino acid substitutions in the coding sequences of esterase genes has been found in *Culex* strains (Cui et at., 2007a).

Recently a specific CCE (CCEae3a) in *Aedes* mosquitoes has been implicated directly in metabolizing OPs (Grigoraki et al., 2016). Increased CCEae3a activity is associated with amino substitutions in *CCEae3a* gene, in addition to gene amplification mechanism (Poupardin et al., 2014).

Enhanced activity of esterase has been also associated with CAs and PYs resistance in *Anopheles* (Aïzoun et al., 2013; Beach et al., 1989; Brogdon and Barber, 1990; Vulule et al., 1999) and *Culex* mosquitoes (Gordon and Ottea 2012). Evidence in permethrin-resistant *Ae. aegypti* is also reported (Mourya et al., 1993). Recently, the capacity of *Ae. aegypti* CCEs to metabolize PYs has been demonstrated in vitro (Somwang et al., 2011). However, no specific mosquito CCE has yet been validated as a PY metabolizer.

Cytochrome P450s. The cytochrome P450s (also termed mixed-function oxidases or monooxygenases) constitute a ubiquitous and complex superfamily of hydrophobic, heme-containing enzymes, which act as the terminal oxidase in monooxygenase systems (Hlavica, 2011; Scott, 1999). P450-based metabolic resistance confers cross resistance to broad group of insecticides but are particularly important in PY resistance (Casida y Quistad, 1998; David et al., 2013). In addition, P450s are responsible for the bioactivation of many OPs. It is also possible that resistance could be achieved through decreased activation, but it does not appear to be a common mechanism of resistance (reviewed by Scott, 1999). In *Ae. aegypti,*

160 P450-encoding genes have been identified, considerably more than found in *Drosophila melanogaster*, and *Anopheles gambiae* (Feyereisen, 2012; Strode et al., 2008).

Overproduction of P450 enzymes has been identified as a common mechanism of PYs resistance in several species of mosquitoes as result from a change in a regulatory factor, which regulates enzyme expression (Scott, 1999; Waters and Nix, 1988). Most of P450 genes over-expressed in pyrethroid resistant strains of *Ae. aegypti* from South-East Asia, Latin America and Caribbean belong to the *CYP9* and *CYP6* genes (David et al., 2013; Stevenson et al., 2012). But only a subset of *Aedes* P450s (e.g. CYP9J24, CYP9J26, CYP9J28 and CYP9J32) that have been associated with pyrethroid resistance has been confirmed to be able to metabolise pyrethroids by functional in vitro studies (Reviewed by Vontas et al., 2012) P450 gene duplication has also been reported in *Ae. aegypti* (Bariami et al., 2012).

In *Ae. aegypti*, both P450 (CYP6Z8) and CCE (CCEae3a) enzymes were found overexpressed together in PY and OP resistant populations supporting the possible coordinated role of theses enzymes in insecticide detoxification (Marcombe et al., 2009; Marcombe et al., 2012; Poupardin et al., 2014). Particular P450 enzymes from *Ae. aegypti* (CYP6Z8) and *An. gambiae* (CYP6Z2) have been shown to metabolize PY metabolites produced by CCE, and it is possible that elevated levels of this enzyme is an important secondary resistance mechanism (David et al., 2013; Poupardin et al., 2014).

Increased levels P450 enzymes in *Anopheles* pyrethroid-resistant populations, may also confer cross-resistance to CAs (Brooke et al., 2001). Recent studies suggests that an upregulated P450 belonging to CYP6 subfamily could be associated with bendiocarb resistance in *Cx. quinquefasciatus* (Martins, 2014) and *An. gambiae* (Edi, 2014).

Glutathione S-transferases (GSTs). At least six classes of cytosolic GSTs are present in insects (Ding et al., 2003; Enayati et al., 2005; Ranson et al., 2002). GSTs enzyme families are mostly associated with DDT resistance, although specific genes belonging to GST families are also frequently found over-expressed in pyrethroid resistance populations. There are 26 GST-encoding genes in *Ae. aegypti* compared to 28 in *An. gambiae* (Strode et al., 2008).

Overproduction of GST enzymes is a major mechanism of DDT-resistance in mosquitoes (Ding et al., 2005; Enayati et al., 2005; Hemingway et al., 2004; Ranson et al., 2001). GSTE2 has been confirmed to have DDTase activity in *Anopheles* (Ortelli et al., 2003; Ranson et al., 2001) and *Aedes* mosquitoes (Lumjuan et al., 2005). Recently high level of DDT resistance was associated a single amino acid change (L119F) in the binding pocket of GSTE2 in *An. funestus* field populations in addition to gene increased transcription mechanism (Mulamba et al., 2014; Riveron et al., 2014). Similarly, in *Ae. aegypti* the GSTE2

isoform from the DDT resistant strain (which differs at five residues compared with the susceptible strain) has higher affinity for DDT (Lumjuan et al., 2011).

The GST role in the detoxification of PYs has been attributed to its capacity to reduce the peroxidative damage induced by PYs, mainly by detoxifying lipid peroxidation products (Vontas et al., 2001). Although some evidences were previously showed in *Ae. aegypti* (Lumjuan et al., 2011), recently more evidence in *Anopheles* (Riberon et al., 2014) and *Culex* (Huang et al., 2012) implicate to particular GST (*GSTe2* and *CpGSTD1* genes respectively) directly in metabolizing PYs, but the mechanism by which this occur still remains to be resolved.

It is suggested that GSTs may also protect against PY toxicity through a passive sequestrating process (Kostaropoulos et al., 2001) and some *Anopheles* GSTs have been shown to bind PYs (Jirajaroenrat et al., 2001; Prapanthadara et al., 1998, 2000; Udomsinprasert and Ketterman, 2002).

For some insecticide classes, the GST detoxification mechanism acts as a secondary resistance mechanism in conjunction with a P450 or esterase based resistance mechanism (Hemingway et al., 1991).

2.2.3.2. Target-site insensitivity.

Alterations in the amino acid sequence of the insecticide-target proteins (the voltage gated sodium channel and insect AChE) can cause the insecticide to be less effective or even ineffective (Figure 1).

Voltage gated sodium channel. Mutations in the voltage-dependent sodium channel gene have been associated with knockdown resistance (kdr).

The molecular basis of kdr was first recognised in *Musca domestica*: point mutations in the S6 segment (L1014F) and in the S4-S5 linker (M918T) of domain II (Williamson et al., 1996) were linked to pyrethroid resistance. Since then kdr-like mutations at codon 1014 (L to F/H/S mutation in IIS6) have been reported in various insect species including mosquitoes (Martinez-Torres et al., 1998 & 1999).

The *Ae. aegypti* mosquitoes do not present any substitution in the classic 1014 kdr site, because codon usage of the *Ae. aegypti* sodium channel does not favour substitutions at residue 1014 (Martins et al., 2009b; Saavedra-Rodriguez et al., 2007). Instead, several mutations in different positions have been observed in *Ae. aegypti* populations from Latin America and Southeast Asia. One of these mutations I1011M occurs in South American populations. In Brazil, the I1011M frequency was associated with cypermethrin-resistant field population (Lima et al., 2011; Martins et al., 2009a). However, for the most of mutations

identified for *Ae. aegypti*, there is little evidence associating these mutations with resistance. To date only the V1016I/G and F1534C sodium channel mutations in *Ae. aegypti* have been clearly associated with resistance to insecticides and they have been proven to reduce sodium channel sensitivity to pyrethroids to DDT and PYs (Brengues et al., 2003; Du et al., 2013; Hirata et al., 2014; Hu et al., 2011).

In Mexico (Ponce-García et al., 2009) and Cayman Islands (Harris et al., 2010) V1016I has been associated with permethrin resistance. An alternative mutation at this codon, the V1016G mutation, has been reported in Asian populations of *Ae. aegypti* from Indonesia (Brengues et al. 2003), Thailand (Rajatileka et al, 2008; Srisawat et al., 2010; Stenhouse et al., 2013), Vietnam (Kawada et al, 2009) and Taiwan (Chang et al., 2009) and it has been associated with resistance to deltamethrin (Srisawat et al., 2010; Stenhouse et al., 2013).

The F1534C mutation is associated with resistance to permethrin and DDT. Electrophysiological experiments showed F1534C mutation drastically reduced channel sensitivity to type I PYs, but not to type II PYs (Hu et al., 2011). Mosquitoes with the homozygous F1534C mutation are generally susceptible to deltamethrin. In Thailand *Ae. aegypti* populations where this mutation is widely distributed (Yanola et al., 2011), mutant homozygous 1534C mosquitoes were always expressed with wild-type V1016, and the presence of the F1534C mutation was not associated with deltamethrin resistance (Stenhouse et al., 2013). In the Cayman Islands a number of mosquitoes were homozygous for both resistant allele, 1016I and 1534C, the first one was associated with permethrin survival, and 1534C was strongly associated with survival to both insecticides permethrin and DDT (Harris et al., 2010).

F1534C and V1016I mutations frequencies responded to deltamethrin selection, but in diferent levels. Studies carried out with a field strain from Venezuela, which was selected in the laboratory for 15 generations with deltamethrin, showed that the frequency of V1016I increased from 0.02 in F1 up to 0.5 in F15 (Alvarez et al., 2014). Similary *Ae. aegypti* from Santiago de Cuba selected with deltamethrin for 12 generations, the V1016I frequency increased from 0.033 in the original generation to 0.565 in F12 (Saavedra-Rodríguez et al. 2007). Interestingly for the first study, frequency of F1534C increased from 0.35 up to fixation, showing that deltamethrin select the F1534C mutation more rapidly than V1016I (Alvarez et al., 2014). The participation of this mutation in deltamethrin resistance needs to be investigated, considering the previous evidence about F1534C has been correlated with resistance to type I but not type II pyrethroids (Hu et al., 2011).

In addition to the fitness cost (for more discussion see Ffrench-Constant 2013) the type of kdr-mutation seems to depend of the kind of insecticide selection pressures (e.g. exposure to either type-I or type-II PYs or DDT). A particular kdr residue may be more effective at reducing susceptibility to one chemical structure than another. The recent discovery of a second putative PY receptor site in sodium channel in insects suggest that simultaneous binding of PY to two receptor sites in a four-domain sodium channel is necessary to efficiently lock sodium channels in the open state and, thereby, to exert the highly insecticidal action (Du et al., 2013). Specific mutations and their ubication in these two receptor sites could interact in conferring resistant to specific or general types of PYs.

Acetylcholinesterase (AChE). In insects AChE is a glycosylated dimer which is attached to a membrane via a glycolipid anchor (Figure 1). In mosquitoes there are two genes, *ace-1* and *ace-2*, coding for AChE1 and AChE2, respectively. The mosquitoes *An. gambiae* and, *Ae. aegypti* contains these two *ace* genes, in contrast with *D. melanogaster* which possess a single AChE encoded by *ace-2* (Strode et al., 2008).

In mosquito species, only three mutations on the *ace-1* gene have been linked to insensitive AChE mediated resistance: G119S, F290V and F331W (reviewed by Labbé et al., 2011). The most common resistance mutation (G119S) in the *ace-1* gene is situated near the active site "gorge", and confers high resistance to OPs in *Cx. pipiens* and *An. gambiae* (Weill et al., 2003, 2004). This mutation has not yet been observed in *Ae. aegypti* (Grisales et al., 2013; Weill et al., 2004), probably because this mutation is unlikely to occur spontaneously as it would require two mutation steps (Weill et al., 2004).

Duplication of resistant alleles of the *ace-1* gene has also been described in mosquitoes (Edi, 2014; Labbé et al., 2011; Liebman et al., 2015).

2.2.4. Insecticide resistance mechanism in *Aedes aegypti* populations from Mexico.

In Mexico the extensive use of DDT between 1950-1987 and the long and intensive use of pyrethroids in public health during the 1990´s, promoted an intense selection pressure for the evolution of resistance in *Ae. aegypti* (Brengues et al., 2003), and resulted in dramatic increases on the frequency of the 1016I kdr allele from 1996 to 2009 (Saavedra-Rodríguez et al., 2007; Ponce-García et al., 2009; Bobadilla-Utrera, 2010; Siller et al., 2011; Loroño-Piña et al 2013). More recently the 1534C kdr allele, originally reported in Asia (Kawada et al., 2009; Yanola et al., 2010, 2011) and in the Caribbean (Harris et al., 2010), has been reported to be common in permethrin-resistance *Ae. aegypti* populations from pacific coast of Mexico (Aponte et al., 2013; Penilla-Navarro et al., 2013).

Metabolic resistance has been also reported for Mexican *Ae. aegypti* populations. Elevated esterase levels were identified as the primary detoxifying mechanism in permethrin-selected populations from Quintana Roo State in the Peninsula of Yucatan (Flores, et al., 2006); esterase-based dextofication have been also reported as important resistance mechanism for permethrin in north of Mexico (Flores et al., 2005; Flores et al., 2009). The GST and esterase-based mechanism have been reported in pyrethoid resistant populations from Guerrero State in the pacific coast of Mexico (Aponte et al., 2013).

Resistance to OPs (e.g. chlorpyrifos, currently used in Mexican Anti-*Aedes* campaign) is suggested to be confered by esterase-based mechanism (Lopez et al., 2014). Theses studies showed no indication of involvement of insensitive AChE in resistance in Mexican populations (Flores et al. 2005, 2006, Lopez et al., 2014).

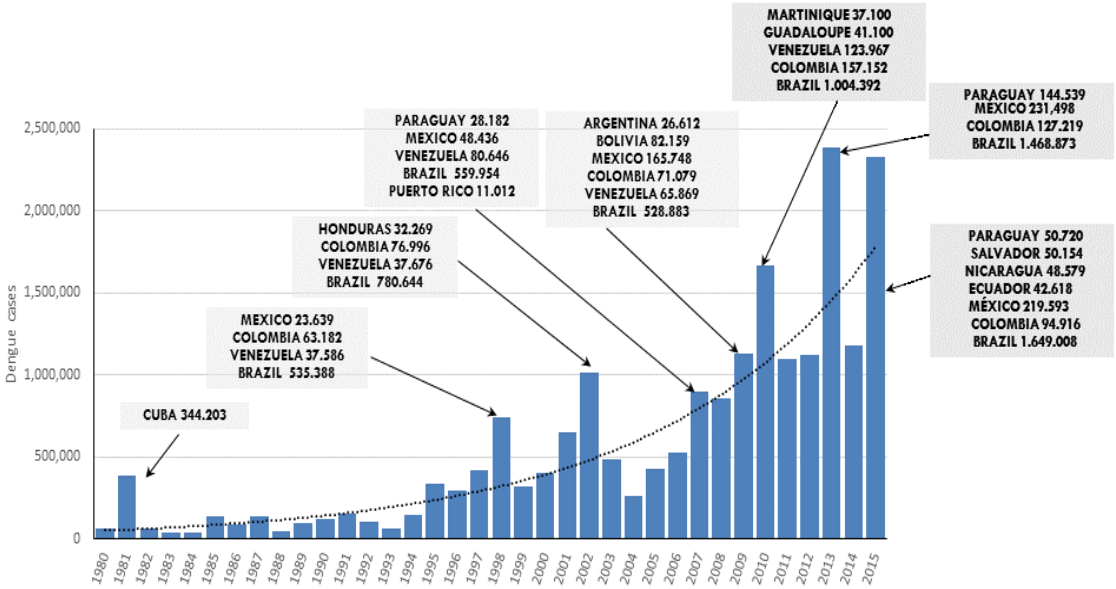
2.3 Epidemiology of Dengue

2.3.1. Dengue transmission in the Americas

Dengue has emerged as the most important vector-borne disease in the Americas because it threatens the health of millions of people living in urban, suburban and even rural environments (Tapia-Conyer, et al 2012). Dengue represents an enormous burden for clinical and health services in endemic regions which have been unable to reduce significantly this disease. The PAHO launched an *Ae. aegypti* eradication campaign in the 1950s and 1960s that came close to eliminating the vector from the continent (see section 2.1.3). The initial impact of the eradication campaign created the false impression that any vector control strategy could or should achieve similar reductions. The current distribution and incidence of the dengue in the Americas is increasing (Figure 7A) especially due to the deteriorating social, environmental and economic conditions that have made vector control a more challenging goal nowadays than it was in the past (Dantes et al., 2014). For example, the ecological setting for *Ae. aegypti* development and dengue transmission are urban centers in tropical and subtropical regions in the continent and, in the last 40 years, there has been intensive urbanization in Latin America and the Caribbean that is expected to reach 84% of the population by 2030; by then this region will be the second most urbanized in the world (Gomez-Dantes and Ramsey-Willoquet, 2009).

In the Americas region, a few countries, mainly Brazil, Mexico and Venezuela, represented above 80% of dengue cases reported from last 10 years (Figure 7 A-B). Mexico contributes to the high number of dengue cases in the Americas (San-Martin et al., 2010) and provides favourable conditions for the spread of dengue disease.

A



B

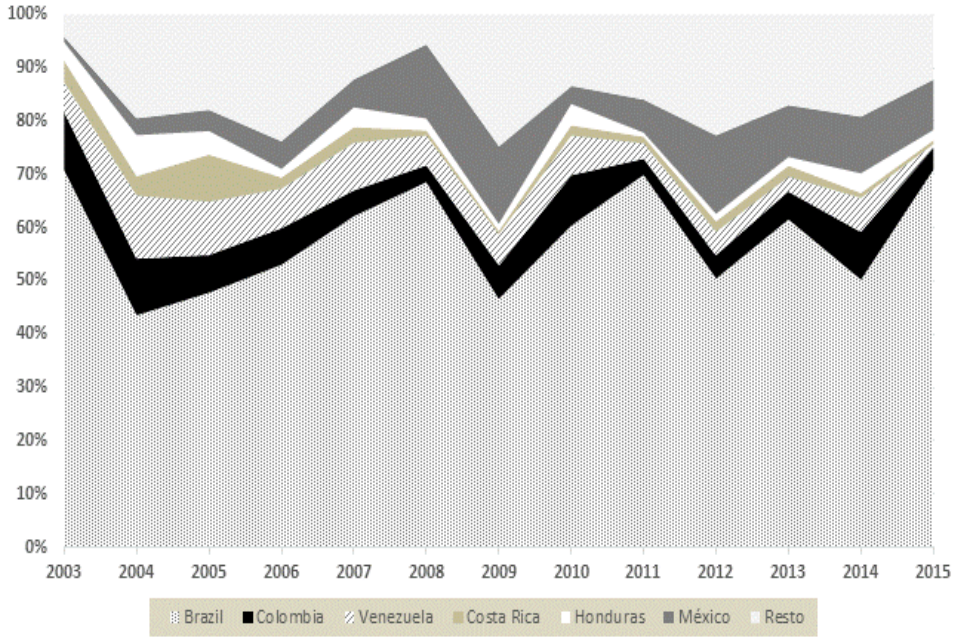


Figure 7. Dengue transmission in the Americas. Number of clinical dengue cases and main outbreaks in the region of the Americas, 1980–2015 (A). Proportion of clinical dengue cases reported in the region of the Americas 2003-2015 (B). Epidemiological Data were obtained from PAHO web page: http://www.paho.org/hq/index.php?option=com_topics&view=article&id=1&Itemid=40734.

2.3.2. Dengue disease patterns in Mexico.

In terms of morbidity, mortality, and economic costs, dengue disease is the most important mosquito-borne viral disease of Mexico. The first official reports of dengue transmission in Mexico date from the 1940s (Narro-Robles & Gomez-Dantes, 1995), when 6,955 cases were reported, but decreased as the "*Ae. aegypti* eradication" progressed (see

section 2.1.3). Dengue transmission reappeared in Mexico in 1978, having been absent for almost twenty years (CDC, 1979, 1980). The major epidemics were in 1979-1980, 1982, 1984, 1995-1999, 2007-2010 and 2012-2014. All DENV serotypes have circulated during this 37-year period (Figure 8). Overall, the total dengue cases (both dengue fever-DF- and dengue haemorrhagic fever –DHF- confirmed by laboratory) reported in Mexico were 876,528 (767,296 DF and 109,232 DHF respectively).

The period from 1979-1993 was characterised by an oscillation between epidemics caused by a single serotype to the simultaneous circulation of multiple serotypes in the last years (Figure 8). The circulating serotype reported most frequently during the 80s was DENV-1, followed by DENV-2 and DENV-4. These three serotypes were observed from 1983 to 1985, each causing significant numbers of cases and over a wide geographic distribution: in nearly all (>85%) of the states in the country.

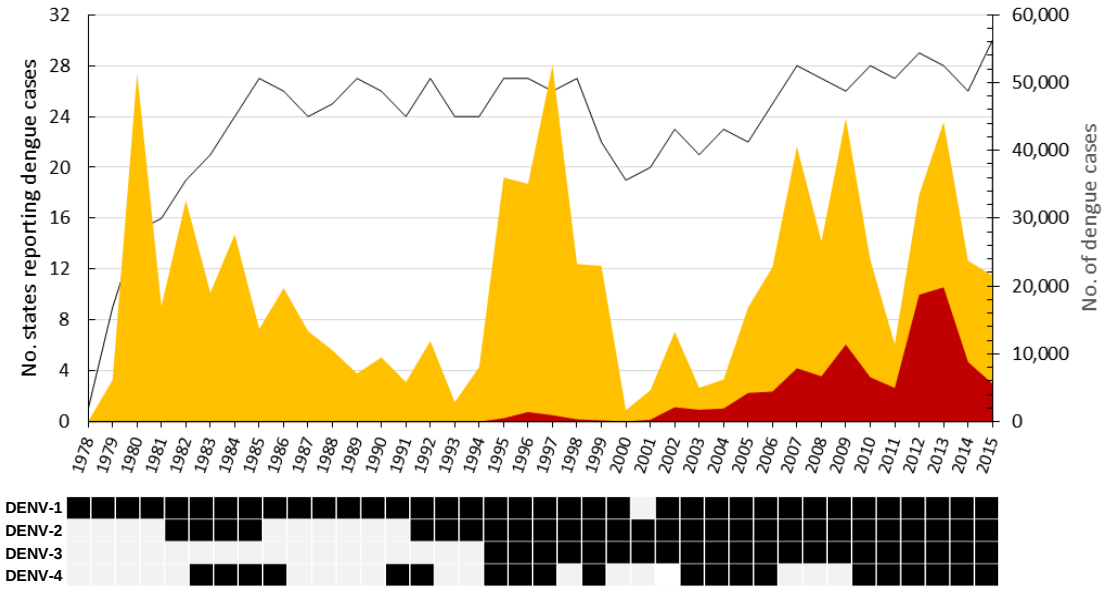


Figure 8. Total confirmed dengue cases, DF (yellow line) and DHF (red line), and DENV serotypes circulating reported in Mexico from 1978 to 2015. The grey line represent the number of Mexican states reporting dengue cases throughout the period. Epidemiological data before 2009 were obtained from the National Centre of Epidemiological Surveillance of the General Directorate of Epidemiology (DGE), Mexican Ministry of Health. Data from next years were obtained from nation-wide, web-based epidemiological surveillance system (SINAVE-DGE).

This pattern changed during the 90s when DENV-2, DENV-3 and DENV-4 were more regularly reported (Figure 8). However, DENV-1 has been associated with most of the DF cases reported. Changes in the incidence and severity of dengue in Mexico suggest an association with the introduction and circulation of different serotypes and genotypes –and some particularly virulent strains- of DENV (Diaz et al., 2006). The severity of dengue in

Mexico seems to have increased with the introduction of DENV-3 and/or the circulation of DENV-2 non-American genotype strains.

Historically, DF cases were distributed as follows: 212,178 (317/100,000 inhabitants) during the 80s, 208,196 (256/100,000) during the 90s, 184,450 (187/100,000) during 2000s and 158,249 (141/100,000) during the period 2010-2015. The last 10 years have witnessed large dengue outbreaks with 216,779 cases from 2006 to 2011 totalling, and 175,972 cases from 2012-2015.

The first DHF cases in Mexico occurred in 1994, and increased over time from 3,607 (4/100,000) during the 90s, to 41,018 (42/100,000) during the 2000s and to 64,598 (58/100,000) during the period 2010-2015. Similarly to DF, there was an increase in the number of cases reported from 2005 to 2015 totalling 99,343 cases (90.9% of DHF reported in more than 20 years). The DHF cases as a percentage of total dengue cases also increased from <2% in the 90s to 18-28% in the 2000s and the period 2010-2015 respectively.

Fatalities caused by dengue have been negligible except for 490 deaths reported during 2009-2015, with a lethality less than 1%.

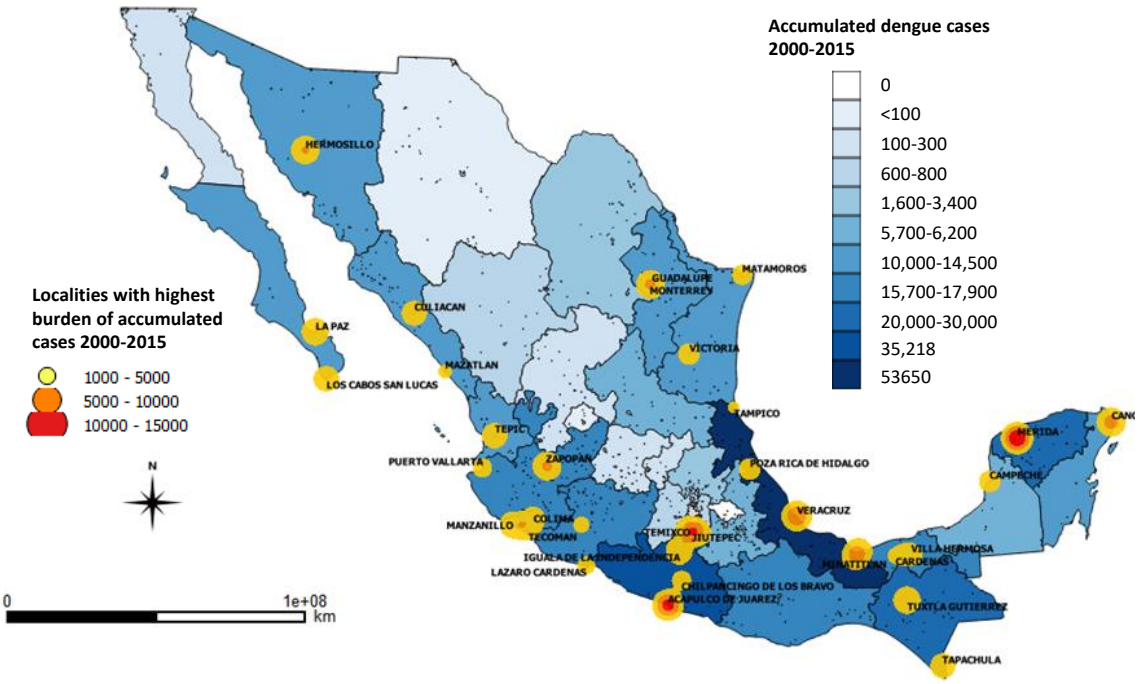


Figure 9. Geographical distribution of dengue cases in Mexico 2000–2015. Mexico is divided into 31 states and one federal district that contains the capital, Mexico City. The map show the 37 localities/cities with high concentrations of cases. Source: DGE technical reports and SINAVE-DGE.

Dengue is hyperendemic across the country with 30 states out of 32 reporting DENV transmission, (Figure 9) but levels of DHF endemicity are quite variable within the country

with most Mexican states having only low or moderate incidence. Almost 41% of dengue cases reported in the last 15 years are concentrated in 37 localities ranging from 38,000 to 1,700,000 inhabitants (Figure 9). Most of these localities/cities are important tourist and commercial centres. Three cities (above of 800,000 inhabitants) that have reported the highest proportion of cases in the last 15 years are Merida (3.7%), Acapulco (3.1%) and Veracruz (2.4%).

These three cities are dengue transmission hot spots, consistently reporting more than 30%-60% of all annual cases within their respective states, with continuous dengue transmission throughout all the year (over 90% of the weeks with dengue cases) but increased transmission (most of cases 82% approximately) occur in the second half of the year during the rainy season (Figure 10). The Ministry of Health recognises these cities as a high-risk area within high-risk municipalities, located in a high-risk state (CENAPRECE, 2014).

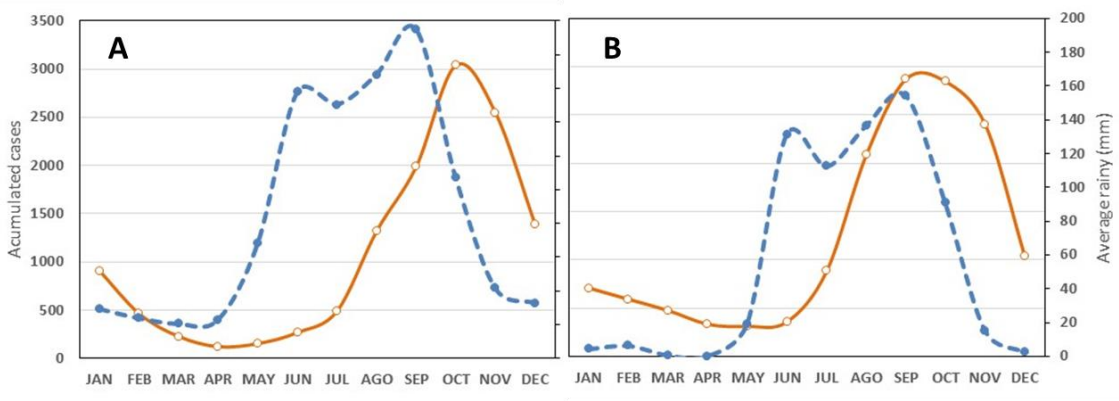


Figure 10. Multiannual behaviour of dengue cases (orange line) and rainfall (blue line) in Mérida (A) and Acapulco (B) Mexico. Monthly average rainfall (1950-2011 for Merida; 1980-2009 for Acapulco) is overlayed to the number of accumulated dengue cases reported per month (2001-2009 for Merida; 2003-2009 for Acapulco). Source: SINAVE-DGE and CONAGUA technical reports.

2.4 Aedes aegypti bionomics

2.4.1. Taxonomic classification and life cycle.

The mosquito *Aedes aegypti* is an insect of the order Diptera, suborder Nematocera, Family Culicidae, subfamily Culicinae, Tribe Aedini, Genus *Aedes*, and subgenus *Stegomyia* (Harbach, 2007; Savage, 2005). There are three recognised subspecies: *Ae. aegypti aegypti*, widely-distributed in tropical and subtropical regions and typically associated with humans; *Ae. aegypti formosus*, the presumed ancestral form, sylvatic and limited to sub-Saharan Africa; and *Ae. aegypti queenslandensis*, now apparently eradicated from the Mediterranean Basin (Powell and Tabachnick, 2013).

The life cycle of *Ae. aegypti* comprises four well-defined stages: egg, larvae, pupae and adult. The first three stages develop in the aquatic environment; and those bodies of water (habitats) where the immature stages develop are commonly called "breeding sites". The life cycle of *Ae. aegypti* can be completed within one-and-a-half to three weeks

Female *Ae. aegypti* lay eggs individually a few centimetres above the water level of the containers that can store water in the domestic and peridomestic environment (Nelson, 1986), each gravid female can distribute her eggs among several oviposition sites (skip oviposition behaviour), so each container can contain a mixture of eggs of different females (Apostol et al., 1994; Colton et al., 2013; Reiter et al., 1995). A single female usually produce on average 100-120 eggs per batch (Apostol et al., 1994). The oviposition period lasts several days and the diel patterns of oviposition both indoors and outdoors are bimodal with consistent peaks at 06:00-08:00 h and 16:00-18:00 h (Chadee and Corbet, 1989). The oviposition activity intensifies during the rainy season due to increased water containers available and growth of the mosquito population. Females are able to fly distances ca. 800 m to oviposit (Honorio et al, 2003; Reiter et al., 1995), and if they do not have access to oviposition sites, can retain their eggs for several days (Chadee, 1997).

In warm climates, embryonic development is completed in 48 hours and larval hatching can occur at any time depending on the temperature and oxygen concentration. In unfavourable conditions, eggs can survive for very long periods (diapause), even for more than a year (Christophers, 1960; Nelson, 1986). The larvae moult four times (four instars), and usually, larval development is completed in one week (Christophers, 1960; Grench et al., 2010). The pupal stage lasts 48 -72 hours (Grench et al., 2010).

The adult mosquitoes usually emerge at a 1:1 ratio of male and female (Grench et al., 2010), with males the first to emerge, and spend their first 24 hours resting, perching on vertical shaded surfaces close to the breeding site. Males begin a short-flight period searching for females to copulate and, females, fly to look for hosts to feed (host-seeking behaviour) between 24 and 72 hours old after emergence (Sanchez-Hernandez, 2011). At both indoor and outdoor sites the copulation periodicity has two significant peaks at 06:00-08:00 h and 16:00-18:00 h. The copulation encounters often occur in and around breeding containers and within houses in close proximity to human bait (Chadee and Gilles, 2013).

Once females take a first blood meal (biting activity), after 48 to 72 hours, they are ready to oviposit. After oviposition, the female restarts host-seeking behaviour for the next batch of eggs (Klowden, 1990). The host-seeking behaviour and biting activity of *Ae. aegypti* are closely related, therefore, both events described biorhythms that overlap, showing two

consistent peaks at 06:00-08:00 h and 16:00-18:00 h in intra, peri, and extradomiciliary sites (Casas-Martinez et al., 2013; Corbet and Smith, 1974; Trpis et al., 1973).

The period between host-seeking and oviposition behaviour defines the start and end of the gonotrophic cycle. Studies in the south of Mexico report that the time required to complete the first gonotrophic cycle in *Ae. aegypti* averages 2.8 days at an average temperature of 26.2°C (Tamayo-Dominguez, 2011).

The adult stage can range from two weeks to a month depending on environmental conditions (Grench et al., 2010).

The mosquito *Ae. aegypti* is an antropophilic, endophilic, endophagic and day-biting species, it has an eminently domestic behaviour and closely related to humans (synanthropic). Residential premises (house and peridomicile) offer important habitats for *Ae. aegypti*; female mosquitoes emerging from productive breeding-sites move in and out the houses in search of food (human blood), refuge and mating and oviposit at the suitable breeding-sites to complete their life cycle. Epidemiologically, this is particularly important to disrupt vector-borne transmission of disease, overall if most transmission occurs indoors. In particular, the prevention of endophagy by *Ae. aegypti* is obviously important to stop transmission of virus from infected mosquitoes to susceptible humans, but also to stop *Ae. aegypti* from feeding upon infected humans.

2.4.2. *Aedes aegypti* in Mexico.

The mosquito *Ae. aegypti* was very probably introduced to Mexico on ships after first Europeans arrived in the early 16th century. The first confirmed outbreak of yellow fever in the New World occurred in the Yucatan, Mexico in 1648 (Powell and Tabachnick, 2013). This vector is widely distributed in Mexico predominantly at low level elevations (< 610 m) above sea level (Ibanez-Bernal and Gomez-Dantes, 1995). However, it can be found at higher elevations. For example, well-established populations of *Ae. aegypti* have been reported at 1,630 m in Tlacapayan, state of Morelos (Ibañez-Bernal, 1987) and up to 2130 m at Puebla City (Lozano-Fuentes et al., 2012) which is the highest elevation record. The great plasticity of this species and its ability to colonise new environments increases the risk of DENV outbreaks. Nowadays, autochthonous DENV cases are reported in 30 of the 32 states (Figure 9).

Based on the results of the larval surveys and characterization of *Ae. aegypti* breeding-sites, it was found that the importance of containers is related with two characteristics, mainly, preference as oviposition site (availability/operation) and mosquito production (female/type of container) (Ordoñez-Torres, 2004). Studies in Mexico on productive container types for

Ae. aegypti immatures have incriminated as the most important breeding-sites: disposable containers (i.e. cans, tires, bottles, vases, scrap metal, etc.), and buckets/pots, mostly related with rain-filled objects left in the backyards (Garcia-Rejon et al., 2011; Manrique-Saide et al., 2008 & 2011; Winch et al., 1992); and large containers, such as tanks and drums, mainly in localities where public services of piped-water is limited (Ulloa et al, 2010). The variation in the availability and productivity of the different types of useful and disposable containers as sites for oviposition female *Ae. aegypti* depends on cleaning habits of the local human population, degree of urbanization and the season (Garcia-Rejon et al, 2011; Rubio et al., 2011).

The dispersal of *Ae. aegypti* throughout Mexico probably occurs mainly through transport of eggs, larvae, and adults in discarded bottles, cans, appliances, tires, and other type of containers along commercial and human migration routes (Winch et al., 1992). In terms of its distribution and gene flow, the north-eastern Mexican populations of *Ae. aegypti* are genetically different from and had lower genetic diversity than Yucatan and Pacific coastal population. While Yucatan and Pacific populations are genetically more homogeneous. In general it is suggested that under distances of 150 km, Mexican populations of *Ae. aegypti* can be expected to remain genetically uniform (Gorrochotegui-Escalante, 2002), in others words, the gene flow among populations decreases with increasing geographic distances >150 km suggesting that genes, for example, affecting DENV susceptibility or insecticide resistance could remain uniformly spread within 150 km area by high rates of gene flow. Nevertheless, recently studies in Mexico about *Ae. aegypti* insecticide resistance gene-frequencies showed evidence that they are not uniform among populations within <150 km of one another (Saavedra-Rodriguez et al., 2014) and even more, among blocks within the same locality (Deming et al., 2016). Despite high rates of gene flow, insecticide resistance evolution occurs locally, probably driven by local selection, i. e. local insecticide pressure (Saavedra-Rodriguez et al., 2014).

On a smaller scale, it has been demonstrated that adult *Ae. aegypti* dispersion occurs at relatively short distances (Getis et al., 2003; Harrintong et al. 2005; Scott & Morrison, 2002). Studies in Mexico report, for example, females of *Ae. aegypti* are dispersed on average 30.5 m to a maximum distance of 120 m in an urban environment (Ordoñez- Gonzalez et al., 2001), while males of the same species were dispersed between 12 and 166 m from the point of release in a rural environment (Valerio et al., 2011). Other studies in Mexico suggest that dengue transmission is, at least initially, peridomestic; the highest risk is inside the first 50 meters of an index case (vicinity) (Martinez-Vega et al., 2015). This is because the vector is

essentially static, spends most of its adult life within or in the close vicinity of human habitations. In one field study in the south of Mexico, the majority of adult, *Ae. aegypti* females and males, were found inside the houses (95% and 92% respectively, compared with the peridomicile), most commonly (60-63%) resting in the bedrooms (Garcia-Rejon et al., 2008). Moreover, DENV-infected *Ae. aegypti* females were collected from homes of dengue patients up to 27 days after the onset of symptoms (Garcia-Rejon et al., 2008). This has clear epidemiological implications, suggesting that humans rather than mosquitoes are the primary mode of DENV dissemination within and among localities; and the house as an important place for human-vector contact and an epidemiologically significant point of contact for DENV transmission.

2.5 Chemical control strategies

Chemical control of *Ae. aegypti* mosquitoes is an important part of integral strategies for dengue prevention and control in all countries worldwide, including Mexico. Dengue control programs in Mexico employ a range of chemical interventions for *Ae. aegypti* control: 1) "Abatización" with 1% granules of Temephos® used as a larvicide for treating permanent breeding-sites which cannot be eliminated (although others options are available, such as Spinosad, Bti and IGRs); 2) ground vehicle-mounted ULV space spraying using a variety of insecticide chemical groups (mainly PYs and OPs) in areas/clusters with outbreaks and/or high entomological risk; 3) focal intra-domiciliary spraying (IS) using motorized-portable equipment at houses of probable dengue cases with a variety of potential insecticides (mainly PYs and some CAs) (Figure 11).

There is a controversy over the impact of traditional chemical interventions on *Aedes* abundance and DENV transmission. Most authors conclude that there is no solid evidence to support the effectiveness of mosquito control by local vector control programmes (Ballenger-Browning and Elder, 2009; Bowman et al., 2016). However it is agreed that the maximum impact in reducing vector populations is achieved when control interventions are implemented with high coverage and integrated approach, in combination with multiple control strategies, including clinical trainning, educational programmes, community-based intervention (Erlanger et al., 2008; Pilger et al., 2010).

In this section a brief review of chemical control strategies for *Aedes* control worldwide and particularly in Mexico is given.

2.5.1. Traditional chemical methods for vector control.

Adult space-spraying. The rational of adult space-spraying for dengue control is that it kills adult mosquitoes and therefore, has an immediate impact on population numbers, and so rapidly reduces transmission. In epidemic situations this measure is used to reduce the populations of adult mosquitoes rapidly by outdoor space-spraying with insecticides using thermal/cold fogging in ULV aerosols (WHO, 2003). Giglioli (1979) stated that an immediate reduction of 97% in the vector abundance is required to achieve effective control of a dengue epidemic.

However, there is much controversy over the efficacy of ULV space spraying from truck-mounted equipment for control of *Aedes* mosquito populations (Esu et al., 2010; Pilger et al., 2010). While some argue that ULV space-spraying has an insignificant effect on the abundance and dynamics of mosquito populations, others consider that ULV is the last resort for combating mosquitoes and hence dengue transmission, providing rapid and effective emergency control at the time of outbreaks of disease in urban and periurban areas. The experts conclude that its impact is, at best, limited and of short duration (Esu et al., 2010; Pilger et al., 2010). Nevertheless, this method is still utilised regularly for seasonal control of *Ae. aegypti* and other mosquito species in many places including Mexico.

Preferred insecticides for this type of treatments are OPs and PYs (WHO, 2006). Currently the Mexican National Programme for Dengue Vector Control approves the use of the OPs (chlorpyriphos and malathion) and PYs (Bifenthrin and Sumithrin) for ULV space-spraying applications (SSA, 2014).

Intra-domiciliary spraying. Indoor residual spraying (IRS) – defined by the World Health Organization as the application of long-acting chemical insecticides on the walls and roofs of houses and domestic animal shelters – is widely used for the control of some vector borne diseases such as Chagas disease (Gürtler and Yadon, 2015). Particularly for mosquitoes is a proven method for controlling adult resting indoors (Mani et al., 2005; Dzul-Manzanilla et al., 2014).

In Mexico the intra-domiciliary spraying on walls using a motorized portable equipment is preferred to the traditional Hudson X-Pert equipment because of its simplicity. The list of insecticides approved for this method in Mexico includes PYs (cyfluthrin, γ-cyhalothrin, α-cypermethrin, deltamethrin, cyfluthrin and bifenthrin) and CAs (propoxur and bendiocarb) (SSA, 2014).

Larvicidal treatment. Perhaps the most effective preventative measures aim at reducing the population density of the vector *Ae. aegypti*. Their efficacy is maximal when larvae are restricted to breeding sites accessible and limited in size and numbers. Various insecticides are used (oils, Bti, IGRs, OPs) for larvicidal trearment in mosquito breeding sites (WHO, 2006).

"Traditional" control of *Aedes* breeding-sites by the Mexican Ministry of Health included for 30 years short clean-up campaigns called "descacharrizacion", generally at the start of the rainy season, considered locally as the "dengue risk season" which incorporate messages transmitted on television, radio and newspapers announcing special day(s) for refuse collections. In addition, vector control personnel performed city-wide chemical interventions: the "Abatización" (1% granules of Temephos) for permanent breeding-sites. As there is no evidence of temephos resistance in *Ae. aegypti* populations from Mexico, this OP is the insecticide of choice. However, the use of Bti, IGRs and spinosad are also available (SSA, 2014).

2.5.2. Vector control programme in Mexico.

The Mexican dengue vector control program includes "integrated chemical vector control" and "targeting households and areas at risk" (Figure 11). The program still employs today "Abatización" and ULV space-spraying, but actions are synchronised and target areas/clusters with outbreaks and/or entomological risk. The local vector control programmes use the epidemiological and entomological information available within the web-based, geographically enabled, dengue integral surveillance system (Hernandez-Avila et al., 2013) to evaluate, identify and prioritize risk areas to implement vector control interventions. In addition, focal IS at houses with clinical dengue cases (probable dengue cases) has been introduced. When a clinical case is reported within the web-based dengue surveillance system, the local vector control programme implement IS in all houses surrounding the home of the positive case and in the home of the positive case, in combination with the application of adulticide space-spraying with truck-mounted equipment and the active distribution of larvicide in all blocks surrounding the block where the home of the positive case is located, including the block of positive case.

However, the costs associated with the maintenance of the current program have increased. In terms of environmental management, the "descacharrizacion" is still performed; but more recently, a strategy known as "Patio limpio" (hereafter referred as tidy backyard) has been encouraged for the control of dengue vector breeding sites with community participation. Tidy backyard includes rubbish elimination and destruction of

containers that are potential mosquito breeding-sites and the integration of covering/storing/impeding techniques for domestic water storage containers and water for human consumption. Nevertheless, tidy backyard, as a community-based environmental approach for integrated vector management, is in an initial phase of implementation and yet still far from ideal results.

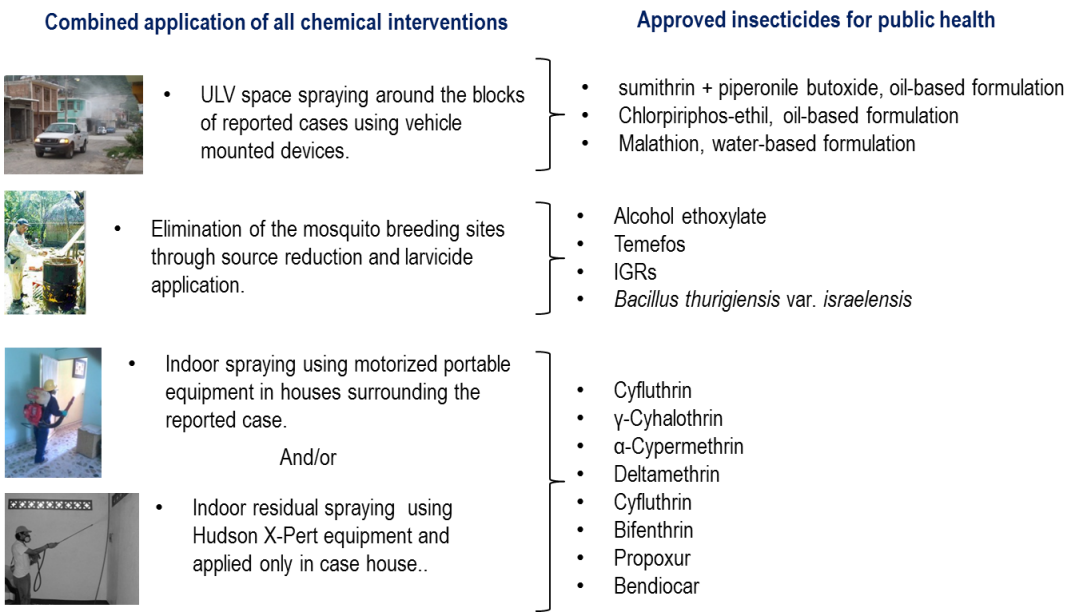


Figure 11. Major chemical strategies for dengue control in Mexico. Combined application of all chemical interventions are focused on houses of clinical cases. Synchronised interventions are also applied by targeting areas/clusters with outbreaks and/or entomological risk.

However, these routine control interventions have not had the required results, not only in Mexico but elsewhere (Bowman et al., 2016; Horstick et al., 2010), due to insufficient coverage, transient effects, failure to implement as an integrated strategy, and last but not least, they were usually intense only during but often behind transmission periods e. g. in emergency situations. Program sustainability is key, as mosquitoes rapidly return once prevention methods are relaxed (Nathan, 2012). The evidence states that to ensure the success and the long term sustainability of an *Ae. aegypti* control programme it is essential that there is a partnership between government control agencies and the affected communities (Espinoza-Gomez et al., 2002; Pilger et al., 2010).

2.5.3. Insecticide-treated material (ITMs).

The ITMs are tools that have been investigated in recent years for targeting the adult mosquitoes. ITMs are a highly effective, safe, affordable, low-tech, long-lasting and simple intervention which has been shown to prevent the transmission of a variety of vector-borne

diseases by having an individual effect (i.e. bed nets preventing the vector from blood feeding) and having a community effect (i.e. by reducing the vector lifespan and population). Based on the successful control demonstrated against nocturnal endophilic vectors *Anopheles* spp and protective efficacy of ITMs (in the form of -treated bednets (ITNs)), in reducing malaria transmission (Gu and Novak, 2009; Lengeler, 2009; Rafinejad et al., 2008; WHO, 2005) the WHO Dengue Scientific Working-Group of 2006 identified the development/evaluation of ITMs as a primary global research stream (McCall and Kittayapong, 2007).

Initial ITN technology was dramatically improved with the development of technologies to produce long-lasting insecticidal nets (LLIN) that do not require retreatment. LLINs have contributed largely in the reduction of malaria cases, estimated around 68% (Bhatt et al., 2015). However, considering the diurnal activity patterns of *Aedes* mosquitoes LLINs are not expected have the same effect as in malaria vectors.

In the case of dengue vectors, it´s argued that a major factor in the failure of previous prevention methods is their focus on eliminating immature forms of *Ae. aegypti*, rather than targeting the adult mosquitoes that actually transmit the disease (Morrison et al., 2008). The dengue vector *Ae. aegypti* is a highly synanthropic mosquito living in close-dependence with human-made ecosystems (Getis et al., 2003; Scott and Morrison, 2002). The challenge is to reduce the infected adult vector populations and/or their interaction with humans affecting dengue-virus transmission (Achee et al., 2015; Morrison et al., 2008).

2.5.4. Long-lasting insecticidal mosquito nets (LLINs) for dengue control.

The LLINs have been evaluated for their protective efficacy against dengue. In Haiti, LLIN bednets showed an immediate effect on immature based indicators and dengue transmission, and extended for the following 5–12 months after their deployment (Lenhart et al., 2008). Recent studies have suggested the potential of LLIN as window curtains (Insecticide Treated Curtains (ITC)), to reduce dengue vector densities to low levels and potentially impact on dengue transmission. In Thailand, ITCs showed immediate effects on immature based indicators at 6 months (Vanlerberghe et al., 2013). However, in some cases housing style could affect the ITC interventions favoring the entrance of mosquitoes and move through houses without ever coming into contact with insecticide (Lenhart et al., 2013). In a field trial carried out in Mexico, ITC interventions did not affect the indoor adult population, but it seemed to reduce the number of DENV infected females and the human infection prevalence in some areas (Loroño-Pino et al., 2013). Combining ITCs with targeting productive breeding-sites in Mexico (Kroeger et al., 2006), Venezuela (Kroeger et

al., 2006; Vanlerberghe et al 2011a) and Guatemala (Rizzo et al., 2012) improved the impact on *Ae.* ae*gypti*.

However, two key challenges have emerged from the initial field trials. First, coverage of the interventions based on ITC typically falls dramatically over time (Tun-Lin, et al., 2009; Vanlerberghe et al., 2011b, 2013), undoubtedly compromising efficacy throughout the community, and a problem common to many control strategies. In Guatemala (Rizzo et al. 2012) and Mexico (Loroño-Pino et al., 2013) also found this, noting that families would removed or tie back the curtains to increase ventilation during the day, compromising the utility of the intervention.

Secondly, as PYs are the only insecticide class recommended by WHO for the impregnation of insecticide nets (WHO, 2014), the development of PY resistant *Ae. aegypti* populations is a major concern. However, the impact of PY resistance on the efficacy of LLINs in preventing malaria has proven difficult to determine, partly because it will depend on the strength of resistance in the population (Ranson and Lissenden, 2016)

2.5.5. House screening.

Protection against mosquito bite and disease transmission with mosquito netting in houses has been historically observed as fundamental technique of mosquito-borne diseases control in the early 1900s. In the 1880s, in Cuba, Carlos Finlay recommended using physical measures as a barrier to the mosquitoes that he assumed were transmitting yellow fever (reviewed by Ferroni et al., 2011). However, the first published work evaluating the screening houses as physical measures to prevent mosquito-borne diseases was reported by Celli in Italy for malaria control (Celli, 1900). The Italian experience led to widespread screening of houses against mosquitoes in malarious areas, not only in Italy, but around the world (reviewed by Lindsay 2002).

Using netting to screen the most important points of entry into a house, such as windows and doors, prevents the entry of adult mosquitoes (Schofield et al 1990). "Mosquito-proofing" of houses is a form of environmental management based on changes to human habitation to exclude vectors and reduce man-vector-pathogen contact (WHO, 1982). House screens have been shown to provide protection against malaria (Kirby et al., 2008; Lindsay et al., 2003; Walker, 2010) and to be widely accepted by communities (Kirby et al. 2010).

The integration of house-screening with LLIN (LLIS) to dengue control programs has been evaluated in Vietnam in 500 households. Nguyen et al. (1996) and Igarashi (1997) evaluated an intervention with permethrin nets covering all openings of houses (in addition to routine anti-*Aedes* health education and control measures) and reported a significant

reduction (close to 100%) in the number of houses positive for dengue vectors. Furthermore indoor *Ae. aegypti* were undetectable levels for seven months, while in the control group infestation gradually increased during the epidemic season and a positive impact in preventing DENV transmission during the epidemic season (at 6 months after intervention) was observed (Igarashi, 1997; Nguyen et al. 1996). At the start of the current study, this Vietnam trial was the only published study on the effect of LLIS on dengue parameters. In the following chapters, the results from trails of LLIS in two Mexican cities are reported. As PY resistance is known to be prevalent in dengue vectors in Mexico, it was also necessary to quantify the level of resistance in the study sites prior to the trial and to see if the resistance levels were altered by the introduction of LLIS.

Chapter 3: Studies on the susceptibility status and resistance mechanisms to insecticides in *Aedes aegypti* populations from Acapulco Guerrero and Mérida Yucatan, Mexico.

3.1 Context of the Study

Insecticide resistance, particularly to pyrethroids that have been widely used, is a significant threat to the success of dengue-vector control programmes. The development of insectice resistance by *Aedes* mosquitoes was first documented in 1947 when the salt-marsh mosquitoes *Ae. taeniorhynchus* and *Ae. sollicitans* began to show resistance to DDT in Florida, USA (Brown, 1958; Brown, 1986). Later, the first indication or evidence of DDT-resistance was recorded for a Surinam, Dominican Republic, Venezuela and Trinidad strain of *Ae. aegypti* between 1953 and 1955 respectively (reviewed for Brown, 1958). The *Ae. aegypti* populations were very susceptible to DDT, and house-spraying with this insecticide which began in 1948 virtually eliminated this species in several countries of Americas, including Mexico, until the late 1970´s (Brown, 1958; Torres, 1995). Today resistance to many of the insecticides used in control programmes have been well documented in *Ae. aegypti* populations from many countries (Vontas et al., 2012).

In Latin American resistant populations of *Ae. aegypti* have been detected in Cuba, Brazil, Puerto Rico, Perú, Panamá, Venezuela and Colombia (Alvarez et al. 2006; Aparecida et al. 2004; Beserra et al. 2007; Chavez et al. 2005; Macoris et al. 2003; Pereira-Lima et al. 2006; Prieto et al. 2002; Rawlins 1998; Rodríguez et al. 2004; Rodriguez et al., 2007; Salazar et al. 2007; Vargas et al. 2006).

In Mexico the extensive and intense use of DDT for agriculture industry since 1950-1987 and the long and intensive use of pyrethroids in public health during 90´s, particularly permethrin-based formulations, promoted an intense selection pressure for the evolution of resistance in *Ae. aegypti* (Brengues et al., 2003). Dramatic increases in the frequency of the 1016I mutation were recorded from 1996 to 2009 (Bobadilla-Utrera, 2010; Loroño-Piña et al 2013; Ponce-Garcia et al., 2009; Saavedra-Rodriguez et al., 2007; Siller et al., 2011). Permethrin has been prohibited since then and it has been replaced by others pyrethroid-based formulations, i.e. deltamethrin, sumithrin, bifenthrin, cyfluthrin and lamda-cyhalothrin are commonly applied for adult mosquito control in Mexico (SSA, 2008b).

Metabolic resistance has been also reported for Mexican *Ae. aegypti* populations. Elevated esterase levels are found in permethrin-selected populations from Quintana Roo State in the

Peninsula of Yucatan (Flores, et al., 2006); esterase and oxidases-based dextofication have been also reported as important resistance mechanism for permethrin in the north of Mexico (Flores et al., 2005; Flores et al., 2009). GST and esterase-based mechanism have been reported in pyrethoid resistant populations from Guerrero State in the pacific coast of Mexico (Aponte et al., 2013).

There is strong evidence of resistance to pyrethroids in multiple *Ae. aegypti* populations in Mexico, including cities of epidemiological importance for dengue transmission as Merida and Acapulco (Gonzalez et al., 2012; Ponce-García et al., 2009; Rodríguez et al., 2010; Saavedra-Rodríguez et al., 2007; Siller et al., 2009) and this is a major concern for the Mexican dengue control program.

Detecting resistance at an early stage in the selection process, continuously monitoring the effect of control strategies on resistance, and providing baseline data for program planning and pesticide selection before the start of control operations are important strategies for insecticide resistance management (Brogdon and McAllister, 1998a). This chapter establishes baseline data on insecticide resistance in sites targeted for new insecticide based interventions.

3.2 Materials and methods

3.2.1. Study sites.

The study took place in the urban area of the municipalities of Merida, located in the Peninsula of Yucatan (20º 45' and 21º 15' North, 89º 30' and 89º 45' West) and Acapulco, located in Guerrero State (17° 36' North, 99°57' West), on the South of Pacific Coast and South East of Mexico respectively (Figure 12). Both localities have received regular vector control, including ULV with an organophosphate (i.e. chlorpyrifos) and intradomiciliary space spraying with a carbamate (i.e. propoxur and bendiocarb), and temephos for breeding sites control (according to Ministry of Health normativity) since they are *Aedes*-endemic Mexican cities and considered important epidemiologically for dengue transmission (SSA, 2008a).

Merida study site. Merida, the Capital of Yucatan State, has 814,435 inhabitants who live in 272,418 households distributed in approximetly 485 neighbourhoods (INEGI, 2010). Overall, infrastructure and public services of piped-water is good in the north of the city (which represent the 23% of all houses in the city) where 91% of the houses have regular supply of piped-water. Coverage is less complete in the south side of the city, where the 75%

have piped-water coverage 83% of the time and 2% have water just 12.5% of the time (Domínguez-Aguilar, 2009).

The average altitude of the city is nine metres above sea level. The climate in Merida is mainly warm with an annual average temperature of 26°-27°C (36°C max- 18°C min). Two seasons can be clearly distinguished: a rainy season, in May to October (with most of the rainfall from June-October) and a dry season from November to April. The rainy season is the *dengue risk* season (the transmission increase 80% approximately) and marks the starting point for major vector control activities. Over half of all dengue cases in Yucatan in the last 6 years have occurred in Merida, with continuous dengue transmission throughout all the year (over 90% of the weeks with dengue cases). Therefore, the majority of vector control actions implemented for local Ministry of Health (MoH) are focused in Merida.

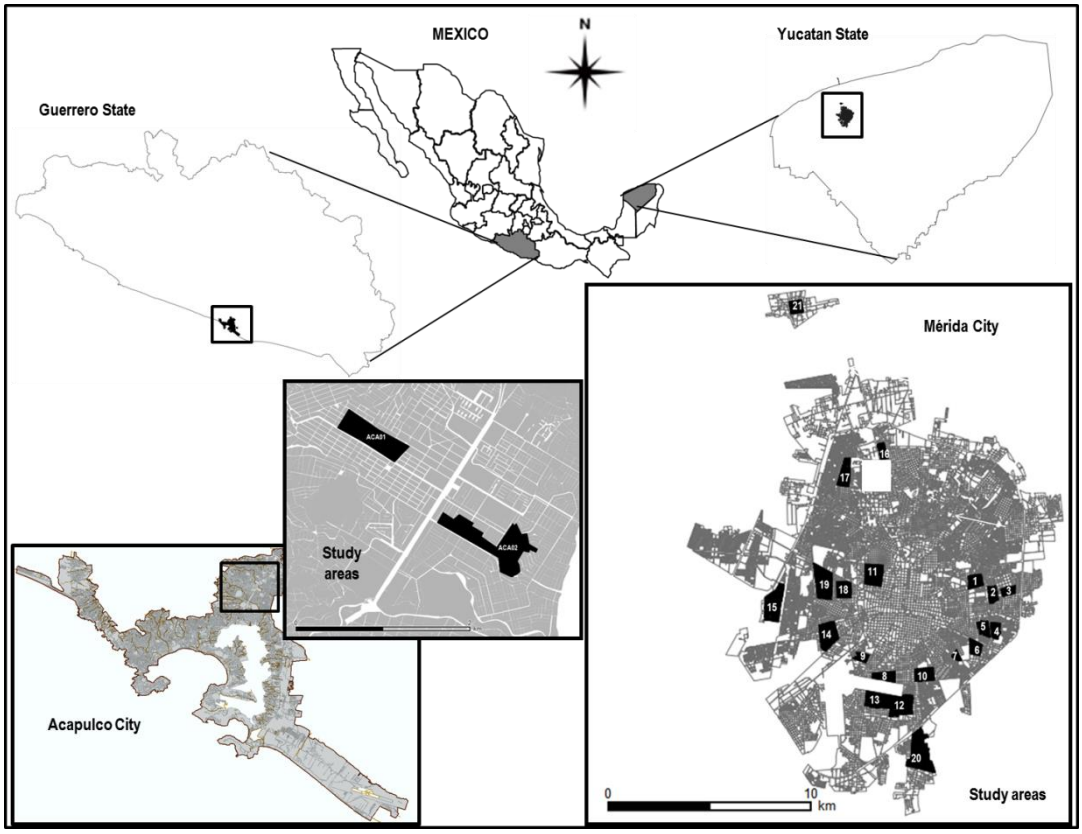


Figure 12. Study areas, showing the location of the study sites. In Acapulco, two sites were located in the "Ciudad Renacimiento" neighbourhood. In Merida, 21 sites were distributed throughout the city: 1. Manuel A. Camacho; 2. Pacabtun; 3. Fidel V.; 4. Vergel III; 5. Vergel II; 6. San A. Kahua; 7. U.H. Morelos; 8. Castilla C.; 9. Manzana 115; 10. Cinco C.; 11. Centro; 12. San J. Tecoh; 13. San A. Xluch; 14. Mulsay; 15. Juan Pablo II; 16. Cordemex; 17. Francisco M.; 18. Bojorquez; 19. Yucalpeten; 20. Plan de A.; 21.Dzitya.

Temephos was historically used for larval control activities, but this practice is no longer widespread due to the type of breeding-sites present in Merida (previous studies on

productive container types for *Ae. aegypti* immatures have incriminated disposable containers and buckets/pots, mostly related with rain-filled objects left in the backyards [Garcia-Rejon et al., 2011; Manrique-Saide et al., 2008; Winch et al., 1992]). Pyrethroid (PY) adulticides, such as permethrin, were used for ULV space spraying from 1998 to 2010, and later replaced by the PY sumithrin in 2011. Since January 2012 the MoH of Yucatan replaced the use of PYs and started using the organophosphate (OP) chlorpyrifos for ULV space spraying in the urban area of Merida city. Eventually the MoH stopped using PYs for indoor spraying and they were replaced for the CAs propoxur. This as result of the evidence for PY resistance reported for the Yucatan Peninsula (Ponce-García et al., 2009; Siller et al., 2011).

Acapulco study site. The municipality and city of Acapulco is the major seaport in the state of Guerrero on the Pacific coast of Mexico (Figure 12) and is a very popular tourist beach resort destiny. Acapulco has warm to hot weather throughout the year, with an annual average temperature of 27.9°C (31.3°C max- 24.5°C min). The rainy season is from June to October. As in Merida, the rainy season is considered the *dengue risk* season and therefore, the majority of vector control actions are implemented during this season.

Within the city, the neighbourhood of Ciudad Renacimiento is a dengue endemic community that has been deemed a high priority for dengue control by the local MoH authorities because it reports above 10% of all annual dengue cases from Acapulco. Ciudad Renacimiento neighbourhood has a total 48,460 inhabitants (6.55% of total population of Acapulco) and 11,725 premises (311 ha.). While the neighbourhood is primarily residential, it also contains an abundance of small businesses, schools, markets; automobile/tyre repair shops and churches (INEGI, 2005). However, houses were constructed on ground prone to flooding with deficient drainage (Salgado, 2005) and other infrastructure and public services deficiencies i.e. water supply (62% of the houses have piped-water on the premise but only 29% have direct water supply within the house). Therefore, productive and important breeding-sites identified in Ciudad Renacimiento include: 200L water-drums, large cement washbasins and buckets (Internal reports, data no published).

Temephos historically has been used for larval control activities. PY adulticides, mainly permethrin, were used for both outdoor ULV space spraying and indoor spraying from 1998 to 2009 and later replaced by the PY sumithrin (for outdoor ULV space spraying) and other types of PY such as lamda-cyhalothrin and deltamethrin (for indoor spraying). In 2010 the local MoH decided to replace PYs due to evidence (Aponte et al., 2013) for high levels of permethrin and deltamethrin resistance and the presence of kdr mutation (1016I) in several *Ae. aegypti* populations from Guerrero, including Acapulco. Currently the OP chlorpyrifos is

used for outdoor ULV space spraying and a CA (bendiocarb initially, and later propoxur) for indoor spraying in Acapulco.

3.2.2. Mosquito strains/samples collection.

A cross-sectional entomological survey was carried out during the middle and end of the 2012 rainy season in both study sites; Merida in July to September 2012, prior to installing window/door screening with LLINs and Acapulco in August-November 2012, during installing LLINs screening (hereafter LLIS). Mosquito specimens were emerged from egg batches collected from a network of weekly-serviced ovitraps along 21 locations in Merida and 2 locations (covering three clusters for both arms, no-intervention and LLIS arm) in Acapulco (Figure 12). Briefly, field-collected eggs were transferred to rearing trays in UCBE-UADY insectary and allowed to hatch. Eggs from multiple ovitraps in the same clusters were pooled (minimum 2 ovitraps). Larvae were reared according to standard procedures (Gerberg et al., 1994) until adult emergence in order to obtain 1-3 day-old F1 adult generation of each egg batch of clusters.

Batches of 1-3 day-old female mosquitoes were subjected to standard CDC bottles bioassays (see section 3.2.3). 24 h survivors and dead mosquitoes from CDC insecticide susceptibility tests with 15 µg/mL permethrin were stored separately in properly labelled Eppendorf tubes containing silica gel and maintained at –20 °C. Other batches of female mosquitoes from ovitrapping were maintained and stored separately at –70 °C. All samples were sent to the CDC, Atlanta, USA on gel ice packages for molecular and biochemical analysis respectively.

The New Orleans and Rockefeller strain of *Ae. aegypti*, were kindly provided by Centre for Disease Control (CDC), Atlanta, USA and they were used as the standard susceptible strain for all assays.

3.2.3. CDC bottles bioassays.

All CDC bottle tests were performed in UCBE-UADY in Merida, Yucatan. Bioassays were conducted on mosquitoes from clusters and from the external controls "Tres Palos" 10 km from Renacimiento neighbourhood in Acapulco, and "Dzitya" 6 km from the nearest Merida´s cluster (Figure. 12). The external controls were small localities (less than 4,000 inhabitants and less than 1000 households) considered historically by the local Ministry of Health to have low risk areas of dengue transmission, and consequently they receive less pressure of insecticide use (chemical interventions for vector control) in comparison with Acapulco and Merida.

Each population was evaluated against permethrin (15 µg/mL), alpha-cypermethrin (10 µg/mL), propoxur (12.5 µg/mL) and chlorpyrifos (14 µg/mL), using the suggested diagnostic doses (DD) and diagnostic times (DT) previously established for the Rockefeller susceptible strain (CDC, 2010). In the case of propoxur the DD and DT used were based on a study by Fonseca-González (2008) using the Rockefeller strain. Chlorpyrifos 14 µg/ml/bottle was used as recommended by Regional Centre of Public Health Research (CRISP) from Tapachula. In order to confirm the chlorpyrifos DD a range of five different concentrations of this insecticide (including the suggested DD) were used per bottle (e. g. 2.5, 5, 14, 17, 25 µg/mL), as outlined in the CDC guideline. Using adult mosquitoes from the susceptible reference strain New Orleans the concentration 14 µg/mL killed 100% representing the saturation point (the lowest doses that reached 100% of mortality at 30 min, and above which the mortality remained the same).

Knock-down (KD) times resulting from tarsal contact with treated surface were measured. Briefly, 1-3 day old female *Ae. aegypti* mosquitoes (at least 10 per bottle, four replicates per test) were exposed to the different insecticides according to DD and DT. The KD effect on mosquitoes was recorded every 10 minutes until 100% KD was recorded or up to 2 hours. A mosquito was considered knocked down if it was unable to stand or fly in a coordinated way KD at 30 minutes was used as indicator of the population susceptibility. With the aim to provide evidence for additive, possibly metabolic resistance mechanisms, survival status after 24 h recovery period was also considered. For this purpose, all mosquitoes were transported to recovery cups at the end of the 2 hour exposure, and provided with a source of sugar liquid. Additionally, a control bottle test was set up in which mosquitoes were only exposed to bottle´s surface treated with acetone alone (without insecticide active ingredient). If KD between 3% and 10% was observed in the controls, the percentage was recalculated using Abbott's formula (Abbot, 1925)

The intensity of resistance was evaluated for those *Ae. aegypti* population which showed KD resistance to DD of insecticide tested (<90%). The resistance intensity was established using CDC bottles (four replicates per dose) treated with 2, 5 and 10 fold the diagnostic dose of insecticide plus a control.

New Orleans was used as the standard susceptible strain. For every 4-6 CDC bottle tests using field strains, a set of CDC bottles bioassays was performed using the susceptible strain as control test.

3.2.4. Biochemical assays.

Mosquito specimens were emerged from eggs collected by ovitraps. Egg batches came from a subsample of 10 clusters selected for all of 20 clusters from Merida: five cluster from intervention arm (MER01 [Manuel A. C.], MER03 [Fidel V.], MER07 [U.H. Morelos], MER09 [Manzana 115], MER16 [Cordemex]) and five cluster from no-intervention arm (MER04 [Vergel III], MER08 [Castilla C.], MER13 [San A. Xluch], MER17 [Francisco de M.], MER20 [Plan de A.]) including the external control (MER21 [Dzityia]). For Acapulco egg samples were collected from three cluster for both, LLIS intervention and no-intervention arm (all ovitrap subtrates of each were put together by arm).

Batches of 30 unfed one-day-old frozen female mosquitoes of the F1 generation per location were individually homogenized in 100 µl 0.01 M KPO4 buffer (pH 7.2), and then diluted to 2 ml with the same buffer. Aliquots of 100 µl in triplicate were transferred to individual wells of a 96 well microtiter plate. Biochemical assays were performed according to Brogdon and McAllister (1988, 1998b, 1997) to evaluate activities of three different enzyme families (oxidases, esterase, glutathione-S-transferase [GST]) and to measure the insensitivity to acethylcholinesterase (iAChE). The enzyme reactions were calculated by reading absorbance in a Biochemical spectra MAX 340 microtitre plate reader (Molecular Devices) connected to a computer through the SoftMax Pro 3.0 software. Results were expressed as a frequency distribution of the absorbance values. The absorbance values of the susceptible Rockefeller strain was considered the resistance threshold. Biochemical assays were performed in CDC laboratory from Atlanta, U.S.A.

Esterase assay. One hundred µl of β-naphthyl acetate solution (56 mg of β-naphthyl acetate diluted in 20 ml of acetone in 80 ml of potassium phosphate [KPO_4] buffer pH 7.2) were added to separate replicates of homogenate. The enzyme reaction was left for 20 mins at room temperature before the addition of 100 µl of fast blue stain solution (100 mg fast blue Sigma-Aldrich™ in 100 ml distilled water) to stop the reaction. Three negative controls were prepared for each plate with 100 µl of KPO_4 buffer, 100 µl of β-naphthyl acetate solution and 100 µl of stain. An additional three wells containing 100 µl of β-naphthol (50 mg β-naphthol in 10 ml of acetone, in 90 ml of KPO_4, and diluted 1:70 with KPO_4), 100 µl of β-naphthyl acetate and 100 µl of stain were run as positive controls. The amount of product from the enzyme reaction was calculated by reading absorbance at 540 nm in the thermoMax plate reader as an end point.

Oxidase assay. The total amount of haem containing protein in each mosquito was titrated using the haem-peroxidase assay (Brogdon et al. 1997). Two hundred µl of TMBZ

solution (50 gm of 3,3',5,5'-tetramethyl benzidine dihydrochloride in 25 ml of absolute methanol mixed with 75 ml of 0.25 M sodium acetate buffer pH 5.0) were added to 100 µl aliquots of mosquito homogenate. Twenty-five µl of 3% hydrogen peroxide (H_2O_2) was added and the mixture left for 10 minutes at room temperature. Three negative controls per plate were prepared with 100 µl of KPO_4 buffer, 200 µl of the solution TMBZ and 25 µl of 3% H_20_2. Other three wells containing 100 µl of KPO^4 buffer, 200 µl of the solution TMBZ and 100 µl of Cytochrome (10 mg Cytochrome-C [from bovine heart] dissolved in 100 ml sodium acetate buffer pH 5, and diluted 1:110 with KPO_4) were run as positive controls. Samples were read at 620 nm.

Insensitive acetylcholinesterase (iAChE) assay. To each replicate aliquot of 100 µl mosquito homogenate was added 100 µl of DTNB solution (13 mg of dithiobis 2-nitrobenzoic acid in 100 µl of KPO_4 buffer pH 7.2) and 100 µl of the substrate ATCH (75 mg acetylthiocholine iodide in 10 ml acetone, dissolved in 90 ml KPO_4 buffer pH 7.2) to initiate the reaction. The latter solution was substituted by 100 µl of the substrate ATCH containing 0.2% of the inhibitor propoxur (21 mg propoxur 0.1 M added to 100 ml of ATCH solution) for iAChE assays. Six negative control wells contained 100 µl of KPO_4 buffer, 100 µl DTNB solution and 100 µl ATCH solution without and with propoxur respectively. The plates were read at 414 nm immediately (T_0) and 20 min (T_{20}) after enzyme reaction. Subtraction of T_0 reading from T_{20} reading was used for statistical analysis.

Glutathione S-transferase assay. Two hundred µl of GSH/CDNB working solution (61 mg reduced glutathione prepared in 100 ml of KPO_4 buffer pH 7.2, and 20 mg 1-chloro-2,4'-dinitrobenzene diluted in 10 ml acetone dissolved in 90 ml of KPO_4 buffer respectively) were added to each replicate. Six blanks were prepared for each plate with 100 µl of KPO_4 buffer and 200 µl GSH/CDNB working solution. Rates for the enzyme reaction were measured at 340 nm immediately (T_0) and 10 min (T_{10}) later. Subtraction of T_0 reading from T_{10} reading was used for statistical analysis.

Protein assay. Two hundred µl of BIO Rad protein reagent solution, prepared as a 1:4 dilution in distilled water, and 80 µl of KPO4 buffer were added to 20 µl of the crude homogenate. Six blanks were prepared for each plate with 20 µl of distilled water and 300 µl of BIO Rad solution and 80 µl of KPO4 buffer. The reaction was read at 620 nm immediately (T0). This assay was ran with bovine serum albumin to make a standard curve in order to relate the observed absorbance of the different enzymes to the amount of protein present in each mosquito.

3.2.5. Molecular assays.

Individual mosquitoes were separated according to the recovery status at 24 h post-exposure to permethrin DD (15 μg/mL), as survivors and dead mosquitoes. A total of 117 and 103 mosquitoes (at least 10% of the dead and survivor mosquitoes recovered from bioassays) were genotyped for both mutation 1016I and 1534C respectively. A negative and positive control using Rockefeller and MF5 strains respectively was used. MF5 is a field strain molecularly characterized (with kdr mutation) collected in Merida and maintened in insectary condition. All molecular assays were performed in CDC laboratory from Atlanta, U.S.A.

Genomic DNA extraction. DNA was extracted from single mosquitoes using the protocol of Collins et al. (1987). Individual mosquitoes were homogenized in 100 μl of Grind buffer (0.08M NaCl, 0.16M sucrose, 0.06M EDTA, 0.1M Tris-HCl pH 9.0, 0.5% SDS) pre-heated to 65°C. Following incubation at 65°C for 30 minutes in water bath, 13 μl of 8M K-acetate was added to achieve a final concentration of 1M, and the samples were incubated on ice for 2 h. Homogenates were spun at 14 000 rpm for 20 min and the supernatants placed in clean tubes. The DNA was precipitated by adding 200 μl of ice-cold 100% ethanol. Tubes were placed in freezer (-20° C) overnight. After centrifugation at 14 000 rpm in the refrigerated microcentrifuge for 20 min the ethanol was removed and the DNA pellets were rinsed with 200 μl of ice-cold 70% ethanol and spun at 14 000 rpm for 5 min. The ethanol was decanted and the DNA pellets were dried in the SpeedVac (aproximatly for 20-40 minutes) with the lid of the eppendorf opened. The DNA was suspended in 25 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

Polymerase chain reaction (PCR). PCR-melting curves were conducted to identify the two kdr mutations using a Bio-Rad CFX96 Real-Time System C1000 thermal cycler. iQ™ SYBR® Green Supermix (Bio-Rad 170-8880) was used to carry out the PCR reaction. The primers used to amplify the region IIS6 of the mutation 1016I (designed by Dr. Karla Saavedra, 2012) were: Val1016f Forward (5'-GCGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCCACCCGCACCGG -3'), Ile1016f Forward (5'-GCGGGCACAAATTGTTTCCCACCCGCACTGA-3'), Ile1016r Reverse (5'- TGATGAACCSGAATTGGACAAAAGC -3'). Reaction conditions were: 95 ° C for 3 min, 40 cycles of 95 ° C for 10 sec, 60 ° C for 10 sec, 72 ° C for 30 sec, and finally 95 ° C for 10 sec. With a fusion curve of 65 ° C to 95 ° C and 0.2 ° C increments every 10 sec.

The primers used to amplify the region IIIS6 of the mutation 1534C (Yanola et al, 2011) were: Cys1534+ Forward (5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG -3'), Phe1534+ Forward (5'- GCGGGCTCTACTTTGTGTTCTTCATCATATT -3'), 1534- Reverse (5'- TCTGCTCGTTGAAGTTGTCGAT -3'). Reaction conditions were: 95 ° C for 3 min, 40 cycles of 95 ° C for 10 sec, 57 ° C for 10 sec, 72 ° C for 30 sec, and finally 95 ° C for 10 sec. With a fusion curve of 65 ° C to 95 ° C and 0.5 ° C increments every 5 sec.

For the 1016I mutation two different melting curve peaks are observed: 79 °C corresponding to the product amplified with the short primer, which is specific for allele A (ATA codes for Isoleucine or mutant type); and 85 °C corresponding to the product amplified with the long primer, which is specific for allele G (GTA codes for Valine or susceptible type) (Figure 13A).

For 1534C mutation two different melting curve peaks are observed: 80 °C corresponding to the product amplified with the short primer, which is specific for allele T (TTC codes for Phenylalanine or susceptible allele); and 85 °C corresponding to the product amplified with the long primer, which is specific for allele G (TGC codes for Cysteine or resistant allele) (Figure 13B).

3.3 Data management and analysis

Descriptive analyses of KD were obtained from different exposures to insecticides. Histograms and other graphs for each enzyme assay were plotted in SPSS 17.0 software. Bottle bioassays carried out with the DD were scored at the DT of 30 minutes and classified according to following criteria (CDC, 2010): 98-100% indicates susceptibility; 90-97% suggests resistance may be developing; less than 90% indicates resistance. Times after which 50% of mosquitoes were knocked down (half knock-down time, KDT_{50}) and their 95% confidence intervals (C.I.) for adults were estimated with Probit analysis using SPSS 17.0 software. The knockdown resistance ratio ($RRKDT_{50}$) was calculated by dividing the KDT_{50} of each population by the KDT_{50} of the New Orleans reference susceptible strain.

For the biochemical assays, protein concentration was determined to correct for size variation among the specimens (Brogdon, 1984). Statistical analysis was performed with an analysis of variance and the Scheffe multiple comparison tests was used; a significance level of $P \leq 0.05$ was used to compare the mean absorbance values between the field strains and the susceptible strain. Populations with altered activity of enzymes were submitted to Spearman correlation (r_s) to test the relationship between enzyme activity and survival.

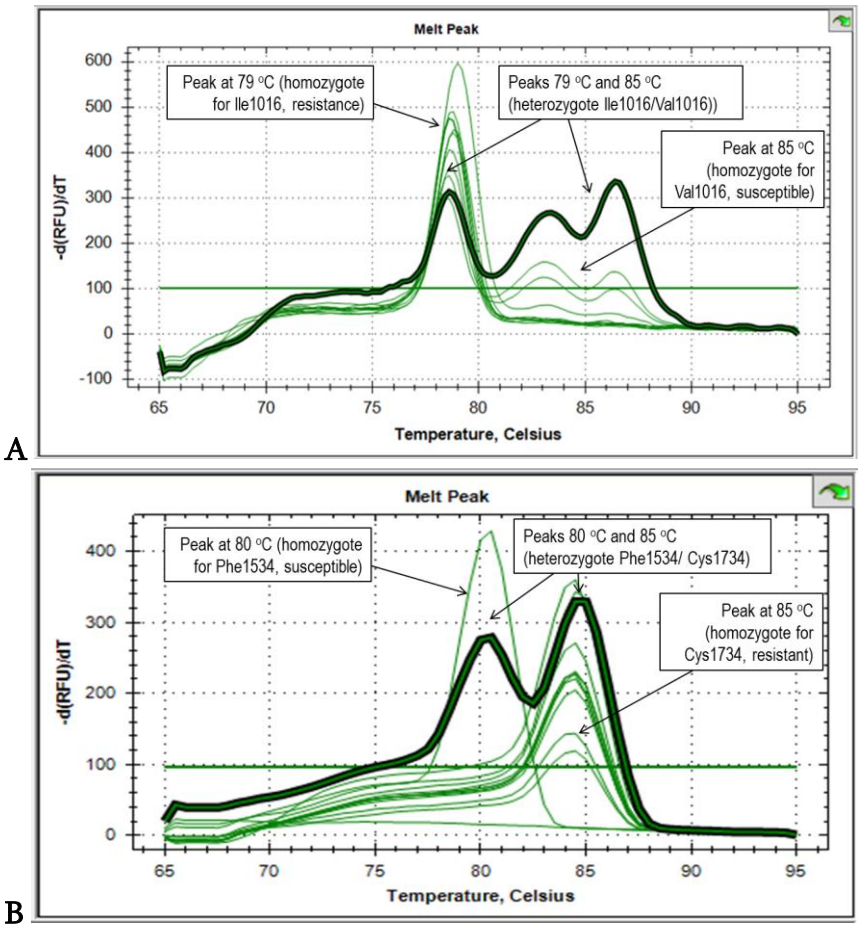


Figure 13. PCR-melting curves to identify the two mutations in the voltage-gated sodium channel gene domain II (A) and III (B) of segment 6.

The frequencies of the 1016I and 1534C alleles were calculated using the following equation: [n heterozygotes +2(n homozygotes)]/2(total n mosquitoes analyzed)

Fisher exact tests were implemented to test the association of each mutation with survival. Statistical analyses were performed using Stata 12.0 software. A P value of 0.05 or less was considered as significant.

In order to explore spatial analysis of *Ae. aegypti* insecticide resistance, the KD dataset from CDC bottles tests using alpha-cypermethrin and the 1016I genotyping performed in the 21 neigbourhoods from Merida were considered. The KD was considered as a binary (1=case, 0=control) variable based on the CDC resistance criteria: it was defined as a case if mortality <90% (n=8), and as control if mortality ≥ 90% (n=13). For 1016I kdr frequency, it was defined as a case if the frequency was 0.9 or above (n=12); anything below was a control. Results of KD using permethrin and 1534C genotyping (19 out 21 sites showed frequencies) were uninformative for this analysis as 19 of the 21 sites were classified as

permethrin resistant and had 1534C frequencies ≥0.9. Spatial analysis tests were applied to detect local hotspots of high phenotypic insecticide resistance to identify the spatial scales up to which the resistant *Ae. aegypti* phenotypes clustered (i.e. case-control K-Function) (Waller, 2004). Statistical analyses were performed with the package spdep from the R statistical computing software (http://www.r-project.org/).

3.4 Results

3.4.1. CDC bottles bioassays.

A total of 178 CDC bottle bioassays were performed using a total of 7,401 *Ae. aegypti* females (an average of 10.31 mosquitoes per bottle) for all clusters from both localities. For the susceptible strain New Orleans all tested insecticide results in 100 % KD at 30 min and mortality of 100%.

Pyrethroid susceptibility status. Figure 14 shows KD results using permethrin (15 μg/mL) for Merida field populations of *Ae. aegypti*.

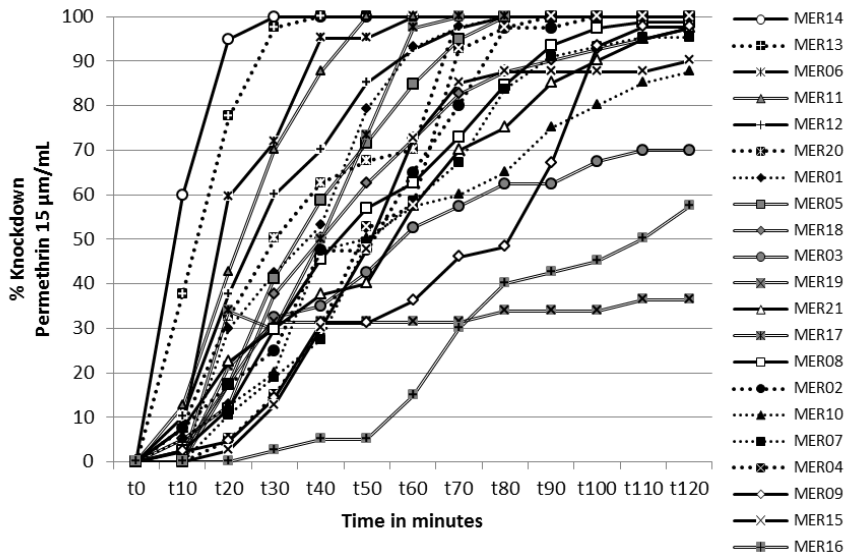


Figure 14 Time-knockdown data measured for 1-3 day old adult female *Ae. aegypti* exposed to permethrin diagnostic dose (15 μg/mL) using CDC bottles method. Results are means for 4 replicates of at at least 10 mosquitoes per bottle.

Resistance to KD was observed for 19 out of 21 cluster populations evaluated for Merida (Table 1). Only the clusters MER14 (Mulsay) and MER13 (San A. Xluch) showed susceptibility to KD (KDT_{50} 7.6 minutes C.I.=5.7,10.0, and 9.8 minutes C.I.=7.6, 12.4 respectively) with a $RRKDT_{50}$ of 0.8-1.1 respect to susceptible strain (KDT_{50} 8.9 minutes C.I.=6.9,11.5). While the remaining clusters showed $RRKDT_{50}$ from 2- to 13-fold resistance

to permethrin KD (confidence intervals of their KDT_{50} didn't overlap with the corresponding values of susceptible strain).

For the majority of the strains tested, the mosquitoes that were knocked down at 30 min never recovered. In general, the percent mortality at 24 h was higher than %KD (at diagnostic time of 30 min), with four exceptions MER06 (San A. Kahua), MER13 (San A. Xluch), MER14 (Mulsay) and MER 19 (Yucalpeten) with a mean of 3.3 (± 1.2 S.D.) fewer mosquitoes dead than knocked down (Table 1). A moderate but significant negative correlation was observed between the mortality frequency and KDT_{50} for permethrin DD (Spearman correlation coefficient, rs=-0.567, y = -0.5094x + 96.356, P=0.004) (Figure 15).

Table 1. Mean percent knockdown at 30 minutes (KD), 24 h mortality, half knockdown time (KDT_{50}) and knockdown resistance ratio at 1 h of exposure (RRKD50) of permethrin (15 μg/mL) against *Ae. aegypti* females from Merida, Mexico during wet season 2012. The 95% confidence intervals (C.I.) were calculated in order to see differences with the susceptible reference strain (differences marked with asterisk).

Study sites	KD		24h mortality		TKD_{50}		
	%	SE	%	SE	Minutes	95 % C.I.	$RRKDT_{50}$
MER14	100.0	0.0	97.5	2.5	7.6	(5.7, 10.0)	0.8
MER13	97.5	2.5	95.0	2.9	9.8	(7.6, 12.4)	1.1
MER06	71.8	6.2	68.4	8.8	19	(15.7, 22.9) *	2.1
MER11	70.0	4.1	82.5	6.3	19.5	(16.1, 23.6) *	2.2
MER12	60.0	9.1	90.0	4.1	25.8	(21.6, 30.8) *	2.9
MER01	50.0	18.3	82.5	10.3	38.3	(32.3, 45.5) *	4.3
MER17	42.2	13.4	67.7	12.3	32.6	(27.6, 38.5) *	3.7
MER05	41.1	10.9	92.2	4.8	37.8	(31.6, 45.1) *	4.2
MER18	37.5	10.3	82.5	7.5	38.2	(32.1, 45.6) *	4.3
MER20	32.5	20.2	40.0	12.2	44.7	(37.5, 53.3) *	5
MER02	31.4	9.7	26.4	9.5	54.7	(45.8, 65.7) *	6.1
MER03	30.0	7.1	90.0	4.1	47.1	(39.6, 56.3) *	5.3
MER08	29.8	5.9	97.9	2.1	35	(29.6, 41.5) *	3.9
MER21	29.7	10.6	88.6	5.5	45	(39.6, 51.2) *	5.1
MER10	25.0	6.5	87.5	9.5	44.2	(37.1, 52.8) *	5
MER15	20.0	9.1	87.5	7.5	47.2	(39.5, 56.5) *	5.3
MER04	19.0	3.6	74.4	11.1	54.2	(47.7, 61.9) *	6.1
MER07	15.0	2.9	87.5	2.5	48.6	(40.5, 58.4) *	5.5
MER19	14.3	2.8	26.4	5.0	74.9	(61.5, 91.9) *	8.4
MER09	12.5	4.8	52.5	8.5	47.8	(39.8, 57.5) *	5.4
MER16	2.5	2.5	50.0	12.2	124.1	(93.6, 166.7) *	13.9
Susceptible	100	0	100	0	8.9	(6.9, 11.5)	1

*MER01 Manuel A. Camacho; MER02 Pacabtun; MER03Fidel V.; MER04 Vergel III; MER05 Vergel II; MER06 San A. Kahua; MER07 U.H. Morelos; MER08 Castilla C.; MER09 Manzana 115; MER10 Cinco C.; MER11 Centro; MER12 San J. Tecoh; MER13 San A. Xluch; MER14 Mulsay; MER15 Juan Pablo II; MER16 Cordemex; MER17 Francisco M.; MER18 Bojorquez; MER19 Yucalpeten; MER20 Plan de A.; MER21 (Dzitya).

Higher levels of KD at DT (30 minutes) were observed using alpha-cypermethrin DD (10 µg/mL) for *Ae. aegypti* populations from Merida (Figure 16). Resistance to alpha-cypermethrin was observed in 38% (8/21) of the sampled sites and 14% (3/21) were developing resistance with $RRKDT_{50}$ from 1.2- to 5.5-fold resistance (Table 2). Complete susceptibility to alpha-cypermethrin was observed in 48% (10/21) of the Merida sites (Table 2).

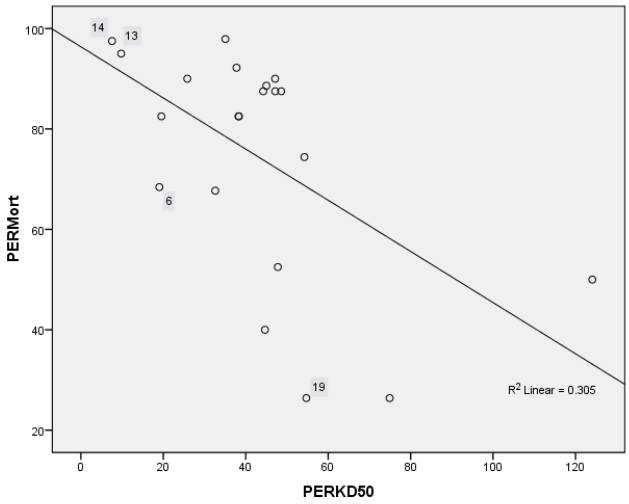


Figure 15. Correlation of percentage of mortality 24h (PERMort) and KDT_{50} (PERKD50) for permethrin diagnostic dose (15 µg/mL) in *Ae. aegypti* populations from Merida, Mexico during rainy season of 2012. The points labelled with numbers correspond to clusters with a fraction of knocked down mosquitoes (at 30 minutes) than were recovered.

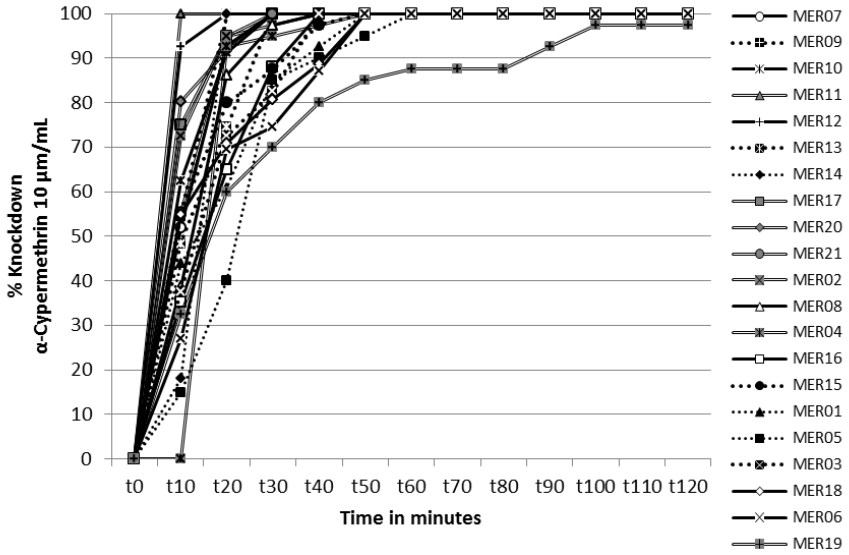


Figure 16. Time-knockdown data measured for 1-3 day old adult female *Ae. aegypti* exposed to alpha-cypermethrin diagnostic dose (10 µg/mL) using CDC bottles method. Results are means for 4 replicates of at at least 10 mosquitoes per bottle.

Table 2. Mean percent knockdown at 30 minutes (KD), 24 h mortality, half knockdown time (KDT_{50}) and knockdown resistance ratio at 1 h of exposure (RRKD50) of alpha-cypermethrin (10 μg/mL) against *Ae. aegypti* females from Merida, Mexico during wet season 2012. The 95% confidence intervals (C.I.) were calculated in order to see differences with the susceptible reference strain (differences marked with asterisk).

Study sites	KD		24h mortality		KDT_{50}		
	%	SE	%	SE	Minutes	95 % C.I.	$RRKDT_{50}$
MER11	100.0	0.0	100.0	0.0	4.8	(2.9, 7.7)	1.1
MER02	100.0	0.0	79.2	4.8	5.7	(3.6, 8.8)	1.3
MER14	100.0	0.0	100.0	0.0	6.5	(4.2, 9.6)	1.5
MER03	100.0	0.0	64.3	13.5	6.6	(4.3, 9.8)	1.5
MER15	100.0	0.0	100.0	0.0	7.2	(4.9, 10.3)	1.6
MER21	100.0	0.0	100.0	0.0	7.2	(4.7, 10.7)	1.7
MER10	100.0	0.0	97.5	2.5	7.2	(4.7, 10.8)	1.7
MER20	100.0	0.0	50.5	5.5	8.1	(6.0, 10.7)	1.9
MER12	100.0	0.0	77.3	9.5	10.5	(7.3, 14.7)*	2.4
MER17	100.0	0.0	100.0	0.0	12	(8.5, 16.5)*	2.8
MER07	97.5	2.5	90.0	5.8	5.4	(3.4, 8.4)	1.2
MER08	97.5	2.5	76.3	10.7	8.5	(6.3, 11.2)	2
MER06	95.0	2.9	50.0	9.1	10.6	(7.2, 15.0)	2.4
MER09	88.1	6.9	88.7	3.8	12	(9.2, 15.3)*	2.7
MER04	87.5	7.5	100.0	0.0	6	(3.9, 9.0)	1.4
MER16	85.0	6.5	97.5	2.5	18.1	(13.1, 24.7)*	4.1
MER13	85.0	2.9	95.0	2.9	22.3	(16.2, 30.4)*	5.1
MER18	82.5	11.8	57.5	14.9	5.9	(3.8, 8.8)	1.4
MER01	80.7	10.9	100.0	0.0	14.1	(10.2, 19.0)*	3.2
MER05	74.4	10.3	89.7	4.1	8.6	(5.8, 12.4)	2
MER19	70.0	20.4	52.5	15.5	23.8	(17.5, 32.2)*	5.5
Susceptible	100	0	100	0	4.4	(2.5, 7.2)	1

*MER01 Manuel A. Camacho; MER02 Pacabtun; MER03 Fidel V.; MER04 Vergel III; MER05 Vergel II; MER06 San A. Kahua; MER07 U.H. Morelos; MER08 Castilla C.; MER09 Manzana 115; MER10 Cinco C.; MER11 Centro; MER12 San J. Tecoh; MER13 San A. Xluch; MER14 Mulsay; MER15 Juan Pablo II; MER16 Cordemex; MER17 Francisco M.; MER18 Bojorquez; MER19 Yucalpeten; MER20 Plan de A.; MER21 (Dzitya).

Almost half (10/21) of the Merida sites showed lower mortality at 24h than KD at 30 min, with a mean difference of 24.7% (± 14.9 S.D). No significant correlation was observed between the frequency of mortality and TKD50 for alpha-cypermethrin (rs=-0.148, y = -0.3492x + 87.597, P= 0.261) (Figure 17).

To examine the relationship of 24 h mortality and KDT_{50} between PYs, additional correlations were implemented comparing permethrin and alpha-cypermethrin. However, no significant correlation was observed for 24 h mortality (rs=0.326, y = 301.92x - 235.31, P=0.074) and KDT_{50} (rs=0.173, y = 0.4462x + 37.096, P=0.227) between both PYs (Figure 18).

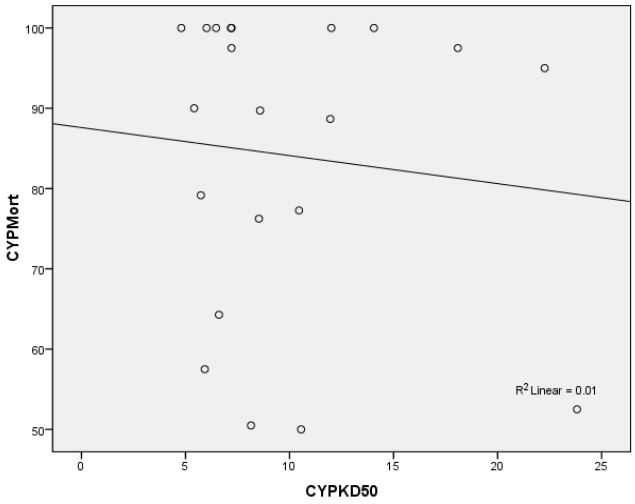
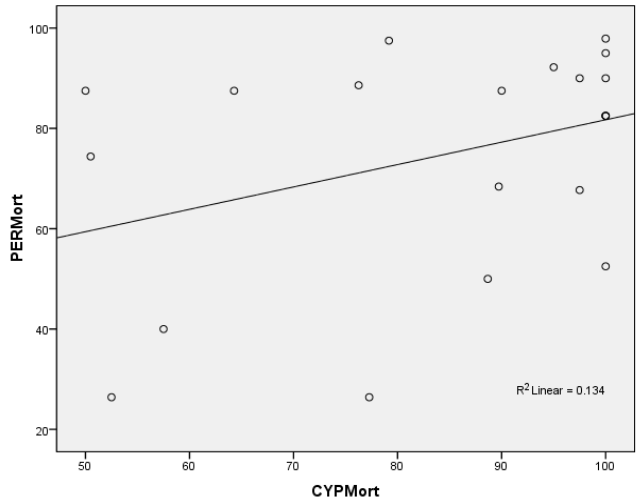


Figure 17. Correlation of percentage of mortality 24h (CYPMort) and KDT_{50} (CYPKD50) for alpha-cypermethrin diagnostic dose (10 μg/mL) in *Ae. aegypti* populations from Merida, Mexico during rainy season of 2012.

A



B

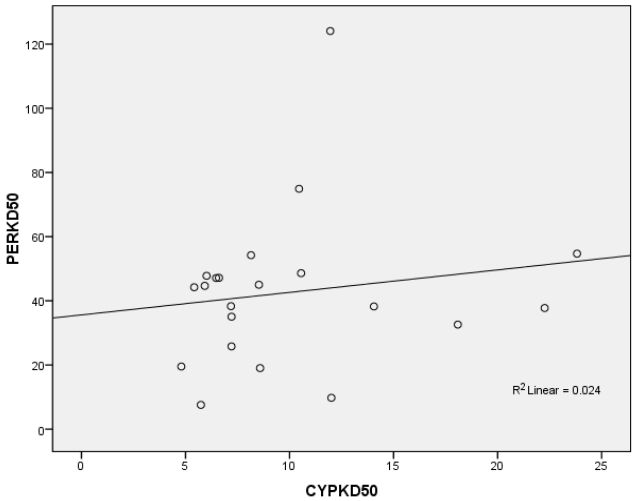


Figure 18. Correlation of mortality 24h (A) and KDT_{50} (B) between permethrin (15 μg/mL) and alpha-cypermethrin (10 μg/mL) in *Ae. aegypti* populations from Merida, Mexico during rainy season of 2012.

For Acapulco evaluations were performed using wild strains of *Aedes* adults emerged of eggs collected from: i) three clusters that have been assigned to the intervention with LLIS (ACA01) and ii) three clusters that will not receive the LLIS (ACA02). For both Acapulco groups, ACA01 and ACA02, a lower KD effect (at 30 minutes) was observed for permethrin DD than alpha-cypermethrin DD (Figure 19). The two sites evaluated in Ciudad Renacimiento were largely susceptible to alpha-cypermethrin, with KD at 30 minutes ranging from 93-100%. However, values for RRKDT50 ranged from 1.7- to 2.2-fold, suggesting that resistance may be developing in these populations. Mortality at 24 h was 87-100% for both alpha-cypermethrin and permethrin in Ciudad Renacimiento (Table 3).

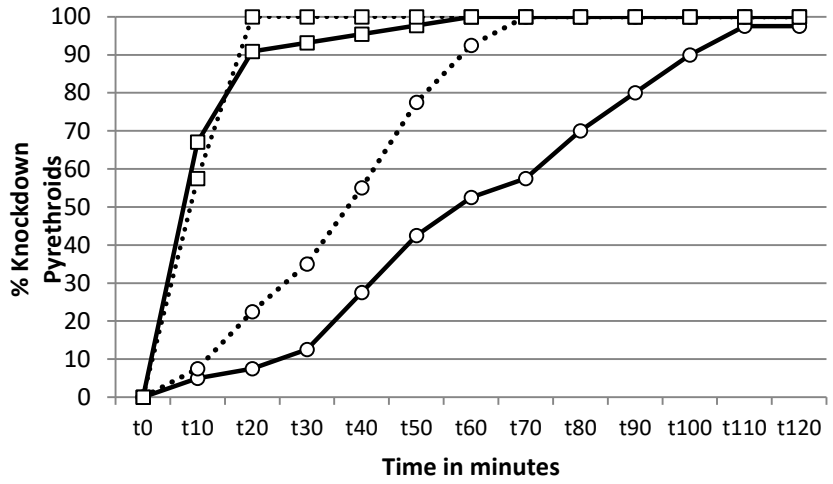


Figure 19. Time-knockdown data measured for 1-3 day old adult female *Ae. aegypti* exposed to permethrin (15 μm/mL) and alpha-cypermethrin (10 μm/mL) diagnostic dose using CDC bottles method. Results are means for 4 replicates of at at least 10 mosquitoes per bottle. The solid and dotted lines represent the cluster ACA01 and ACA02; the dots and squares represent the insceticides permethrin and alpha-cypermethrin respectively.

Table 3. Median knockdown time (KDT_{50}) and knockdown resistance ratio at 1 h of exposure (RRKD50), and mortality at 24 h of permethrin (15 μg/mL) and alpha-cypermethrin (10 μg/mL) against *Ae. aegypti* females from Acapulco, Mexico during wet season 2012. For TKD50 the 95% confidence intervals (C.I.) were calculated in order to see differences with the susceptible reference strain (differences marked with asterisk).

Study sites	KD		24h mortality		KDT_{50}			
	%	SE	%	SE	Minutes	95 % C.I.		$RRKDT_{50}$
Permethrin								
ACA01	12.5	6.3	87.5	4.8	55.1	(44.8,	71.3)*	5.716
ACA02	35	2.9	87.5	7.5	32.3	(26.4,	39.5)*	3.3572
Susceptible	100	0	100	0	9.63	(6.63,	12.9)	
alpha-Cypermethrin								
ACA01	93.2	4.4	100	0.0	8.6	(6.2,	10.7)*	2.1916
ACA02	100	0.0	92.5	4.8	6.7	(4.7,	8.7)*	1.727
Susceptible	100	0	100	0	3.9	(2.3,	5.8)	

Carbamates and organophosphate susceptibility status. After 30 minutes of propoxur (12.5 µg/mL) exposure just one cluster MER15 (Juan Pablo II) from Merida did not record 100% of mortality (mean 87.5 C.I. 57.4, 117.6). No recovery was observed, e.g. the mortality at 24 hours was 100% for all cluster of Merida (Figure 20).

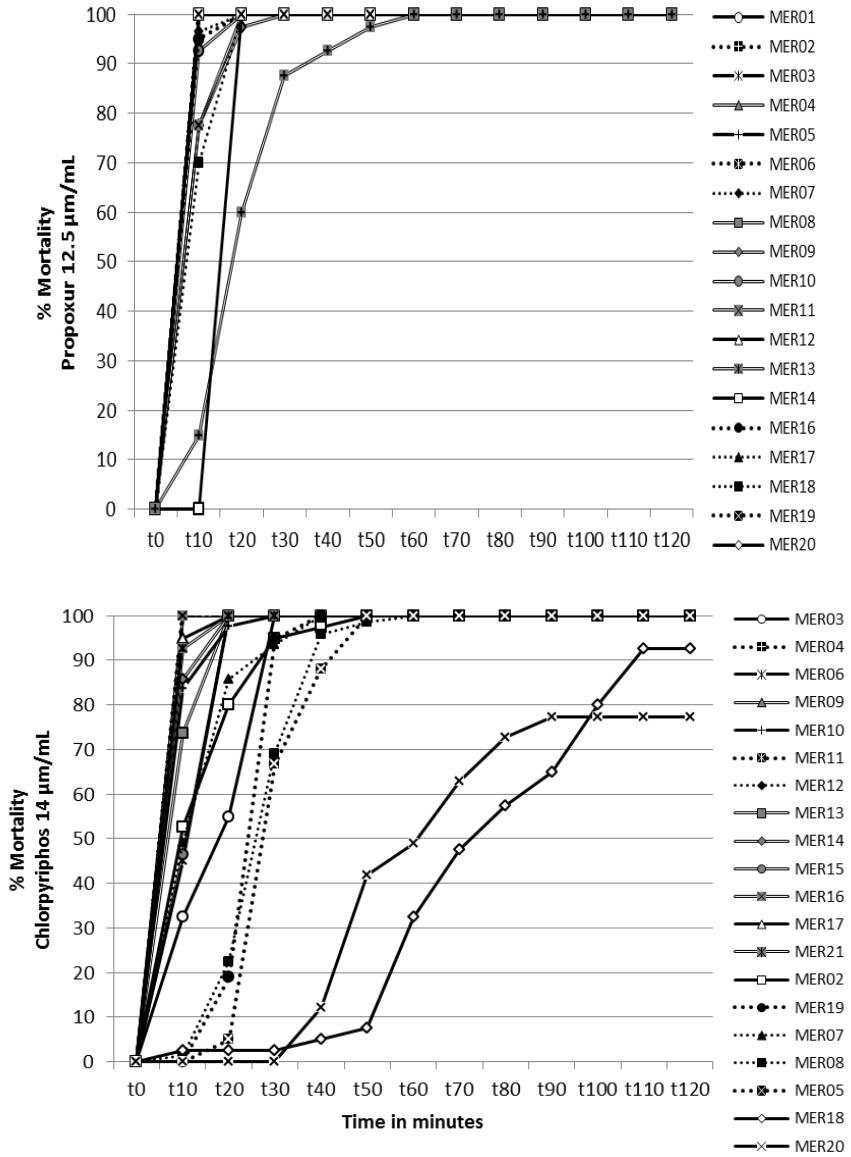


Figure 20. Time-mortality data measured for 1-3 day old adult female *Ae. aegypti* exposed to propoxur (12.5 µg/mL) and chlorpyrifos (14 µg/mL) diagnostic dose using CDC bottles method. Results are means for 4 replicates of at at least 10 mosquitoes per bottle.

In the case of chlorpyrifos (14 µg/mL), 65% (13/20, insufficient number of mosquitoes were available for one site, Manuel A. Camacho) of the sites showed complete susceptibility at 30 min, three sites indicated that resistance may be developing (Pacabtun, mean KD 95%, C.I. 79.1-110.9; Yucalpeten, mean KD 94.7%, C.I. 84.9-104.4; and U.H. Morelos, mean KD 93.5%, C.I. 85.5-101.5), and four sites were classified as resistant (Castilla C., mean KD

68.9%, C.I. 46.6-91.3; Vergel II, mean KD 66.7%, C.I. 39.6-93.7; Bojorquez, mean KD 2.5%, C.I. 5.5-10.5; and Plan de A. 0% KD) (Figure 20).

For the 2 sites in Ciudad Renacimiento, all showed complete susceptibility to both insecticides. Mortality after 24 hours was 100% for all sites in both cities, and no recovery from knockdown was observed.

Intensity of resistance. When exposed to varying multiples of the DD of permethrin, the majority of sites in Merida reached knockdown greater than 90% (resistance threshold) only when they were exposed to 5-fold the DD of permethrin (Figure 21). One site (Bojorquez) did not achieve this level of knockdown until exposure to 10-fold the DD.

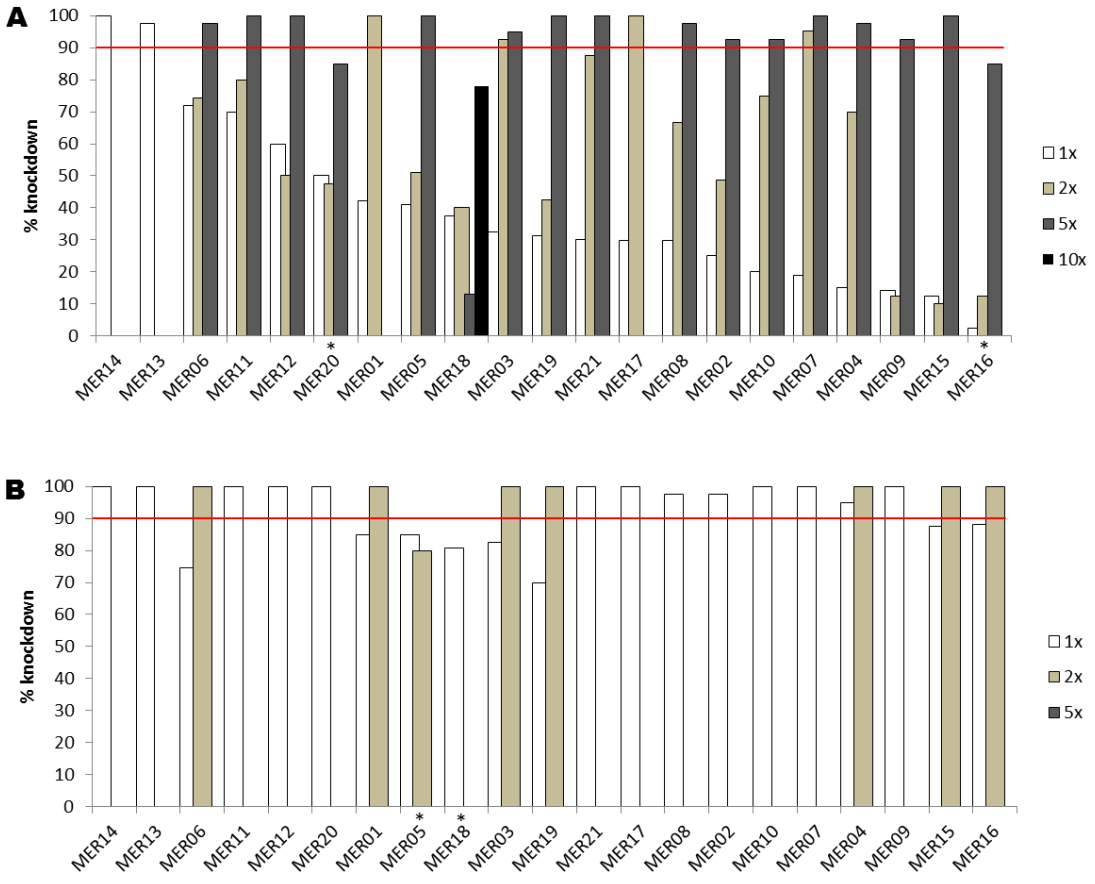


Figure 21. *Ae. aegypti* resistance intensity to permethrin (A) and alpha-cypermethrin (B) in Merida during rainy season 2012. The bars indicate the percent knockdown at the diagnostic time (30 min) to the diagnostic dose of the insecticide, as well as multiples thereof (2x, 5x and 10x). The red line represents the knockdown resistance threshold of 90%. *Sites with an insufficient number of females were unable to be studied.

The intensity of permethrin resistance was higher in Ciudad Renacimiento (Acapulco) than Merida. Of the two sites tested, one site required exposure to 5-fold the DD to achieve >90%

KD (1x=35, 2x=72.5, 5x=92.5), while exposure to 5-fold the DD at the other site only achieved >70% KD (1x=12.5%, 2x=45%, 5x=75%).

On the other hand, the intensity of resistence to alpha-cypermethrin occurred at a much lower frequency (Figure 21). A total of 57% (12/21) of sites in Merida were close to or above the susceptible threshold (≥98% KD) at the DD of alpha-cypermethrin (Table 2). The remaining areas came above 90% KD when they were exposed to 2-fold the DD (with the exceptions of Vergel II and Bojorquez, where insufficient numbers of mosquitoes were available). In Ciudad Renacimiento, the diagnostic dose of alpha-cypermethrin was sufficient to reach mortalities of 93% and 100% for both sites.

3.4.2. Biochemical assays.

For purposes of this study were selected a subsample of clusters for both arms LIS intervention and no-intervention arm, which will be followed-up in next surveys. For Merida eleven clusters were selected according to their susceptibility status to PYs (section 3.3.1). The external control MER21 (Dzitya) was also considered. The clusters selected and their susceptibility status is presented in the following list:

Clusters selected	Treatment	Suceptibility status (KD)
MER04 (Vergel III)	No-intervention	Resistance to permethrin & alpha-cypermethrin
MER01 (Manuel A. C.)	LLIS	
MER09 (Manzana 115)	LLIS	
MER16 (Cordemex)	LLIS	
MER08 (Castilla C.)	No-intervention	Susceptible to alpha-cypermethrin & resistance to permethrin
MER17 (Francisco de M.)	No-intervention	
MER20 (Plan de A.)	No-intervention	
MER03 (Fidel V.)	LLIS	
MER07 (U.H. Morelos)	LLIS	
MER13 (San A. Xluch)	No-intervention	Susceptible to permethrin & resistance to alpha-cypermethrin
MER 21 (Dzitya)	External control	Susceptible to alpha-cypermethrin & resistance to permethrin

For Acapulco the same populations (ACA01 and ACA02) tested on section 2.6.1 were considered.

Enzyme assays for Merida populations. The mean values of absorbance detected in the oxidase assay for 73% (8/11) of Merida sites were significantly elevated compared to the susceptible strain (Figure 22). The highest levels of oxidase activity were detected in MER03 (Fidel V.), MER04 (Vergel III), MER16 (Cordemex) ($P<0.001$), MER13 (San A. Xluch), MER17 (Francisco Montejo), MER09 (Manzana 115), MER07 (U.H. Morelos), and MER20 (Plan de Ayala) ($P<0.0001$). Only three sites MER01 (Manuel A. Camacho, $P=0.063$), MER08 (Castilla Camara, $P=0.37$) and MER21 (Dzityia, $P=0.99$) showed no difference in oxidase activity as compared with the susceptible strain. The highest oxidase activities observed for five clusters MER13 (San A. Xluch), MER17 (Francisco Montejo), MER09 (Manzana 115), MER07 (U.H. Morelos), and MER20 (Plan de Ayala) presented most of individuals (83%-96.7%) having an increase in oxidases activity (Figura 23).

Analysis of esterase activity showed mean absorbance values that were significantly higher than the susceptible strain only for two sites of Merida (Figure 24), MER04 (Vergel III, $P=0.012$) and MER17 (Francisco Montejo, $P<0.0001$), but little more than one quarter of individuals (26.7% and 33.3% respectively) had increased esterase activity (Figure 25). The rest of sites did not show significant differences (MER16: Cordemex, $P=0.193$; MER20: Plan de Ayala, $P=0.993$; MER03: Fidel V., $P=0.972$; MER21: Dzitya, $P=0.958$; MER01: Manuel A. Camacho, $P= 0.911$) or showed significantly lower activity than the susceptible strain (MER09: Manzana 115, $P=0.032$; and MER09: U.H. Morelos, $P=0.001$).

GST analyses showed values that were significantly higher than the susceptible strains in the 64% (7/11) of Merida sites (Figure 26), particularly from MER21 (Dzitya, $P=0.024$), MER16 (Cordemex, $P=0.002$), MER09 (Manzana 115), MER20 (Plan de A.), MER07 (U.H. Morelos), MER04 (Vergel III), MER13 (San A. Xluch) ($P<0.0001$ each one). Most of individuals of theses clusters (60-100%) showed an increase in GST activity (Figure 27). In contrast, MER17 (Francisco de Montejo, $P=0.64$), MER03 (Fidel V., $P=0.47$), MER08 (Castilla Camara, $P=0.55$) y MER01 (Manuel A. Camacho, $P=0.14$) did not exhibit increased GST activity (Figure 26) and showed between 16%-43% of individuals with elevated GST activity (Figure 27).

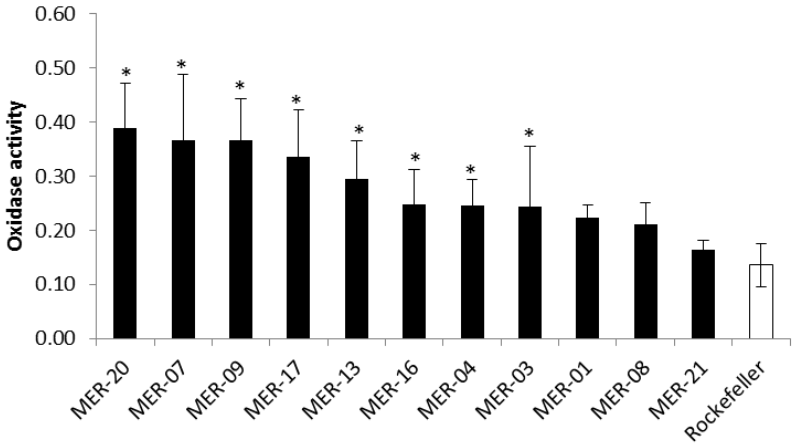


Figure 22. Oxidase activity (at absorbance 620 nm) for different *Ae. aegypti* populations from Merida, Mexico during rainy season 2012. Bars represent mean values and their error bars. Asterisk denote significantly higher mean values of absorbance that Rockeller strain (white bar).

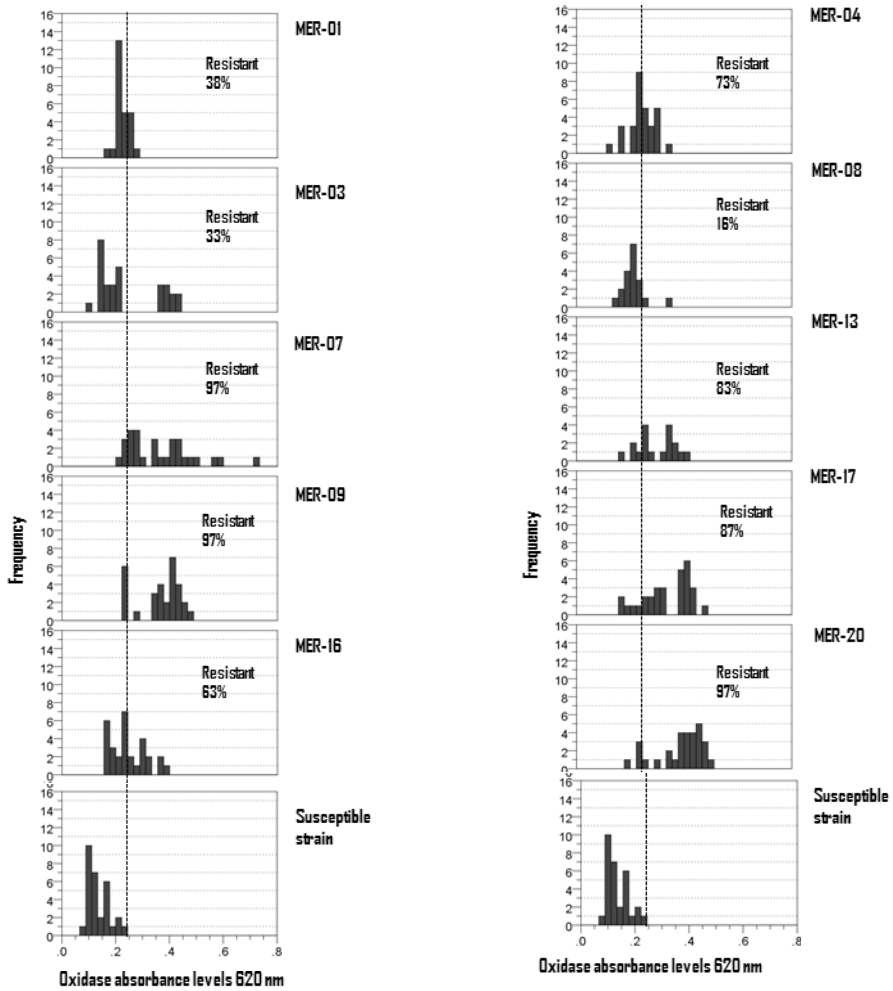


Figure 23. Frequencies (at absorbance 620 nm) of oxidase assay data for different *Ae. aegypti* populations from Merida, Mexico during rainy season 2012. The frequencies were based on three replicates and one control replicate per mosquito. The dotted line represents the susceptibility threshold established in Rockeller strain and percentage of individuals that exceeded this threshold is showed.

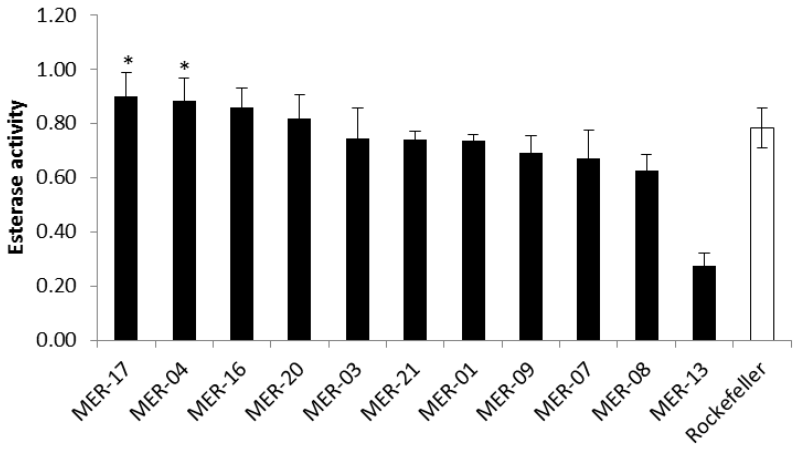


Figure 24. Esterase activity (at absorbance 540 nm) for different *Ae. aegypti* populations from Merida, Mexico during rainy season 2012. Bars represent mean values and their error bars. Asterisk denote significantly higher mean values of absorbance that Rockeller strain (white bar).

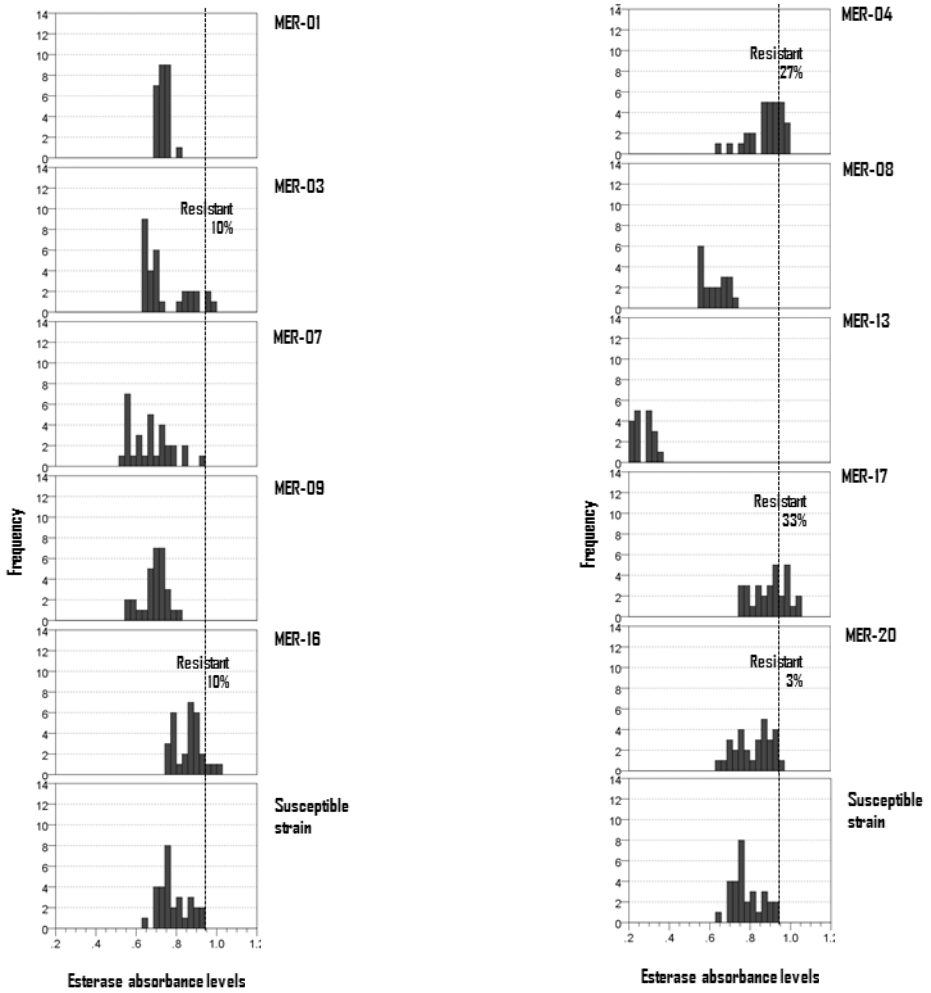


Figure 25. Frequencies (at absorbance 540 nm) of esterase assay data for different *Ae. aegypti* populations from Merida, Mexico during rainy season 2012. The frequencies were based on three replicates and one control replicate per mosquito. The dotted line represents the susceptibility threshold established in Rockeller strain and percentage of individuals that exceeded this threshold is showed.

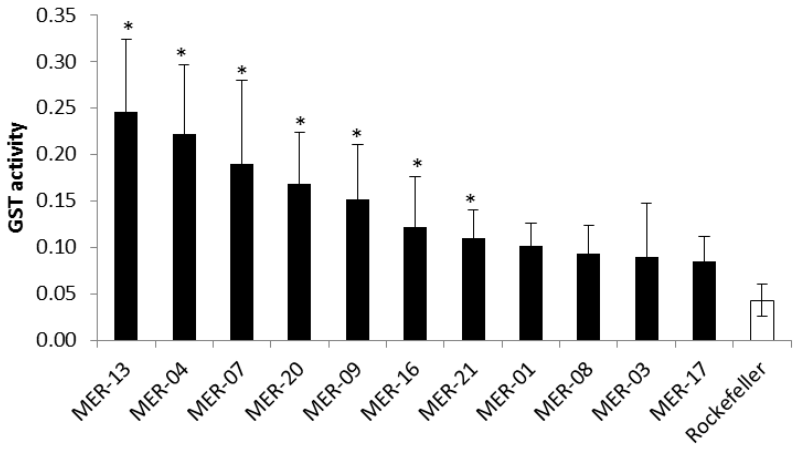


Figure 26. GST activity (at absorbance 340 nm) for different *Ae. aegypti* populations from Merida, Mexico during rainy season 2012. Bars represent mean values and their error bars. Asterisk denote significantly higher mean values of absorbance that Rockeller strain (white bar).

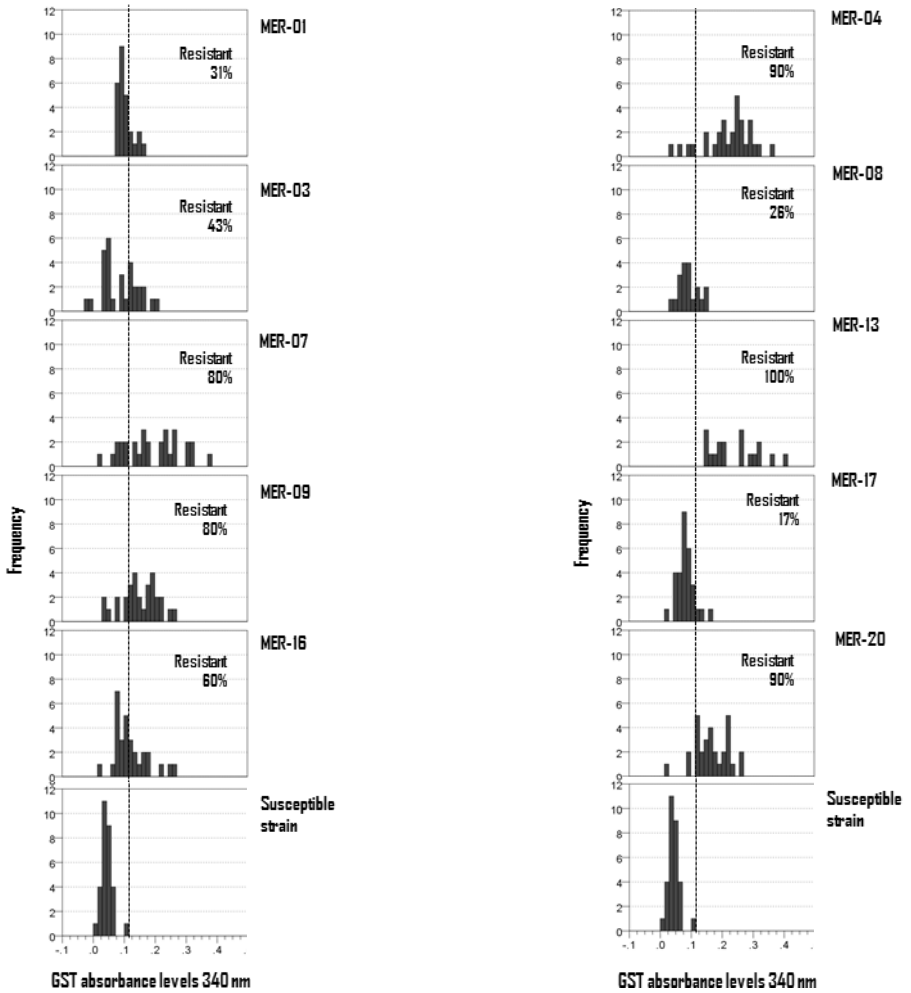


Figure 27. Frequencies (at absorbance 340 nm) of GST assay data for different *Ae. aegypti* populations from Merida, Mexico during rainy season 2012. The frequencies were based on three replicates and one control replicate per mosquito. The dotted line represents the susceptibility threshold established in Rockeller strain and percentage of individuals that exceeded this threshold is showed.

For the insensitive acetylcholine assay remaining AChE activity was less than 30% for almost all clusters in Merida, with some clusters presenting a low percentage of individuals with AChE activity above 30% (MER01 Manuel A. Camacho 3.8%, MER07 U.H. Morelos 3.3%). Only one cluster MER16 (Cordemex) showed highest AChE activity with 97% of female individuals above threshold of 30% (Figure 28).

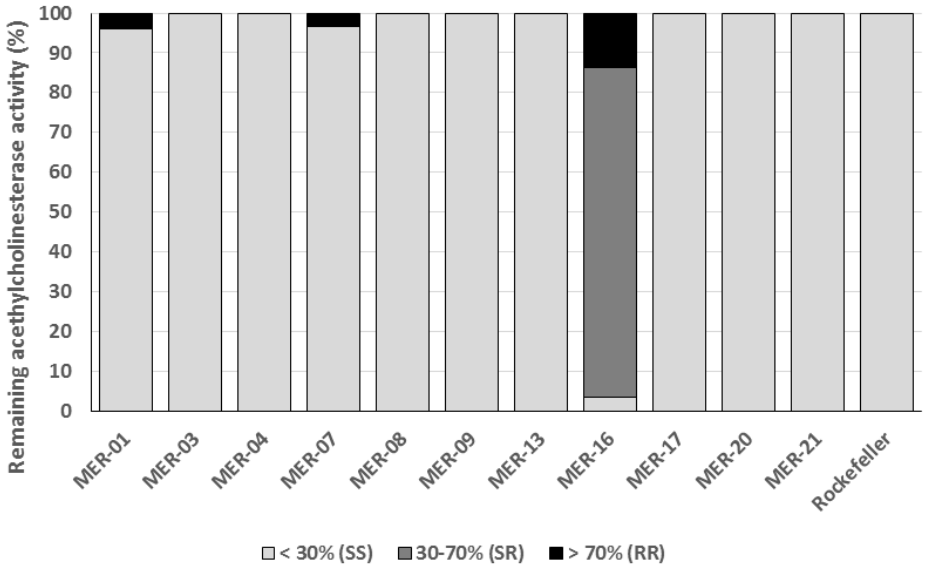


Figure 28. Percentage remaining activity of acetylcholinesterase in the presence of propoxur in *Ae. aegypti* population from Merida, Mexico during rainy season of 2012. <30%=homozygous susceptible (SS), 30–70%=heterozygous (RS), >70%=homozygous resistance (RR).

Enzyme assays for Acapulco populations. For Acapulco both sites evaluated showed oxidase, esterase and GST activity levels significantly higher than the susceptible strain ($P<0.0001$) (Figure 29).

Most of individuals of ACA01 and ACA02 (93% and 97% respectively) had an increase in oxidases activity. Most of individuals of ACA02 (97%) exceed the threshold for GST activity, compared with 80% of ACA01. While for esterase activity for same populations the 40% and 37% of individuals exceed the New Orleans strain threshold respectively (Figure 30).

AChE activity was less than 30% for all individuals for both cluster, i.e. the AChE was inhibited by carbamate propoxur suggesting that insensitive acetylcholinesterase is not present in Acapulco populations.

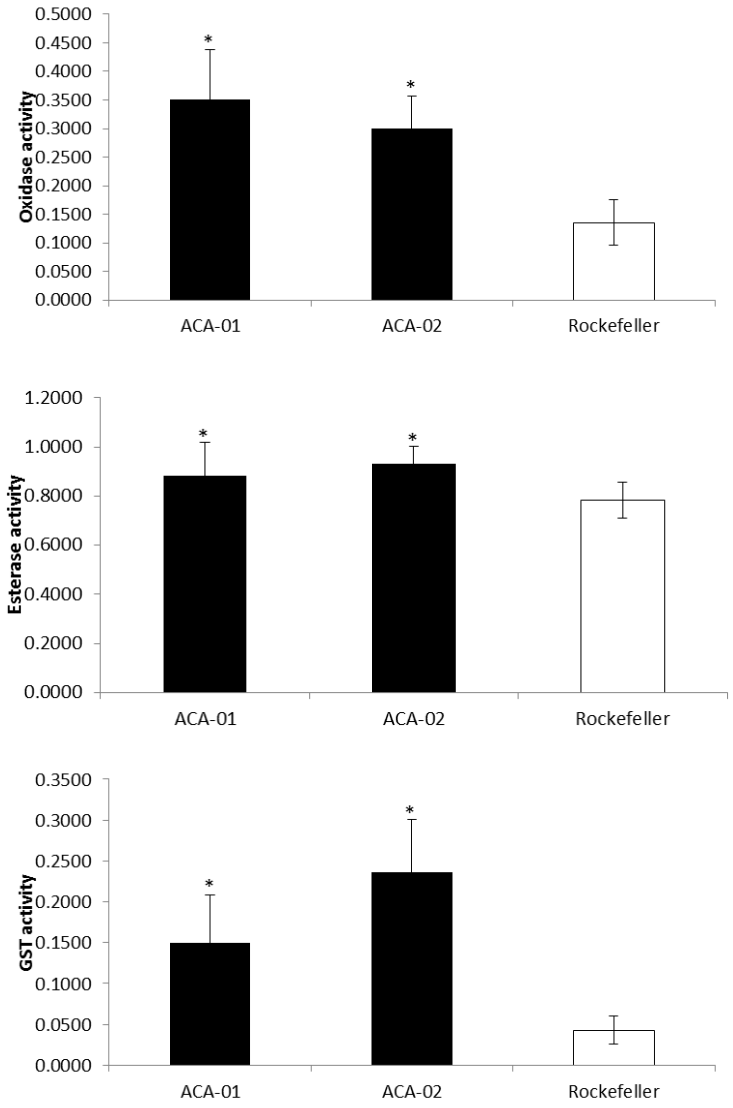


Figure 29. Oxidase (at absorbance 620 nm), esterase (at absorbance 540 nm) and GST activity (at absorbance 340 nm) for *Ae. aegypti* populations from Acapulco, Mexico during rainy season 2012. Bars represent mean values and their error bars. Asterisk denote significantly higher mean values of absorbance that Rockeller strain (white bar).

Correlations of enzyme activity and pyrethroid resistance. To explore associations between resistance phenotype from the permethrin and and alpha-cypermethrin bioassays and each enzyme system correlation analyses were carried out with data from the Merida sites. No significant correlation was found between enzyme activities and KDT50 or 24 mortality for either pyrethroid.

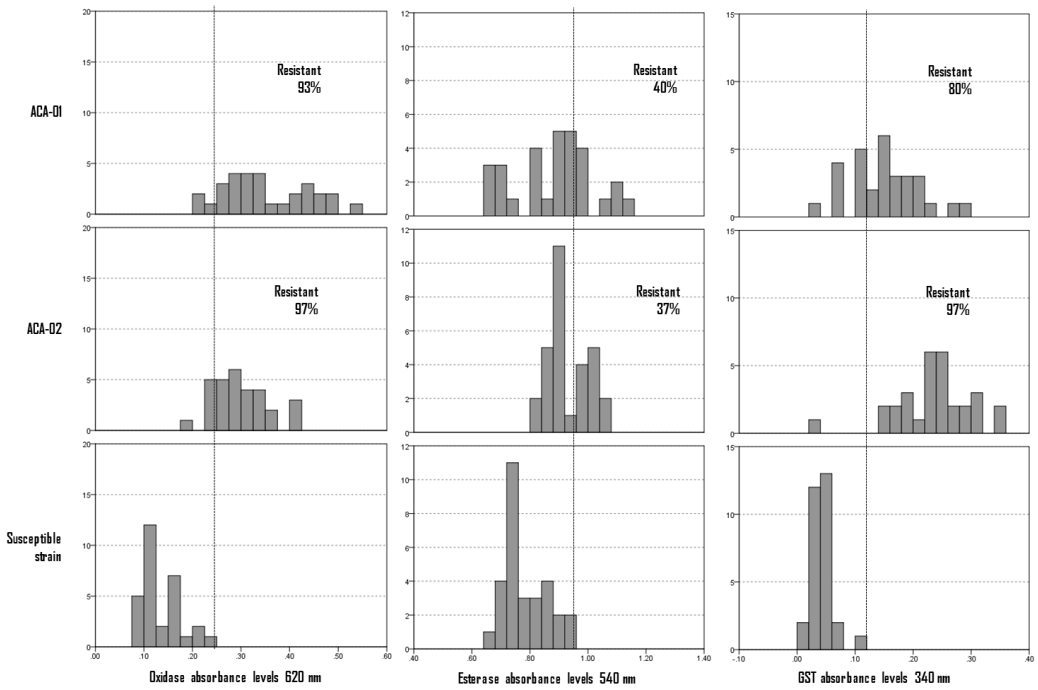


Figure 30. Frequencies oxidase (at absorbance 620 nm), esterase (at absorbance 540 nm) GST (at absorbance 340 nm) assay data for *Ae. aegypti* populations from Acapulco, Mexico during rainy season 2012. The frequencies were based on three replicates and one control replicate per mosquito. The dotted line represents the susceptibility threshold established in Rockeller strain and percentage of individuals that exceeded this threshold is showed.

3.4.3. Molecular assays.

The positive control using Rockefeller strain showed two peaks in the melting curve at temperatures 83.2° C and 86.6° C for V106I and 80-80.5° C for 1534C respectively, being classified as susceptible homozygote for both kdr mutation. The negative control using MF5 strain showed two peaks in the melting curve at temperatures 78.6° C and 86.4° C for V106I and 80.5° C and 84.5° C for 1534C, being classified as heterozygote for both kdr mutation.

Table 4 shows the number of mosquitoes of each genotype, and frequency of both kdr mutations, 1016I and 1534C for dead and surviving mosquitoes after a 24 h recovery period following exposure to the permethrin diagnostic dose. The homozygous wild-type genotypes 1016V/1016V and 1534F/1534F were absent in the survivor mosquitoes from Merida and the homozygous mutant genotype 1016I/1016I and 1534C/1534C predominated at 67% and 96% respectively.

There was no significant difference in kdr genotype between dead and surviving mosquitoes for either 1016I (Fisher exact test p=0.075) or 1534C loci (Fisher exact test p=0.501). At the allelic level, the frequency of the 1016I allele was significantly higher in survivors (Fisher exact test p=0.014), but there was no significant difference in the frequency of the 1534C allele between dead and surviving mosquitoes (Fisher exact test p=0.501 and

p=0.108 respectively). In mosquitoes genotyped for both loci (n=74), 83% of mosquitoes (24/29) of survivors were homozygous for both resistant alleles compared to 53% (24/45) observed in dead individuals.

In Acapulco the homozygous mutant genotype at the 1016 locus predominated in survivors (80%). However, there was not a significant difference in kdr-1016I genotype (Fisher exact test p=0.53) and allelic frequency (P=0.52) between dead and surviving mosquitoes. For the 1534 locus, the predominance of the homozygous mutant genotype was 100% for both dead and survivor individuals. The percentage of resistant homozygotes for both mutations was similar for dead (64%) and survivor (60%).

3.4.4. Spatial Analysis.

Case-control K-functions showed no significant clustering for alpha-cypermethrin (Figure 31A). In contrast for 1016I the probability of finding clusters of 1016I frequencies below 0.9 was significantly higher than random up to 1 km (Figure 31B) although it is difficult to interpret the significance of this result given that sample sizes were very low (3-5 per site) an included both mosquitoes that were surviving or dead mosquitoes 24 h post-exposure to permethrin diagnostic dose.

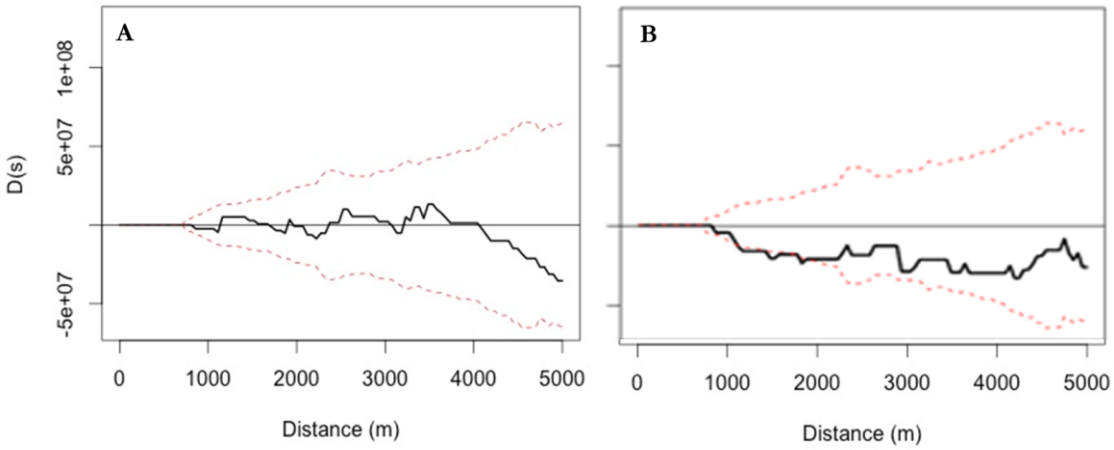


Figure 31. K functions (solid) for cases and controls with confidence bands (dashed) and distance in meters. Results are shown for pyrethroid alpha-cypermethrin (A) and 1016 frequecies (B). Pyrethroid alpha-cypermethrin showed no statistically significant difference from randomness.

Table 4. Number of mosquitoes by genotype (SS, SR and RR) and allelic frequencies of kdr mutation 1016I and 1534C for *Ae. aegypti* from Merida and Acapulco, Mexico during rainy season 2012. All samples (individual mosquitoes) were collected from bottle bioassays (at least 10% of 24 h dead and survivor mosquitoes) for Permethrin diagnostic dose (15 µg/ml). 1016V/1016V and 1534F/1534F are SS (homozygous susceptible); 1016V/1016I and 1534F/1534C are SR (heterozygotes); and 1016I/1016I and 1534C/1534C are RR (homozygous resistant).

Site	Phenotype (24 h recovery)	V1016I						F1534C						Double homozygotes		
		n	1016V	V1016I	1016I	Freq. 1016I	P	n	1534F	F1534C	1534C	Freq. 1534C	P	n	SS	RR
Merida	Dead	60	10% (6)	25% (15)	65% (39)	77.5% (93)		45	2.22% (1)	11.1% (5)	86.7% (39)	92.2% (83)		45	0	24
	Survivor	32	0	33.3% (5)	66.7% (27)	83.3% (59)	**0.014**	29	0	3.5% (1)	96.5% (28)	98.8% (57)	**0.108**	29	0	24
	Total	**92**	**6.5% (6)**	**21.7% (20)**	**71.7% (66)**	**82.6% (152)**		**74**	**1.3% (1)**	**8.1% (6)**	**90.5% (67)**	**94.6% (140)**		**74**	**0**	**48**
Acapulco	Dead	15	0	33.3% (5)	66.7% (10)	83.3% (25)		17	0	0	100% (17)	100% (17)		14	0	9
	Survivor	10	0	40% (4)	60% (6)	80% (16)	**0.523**	12	0	0	100% (12)	100% (12)	---	10	0	6
	Total	**25**	**0**	**26.5% (9)**	**73.5% (16)**	**82% (41)**		**29**	**0**	**0**	**100% (29)**	**100% (29)**		**24**	**0**	**15**

3.5 Discussion

The aim of this study was characterize the susceptibility of *Ae. aegypti* to the key insecticides used in Mexico's national dengue vector control programme in two cities considered epidemiologically important for dengue transmission by the Mexican Ministry of Health.

3.5.1. Susceptibility and resistance mechanism to pyrethroids.

This study demonstrated high levels of resistance to PY insecticides, particularly permethrin, in both Merida and Acapulco. The increase in median knockdown time (a good indication for fast acting insecticides) as compared to a susceptible reference strain was more marked with permethrin than with alpha-cypermethrin. Furthermore insecticide concentrations 5 to 10-fold higher than the diagnostic dose of permethrin were required to knock down all mosquitoes after 30 minutes. Flores et al. (2013) detected varying degrees of resistance intensity to permethrin and alpha-cypermethrin in several neighbouring locations in the Mexican state of Veracruz, and they estimated that it would be necessary to increase by twice or more the concentration of those insecticides to effectively knockdown and eventually kill those mosquitoes.

These differences between these two different types of PYs can be attributed partially to their chemical propiertes and modes of action. Permethrin is a Type 1 pyrethroid, group that include PYs containing descyano-3-phenoxybenzyl[1] or other alcohols (DDT and its analogues also have a similar mode of action). The Type 2 PYs specifically contain an α-cyano-3-phenoxybenzyl alcohol, which increases insecticidal activity about 10-fold (Bloomquist, 1999). The toxic propiertes of different PYs have been evaluated in PY-susceptible and PY-resistant (permethrin/kdr) strains of *Anopheles* mosquitoes, where alpha-cypermethrin had the fastest knock-down of the tested pyrethoids and showed the highest increased biological activity on PY-resistant strains (mortality 94% using the WHO diagnostic doses) (Hougar et al., 2003).

The advantages of alpha-cypermethrin over other PYs could make it a promising candidate in areas with permethrin resistance. However, additional tests would be necessary in order to confirm its suitability for widespread use in Merida and Acapulco, as cross-resistance between alpha-cypermethrin and other pyrethroids, including permethrin, has been reported in *Ae. aegypti* on Atlantic Coast of Mexico (Flores et al., 2013). However, the

[1] Older non-phenoxybenzyl Type 1 compounds include pyrethrins, allethrin, and tetramethrin. They are unstable under UV light and this characteristic prevents their use for residual spraying and then on many crops.

present study didn´t find evidence of relationship between alpha-cypermethrin and permethrin resistance.

Other factor to be considered in the resistance to PY, is the rapid alteration in the susceptible status to PYs of *Ae. aegypti* (Montella et al., 2007), in contrats to the slow acquisition of resistance to OPs (Hemingway et al., 2013; Mazzarri and Georghiou, 1995). Rapid resistance evolution to PYs (e.g. cypermethrin) has been documented in some *Aedes* populations from Brazil (da-Cunha et al., 2005). In this country decrease in cypermethrin susceptibility was noted after two continuos years of insecticide pressure (da-Cunha et al., 2005), without evidence of revertion of resistance in the field for almost 9 years (Lima et al., 2011).

Detoxification enzymes typically linked to PYs resistance in mosquitoes include the oxidases or cytochrome P450 monooxygenases (Brogdon et al., 1997; Beach et al., 1999), the esterases (Brogdon and Barber 1990; Fonseca-González, 2011; Soderlund et al., 1983) and GSTs (Lumjuan et al., 2005; Lumjuan 2011; Vontas et al., 2001, 2002).

Higher levels of esterese activity were detected in Acapulco compared with Merida. Metabolic resistance of PYs in *Ae. aegypti* is reported to be mediated in part by esterases or lesser extent oxidases in the north and Atlantic coast of Mexico (Flores et al. 2005, 2006, 2009). Aponte et al (2013) also reported high levels of GST, esterase, and to a lesser extent oxidase activity in permethrin/deltamethrin/DDT resistance populations of *Ae. aegypti* from Pacific coast of the country, including the locality of Acapulco.

In this study, significantly elevated levels of oxidase and GST activity were observed in both study areas. Although absorbance means analysis did not reveal any correlation between enzyme activities and permethrin and alpha-cypermethrin resistance in *Ae. aegypti* from Merida metabolic resistance mechanism should not be discarded considering that this type of resistance could have a role in the 24 h recovery (see discussion below). Oxidases have been commonly associated with the metabolic resistance to PYs in mosquitoes (Hemingway et al., 1991). The GST enzyme has been more commonly associated with metabolic-based resistance to DDT (Brown, 1986; Hemingway, 2000), but they also play a role in resistance to pyrethroid insecticides (Enayati et al., 2005; Lumjuan et al., 2005; Lumjuan et al., 2011; Vontas et al., 2012). This elevated GST activity could be also associated with DDT resistance, given that DDT was widely used for more than 50 years in the southern regions of Mexico.

Both 1016I and 1534C kdr alleles were found at high frequencies in Merida and Acapulco. In Merida, the 1016I allele was associated with permethrin survival, but not in Acapulco. An increase in the frequency of the 1016I allele in field populations of Merida has been detected

in recent years, from a frequency of 0 in 1999 to 0.72 to 0.72-0.90 in 2009 (Loroño-Pino et al., 2013; Ponce-García et al., 2009; Siller et al., 2011). In 2012 (this study) the frequency was 0.83 and 0.77 among survivor and dead individuals respectively. In Acapulco, the frequency of the 1016I allele was estimated to be 0.97 in 2009 (Siller et al., 2011) and the current study found a frequency of 0.80 and 0.83 among survivor and dead individuals respectively.

The frequency of the 1534C allele was very high in both study sites and no significant associations between 1534C frequency and resistance to permethrin were observed in either population. The 1534C allele has been reported previously in mosquito populations from Guerrero (Aponte et al., 2013) and Merida (Saavedra-Rodríguez et al., 2014). Saavedra-Rodríguez and colleagues (2014) detected a 1534C frequency of 0.79-1 for five areas of Merida, with evidence both 1016I and 1534C co-occurred in 81.2% of the individuals tested (Saavedra-Rodríguez et al., 2014). Co-ocurrence of more than one kdr mutation can have an additive and/or synergistic effect on sodium channel sensitivity (Hu et al., 2011). The present study found resistant homozygotes for both mutations (1016I and 1534C), as well the heterozygote for 1534C and homozygote for 1016I. Double homozygous mutants for these kdr mutations are reported for *Ae. aegypti* populations from Caribbean and South America (Alvarez et al., 2014; Harris et al., 2010; Linss et al., 2014) including Mexico (Aponte et al 2013).

In an environment that is favorable to the proliferation of *Ae. aegypti* throughout the year, with exposure of survivors to PYs over a period of 10 years, and the additive effect of allelic resistance (Ponce-García et al., 2009; Barbosa et al., 2011), it is not surprising that both mutation 1016I and 1534C have almost fixed. The presence of both mutations in Merida and Acapulco could, at least in part, explain the high levels of permethrin resistance observed.

3.5.2. Susceptibility and resistance mechanism to Carbamates and Organophosphates.

The OP and CA resistance in *Ae. aegypti* is widespread in other countries of the Americas. Resistance to temephos (larvae tests) is reported for several localities of the Caribbean Region (Bisset et al., 2001; Polson et al., 2012; Rawlins et al., 1998; Vaughan et al., 1998; Wirth and Georghiou, 1999), Central (Bisset-Lazcano et al., 2009) and South America (Bisset et al., 2001, 2007; Mazzarri and Georghiou, 1995); CAs resistance (adult tests) such as propoxur and bendiocarb is reported in Colombia (Maestre et al., 2010; Ocampo et al., 2011); resistance to several OPs (adult tests) such as chlorpyrifos is found in Cuba (Bisset et al., 2001), fenitrothion in Guatemala (Brogdon and Barber, 1990) and Colombia (Fonseca-González, 2011; Maestre et al, 2010), and pirimiphos methyl in Cuba and Venezuela (Bisset et al., 2001).

In Mexico the evidence OP resistance is reported in temephos resistant populations from several regions of Mexico (Lopez et al., 2009), and seems to be a consequence of uninterrupted use of temephos since 1968 for larval of *Ae. aegypti* in Mexico; and in chlorpyrifos resistance populations of *Ae. aegypti* from the Atlantic coast of Mexico collected in 2009 (before the widespread use of this insecticide), and attributed this to the extensive use of this insecticide in agriculture activities (Lopez et al., 2014). In both cases evidence of esterase-based mechanism is suggested to confer OP resistance.

In this study, both sites were susceptible to CAs but some resistance to the OP chorpyrifos was detected. OPs (i.e chlorpyrifos) and CAs (propoxur and to a lesser extent bendiocarb) were introduced in Acapulco to control mosquito adults since 2010, and more recently in 2012 in Merida. Prior to this, as part of national malaria control strategy, the OP malathion was used for specific foci of malaria transmission, which included the State of Guerrero (Gómez-Dántes and Birn, 2000) from 1996-1999 malathion-based formulations were applied across wider areas for dengue (Espinoza-Gómez et al., 2002; SSA, 2001). Susceptibility to OPs and CAs may be consequence of the lack of an intense insecticide-based selection pressure since 1999, in addition to low resistance evolution reported for OPs and CAs in mosquito field populations (Montella et al., 2007; Hemingway et al., 2013). For example, Hemingway et al. (2013) reported no evidence of CAs resistance selection after 7 years of application.

In addition to the lack of complete phenotypic evidence of OP and CA resistance, there was no mechanistic evidence to suggest resistance to these acethycholine esterase inhibitors. There was no evidence of insensitive AChE (with exception of *Ae. aegypti* Cordemex from Merida which was susceptible to OPs and CAs) and no evidence of esterase-based mechanisms.

This study detected decreased susceptibility to chlorpyrifos in 35% of sites in Merida but the diagnostic dose (14 μg/mL/bottle at 30 min) was 6 times lower than the diagnostic dose used by Lopéz et al. (2014) (85 μg/mL/bottle at 30 min). It is therefore possible that this study may have underestimated the susceptibility to this insecticide.

3.5.3. Immediate knockdown (KD) and 24-h post recovery.

Compared with OPs and CAs (slow acting insecticides), which must penetrate into the central nervous system (to achieve the cholinergic synapses), and must be bioactivated in the case of some OPs, the PYs (fast acting insecticides) have a rapid action because of they act on the voltage gated sodium channels of both, peripheral and central nervous systems. After binding of PYs, the sodium channels in the neurons are maintained for a longer length of

time in their opened conformation, which results in a continuous nervous impulse. The PYs exert a sublethal incapacitating effect on insects known as knockdown effect (KD), characterized by the inability of a mosquito to coordinate its normal movement, e. g. fly or stand upon acute exposure, lead to paralysis and death if prolonged (reviewed by Suppiramaniam et al., 2010). Depending on the insecticide's dosage, this KD effect is reversible if contact with the insecticide is interrupted. In natural conditions, it is assumed that the wild mosquitoes in KD condition would likely be caught and eaten by predators and ants or die because of desiccation, being crushed or damaged (WHO, 2013). So the time taken by the mosquito to exhibit KD behaviour is an essential characteristic in personal protection against mosquito bites.

In this study, with the concentrations of insecticide used, the KD effect of alpha-cypermethrin was better than permethrin. Type II PYs hold the channels open for a longer time than type I, and are therefore expected to provide a better kill by causing irreversible depolarization of the nerve axons and terminals and consequently a pronounced convulsive phase (Suppiramaniam et al., 2010). However, the results suggest that at least a proportion of knocked down mosquitoes (10/21 tests) may recover from initial exposure to alpha-cypermethrin. In contrast, despite the lower KD effect observed with permethrin, only in (3/21) of *Aedes* population some mosquitoes recovered at 24 h post-exposure. A further observation of this study, which has also been reported for *Anopheles* (Owusu et al., 2015), was that time-to-knockdown in CDC bottle test is unreliable predictor of 24 h mortality.

Other studies on *Ae aegypti* have shown 24 hour recovery following knockdown (Flores et al., 2013; Saavedra-Rodriguez et al., 2007). Failure to be 'knocked down' is a good indicator of the presence of target site mutations. In contast, if a mosquito is knocked down at the end of an exposure but subsequently recovers this may indicate metabolic resistance. Recording mosquitoes for a standard recovery period, could give us additional information about the role of metabolic based-resistance (Bagi et al., 2005).

3.5.4. Spatially heterogeneous insecticide resistance patterns in *Aedes aegypti* populations from Merida.

The focal nature of insecticide resistance has been documented in several studies. For example, in Guatemala, sampling sites for *An. albimanus* only a few kilometers apart varied not only in the presence or absence of resistance, but also in the level of resistance and in the dominant mechanism responsible for resistance (Brogdon et al., 1988). Rawlins (1998) noted this difference in *Ae. aegypti* in some villages of different Caribbean countries: villages separated by as little as 0.5 km showed high differences between their resistance ratios. He

attributed this phenomenon to the nature of *Ae aegypti* discrete populations, as reported by Harrintong and cols (2005), suggesting gene flow between *Ae. aegypti* populations is low, despite their close proximity. Several studies would support this assumption (Ayres et al., 2004; Getis et al., 2003; Honorio et al., 2003; Reiter et al., 1995). However, one study carried out in Mexico reports evidence of extensive gene flow among localities winthin 130-180 km of one another (Gorrochotegui-Escalante et al., 2002). Additionally a recent study carried out in 16 localities separated by as much or more as 2.5 km of one another, suggested high gene flow among populations (Marcombe et al., 2013).

In Merida the populations that were very nearly adjacent to one another had significantly different alpha-cypermethrin resistance profiles. The focal nature of insecticide resistance may be associated with heterogeneities in insecticide selection pressure. The sites within Merida that were sampled for this study were separated by a minimum of 200 m and up to 22 km. Previous evidence suggests that local insecticide pressure, rather than migration of mosquitoes, drives pyrethroid resistance evolution in *Ae. aegypti* in the Yucatan (Saavedra-Rodríguez et al., 2014). In Merida, all the populations tested came from neigbourhoods with different epidemiological backgrounds. Therefore, the historical insecticide selection pressure has not been homogeneous, as chemical control activities are implemented according to the location of reported dengue cases, resulting in heterogeneous intensities of insecticide application across the city. Another factor contributing to insecticide resistance at a micro-geographical level could be the domestic use of commercial pyrethroid-based aerosols (Ranson et al., 2010).

Although further works are needed to monitor the resistance profiles over time, the phenomenon of focal nature of resistance in *Aedes* and particularly for this study, generate some operative questions for vector control. How do these variations in resistance affect the operational impact of vector control strategies? Which type of resistance management strategies (rotation, alternation, mosaic, etc) would be recommended to mitigate the resistance to PYs and improve the vector control?

The present study established baseline insecticide resistance data in sites targeted for screening based intervention using LLIS. Evaluations over time on the efficacy of LLIS in areas where insecticide resistance is already present will help us to understand the operational impact of insecticide resistance. Subsequent chapters will include the evaluation of the LLIS intervention in the context of the insecticide susceptibility over almost two years period.

Chapter 4: A field trial evaluating the efficacy of insecticide-treated door and window screens in combination with targeted control of productive breeding-sites, for control of *Aedes aegypti* populations in Acapulco, Guerrero state, Mexico.

4.1 Context of the Study

Insecticide treated materials (ITMs) are an effective, safe and simple tool with the potential to prevent the transmission of a variety of vector-borne diseases (Wilson et al., 2014) through an individual effect (i.e. bed nets preventing the vector biting and blood feeding) and/or by a community effect (i.e. by reducing the vector lifespan and population abundance/density). Based on the successful control of insecticide-treated bednets (ITNs) against nocturnal endophilic vectors *Anopheles* spp. malaria transmission (Gu & Novak, 2009; Lengeler, 2009; Rafinejad et al., 2008; WHO, 2005a), the WHO Dengue Scientific Working-Group, identified the development/evaluation of ITMs as a primary global research stream (McCall & Kittayapong, 2007).

After the discovery that the effectiveness of a net (physical barrier) improved through the addition/treatment with chemicals on it, to kill or repell insect vectors, the use of ITNs increased in popularity during the mid 80s (Reviewed by Takken, 2002) and massively aumented with the development of long-lasting insecticidal nets (LLINs) that do not required retreatment. LLINs incorporate insecticide (all of them pyrethroids) to the polystyrene fabric or in a resin "coat" on the fibre, achieving a residual insecticidal effect for 1-2 years. When used as a physical barrier, LLINs are expected to reduce human–vector contact by physically blocking the entry of mosquitoes plus the insecticidal and/or irritating/deterrence effect on mosquitoes eventually killing them or reducing their life expectancy (Takken, 2002; Vanlerbeghe et al., 2011a). The LLINs deployed as bednets in several African trials reduced malaria morbidity and mortality (Enayati & Hemingway, 2010).

For dengue and its vector *Aedes aegypti*, early studies during the last decade showed that the use of LLINs reduced vector densities to low levels and that had the potential to impact dengue transmission. In Haiti, LLINs (bed nets) showed an immediate effect on adult populations of *Ae. aegypti* and dengue transmission, and extended for the following 5–12 months after their deployment (Lenhart et al., 2008).

Targeted interventions in the most productive container types has been also promoted by WHO-TDR over the last decade, based on multi-centre studies on pupal survey techniques

and the cost-effectiveness of targeted interventions versus holistic or blanket interventions (Focks, 2003; Focks y Alexander, 2006; McCall y Kittayapong, 2007; McCall et al., 2009; Quintero et al., 2014; Tun Lin et al. 2009). Targeted treatment (TT) of productive *Ae. aegypti* breeding-sites focuses on the appropriate control (by environmental/water management, behavioural interventions and judicious chemical vector control) of water containers that produce the greatest number of pupae (and therefore, by proxy, the greatest number of adult mosquitoes) in the domestic environment (Focks & Alexander, 2006). This can lead to focused vector control activities targeting only or in a particularly effective manner those containers of greatest epidemiological importance, particularly in high dengue transmission risk areas (Manrique-Saide et al., 2011).

Both TT and LLINs in combination have been scarcely tested in field conditions, as an integrated environmental management approach to complement and enhance current dengue vector control. For example, LLINs deployed as curtains (ITC) and water container covers of the most productive breeding-sites in Veracruz (Mexico) and Trujillo (Venezuela) suggested a maximised effect on the reduction of *Ae. aegypti* populations (Kroeger et al., 2006).

However, two key challenges emerged from the initial field trials using LLINs. First, coverage/proper use of the interventions with LLINs as curtains typically declined over time (Tun-Lin, et al., 2009; Vanlerberghe et al., 2011b, 2013). For example, studies in Guatemala (Rizzo et al., 2012) and Mexico (Loroño-Pino et al., 2013) reported that families removed or tied back the curtains to increase ventilation during the day, compromising the utility of the intervention. Secondly, LLINs with pyrethroids (PYs) have to meet the challenge of resistant populations of the vectors, mostly PYs which are still the only insecticide class recommended and available for LLINs (Hemingway et al., 2006; Hougard et al., 2003).

This chapter present the results of field and laboratory evaluations of LLINs as framed mosquito screens permanently fit on doors and windows of houses (hereafter LLIS) on PY-resistant *Ae. aegypti* populations in the dengue endemic Mexican city of Acapulco. The project developed a package of novel interventions for dengue control based on the situation analysis and particularly factors associated with vector breeding and indoor infestation with mosquitoes at household-level, which also included the targeted treatment (TT) of the most productive *Ae. aegypti* breeding sites delivered in a community development approach working together with local authorities.

4.2 Materials and methods

4.2.1. The study site.

The study was carried out in the city of Acapulco in the state of Guerrero on the Pacific coast of Mexico as described in Chapter 1. The state of Guerrero has one of the highest levels of dengue in Mexico; over the past three years, it has been in the top three states in terms of reported dengue cases. Particularly Acapulco, reported >30% of the total cases of dengue in Guerrero in the last decade, but in some years exceeding 40-50% (2002, 42%; 2005, 47%; 2006, 50%; 2009, 52%). The neighborhood Ciudad Renacimiento is located on the north side of Acapulco. Epidemiologically, Ciudad Renacimiento and surrounding environs are considered a high-risk area for dengue transmission, with a continuous report of dengue throughout the year.

4.2.2. Study design.

The study design consisted on a cluster-randomized sampling design with cross-sectional surveys. The study followed a TDR-IDRC protocol following a proposal development/study design/methods workshop on (Quintero et al., 2014). Briefly, a cluster in this project was defined as an area of >100 buildings, including at least 100 private households. To obtain a sample of 20 clusters, a map of the study area using Google Earth software (Google Inc., Mountain View, CA, United States of America) was created (Troyo et al., 2008) and digitally overlaid a grid on it with 100 squares and used simple random numbers to select 20 squares (from the total of 30 identified) (Figure 32).

The sample size was calculated as required for the cluster randomized intervention studies to be also conducted during phase II of this research project. It was based on a post-intervention cross-sectional comparison of the number of pupae per person in the intervention and no-intervention clusters using a two-level hierarchical model with clustering at the cluster level. The sample size reflected a desired power of 80% with the significance level set at 5%. The mean number of pupae per person in no-intervention and LLIS treated clusters was assumed to be 3.0 and 0.3, respectively, based on previous studies (Kroger et al., 2006, Tun-Lin et al., 2009, Arunachalam et al., 2010). For a negative binomial distribution with a dispersion coefficient of 0.02 and an intra-cluster coefficient of 0.05, 8.9 clusters with 100 households per cluster were required per study arm, and the number was increased to 10 per study arm (i.e. 20 clusters for the study site). A negative binomial distribution to ensure a large enough sample was assumed, even if it was not clearly needed. For analysis at the household level, this sample size would yield short 95% confidence intervals (C. I.).

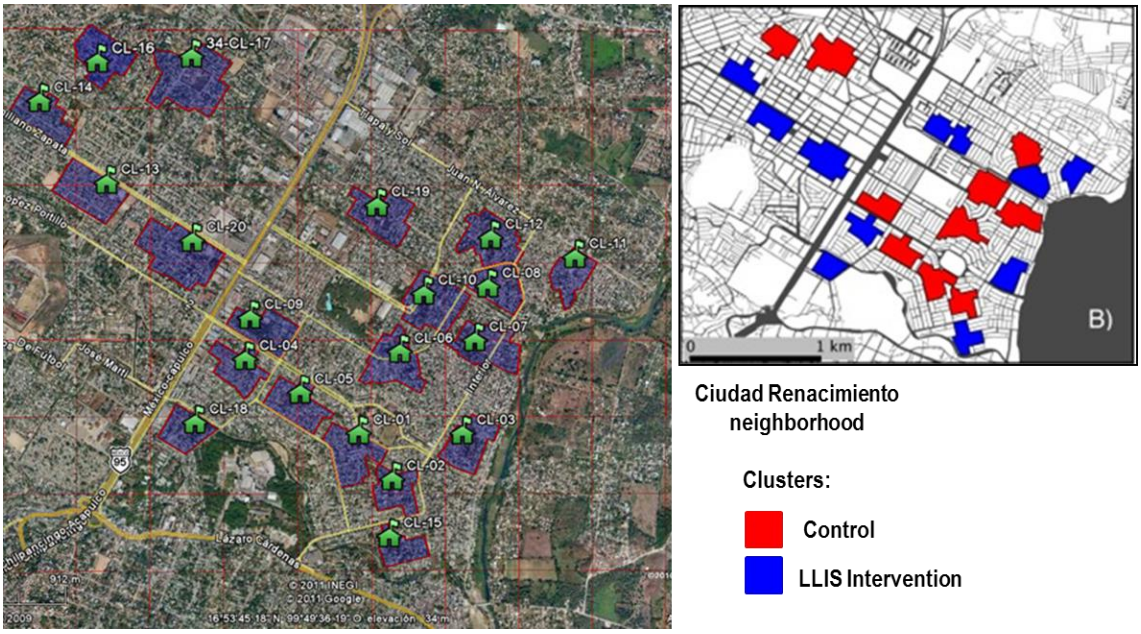


Figure 32. Distribution of the study clusters within the study site Ciudad Renacimiento neighborhood in Acapulco Mexico. The grid used to select randomly 20 squares is showed (left). The final clusters with and without LLIS interventions are showed in blue and red colours respectively (right).

4.2.3. The interventions.

Insecticide-treated house screening (LLIS). The impact on vector infestation of Duranet® screens (0.55% w.w. alpha-cypermethrin-treated non-flammable polyethylene netting [145 denier; mesh = 132 holes/sq. inch]; Clarke Mosquito Control, IL, USA; WHOPES approved) mounted in aluminum frames custom-fitted to doors and windows of residential houses was investigated in Acapulco city in Guerrero state (Figure 33).

The installation, in collaboration with a local small business from the locality and the Ministry of Health (MoH), started on April 2012 and by August 2012 it was finished in 586 households from 9 intervention clusters. For January 2013 the final coverage of intervention was 780 households intervened. During distribution, at least one person in every household received information on the use and maintenance of the through person-to-person communication.

Targeted treatment (TT) of the most productive *Ae. aegypti* breeding sites. A second intervention was implemented 14 months after the beginning of LLIS installation (April 2012) based on targeted treatment (TT) of the most productive *Ae. aegypti* breeding sites every two months (the first TT intervention was performed in June 2013).

Pupal productivity surveys were carried out during the dry and wet season of 2012 in order to identify all potentially productive containers (see section 3.4.4. Entomological surveillance). Targeted interventions may influence vector-breeding patterns over time, hence the pupal productivity survey was repeated after a determined interval to establish newly important or alternative vector breeding sites.

Figure 33. The LLIS intervention (Duranet®) set as framed mosquito screens on windows an external doors of treated houses in Ciudad Renacimiento, Acapulco, Mexico.

The TT included the use of Spinosad, (Natular®) a biological and environmentally friendly larvicide, on the most productive containers identified during the pupal productivity surveys e.g. tanks and 200L drums/barrels in Acapulco. The dose applied was the recommended by the manufacturer, 1 tablet per every 200 lt. in large containers i.e. barrels, water storage tubs and tanks.

During the first cycle (June 2013) 1,789 water tanks and 200 L drums/barrels (Figure 34) in the households of intervention clusters were treated with larvicide Spinosad (Natular®). The following application was performed at the end of the dry season in 2013 (September n=1791 tanks and barrels) and was repeated every two months until March 2014 (November 2013 n=1686, January n=1658, March 2014 n=1595).

Figura 34. Targeted treatment (TT) based on Natular® DT (Spinosad 7.48%) in Acapulco, Guerrero, during the first cycle of application (June 2013).

Routine *Ae. aegypti* control activities by the local vector control program: adulticiding (outdoor and indoor spraying with Chlorpyrifos and Propoxur respectively) and larviciding (Abate and Spinosad) (http://www.cenavece.salud.gob.mx/programas/interior/vectores/dengue/guias_operativas.html), were carried out in untreated houses. Nevetheless, Renacimiento as a whole is a dengue endemic area and activities were also performed in the areas with interventions. These activities are performed periodically where a high risk of dengue transmission is detected using the National Platform for Entomological Surveillance and Integral Vector Control described by Hernández- Avila et al., (2013). Activities affecting both LLIS and no-intervention arms were performed on February-April 2013 (Integrated Vector Control in response to risk indices IVCRI); a "MegaOperativo" was performed during 3-7 July 2013. By September 2013, after tropical storms Manuel and Ingrid, the MoH performed ULV

spraying from vehicles and by airplane from 26 Sep-20 Oct 2013. During 2014, IVCRI were performed in both areas during 28 April- 4 May, 12-18 May and 26 May-1 June.

Figure 35 shows the trial design for Acapulco.

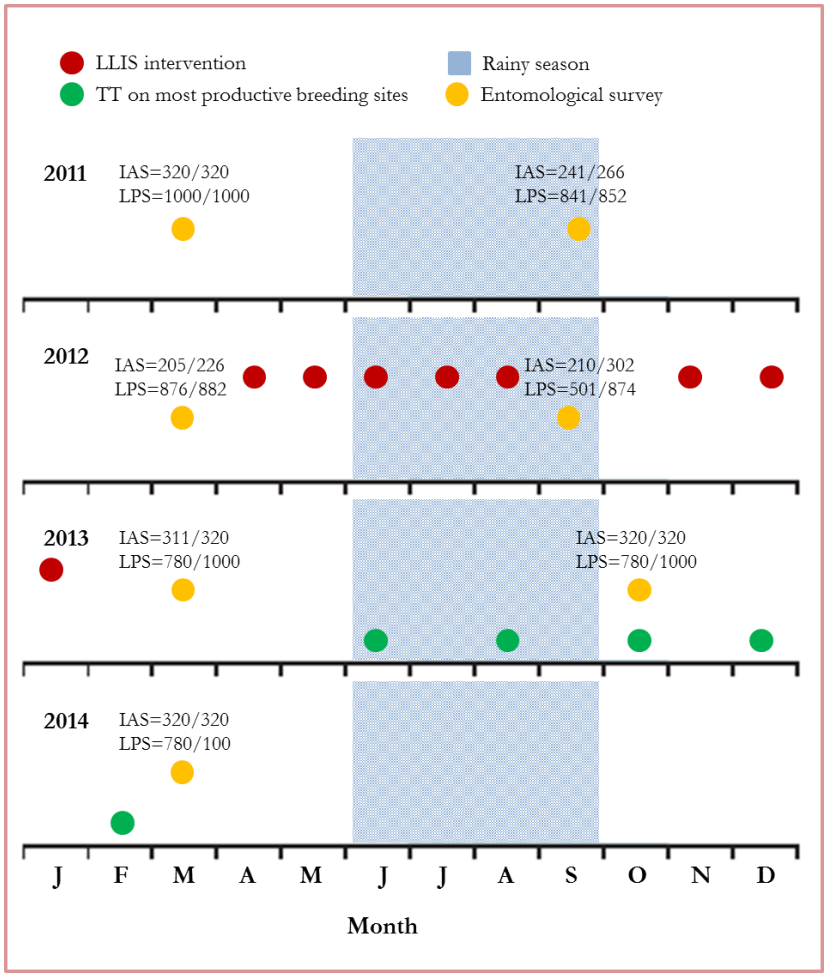


Figure 35. Trial design for Acapulco study site. The number of houses surveyed in the indoor adult survey (IAS) and larval&pupal survey (LPS) is given for both group of houses (LLIS treated/untreated houses). These numbers represent the houses where the entrance was permitted.

4.2.4. Entomological surveillance.

Seven cross-sectional entomological surveys were conducted: before (March 2011, September 2011, March 2012) and at 5, 12, 18 and 24 months (September 2012, March 2013, October 2013, March 2014; wet, dry, wet and dry seasons respectively) post-intervention (PI). See figure 35. In each survey, the number of inhabitants per house was registered.

Indoor-adult surveys. A sub-sample of 32 houses from all the clusters was selected through systematic random sampling for each cross-section entomological survey during dry and wet season of 2011, 2012 and 2013 and for the dry season 2014. In first post-intervention

survey (which corresponded to the rainy season 2012), a sample of 210 houses from 9 clusters[1] with LLIS was monitored because their installation in the study site was in progress (75% of advance; 586/780 of houses to be intervened and distributed only in 9/10 clusters). This entomological survey took place 1 year after the initial baseline study and 1-5 months after LLIS was installed to households that had previously acted as controls (Figure 35).

In each survey indoor-adult mosquito collections were conducted inside of houses using modified CDC backpack aspirators (John W. Hock Company, Gainesville, Florida, USA) for 15 minutes that was sufficient time to ensure complete coverage of the lower level of the house (Williams et al., 2006). Collections from all selected houses within each cluster were made on the same day between 09.00-15.00 hrs. Mosquitoes collected were identified to species and sex.

Larval and pupal surveys. Cross-sectional larval and pupal surveys were conducted in all the houses (n=2,000) during both dry and rainy seasons of 2011, 2012 and 2013 according to a standard protocol for pupal surveys (Manrique-Saide et al., 2011) by 10 trained university or vector control staff members. In each cluster, intradomestic and peridomestic spaces of residential premises were inspected. Containers were classified according to type, source of water, capacity, presence of a proper lid, proximity to shrubbery, and presence of larval control measures. Only water holding containers (WC) were examined. The surveyor determined the presence or absence of *Ae. aegypti* immature (larvae and pupae) in each container and collected and counted all the pupae or a took a sample in large containers were collection of all pupae was unfeasible according to recommendations of the standard protocol. A sample of the pupae thus obtained was examined in the laboratory and left to develop into adult mosquitoes, which were then identified by species and sex.

4.2.5. Monitoring the durability of LLIS under operational conditions.

Standard World Health Organization cone bioassays (WHO, 2005b) were performed in order to determinate the insecticidal activity (bioefficacy) of new, non-exposed LLIS samples and samples of LLIS after 6, 12, 18 and 24 months under operational conditions.

Groups of five non-bloodfed, 1-3 day old *Ae. aegypti* (New Orleans and local strains) were exposed to netting materials (25 cm x 25 cm) for 3 minutes (10 replicates for each sample), under WHO cones and held for 24 h in paper cups with access to a 10% sucrose solution.

[1] The LLIS intervention was not yet ready in one cluster (Cluster 15) at the time of 4th entomological survey (September 2012); the LLIS installation in that cluster was finished until December 2012.

The knock down effect at 30 min and 1 hour after the 3-minutes exposure was determined. Mortality was recorded after 24 h (Figure 36).

The *Ae. aegypti* susceptible and local strains kept under laboratory conditions were used in biossays. Eggs were obtained from the neighbourhood Ciudad Renacimiento from ovitraps deployed during March 2013. Briefly, eggs collected from the filed site were sent to the insectary of the Unidad Colaborativa para Bioensayos Entomologicos of the Universidad Autonoma de Yucatan Mexico (UCBE-UADY) and allowed to hatch. Larvae were reared according to standard procedures and laboratory conditions until adult emergence to obtain 1-3 day-old F1 adult generation. The New Orleans (NO) strain of *Ae. aegypti* provided by Centre for Disease Control (CDC), Atlanta, USA was used as the standard susceptible strain.

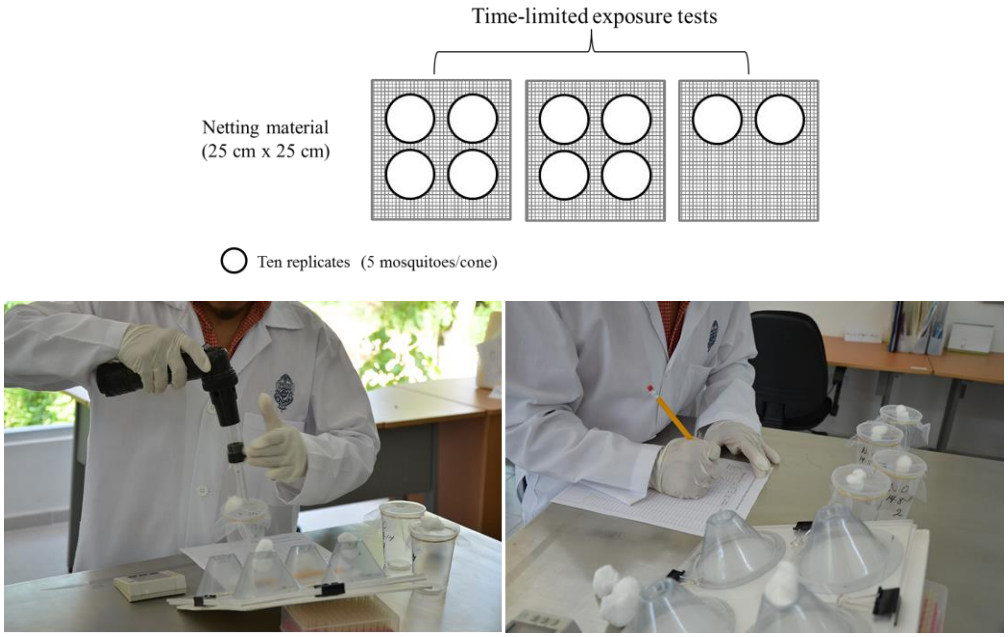


Figure 36. WHO cone test. For evaluate de bioefficacy of LLIS, time-limited exposure tests were carried out. Batches of mosquitoes (5 per cone) were tested on treated netting for 3 min (10 replicates), knockdown scored after 30 min and 1 h and mortality after 24 h.

Three randomly selected houses in each intervention clusters were visited and sampled per house 1 of the LLIS (3 households x 10 cluster) at 6, 12, 18 and 24 months after they were installed. The installation of LLIS started on April 2012 and was completed in January 2013. As different nets had different times of deploying/exposure, the age (deploying time) of nets distributed were identified. Particularly, damaged screens were removed completely and replaced for new ones. Each entire net were transported separately from the field to the laboratory, cut immediately (pieces of 25 cm x 25 cm) and wrapped in aluminium foil and stored at 4 ºC (Figure 37). Theses net samples were used for bioefficacy bioassays.

Figure 37. Sampling netting material under operational conditions. The LLIS used for the bioassays were collected directly from households throughout the 10-intervention clusters from neighbourhood Ciudad Renacimiento (3 households x 10 cluster) at 6, 12, 18 and 24 months after their installation. The nets collected were packaged individually and labelled to avoid mixture with other type of nets.

All these pieces of netting materials were posteriorly classified according to their level of soiling. The level of soiling of each net was categorized according to a gray color palette (Table 5).

Table 5. Values scales for level of soiling.

LLIS samples	Categories	Example of netting material	Gray color palette
DuraNet®	New		
MER03-4	Clean		
MER01-3			
MER01-6	Soiled		
MER10-6			
MER016-3	Very soiled		
ACA08-95			
ACA20-76B	Extremely soiled		
ACA04-9			

The category for each netting sample was defined in consensus among three different members of the working team staff. The effect of the different soiling levels (if any) was explored as another variable on the efficacy of LLIS.

4.3 Data management and analysis

4.3.1. Entomological indicators.

For adult collections the following indicators were calculated: a) Houses positive for female *Aedes* (%), b) Houses positive for blood-fed females (%), c) Houses positive for male *Aedes* (%), d) Number of female *Aedes* per positive house, e) Number of blood-fed female *Aedes* per positive house, f) Number of male *Aedes* per positive house.

For immature collections were calculated: a) Breteau Index (BI)= (number of *Aedes* positive containers x 100)/number of inspected houses); b) House positive for immature *Aedes* (%); c) House positive for *Aedes* larvae (%); d) number of *Aedes* larvae per house; e) House positive to pupae *Aedes*; f) number of *Aedes* pupae per house; g) Pupae per Person Index (PPI)= total number of *Aedes* pupae/registered number of inhabitants.

The houses where the householder did not permit the entrance were not considered for these calculations (see figure 35).

For WHO cone tests, data was pooled and the percent of knockdown and mortality were calculated and corrected when the mortality in control replicates was >5 and <20% using Abbott's formula (Abbott 1925).

4.3.2. Statistic analysis.

Aedes sp. infestation levels were the outcome measures. The BI was calculated per cluster and survey round. For presence-absence data, logistic regression models accounting for each house membership in a given sampling cluster (cluster-robust SE calculation) were performed. Odds ratios and 95% confidence intervals were calculated. Over-dispersed index data were compared between arms using the Mann-Whitney test. The impact of treatment on each metric was analyzed by negative-binomial regression using treatment as predictor variable. Negative binomial models also accounted for membership of a house in a sampling cluster (cluster-robust SE calculation). Odds ratios and incidence rate ratios with 95% confidence intervals (C. I.) were assessed and significance expressed at the 5% level. Analyses were performed using STATA 12.0 (Stata Corp, College Station, TX).

A Generalized Additive Mixed Model (GAMM) (Zuur et al. 2009) was applied to determine the association between various entomologic indicators and the time (in days)

since the installation of the window and door screens. Time to intervention (t_i) was calculated by estimating the number of days that elapsed between the installation of the LLIS and the entomologic survey of each treatment house. The untretaed houses were excluded the from this analysis because analyses aimed at quantifying the temporal effect of LLIS. The full model had the form: $Y_{Aedes} = \alpha + f(t_i) + Z(cluster_i) + \varepsilon_i$. Where Y_{Aedes} is the entomologic measure and $Z(cluster_i), \varepsilon_i \sim N(0, \sigma^2)$, represents a random effects term associated with observations from the same cluster. A negative binomial or binomial link functions was used depending if Y_{Aedes} was based on counts or binary values, respectively. The (possibly) non-linear relationship between the response variable and time since LLIS installation was quantified by incorporating a smoothing function ($f(t_i)$) representing the additive component (Zuur et al. 2009). We fitted $f(t_i)$ by applying a penalized cubic spline function to the data (Zuur et al. 2009). The importance of time since the installation of LLIS was assessed by evaluating the significance of the $f(t_i)$ term. Akaike Information Criterion (AIC) scores were used to compare the full model with a GAM model without random effects. A model with ΔAIC = 2 or more units lower than any other model was considered the best. Once the best model was identified, we plotted each predicted $f(t_i)$ as either a curve (if $f(t_i)$ was significant) or a line (if $f(t_i)$ was not significant). Analyses were performed using the mgcv package from the R statistical software.

The proportion of mosquitoes that had died at 24 h of the total number exposed to the LLIS/netting materials collected at different deploying times was calculated. To estimate the effect of LLIS exposure factors such as soiling on the susceptible mosquito survival rate, Poisson regression models was contructed with survival as dependent and soiling as independent variable. Survival rate ratios and 24-h mortality (and 95% C. I.) were obtained from these models. Analyses were performed using STATA 12.0 (Stata Corp, College Station, TX).

4.4 Ethical aspects

This study received clearance from the ethical committee of the Mexican Ministry of Health of Guerrero and the ERC (Ethical Review Committee) of WHO. The LLIS were made from material that is approved by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for bed net use. Each householder approved the intervention and written informed consent was obtained from each individual household included in the study.

4.5 Results

4.5.1. Vector breeding and productive containers in the dry and wet season 2012.

A total of 10,501 containers holding water were identified during the dry season 2012 in Acapulco. Their characteristics are shown in Table 6. The majority (98%) were found outdoors and 3% were positive for *Ae. aegypti* immature (larva or pupae). The *Ae. aegypti* pupae were collected in 11 different types of containers (Table 6). The most productive containers in Acapulco during the dry season 2012 were: tanks (46.3% of total pupae collected), followed by plastic barrels (35.5%) and bucket/pots (8.7%) accounting for the 90.5% of the pupae collected (Figure 38).

Figure 38. Examples of containers that were productive for pupae in Acapulco, 2012. A) Tanks, B) Plastic barrels, C) Bucket/pots.

A total of 11,719 containers holding water were identified during the rainy season 2012 in Acapulco. Their characteristics are shown in Table 6. The majority (98%) were found outdoors and 3% were positive for *Ae. aegypti* immature (larva or pupae). *Ae. aegypti* pupae were collected in 13 different types of containers which represented only 1.6% (175) of the total number of containers holding water (Table 6). The most productive containers in Acapulco during the rainy season 2012 were plastic barrels (28.9% of total pupae collected), followed by tanks (22.6%) and bucket/pots (19.9%) and assorted small items (15.5%) accounting for the 86.9% of the pupae collected (Figure 38).

Table 6. Frequency of water-holding containers (WC) and the pupal productivity (# PU= number of pupa, % PU=contribution of pupae collected) in 2,000 houses in Acapulco Mexico during the dry and rainy seasons of 2012.

	WC		#PU		%PU	
Category	Dry	Rainy	Dry	Rainy	Dry	Rainy
Tanks	1340	1280	557	86	46.30%	22.60%
Plastic barrels	1697	1800	427	110	35.50%	28.90%
Buckets/pots	5495	5359	105	76	8.70%	19.90%
Large water tubs	164	22	72	10	6.00%	2.60%
Small tanks	109	35	17	0	1.40%	0.00%
Flower pots	119	167	12	21	1.00%	5.50%
Kitchen/laundry containers	35	75	5	1	0.40%	0.30%
Plant pots	306	518	4	4	0.30%	1.00%
Pools	1	0	2	0	0.20%	0.00%
Ground tanks	628	598	2	0	0.20%	0.00%
Broken domestic appliances	7	6	1	6	0.10%	1.60%
Bathroom containers	8	10	0	0	0.00%	0.00%
Drinking troughs	410	408	0	2	0.00%	0.50%
Cisterns	27	18	0	0	0.00%	0.00%
Assorted small	106	522	0	59	0.00%	15.50%
Assorted large	1	107	0	0	0.00%	0.00%
Fountains	8	2	0	0	0.00%	0.0%
Other (Leaf, coconuts)	0	20	0	0	0.00%	0.0%
Puddles	0	12	0	0	0.00%	0.0%
Bottles	0	276	0	0	0.00%	0.0%
Cans	0	10	0	0	0.00%	0.0%
Tires	6	39	0	4	0.00%	1.00%
Wells	34	8	0	1	0.00%	0.30%
TOTAL	**10501**	**11292**	**1,204**	**380**	**100%**	

4.5.2. First intervention: insecticide-treated house screening (LLIS).

Impact on indoor adult mosquitoes. Adult-based entomological indicators are summarized on Table 7.

During the pre-intervention surveys (March and August 2011 and March 2012) adult-based entomological indicators showed similar seasonal patterns of infestation in both study arms, showing an increase of infestation levels during rainfall and a decrease during the dry season (Figure 39).

The results from post-intervention (PI) surveys, 5 months (September 2012) and 12 months (March 2013) after the intervention was in place, showed clearly that all indicators of adult infestation in the houses protected with LLIS during the rainy and dry season were lower than in the previous dry season in the same year (Table 7 and Figure 39).

At five months PI; significantly fewer treated houses were infested with *Ae. aegypti* adult females (OR=0.38, 95% C. I. 0.21–0.69), blood-fed females (OR=0.36, 95% C. I. 0.21–0.60) and males (OR=0.39, 95% C. I. 0.19–0.77). A significant impact was still seen at 12 months PI for adult females (OR=0.41, 95% C. I. 0.25–0.68) and males (OR=0.41, 95% C. I. 0.27-0.64) but not for blood-fed females (OR=0.51, 95% C. I. 0.24-1.05) (Figure 39).

Analyses of infestation density showed a similar trend with significant reductions in mean *Ae. aegypti* abundance in treated houses: adult females at 5 (IRR=0.37, 95% C. I. 0.27–0.49) and 12 (IRR = 0.40, 95% C. I. 0.23-0.70) months PI; males at 5 (IRR=0.39, 95% C. I. 0.28–0.54) and 12 (IRR=0.49, 95%C. I. 0.33-0.72) months; blood-fed females at 5 (IRR=0.32, 95% C. I. 0.23–0.45) but not at 12 (IRR=0.49, 95% C. I. 0.23-1.05) months (Figure 39).

Comparing wet season data from treatment houses before (Aug 2011) and after (Sep 2012) intervention, *Aedes* female and blood-fed female numbers were significantly lower after intervention (Wilcoxon Matched Pairs, W = 30706, z=3.717, $p<0.05$ and W = 20706, z=3.146, $p<0.05$), but male abundance did not change (W = 20706, z=1.385, $p>0.05$).

In addition, the results also indicated that even if adult infestation in both arms (LLIS and no-intervention arms) was lower during the rainy season 2012 than in the rainy season in the previous year, the infestation in houses protected with LLIS were significantly much lower (Figure 39).

Impact on breeding sites and immature mosquito stages. Immature-based entomological indicators are summarized on Table 8.

In August 2011 (before the intervention) the proportion of houses positive for immature *Aedes* was 20% (IC95%=15.68-25.21) in the treated arm and 27% (IC95%=21.18-31.81) in the untreated arm (Table 8). During this survey, significant differences were observed between both arms, treated and untreated (OR=0.71, 95% C. I. 0.51-1.00). Significant differences were also observed for house positive for larvae *Aedes* (OR=0.71, 95% C. I. 0.50-1.00).

At the first PI survey (5 months after the LLIS intervention was started) both arms of houses, treated and untreated, showed a decrease on larvae/pupae indicators in comparison with the baseline survey. At that moment, the installation of LLIS was in progress, with a 75% of coverage (586/780 of the total of houses in which the intervention was accepted).

At the second PI survey (12 months), all immature indicators kept decreasing in the LLIS arm; but contraily, pupae-based indicators increased in the no-intervention arm. Significant differences were observed between LLIS and no-intervention arms at 12 months PI for all pupae-based indicators (house positive to *Aedes* pupae OR=0.56, 95% C. I. 0.33-0.96; number of Aede pupae s per house IRR=0.29, 95% C. I. 0.12-0.70; PPI IRR=0.31, 95% C. I. 0.11-0.86). No significant differences were observed on houses positive for immature *Aedes* (IRR=0.68, 95% C. I. 0.38-1.22), houses positive for larvae (IRR=0.69, 95% C. I. 0.38-1.28) and BI (IRR=0.68 95% C. I. 0.37-1.22).

4.5.3. Second intervention: TT of the most productive *Aedes aegypti* breeding sites.

Impact on indoor adult mosquitoes. Significantly fewer treated houses were infested at 18 months PI with *Ae. aegypti* adult females *(*OR=0.07, 95% C. I. 0.05-0.10); but not for blood-fed females (OR=0.63, 95% C. I. 0.36-1.09) or males (OR=1.19, 95% C. I. 0.84-1.7) (Figure 39). At 24 months PI significant differences were observed between untreated and treated houses in the presence of adult females (OR=0.44, 95% C. I. 0.20-0.95), blood-fed females (OR=0.28, 95% C. I. 0.10-0.74) and males (OR=0.44, 95% C. I. 0.27-0.71).

Analyses of infestation density based on adult catches showed a similar trend with a significant reduction in adult females (IRR=0.12, 95%C. I. 0.08-0.19) at 18 months PI; but not for blood-fed females (IRR=0.54, 95% C. I. 0.29-1.0 or males (IRR=0.93, 95% C. I. 0.72-1.22) (Figure 39). At 24 months PI significant differences were observed on the number of indoor adult females (IRR=0.04, 95%C. I. 0.21-0.98); blood-fed females (IRR=0.25, 95% C. I. 0.09-.070) and males (IRR=0.48, 95% C. I. 0.27-0.86).

Table 7. *Aedes aegypti* adult infestation indicators by group (LLIS treated and untreated) of houses and entomological survey in Acapulco, Guerrero.

Group	Pre-Intervention (Dry season 2011)	Pre-Intervention (Wet season 2011)	Pre-Intervention (Dry season 2012)	5 months PI (Wet season 2012)	12 months PI (Dry season 2013	18 months PI (Wet season 2013)	24 months PI (Dry season 2014)
House positive for Females							
Treated LLIS	19%	64%	49%	34%	18%	13%	8%
Untreated	20%	64%	54%	56%	35%	68%	17%
House positive for Blood Fed Females							
Treated LLIS	14%	54%	43%	24%	11%	7%	2%
Untreated	16%	58%	47%	46%	20%	11%	8%
House positive for Males							
Treated LLIS	28%	64%	56%	38%	22%	28%	9%
Untreated	27%	61%	58%	60%	40%	24%	19%
Number of Females/positive house							
Treated LLIS	0.331	2.365	1.234	0.543	0.302	0.263	0.122
Untreated	0.341	2.169	1.350	1.477	0.747	2.056	0.266
Number of Blood Fed Females/positive house							
Treated LLIS	0.244	1.564	1.000	0.333	0.164	0.094	0.022
Untreated	0.253	1.628	0.934	1.026	0.331	0.172	0.088
Number of Males/positive house							
Treated LLIS	0.478	2.212	1.459	0.671	0.370	0.619	0.147
Untreated	0.453	1.812	1.726	1.728	0.750	0.659	0.303

Impact on and breeding sites and immature mosquitoes. Treated houses showed significant lower levels of infestation and infestation density in comparison with untreated houses at 18 months PI for all *Aedes* indicators (Figure 40) i.e. BI (IRR=0.43, 95% C. I. 0.26-0.71), houses positive for immature (OR=0.44, 95% C. I. 0.26-0.75), houses positive for

larvae (OR=0.44, 95% C. I. 0.26-0.75), number of larvae per house (IRR=0.36, 95% C. I. 0.20-0.66), houses positive for pupae (OR=0.44, 95% C. I. 0.23-0.82), number of pupae per house (IRR=0.22, 95% C. I. 0.08-0.57) and pupae per person (IRR=0.33, 95% C. I. 0.13-0.82). At 24 months PI significant differences were observed on infestation density indicators but not for infestation indicators, i.e. number of larvae per house (IRR=0.33, 95% C. I. 0.13-0.83), number of pupae per house (IRR=0.26, 95% C. I. 0.10-0.68), and number of pupae per person (IRR=0.30, 95% C. I. 0.10-0.88).

Table 8. *Aedes aegypti* immature infestation indicators by group (LLIS treated and untreated) of houses and entomological survey in Acapulco, Guerrero.

Group	Pre-Intervention (Dry season 2011)	Pre-Intervention (Wet season 2011)	Pre-Intervention (Dry season 2012)	5 months PI (Wet season 2012)	12 months PI (Dry season 2013	18 months PI (Wet season 2013)	24 months PI (Dry season 2014)
Breteau Index (BI)							
Treated LLIS	5.5	31.7	20.4	9.2	7.3	4.5	5.0
Untreated	5.3	36.5	19.2	11.2	10.8	10.4	7.0
House positive for immature *Aedes*							
Treated LLIS	4%	20%	16%	8%	6%	4%	4%
Untreated	5%	27%	15%	9%	9%	10%	6%
House positive for larvae *Aedes*							
Treated LLIS	4%	20%	16%	7%	6%	4%	4%
Untreated	5%	26%	15%	9%	9%	9%	6%
Number of larvae *Aedes* per 100 house							
Treated LLIS	34.9	803.7	270.2	116.6	80.0	41.9	42.3
Untreated	74.1	702.6	256.3	103.8	143.6	116.0	126.5
House positive to pupae *Aedes*							
Treated LLIS	2%	10%	9%	3%	3%	2%	2%
Untreated	3%	12%	9%	4%	5%	5%	2%
Number of pupae *Aedes* per 100 house							
Treated LLIS	2.5	13.3	10.0	3.4	2.9	2.4	2.2
Untreated	2.7	14.3	10.7	4.3	5.2	5.8	2.9
Pupae per Person Index (PPI)							
Treated LLIS	0.025	0.203	0.171	0.041	0.028	0.023	0.018
Untreated	0.031	0.214	0.150	0.049	0.102	0.105	0.071

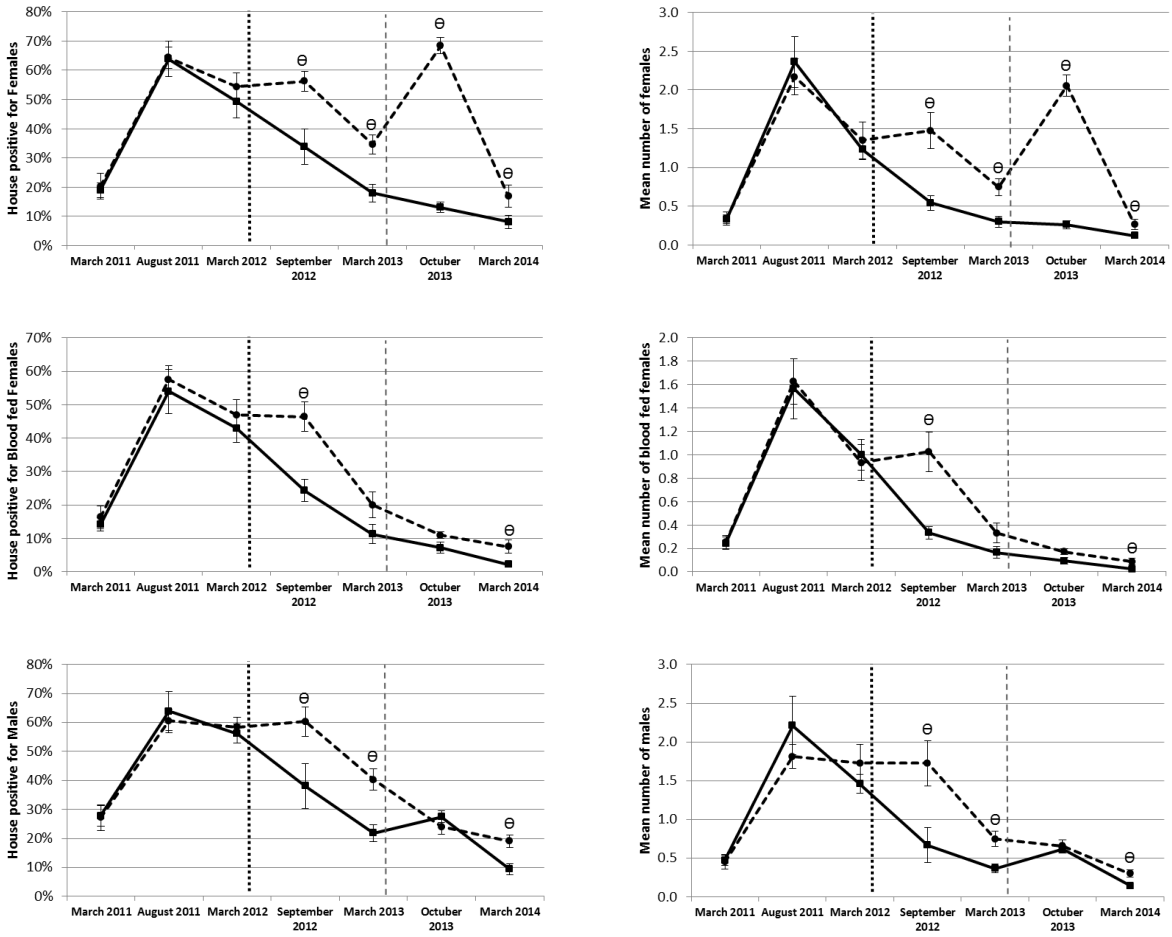


Figure 39. Comparison between treated (solid line) and untreated (broken line) arms of percentage of infested houses (left) and infestation density (right) for *Ae. aegypti* in Acapulco, Guerrero. The vertical dotted and dashed lines represent the start of LLIS and TT interventions respectively. The symbol denotes dates when the index was significantly different between LLIS and no-intervention arms on that date. Error bars show the standard error of the mean.

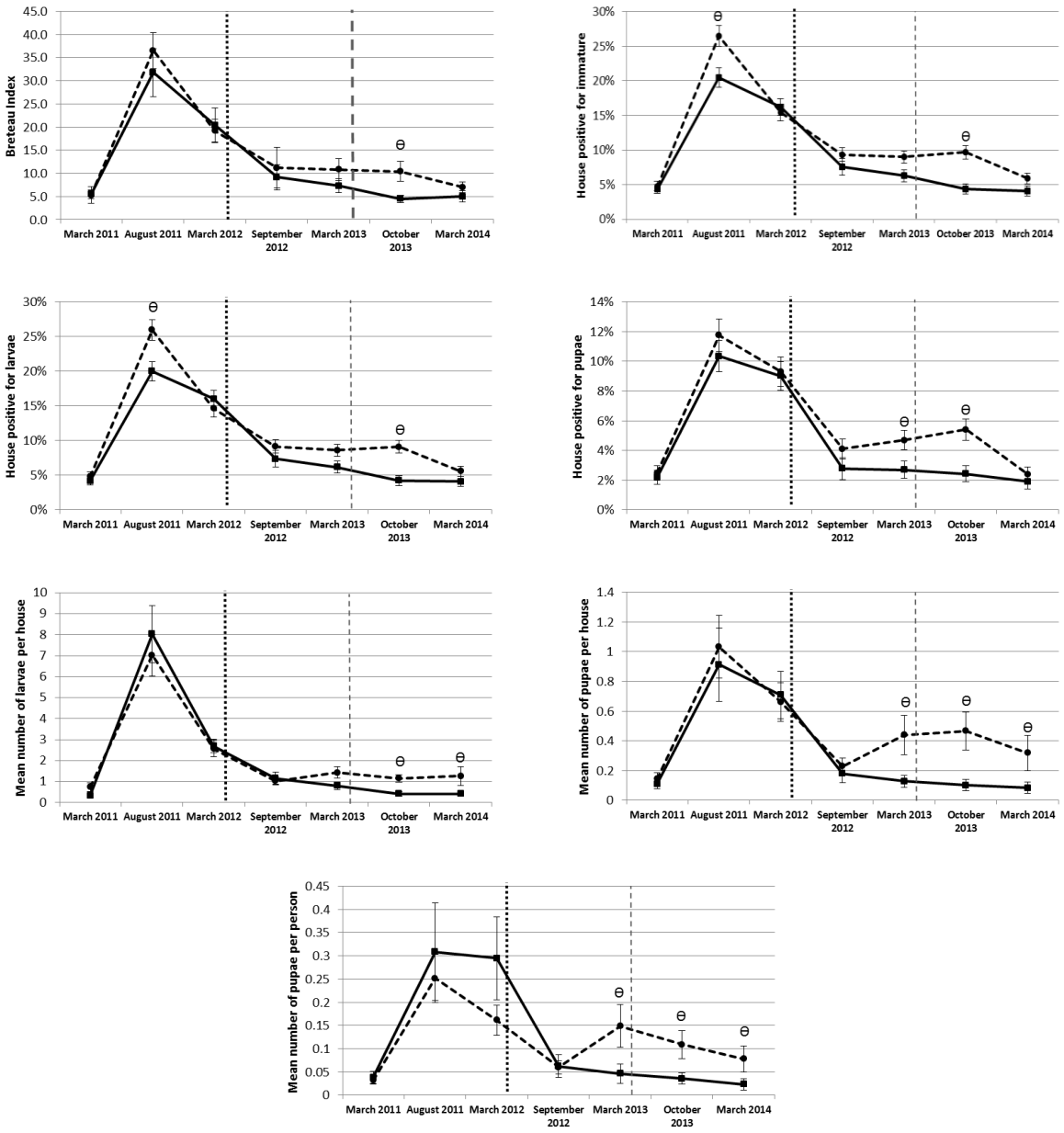


Figure 40. Comparison between treated (solid line) and untreated (broken line) arms of *Ae. aegypti* immature-based indicators for in Acapulco, Guerrero. The vertical dotted and dashed lines represent the start of LLIS and TT interventions respectively. The symbol denotes dates when the index was significantly different between LLIS and no-intervention arms on that date. Error bars show the standard error of the mean.

4.5.4. Temporal persistence of interventions.

Figure 41 shows the plot of f(ti) for each entomologic indicator (immatures and adults). The y-axis of figure 41 can be interpreted as the effect of time since LLIS installation on each entomologic measure. When the predicted value and its 95% credible interval are negative it means that there is a protective effect of LLIS for that factor. In all cases, LLIS achieved a

protective effect for at least 600 days post installation. Adult indices (presence and abundance) showed a second reduction at 500 days post intervention, coincidentally with the introduction of the TT strategy. Figure 39-40 and table 9.

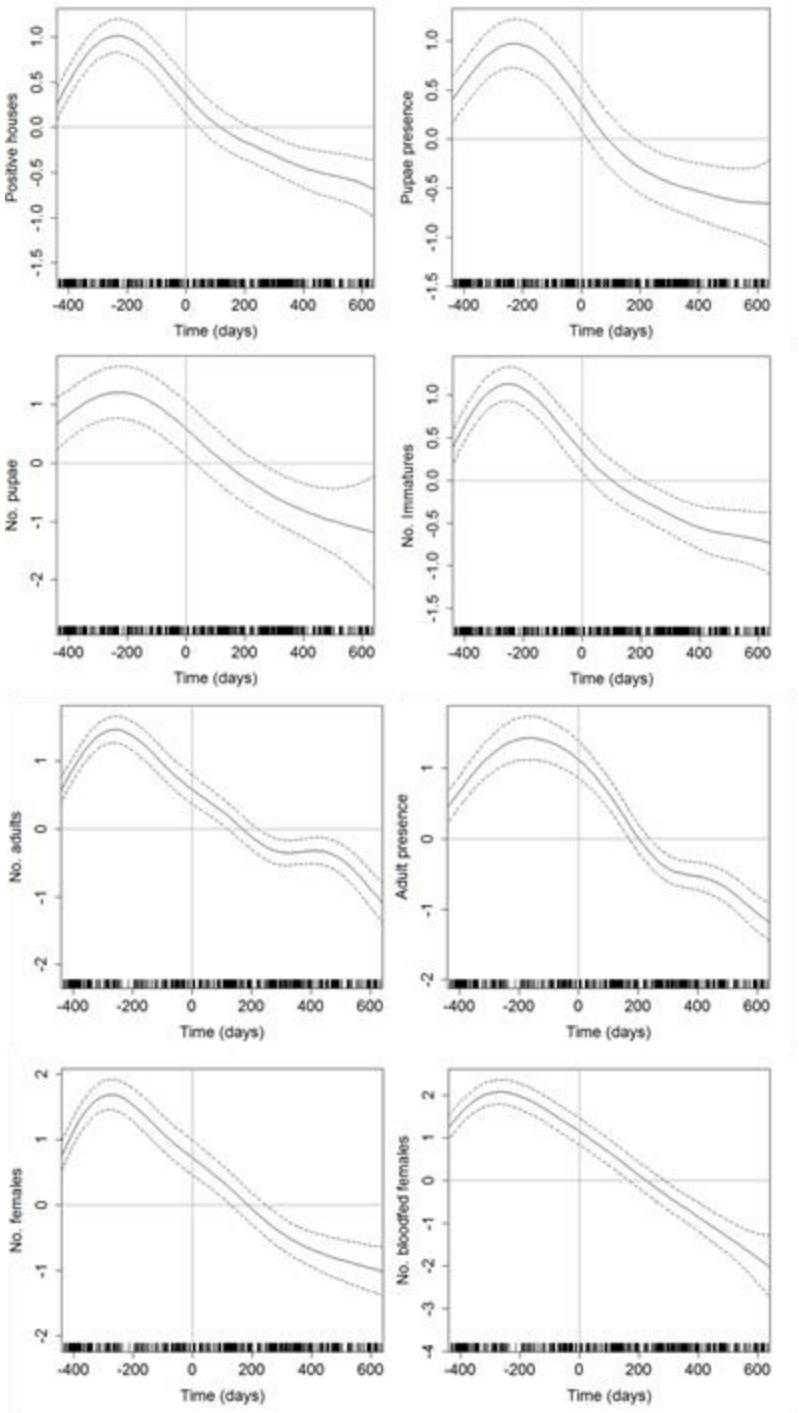


Figure 41. Predicted values for the best GAMM showing the association between the time since LLIS installation and each entomologic indicator for Acapulco, Mexico. Horizontal line shows the area of no difference and vertical line the time when LLIS were installed. The solid line is the predicted value of the dependent variable as a function of the x axis. Dashed lines represent 95% confidence intervals. Places where the confidence bands enclose the horizontal line indicates predicted values where the overall pattern is not significant

Table 9. Parameter value and significance of non-linear parameter ($f(t_i)$) on GAMM models estimating the association between entomologic indices and the time since LLIS installation for Acapulco, Mexico. ΔAIC represents the difference between AIC values of a model excluding (AIC_{GAM}) and including (AIC_{GAMM}) a random effect associated with each cluster.

Life stage	Indicator	Estimated degrees of Freedom	*F*	*P*	ΔAIC (AIC_{GAM} - AIC_{GAMM})
Immature	No. Immatures	5.32	26.1	<0.0001	105
	No. pupae	3.33	9.5	<0.0001	60
	Positive houses	5.35	23.0	<0.0001	956
	Pupae presence	4.55	15.8	<0.0001	1026
Adult	No. females	5.13	39.5	<0.0001	25
	No. bloodfed females	6.49	43.7	<0.0001	4
	No. adults	6.49	43.7	<0.0001	23
	Presence of adults	5.62	40.1	<0.0001	21
	Presence of females	5.29	39.2	<0.0001	2

4.5.5. Bioefficay of LLIS under operational conditions.

Soiling condition of used LLIS. A total of 121 LLIS were sampled in the intervened households with an average fo 30 nets sampled by deployed time. Above 50% of LLIS were categorized as very to extremely soiled (63%, 55%, 59% and 76% for 6, 12, 18 and 24 months PI sampling), and less than 7% as soiled and the rest (24-39%) were classified as clean LLIS (Figure 42).

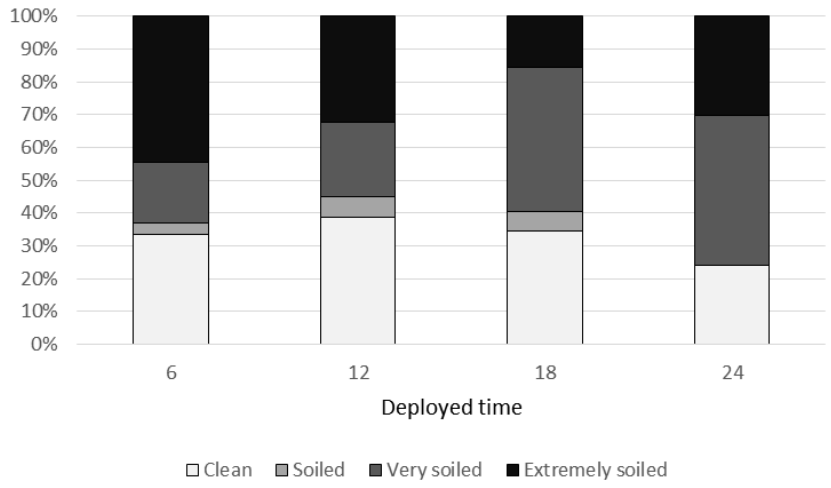


Figure 42. Proportions of net soiled condition by deployed time of LLIS sampled in Ciudad Renacimiento, Acapulco between 2012-2014.

Bioassays. According to bioassays results using the New Orleans susceptible strain (Figure 43A), the residual activity of insecticide on the screens remained consistently high when new (time 0, non-exposed nets). The KD and mortality on new non-exposed nets was 100% and 98% respectively for susceptible strain (the cut off efficacy criteria of WHO for KD is 95% and for mortality is over the 80%). These variables showed a tendency to decrease at 6 months, and then, a gradual increase at 12-18 months of exposure under operational condition, and then again a decrease after 24 months, but always achieving KD and mortality rates below to the cut off efficacy WHO criteria. Overall, very low KD effect and mortality were observed with the wild Renacimiento strain (<30% including new non-exposed nets) (Figure 43B).

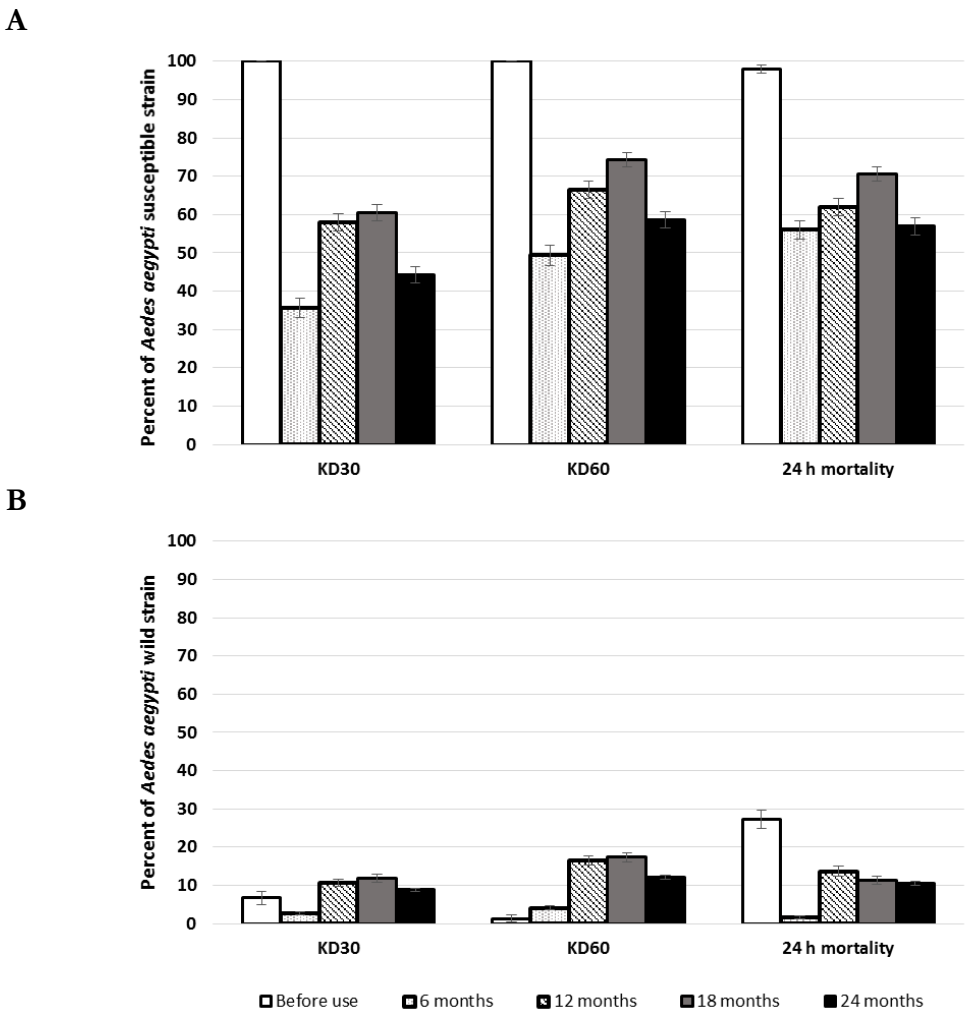


Figure 43. Bioefficacy test on different DuraNet® LLIS samples exposed 6, 12, 18 and 24 months under operational conditions in Acapulco. Results after WHO cone bioassays after 3 min. exposure: knockdown at 30 and 60 minutes (KD30 and KD60) and 24 h mortality and their standard errors of mean (SE) are showed for both, New Orleans susceptible strain (A), and field collected strain (B). Result for a new, non-exposed DuraNet® LLIS sample is also showed (white bar).

When the exposure factor "LLIS age" is considered the chance to survive of susceptible *Aedes* mosquitoes decreases from 21.9 times for the first 6 months of use to 19-14.7 times for the next 18 and 24 months of use respectively; but increases (21.5 times) at 24 months of use. The soiled condition was also significantly associated with increased mosquito survival with 19-40 time more chance to survive when exposed to very to extremely soiled LLIS (Table 10).

Table 10. LLIS exposure factor analysis using poisson regression models contructed with survival as dependent variable, and deployed time and soiling as independent variables for Acapulco net sampling.

LLIS exposure factor	24-h mortality (95% C. I.)	24-h survival rate ratio*	P-value
New Orleans susceptible strain			
LLIS Age			
New, non-exposed	98% (95.7-100.3)	1	
6 months use	56.1% (51.4-60.8)	21.9 (17.0-28.3)	
12 months use	62% (57.5-66.5)	19 (14.7-24.5)	<0.001
18 months use	70.6% (66.9-74.4)	14.7 (11.4-18.9)	<0.001
24 months use	56.9% (52.7-61.2)	21.5 (16.7-27.8)	<0.001
Soiled condition			
New	98% (95.7-100.3)	1	
Clean	96.08% (94.7-97.5)	1.9 (1.5-2.5)	<0.001
Soiled	80% (73.7-86.3)	10 (7.7-12.9)	<0.001
Very soiled	62.5% (59.5-65.5)	18.8 (14.624.2)	<0.001
Extremely soiled	20.2% (17.6-22.7)	39.9 (30.9-51.4)	<0.001

*Estimated with poisson regression models.

4.6 Discussion

The data presented here are the first showing that mosquito-proofing houses with LLIS can reduce the infestation with dengue vectors in houses and that can potentially reduce disease transmission.

The protection of houses with LLIS in an *Aedes*-dengue endemic area of Acapulco substantially reduced indoor adult vector infestation during the rainy, commonly the most favorable season for mosquito abundance. Before the intervention, exposure at the house level to mosquito contact (and biting) in the study site seemed very likely: indoor mosquito collections during the baseline surveys showed the presence of adult *Aedes* in 38% of the houses during the dry season and reached 77% during the rainy season; not surprising since 60-70% of the houses had openings/incomplete walls and unprotected windows/doors open

during the day (data not shown). Nevertheless, after the intervention with LLIS, levels of infestation were significantly lower than in the dry season the same year.

In general, the prevention of adult mosquitoes entering the houses has been a public health measure suggested and employed historically in places where mosquito nuisance and disease transmission are a problem (Lindsay et al., 2002). Basically, screening with mosquito nets the most important points of entrance into a house, such as windows and doors, prevents the entry of adult mosquitoes (Schofield et al., 1990). "Mosquito-proofing" of houses is a form of environmental management based on changes to human habitation to exclude vectors and eventually reducing man-vector-pathogen contact (WHO, 1982).

Research on the efficacy of LLINs in controlling diurnally active *Ae. aegypti* has being encouraged by WHO (McCall et al., 2009). The LLINs, used as single or combined interventions, have been field-evaluated in different settings worldwide as an integrated environmental management approach to complement and enhance current dengue vector control. Some degree of success has been reported after interventions with LLINs against dengue vectors (measured with immature based indicators) when used as a physical barrier on breeding-sites to block oviposition (Kroeger et al., 2006; Seng et al, 2008; Tsunoda et al., 2013) or to reduce human-contact and provide personal protection in the home as bednets (Lenhart et al., 2008) or as curtains hanged on windows and doors (Igarashi, 1997; Kroeger et al. 2006; Lenhart et al., 2013; Nguyen et al., 1996; Rizzo et al., 2012; Vanlerberghe et al., 2011; Vanlerberghe et al., 2013).

A known challenge in reducing dengue-virus transmission is to reduce infected adult vector populations and/or their interaction with humans to levels below that which can sustain an epidemic (Morrison et al., 2008). Residential premises (house and peridomicile) offer important habitats for *Ae. aegypti*. Female mosquitoes emerging from productive breeding-sites move in and out the houses in search of food (human blood), refuge and mating and oviposit at the suitable breeding-sites to complete their life cycle. *Ae. aegypti* is an antropophilic, endophilic and endophagic species which spends most of its adult life within or in the close vicinity of human habitations, as has been demonstrated in studies reporting evidence about *Aedes* adults do not fly away from their habitats where they were developed (Getis et al., 2003; Scott & Morrison, 2002). The house is an important place for human-vector contact because but also the epidemiologically most significant point of contact for dengue virus transmission. In particular, the prevention of endophagy by *Ae. aegypti* is obviously important to stop transmission of virus from infected mosquitoes to susceptible humans, but also to stop *Ae. aegypti* from feeding upon infected humans, to stop mosquitoes

becoming infected, and last but not least, to stop transmission of the virus to new susceptible humans (Beaty et al., 2010).

All above described has been indeed considered for routine control, via ULV or residual spraying of chemical insecticides, as an attempt to prevent the vector from entering and/or attack the vector in the houses. The incorporation of the insecticide with a more judicious use of insecticides to materials (mostly textiles), initially as ITMs and more recently in LLINs, deployed as curtains has been under evaluation, alone or in combination with other methods to prevent *Ae. aegypti* from the indoor home environment, kill a proportion of the population and eventually reduce dengue virus transmission (Igarashi, 1997; Kroeger et al., 2006; Lenhart et al., 2013; Loroño-Pino et al., 2013; Madarieta et al., 1999; Nguyen et al., 1996; Rizzo et al., 2012; Vanlerberghe et al., 2010; Vanlerberghe et al., 2011; Vanlerberghe et al., 2013).

The degree of protection in this study and measured on indoor adult *Aedes* is well greater when compared with a recent study using deltamethrin-treated window curtains (Loroño-Pino et al., 2013). These authors reported 27% of reduction on abundance of adult *Aedes* in houses with ITC and only sustained for a short period of time after their installation. Nevertheless, the authors also reported that houses with ITC were significantly less likely to experience multiple DENV infections in humans and that Dengue virus–infected *Ae. aegypti* females were reduced in houses where curtain use was highest.

Nguyen et al., (1996) and Igarashi (1997) evaluated earlier an intervention with permethrin nets set up covering all openings of houses (in addition to routine anti-*Aedes* health education and control measures) in Hai Hung Province, northern Vietnam and reported a significant reduction (close to 100%) on the houses positive and the abundance of indoor *Ae. aegypti* to undetectable levels for six months, while in the no infestation gradually increased during the epidemic season. Even more, the intervention seemed to effectively prevented DENV transmission in the treated area after the epidemic season (anti-dengue IgM positive rates between the study and control areas). No other experiences relating this level of reduction and duration are available.

Other studies available and showing success after interventions with LLINs against dengue vectors have measured immature based indicators i.e. House, Bretaeu and Pupal indices (Lenhart et al., 2008; Nguyen et al., 1996; Rizzo et al., 2012; Seng et al, 2008; Tsunoda et al., 2013; Vanlerberghe et al., 2011, Vanlerberghe et al., 2013). Thus, no other entomological data based on adult collections of dengue vectors is available for comparison.

A couple of key challenges have emerged from these field trials above mentioned and can be addressed by installing permanently insecticide-treated nets on windows and doors or LLIS. Sometimes curtains are not used as intended for their maximum efficacy i.e. are removed or tied up (Loroño-Pino et al., 2013) and the coverage of the interventions based on LLINs typically falls dramatically over time (Tun-Lin, et al., 2009; Vanlerberghe et al., 2011; Vanlerberghe et al., 2013). The LLIS, are 'user-friendly', requiring little additional work or behavioral change by householders and are well accepted by communities, as their perceived efficacy is reinforced by the reduction in other biting insects, cockroaches, houseflies and other pests (Fig. 33). Additionally, this shows that insects, including mosquitoes, definitely contact LLIS when they are trying to enter and move through houses, contrarily to what has been reported for ITC (Lenhart et al., 2013).

Results of this study compare well with the ca. 50% reduction recently reported by Manrique-Saide et al., (2014). These authors reported, during entomological collections describing levels of *Aedes* infestation in Merida Mexico, that the presence of window screening significantly decreased both the odds of having *Aedes* adult mosquitoes inside the house (13.6% of unscreened houses vs. 8.5% of the screened houses positive for female *Aedes*) and of the number of females found indoors (means of 0.24 and 0.13 for unscreened and screened houses respectively) (OR = 0.59; 95% C. I. = 0.378 - 0.933; P = 0.02; IRR = 0.52; 95% C. I. = 0.330 - 0.824; P= 0.005). The LLIS incorporate insecticide (pyrethroids) to the polystyrene fabric or in a resin "coat" on the fibre. When used as a physical barrier, they are expected to directly target the adult mosquitoes/reduce human–vector contact by repellence or eventually can kill the vectors that come into sufficient contact with the LLIS, achieving a residual effect for 1-2 years, which is longer than any other applied *Aedes* control tool (Rizzo et al., 2012; Vanlerberghe et al., 2010). Therefore, the location on doors and windows, LLIS theoretically has an effect on the number of mosquitoes entering the house but is also expected to have a potential effect on the survival of those attempting to exit (Kirby et al., 2009; Ogoma et al., 2010).

Protection against mosquito bite and disease transmission with mosquito netting in houses has been historically observed as fundamental technique of malaria control in the early 1900s (Lindsay et al., 2002; Manson, 1900; Ross, 1913). A resurgence of this approach -modifying or improving current housing designs with screens- has shown that provided protection against malaria by reducing the exposure to malaria parasites (Kirby et al., 2008; Lindsay et al., 2003; Walker, 2010) and a well-appreciated and durable vector control (Kirby et al., 2010). Why not for dengue? and against other vector-borne diseases? The integration

of house-screening with insecticide-treated nets to vector-borne-disease control programs merits to be evaluated.

The combination of LLIS with targeted interventions in productive container types was successful in continuing reducing the number of *Aedes* pupae and consequently of adult dengue vectors. The integration with TT is because it is also desirable want to reduce significantly the recruitment *Aedes* populations. The effect was achieved because was applied in the largest coverage possible and at least every two months. If the reduction of the vector density achieved by our intervention is sufficient for reducing or interrupting dengue transmission is unclear. The efficacy of LLIS has at this moment shown to reduce the numbers of mosquitoes that enter a house, but would only kill a proportion of adults (if they are susceptible to the insecticide) that contact the materials. The LLIS alone would not totally suppress adults *Ae. aegypti* because continued recruitment of individuals via adults surviving insecticide contact, plus adults never contacting the materials and adults emerging from breeding sites. Furthermore, this integrated approach with a rational use of insecticides could address positively the high cost of control programs and vector resistance to insecticides.

Bioassays to determine residual effect of the nets after their deployment and operational conditions did not showed very satisfactory results. DuraNet® screens showed low residual insecticidal activity after a year (<63% of 24 h mortality using a susceptible strain), with best results (70% of 24 h mortality) at 18 months of exposure. Other studies have shown better results using the same approach (WHO standard cone bioassays) on susceptible *Ae. aegypti* strains exposed i.e. to deltamethrin-treated curtains (PermaNet®) whose residual insecticidal effectiveness was 98-100% after 12 months (Rizzo et al., 2012; Vanlerberghe et al., 2010). However some variation in bioassay results may to be due to the exposure of LLIS to dust, as evidenced in this study where most of the net samples (>60%) were covered with dust and different levels of soiling. The evidence reported from some studies suggests that dust is not a factor affecting the bioefficacy of long-lasting insecticidal nets (Kayedi et al., 2008; Vanlerberghe et al., 2010); but results associated in this study with mosquito survival showed more likely to survive when the soiled condition level increases.

The field population (wild Renacimiento strain) showed mortalities <30%, for both the new and non-exposed net samples. This can be explained because the locally documented level of resistance to pyrethroids, mainly to permethrin and deltamethrin, and high frequencies of kdr mutation in *Ae. aegypti* populations from Guerrero, including Acapulco (Aponte-Hincapie et al., 2013; Siller et al., 2011). However, recently new evidence reporting resistance to PYs Type I, but susceptibility to PYs Type II (i.e. lamda-cyhalothrin and alpha-

cypermethrin) (Dzul-Manzanilla et al., 2014) in the same *Aedes* population from Acapulco, contrasting with the low mortality obtained in this study from cone bioassays. Some authors state that the efficacy of treated nets can be underestimated if judged only on standard cone bioassays, so these results should be taken with caution (Itoh, 2005). A detailed discussion of the implication of insecticide resistance on field efficacy of LLIS in this study will be presented in the chapter 6 and 7.

The expected effect of protecting houses with LLIS was to work as a mechanical but also as a chemical barrier. Although LLIS materials could be potentially compromised by degradation of insecticide and/or resistance of *Aedes* populations to pyrethoird-based insecticides, the effect of a physical barrier and/or the re-impregnation with different groups of insecticides would still provide protection.

Chapter 5: Efficacy of insecticide-treated screening of houses on *Aedes aegypti* populations from Merida Yucatan, Mexico.

5.1 Context of the Study

In the previous chapter, long-lasting insecticidal nets, fitted as screens (LLIS) on doors and windows in the city of Acapulco showed an immediate and significant effect on indoor-adult *Aedes* infestations which extended for two years, despite the *Aedes* populations being resistant to PYs (Che-Mendoza et al. 2015; Manrique-Saide et al., 2015). The intervention designated as "*Aedes* proof housing", was well accepted by study participants and considered potentially suitable for other regions at risk from dengue worldwide (Jones et al. 2014). However, in the Acapulco study, a second intervention based on targeted treatment (TT) of the most productive *Ae. aegypti* breeding sites was implemented 14 months after the installation of LLIS and therefore, the overall protection conferred was explained by the accumulative effect of the combination of the two interventions.

House screening, as a physical barrier, confers protection against mosquito bites and eventually disease transmission preventing or restricting insects to acquire new infections from infected hosts (Kirby et al., 2008; Kirby, 2010; Lindsay et al., 2003; Walker, 2010). Manrique-Saide et al. (2014) reported in Merida Mexico, that the presence of untreated window screening significantly decreased both the odds of having *Aedes* adult mosquitoes inside the house and of the number of females found indoors. The use of LLIS should provide a mechanical but also a chemical barrier for mosquitoes. The pyrethroid insecticide reduces the number of vectors entering the house and potentially reduces the survival of those attempting to exit (Kirby et al. 2009; Ogoma et al. 2010).

The Mexican Ministry of Health is currently interested in promoting the use of house screening for dengue vector control as part of improving house programs "*Aedes* proof housing" (Official Regulations of Mexico, 2014). Additional evidence is needed to evaluate the efficacy of this tool in other dengue vector endemic scenarios in Mexico and elsewhere. The present study shows the results of the effect of LLIS in controlling local *Ae. aegypti* populations in the dengue endemic Mexican city of Merida.

5.2 Materials and methods

5.2.1. Study site.

The study took place in the urban area of the municipalities of Merida, located in the Peninsula of Yucatan as described in Chapter 3. Merida, the Capital of Yucatan State, has been the site of >50% of all cases of Yucatan in the last 6 years, with continuous dengue transmission throughout all the year (over 90% of the weeks with dengue cases) but increased transmission (70% approximately) during the rainy season (July-November). Merida is the major human settlement with 40% of the state population (814,435 inhabitants), and up to 50% if we consider the con-urban area (ca. 200,000 inhabitants) (García et al 2012). The number of houses in Merida (272,418 households) represents above 50% of houses in the state. It is also the most important city in terms of economic activity concentrating 50% of industrial activity. Tourism also represents an important economic activity and approximately 1 million and 250,000 national and international tourists visit Merida every year respectively.

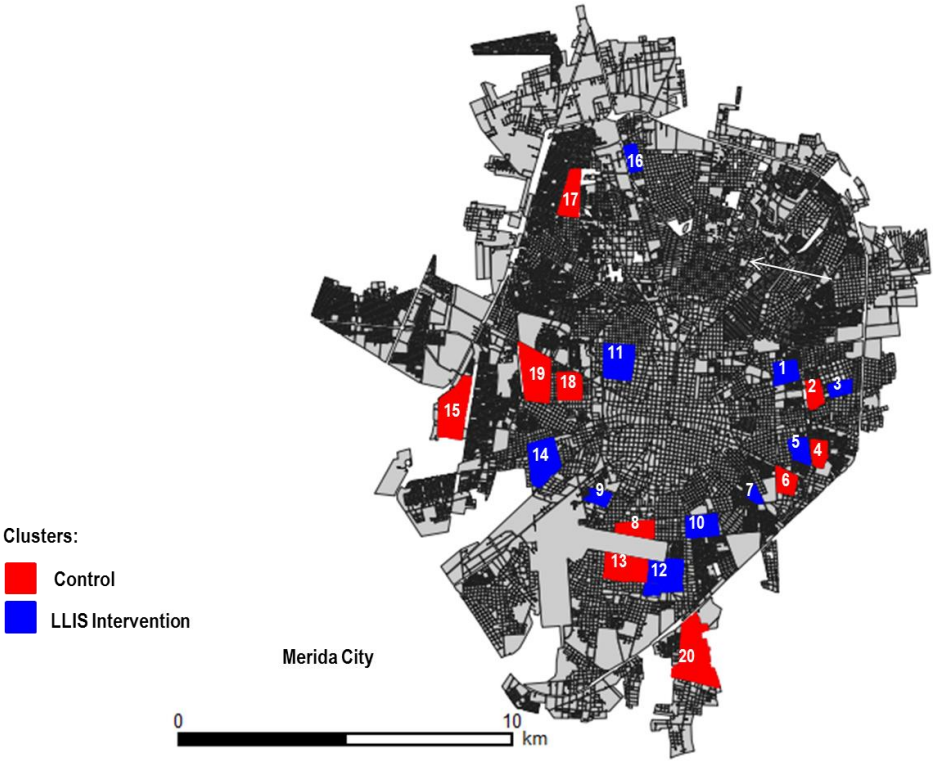


Figure 44. Study site, showing the location of the study areas in the city of Merida. All of the 20 clusters are distributed throughout the city extension. The clusters with and without LLIS interventions are showed in blue and red colours respectively. 1. Manuel A. Camacho; 2. Pacabtun; 3. Fidel V.; 4. Vergel III; 5. Vergel II; 6. San A. Kahua; 7. U.H. Morelos; 8. Castilla C.; 9. Manzana 115; 10. Cinco C.; 11. Centro; 12. San J. Tecoh; 13. San A. Xluch; 14. Mulsay; 15. Juan Pablo II; 16. Cordemex; 17. Francisco M.; 18. Bojorquez; 19. Yucalpeten; 20. Plan de A.

5.2.2. Study design.

This study followed a core protocol suggested by TDR-IDRC (Quintero et al. 2014) as described in Chapter 4. Briefly, a cluster-randomized sampling design with cross-sectional entomological surveys was performed in 20 geographic clusters (each one corresponding to different neighborhoods) of 100 households each, with 10 randomly assigned to either intervention or control treatments (no-intervention), over 24 months. The neighborhoods selected, in consensus with the local MoH, were all of epidemiological importance for the local dengue control program (Figure 44).

5.2.3. The LLIS intervention.

As described in Chapter 4, Duranet® screens (Clarke Mosquito Control, IL, USA) containing 0.55% w.w. alpha-cypermethrin were mounted in aluminum frames custom-fitted to doors and windows of residential houses (Figure 45). The intervention started in October 2012 and was completed by June 2013.

Figure 45. Photographs show the long-lasting insecticide-treated screens (LLIS; Duranet®) mounted on aluminum frames and fixed to windows and external doors of treated houses in Merida, Mexico.

No interventions were delivered by the project to the untreated clusters. However, routine vector control activities were periodically undertaken throughout the study by MoH according to national policy (Hernandez-Avila et al., 2013). These activities included

adulticiding (outdoor spraying with Chloropyrifos or Malathion and indoor spraying with Propoxur or Deltamethrin, respectively) and larviciding (Temephos) in response to elevated dengue and entomologisal risk indices.

Additionally since 2013, the local Government implemented the program "Recycle for your welfare" (RxB); a mulsectorial program based on a media campaign to promote (in one-day activities) separation of non-useful solid waste in the household environment, this include removing of containers that can be *Aedes* breeding sites (Barrera-Pérez et al., 2014). During June-September 2013 90% of intervention clusters (9 out of 10 clusters) and 50% of no-intervention clusters (5/10) received RxB. For the same period of 2014, 60% and 90% of LLIS intervention and no-intervention clusters received RxB respectively. The short-term impact of RxB strategy was evaluated in a previous study (Barrera-Pérez et al., 2014). For this study it was not considered to impact on the evaluation of LLIS since it was not applied systematically or continuously.

5.2.4. Entomological surveillance.

Five cross-sectional entomological surveys were conducted in LLIS and no-interventio arms as described in Chapter 4. Adult and larval/pupal entomologic surveys were performed in a sub-sample of 30 houses from each cluster. The baseline survey (September 2012) and the follow up surveys at 8, 13, 19 and 25 months (March 2013, October 2013, March 2014, October 2014) post-intervention (PI) correspond to wet, dry, wet, dry and wet seasons respectively.

Figure 46 shows the trial design for Merida.

5.2.5. Monitoring the durability of LLIS under operational conditions.

In order to determinate insecticidal activity of LLIS under operational conditions after 6, 12, 18 and 24 months standard World Health Organization cone bioassays (WHO, 2005b) were performed as described in Chapter 4.

The susceptible strain New Orleans (provided by CDC, Atlanta, USA) and two field local mosquito strains kept under laboratory conditions (UCBE insectary, Mexico) were used in theses biossays. For wild strains, eggs were obtained from the clusters Manzana 115 (MER09) and San Antonio Xluch (MER13) from ovitraps deployed during July to September 2012. The selection of theses strains was based on the results of CDC bottle bioassays where MER09 and MER13 strains were shown to be resistant and susceptible to permethrin respectively. Both populations were resistant to alpha-cypermethrin (see Chapter 3).

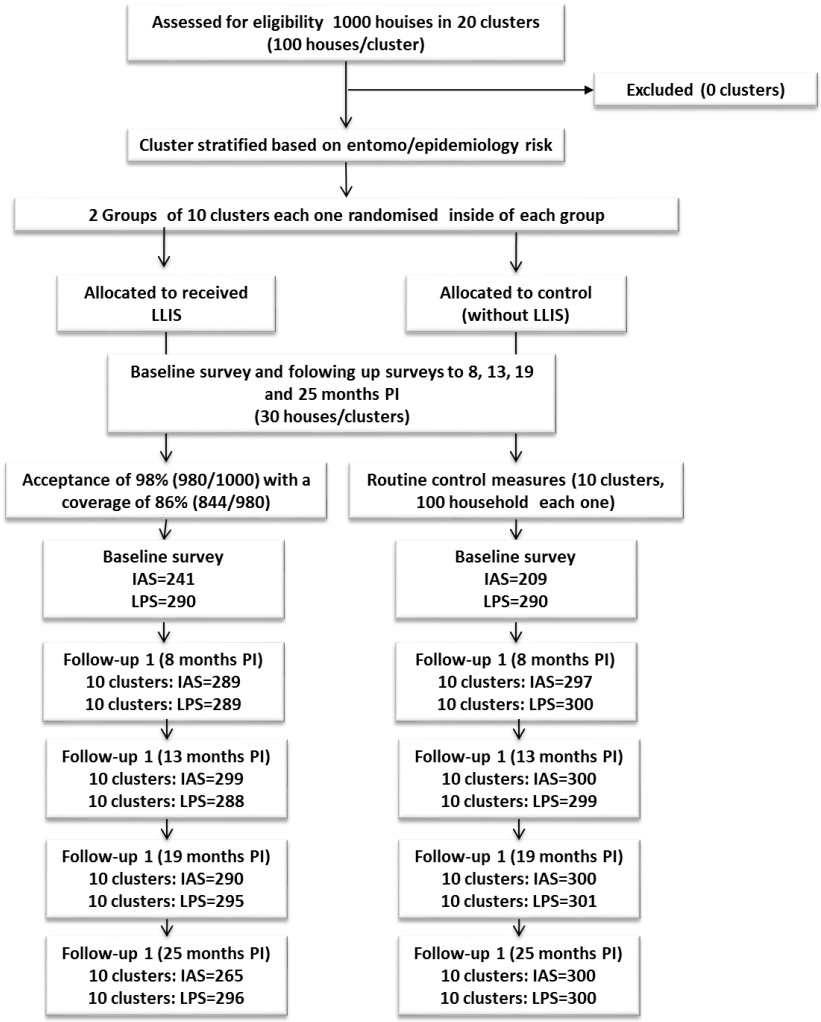


Figure 46. Flow of households through the study. The baseline survey was carried out in wet season of 2012 and post-intervention (PI) surveys were carried out during dry and wet season of 2013 and 2014. The number of houses surveyed in the indoor adult survey (IAS) and larval&pupal survey (LPS) is given for both group of houses, LLIS treated and untreated houses. These numbers represent the houses where the entrance was permitted.

5.3 Data management and analysis

5.3.1. Entomological indicators.

From indoor-adult collections a) House positive for *Aedes* adults, b) Houses positive for female *Aedes* (%), c) Houses positive for male *Aedes* (%), d) Number of *Aedes* adults per house, e) Number of *Aedes* females per house, and f) Number of *Aedes* males per house were calculated.

From immature collections data were collected on: a) the Breteau Index (BI), representing the number of containers positive for *Ae. aegypti* immatures/houses inspected)×100; b)

House positive for inmature *Aedes* (%); c) Houses positive for *Aedes* larvae (%); d) Houses positive for *Aedes* pupae (%); e) Number of *Aedes* larvae per house; f) Number of *Aedes* pupae per house; and g) the Pupae per Person Index (PPI) which is the ratio between pupae and persons living in each cluster.

For WHO cone tests, data was pooled and the percent of knockdown at 30 and 60 minutes and mortality at 24 hours were calculated and corrected when the mortality in control replicates was >5 and <20% using Abbott's formula (Abbott 1925).

5.3.2. Statistic analysis.

Logistic regression models (for presence-absence data) and negative binomial models (for count data) accounting for each house membership in a given sampling cluster were performed for each cross-sectional entomological evaluation survey as described in Chapter 4. Odds ratios (OR) and incidence rate ratios (IRR) with 95% confidence intervals (C. I.) were assessed and significance expressed at the 5% level.

A Generalized Additive Mixed Model (GAMM) (Zuur et al. 2009) was also applied to determine the association between various household-level entomologic indicators and the time (in days) since the installation of the LLIS. See details in Chapter 4.

To estimate the effect of LLIS exposure factors such as soiling on the susceptible mosquito survival rate, Poisson regression models was contructed as described in Chapter 4.

Analyses were performed using STATA 12.0 (Stata Corp, College Station, TX) and the mgcv package from the R statistical software.

5.4 Ethical aspects

This study received clearance from the ethical committee of the Ministry of Health of Yucatan. Written informed consent was obtained for each participating household.

5.5 Results

A total of 2,790 and 2,948 houses in 20 clusters participated in the trial for collecting adults and immatures respectively. A total of 844 households from intervention clusters (86% of coverage of houses which accepted to be intervened) were protected with Duranet® screens. An average of 1.9 and 4.9 doors and windows by houses respectively were registered in each intervention cluster.

5.5.1. Impact of insecticide-treated house screening (LLIS) on adult indoors.

The impact on adult-based entomological indicators is shown in the figure 47.

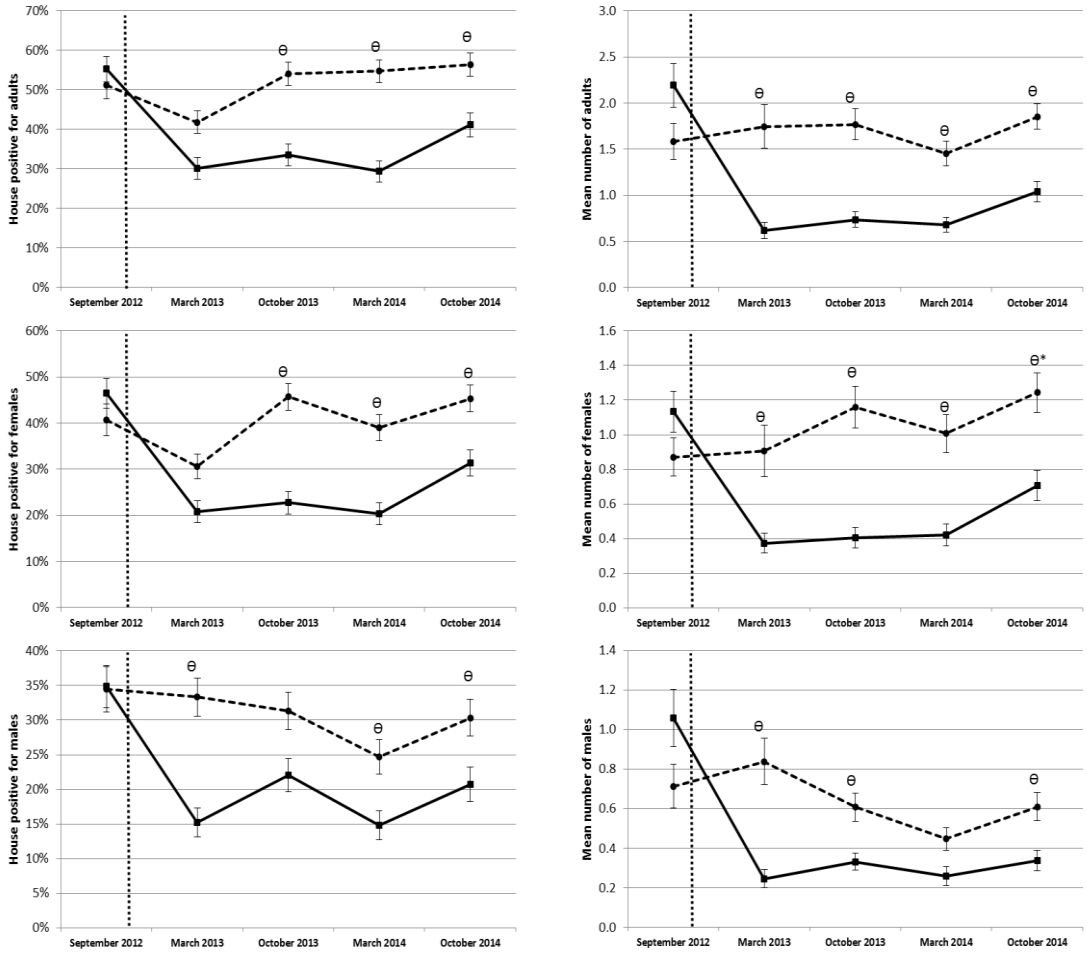


Figure 47. Comparison between treated (solid line) and untreated (broken line) arms of percentage of infested houses (left) and infestation density (right) for *Ae. aegypti* in Merida, Yucatan, Mexico. The vertical dotted line represents the start of LLIS intervention. The symbol θ denotes dates when the index was significantly different between LLIS and no-intervention arms on that date (with α=0.05). Asterisk denotes marginally significant. Error bars show the standard error of the mean.

During the pre-intervention survey (September 2012, wet season) adult-based entomological indicators showed similar infestation in both study arms. At 8 months PI, significantly fewer treated houses were infested with *Ae. aegypti* adult males (OR=0.36, 95% C. I. 0.20–0.66), but not for adult females (OR=0.59, 95% C. I. 0.28–1.27) or total adults (OR=0.60, 95% C. I. 0.30–1.20). One year after the LLIS intervention was implemented marked differences were observed in house positivity for total adults (13 months OR=0.43, 95% C. I. 0.21–0.89; 19 months OR=0.34, 95% C. I. 0.21–0.56; 25 months OR=0.54, 95% C. I. 0.34–0.86), and female adults (13 months OR=0.35, 95% C. I. 0.17–0.72; 19 months OR=0.40, 95% C. I. 0.23–0.69; 25 months OR=0.55, 95% C. I. 0.32–0.93). And for male adults marked differences were observed in all following up surveys, excepting to 13 months

PI (13 months OR=0.62, 95% C. I. 0.33–1.17; 19 months OR=0.53, 95% C. I. 0.25–1.15; 25 months OR=0.60, 95% C. I. 0.41–0.89).

Analyses of infestation density showed significant reductions on the mean abundance of indoor *Ae. aegypti* in houses with LLIS during the 2 years in all indicators, except for male adults: total adults at 8 (IRR=0.36, 95% C. I. 0.16–0.79), 13 (IRR=0.42, 95% C. I. 0.22-0.77), 19 (IRR=0.47, 95% C. I. 0.28-0.78) and 25 (IRR=0.56, 95% C. I. 0.35-0.90) months PI; female adults at 8 (IRR=0.41, 95% C. I. 0.18–0.95), 13 (IRR=0.35, 95% C. I. 0.18-0.69), 19 (IRR=0.42, 95% C. I. 0.21-0.82) and 25 (IRR=0.57, 95% C. I. 0.31-1.04) months PI; male adults at 8 (IRR=0.29, 95% C. I. 0.13–0.66), 13 (IRR=0.55, 95% C. I. 0.31-0.96), 19 (IRR=0.58, 95% C. I. 0.26-1.27) and 25 (IRR=0.55, 95% C. I. 0.36-0.84) months PI.

5.5.2. Impact of insecticide-treated house screening (LLIS) on immature populations.

At 8 months PI with LLIS, significant differences between treated and untreated houses were observed in the immature-based indicators, including all the *Stegomyia* indices (Figure 48): Houses infested for larvae (OR=0.26, 95% C. I. 0.07–1.00) and pupae (OR=0.23, 95% C. I. 0.06–0.88); abundance of larvae (IRR=0.23, 95% C. I. 0.61-0.92) and pupae (IRR=0.11, 95% C. I. 0.01-1.09); HI (OR=0.29, 95% C. I. 0.08-1.03), BI (IRR=0.28, 95% C. I. 0.09-0.90), and PPI (IRR=0.11, 95% C. I. 0.01-1.00). However, at 13 months PI a significant impact was only seen on *Aedes* pupae (OR=0.57, 95% C. I. 0.34-0.96). When analyzing the rest of larval-based indicators, these were lower in the clusters with the intervention, but the differences were not statistically significant (Figure 48). Nineteen months after the installation of LLIS, significant differences were only observed on the mean number of larvae per house (IRR=0.28, 95% C. I. 0.08-0.96) between LLIS and no-intervention arms.

5.5.3. Temporal persistence of interventions.

The effect of time since the installation of LLIS (y-axis) on each entomologic indicator (f(ti)) are showed in the figure 49. The figure shows a protective effect of LLIS (predicted value and its 95% credible interval are negative) for *Aedes* indicators (adults and immatures) for at least 600 days PI. The non-linear relationship between the abundance of pupae and time since LLIS installation was not significant (P=0.55). Table 11.

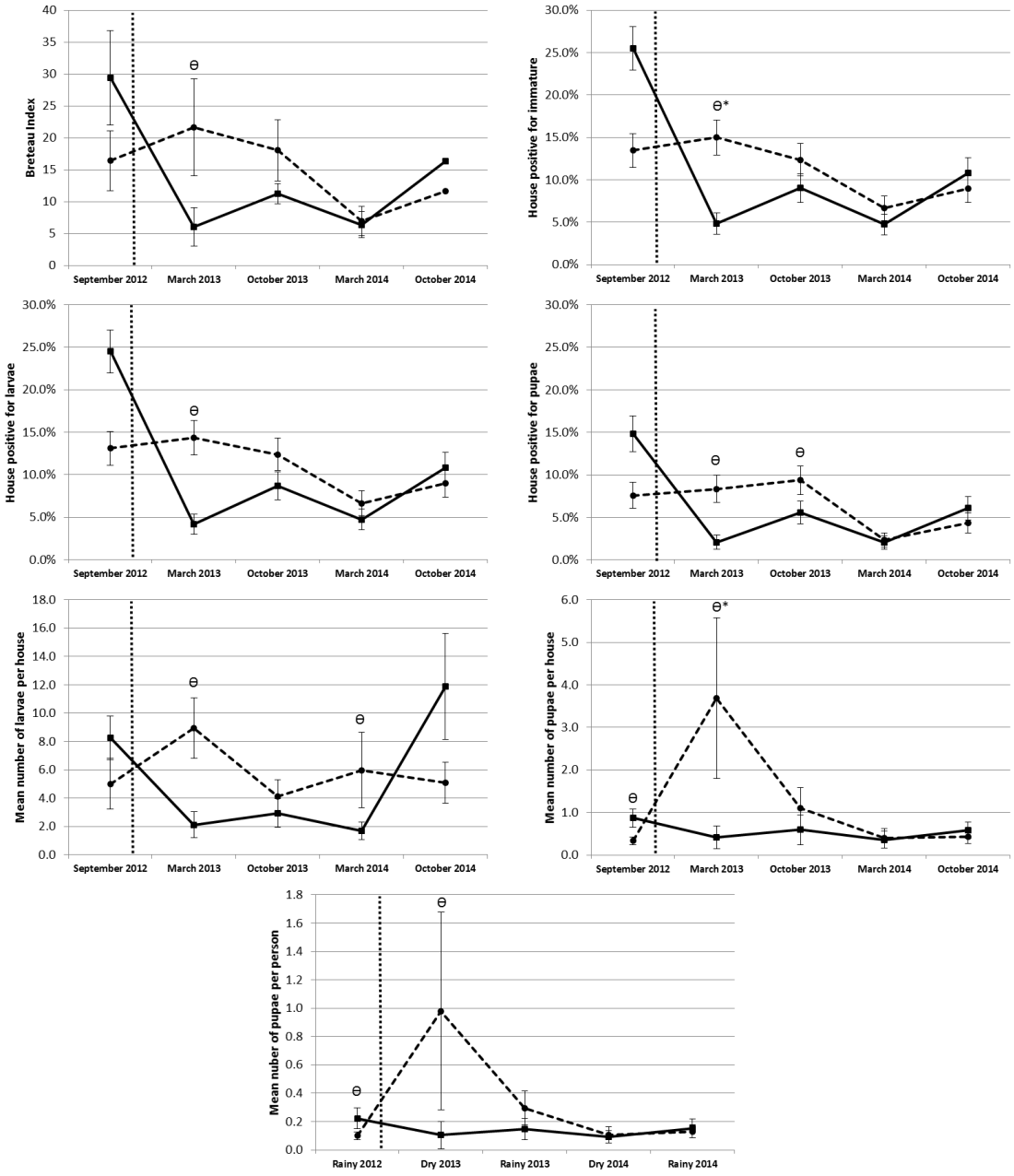


Figure 48. Comparison between treated (solid line) and untreated (broken line) arms of *Ae. aegypti* immature-based indicators for Merida, Yucatan, Mexico. The vertical dotted line represents the start of LLIS intervention. The symbol θ denotes dates when the index was significantly different between LLIS and no-intervention arms on that date (with α=0.05). Asterisk denotes marginally significant. Error bars show the standard error of the mean.

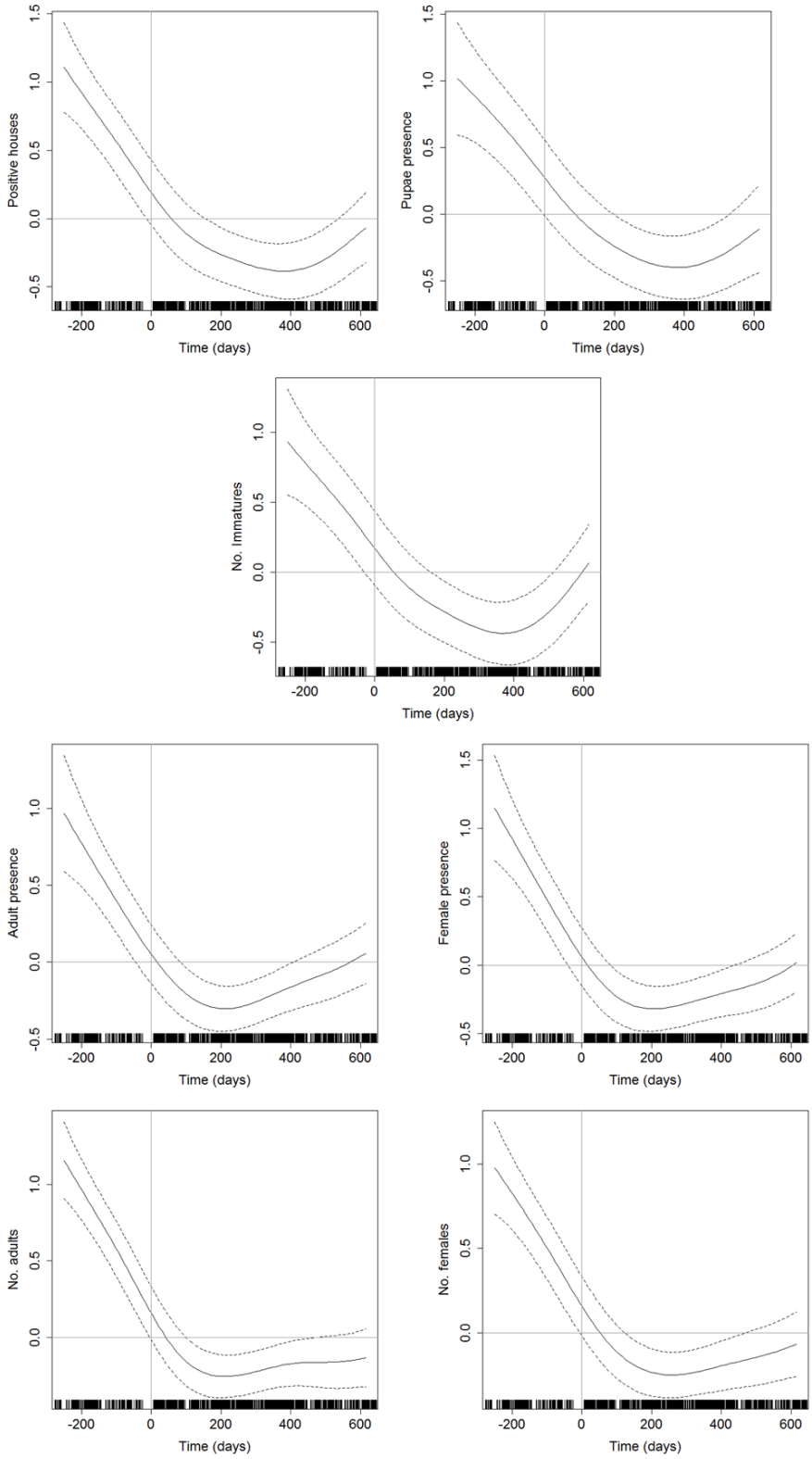


Figure 49. Predicted values for the best GAMM showing the association between the time since LLIS installation and each entomologic indicator for Merida, Mexico. Horizontal line shows the area of no difference and vertical line the time when LLIS were installed. The solid line is the predicted value of the dependent variable as a function of the x axis. Dashed lines represent 95% confidence intervals. The small lines along the x axis represent the days since the installation of LLIS (independent variable). The y axis is in the predicted outcome (dependent variable), and extend to both positive and negative values. Places where the confidence bands enclose the horizontal line indicates predicted values where the overall pattern is not significant.

Table 11. Parameter value and significance of non-linear parameter ($f(t_j)$) on GAMM models estimating the association between entomologic indices and the time since LLIS installation for Merida, Mexico. ΔAIC represents the difference between AIC values of a model excluding (AIC_{GAM}) and including (AIC_{GAMM}) a random effect associated with each cluster.

Life stage	Indicator	Estimated degrees of Freedom	*F*	*P*	ΔAIC (AIC_{GAM} - AIC_{GAMM})
Immature	No. Immatures	3.1	11.1	<0.0001	28
	No. pupae	1	0.357	0.55	284
	Positive houses	3.1	17.4	<0.0001	297
	Pupae presence	2.6	10.2	<0.0001	113
Adult	No. females	3.2	19	<0.0001	51
	No. adults	3.8	26.9	<0.0001	58
	Presence of adults	3.3	11.2	<0.0001	57
	Presence of females	3.4	13.9	<0.0001	49

5.5.4. Bioefficay of LLIS under operational conditions.

Soiling condition of used LLIS. A total of 98 LLIS were sampled in the intervention households with an average of 25 nets sampled by deployed time. At 6 months the proportion of clean, soiled and very soiled LLIS was similar between all theses categories (30%, 33% and 37% respectively). At 12 months the very soiled LLIS covered the 58% of the total of nets sampled. Extremely soiled LLIS were sampled at 18 and 24 months PI covering the 30% of all net samples (Figure 50).

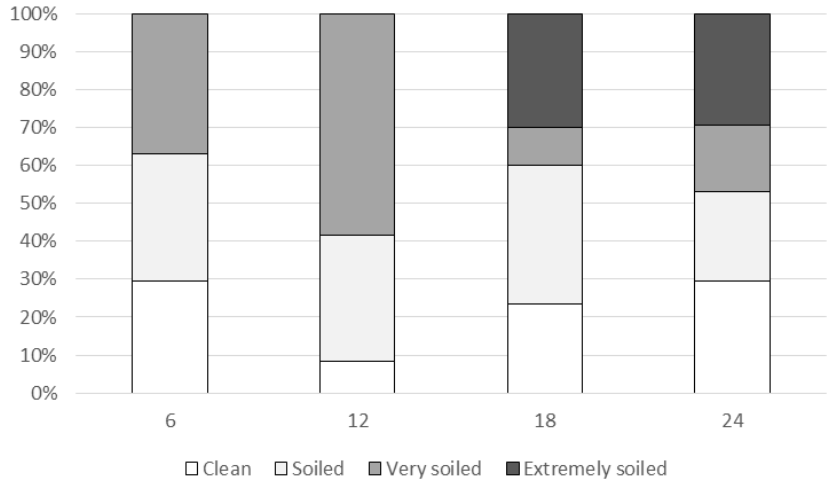


Figure 50. Proportions of net soiled condition by deployed time of LLIS sampled in Merida between 2013-2014.

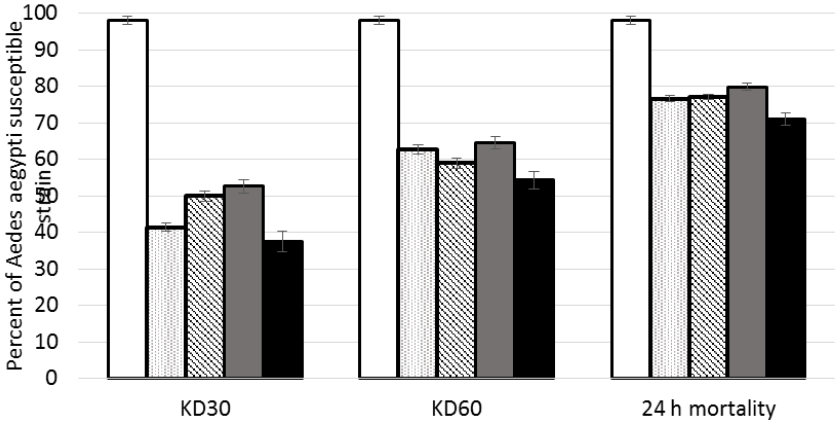
Bioassays. The efficacy of 98 LLIS samples was assessed on the *Ae. aegypti* New Orleans susceptible strain and two wild strains (MER09 and MER13), both showed moderate resistant to alpha-cypermethrin (80% KD), but MER13 was more susceptible to permethrin (97.5% KD) than MER09 (12% KD) (see Chapter 3).

For the susceptible strain, the highest KD and mortality rates were observed on new non-exposed nets (98% for both), according to cut off efficacy criteria of WHO for KD (≥95%) and mortality (≥80%). Overall, none of the net samples collected at 6, 12, 18 and 24 months passed the WHO KD efficacy criteria when tested against the susceptible strain (Figure 51A); in the case of mortality, only nets collected at 18 months achieved 80%, and the rest of nets tested in this study caused a range of mortality between 71-77%.

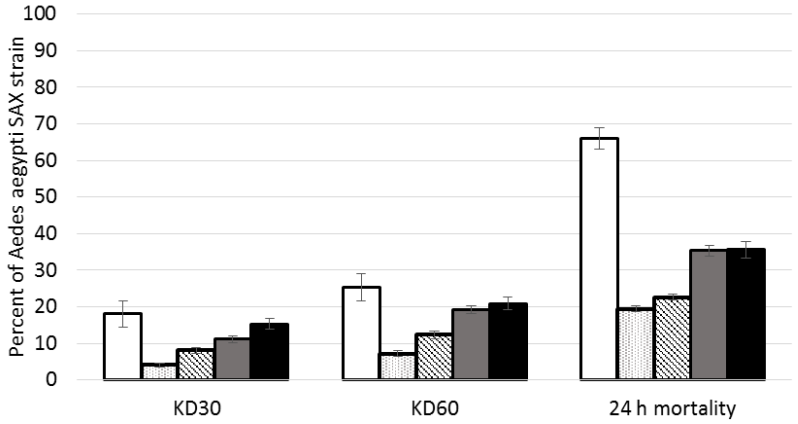
For wild strains, MER09 and MER13 none of the nets tested caused KD 95%. The highest mortality rate recorded was for new non-exposed nets achieving 66% and 51% in MER13 and MER09 respectively; the rest of the nets showed less than 36% of mortality for both strains (Figure 51B-C) with a tendecy to increase over the time of use. The MER13 strain showed significant higher values of KD at 30/60 minutes and 24 h mortality than MER09 strain in all the cases ($P<0.05$); except for KD when they were exposed to new non-exposed nets (KD30 95% C. I. 10.8-25.2 and KD60 95% C. I. 18-32.7 for MER13 strain; KD30 95% C. I. 19.5-33.8 and KD60 95% C. I. 20.8-35.2 for MER09 strain).

LLIS exposure factor analysis using poisson regression models showed that the time after deployment and soiling levels were significantly associated with survival in the susceptible strain (in all the cases $P<0.001$). The probability of susceptible mosquitoes surviving exposure was between 10-11 times for the first 6-18 months of use and, increase to 14.5 times for the next 24 months of use; considering the soiled or dusty condition the chance to survive increases with the level of soiling from 10% and 14% when exposed to soiled and very soiled LLIS respectively, to 22% for extremely soiled LLIS (Table 12).

A



B



C

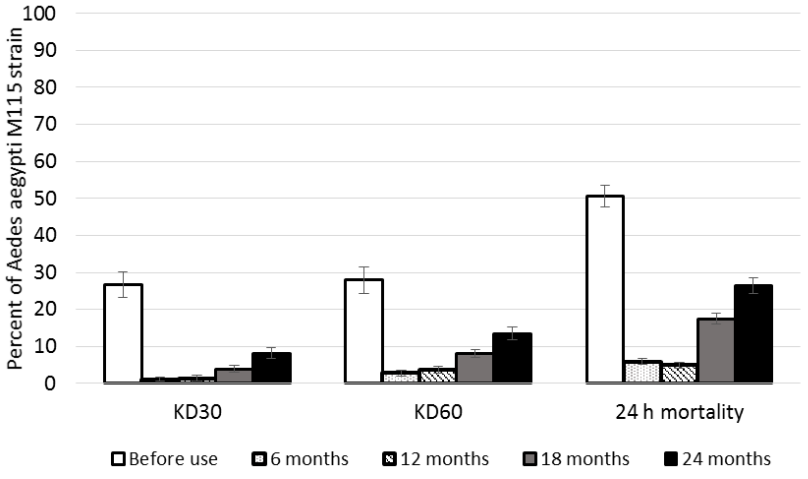


Figure 51. Bioefficacy test on different DuraNet® LLIS samples exposed 6, 12, 18 and 24 months under operational conditions in Merida. Results after WHO cone bioassays after 3 min. exposure: knockdown at 30 and 60 minutes (KD30 and KD60) and 24 h mortality and their standard errors of mean (SE) are showed for both, New Orleans susceptible strain (A), and field collected MER13 (B) and MER09 strains (C). Result for a new, non-exposed DuraNet® LLIS sample is also showed (white bar).

Table 12. LLIS exposure factor analysis using poisson regression models contructed with survival as dependent variable, and deployed time and soiling as independent variables for Merida nets sampling.

LLIS exposure factor	24-h mortality (95% C. I.)	24-h survival rate ratio*	P-value
New Orleans susceptible strain			
LLIS Age			
New, non-exposed	98% (95.7-100.3)	1	
6 months use	76.6% (75-78.1)	11.7% (9.1-15.1)	<0.001
12 months use	77.1% (75.5-78.7)	11.5% (8.9-14.8)	<0.001
18 months use	79.9% (77.7-82)	10.1% (7.8-13)	<0.001
24 months use	71.1% (67.7-74.4)	14.5% (11.2-18.7)	<0.001
Soiled conditions			
New	98% (95.7-100.3)	1	
Clean	91.7% (90.1-93.3)	4.2% (3.2-5.4)	<0.001
Soiled	79.4% (77.8-80.9)	10.3% (8-13.3)	<0.001
Very soiled	72.3% (70.8-73.8)	13.9% (10.8-17.9)	<0.001
Extremely soiled	56.9% (54.6-59.1)	21.6% (16.7-27.8)	<0.001

*Estimated with poisson regression models.

5.6 Discussion

For the particular case of dengue vectors, it has been argued that a major factor in the failure of previous prevention methods is their focus on eliminating immature forms of *Ae.aegypti*, rather than targeting the adult mosquitoes that actually transmit the disease (Morrison et al. 2008). The dengue vector *Ae. aegypti* is a highly synanthropic mosquito living in close-dependence with human-made ecosystems (Getis et al., 2003; Scott and Morrison, 2002). The contemporary challenge is precisely to reduce infected adult vector populations and/or their interaction with humans affecting DENV transmission (Morrison et al. 2008).

Screening with the most important points of entry into a house, such as windows and doors, with netting prevents the entry of adult mosquitoes (Schofield et al 1990). "Mosquito-proofing" of houses is a form of environmental management based on changes to human habitation to exclude vectors and eventually reducing man-vector-pathogen contact (WHO, 1982). The theory behind house screening and improvement is simple: a physical barrier will prevent vectors from entering houses or restrict them to a part of the house where there is no access to hosts (Kirby, 2013).

In the present study we observed an important reduction in the dengue vector density in houses protected with LLIS in Merida city. Similar effects are reported in previous studies carried out in a dengue endemic city in Mexico (Che-Mendoza et al., 2015). In Acapulco city, LLIS achieved a protective effect for at least 600 days post installation for both adult- and immature-based indicators but, in this study a second intervention was implemented 14 months after the beginning of LLIS installation, based on targeted treatment (TT) of the most productive *Ae. aegypti* breeding sites (Che-Mendoza et al., 2015). The LLIS protection conferred for at least 2 years could be explained by the cumulative effect of the combined interventions. In the present study, the LLIS showed a protective effect for the same period for adult indicators, but not for immature-based indicators. In addition to the reduction in number of adults per house, the number of houses infested was also reduced and this effect was more pronounced for females, which are most epidemiologically important. This demonstrates that LLIS acted as barrier preventing the entry of mosquitoes inside the houses in the intervention areas. The indoor mosquitoes in the treated houses probably were not completely suppressed, because adults survived insecticide contact or because not all adults contacted the screened surfaces, maintaining the house positive. It's suggested that daily contact with treated screened surfaces would be lower than that experienced by bed net users (Kirby, 2013), and this could explain in part the partial reduction of indoor mosquitoes but not their complete suppression.

In Haiti, insecticide-treated bednets showed an immediate effect on immature based indicators, and extended for the following 5–12 months after their deployment (Lenhart et al., 2008). In Thailand, ITC showed immediate effect on immature-based indicators at 6 months (Vanlerberghe et al., 2013). Most of these studies evaluated the impact of this type of interventions on *Aedes* immature indicators, but not on indoor adult density. Housing style could affect this intervention, such as ITC interventions, favoring the entrance of mosquitoes and movement through houses without ever coming into contact with insecticide (Lenhart et al., 2013). In a field trial carried out in Mexico, ITC interventions did not affect the indoor adult population, but it seemed to reduce the number of DENV infected females and the human infection prevalence in some areas (Loroño-Pino et al., 2013).

Nguyen et al. (1996) and Igarashi (1997) evaluated earlier an intervention with permethrin nets set up covering all openings of houses (in addition to routine anti-*Aedes* health education and control measures) and reported a significant reduction (close to 100%) on the houses positive and the abundance of indoor *Ae. aegypti* to undetectable levels for seven months, while in the control group infestation gradually increased during the epidemic season. This

study show evidence that insecticide-treated house screening (LLIS) reduced significantly the indoor *Aedes* density for at least 1 year. No other experiences relating this level of reduction in adult density and duration are available.

We observed an immediately effect of LLIS intervention on all the immature indicators evaluated. However, the protection was not consistently extended for more than 8 months. Sustainable interventions on larvae/pupae habitats can contribute to reducing the breeding sites and eventually the recruitment of individuals emerging from breeding sites (Che-Mendoza., et al., 2015). The government campaign to eliminate the potential *Aedes* breeding sites in the most important areas for dengue transmission has demonstrated a short term protective effect (Barrera et al., 2014), but it was not possible confirm its contribution to reducing the entomological indicators in this long term study. ITC interventions in combination with targeting productive breeding-sites in Mexico (Kroeger et al., 2006), Venezuela (Kroeger et al., 2006; Vanlerberghe et al 2011) and Guatemala (Rizzo et al., 2012) have also indicated a synergistic effect on *Ae. aegypti* control.

To attempt to measure the killing effect of LLIS, as opposed to their physical barrier, the insecticidal activity of LLIS was assessed using a susceptible strains. The residual activity of insecticide on the LLIS was high against new nets, meeting WHO efficacy criteria. The KD effect was low for the rest of deployed times of LLIS, but mortality rates were close to the 80% WHO efficacy criteria (71-80%). However the LLIS were not efficient against field collected resistant strains, although their efficacy varied according to the resistance level of the local mosquitoes populations, as was demonstrated in this study (differences in KD and mortality between SAX and M115 strains). To get a better understanding of levels of insecticide resistance and LLIS protection effects, a possible alternative design would have been to randomly allocate treated and untreated nets to households in areas of known insecticide resistance to see whether the treated nets still conferred additional protection compared to untreated nets. This would be based on the assumption that long-lasting insecticidal nets in the presence of resistance are more effective than untreated nets (Strode et al., 2014).

The PYs are still the only insecticide class recommended and available for LLIS. Insecticide resistance to PYs in *Ae. aegypti* populations in Mexico has increased during the last decade (González et al., 2012; Ponce-García et al., 2009; Rodríguez et al., 2010; Saavedra-Rodríguez et al., 2007) and is established in Merida populations (Chapter 3). Based on the WHO efficacy criteria none of LLIS tested were efficient against pyrethroid resistant wild strains in the current study. The implications of these results are unknown and may not be

predictive of field efficay (Itoh, 2005). Additional information of LLIS effect on blood feeding inhibition and its excito-repellent effect needs to be determinated. As observed in Acapulco (see Chapter 4) one important factor affecting the bioefficacy and variability winthin LLIS under operational conditions seems to be the levels of soiling. In addition, the lifespan in field may be reduced.

Although interventions like LLIS are more difficult to implement and maintain than other alternative approaches (i.e. insecticide spraying) their protective effect on *Aedes* density is extended until for 600 days, as it has been shown in previous studies in Mexico and by this study. However, LLIS have to meet the challenge of resistant populations of the vectors, mostly to PY which are still the only insecticide class recommended and available for LLIS. In this point, new alternatives to PY insecticides are urgently needed for screens in order to counteract the emergence of resistance in field operational conditions.

Chapter 6: Changes in *Aedes aegypti* insecticide resistance profiles in response to LLIS intervention in Acapulco Guerrero and Mérida Yucatan, Mexico.

6.1 Context of the Study

The mosquito *Aedes aegypti* has experienced an escalating trend in the intensity and geographic distribution of insecticide resistance over the past decade. As dengue has re-emerged on a global scale (Messina et al., 2014), the subsequent rise in chemical interventions in response to large and recurrent outbreaks coupled with the long-term reliance on PY insecticides for urban vector control have been key drivers of rapid and widespread increase in insecticide resistance (Ranson et al., 2010; Vontas et al., 2012). The molecular mechanisms underlying insecticide resistance in *Ae. aegypti* are thought to be primarily increased metabolic activity and point mutations on the target-sites of insecticides (Brogdon and McAllister, 1998; Hemingway et al., 2004; Rinkevich et al., 2013).

In Mexico, as a result of the historical reliance on DDT to control *Ae. aegypti* during the yellow fever campaigns and the intense use of PY in recent years, kdr-mediated resistance to PY/DDT is now widespread in many regions (Ponce-García et al., 2009; Saavedra-Rodríguez et al., 2014; Siller et al., 2011). This evidence led to Public Health Mexican authorities to ban the use of permethrin (a PY used almost exclusively for *Aedes* control for more than 10 years), but a large list of pyrethroid-based formulations (i.e. sumithrin, deltamethrin, bifenthrin, lambda cyhalothrin, alpha cypermethrin, and cyfluthrin) are still available to be applied for adult mosquito control in Mexico (SSA, 2015).

As a result of potential transmission of DENV-CHIKV-ZIKV in several Mexican urban centers, the National MoH is currently interested in promoting "Vivienda Segura" (Safe household) with the use of LLIS for disease prevention (Official Regulations of Mexico, 2014). In previous chapters evidence of the entomological efficacy of LLIS in two Mexican dengue endemic cities, where resistance to PY has been confirmed, was presented.

This chapter show the results from monitoring changes in resistance profile during the two years of the LLIS field trials.

6.2 Materials and methods

A detailed description of the sample collections and study site areas is given in previous chapters. In Merida 20 locations were monitored during thebaseline survey, and half of the locations from both study arms (i.e. no-intervention and LLIS arms), were considered for the next following-up surveys. The five intervention clusters monitored in Merida were: 01 (Manuel A. Camacho), 03 (Fidel V.), 07 (U.H. Morelos), 09 (Manzana 115), 16 (Cordemex); and the five untreated clusters were: 04 (Vergel III); 08 (Castilla C.) 13 (San A. Xluch), 17 (Francisco M.) and 20 (Plan de A). In a similar way, three clusters for each arm were selected for follow up in Acapulco: clusters 08, 14 and 18 from the intervention clusters; and cluster 02, 06, 09 for untreated clusters.

In addition to cross-sectional entomological surveys (using ovitraps) carried out during the baseline period in the middle and end of the 2012 rainy season in both study sites (baseline study, see Chapter 3); four and three following up surveys were performed for Merida and Acapulco respectively during the dry and wet season of 2013 to 2014 (March 2013, October 2013, March 2014, October 2014). Mosquito specimens were emerged from egg batches (a pool eggs from each cluster) collected from a network of weekly-serviced ovitraps along the clusters selected in both study sites. Batches of 1-3 day-old female mosquitoes were subjected to standard CDC bottles bioassays (see details in Chapter 3). A separate cohort (at least 30 unfed one-day-old females of the F1 generation/cluster) of mosquitoes from ovitrapping were maintained and stored separately at –70 °C for molecular and biochemical analysis.

To monitor changes in the insecticide susceptibility and resistance mechanisms of *Ae. aegypti* field populatios, CDC bottle bioassays (susceptibility and intensity of resistance tests), biochemical and molecular assays were conducted on mosquito samples from each collection as described in the Chapter 3. The same assays were conducted on mosquitoes from the external controls "Tres Palos" and "Dzitya" located 10 km from Renacimiento neighbourhood in Acapulco, and 6 km from the nearest Merida´s cluster respectively (see Figure 12 in Chapter 3). The external controls were small localities (less than 4,000 inhabitants and less than 1000 households) considered historically by the local MoH to have low risk areas of dengue transmission, and consequently they receive less pressure of insecticide use (chemical interventions for vector control) in comparison with Acapulco and Merida.

Genomic DNA was extracted from single mosquitoes or from a body part in a solution of 45 µl of H_2O and 5 µl of Promega Taq DNA Polymerase10x Buffer with $MgCl_2$ (Madison,

WI) in a 96 well PCR plate. Samples were incubated at 95 °C in a BioRad icycler thermocycler for 15 minutes.

All CDC bottle tests were performed in UCBE-UADY in Merida, Mexico, and all biochemical and molecular assays were performed in CDC laboratory from Atlanta, U.S.A.

The New Orleans and Rockefeller susceptible strains of *Ae. aegypti* were used as references for all CDC bottle and biochemical assays respectively. Genomic DNA from the Rockefeller strain was used as a susceptible (wild-type) control and DNA from previously genotyped individuals was used as positive controls for both kdr mutations.

To evaluate the impact of LLIS intervention on number of DENV cases, data from 2009-2015 years were obtained from SINAVE-DGE (Hernandez-Avila et al., 2013). All DENV case addresses were geocoded in R (http://www.r-project.org/) using the geocode() function from the ggmap library. The geocode function uses Googles Geocoding API to turn addresses from text to latitude and longitude pairs. Once geocoded the csv files were exported to Quantum Geographic Information System software (QGIS - project http://www.qgis.org/es/site/). Then the DENV cases overlapping on clusters of the study sites were extracted using this software.

6.3 Data management and analysis

For CDC bottles bioassays data from all five clusters in each arm was pooled and the mean KD rate (at 30 minutes) was calculated per insecticide for each arm and period (survey), and compared using Fisher's exact test two-tailed P value. For analysis of intensity of resistance assays probit analysis using R software (The R Project for Statistical Computing – http://www.r-project.org/) was performed to plot the predicted probabilities and 95% confidence interval. This analysis was only performed for permethrin, due to the limited data available for alpha-cypermethrin (in most of cases, the intensity of resistance reached no more than twice the diagnostic doses). For alpha-cypermethrin the binomial confidence intervals of KD rates were calculated and plotted. The Abbott's correction was not required as the mortality in all the control used was under 5%.

For the biochemical assays, the mean absorbance values were calculated for mosquitoes from arms of clusters and plotted by period for each study arm. A one-way ANOVA and the Scheffe multiple comparison were used to test differences in enzyme activities between arms and periods.

The frequencies of the 1016I and 1534C alleles were calculated using the following equation: [n heterozygotes +2(n homozygotes)]/2(total n mosquitoes analyzed). Fisher exact tests were implemented to test any association of each mutation with LLIS intervention.

Statistical analyses were performed using Stata 12.0 software. A P value of 0.05 or less was considered as significant.

For statistical analysis between LLIS and non-intervention arms, the cases per cluster/season were the outcome measures. Logistic regression models and negative-binomial regressions were performed to evaluate the impact of LLIS intervention on presence/absence (binary categorical variable) and abundance of cases between arms respectively, using LLIS as predictor variable with 95% C. I. and significance expressed at the 5% level. Analyses were performed using STATA 12.0 (Stata Corp, College Station, TX).

6.4 Results

6.4.1. Monitoring the suceptibility to insecticides.

A total of 362 sets of CDC susceptibility tests (4 replicates for each set, 1,448 bottles in total) were performed using a total of 19,116 *Ae. aegypti* females (an average of 13 mosquitoes per bottle) for all insecticides tested and all the clusters from both localities during all the study period. For the susceptible strain New Orleans all tested insecticide results in 100 % KD at 30 min.

Changes in carbamates and organophosphate susceptibility status. No changes in the susceptibility status to the carbamate propoxur were observed in the diferent study arms for both study sites. Complete susceptibility to propoxur was observed in both study arms with a KD ranging between 98-100% in Merida, 100% in Acapulco and the external controls achieved 100% KD during all periods of the study (baseline and the next four follow up surveys).

Decreased susceptibility to for the organophosphate chlorpyrifos was observed in all groups at differing levels; in Merida KD ranged from 46-100% and in Acapulco from 15-100%.

In the case of Merida the susceptibility levels to chlorpyrifos showed significant differences ($P<0.0001$) between the LLIS and no-intervention arms in almost all the periods of the study, with the mosquitoes in the LLIS arm generally being more susceptible to the organophosphate than those in the no-intervention arm. In the LLIS arm KD rates were 96% in the baseline survey and 100% KD in the next 6 months (Figure 52). However, a decrease on chlorpyriphos susceptibility was consecutively observed in the next periods. The corresponding external control showed full susceptibility to chlorpyriphos, with the exception of the last period (at 24 months post-intervention) with 84% KD, suggesting the emergence resistance to this insecticide in this locality.

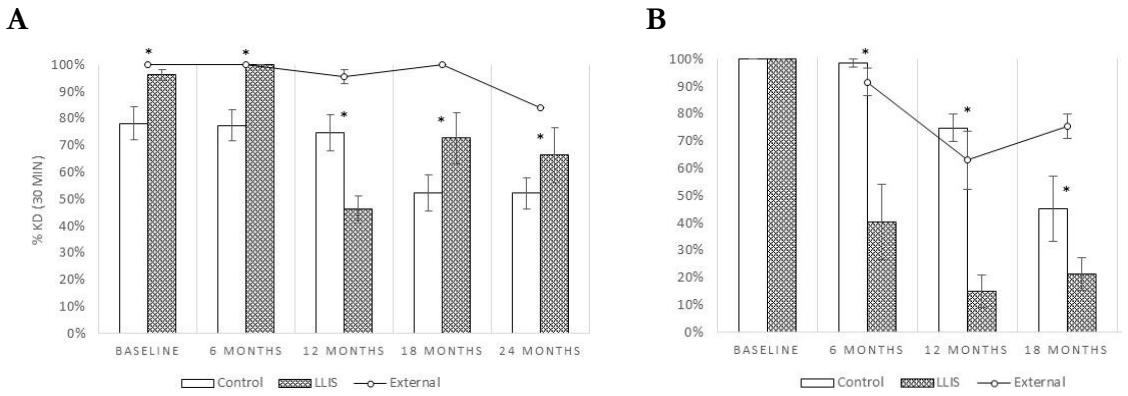


Figure 52. Average knockdown effect at 30 minutes of 1-3 day old adult female *Ae. aegypti* from LLIS intervention arm, no-intervention arm, and external control of Merida (A) and Acapulco (B) exposed to chlorpyrifos using CDC bottles method. Results are means for 4 replicates (average number of mosquitoes per cluster/per time point = 58, range = 45-84) for each cluster evaluated. Significant differences (p=<0.05) from comparison of the mean KD rate between LLIS and no-intervention arms are denoted by an asterisk (*) in each period. Baseline=baseline study; following up surveys were carried out approximately at 6 intervals.

In Acapulco complete susceptibility to the DD of chlorpyrifos were observed in the baseline study. For subsequent months a marked decrease in susceptibility was observed in the LLIS arms and the no-intervention arm but with the loss of susceptibility more rapid compared with the no-intervention arm (Figure 52). The external control also show decreasing susceptibility to chlorpyrifos.

In general the susceptibility to chlorpyrifos decreased in all groups in both study sites during with the most dramatic reduction beign observed in the Acapulco intervention arm.

Changes in pyrethroid susceptibility status. High resistance to permethrin (KD <40%) was observed during the baseline survey in Merida, with no statistically significant differences between no-intervention and LLIS arms (P=1.00). However, in the following 6, 12 and 18 months post-intervention (PI), significant differences were observed between no-intervention and LLIS arms, with a decline in susceptibility in the LLIS arm compared with the no-intervention arm (Figure 53A). At 24 months PI no significant differences in the susceptibility were observed between arms (P=0.58), but a significant reduction in the susceptibility was observed when each arm was compared with its respective baseline (no-intervention arm, P=0026; LLIS arm, P<0.001). The external control varied over the course of the study making it difficult to ascertain trends.

For alpha-cypermethrin both experimental arms in Merida, showed similar tendency with a gradual decrease in the susceptibility from 90-95% in the baseline (significant differences observed P= 0.0097) to <66% at 6-18 months PI, being lower in the LLIS arm than no-intervention arm with significant differences also observed at 6 and 18 months PI

(P=0.0001). A recovery of susceptibility (78-85% KD) is observed at 24 months PI with significant differences between LLIS and the no-intervention arm (P= 0.0299). The KD rates differed signicantly between baseline and final monitoring point in LLIS arm (P<0.001), and marginally significant in no-intervention arm (P=0.052). The corresponding external control showed almost consistently complete susceptibility during all the periods of the study (Figure 53B).

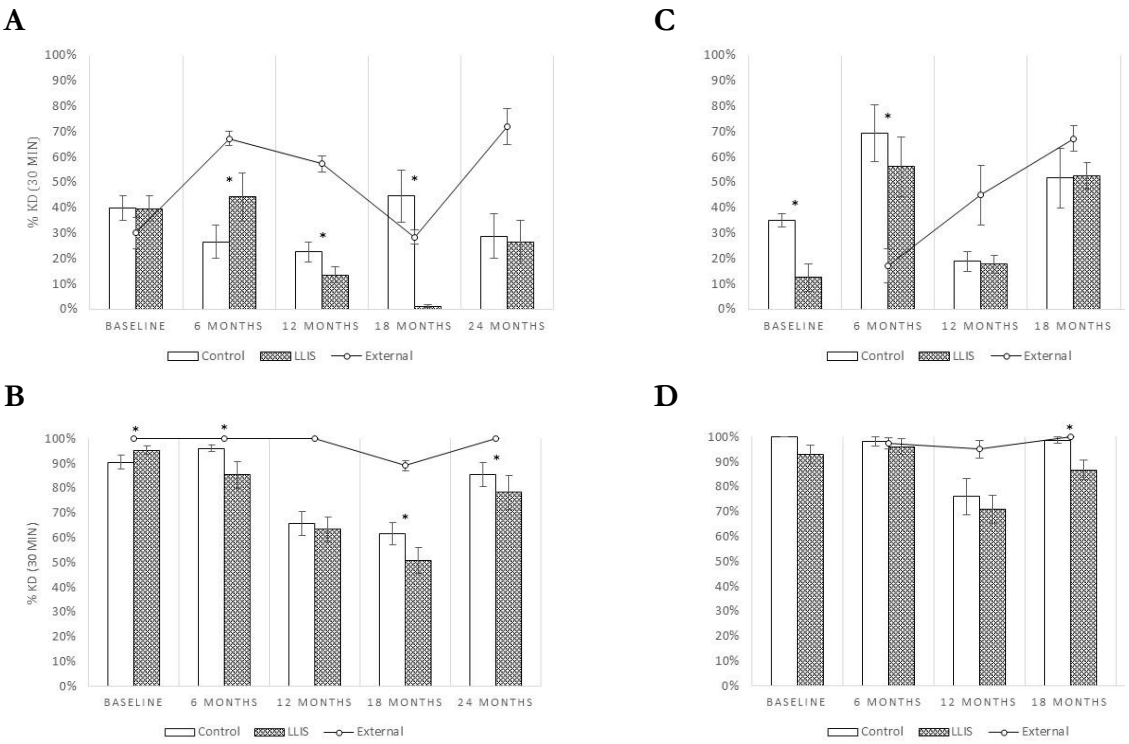


Figure 53. Average knockdown effect at 30 minutes of 1-3 day old adult female *Ae. aegypti* from LLIS intervention arm, no-intervention arm, and external control of Merida (left A&B) and Acapulco (right C&D) exposed to permethrin (A-C) and alpha-cypermethrin (B-D) using CDC bottles method. Results are means for 4 replicates (average number of mosquitoes per cluster/per time point = 61, range = 50-84) for each cluster evaluated. Significant differences (P<0.05) from comparison of the mean KD rate between LLIS and no-intervention arms are denoted by an asterisk (*) in each period. Baseline=baseline study; following up surveys were carried out approximately at 6 intervals.

For Acapulco there was little difference in the susceptibility to either pyrethroid tested between the no-intervention and LLIS intervention arms in any of the follow up surveys from 6-18 months PI (the survey at 2 years was no completed). High levels of permethrin resistance is observed, but without a defined pattern in the changes of susceptibility and KD levels were higher at the end of the study than at baseline in LLIS arms (P<0.001), but not in no-intervention arm (P=0.76). The corresponding external control shows a recovery of susceptibility to permethrin from 13% KD at 6 months PI to 67% KD at 12 months PI. Baseline data is not available for this population (Figure 53C).

For alpha-cypermethrin high susceptibility was observed for both study arms during the baseline and at 6 months PI; showing a decrease to 70-76% KD in the next periods (with not significant differences between study arms in all theses periods). A recovery of the susceptibility to levels of 87-99% was observed in last period, with a significantly lower KD in the LLIS arm compared to the no-intervention arm ($P<0.001$). Again, there was no signficiant difference in the cypermethrin KD rates between baseline and the final monitoring period (18 month PI) for either study arm in Acapulco ($P>0.40$). The external control showed high susceptibility to alpha-cypermethrin during all the periods (Figure 53D).

Monitoring changes the intensity of resistance. In order to determine the intensity of resistance 404 additional sets of CDC susceptibility tests (4 replicates for each set, 1,616 bottles in total) were performed using a total of 21,956 *Ae. aegypti* females (an average of 13.5 mosquitoes per bottle).

High levels of intensity of resistance to permethrin were observed for permethrin in both study arms. In Merida (Figure 54A) the external control and LLIS arm showed similar intensity of resistance during the baseline study, requiring 5-fold the DD of permethrin to reach KD greater than 90% (resistance threshold). In contrast the no-intervention arm required 10-fold the DD to reach 90% KD for the same period. For the LLIS arm the 90% KD threshold was not reached even when using 10-fold DD whereas ≥90% KD was always reached in the no-intervention arm with 10-fold DD (with the exception of the following up survey at 24 months PI). Therefore, resitance seems to be stronger in LLIS arms. Similarly, in Acapulco, the LLIS arm always required exposure to higher dose of permethrin to reach 90% KD threshold than in the no-intervention arm. With the exception of the following up survey at 12 months, no-intervention arm was more resistant than external (Figure 55A).

For both study sites the intensity of resistance to alpha-cypermethrin was always lower that permethrin in all the periods. The 90% KD threshold was achieved at either 1- or 2-fold the DD in all arms and time periods in both cities, with the exception of survey at 18 months PI in Merida in the LLIS arm which required exposure to 5-fold DD to achieve > 90% KD. The external control showed > 90% KD at the alpha-cypermethrin DD and so intensity assays were not needed. In general the intensity of resistance in Merida increase gradually from baseline to 18 months PI in both, no-intervention and LLIS arms, until 5-fold DD for this last one (Figure 54B), but returned to levels of 2-fold DD to next period. Similarly in Acapulco the intensity was low in the first two periods (baseline and at 6 months PI), but increased to 2-fold DD in the next two periods, mainly in the LLIS arm (Figure 55B).

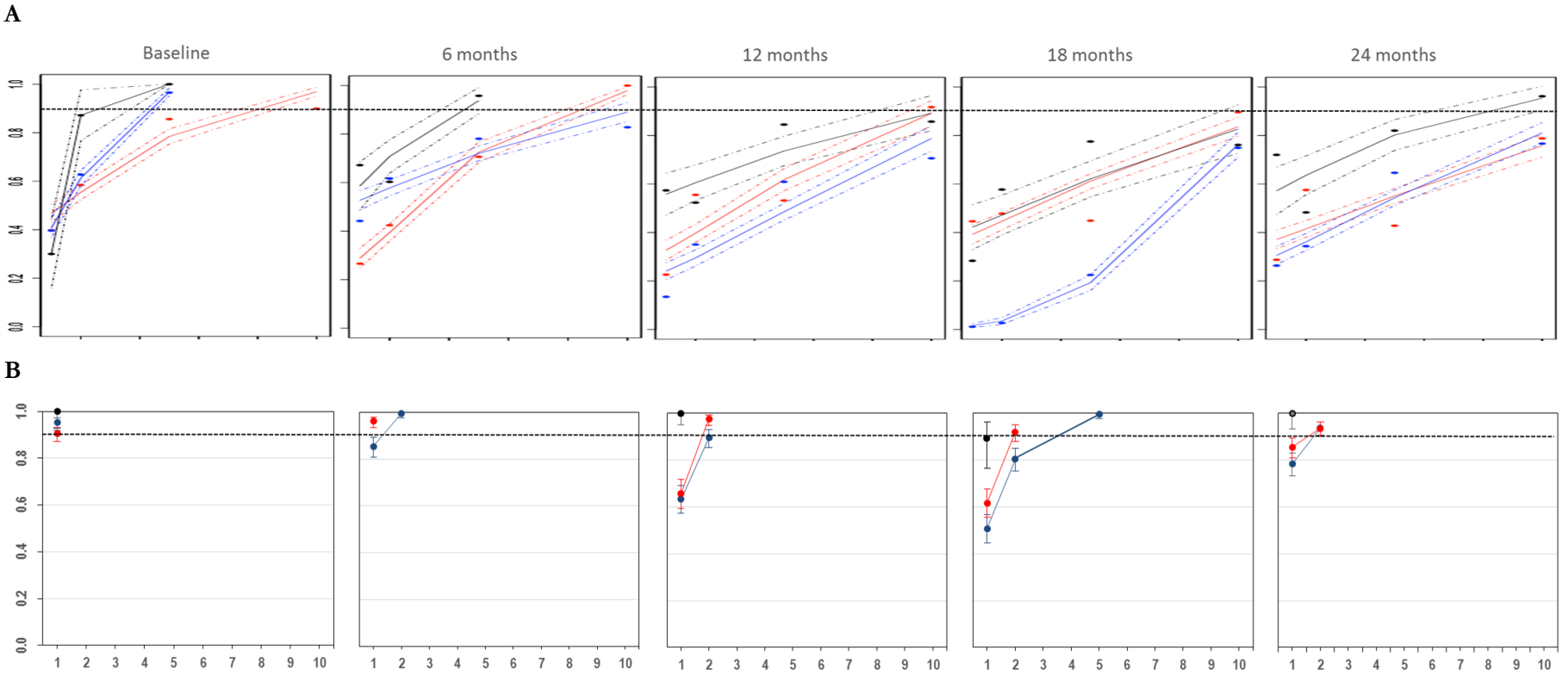


Figure 54. *Aedes aegypti* resistance intensity to pyrethroids in Merida during 2012-2014. A) Predicted probabilities and 95% confidence intervals of probit against different doses of permethrin. B) Means of knockdown (±binomial confidence interval) against different doses of alpha-cypermethrin. The data come from knockdown observed at the diagnostic time (30 min) to the diagnostic dose of the insecticide (1), as well as multiples thereof (2, 5 and 10). Red, blue and black lines and circles represent the no-intervention arm, LLIS arm, and the external control. Baseline=baseline study; following up surveys were carried out approximately at 6 monthly intervals. The dotted line represents the knockdown resistance threshold of 90%.

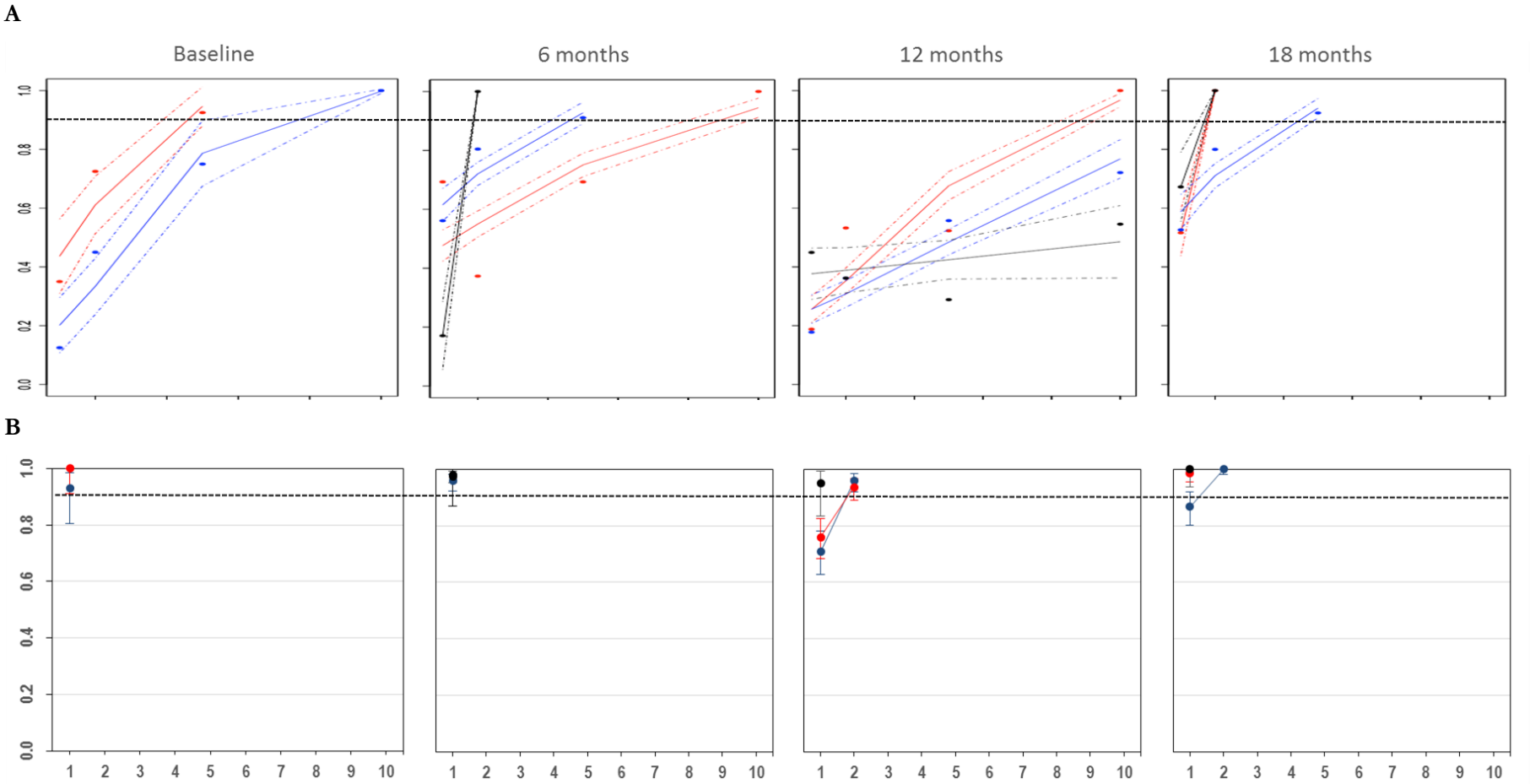


Figure 55. *Aedes aegypti* resistance intensity to pyrethroids in Acapulco during 2012-2014. A) Predicted probabilities and 95% confidence intervals of probit against different doses of permethrin. B) Means of knockdown (±binomial confidence interval) against different doses of alpha-cypermethrin. The data come from knockdown observed at the diagnostic time (30 min) to the diagnostic dose of the insecticide (1), as well as multiples thereof (2, 5 and 10). Red, blue and black lines and circles represent the no-interventio arm, LLIS arm, and the external control. Baseline=baseline study; following up surveys were carried out approximately at 6 monthly intervals. The dotted line represents the knockdown resistance threshold of 90%.

6.4.2 Monitoring changes in the resistance-related enzymes activities.

A laboratory susceptible (Rockefeller strain) of *Ae. aegypti* was used as a reference strain for all the biochemical assays. The statistics for all enzymes in the Rockefeller strain are given in Table 13.

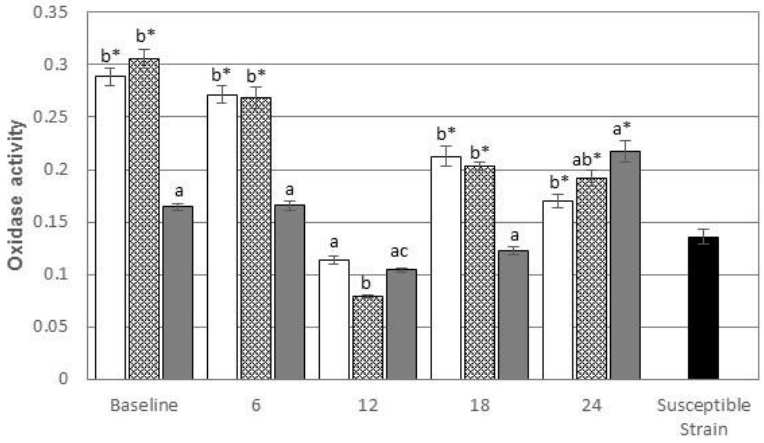
Table 13. Summary statistics for absorbances of the different enzyme baselines in the Rockefeller susceptible strains.

Mechanism	N	Mean	SE	Min.	Max.
Oxidase activity	30	0.13559	0.00721	0.08533	0.22633
Esterase activity	30	0.78403	0.01356	0.65557	0.93943
GST activity	30	0.04270	0.00316	0.01313	0.10406
AChE inhibition	30	94.7812	1.62590	63.0854	100

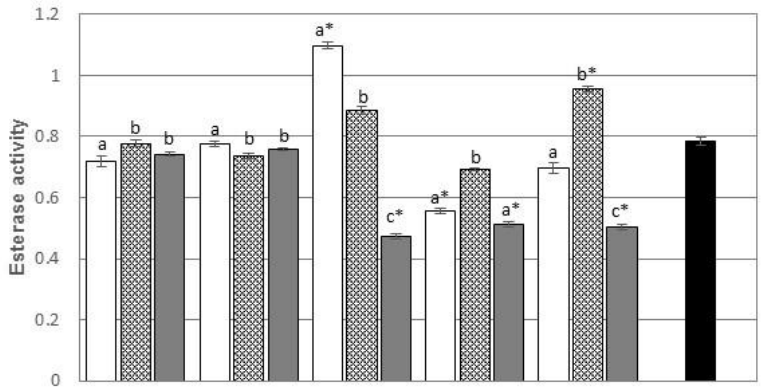
Enzyme activities for Merida populations. Levels of oxidases from LLIS and no-intervention arms were higher than those for the susceptible strain ($P < 0.0001$) in all the periods, with exception of 12 months PI when both experienced a decrease (levels similar to the susceptible one). This is indicative of an elevated P450-based resistance mechanism in the field population. In contrast to LLIS and no-intervention arms, the corresponding external control showed oxidase levels similar to the susceptible strain during the baseline to 18 months PI, but suddenly showed a significant increase ($P<0.0001$) at 24 months PI (Figure 56A). No statistical differences between study arms (LLIS vs no-intervention arms) were observed (Figure 5A), indicating similar oxidase content between arms. The only exception was at 12 months PI, when both arms showed similar oxidase levels to the susceptible strain (with significantly higher levels in the no-intervention arm vs LLIS ($P<0.0001$). The external control showed oxidase levels significantly lower than the LLIS and no-intervention arms in most of periods ($P<0.0001$)

Esterase activities were not significantly higher than the laboratory susceptible strain in the baseline or in the majority of follow up surveys (Figure 56B) The exception was at 12 and 24 months PI, when the no-intervention arm and the LLIS arm showed significantly higher rates of esterase activity than the susceptible strain respectively ($P<0.0001$, Figure 5B). The results demostrate that elevated esterase-based resistance mechanism was not prevalent in the field population.

A



B



C

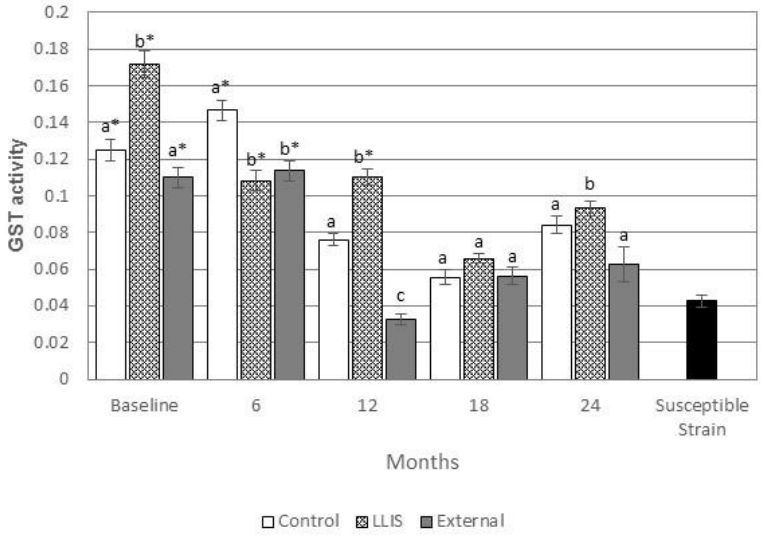


Figure 56. Oxidase (at absorbance 620 nm), esterase (at absorbance 540 nm) and GST activity (at absorbance 340 nm) for *Ae. aegypti* populations from Merida, during 2012-2014. White bars represent mean values (±SE) of the no-intervention arm, textured bars are means of LLIS arm, gray bars are means of external control and the black bar represent the mean of susceptible strain. Different letters mean significant difference between study groups within the sampling round. Asterisk denote significantly higher mean values ($P<0.05$) of absorbance that Rockeller strain (black bar).

GST activities in mosquitoes from all the study groups were significantly higher than in the susceptible strain during the baseline and at 6 months PI (P<0.0001, Figure 56C). However, a reduction in the GST levels was observed in each following period, reaching similar levels to the susceptible strain at 12 to 24 months PI. Among groups there was no clear trend (Figure 56C).

For AChE assays, in all periods the range of percentage inhibition of AChE activity by propoxur for the field collected *Ae. aegypti* mosquitoes was >90%, ranging from 90% to 98%, demonstrating that the AChE-based resistance gene was not present in the study groups.

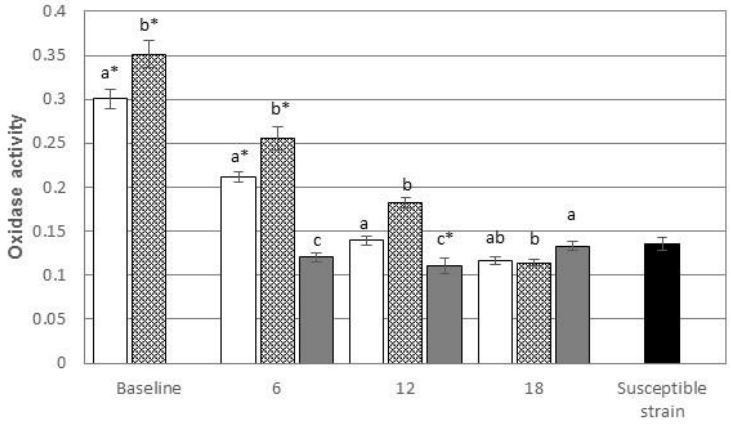
Enzyme activities for Acapulco populations. During the baseline and at 6 months PI the average oxidase content of the mosquitoes from LLIS and no-intervention arms was higher than the susceptible strains (Figure 57A, P<0.0001), but levels were decreasing in each following period until reaching similar levels to the susceptible one at 12-18 months. In most time points the LLIS arm showed higher rates of oxidase activities than no-intervention arm (P<0.0001). The corresponding external control had oxidase levels similar to the susceptible strain.

Esteras-based resistance mechanism seems not be important in the field population (Figure 57B). Only the no-intervention arm during the baseline showed higher levels of esterase activities than susceptible strain (P<0.0001).

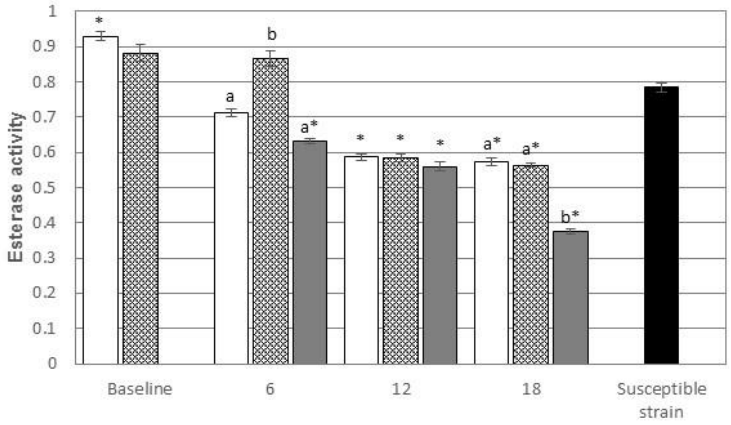
GST activities in mosquitoes from the LLIS and no-intervention arms were significantly higher than in the susceptible strain during the baseline and at 6 months PI (P<0.0001, Figure 57C). For both groups a reduction in the GST levels was observed in each following period, until reaching similar levels to susceptible one at 12-18 months PI, with exception of LLIS arm which continued showing high levels (P<0.0001) at 12 months PI. Among groups most of the significant differences were observed between LLIS and no-intervention arms, with the no-intervention arm typically having lower GST levels than LLIS (Figure 57C). The corresponding external control showed always levels similar to the susceptible strain.

The percentage inhibition of AChE activity by propoxur in field collected mosquitoes ranged from 100% to 92% in all the periods and study groups, indicating that theses populations does not carry the altered AChE gene.

A



B



C

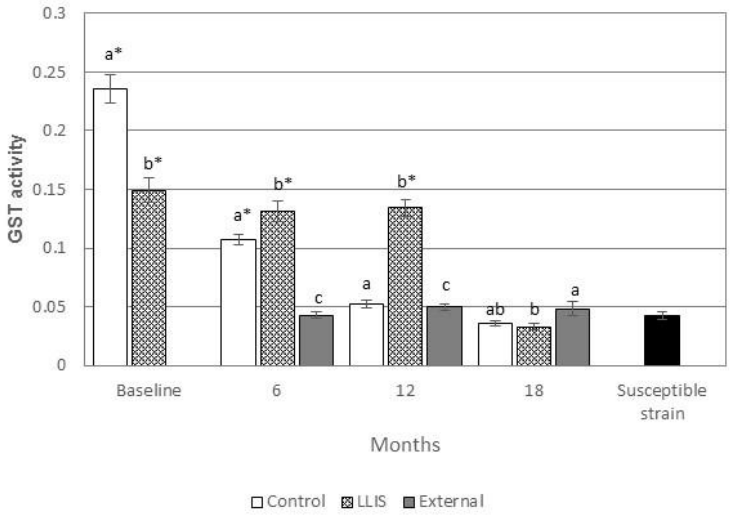


Figure 57. Oxidase (at absorbance 620 nm), esterase (at absorbance 540 nm) and GST activity (at absorbance 340 nm) for *Ae. aegypti* populations from Acapulco, during 2012-2014. White bars represent mean values (±SE) of the no-intervention arm, textured bars are means of LLIS arm, gray bars are means of external control and the black bar represent the mean of susceptible strain. Different letters mean significant difference between study groups within the sampling round. Asterisk denote significantly higher mean values of absorbance that Rockeller strain (black bar).

6.4.3. Molecular assays.

Table 14 and 15 shows the number of mosquitoes of each genotype, and frequency of both kdr mutations, 1016I and 1534C for each study arm and period of survey.

In Merida the average frequency of the homozygous wild-type genotypes 1016V/1016V and 1534F/1534F (mean of all surveys) was 3.5% (0-7.5%) and 3.8% (1-9%) respectively, and the homozygous mutant genotype 1016I/1016I and 1534C/1534C predominated at 49.2 (37-59%) and 88.5% (84-92%) respectively.

The frequency of 1016I kdr allele in Merida differed significantly between LLIS and no-intervention arms in all the periods (Fisher's exact test, $P<0.05$), except the final survey at 24 months. The frequency of 1016I in the external control varied significantly respect to LLIS and no-intervention arm in each period ($P<0.04$), except at 24 months PI, when only showed a frequency significantly lower that LLIS arm ($P=0.016$). In the no-intervention arm the 1016I frequencies over time were similar, fluctuating between 0.696 to 0.723, only showing a significant peak at 18 monts PI (0.891, $P<0.0001$). In contrast, the frequency in LLIS arm showed two significant peaks (compared with the rest of periods, $P<0.008$), in the beginning and in the end of the surveys (0.822 and 0.761 respectively, with no significant differences). The external control showed a gradual decrease in the 1016I frequency in the following periods after baseline (from 0.75 to 0.50), but did not differ significantly between surveys. See Figure 58A.

The frequency of the 1534C kdr allele in Merida differed significantly between LLIS and no-intervention arms at 12 and 24 months PI ($P<0.004$). The frequency of 1534C in the external control was always significantly lower that both LLIS and no-intervention arm at 12 and 18 months PI ($P<0.021$), and at 24 months PI only showed significant differences to no-intervention arm ($P=0.005$). In the no-intervention arm the 1534C frequencies over time were similar, fluctuating between 0.87 to 0.92, and not differ significantly between periods. The frequency in LLIS arm fluctuated between 0.91-0.99, the highest recorded at 24 months which was significant differences to baseline and 12 months PI. In contrast to 1016I, after baseline the frequency of 1534C in the external control showed a gradual increase showing a significant peak (0.75 to 1) at 24 months PI, compared with the baseline and second survey ($P<006$). See Figure 58B.

In Acapulco the average frequency of the homozygous wild-type genotypes 1016V/1016V and 1534F/1534F (mean of all surveys) were 4.5% (1-8%) and 1.3% (1-2%) respectively, and the homozygous mutant genotype 1016I/1016I and 1534C/1534C predominated at 49 (41-62%) and 94% (85-99%) respectively.

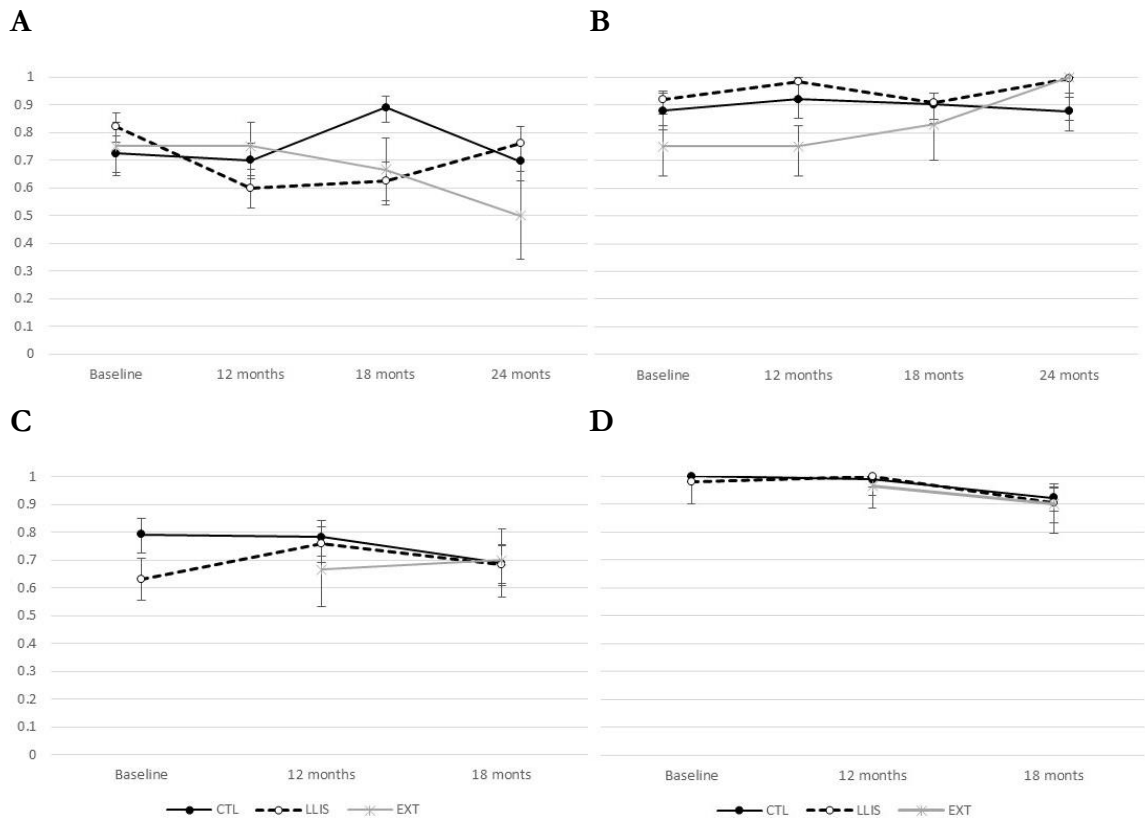


Figure 58. Kdr allelic frequency for 1016I (A, C) and 1534C (B, D) in Merida (A-B) and Acapulco (C-D) Mexico, during 2012-2014 for the different study groups, LLIS arm, no-intervention arm and external control.

The frequency of the 1016I kdr allele in Acapulco differed significantly between the LLIS and no-intervention arms only in the baseline (P=0.001). The frequency of 1016I in the external control was not significantly different to either the LLIS or no-intervention arm in any period. In the no-intervention arm the 1016I frequencies over time showed a significant reduction (from 0.79 to 0.69) compared to baseline and 12 months PI (P<0.08). The frequency in LLIS arm showed a significant peaks at 12 months PI (compared with the baseline from 0.63 to 0.76, P<0.014), but showed a reduction in the following period (0.68). The external control showed a gradual increase in the 1016I frequency (from 0.67 to 0.70), but did not differ significantly between surveys. See Figure 58C.

The frequency of the 1534C kdr mutation in Acapulco differed significantly between LLIS and no-intervention arms only in the baseline (P=0.055). The frequency of 1534C in the external control did show significant differences with either LLIS or no-intervention arm in any period. In the no-intervention arm the 1534C frequencies over time were similar between baseline and 12 months PI (from 1 to 0.99), but showed a significant drecrease at 18 months PI (0.92, P<0.004). In similar way the frequency in LLIS arm fluctuated between 0.98 to 1 during the

Table 14. Number of mosquitoes by genotype (SS, SR and RR) and allelic frequencies of kdr alleles 1016I and 1534C for *Ae. aegypti* from Merida, Mexico during 2012-2014. All samples (individual mosquitoes) were emerged from egg batches collected from ovitraps along the clusters selected in each study arm. 1016V/1016V and 1534F/1534F are SS (homozygous susceptible); 1016V/1016I and 1534F/1534C are SR (heterozygotes); and 1016I/1016I and 1534C/1534C are RR (homozygous resistant). P values < 0.05 indicate significant differences between arms.

		V1016I						F1534C						Percentage of double homozygotes		
Survey	**Arms of study**	**n**	**SS**	**SR**	**RR**	**Freq. R**	**P**	**n**	**SS**	**SR**	**RR**	**Freq. R**	**P**	**n**	**RR**	**SS**
Baseline	Untreated	94	0.47% (1)	23.9% (50)	20.5% (43)	0.72 (136)	**0.018**	144	5.42% (16)	1.01% (3)	42.3% (125)	0.87 (253)	**0.100**	94	44.7%	0.0%
	LLIS	115	0.95% (2)	17.7% (37)	36.3% (76)	0.82 (189)		151	3.72% (11)	0.67% (2)	46.7% (138)	0.92 (278)		115	60.0%	0.0%
12 months	Untreated	100	3.5% (7)	23% (46)	23.5% (47)	0.70 (140)	**0.046**	100	1.5% (3)	5% (10)	43.5% (87)	0.92 (184)	**0.004**	100	47.0%	1.0%
	LLIS	100	4% (8)	32% (64)	14% (28)	0.60 (120)		100	0% (0)	1.5% (3)	48.5% (97)	0.98 (197)		100	28.0%	0.0%
18 months	Untreated	96	0% (0)	10.9% (21)	39.0% (75)	0.89 (171)	**>0.001**	96	0% (0)	9.89% (19)	40.1% (77)	0.90 (173)	**1.000**	96	68.8%	0.0%
	LLIS	96	0% (0)	37.5% (72)	12.5% (24)	0.62 (120)		96	3.64% (7)	2.08% (4)	44.2% (85)	0.90 (174)		96	21.9%	0.0%
24 months	Untreated	97	2.09% (4)	26.7% (51)	21.9% (42)	0.69 (135)	**0.169**	98	1.01% (2)	10.1% (20)	38.3% (76)	0.87 (172)	**>0.001**	97	39.2%	0.0%
	LLIS	94	3.14% (6)	17.2% (33)	28.7% (55)	0.76 (143)		100	0% (0)	0.50% (1)	50% (99)	0.99 (199)		94	57.4%	0.0%

Table 15. Number of mosquitoes by genotype (SS, SR and RR) and allelic frequencies of kdr mutation 1016I and 1534C for *Ae. aegypti* from Acapulco, Mexico during 2012-2014. All samples (individual mosquitoes) were emerged from egg batches collected from ovitraps along the clusters selected in each study arm. 1016V/1016V and 1534F/1534F are SS (homozygous susceptible); 1016V/1016I and 1534F/1534C are SR (heterozygotes); and 1016I/1016I and 1534C/1534C are RR (homozygous resistant). P values < 0.05 indicate significant differences between arms.

		V1016I						**F1534C**						**Percentage of double homozygotes**		
Survey	**Arms of study**	**n**	**SS**	**SR**	**RR**	**Freq. R**	**P**	**n**	**SS**	**SR**	**RR**	**Freq. R**	**P**	**n**	**RR**	**SS**
Baseline	Untreated	89	0% (0)	21.5% (37)	30.2% (52)	30.2% (52)	**0.001**	99	0% (0)	0% (0)	51.2% (99)	1 (198)	**0.055**	89	58.4%	0.0%
	LLIS	83	1.16% (2)	33.1% (57)	13.9% (24)	13.9% (24)		94	1.03% (2)	0% (0)	47.6% (92)	0.97 (184)		83	28.9%	0.0%
12 months	Untreated	90	3.88% (7)	13.8% (25)	32.2% (58)	32.2% (58)	**0.706**	90	0.55% (1)	0% (0)	49.4% (89)	0.98 (178)	**0.499**	90	64.4%	0.0%
	LLIS	90	4.44% (8)	15% (27)	30.5% (55)	30.5% (55)		90	0% (0)	0% (0)	50% (90)	1 (180)		90	61.1%	0.0%
18 months	Untreated	90	2.22% (4)	26.6% (48)	21.1% (38)	21.1% (38)	**1.000**	90	1.11% (2)	5.55% (10)	43.3% (78)	0.92 (166)	**0.708**	90	38.9%	1.1%
	LLIS	90	1.66% (3)	28.3% (51)	20% (36)	20% (36)		90	1.11% (2)	7.22% (13)	41.6% (75)	0.90 (163)		90	33.3%	0.0%

baseline and at 12 months PI respectively, but showed a significant decrease at 18 months PI (0.91, P<0.006). The external control showed significant decrease from 0.97 to 0.90 (P=0.006) in the period from 12 to 24 months PI. See Figure 58D.

6.4.4. Impact of LLIS intervention on dengue cases.

A total of 943 DENV cases were reported in Mérida (479 cases in no-intervention arm: 464 in LLIS arm) and 109 in Acapulco (57:52) during the periods of 2011-2015 and 2009-2015 respectively (Table 16 and 17). No significant differences were observed in the number of cases between arms in any of the periods evaluated in both study sites, Merida and Acapulco (negative-binomial regressions P≥0.18). To evaluate if the LLIS are an important prognostic factor for the presence/absence of cases, the number of clusters with and without cases were identified in each period. A total of 131 events (positivity in clusters, considering that a cluster was positive when at least one case was identified within its boundaries) were reported in Mérida (67:64) and 62 in Acapulco (34:28) during the same periods; however no significant differences were observed in the cluster positivity between arms, in any of study sites during the periods evaluated.

Table 16. Number (mean ± standard error) of DENV cases reported in the clusters and cluster positivity to DENV cases for both study arms, no-interventio and LLIS arm in Merida from 2011-2015. Pre, pre-intervention or baseline; Int, intervention deploying; Post, post-intervention period.

	Season	No-intervention arm	LLIS arm
Number of cases			
Pre	Rainy 2011	234 (23.4 ± 6.12)	291 (29.1 ± 3.63)
	Dry 2012	46 (4.6 ± 0.93)	40 (4 ± 1.00)
Int.	Rainy 2012	96 (9.6 ± 2.83)	59 (5.9 ± 1.35)
	Dry 2013	20 (2 ± 0.80)	13 (1.3 ± 0.56)
Post	Rainy 2013	49 (4.9 ± 1.23)	29 (2.9 ± 0.90)
	Dry 2014	9 (0.9 ± 0.41)	10 (1 ± 0.49)
	Rainy 2014	14 (1.4 ± 0.86)	10 (1 ± 0.39)
	Dry 2015	4 (0.4 ± 0.16)	2 (0.2 ± 0.13)
	Rainy 2015	7 (0.7 ± 0.33	10 (1 ± 0.21)
Cluster positivity*			
Pre	Rainy 2011	10 (1 ± 0.00)	10 (1 ± 0.00)
	Dry 2012	10 (1 ± 0.00)	9 (0.9 ± 0.10)
Int.	Rainy 2012	10 (1 ± 0.00)	9 (0.9 ± 0.10)
	Dry 2013	9 (0.9 ± 0.10)	7 (0.7 ± 0.15)
Post	Rainy 2013	9 (0.9 ± 0.10)	8 (0.8 ± 0.13)
	Dry 2014	5 (0.5 ± 0.17)	5 (0.5 ± 0.17)
	Rainy 2014	6 (0.6 ± 0.16)	6 (0.6 ± 0.16)
	Dry 2015	4 (0.4 ± 0.16)	2 (0.2 ± 0.13)
	Rainy 2015	4 (0.4 ± 0.16)	8 (0.8 ± 0.13)

Table 17. Number (mean ± standard error) of DENV cases reported in the clusters and cluster positivity to DENV cases for both study arms, no-intervention and LLIS arm in Acapulco from 2009-2015. Pre, pre-intervention or baseline; Int, intervention deploying; Post, post-intervention period.

	Season	No-intervention arm	LLIS arm
Number of cases			
Pre	Dry 2009	1 (0.1 ± 0.10)	2 (0.2 ± 0.20)
	Rainy 2009	16 (1.6 ± 0.37)	18 (1.8 ± 0.59)
	Dry 2010	8 (0.8 ± 0.51)	9 (0.9 ± 0.28)
	Rainy 2010	2 (0.2 ± 0.13)	0 (0 ± 0.00)
	Dry 2011	0 (0 ± 0.00)	1 (0.1 ± 0.10)
	Rainy 2011	1 (0.1 ± 0.10)	0 (0 ± 0.00)
Int.	Dry 2012	1 (0.1 ± 0.10)	0 (0 ± 0.00)
	Rainy 2012	12 (1.2 ± 0.33)	16 (1.6 ± 0.78)
	Dry 2013	1 (0.1 ± 0.10)	1 (0.1 ± 0.10)
Post	Rainy 2013	3 (0.3 ± 0.21)	5 (0.5 ± 0.27)
	Dry 2014	2 (0.2 ± 0.13)	1 (0.1 ± 0.10)
	Rainy 2014	0 (0 ± 0.00)	2 (0.2 ± 0.13)
	Dry 2015	2 (0.2 ± 0.13)	2 (0.2 ± 0.20)
	Rainy 2015	3 (0.3 ± 0.21)	0 (0 ± 0.00)
Cluster positivity			
Pre	Dry 2009	1 (0.1 ± 0.10)	1 (0.1 ± 0.10)
	Rainy 2009	8 (0.8 ± 0.13)	7 (0.7 ± 0.15)
	Dry 2010	3 (0.3 ± 0.15)	6 (0.6 ± 0.16)
	Rainy 2010	2 (0.2 ± 0.13)	0 (0 ± 0.00)
	Dry 2011	0 (0 ± 0.00)	1 (0.1 ± 0.10)
	Rainy 2011	1 (0.1 ± 0.10)	0 (0 ± 0.00)
Int.	Dry 2012	1 (0.1 ± 0.10)	0 (0 ± 0.00)
	Rainy 2012	9 (0.9 ± 0.10)	5 (0.5 ± 0.17)
	Dry 2013	1 (0.1 ± 0.10)	1 (0.1 ± 0.10)
Post	Rainy 2013	2 (0.2 ± 0.13)	3 (0.3 ± 0.15)
	Dry 2014	2 (0.2 ± 0.13)	1 (0.1 ± 0.10)
	Rainy 2014	0 (0 ± 0.00)	2 (0.2 ± 0.13)
	Dry 2015	2 (0.2 ± 0.13)	1 (0.1 ± 0.10)
	Rainy 2015	2 (0.2 ± 0.13)	0 (0 ± 0.00)

6.5 Discussion

The main objective of this part of the study was to determine if there was any change in the susceptibility and resistance mechanisms in *Ae. aegypti* after the implementation of LLIS.

The study areas selected in this study are cities classified for the Ministry of Health as high level of risk for dengue transmission, and one of the most important in terms of historic number of severe dengue cases reported (Dantes et al., 2014). Furthermore, the locations where the study took place represent neighbourhoods catalogued as high priority by local

authorities, considering the historical records of dengue cases reported and the outbreaks experienced (large outbreaks occured recently in 2011 in Merida and in 2009-2010 in Acapulco) in the whole city (SINAVE, 2015). According to the national strategies for vector chemical control in response to cases (Hernandez-Avila et al 2013), it is expected that these locations have experienced an intensive and extensive use of insecticides. So routine *Ae. aegypti* control activities by the local vector control program (outdoor and indoor spraying mainly with Chlorpyrifos/Malathion and Propoxur/Bendiocarb/Deltamethrin, respectively) (CENAPRECE, 2014) were carried out in both the untreated and intervened houses during the period when the study took place.

Regarding the susceptibility to carbamates and organophosphates, all the study groups showed complete susceptibility to propoxur and decreased susceptibility to chlorpyrifos over the study period. To date only one report about the susceptibility to carbamates in *Ae. aegypti* populations from Mexico have been published (Deming et al., 2016). Deming and cols. (2016) reported high susceptibility to carbamate bendiocarb in several small localities close to Merida. Carbamates (bendiocarb and propoxur) where reciently approved in Mexico to be used in public health. They were gradually introduced in Acapulco and Merida since 2010 and 2012 respectively (more reciently the use of deltamethrin returned in 2014 replacing the use of carbamates particularly in Merida). Therefore, the selection pressure by these insecticides has been low, considering also that use of carbamate is based on the focal application compared with other insecticides applied extensively, such as ULV applications (i.e. chlorpyrifos). A few studies report a decreased susceptibility to chlorpyrifos in several *Aedes* population from Mexico (Lopez et al., 2014; Deming et al., 2016), attributed to increased level of esterase activity (Lopez et al., 2014). High resistance to this insecticide across the region seems to be a consequence of its extended use since more than 3 years. The higher levels of chlorpyrifos resistance in Acapulco than Merida could be associated with the historical use of this inseciticide in the two cities. The chlorpyriphos was used for first time in Acapulco in 2010 (to present); whereas in Merida the chlorpyriphos was introduced in the begining of 2012 and changed to malathion in 2013 (to present). However, as discussed in Chapter 3, its important to note that the diagnostic dose used in this study (14 μg/mL/bottle at 30 min) was 3-6 times lower than the diagnostic dose calculated independently by others authors (Deming et al., 2016; Lopez et al., 2014). According to results, the AChE and esterases profiles were not altered throughout the study. Therefore, the observed resistance is likely due to an alternative mechanism since cross-resistance between carbamates and organophosphates was not observed. The previous report of

esterase-based mechanism in confering chlorpyrifos is not discarted, but studies that are more specific must be carried out in order to clarify if specific esterases are involved in chorpyrifos resistance.

The reason to focus on evaluating resistance to permethrin and alpha-cypermethrin (and not others PYs) in this study was in first instance, due to the historical use of pyrethroids and the current insecticide application strategy in both study sites. From 1998 to 2009, pyrethroids were the primary insecticides used for outdoor and indoor spraying to control adult mosquito, mainly using permethrin-based formulations, and less frequently deltamethrin-based formulation for indoor residual spraying. And secondly because the alpha-cypermethrin is the active ingredient of the LLIS evaluated in this study.

In this context, the results from this study show that after a switch away from pyrethroids for over 4 years, phenotypic resistance to permethrin and a high frequency of kdr alleles still remain in the vector population. The results in this study show that metabolism-based resistance could be involved in confers PY resistance, as oxidase and GST activities were generally higher than in the control. It was very hard to see any clear trends in the levels of enzyme acitivity between the different study arms and between different surveys. This may reflect the fact that additional selection pressures are involved. For example, deltametrhin replaced carbamates in Merida in 2014 as the insecticide selected for indoor spraying for mosquito control in response to dengue cases. The results show that oxidase and GST-based insecticide resistance are established in the *Ae. aegypti* population of Acapulco and Merida which agree with previous reports in the same populations (Aponte et al., 2013). The maintenance of this mechanism in field population probabbly reflects the historic and continuos use of pyrethroids in both cases, in public health intervention and by householders.

Another interesting finding is the temporal variation in the frequency of the kdr alleles. After the withdrawel of pyrethroids in 2009 the frequency of the 1016I and 1534C kdr alleles in the no-intervention arm might be expected to decrease. However, both 1016I and 1534C kdr alleles were found at high frequencies (from 0.50 to 1) in Merida and Acapulco, but these resistance mechanism was not homogeneously spread in the mosquito population over time. The frequency of 1534C kdr was always higher than 1016I. This coincide with the findings of more recent studies carried out in the same localities. The 1534C has been reported almost fixed (0.99-1) in two different locations from Acapulco using mosquitoes emerged from eggs collected in 2009-2010, compared with frequencies reported for 1016I (0.77-0.93) in the same study (Penilla-Navarro et al., 2013). In Merida previous studies detected a 1534C

frequency of 0.79-1, and a 1016I frequency of 0.60-0.91 on larvae collected between 2010-2011 in five locations in the city (Saavedra-Rodríguez et al., 2014). The 1016I mutation is known to be associated with resistance to type I and II pyrethroids (Deming et al., 2016; Saavedra-Rodriguez et al., 2007), while the 1534C mutation is known to be associated most strongly with resistance to type I pyrethroids such as permethrin (Harris et al., 2010; Hirata et al., 2014). It is suggested that pyrethroid resistance requires the sequential evolution of the two mutations and that 1534C must occur first and appears to enable the 1016I mutation to survive (Vera-Maloof et al., 2015). In addition to metabolic-based resistance, the presence of the two mutations could be confering the high resistance to PY observed in the study (low KD rate observed for permethrin and high 24 h recovery observed for alpha-cypermethrin), considering that double mutants have higher pyrethroid resistance than mutants in either domain alone (Hu et al., 2011).

An external control was included in the study to record the general trends in resistance in areas under less extensive insecticide pressure. However, the levels of intensity of resistance and kdr frequencies in the external controls were similar to the other study groups. The kdr alleles 1016I&1534C have a fitness cost in *Ae. aegypti* (Brito et al., 2013), so their maintenaince in the populations after years of no applications of PY by local Ministry of Health, suggest a additional source of insecticide use pressure on the mosquitos populations, such as the pressure exerted by the use of insecticide in the household level. Some evidence of extensive use of commercial household pyrethroid products (such as aerosol cans) is reported at least for Merida (Loroño-Pino et al., 2014). However, no systematic studies has been carried out in order to evaluate the impact of household use of insecticides in maintaining pyrethroid pressure.

The kdr genotyping studies were carried out using mosquitos adults emerged from eggs. Since a single egg batch may be derived from just a few adult females (Apostol et al., 1994), in this study the eggs from multiple ovitraps in the same clusters were pooled, in order to provide a sufficient number of genomes as is recommended in the standard procedures (WHO, 2013) and to ensure a large enough number of mosquitos for the different aasays (as a minimum at least 2 ovitraps located in differents blocks of the clusters were used). For external controls 10 ovitraps deployed in the differents block were used.

The external controls showed low levels of detoxification enzymes similar to the laboratory susceptible strain during the most part of the study but did have high levels of the kdr alleles (0.67-1). Studies of resistance intensity found a lower level of resistance in the external controls than in the study area, which may suggest that both kdr and metabolic

resistance mechanisms are needed to confer very high level of pyrethroid resistance as has been proposed previously (Brito et al., 2013; Hu et al., 2011).

In contrast to the low KD observed in all study groups tested for permethrin, alpha-cypermethrin KD was > 90% at the beginning of the study. Within the study area, susceptibility to the alpha-cypermethrin did decrease over the time with a subsequent increase in the intensity of resistance but the external control was susceptible to this insecticide in most of the time that study took place. The Mexican Health authorities approve the use of alpha-cypermethrin-based formulations for indoor residual spraying (CENAPRECE, 2015), but in reality it is rarely used by the local vector control programmes in Merida and Acapulco. Any commercial household insecticide product contain alpha-cypermethrin as an active ingredient (See Annexes). Therefore, the low selection pressure by this insecticide could explain the levels of susceptibility observed in *Aedes* field population but only if the mechanisms conferring resistance to alpha-cypermethrin differ from those involved in resistance to other pyrethroid insecticides; this has not yet been evaluated

In previous chapters, it was demonstrated that the protective effect of LLIS last up to 600 days. Evaluation on the efficacy of LLIS in the context of the insecticide susceptibility over almost a two year period did not reveal clear patterns (in susceptibility status, kdr frequencies, metabolic alteration levels) that leads to the conclusion that LLIS could be affecting the resistance profiles in *Ae. aegypti* field population. But the intensity of resistance does suggest that the intervention arm has selected for higher levels of permethrin resistance than in the no-intervention arm. Whether the physical barrier or insecticidal effect or both drove the success of LLIS will be discussed in the last chapter.

Finally, in order to evaluate if LLIS may have protected against DENV transmission, confirmed DENV cases through National Surveillance System SINAVE-DGE (Hernandez-Avila et al., 2013) were identified for both study sites, and they were geocoded to determine if they fell within the boundaries of the clusters of both arms. However, in this initial analysis there was no association between LLIS and DENV cases in this study. It is important to mention that the analysis was based on passive surveillance information obtained from SINAVE-DGE system, where regularly is reported the suspected DENV cases (based on fever) from all potential reporting health care workers. Moreover, only a small percentage of the suspected cases (approximately 30%) are confirmed in the laboratory (according to the national guidelines of epidemiological surveillance of DENV). Future studies in this field should focus on the evaluation of effectiveness against DENV incidence of LLIS, based on

active search for cases in house level, including laboratory confirmation of all suspected cases, in order to generate evidence of LLIS "home residents protection" and "mass effect".

Chapter 7: Conclusions and recommendations

7.1 Long-lasting insecticide-treated house screening confers long-term house protection against entry by *Aedes aegypti* mosquitoes

In 2012, cluster randomised controlled trials were conducted in two Mexican cities - Acapulco (Guerrero state) and Merida (Yucatan state)- to test the efficacy of a novel *Aedes aegypti* intervention. The study compared ten control and ten intervention areas of 100 households each across both cities. Routine vector control activities -as implemented by the local Ministry of Health- were performed in control clusters. Intervention clusters included insecticide treated window and door screens (Acapulco and Merida) and targeted interventions in the productive water container types (in Acapulco only). The main outcome metrics were the reduction of vector densities.

The use of long-lasting insecticide-treated house screening (LLIS) protected houses against the entry of *Ae. aegypti* mosquitoes: significant reduction of indoor-resting adults by approximately 50% on the presence ($OR \leq 0.62$, $P < 0.05$) and abundance ($IRR \leq 0.58$, $P < 0.05$). The combination of LLIS with interventions targeting productive container types was successful in continuing reducing the number of *Aedes* pupae and consequently of adult dengue vectors. The rationale of targeted intervention is because the efficacy of LLIS on the control of *Aedes* populations depends on the proportion of adults that contact the materials and die. LLIS alone would not totally suppress adults *Ae. aegypti* because continued recruitment of individuals via adults surviving insecticide contact, plus adults never contacting the materials and adults emerging from breeding sites.

Two key results stand out from this study: i) an immediate significant effect on indoor-adult *Aedes* infestations was seen in houses protected with LLIS but not in controls, despite the fact that the *Aedes* populations at both sites were resistant to pyrethroids, and ii) this protection was sustained beyond 24 months when LLIS was combined with targeted treatment of productive breeding-sites (as demonstrated in Acapulco, where the combined intervention maintained a statistically significant protective effect on *Aedes* adult and immature stages until the end of the study, approximately 600 days after LLIS installation).

Previous cross sectional and case-control studies that measured the impact on numbers of indoor adult *Aedes* mosquitoes of screened houses (with permethrin nets) compared to houses without screens, have reported protective levels of around 50-100% for mosquito infestation (Igarashi, 1997; Nguyen et al. 1996; Manrique-Saide et al. 2014) and reductions around 40% for dengue incidence (Ko et al., 1992; McBride et al., 1998). These few examples,

and the findings from the present study, are indeed very valuable and promising; but there still is a definitive need to develop more field-trials in different contexts (Wilson et al., 2014; Achee et al., 2015; Bowman et al., 2016), particularly with respect to scaling-up as part of institutional programs i.e. by the Ministries of Health and to assessing the impact on disease transmission.

The present study provides valuable and unique information on the use of house screening within cities endemic for mosquito-borne diseases, and at the time of writing, is unique in supporting the feasibility and potential benefit of this method for the simultaneous prevention and control of DENV/CHIKV/ZIKV transmission.

Modifying or improving current housing designs with screens has been shown to be an effective way of preventing malaria (Kirby et al., 2008; Lindsay et al., 2002&2003; Walker, 2010), and other vector borne-diseases such as lymphatic filariasis, Japanese encephalitis, cutaneous leishmaniasis and other arboviruses (Ogoma et al., 2010; Wilson et al., 2014). The benefits of house screening, as a physical barrier, rely on its efficacy to exclude mosquitoes and eventually protect against mosquito bites, which is epidemiologically relevant if most transmission occurs indoors. From an environmental health perspective, residential premises (house and peridomicile) offer important habitats for supporting populations of *Ae. aegypti* as they emerge from productive breeding-sites and move in and out houses in search of food (human blood), refuge and mating and oviposit at the suitable breeding-sites to complete their life cycle. *Aedes* is an anthropophilic, endophilic and endophagic species and the house is the epidemiologically most significant point of vector-human contact for arbovirus transmission.

The adaptation of long-lasting insecticide nets permanently fitted as mosquito screens on windows and doors may be advantageous over other approaches (such as bednets and curtains) because these interventions are in place permanently and require little additional work or behavioural change by householders. Although they could be intrusive at first, overall satisfaction and acceptance levels are very high (Jones et al., 2014). Therefore the development of an effective house screening design is feasible for malaria (Kirby, et al., 2009) and dengue vectors (Jones et al., 2014), potentially protecting householders from multiple vector-borne diseases.

The level and duration of the protection against mosquitoes reported in this study can be compared with indoor residual spraying (IRS), historically the most effective and long-lasting method for killing indoor-mosquitoes (Najera et al., 2011). The IRS with DDT was the primary malaria control method used since 1946 and was the main prevention strategy of

malaria elimination efforts (1955-1969), eliminating malaria from several areas and sharply reducing the burden of malaria disease in others (Najera et al., 2011). However, the campaign collapsed and due to economic constraints, environmental concerns about the use of DDT and later the emergence of insecticide resistance, to DDT, malaria soon returned to pre-campaign levels in many locations. Programmes of indoor residual spraying that have responded to pyrethroid resistance by switching to alternative insecticides (such as carbamates) have shown a substantial fall (by more than 80%) in cases of malaria (Hemingway et al., 2013, 2016), demonstrating the potential of IRS programmes for malaria vector control.

Although IRS is not a standard recommended method for control of *Aedes* mosquitoes, when properly performed, it can have both an impact on *Ae. aegypti* infestation and dengue transmission (Vazquez-Prokopec et al., 2010). Today, in response to the Zika crisis, WHO recommend targeted IRS of resting sites of *Aedes* spp. (18 March 2016: http://www.who.int/neglected_diseases/news/mosquito_vector_control_response/en/). IRS, is time consuming and expensive to implement and requires regular retreatment. In contrast, implementation of house screening would only be performed once every two years.

The encouraging results from trials using house screening/full screening of windows/doors suggest that excluding the vector *Ae. aegypti* from the home may prove to be an innovative approach in terms of environmental management (changes to human habitation), if it is proven ultimately to reduce transmission of the pathogens to humans. With simultaneous potential transmission of DENV/CHIKV/ZIKV a reality in many countries of the Americas region today, and the potential for urban transmission by the same vector of additional re-emerging and emerging arboviruses, such as yellow fever and Mayaro virus (Moraes-Figueiredo, 2007), this simple classic method of vector control should be considered by National Ministries of Health. The resurgence of this approach becomes more important considering the emerging and re-emerging of multiple *Aedes*-borne diseases. At the time this thesis was written, Angola experienced an outbreak of yellow fever (http://wwwnc.cdc.gov/travel/notices/alert/yellow-fever-angola).

7.2 LLIS and insecticide resistance

One of the most interesting findings of this study was the encouraging observation that insecticide-treated house screening remained effective despite the high levels of insecticide resistance in local *Ae. aegypti* populations, as demonstrated by the baseline data from insecticide resistance monitoring (see section 3.4 of Chapter 3).

Insecticide-treated nets are more protective than untreated nets (Strode et al., et al., 2014). However, the efficacy of insecticidal properties can be compromised by the evolution of insecticide resistance. The present study also explored trends in the selection of insecticide resistance phenotypes in *Ae. aegypti* during a two-arm cluster randomised trial in which one study arm received LLIS and the other was considered control (without LLIS intervention). In this study, the 100-house clusters were located in different neighbourhoods in two cities, capturing differences in terms of geography, population, housing and health, economic, cultural, and social aspects. A paired design ensured a high consistency of key variables in intervention and control clusters.

Overall, the intensity of resistance was higher to permethrin than alpha-cypermethrin. In the baseline study, high levels of permethrin resistance were detected in most locations, but mosquito populations were moderately resistant or completely susceptible to alpha-cypermethrin. In both study sites, Merida and Acapulco, kdr frequencies were very high, close to 0.80 for 1016I and above 0.90 for 1534C. High levels of GST and oxidase activity were the most common metabolic mechanism detected. When clusters were grouped according to the arm of study to which they belonged, these resistance profiles did not change (the kdr frequencies, calculated for the field collected populations, were between 70-80% for 1016I and close to 90 for 1534C). As discussed in previous chapters this status of resistance is most likely the result of historic selection pressure experienced in the last 15 years, mainly by the use of pyrethroids in response to dengue cases and outbreaks in theses localities.

In order to determinate if there was any change in the resistance profiles attributable to the instalment of LLIS, resistance was monitored every 6 months during almost two years. There is no clear effect from the studies on mechanism of insecticide resistance (kdr frequencies and levels of enzyme activity), but the intensity of resistance suggests that the LLIS intervention arm was selecting for higher levels of permethrin resistance in comparison to the control arm in both study sites (see section 6.4, Chapter 6). For the LLIS arm, in most of cases the 90% knockdown threshold was not reached even when using 10x diagnostic doses whereas, threshold knockdown was reached in control arm with 10x. Therefore, resistance seemed to be stronger in LLIS arms after the intervention. Nevertheless, it is not possible to confirm if this selection pressure was exerted mainly by the use of LLIS, because additional selection pressures probably were involved. Differences in the selection pressure by use of pyrethroids for local ministry of health (for example, deltamethrin replaced carbamates in Merida in 2014) and householders (the use of commercial pyrethroid-based

aerosol sprays) must be considered. Probably much clearer effects on mechanism of insecticide resistance could be observed in longer periods and at larger-scales.

Temporal and spatial trends in *Anopheles* resistance (temporal increases in metabolic resistance and widespread distribution of knock-down resistance –kdr- mutations) throughout eastern and western Africa (Knox et al., 2014) match with long-lasting pyrethroid treated bednet coverage (Hemingway et al., 2016). Particularly increases in the allelic frequency of kdr mutations have been linked to the increased coverage of insecticide treated bednets (Ranson et al., 2011), which -in some cases- may have resulted in reduced impact of vector control interventions (WHO, 2012).

In the present study low knockdown and mortality of pyrethroid resistant field strains following exposure to LLIS was observed (even new non-exposed nets only resulted in <30% knockdown and <65% mortality). The field populations used were locally collected during the baseline in the study sites and maintained in insectary conditions. They were characterized in the baseline study as highly (M115 and Renacimiento strains) or moderately (SAX strain) resistant to permethrin, but generally showing moderate susceptibility to alpha-cypermethrin in the CDC bottles bioassays. In addition, the LLIS lifespan in field may be reduced, considering the low efficacy showed against the susceptible strain, which was constant throughout the study period. Although these results may be explained in part by limited bioavailability of active ingredient on the LLIS surface (the dust being an important factor affecting the bioefficacy of LLIS), the physiological resistance of mosquitoes to the insecticide limiting the performance of the tool is not ruled out. Many of the studies relating pyrethroid resistance to the bio-efficacy of standard long-lasting insecticidal nets have been performed on malaria vector populations (reviewed by Strode et al., 2014), with controversial results (Enayati and Hemingway, 2010; Strode et al., 2014).

On the other hand, the study shows that susceptibility tests cannot be extrapolated to expected results from cone bioassays. Such was the case of alpha-cypermethrin, which demonstrated high levels of efficacy in CDC bottles test when challenged with field strains, but failed in the bio-efficacy bioassays using LLIS (whose active ingredient is precisely the alpha-cypermethrin). Similar results have been reported in malaria vectors (Okia et al., 2013). This is not unexpected as the doses and exposure time vary between assays. In the CDC bottle assay the dose (10 μg/ml) and the exposition time (30 minutes) used is almost twice and ten times higher than the LLIS target dose (5.8 μg/mg) and the exposition time used in cone test (3 minutes) respectively.

This study represents one of the first studies that link the pyrethroid resistance to the field efficacy of LLIS for dengue vector control. Many studies evaluating the LLIS for dengue vector control overlook studies on profiles of resistance in the target population, and/or are have been limited to bioefficacy bioassays on nets (Lenhart et al., 2008; Rizzo et al., 2012; Seng et al., 2008; Vanlerberghe et al., 2010) or susceptibility tests (Vanlerberghe et al., 2013). A few studies considered additional tests such as target-site resistance testing (Loroño-Pino et al., 2013). Although there are not clear guidelines for measuring the efficacy of this kind of intervention against resistant mosquitoes, it is always desirable measure at the time of the study the phenotypic resistance through bioassays (susceptibility test and bio-efficacy bioassays), in addition to genotyping studies (for target-site and metabolic resistance) and biochemical assays (Strode et al., 2014).

In addition, the present study included the monitoring of intensity of resistance, in order to see differences in trends in insecticide resistance profiles that is not possible see using the diagnostic doses (Bagi et al., 2015). The inclusion of an external control (where the selection pressure by public health use of insecticide is lower than in the study arms) was designed to see if the changes in insecticide resistance observed are part of natural fluctuations not driven by the selection pressure related to the intensive use of insecticides. What we observed is that in general, the fluctuation or patterns in the profiles of resistance in the external control were similar to observed in the study arms, although with less intensity in some cases (i.e. showing highest susceptibility to alpha-cypermethrin and lowest levels of enzyme activity), clearly associated with the level of insecticide selection pressure. Unfortunately, the present study failed to assess the LLIS bio-efficacy on an external control.

Pyrethroid resistance should be considered as a key factor that may affect the implementation of control programmes using long-lasting insecticidal nets. These results underline the need to monitor the profiles of insecticide resistance and the inclusion of monitoring the intensity of resistance should be widely adopted, as it provides valuable information in addition to that obtained in the studies on resistance mechanism.

7.3 Final considerations

Currently, most national vector control programs rely heavily on chemical control methods for dengue prevention and control. Operationally, the majority of these programs provide emergency response to outbreaks and are unable to achieve sustained prevention by controlling the proliferation of the mosquito. In recent years, consensus has increased over the urgent need for effective interventions that impact on adult vector populations and/or

their interaction with humans in order to reduce virus transmission (Achee et al., 2015; Morrison et al., 2008).

The promising results obtained at a small scale in the present study indicate that interventions incorporating LLIS can be effective in controlling endophagic/endophilic *Aedes sp.* populations. Furthermore, an integrated approach with a rational use of insecticides could address positively the high cost of control programs and incipient vector resistance to insecticides. Where pyrethroid and organophosphate resistance has been demonstrated (as reported in this study), vector control programmes should consider implementing chemical control strategies based on the use of carbamates. Experience in Malaria control programs (Matowo et al., 2015), may provide synergistic interaction with LLIS. In any case, projects of this nature it deserves and needs to be evaluated for *Aedes* vector.

The results justify a second phase of research in which the intervention is scaled up to much larger geographic scales (thousands of households) in order to, a) broaden the evidence base for the feasibility of the proposed intervention; b) better quantify the effectiveness of the intervention in both reducing *Ae. aegypti* infestations and, ultimately, impacting virus transmission. It is generally recognised that greater coverage of the intervention will result in mass protection, reduced mosquito biting, and greater reductions in transmission; i.e. a community level effect. Another approach is in combination with identification of areas likely to be at risk of dengue outbreaks, as defined by risk mapping, such strategies could bring great health benefits by preventing outbreaks and reducing numbers of cases, with consequent reductions in overall morbidity and mortality. Although findings suggest that substantial virus transmission occurs away from the home, the inclusion of public locations (such as schools) could be an excellent opportunity to evaluate the efficacy of LLIS on dengue virus transmission.

While the data described here suggest that LLIS provided a measure of protection against *Aedes* mosquitoes, further research is clearly necessary to characterize in more detail the nature of their effect. It is unlikely that LLIS, as used in this study, provided a protective effect by killing pyrethroid-resistant mosquitoes. It is more likely that they functioned simply as a physical barrier to prevent *Aedes sp.* from entering houses. Follow-up projects at University of Yucatan in Merida, Mexico are currently underway to answer some questions about LLIS effects on behaviours such as blood feeding inhibition and its excito-repellent effect.

In conclusion, the results presented in this thesis indicate that Long-lasting insecticidal screens (LLIS) are a simple, easily implemented method of control of *Ae. aegypti*. They have

the potential to be effective even in areas where populations of this vector are pyrethroid-resistant with evidence indicating good potential for sustainability, given the high levels of acceptance and popularity among targeted communities. Although their effectiveness in reducing transmission of arboviruses remains to be confirmed, the significant impact of a single installation of LLIS on populations of the primary vector of dengue, chikungunya, Zika, Yellow fever and Mayaro viruses, provides good evidence that this method must be considered a strategy meriting incorporation into integrated vector management approaches both in Mexico and other suitable locations.

References

Abbot W. S. 1925. A method for computing the effectiveness of an insecticide. J. Econ Entomol. 18: 265-7.

Achee NL, Gould F, Perkins TA, Reiner RC, Jr., Morrison AC, Ritchie SA, Gubler DJ, Teyssou R, Scott TW. 2015. A Critical Assessment of Vector Control for Dengue Prevention. PLoS Negl Trop Dis. 9: e0003655.

Achee NL, Sardelis MR, Dusfour I, Chauhan KR, Grieco JP. 2009. Characterization of spatial repellent, contact irritant, and toxicant chemical actions of standard vector control compounds. J Am Mosq Control Assoc. 25 (2): 156-67.

Aïzoun N, Aïkpon R, Padonou GG, Oussou O, Oké-Agbo F, Gnanguenon V, Ossè R, Akogbéto M. 2013. Mixed-function oxidases and esterases associated with permethrin, deltamethrin and bendiocarb resistance in *Anopheles gambiae s.l.* in the south--north transect Benin, West Africa. Parasit Vectors. 6: 223.

Alvarez L, Briceño A, Oviedo M. 2006. Resistencia al temefos en poblaciones de *Aedes aegypti* (Diptera: Culicidae) del occidente de Venezuela. Rev Colom Entomol. 32: 172-5.

Alvarez LC, Ponce G, Saavedra-Rodriguez K, Lopez B, Flores AE. 2014. Frequency of V1016I and F1534C mutations in the voltagegated sodium channel gene in *Aedes aegypti* of Venezuela. Pest Manag Sci. Pest Manag Sci. 71 (6): 863-9.

Aparecida-Braga I, Pereira-Lima J, Da Silvasoares S, Valle D. 2004. *Aedes aegypti* resistance to temephos during 2001 in several municipalities in the states of Rio de Janeiro, Sergipe and Alagoas, Brazil. Mem Inst Oswaldo Cruz. 99: 199-203.

Aponte HA, Patricia Penilla R, Dzul Manzanilla F, Che Mendoza A, Lopeza AD, Solis F, Manrique-Saide P, Ranson H, Lenhart A, McCalld PJ, Rodrigueza AD. 2013. The pyrethroid resistance status and mechanisms in *Aedes aegypti* from the Guerrero state, Mexico. Pestic Biochem Phys. 107: 226–34.

Apostol B L., Black IV W. C., Reiter P. y B. R. Miller. 1994. Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. J. Trop. Med. Hyg. 51 (1): 89-97.

Apperson CS, Georghiou GP. 1975. Mechanisms of resistance to organophosphorus insecticides in *Culex tarsalis*. J Econ Biochem. 68: 63-78.

Arunachalam N , Tana S, Espino FE, Kittayapong P, Abeyewickreme W, Wai KT, Tyagi BK, Kroeger A, Sommerfeld J, Petzold M. 2010. Eco-Bio-Social determinants of dengue vector breeding: a multi country study in urban and peri-urban Asia. Bull World Health Organ. 88 (3): 173-84.

Ayres CFJ, Melo-Santos MAV, Prota JR, Solé-Cava AM, Regis LN, Furtado AF. 2004. Genetic structure of natural populations of *Aedes aegypti* at the microand macrogeographic levels in Brazil. J Am Mosq Control Assoc. 20: 350-6.

Bagi J, Grisales N, Corkill R, Morgan JC, N'Falé S, Brogdon WG, Ranson H. 2015. When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors. Malaria J. 20;14: 210.

Ballenger-Browning KK, Elder JP. 2009. Multi-modal *Aedes aegypti* mosquito reduction interventions and dengue fever prevention. Trop Med Int Health. 14: 1542-51.

Barbosa S, Black WC, Hastings A. 2011. Challenges in estimating insecticide selection pressures from mosquito field data. PLoS Negl Trop Dis. 5: e1387.

Bariami V, Jones CM, Poupardin R, Vontas J, Ranson H. 2012. Gene Amplification, ABC Transporters and Cytochrome P450s: Unraveling the Molecular Basis of Pyrethroid Resistance in the Dengue Vector, *Aedes aegypti*. PLoS Negl Trop Dis. 6: e1692.

Barrera-Pérez MA, Pavía-Ruz N, Mendoza-Mézquita JE, Torres-Arcila N, Hernández-Hernández R, Castro-Gamboa F, Geded-Moreno E, Cohuo-Rodríguez A, Medina-Barreiro A, Koyoc-Cardeña E, Gómez-Dantés H, Kroeger A, Vázquez-Prokopec G, Manrique-Saide P. 2014. Control de criaderos de *Aedes aegypti* con el programa Recicla por tu Bienestar en Mérida Yucatán México. Salud Publica Mex. 57 (3): 201-10.

Bass C, Field LM. 2011. Gene amplification and insecticide resistance. Mini-review. Pest Manag Sci. 67: 886-90.

Beach RF, Atieli FK, McAllister JC, Brogdon WG, Roberts JM, Mwangi RW, Hawley WA. 1999. Elevated oxidase and esterase levels associated with permethrin tolerance in *Anopheles gambiae* from Kenyan villages using permethrin-impregnated nets. J Med Vet Entomol. 13: 239-44.

Beach RF, Cordon-Rosales C, Brogdon WG. 1989. Detoxifying esterases may limit the use of pyrethroids for malaria vector control in the Americas. Parasitol Today. 5: 326.

Beaty B, Bernhardt S, Black Wet al. Novel Strategies to Control *Aedes aegypti* and Dengue. In: Atkinson PW (editor). Vector Biology, Ecology and Control. Springer: The Netherlands; 2009; p. 99–111.

Berg H van den, Zaim M, Yadav RS, Soares A, Ameneshewa B, Mnzava A, Hii J, Dash AP, Ejov M. 2012. Global trends in the use of insecticides to control vector-borne diseases. Environ Health Persp. 120: 577-82.

Beserra E, Fernández C, De Quiroga M, De Castro F. 2007. Resistance of *Aedes aegypti* (L.) (Diptera: Culicidae) populations to organophosphates temephos in the Paraíba State, Brazil. Neotrop Entomol. 36: 303-7.

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI. 2013. The global distribution and burden of dengue. Nature. 496: 504-7.

Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, Battle KE, Moyes CL, Henry A, Eckhoff PA, Wenger EA, Briët O, Penny MA, Smith TA, Bennett A, Yukich J, Eisele TP, Griffin JT, Fergus CA, Lynch M, Lindgren F, Cohen JM, Murray CL, Smith DL, Hay SI, Cibulskis RE, Gething PW. 2015. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature. 8; 526 (7572): 207-11.

Bisset JA, Rodríguez MM, Fernández D. 2007. Resistencia a insecticidas y mecanismos de resistencia en *Aedes aegypti* (Diptera: Culicidae) de dos provincias del Perú. Rev Cubana Med Trop. 58: 210–6.

Bisset JA, Rodríguez MM, Molina D, Díaz C, Soca LA. 2001. Esterasas elevadas como mecanismo de resistencia a insecticidas organofosforados en cepas de Aedes aegypti. Rev Cubana Med Trop. 53: 37-43.

Bisset-Lazcano JA, Rodríguez MM, San Martín JL, Romero JE, Montoya R. 2009. Evaluación de la resistencia a insecticidas de una cepa de *Aedes aegypti* de El Salvador. Rev. Panam. Salud Públ. 26: 229-34.

Bloomquist JR. 1999. Insecticides: Chemistries and characteristics. In: E. B. Radcliffe and W. D. Hutchison, eds. Radcliffe's IPM World Textbook, URL: http://ipmworld.umn.edu., University of Minnesota, St. Paul, MN.

Bobadilla-Utrera MC. 2010. Perfil de resistencia y mutación "kdr" asociada a insecticidas piretroides en *Aedes aegypti* (L.) (Diptera: Culicidae) de Veracruz, México. UANL. PhD Thesis. 142 pp.

Bowman LR, Donegan S, McCall PJ. 2016. Is Dengue Vector Control Deficient in Effectiveness or Evidence?: Systematic Review and Meta-analysis. PLoS Negl Trop Dis 10(3): e0004551.

Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, Moyes CL, Farlow AW, Scott TW, Hay SI. 2012. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 6: e1760.

Brengues C, Hawkes NJ, Chandre F, McCarroll L, Duchon S, Guillet P, Manguin S, Morgan JC, Hemingway J. 2003. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage gated sodium channel gene. Med Vet Entomol. 17: 87-94.

Brengues C, Hawkes NJ, Chandre F, McCarroll L, Duchon S, Guillet P, Manguin S, Morgan JC, Hemingway J. 2003. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltagegated sodium channel gene J Med Vet Entomol 17: 87-94.

Brito LP, Linss JGB, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JB, Valle D, Martins AJ. 2013. Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. PLOS One 8(4): e60878.

Brogdon WG, Barber AM. 1990. Fenitrothion–deltamethrin cross-resistance conferred by esterases in Guatemalan *Anopheles albimanus*. Pestic Biochem Physiol 37: 130–9.

Brogdon WG, Beach RF, Stewart JM, Castanaza L. 1988. Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan *Anopheles albimanus*. Bull World Health Organ 66: 339-46.

Brogdon WG, McAllister JC, Vulule J. 1997. Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for in- secticide resistance. J Am Mosq Control Assoc. 13 (3): 233-7.

Brogdon WG, McAllister JC. 1998a. Insecticide resistance and vector control synopses. Emerg Infect Dis. 4: 605-14.

Brogdon WG, McAllister JC. 1998b. Simplification of adult mosquito bioassays through use of timemortality determinations in bottles. J Am Mosq Control Assoc.. 14 (2): 159–64.

Brogdon WG. 1984. Mosquito protein microassay. I. Protein determinations from small portions of single mosquito homogenates. Comp Biochem Physiol 79: 457-9.

Brogdon WG. 1984. Mosquito protein microassay. I. Protein determinations from small portions of single mosquito homogenates. Comp Biochem Physiol. 79: 457-9.

Brogdon WG. 1989. Biochemical resistance detection: an alternative to bioassay. Parasitol Today 5: 56-60.

Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, Taylor ME, Small G, Hemingway J, Coetzee M. 2001. Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). Bull Entomol Res. 91: 265–72.

Brown AWA. 1958. Insecticide resistance in arthropods. Monograph Series No. 38. World Health Organization.

Brown AWA. 1986. Insecticide resistance in Mosquitoes. J Am Mosq Control Assoc. 2: 123-140.

Casas-Martinez, M. 2013. Bionomia comparativa de *Aedes aegypti* y *Aedes albopictus* y sus implicaciones en la transmision del dengue en el sur de Mexico. Tesis de Doctorado. El Colegio de la Frontera Sur. Tapachula, Chis., Mexico.

Casida JE, Quistad GB. 1998. Golden age of insecticide research: past, present, or future? Annu Rev Entomol. 43: 1-16.

Casida JE, Quistad GB. 2004. Why insecticides are more toxic to insects tan people: the unique toxicology of insects. J Pestic Sci. 29: 81-6.

Celli A. 1900. La nuova profilassi della malaria nel Lazio [The new prophylaxis against malaria in Lazio]. Supplemento al Policlinico 6: 1601-6.

CENAPRECE. 2014. Prevención y control del dengue. Programas de Acción Específico 2013-2018.

Centers for Disease Control and Prevention (CDC). 1979. Dengue-Mexico. Morbidity and Mortality Weekly Report, 28: 402-4.

Centers for Disease Control and Prevention (CDC). 1980. Follow-up on Dengue-Mexico. Morbidity and Mortality Weekly Report, 29: 169-70.

Centers for Disease Control and Prevention (CDC). 2010. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. Atlanta, Georgia 30333, USA. 56 pp.

Centro Nacional de Prevencion y Control de Enfermedades (CENAPRECE). 2014. Prevencion y Control del Dengue. Programa Sectorial de Salud 2013-2018. Secretaria de Salud. Mexico, D.F. pp. 94.

Chadee DD, Gilles JRL. 2013. The diel copulation periodicity of the mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae) at indoor and outdoor sites in Trinidad, West Indies. Acta Tropica 132S: S91-S95.

Chadee DD. 1997. Effects of forced egg-retention on the oviposition patterns of female *Aedes aegypti* (Diptera: Culicidae). Bull Entomol Research. 87: 649-51.

Chadee, DD, Corbet PS. 1989. Diel patterns of oviposition of the mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae) in Trinidad, W. I.: a preliminary study. Ann Trop Med Parasitol. 84 (1): 79-84.

Chang C, Shen WK, Wang TT, Lin YH, Hsu EL, Dai SM. 2009. A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in *Aedes aegypti.* Insect Biochem Mol Biol. 39: 272-8.

Chavez J, Vargas J, Vargas F. 2005. Resistencia a deltametrina en dos poblaciones de *Aedes aegypti* (Diptera: Culicidae) del Perú. Rev Peru Biol. 12: 161-4.

Che-Mendoza A, Guillermo-May G, Herrera-Bojórquez J, Barrera-Pérez M, Dzul-Manzanilla F, Gutierrez-Castroc C, Arredondo-Jiménez JI, Sánchez-Tejeda G, Vazquez-Prokopec G, Ranson H, Lenhart H, Sommerfeld J, McCall PJ, Kroeger A, Manrique-Saide P. 2015. Long-lasting insecticide treated house screens and targeted treatment of productive breeding sites for dengue vector control in Acapulco, Mexico. Trans R Soc Trop Med Hyg 109: 106-15.

Christophers KM. 1960. *Aedes aegypti* L. The Yellow Fever Mosquito. Cambridge University Press. G.B.

Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V. 1987. A ribosomal RNA gene probe differentiates member species of the Anopheles gambiae complex. Am J Trop Med Hyg 37: 37-41.

Colton YM, Chadee DD, Severson DW. 2013. Natural skip oviposition of the mosquito *Aedes aegypti* indicated by codominant genetic markers. Med Vet Entomol. 17 (2): 195-204.

Corbet PS, Smith SM. 1974. Diel periodicities of landing of nulliparous and parous *Aedes aegypti* (L.) at Dar es Salaam, Tanzania (Diptera: Culicidae). Bull Entomol Res. 64: 111-21.

Corbett JR., Wright K., Ballie AC. 1984. The biochemical mode of action of pesticides. Academic Press, London. pp. 382.

Cui F, Qu H, Cong J, Liu X-L, Qiao CL. 2007a. Do mosquitoes acquire organophosphate resistance by functional changes in carboxylesterases? FASEB J. 21, 3584–91.

Cui F, Weill M, Berthomieu A, Raymond M, Qiao CL. 2007b. Characterization of novel esterases in insecticide-resistant mosquitoes. Insect Biochem Mol Biol. 37 (11): 1131-7.

da-Cunha MP, Lima JB, Brogdon WG, Moya GE, Valle D. 2005. Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* (Diptera: Culicidae) populations collected between 2001 and 2003 Mem Inst Oswaldo Cruz. 100: 441-4.

Dantes HG, Farfan-Ale JA, Sarti E. 2014. Epidemiological Trends of Dengue Disease in Mexico (2000–2011): A Systematic Literature Search and Analysis. PLoS Negl Trop Dis 8(11): e3158.

David J-P, Ismail HM, Chandor-Proust A, Paine MJI. 2013. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. Phil Trans R Soc B. 368: 20120429.

Davies TGE, Field LM, Usherwood PNR, Williamson MS. 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. Critical review. Life 59: 151-62.

Deming R, Manrique-Saide P, Medina Barreiro A, Koyoc Cardeña EU, Che-Mendoza A, Jones B, Liebman K, Vizcaino L, Vazquez-Prokopec G, Lenhart A. 2016. Spatial variation of insecticide resistance in the dengue vector *Aedes aegypti* presents unique vector control challenges. Parasit Vectors 9: 67.

Denholm I, Devine GJ, Williamson MS. 2002. Evolutionary genetics. Insecticide resistance on the move. Comment on Science. 297: 2253-6.

Diaz FJ, Black WC4th, Farfan-Ale JA, Lorono-Pino MA, Olson KE, Beaty BJ. 2006. Dengue virus circulation and evolution in Mexico: a phylogenetic perspective. Arch Med Res 37 (6): 760-73.

Ding Y, Hawkes, N, Meredith, J, Eggleston, P, Hemingway, J, Ranson, H. 2005. Characterisation of the promoters of Epsilon glutathione transferases in the mosquito *Anopheles gambiae* and their response to oxidative stress. Biochem J. 387: 879-88.

Ding Y, Ortelli F, Rossiter LC, Hemingway J, Ranson H. 2003. The Anopheles gambiae glutathione transferase supergene family: annotation, phylogeny and expression profiles. BMC Genomics 4: 1-16.

Dominguez-Aguilar M. 2009. Procesos recientes del acceso al agua potable en el Mexico urbano: el caso de la Zona Conurbada de Merida, Yucatan. Investigaciones Geograficas, Boletin del Instituto de Geografia, UNAM 69: 68-84.

Du Y, Nomura Y, Satar G, Hu Z, Nauen R, He SY, Zhorov BS, Dong K. 2013. Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. Proc Natl Acad Sci USA. 110: 11785-90.

Dzul-Manzanilla F, Gutierrez-Castro C, Hernandez-HerreraL, Ibarra-Lopez J, Bibiano-Marin W, Lopez-Damian L. 2014. Las poblaciones de *Aedes aegypti* de Acapulco, Guerrero son resistentes a piretroides tipo I, pero susceptibles a piretroides tipo II y pseudopiretroide. Entomol Mex. 1: 898-903.

Dzul-Manzanilla F, Gutierrez-Castro C, Lopez-Damian LJ, Ibarra-Lopez J, Bibiano-Marin W, Che-Mendoza A, Manrique-Saide P, Torres-Leyva J, Arredondo-Jimenez JI, Garibay JA. 2014. Integral and targeted control on dengue vector at Iguala, Guerrero, Mexico. *In preparation.*

Ecobichon DJ. 2001.Carbamates insecticides Chapter 52. In Krieger R, eds. Handbook of pesticide toxicology principles. Second edition. Academic Press. San Diego, California, USA.

Edi C. 2014. CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. PGR Student Conference. LSTM. Liverpool 14th April 2014.

Enayati AA, Hemingway J. 2010. Malaria Management: Past, present, and future. Annu Rev Entomol. 55: 569–91.

Enayati AA, Ranson H, Hemingway J. 2005. Mini Review: Insect glutathione transferases and insecticide resistance. Insect Mol Biol. 14:3-8.

Environews Forum. 1997. Mexico moves to phase out DDT and chlordane. Environ Health Perspect. 105 (8): 790-1.

Erlanger TE, Keiser J, Utzinger J. 2008. Effect of dengue vector control interventions on entomological parameters in developing countries: a systematic review and meta-analysis. Med. Vet. Entomol. 22: 203-21.

Espinoza-Gomez F, M Hernández-Suárez, Coll-Cardenas R. 2002. Educational campaign versus malathion spraying for the control of *Aedes aegypti* in Colima, Mexico. J Epidemiol Community Health. 56: 148–52.

Esu E, Lenhart A, Smith L, Horstick O. 2010. Effectiveness of peridomestic space spraying with insecticide on dengue transmission; systematic review. Trop Med lnt Health 15: 619-31.

Ferroni E, Jefferson T, Gachelin G. 2011. Angelo Celli and research on the prevention of malaria in Italy a century ago. JLL Bulletin: Commentaries on the history of treatment evaluation (www.jameslindlibrary.org).

Feyereisen R, 2012. Insect CYP Genes and P450 Enzymes. Chapter 8. In Insect Molecular Biology and Biochemistry. Edited by Gilbert LI. Elsevier B.V.

Ffrench-Constant RH. 2013. The molecular genetics of insecticide resistance. Genetics. 194: 807-15.

Flores A, Albeldaño-Vásquez W, Fernández-Salas I, Badii M, Loaiza-Becerra H, Ponce-Garcia G, Lozano-Fuentes S, Brogdon W, Black W, Beaty B. 2005. Elevated α-esterase levels associated with permethrin tolerance in *Aedes aegypti* (L.) from Baja California, México. Pestic Biochem Phys. 82: 66-78.

Flores A, Grajales J, Fernandez- Salas I, Ponce G, Loaiza M, Lozano S, Brogdon WG, Black IV W, Beaty B. 2006. Mechanisms of insecticide resistance in field populations

of *Aedes aegypti* (L) from Quintana Roo, shouthern Mexico. J Am Mosq Control Assoc. 22: 672-7.

Flores AE, Ponce G, Silva BG, Gutierrez SM, Bobadilla C, Lopez B, Mercado R, Black IV WC. 2013. Wide spread cross resistance to pyrethroids in *Aedes aegypti* (Diptera: Culicidae) from Veracruz state Mexico. J Econ Entomol 106 (2): 959-69.

Flores AE, Reyes G, Fernandez-Salas I, Sanchez FJ, Garcia GP. 2009. Resistance to permethrin in *Aedes aegypti* (L.) in Northern Mexico, Southwest Entomol. 34: 167-77.

Focks D, Alexander N. 2006. Multicountry study of *Aedes aegypti* pupal productivity survey methodology: findings and recommendations. Special Programme for Research and Training in Tropical Diseases (TDR) [Internet] 06.1:1–48. Available from: http://www.who.int/tdr/cd_publications/pdf/aedes_aegypti.pdf.

Focks D. 2003. A Review of Entomological Sampling Methods and Indicators for Dengue Vectors. TDR/IDE/Den/03.1. Geneva: World Health Organization.

Fonseca-Gonzalez I, Quinones ML, Lenhart A, Brogdon WG. 2011. Insecticide resistance status of *Aedes aegypti* (L.) from Colombia. Pest Manag Sci. 67: 430-7.

Fonseca-González I. 2008. Estatus de la Resistencia a Insecticidas de los Vectores Primarios de Malaria y Dengue en Antioquia, Choco, Norte De Santander y Putumayo, Colombia. PhD Thesis. Universidad de Atioquia, Facultad de Ciencias Exactas y Naturales, Instituto de Biología. Medellín, Colombia. 203 pp.

García Gil G, Oliva Peña Y, Ortiz Pech R. 2012. Distribución espacial de la marginación en la ciudad de Mérida, Yucatán, México. Investigaciones Geográficas, Boletín 77: 89-106.

Garcia-Rejon J, Loroño-Pino MA, Farfan-Ale JA, Flores-Flores L, Del Pilar Rosado-Paredes E, Rivero-Cardenas N, Najera-Vazquez R, Gomez-Carro S, Lira-Zumbardo V, Gonzalez-Martinez P, Lozano-Fuentes S, Elizondo-Quiroga D, Beaty BJ, Eisen L. 2008. Dengue virus-infected *Aedes aegypti* in the home environment. Am J Trop Med Hyg. 79 (6): 940-50.

García-Rejón JE, López-Uribe MP, Loroño-Pino MA, Farfán-Ale JA, Najera-Vazquez MR, Lozano-Fuentes S, Beaty BJ, Eisen L. 2011. Productive Container Types for *Aedes aegypti* Immatures in Mérida, México. J Med Entomol. 48: 644-50.

Garcia-Rejon, JE, Lopez-Uribe MP, Loroño-Pino MA, Farfan-Ale JA, Najera-Vazquez MR, Lozano-Fuentes S, Beaty BJ, Eisen L. 2011. Productive container types for *Aedes aegypti* immatures in Merida, Mexico. J Med Entomol. 48 (3): 644-50.

Gatton ML, Chitnis N, Churcher T, Donnelly MJ, Ghani AC, Godfray HC, Gould F, Hastings I, Marshall J, Ranson H, Rowland M, Shaman J, Lindsay SW. 2013. The importance of mosquito behavioural adaptations to malaria control in Africa. Evolution 67 (4): 1218-30.

Georghiou GP. 1965. Genetic studies on insecticide resistance. Adv Pest Control Res. 6: 171.

Gerberg EJ, Barnard DR, Ward RA. 1994. Manual for mosquito rearing and experimental techniques. AMCA Bulletin No. 5 (revised). 98 p.

Getis A, Morrison A, Gray K, Scott TW. 2003. Characteristic of the spatial pattern of dengue vector, *Aedes aegypti*, in Iquitos, Perú. Am J Trop Med Hyg. 69 (5): 491-505.

Giglioli MEC. 1979. *Aedes aegypti* programs in the Caribbean and emergency measures against the dengue pandemic of 1977–1978: a critical review. In: Dengue in the Caribbean. Pan American Health Organisation Scientific Publication, Washington, DC, Vol. 375, pp. 375.

Gomez-Dantes H, Ramsey-Willoquet J. 2009. Dengue in the Americas: challenges for prevention and control. Review. Cad. Saude Publica 25 Sup 1: S19-S31.

Gomez-Dantes, Birn A-E. 2000. Malaria and social movements in Mexico: the last 60 years. Parassitologia 42: 69-85.

Gonzalez G, Ponce G, Quiroz-Martinez H,Garcia JE, Flores AE. 2012. Insecticide resistance mechanisms in *Aedes aegypti* from Merida, Yucatan, Mexico, related with two dengue transmission seasons. J Am Mosq Control Assoc., 28 (2): 103.

Gordon JR, Ottea J. 2012. Association of esterases with insecticide resistance in *Culex quinquefasciatus* (Diptera: *Culicidae*). J Econ Entomol. 105: 971-8.

Gorrochotegui-Escalante N, Gomez-Machorro C, Lozano-Fuentes S, Fernandez-Salas I, Munoz MD, Farfan-Ale JA, Garcia-Rejon J, Beaty BJ, Black WC 4th. 2002. Breeding structure of *Aedes aegypti* populations in Mexico varies by region. Am J Trop Med Hyg 66: 213–22.

Gorrochotegui-Escalante N, Gomez-Machorro C, Lozano-Fuentes S, Fernandez-Salas L, De Lourdes Munoz M, Farfan-Ale JA, Garcia-Rejon J, Beaty BJ, Black WC 4th. 2002. Breeding structure of *Aedes aegypti* populations in Mexico varies by region. Am J Trop Med Hyg. 66 (2): 213-22.

Grech MG, Ludueña-Almeida F, Almirón WR. 2010. Bionomics of *Aedes aegypti* subpopulations (Diptera: Culicidae) from Argentina. J Vector Ecol. 35 (2): 277-85.

Grieco JP, Achee NL, Chareonviriyaphap T, Suwonkerd W, Chauhan K, Sardelis MR, Roberts DR. 2007. A New Classification System for the Actions of IRS Chemicals Traditionally Used For Malaria Control. PLOS One 2(8): e716.

Grigoraki L, Balabanidou V, Meristoudis C, Miridakis A, Ranson H, Swevers L, Vontas J. 2016. Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Insect Biochem Mol Biol. 74: 61-7.

Grisales N, Poupardin R, Gomez S, Fonseca-Gonzalez I, Ranson H, Lenhart A. 2013. Temephos Resistance in *Aedes aegypti* in Colombia Compromises Dengue Vector Control. PLoS Negl Trop Dis 7(9): e2438.

Gu W, Novak RJ. 2009. Predicting the impact of insecticide –treated bed nets on malaria transmission: the devil is the detail. Malaria J. 8: 256.

Gullemaud T, Makate N, Raymond M, Hirst B, Callaghan A. 1997. Esterase gene amplification in Culex pipiens. Insect Mol Biol. 6: 319–327.

Gürtler RE, Yadon ZE. 2015. Eco-bio-social research on community-based approaches for Chagas disease vector control in Latin America. Trans R Soc Trop Med Hyg. 109 (2): 91-8.

Harbach RE. 2007. The Culicidae (Diptera): a review of taxonomy, classification and phylogeny. In: Zhang, Z.-Q. & Shear, W.A. (Eds) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy. Zootaxa 1668: 1–766.

Harrington LC, Scott TW, Lerdhusnee K, Coleman RC, Costero A, Clark GG, Jones JJ, Kitthawee S, Kittayapong P, Sithiprasasna R, Edman JD. 2005. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg. 72 (2): 209-20.

Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, Clark GG, Jones JJ, Kitthawee S, Kittayapong P, Sithiprasasna R, Edman JD. 2005. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg 72: 209-20.

Harris AF, Shavanthi R, Ranson H. 2010. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. Am J Trop Med Hyg 83: 277-84.

Hemingway J, Beaty BJ, Rowland M, Scott TW, Sharp BL. 2006 The innovative vector control consortium: improved control of mosquito-borne diseases. Trends Parasitol. 22: 308-12.

Hemingway J, Hawkes NJ, McCarroll L, Ranson H. 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 34: 653-65.

Hemingway J, Karunaratne SH. 1998. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. Med Vet Entomol 12: 1-12.

Hemingway J, Miyamoto J, Herath PRJ. 1991. A posible novel link between organophosphorus and DDT insecticide genes in *Anopheles*: supporting evidence from fenitrothion metabolism studies. Pestic Biochem Physiol. 39: 49-56.

Hemingway J, Ranson H, Magill A, Kolaczinski J, Fornadel C, Gimnig J, Coetzee M, Simard F, Roch DK, Hinzoumbe CK, Pickett J, Schellenberg D, Gething P, Hoppé M, Hamon N. 2016. Averting a malaria disaster: will insecticide resistance derail malaria control? Lancet. 23;387 (10029):1785-8.

Hemingway J, Vontas J, Poupardin R, Raman J, Lines J, Schwabe C, Matias A, Kleinschmidt I. 2013. Country-level operational implementation of the Global Plan for Insecticide Resistance Management. PNAS USA. 110(23): 9397-402.

Hemingway J. 2000. Mini review: The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. Insect Biochem Mol Biol. 30: 1009-15.

Hernandez-Avila JE, Rodriguez M-H, Santos-Luna R, Sanchez-Castañeda V, Roman-Perez S, Rios-Salgado VH, Salas-Sarmiento JA. 2013. Nation-Wide, Web-Based, Geographic Information System for the Integrated Surveillance and Control of Dengue Fever in Mexico. PLOS One 8(8): e70231.

Hirata K, Komagata O, Itokawa K, Yamamoto A, Tomita T, Kasai S. 2014. A Single Crossing-Over Event in Voltage-Sensitive Na+ Channel Genes May Cause Critical Failure of Dengue Mosquito Control by Insecticides. PLoS Negl Trop Dis. 8(8): e3085.

Hlavica P. 2011. Insect cytochromes P450: Topology of structural elements predicted to govern catalytic versatility. Review. J Inorg Biochem. 105: 1354–64.

Honorio NA, da Costa Silva W, Leite PJ, Goncalves JM, Lounibos LP, de Oliveira RL. 2003. Dispersal of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in an urban endemic dengue area in the state of Rio de Janeiro, Brazil. Mem. Inst. Oswaldo Cruz. 98 (2): 191-8.

Honorio NA, Silva WD, Leite PJ, Goncalves JM, Lounibos LP, Lourenço-de-Oliveira R. 2003. Dispersal of *Aedes aegypti* and *Aedes albopictus* (Diptera : Culicidae) in an urban endemic dengue area in the State of Rio deJaneiro, Brazil. Mem Inst Oswaldo Cruz. 98: 191-8.

Horstick O, Runge-Ranzinger S, Nathan MB, Kroeger A. 2010. Dengue vector-control services: how do they work? A systematic literature review and country case studies. Trans R Soc Trop Med Hyg. 104: 379-86.

Hougard JM, Duchon S, Darriet F, Zaim M, Rogier C, Guillet P. 2003 Comparative performances, under laboratory conditions, of seven pyrethroid insecticides used for impregnation of mosquito nets. Bull World Health Organ. 81: 324–33.

http://www.cenaprece.salud.gob.mx/descargas/pdf/PAE_PrevencionControlDengue2013_2018.pdf

Hu Z, Du Y, Nomura Y, Dong K. 2011. A sodium channel mutation identified in *Aedes aegypti* selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids. Insect Biochem Mol Biol. 41: 9-13.

Huang H, Yao H, Liu JY, Samra AI, Kamita SG, Cornel AJ, Hammock BD. 2012. Development of pyrethroid-like fluorescent substrates for glutathione S-transferase. Anal Biochem. 15;431 (2): 77-83.

Ibanez-Bernal S, Gomez-Dantes H. 1995. Los vectores del dengue en Mexico: una revision critica. Salud Publica Mex. 37: 53–63.

Ibañez-Bernal S. 1987. Nuevo registro altitudinal de *Aedes* (Stegomyia) *aegypti* (Linnaeus, 1762) (Diptera: Culicidae) en Mexico. Fol Entomol Mex. 72: 163-4.

Igarashi A. 1997. Impact of dengue virus infection and its control. FEMS Immunol Med Mic. 18 (4): 291-300.

INEGI. 2005. Conteo de Población y Vivienda 2005. http://www.inegi.org.mx/est/contenidos/proyectos/ccpv/cpv2005/Default.aspx.

INEGI. 2010. Censo de Población y vivienda 2010. http://www.censo2010.org.mx/.

Insecticide Resistance Action Committee (IRAC). 2013. www.irac-online.org.

Itoh T. 2005. Letters to the editors. Tropical Medicine and International Health 10, 1321-6.

Jirajaroenrat K, Pongjaroenkit S, Krittanai C, Prapanthadara L, Ketterman AJ. 2001. Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single *Anopheles* gene. Insect Biochem Mol Biol. 31: 867-75.

Jones C, Benítez-Valladares D, Barrera-Pérez M, Selem-Salas C, Chablé-Santos J, Dzul-Manzanilla F, Che-Mendoza A, Guillermo-May G, Medina-Barreiro A, Sommerfeld J, Kroeger A, Manrique-Saide P. 2014. Use and acceptance of Long Lasting Insecticidal Nets for dengue prevention in Acapulco, Guerrero, Mexico. BMC Public Health 14 (1): 846.

Karunaratne SHPP, Hemingway J, Jayawardena KGI, Dassanayaka V, Vaughan A. 1995. Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide-resistant and susceptible *Culex quinquefasciatus* mosquitoes. J Biol Chem. 270: 31124-8.

Karunaratne, SHPP. 1998. Insecticide resistance in insects a review. Ceylon J. Sci. (Biol. Sci.) 25: 72–99.

Kawada H, Higa Y, Komagata O, Kasai S, Tomita T, Yen NT, Loan LL, Sanchez RA, Takagi M. 2009. Widespread distribution of a newly found point mutation in voltage-gated sodium channel in pyrethroid-resistant *Aedes aegypti* populations in Vietnam. PLoS Negl Trop Dis 3: e0000527.

Kayedi MH, Lines JD, Haghdoost AA, Vatandoost MH, Rassi Y, Khamisabady K. 2008. Evaluation of the effects of repeated hand washing, sunlight, smoke and dirt on the

persistence of deltamethrin on insecticide-treated nets. Trans R Soc Trop Med Hyg. 102 (8): 811-6.

Kirby MJ, Ameh D, Bottomley C, Green C, Jawara M, Milligan PJ, Snell PC, Conway DJ, Lindsay SW. 2009. Effect of two different house screening interventions on exposure to malaria vectors and on anaemia in children in The Gambia: a randomised controlled trial. Lancet. 374: 998-1009.

Kirby MJ, Bah P, Jones CO, Kelly AH, Jasseh M, Lindsay SW. 2010. Social acceptability and durability of two different house screening interventions against exposure to malaria vectors, *Plasmodium falciparum* infection, and anemia in children in the Gambia, West Africa. Am J Trop Med Hyg. 83: 965-72.

Kirby MJ, Green C, Milligan P, Sismanidis C, Jasseh M, Conway D, Lindsay S. 2008. Risk factors for house-entry by malaria vectors in a rural town and satellite villages in The Gambia. Malar J. 7: 2 7.

Kirby MJ. 2013. House screening. Chapter 7. In: Biological and environmental control of Disease Vectors. Edited by Cameron MM and Lorenz LM. CAB International. London, UK. 117-43 pp.

Klowden M. J. 1990. The endogenous regulation of mosquito reproductive behavior. Experientia 46: 660-70.

Knox TB, Juma EO, Ochomo EO, Pates Jamet H, Ndungo L, Chege P, Bayoh NM, N'Guessan R, Christian RN, Hunt RH, Coetzee M: 2014. An online tool for mapping insecticide resistance in major *Anopheles* vectors of human malaria parasites and review of resistance status for the Afrotropical region. Parasit Vectors 7: 76.

Ko YC, Chen MJ, Yeh SM. 1992. The predisposing and protective factors against dengue virus transmission by mosquito vector. Am J Epi. 136 (2): 214–20.

Kostaropoulos I, Papadopoulos AI, Metaxakis A, Boukouvala E, Papadopoulou-Mourkidou E. 2001. Glutathione S-transferase in the defence against pyrethroids in insects. Insect Biochem Mol Biol. 31: 313–9.

Kroeger A, Lenhart A, Ochoa M, Villegas E, Levy M, Alexander N, et al. 2006. Effective control of dengue vectors with curtains and water container covers treated with insecticide in Mexico and Venezuela: cluster randomised trials. BMJ. 332: 1247-52.

Kwiatkowska R, Platt N, Poupardin R, Irving H, Dabire R, Mitchell S, Jones C, Diabaté A, Ranson H, Wondji C. 2013. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in *Anopheles gambiae* s.s., M form, from Vallée du Kou, Burkina Faso. Gene 519 (1): 98-106.

Labbé P, Alout H, Djogbénou L, Pasteur N, Weill M. 2011. Evolution of Resistance to Insecticide in Disease Vectors. Chapter 14. In: Genetics and Evolution of Infectious Disease. Tibayrenc M. eds. Elsevier. Pp: 363-409

Lengeler C. 2009. Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database of Systematic Reviews 2004, Issue 2, CD000363.

Lenhart A, Orelus N, Maskill R, Alexander N, Streit T, McCall PJ. 2008 Insecticide-treated bednets to control dengue vectors: preliminary evidence from a controlled trial in Haiti. Trop Med lnt Health. 13 (1): 56–67.

Lenhart A, Trongtokit Y, Alexander N, Apiwathnasorn C, Satimai W, Vanlerberghe V, Van der Stuyft P, McCall PJ, 2013. A cluster-randomized trial of insecticide-treated curtains for dengue vector control in Thailand. Am J Trop Med Hyg. 88: 254-9.

Lertkiatmongkol P, Pethuan S, Jirakanjanakit N, Rongnoparut P. 2010. Transcription analysis of differentially expressed genes in insecticide-resistant *Aedes aegypti* mosquitoes after deltamethrin exposure. J Vector Ecol. 35 (1): 197-203.

Liebman KA, Pinto J, Valle J, Palomino M, Vizcaino L, Brogdon W, Lenhart A. 2015. Novel mutations on the ace-1 gene of the malaria vector *Anopheles albimanus* provide evidence for balancing selection in an area of high insecticide resistance in Peru. Malar J. 2015; 14: 74.

Lima E, Santos MH, Araujo A, Gomes EV, Da Silva UM, Oliveira LN, Santana AE, Barbosa C, Paiva C, Goulart CM, Wilding CS, Junqueira CF, Melo MA. 2011. Insecticide resistance in *Aedes aegypti* populations from Ceara, Brazil. Parasit Vectors 4-5.

Lindsay SW, Emerson PM, Charlwood JD. 2002. Reducing malaria by mosquito-proofing houses. Trends Parasitol. 18(11): 510-514.

Lindsay SW, Jawara M, Paine K, Pinder M, Walraven G, Emerson PM. 2003. Changes in house design reduce exposure to malaria mosquitoes. Trop Med Int Health. 8: 512-7.

Linss JGB, Brito LP, Azambuja G, Saori A, Vieira R, Bento J, Valle D, Martins A. 2014. Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors 7(25): 2-11.

Lopez B, Gutierrez S, Elizondo D, García J, Farfán JA, Black IV WC, Flores AE. 2009. Temephos and deltamethrin susceptibility and enzymes associated with resistance in *Aedes aegypti* from Mexico. J Am Mosq Control Assoc. 25: 496.

Lopez B, Ponce G, Gonzalez JA, Gutierrez SM, Villanueva OK, Gonzalez G, Bobadilla C, Rodriguez IP, Black WC 4th, Flores AE. 2014. Susceptibility to chlorpyrifos in pyrethroid-resistant populations of *Aedes aegypti* (Diptera: Culicidae) from Mexico. J Med Entomol. 51:644-9.

Loroño-Pino MA, Chan-Dzul YN, Zapata-Gil R, Carrillo-Solis C, Uitz-Mena A, Garcia-Rejon JE, Keefe TJ, Beaty BJ, Eisen L. 2014. Household use of insecticide consumer products in a dengue-endemic area in Mexico. Trop Med Int Health 19 (10): 1267-75.

Loroño-Pino MA, García-Rejón JE, Machain-Williams C, Gomez-Carro S, Nuñez-Ayala G, Nájera-Vázquez Mdel R, Losoya A, Aguilar L, Saavedra-Rodriguez K, Lozano-Fuentes S, Beaty MK, Black WC 4th, Keefe TJ, Eisen L, Beaty BJ. 2013. Towards a Casa Segura: a consumer product study of the effect of insecticide-treated curtains on *Aedes aegypti* and dengue virus infections in the home. Am J Trop Med Hyg. 89 (2): 385-97.

Lozano-Fuentes S, Hayden MH, Welsh-Rodriguez C, Ochoa-Martinez C, Tapia-Santos B, Kobylinski KC, Uejio CK, Zielinski-Gutierrez E, Monache LD, Monaghan AJ, Steinhoff DF, Eisen L. 2012. The dengue virus mosquito vector *Aedes aegypti* at high elevation in Mexico. Am J Trop Med Hyg 87 (5): 902–9.

Lumjuan N, McCarrol L, Prapanthadara L, Hemingway J, Ranson H. 2005. Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. Insect Biochem Mol Biol. 35: 861-71.

Lumjuan N, Rajatileka S, Changsom D, Wicheer J, Leelapat P, Prapanthadara LA, Somboon P, Lycett G, Ranson H. 2011. The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. Insect Biochem Mol Biol. 41(3): 203-9.

Macoris M, Andrighetti M, Takaku L, Glasse C, Garbeloto V, Bracco J. 2003. Resistance of *Aedes aegypti* from the state of Sao Paulo, Brazil to organophosphates insecticides. Mem Inst Oswaldo Cruz. 98: 703-8.

Madarieta SK, Salarda A, Benabaye MR, Bacus MB, Tagle JR, 1999. Use of permethrintreated curtains for control of *Aedes aegypti* in the Philippines. Dengue Bull 23: 51–4.

Maestre RS, Rey GV, De las Salas JA, Vergara CS, Santacoloma LV, Goenaga SO, Carrasquilla MC. 2010. Estado de la susceptibilidad de *Aedes aegypti* a insecticidas en Atlántico (Colombia). Rev Colom Entomol. 36: 242-8.

Mani TR, Arunachalam N, Rajendran R, Satyanarayana K, Dash AP. 2005. Efficacy of thermal fog application of deltacide, a synergized mixture of pyrethroids, against *Aedes aegypti* the vector of dengue. Trop Med lnt Health 10: 1298-304.

Manrique-Saide P, Azael Che-Mendoza, Nidia Rizzo, Byron Arana, Daniel Pilger, Audrey Lenhart, Axel Kroeger. 2011. Operational guide for assessing the productivity of Aedes aegypti breeding sites. Web publication by TDR-WHO Available at http://apps.who.int/tdr/publications/tdr-research-publications/sop-pupal-surveys/pdf/sop-pupal-surveys.pdf.

Manrique-Saide P, Che-Mendoza A, Barrera-Pérez M, Guillermo-May G, Herrera-Bojórquez J, Dzul-Manzanilla F, Gutierrez-Castro C, Lenhart A, Vazquez-Prokopek G, Sommerfeld J, McCall PJ, Kroeger A, Arredondo-Jiménez JI. 2014. Long-lasting insecticide treated house screens reduce domestic infestations of dengue vectors in Mexico. Emerging Infectious Diseases 21: 308-11.

Manrique-Saide P, Che-Mendoza A, Gonzalez-Fernandez MI, Gonzalez-Acosta C, Villegas-Trejo A, Dzul-Manzanilla F, Cruz-Canto W, Guillermo-May G. 2011. Container types and characteristics associated with *Aedes aegypti* pupal productivity in localities of high risk of dengue transmission of Morelos State, Mexico. J Am Mosq Control Assoc. 27 (3): 293-94.

Manrique-Saide P, Coleman P, McCall PJ, Lenhart A, Vázquez-Prokopec G, Davies CR. 2014. Multi-scale analysis of the associations among egg, larval and pupal surveys and the presence and abundance of adult female *Aedes aegypti* (*Stegomyia aegypti*) in the city of Merida, Mexico. Med Vet Entomol. 28 (3): 264-72.

Manrique-Saide P, Davies CR, Coleman PG, Rebollar-Téllez E, Che-Mendoza A, Dzul-Manzanilla F, Zapata-Peniche A. 2008. Pupal surveys for Aedes aegypti surveillance and potential targeted control in residential areas of Merida, Mexico. J Am Mosq Control Assoc. 24: 289-98.

Manson P. 1900. Experimental proof of the mosquito malaria theory. Lancet 1: 923-5.

Marcombe S, Mathieu RB, Pocquet N, Riaz M-A, Poupardin R, et al. .2012. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors. PLOS One 7: e30989.

Marcombe S, Paris M, Paupy C, Bringuier C, Yebakima A, Chandre F, David J-P, Corbel V, Despres L. 2013. Insecticide-Driven Patterns of Genetic Variation in the Dengue Vector *Aedes aegypti* in Martinique Island. PLOS One 8 (10): e77857.

Marcombe S, Poupardin R, Darriet F, Reynaud S, Bonnet J, Strode C, Brengues C, Yébakima A, Ranson H, Corbel V, David JP. 2009. Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). Bmc Genomics 10: 494.

Martinez-Palacios A. 1965. Insecticide resistance in *Anopheles pseudopunctipennis* and its operational significance. Parte II. WHO/Vector Control/105.65. WHO/Mal/482.65.

Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, Guillet P, Pasteur N, Pauron D. 1998. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s.* Insect Mol Biol. 7: 179-84.

Martinez-Torres D, Chevillon C, Brun-Barale A, Bergé JB, Pasteur N, Pauron D. 1999. Voltage-dependent Na+ channels in pyrethroid resistant *Culex pipiens* L mosquitoes. Pestic Sci. 55: 1012-20.

Martinez-Vega RA, Danis-Lozano R, Diaz-Quijano FA, Velasco-Hernández J, Santos-Luna R, Román-Pérez S, Kuri-Morales P, Ramos-Castañeda J. 2015. Peridomestic infection as a determining factor of dengue transmission. PLoS Negl Trop Dis. 9 (12): e0004296.

Martins AJ, Lima JB, Peixoto AA, Valle D. 2009b. Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. Trop Med Int Health. 14: 1351-5.

Martins AJ, Lins RMMA, Linss JGB, Peixoto AA, Valle D. 2009a. Voltage gated sodium channel polymorphism and metabolic resistance in pyrethroid resistant *Aedes aegypti* from Brazil. Am J Trop Med Hyg. 81: 108-15.

Martins S. 2014. Identification of candidate genes associated with carbamate insecticide resistance in the lymphatic filariasis vector *Culex quinquefasciatus*. PGR Student Conference. LSTM. Liverpool 14th April 2014.

Matowo J, Kitau J, Kaaya R, Kavishe R, Wright A, Kisinza W, Kleinschmidt I, Mosha F, Rowland M, Protopopoff N. 2015. Trends in the selection of insecticide resistance in *Anopheles gambiae* s.l. mosquitoes in northwest Tanzania during a community randomized trial of longlasting insecticidal nets and indoor residual spraying. Med Vet Entomol. 29 (1): 51-9.

Matsumura F. 1985. Toxicology of insecticides. 2nd ed. Plenum Press, New York. Pp. 598.

Mazzarri MB, Georghiou GP. 1995. Characterization of resistance to organophosphate, carbamate, and pyrethroid insecticides in field population of *Aedes aegypti* from Venezuela. J Am Mosq Control Assoc.. 11 (3): 315-22

McBride WJ, Mullner H, Muller R, Labrooy J, Wronski I. 1998. Determinants of dengue 2 infection among residents of Charters Towers, Queensland, Australia. Am J Epi. 148 (11): 1111–6.

McCall P, Lloyd L, Nathan M. 2009. Vector management and delivery of vector control services. In: Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control, Geneva, World Health Organization, pp. 57-87.

McCall PJ, Kittayapong K. 2007. Control of Dengue Vectors: Tools and Strategies. Report of the Scientific Working Group Meeting on Dengue, Geneva, 1-5 October 2006. Pp. 110-9.

Méndez-Galván JF, Betanzos-Reyes SF, Velazquéz-Monriy OV, Tapia-Conyer R. 2004. Proyecto DDT/GEF Guía para la implementación y demostración de alternativas sostenibles de control integrado de la malaria en México y América Central.

Secretaría de Saud de México, CCA, GEF/UNEP, PNUMA, OPS. D.F. México. 210 pp.

Messina JP, Brady OJ, Scott TW, Zou C, Pigott DM, Duda KA, Bhatt S, Katzelnick L, Howes RE, Battle KE, Simmons CP, Hay SI. 2014. Global spread of dengue virus types: mapping the 70 year history. Trends microbiol. 22: 138-46.

Montella IR, Martins AJ, Viana-Medeiros PF, Lima JB, Braga IA, Valle D. 2007. Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. Am J Trop Med Hyg 77: 467-77.

Montella IR, Schama R, Valle D. 2012. The classification of esterases: an important gene family involved in insecticide resistance--a review. Mem Inst Oswaldo Cruz. 107: 437-49.

Moraes-Figueiredo LT. 2007. Emergent arboviruses in Brazil. Rev Soc Bras Med Tro. 40 (2): 224-9.

Morrison A., Zielinski-Gutierrez E., Scott T. & Rosenberg R. 2008. Defining Challenges and Proposing Solutions for Control of the Virus Vector *Aedes aegypti*. PLoS Med 5 (3): e68.

Mourya DT, Hemingway J, Leake, CJ. 1993. Changes in enzyme titres with age in four geographical strains of *Aedes aegypti* and their association with insecticide resistance. Med. Vet. Ent. 7: 11-6.

Mulamba C, Riveron JM, Ibrahim SS, Irving H, Barnes KG, Mukwaya LG, Birungi J, Wondji CS. 2014. Widespread pyrethroid and DDT resistance in the major malaria vector Anopheles funestus in East Africa is driven by metabolic resistance mechanisms. PLOS One 9 (10): e110058.

Najera JA, Gonza´lez-Silva M, Alonso PL. 2011. Some Lessons for the Future from the Global Malaria Eradication Programme (1955–1969). PLoS Med 8(1): e1000412.

Narro-Robles J, Gomez-Dantes H. 1995. El dengue en México: un problema prioritario de salud pública. Salud Publica Mex. 37: 12-20.

Nathan M. 2012. Introduction. Pathog Glob Health 106: 427

Nelson MJ. 1986. *Aedes aegypti*: Biology and ecology. Pan American Health Organization, Washington D.C.

Nelson MJ. 1986. *Aedes aegypti*: Biology and Ecology. Pan American Health Organization. E.U.

Nguyen HT , Tien TV , Tien NC , Ninh TU , Hoa NT. 1996 . The effect of Olyset net screen to control the vector of dengue fever in Viet Nam. Dengue Bull 20: 87-92.

Novo S. 1995. Breve historia y antología sobre la fiebre amarilla. Nuestro siglo. Salud Pública Méx 37: 99-102.

O´Reilly AO, Khambay BPS, Williamson MS, Field LM, Wallace BA, Davies TGE. 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochem. J. 396: 255-63.

Ocampo CB, Salazar-Terreros MJ, Mina NJ, McAllister J, Brogdon W. 2011. Insecticide resistance status of *Aedes aegypti* in 10 localities in Colombia. Acta Tropica 118: 37-44.

Official Regulations of Mexico, 2014. NOM-032-SSA2-2014 para la vigilancia epidemiológica, prevención y control de enfermedades transmitidas por vector D.O.F. 16th April 2015. Norma Oficial Mexicana, Mexico City.

Ogoma SB, Lweitoijera DW, Ngonyani H, Furer B, Russell TL, Mukabana WR, Killeen GF, Moore SJ. 2010. Screening mosquito house entry points as a potential method for integrated control of endophagic filariasis, arbovirus and malaria vectors. PLoS Negl Trop Dis. 2010 4(8): e773.

Okia M, Ndyomugyenyi R, Kirunda J, Byaruhanga A, Adibaku S, Lwamafa DK, Kironde F. 2013. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of *Anopheles gambiae* s.s. from different malaria transmission zones in Uganda. Parasit Vectors. 2;6: 130.

Ordoñez-Gonzalez JG, Mercado-Hernandez R, Flores-Suarez AE, Fernandez-Salas I. 2001. The use of sticky ovitraps to estimate dispersal of *Aedes aegypti* in northeastern Mexico. J Am Mosq Control Assoc. 17 (2): 93-7.

Ordoñez-Torres JA. 2004. Niveles de infestación larval y patrones de seleccion de criaderos de mosquitos *Aedes* en el area urbana de Tapachula, sur de México. Tesis de Licenciatura. Universidad Autonoma de Chiapas. Tapachula, Chis., Mexico.

Ortelli F, Rossiter LC, Vontas J, Ranson H and Hemingway J. 2003. Heterologous expression of four glutathione transferase genesgenetically linked to a major

insecticide resistance locus, from the malaria vector, *Anopheles gambiae*. Biochem J. 373: 957-63.

Owusu HF, Jančáryová D, Malone D, Müller P. 2015. Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology? Parasit Vectors 7;8: 357.

Paton MG, Karunaratne SHPP, Giakoumaki E, Roberts N, Hemingway J. 2000. Quantitative analysis of gene amplification in insecticide resistant *Culex* mosquitoes. Biochem. J. Pp. 345.

Peiris HTR, Hemingway J. 1993. Characterizacion and inheritance of elevated esterases in organophosphorus and carbamate insecticide resistant *Culex quinquefasciatus* (Diptera: Culicidae) from Sri Lanka. Bull Entomol Res. 83: 127-32.

Penilla-Navarro RP, Saavedra-Rodríguez K, Dzul-Manzanilla Felipe, Rodríguez-Ramírez AD, López-Solis AD, Black W. 2013. *Aedes aegypti* de Acapulco Guerrero presentó altas frecuencias del gen de resistencia a piretroides "kdr". Entomología Mexicana 12: 896-900.

Pereira-Lima E, De Oliveira-Filho A, De Oliveiralima J, Ramos-Junior A, De Goes-Cavalcanti L, Soares-Pontes R. 2006. *Aedes aegypti* resistance to temephos in counties of Ceará State. Rev Soc Bras Med Trop 39: 259-63.

Pilger DD, De Maesschalck M, Horstick O, San Martin J. 2010. Dengue outbreak response: documented effective interventions and evidence gaps. TropIKA.net Journal 1 (1).

Pimentel D. 2005. Environmental and economic costs of the application of pesticides primarily in the United States. Environ Dev Sustain. 7: 229–52.

Polson KA, Brogdon WG, Rawlins SC, Chadee DD. 2012. Impact of environmental temperatures on re- sistance to organophosphate insecticides in *Aedes aegypti* from Trinidad. Rev Panam Salud Pub. 32: 1-8.

Ponce-García GP, Flores AE, Fernández-Salas I, Saavedra-Rodríguez K, Reyes-Solis G, Lozano-Fuentes S, Guillermo Bond J, Casas-Martínez M, Ramsey JM, García-Rejón J, Domínguez-Galera M, Ranson H, Hemingway J, Eisen L, Black IV WC. 2009. Recent rapid rise of a permethrin knock down resistance allele in *Aedes aegypti* in México. PLoS Negl Trop Dis. 13;3 (10): e531.

Poupardin R, Srisukontarat W, Yunta C, Ranson H. 2014. Identification of carboxylesterase genes implicated in temephos resistance in the dengue vector *Aedes aegypti*. PLoS Negl Trop Dis. 20;8: e2743.

Powell JR, Tabachnick WJ. 2013. History of domestication and spread of *Aedes aegypti*--a review. Mem Inst Oswaldo Cruz. 108 Suppl 1:11-7.

Prapanthadara L, Promtet N, Koottathep S, Somboon P, Ketterman AJ. 2000. Isoenzymes of glutathione S-transferase from the mosquito *Anopheles dirus* species B: the purification, partial characterization and interaction with various insecticides. Insect Biochem Mol Biol. 30: 395-403.

Prapanthadara LA, Ranson H, Somboon P, Hemingway J. 1998. Cloning, expression and characterization of an insect class I glutathione S-transferase from *Anopheles dirus* species B. Insect Biochem Mol Biol. 28: 321–9.

Prieto AV, Suárez MF, González R. 2002. Susceptibilidad de dos poblaciones de *Aedes aegypti* (Diptera: Culicidae) de Cali (Valle, Colombia) a temefos (Abate) y Triflumuron (Starycide). Rev Colomb Entomol 28: 175-8.

Quintero J, Brochero H, Manrique-Saide P, Barrera-Pérez M, Basso C, Romero S, Caprara A, De Lima Cunha JC, Beltrán-Ayala E, Mitchell-Foster K, Kroeger A, Sommerfeld J, Petzold M. 2014. Ecological, biological and social dimensions of dengue vector breeding in five urban settings of Latin America: a multi-country study. BMC Infectious Diseases 14: 38.

Rafinejad J, Vatandoosta H, Nikpoor F, Abai MR, Shaegh iM, Duchen S, Rafi F. 2008. Effect of washing on the bio-efficacy of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs) against main malaria vector *Anopheles stephensi* by three bioassay methods. J Vector Borne Dis. 45: 143–50.

Rajatileka S, Black WC IV, Saavedra-Rodriguez K, Trongtokit Y, Apiwathnasorn C, McCall PJ, Ranson H. 2008 . Development and application of a simple colorimetric assay reveals widespread distribution of sodium channel mutations in Thai populations of *Aedes aegypti*. Acta Trop 108: 54–57.

Ranson H, Burhani J, Lumjuan N, Black IV WC. 2010. Insecticide resistance in dengue vectors. TropIKA.net 1: 1-12.

Ranson H, Claudianos C, Ortelli F, Abgrall C, Hemingway J, Sharakhova MV, Unger MF, Collins FH, Feyereisen R. 2002. Evolution of supergene families associated with insecticide resistance. Science. 298: 179-81.

Ranson H, Lissenden N. 2016. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. Trends Parasitol. 32 (3): 187-96.

Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 27: 91-98.

Ranson H, Rossiter L, Ortelli F, Jensen B, Wang X, Roth CW, Collins FH, Hemingway J. 2001. Identification of a novel class of insect glutathione S-transferases involved in DDT resistance in the malaria vector, *Anopheles gambiae*. Biochem. J 359: 295-304.

Rawlins S. 1998. Distribución espacial e importancia de la resistencia a insecticidas de poblaciones de *Aedes aegypti* en el Caribe. Rev. Panam. Salud Públ. 4: 243-51.

Raymond M, Berticat C, Weill M, Pasteur N, Chevillon C. 2001. Insecticide resistance in the mosquito *Culex pipiens*: what have we learned about adaptation. Genetica 112-113: 287-96.

Reiter P, Amador MA, Anderson RA, Clark GG. 1995. Short report: dispersal of *Aedes aegypti* in an urban area after blood feeding as demonstrated by rubidium-marked eggs. Am J Trop Med Hyg. 52: 177–9.

Reiter P, Amador MA, Anderson RA, Clarke GG. 1995. Dispersal of *Aedes aegypti* in an urban area after blood feeding as demonstrated by rubidium-marked eggs. Am J Trop Med Hyg 52: 177-9.

Rinkevich FD, Du Y, Dong K. 2013. Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids. Pestic Biochem Physiol. 106 (3): 93-100.

Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD, Ismail HM, Hemingway J, Ranson H, Albert A, Wondji CS. 2014. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biol. 25;15 (2): R27.

Rizzo N, Gramajo R, Cabrera-Escobar M, Arana B, Kroeger A, Manrique-Saide P, Petzold M. 2012. Dengue vector management using insecticide treated materials and targetted interventions on productive breeding-sites in Guatemala. BMC Public Heath 12: 931-48.

Roberts DR, Andre RG. 1994. Insecticide resistance issues in vector-borne disease control. Am J Trop Med Hyg. 50: 21-34.

Roberts DR, Chareonviriyaphap T, Harlan HH, Hshieh P. 1997. Methods of testing and analyzing excito-repellency responses of malaria vectors to insecticides. J Am Mosq Contr Assoc. 13: 13-7.

Rodriguez AD, Dzul-Manzanilla F, Penilla-Navarro P, Che-Mendoza A, Lopez-Solis AD, Solís-Santoyo F. 2010. Monitoreo de Resistencia a Insecticidas en *Aedes aegypti* de Acapulco, Guerrero, México. Entomol Mex. vol. 9: 712-5.

Rodriguez M, Bisset J, Fernandez D, Perez O. 2004. Resistencia a insecticidas en larvas y adultos de *Aedes aegypti*: prevalencia de esterasa A4 asociada con la resistencia a temefos. Rev Cubana Med Trop 56: 54-60.

Rodriguez MM, Bisset JA, Fernandez D. 2007. Levels of insecticide resistence and resistance mechanisms in *Aedes aegypti* from some Latin American countries. J Am Mosq Control Assoc. 23: 420-9.

Rosecrans K, Cruz-Martin G, King A, Dumonteil E. 2014. Opportunities for improved chagas disease vector control based on knowledge, attitudes and practices of communities in the Yucatan Peninsula, Mexico. PLoS Negl Trop Dis. 8 (3): e2763.

Ross R. 1913. Malaria prevention in Greece. BMJ 1: 1186

Rubio, A., M. V. Cardo, and D. Vezzani. 2011. Tire-breeding mosquitoes of public health importance along an urbanization gradient in Buenos Aires, Argentina. Mem Inst Oswaldo Cruz. 106 (6): 678-84.

Saavedra-Rodriguez K, Beaty M, Lozano-Fuentes S, Denham S, Garcia-Rejon J, Reyes-Solis G, Machain-Williams C, Loroño-Pino MA, Flores-Suarez A, Ponce-Garcia G, Beaty B, Eisen L, Black IV WC. 2014. Local evolution of pyrethroid resistance offsets gene flow among *Aedes aegypti* collections in Yucatan state, Mexico. Am J Trop Med Hyg 92 (1): 201-9.

Saavedra-Rodriguez K, Suarez AF, Salas IF, Strode C, Ranson H, Hemingway J, Black WC 4th. 2012. Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*. Insect Mol Biol. 21 (1): 61-77.

Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas I, Bisset J, Rodriguez M, Mccall PJ, Donelly MJ, Ranson H, Hemingway J, Black IV WC. 2007. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol Biol 16: 785–98.

Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, Jiwariyavej V, Dulyachai W, Pengsaa K, Wartel TA, Moureau A, Saville M, Bouckenooghe A, Viviani S, Tornieporth NG, Lang J. 2012. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet 3;380: 1559-67.

Sadasivaiah S, Tozan Y, Breman JG. 2007. Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control? Am J Trop Med Hyg. 77: 249-63.

Salazar M., Carvajal A, Cuellar M, Olaya A, Quiñones J, Velásquez O, Viveros A, Ocampo C. 2007. Resistencia a insecticidas en poblaciones de *Aedes aegypti* y *Anopheles* spp. en los departamentos de Huila, Valle, Cauca y Nariño. Memorias XIII Congreso colombiano de parasitología y medicina tropical. Biomédica 27: 177.

Salgado C. 2005. Políticas urbanas de Acapulco 1927-1997, México. Universidad Autónoma de Guerrero, Gobierno del Estado de Guerrero.

San Matin JL, Brathwaite O, Zambrano B, Solorzano JO, Bouckenooghe A, Dayan GH, Guzman MG. 2010. The epidemiology of dengue in the Americas over the last three decades: a worrisome reality. Am J Trop Med Hyg 82: 128–35.

Sanchez-Hernandez, C. 2011. Competencia larvaria interespecifica de *Aedes aegypti* y *Aedes albopictus* en condiciones de insectario en Tapachula. Tesis de Licenciatura. Universidad Autonoma de Chiapas. Tapachula, Chis., Mexico.

Savage HM, 2005. Classification of mosquitoes in Tribe Aedini (Diptera: Culicidae): paraphylyphobia, and classification versus cladistic analysis. J Med Entomol. 42 (6): 923-7.

Schleier III JJ, Peterson RKD. 2011. Pyrethrins and pyrethroid insecticides Chapter 3. In Lopez O and Fernández-Bolaños JG, eds. Green Trends in Insect Control. Royal Society of Chemistry.

Schofield C.J., Briceno-Leon R, Kolstrup N, Webb DJT, White GB. 1990. The role of house design in limiting vector-borne disease. In: Appropriate Technology in Vector Control. C.E Curtis (ed.) CRC Press, Boca Raton, FL. 233 pp.

Scott JG, Cytochromes P450 and insecticide resistance. 1999. Insect Biochem Mol Biol. 29: 757-77.

Scott TW, Morrison AC. 2002. Chapter 14. *Aedes aegypti* and the risk of dengue-virus transmission. In: Takken W. y T. W. Scott, eds. Ecological aspects for application of genetically modified mosquitoes. Proceedings of the frontis workshop on ecological challenges concerning the use of genetically modified mosquitoes for disease control, Wageningen, The Netherlands.

Secretaria de Recursos Hidráulicos (SRH). 1955. Comisión del Papaloapan: Actividades Médico Sanitarias de la Comisión de Papaloapan. Archivo Histórico de la Secretaria de Salud. 1952-4.

Seng CM, Setha T, Nealon J, Chantha N, Socheat D, Nathan MB. 2008. The effect of long-lasting insecticidal water container covers on field populations of *Aedes aegypti* (L.) mosquitoes in Cambodia. J Vector Ecol. 33: 333–41.

Siller Q, Ponce G, Flores AE. 2009. Knockdown resistance (KDR) associated with permethrin resistance in *Aedes aegypti* from Guerrero State on the west coast of Mexico. J Am Mosq Control Assoc. 25 (4): 496.

Siller Q, Ponce G, Lozano S, Flores AE. 2011. Update on the frequency of Ile1016 mutation in voltage-gated sodium channel gene of *Aedes aegypti* in Mexico. J Am Mosq Control Assoc. 27: 357-62.

SINAVE. 2015. Sistema Nacional de Vigilancia Epidemiológica. www.rhove.com.mx

Soderlund DM, Sanborn JR, Lee PW. 1983. Metabolism of pyrethrins and pyrethroids in insects. In: Hutson D, Roberts TR, eds. Progress in pesticide biochemistry and toxicology. Vol. 3. New York: Wiley. p 401–435.

Somwang, P, Yanola J, Suwan W, Walton C, Lumjuan N, Prapanthadara LA, Somboon P. 2011. Enzymes-based resistant mwechanism in pyrethroid resistant and susceptible *Aedes aegypti* strains from northern Thailand. Parasitol Res. 109: 531-7.

Srisawat R, Komalamisra N, Apiwathnasorn C, Paeporn P, Roytrakul S, Rongsriyam Y, Eshita Y. 2012. Field-collected permethrin-resistant *Aedes aegypti* from central Thailand contain point mutations in the domain IIS6 of the sodium channel gene (KDR). Southeast Asian J Trop Med Public Health. 43 (6): 1380-6.

Srisawat R, Komalamisra N, Eshita Y, Zheng M, Ono K, Itoh TQ, Matsumoto A, Petmitr S, Rongsriyam Y. 2010. Point mutations in domain II of the voltage-gated sodium channel gene in deltamethrin-resistant *Aedes aegypti* (Diptera: Culicidae). Appl Entomol Zool. 45: 275–82.

SSA. 2001. Programa de acción: Enfermedades transmitidas por vector. Secretaría de Salud. México, D.F. 73 p.

SSA. 2008a. Secretaría de Salud. Programa de Acción Específico 2007-2012 Dengue. México, D. F.

SSA. 2008b. Lista de materiales de insecticidas aprobados para el 2009. CENAVECE. www.salud.gob.mx

SSA. 2014. Lista de materiales de insecticidas aprobados para el 2013. CENAVECE. www.salud.gob.mx.

SSA. 2015. Lista actualizada de productos recomendados por el CENAPRECE para el Combate de insectos vectores de enfermedades a partir de 2015. www.cenaprece.salud.gob.mx

Stapleton DH. 1998. The dawn of the DDT and its experimental use by the Rockefeller Foundation in Mexico. 1943-1952. Parassitologia 40 : 149-58.

Stenhouse SA, Plernsub S, Yanola J, Lumjuan N, Dantrakool A, Choochote W, Somboon P. 2013. Detection of the V1016G mutation in the voltage-gated sodium channel gene of *Aedes aegypti* (Diptera: Culicidae) by allele-specific PCR assay, and its distribution and effect on deltamethrin resistance in Thailand. Parasit Vectors. 30;6: 253.

Stevenson BJ, Pignatelli P, Nikou D, Paine MJ. 2012. Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, *Aedes aegypti*: developing new tools to combat insecticide resistance. PLoS Negl Trop Dis. 6: e1595.

Stone BF and Brown AWA. 1969. Mechanisms of resistance to fenthion in *Culex pipiens fatigans* Wied. Bull. WHO 40, pp. 401–8.

Strode C, Donegan S, Garner P, Enayati AA, Hemingway J. 2014. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African Anopheline mosquitoes: systematic review and meta-analysis. PLoS Med 11 (3): e1001619.

Strode C, Wondji CS, David J-P, Hawkes NJ, Lumjuan N, Nelson DR, Drane DR, Karunaratne SHPP, Hemingway J, Black IV WC, Ranson H. 2008. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochem. Mol. Biol. 38: 113e123.

Strycharza JP, Laoa A, Lic H, Qiuc X, Leeb SH, Sunc W, Yoona KS, Dohertya JJ, Pittendrighc BR, Clark JM. 2013. Resistance in the highly DDT-resistant 91-R strain of *Drosophila melanogaster* involves decreased penetration, increased metabolism, and direct excretion. Pestic Biochem Physiol. 107: 207–17

Suppiramaniam V, Abdel-Rahman EA, Buabeid MA, Parameshwaran K. 2010. 13.09 Ion Channels. In: Comprehensive toxicology (second edition). Volume 13: Nervous System and Behavioral Toxicology. editor in chief: McQeen CA. Elsevier Ltd. United Kingdom.

Takken W. 2002. Do insecticide-treated bednets have an effect on malaria vectors? Tropical Medicine and International Health 7 (12): 1022–1030.

Tamayo-Dominguez, R. 2011. Comparacion del ciclo gonotrofico de hembras de *Aedes aegypti* Linnaeus, 1762 y *Aedes albopictus* (Skuse, 1894) (Diptera: Culicidae) en condiciones de insectario en el Municipio de Tapachula, Chiapas. Tesis de Licenciatura. Universidad Autonoma de Chiapas. Tapachula, Chis., Mexico.

Tapia-Conyer R, Betancourt-Cravioto M, Mendez-Galvan J. 2012. Dengue: an escalating public health problem in Latin America. Paediatr Int Child Health. 32 Suppl 1: 14-7.

Torres MA. 1995. La fiebre amarilla en Mexico. Erradicacion del *Aedes aegypti*. Salud Publica de Mexico, 37: S103-S110.

Troyo A, Fuller DO, Calderon-Arguedas O, Beier JC. 2007. A Geographical sampling method for surveys of mosquito larvae in an Urban area using high-resolution satellite imagery. J. Vector Ecol.. 33 (1): 1-7.

Trpis M, McClelland GAH, Gillett JD, Teesdale C, Rao TR. 1973. Diel periodicity in the landing of *Aedes aegypti* on man. Bull World Health Organ. 48: 623-9.

Tsunoda T, Kawada H, Huynh TT, Luu LL, Le SH, Tran HN, Vu HT, Le HM, Hasebe F, Tsuzuki A, Takagi M. 2013. Field trial on a novel control method for the dengue vector, *Aedes aegypti* by the systematic use of Olyset® Net and pyriproxyfen in Southern Vietnam. Parasit Vectors 6:6.

Tun-Lin W, Lenhart A, Nam VS, Rebollar-Téllez E, Morrison AC, Barbazan P, Cote M, Midega J, Sanchez F, Manrique-Saide P, Kroeger A, Nathan MB, Meheus F, Petzold M. 2009. Reducing costs and operational constraints of dengue vector control by targeting productive breeding places: a multi-country non-inferiority cluster randomized trial. Trop Med Int Health. 14(9): 1143-53.

Udomsinprasert R, Ketterman AJ. 2002. Expression and characterization of a novel class of glutathione S-transferase from *Anopheles dirus*. Insect Biochem Molec Biol. 32: 425-33.

Ulloa A, Cruz-Magariño J, Danis-Lozano R, Lopez-Ordoñez T. 2010. Cement tank: an artificial water container for *Aedes aegypti*. J Am Mosq Control Assoc. 26 (3): 307.

Valerio L, Facchinelli L, Ramsey JM, Scott TW. 2011. Dispersal of male *Aedes aegypti* in a coastal village in southern Mexico. Am J Trop Med Hyg. 86 (4): 665-76.

Vanlerberghe V, Trongtokit Y, Cremonini L, Jirarojwatana S, Apiwathnasorn C, Van der Stuyft P. 2010. Residual insecticidal activity of long-lasting deltamethrin-treated curtains after 1 year of household use for dengue control. Trop Med Int Health. 15 (9): 1067-71.

Vanlerberghe V, Trongtokit Y, Jirarojwatana S, Jirarojwatana R, Lenhart A, Apiwathnasorn C, McCall PJ, Van der Stuyft P. 2013. Coverage-dependent effect of insecticide-treated curtains for dengue control in Thailand. Am J Trop Med Hyg. 89(1): 93-8.

Vanlerberghe V, Villegas E, Jirarojwatana S, Santana N, Trongtorkit Y, Jirarojwatana R, Srisupap W, Lefèvre P, Van der Stuyft P. 2011b. Determinants of uptake, short term and continued use of insecticide-treated curtains and jar covers for dengue control. Trop Med lnt Health. 16 (2): 162-73.

Vanlerberghe V, Villegas E, Oviedo M, Baly A, Lenhart A, McCall PJ, Van der Stuyft P. 2011. Evaluation of the Effectiveness of Insecticide Treated Materials for Household Level Dengue Vector Control. PLoS Negl Trop Dis. 5 (3): e994.

Vargas F, Cordova O, Alvarado A. 2006. Determinación de la resistencia a insecticidas en *Aedes aegypti*, *Anopheles albimanus* y *Lutzomyia peruensis* procedentes del norte peruano. Rev Peru Med Exp Salud Publica 23: 259-64.

Vaughan A, Chadee DD, ffrench-Constant R. 1998. Biochemical monitoring of organophosphorous and carbamate insecticide resistance in *Aedes aegypti* mosquitoes from Trinidad. Med Vet Entomol. 12: 318–21.

Vaughan A, Hemingway J. 1995. Mosquito carboxylesterase Est alpha 2(1) (A2). Cloning and sequence of the full-length cDNA for a major insecticide resistance gene worldwide in the mosquito *Culex quinquefasciatus*. J Biol Chem. 14;270: 17044-9.

Vazquez-Prokopec GM, Kitron U, Montgomery B, Horne P, Ritchie SA. 2010. Quantifying the Spatial Dimension of Dengue Virus Epidemic Spread within a Tropical Urban Environment. PLoS Negl Trop Dis 4(12): e920.

Vera-Maloof FZ, Saavedra-Rodriguez K, Elizondo-Quiroga AE, Lozano-Fuentes S, Black WC, IV. 2015. Coevolution of the Ile1,016 and Cys1,534 Mutations in the Voltage Gated Sodium Channel Gene of *Aedes aegypti* in Mexico. PLoS Negl Trop Dis. 9 (12): e0004263.

Vontas J, Kioulos E, Pavlidi N, Morou E, della Torre A, Ranson H. 2012. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pestic Biochem Physiol. 104: 126–31.

Vontas JG, Small GJ, Hemingway J. 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. Biochem J. 357: 65-72.

Vontas JG, Small GJ, Nikou DC, Ranson H, Hemingway J. 2002. Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in

insecticide resistance from the rice brown planthopper, *Nilaparvata lugens*. Biochem J. 362: 329-337.

Vulule JM, Beach RF, Atieli FK, McAllister JC, Brogdon WG, Roberts JM, Mwangi RW, Hawley WA. 1999. Elevated oxidase and esterase levels associated with permethrin tolerance in Anopheles gambiae from Kenyan villages using permethrin-impregnated nets. Med Vet Entomol. 13: 239-44.

Walker N. 2010. The Hygienic House: Mosquito-Proofing with Screens Am J Trop Med Hyg. 83 (5): 963-4.

Waller LA, Gotway CA. 2004. Applied spatial statistics for public health data. John Wiley & Sons.

Waters LC, Nix CE. 1988. Regulation of insecticide resistance related cytochrome P450 expression in *Drosophila melanogaster*. Pest Biochem Physiol. 30: 214-27.

Weill M, Berthomieu A, Berticat C, Lutfalla G, Negre V, Pasteur N, Philips A, Leonetti J-P, Fort P and Raymond M. 2004. Insecticide resistance: a silent base prediction. Curr Biol. 14: R552–3.

Weill M, Lutfalla G, Mogensen K, Chandra F, Berthomieu A, Vertical C, Pasteur N, Philips A, Fort P, Raymond M. 2003. Insecticide resistance in mosquito vectors. Nature 423: 136–7.

WHO. 1960. Insecticide resistance and vector control, 10th Report. Technical Report Series No. 191. WHO, Geneva

WHO. 1976. Resistance of vectors and reservoirs of disease to pesticides. 22nd Report of the WHO Expert Committee on Insecticides. Technical Report Series 585. WHO, Geneva.

WHO. 1982. Manual on environmental management for mosquito control, with special emphasis on malaria vectors. WHO Offset Publication No. 66. Geneva.

WHO. 2002. Global insecticide use for vector-borne disease control. Geneva. WHO_CDS_WHOPES_GCDPP_2002.2.

WHO. 2003. Space spray application of insecticides for vector and public health pest control. A practitioner's guide. WHO. Geneva. WHO/CDS/WHOPES/GCDPP/2003.5.

WHO. 2004. Global insecticide use for vector-borne disease control. 2nd ed. Geneva. WHO_CDS_WHOPES_GCDPP_2004.9.

WHO. 2005a. Malaria control today: Current WHO recommendations. Geneva, Switzerland: Working document Roll Back Malaria Department. 75 pp.

WHO. 2005b. Guidelines for laboratory and field testing of long-last- ing insecticidal mosquito nets. In Document WHO/CDS/WHOPES/ GCDPP/2005.11 World Health Organisation, Geneva.

WHO. 2006. WHO. Pesticides and their application for the control of vectors and pests of public health importance .6th ed. Geneva. WHO/CDS/NTD/WHOPES/GCDPP/2006.1

WHO. 2007a. The use of DDT in malaria vector control WHO position statement. Global Malaria Programme, WHO. Geneva.

WHO. 2007b. Global insecticide use for vector-borne disease control. 3rd ed. Geneva. WHO_CDS_NTD_WHOPES_GCDPP_2007.2.

WHO. 2009a. Dengue-Guidelines for diagnosis, treatment, prevention and control. WHO/HTM/NTD/DEN/2009.1.

WHO. 2009b. Global insecticide use for vector-borne disease control, 4th ed. Geneva. WHO/HTM/NTD/WHOPES/GCDPP/2009.6.

WHO. 2011. Global insecticide use for vector-borne disease control. A 10-year assessment (2000–2009). 5th ed. Geneva. WHO/HTM/NTD/VEM/WHOPES/2011.6.

WHO. 2012. Global plan for insecticide resistance management in malaria vectors. Geneva.

WHO. 2012. Global Strategy for dengue prevention and control, 2012–2020. Geneva. WHO/HTM/NTD/VEM/2012.5.

WHO. 2013. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. WHO, Geneva.

WHO. 2014. WHO-Recommended Long-Lasting Insecticidal Mosquito Nets, February 2014.

Williams CR, Long SA, Russell RC, Ritchie SA. 2006. Field efficacy of the BG-Sentinel compared with CDC Backpack Aspirators and CO2-baited EVS traps for collection

of adult *Aedes aegypti* in Cairns, Queensland, Australia. J Am Mosq Control Assoc. 22(2): 296-300.

Williams LL. 1964. Pesticide: a contribution to public health. Am J Public Health N. 54: 32-6.

Williamson MS, Martinez-Torres D, Hick CA, Devonshire AL. 1996. Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Mol. Gen. Genet. 252: 51-60.

Wilson AL, Dhiman RC, Kitron U, Scott TW, van den Berg H, Lindsay SW. 2014 Benefit of Insecticide-Treated Nets, Curtains and Screening on Vector Borne Diseases, Excluding Malaria: A Systematic Review and Meta-analysis. PLoS Negl Trop Dis 8 (10): e3228.

Winch PJ, Barrientos-Sanchez G, Puigserver-Castro E, Manzano-Cabrera L, Lloyd LS, Mendez-Galvan JF. 1992. Variation in *Aedes aegypti* larval indices over a one year period in a neighborhood of Merida, Yucatan, Mexico. J Am Mosq Control Assoc. 8 (2): 193-5.

Winch PJ, Barrientos-Sanchez G, Puigserver-Castro E, Manzano-Cabrera L, Lloyd LS, Mendez-Galvan JF. 1992. Variation in *Aedes aegypti* larval indices over a one year period in a neighborhood of Merida, Yucatan, Mexico. J Am Mosq Control Assoc. 8: 193-5.

Wirth MC. Georghiou GP. 1999. Selection and characterization of temephos resistance in a population of *Aedes aegypti* from Tortola, British Virgin Islands. J Am Mosq Control Assoc. 15: 315-20 .

Yanola J, Somboon P, Walton C, Nachaiwieng W, Prapanthadara L. 2010. A novel F1552 / C1552 point mutation in the *Aedes aegypti* voltage-gated sodium channel gene associated with permethrin resistance. Pestic Biochem Physiol 96: 127–31.

Yanola J, Somboon P, Walton C, Nachaiwieng W, Somwang W, PrapanthadaraL. 2011. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant *Aedes aegypti* and the distribution of this mutation throughout Thailand. Trop Med Int Health. 16: 501–9.

Yap H.H. 1999. Insecticide Usage in the Urban Environment. In: Lee C.Y., Yap H.H., Chong N.L. and Jaal Z., eds, Urban Pest Control, A Malaysian Perspective. Universiti Sains Malaysia. 8-14 pp.

Yap H.H., Y.W. Lee and J. Zairi. 1999. Chemical control of mosquitoes: fogging and personal protection measures. In: Proceeding of Symposium on 'Towards a Mosquito-Safe Environment' Academy of Sciences Malaysia, 29th - 30th June 1999.

Zlotkin E. 1999. The insect voltage-gated sodium channel as target of insecticides. Annu Rev Entomol. 44: 429–55

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM: 2009. Mixed Effects Models and Extensions in Ecology with R. New York: Springer.

ANNEXES

Trans R Soc Trop Med Hyg 2015; **109**: 106–115
doi:10.1093/trstmh/tru189

ORIGINAL ARTICLE

Long-lasting insecticide-treated house screens and targeted treatment of productive breeding-sites for dengue vector control in Acapulco, Mexico

Azael Che-Mendoza[a], Guillermo Guillermo-May[b], Josué Herrera-Bojórquez[b], Mario Barrera-Pérez[b], Felipe Dzul-Manzanilla[c], Cipriano Gutierrez-Castro[c], Juan I. Arredondo-Jiménez[d], Gustavo Sánchez-Tejeda[d], Gonzalo Vazquez-Prokopec[f], Hilary Ranson[e], Audrey Lenhart[g], Johannes Sommerfeld[h], Philip J. McCall[e], Axel Kroeger[h] and Pablo Manrique-Saide[b,*]

[a]Servicios de Salud de Yucatán, Gobierno del Estado de Yucatán, Mérida C.P. 97000, Mexico; [b]Universidad Autónoma de Yucatán, Mérida C.P. 97000, Mexico; [c]Servicios Estatales de Salud de Guerrero, Chilpancingo C.P. 39090, Mexico; [d]Centro Nacional de Programas Preventivos y Control de Enfermedades, Secretaria de Salud, Mexico; [e]Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK; [f]Department of Environmental Sciences, Emory University, Atlanta, GA, 30322, USA; [g]US Centers for Disease Control and Prevention, Atlanta, GA30329, USA; [h]Special Programme for Research and Training in Tropical Diseases (TDR), World Health Organization, 1211 Geneva 27, Switzerland

*Corresponding author: Present address: Unidad Colaborativa para Bioensayos Entomológicos, Departamento de Zoología, Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán, Carretera Mérida-Xmatkuil Km. 15.5, Mérida C.P. 97315, Mexico. Tel: +52 9999 423200; E-mail: pablo_manrique2000@hotmail.com

Received 13 August 2014; revised 9 October 2014; accepted 29 October 2014

Background: Long-lasting insecticidal net screens (LLIS) fitted to domestic windows and doors in combination with targeted treatment (TT) of the most productive *Aedes aegypti* breeding sites were evaluated for their impact on dengue vector indices in a cluster-randomised trial in Mexico between 2011 and 2013.

Methods: Sequentially over 2 years, LLIS and TT were deployed in 10 treatment clusters (100 houses/cluster) and followed up over 24 months. Cross-sectional surveys quantified infestations of adult mosquitoes, immature stages at baseline (pre-intervention) and in four post-intervention samples at 6-monthly intervals. Identical surveys were carried out in 10 control clusters that received no treatment.

Results: LLIS clusters had significantly lower infestations compared to control clusters at 5 and 12 months after installation, as measured by adult (male and female) and pupal-based vector indices. After addition of TT to the intervention houses in intervention clusters, indices remained significantly lower in the treated clusters until 18 (immature and adult stage indices) and 24 months (adult indices only) post-intervention.

Conclusions: These safe, simple affordable vector control tools were well-accepted by study participants and are potentially suitable in many regions at risk from dengue worldwide.

Keywords: *Aedes aegypti*, Control, Dengue, LLIS, Mexico, Targeted treatment

Introduction

The dengue vector *Aedes aegypti* is a highly anthropophilic, endophilic and endophagic mosquito and has successfully exploited human-made ecosystems more than any other vector. Traditional *Ae. aegypti* interventions that are based on insecticide application such as indoor or outdoor space-spraying (or fogging) and larviciding, although effective in some settings, have shown limitations in terms of spatial and temporal coverage, residual power, sustainability and effectiveness in many contexts.[1] There is a pressing need from vector control programmes worldwide, for better dengue vector control tools that can achieve sustained reduction of dengue virus transmission by impacting the adult vector populations and/or interrupting their interaction with humans.[2]

Ecosystem management interventions such as the deployment of insecticide treated materials (ITMs) as window/indoor net curtains in houses, and the targeted treatment (TT) of productive breeding-sites have shown potential for integrated

dengue vector control in many geographical contexts.[1,3–6] Long-lasting insecticidal net screens (LLIS) are factory-produced mosquito nets pre-loaded with synthetic pyrethroid insecticide that is intended to retain its biological activity for at least 20 standard washes under laboratory conditions and 3 years of recommended use under field conditions.[7] Deployed as bednets, LLIS potentially can impact vector longevity at both household and community levels by reducing human biting rates.[4] Encouraging results have also been shown when LLIS are deployed as window or door curtains or as water jar covers for dengue control, particularly in Latin America[5,6] though the magnitude of such effect was sometimes dependent on the coverage attained, which could decline rapidly over time.[5] Targeting treatment of productive breeding-sites is a strategy that aims to impact vector populations by treating only water containers that produce the greatest number of pupae,[8] and also has potential for effective community-level dengue control.[1,9,10]

There is a need for more studies to comprehensively assess the long-term impact and cost-effectiveness of LLIS and TT in controlling local mosquito populations and reducing dengue transmission, particularly if both could be deployed simultaneously. The present study aimed to assess the long-term (over 2 years) impact of LLIS and TT in controlling domestic *Ae. aegypti* infestations, when deployed simultaneously, in an urban environment with perennially high dengue transmission in Mexico. The data reported here build upon the findings of the initial phase of a cluster randomized trial which investigated the impact of LLIS alone on adult vector indices.[11]

Materials and methods

This study formed part of a multi-country effort with a universal initial core protocol developed during a TDR-IDRC proposal development workshop in 2009. An earlier situational analysis of randomly selected study clusters (neighborhoods) in urban environments,[12] provided the initial information on which this intervention study was designed.

Study site

Ciudad Renacimiento (here after called Renacimiento) in Acapulco, is in Guerrero state, Mexico (Figure 1). Guerrero has one of the highest levels of dengue in Mexico, Acapulco reported >30% of the total dengue cases in Guerrero in the last decade.[13] Renacimiento is a high-risk area for dengue transmission: in 2011, entomological surveys found adult Aedes in 40% and 85% of houses during the dry and rainy seasons respectively, and over 70% of houses had incomplete walls, unprotected windows and/or open doors during daytime; although all households reported receiving water supply, 98% stored water in tanks (1000 litres) or barrels (100–200 litres), which produce 89% of total pupae in the study area.[12] Such large and highly productive containers are the focus of a targeted control effort by the local ministry of health (MoH) using larvicide (Temephos).

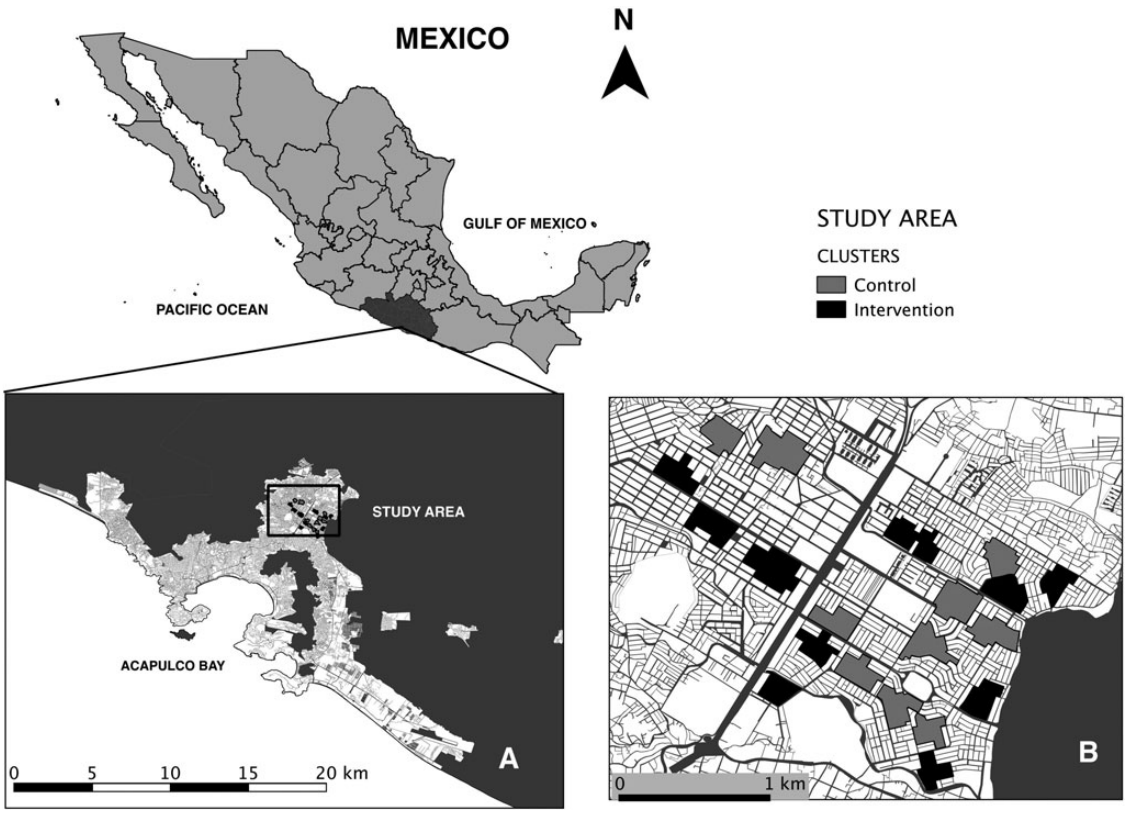


Figure 1. Study site. Location of Acapulco in Mexico and (A,B) the particular study area within Acapulco city showing the distribution of the study clusters with and without interventions.

Study design

A cluster-randomized sampling design with cross-sectional entomological surveys[12] was performed in 20 geographic clusters of 100 households each, with 10 randomly assigned to either intervention or control treatments, over 24 months. Procedures for selection of clusters, random assignment of treatments and statistical power calculations were described previously.[11]

House screening with LLIS

Duranet® screens (0.55% w.w. alpha-cypermethrin-treated non-flammable polyethylene netting [145 denier; mesh=132 holes/sq. inch]; Clarke Mosquito Control, Roselle, IL, USA; WHOPES approved for LLIS use) were mounted in aluminum frames custom-fitted to doors and windows of residential houses (Figure 2 A,B). The installation in 586 households from nine intervention clusters, carried out in collaboration with a local small business and the MoH, started in April 2012 and was finished by August 2012. In January 2013 the coverage of intervention was 78.0% of the households (780/1000) in the intervention clusters. During the installation, at least one person in every household received information on the use and maintenance of the LLIS through person-to-person communication.

Targeted treatment of the most productive *Ae. aegypti* breeding sites

Targeted treatment to prevent *Ae. aegypti* breeding in the most productive sites, was implemented 14 months after the beginning of LLIS installation (June 2013). All 1789 water tanks and 200 litre drums/barrels (Figure 2 C,D) in the households of intervention clusters, which were the most productive type of containers in baseline pupal surveys, were treated with the environmentally friendly larvicide Natular® DT (Spinosad 7.48%; Clarke Mosquito Control; WHOPES approved), delivering 1 tablet per 200 litres. The first cycle of application was performed at the end of the dry season in 2013 (September, n=1791 tanks and barrels) and was repeated every two months until March 2014 (November 2013 n=1686, January 2014 n=1658, March 2014 n=1595).

No interventions were delivered to the control clusters. However, existing routine vector control activities the local vector control program continued in both intervention and control clusters throughout the study. These included adulticiding (outdoor and indoor spraying with Chloropyrifos and Propoxur, respectively) and larviciding (Abate and Spinosad) in response to elevated dengue and entomologisal risk indices.[14] Notable emergency vector control activities occurred in February to April 2013 with a breeding-site reduction campaign all over the city called 'Megaoperativo' in July and ULV spraying from vehicles and airplanes in September 2013 (after tropical storms 'Manuel' and 'Ingrid').

Figure 2. Photographs show (A,B) the long-lasting insecticidal net screens ([LLIS]; Duranet® [Clarke Mosquito Control, Roselle, IL, USA]) mounted on aluminum frames and fixed to windows and external doors of treated houses and (C,D) the targeted treatment (TT) of the most productive *Aedes aegypti* breeding sites with the larvicide Natular® DT (Spinosad 7.48%) in Acapulco Mexico.

Entomological surveillance

Seven cross-sectional entomological surveys were conducted in treatment and control clusters: before (March 2011, September 2011, March 2012) and at 5, 12, 18 and 24 months (September 2012, March 2013, October 2013, March 2014; wet, dry, wet and dry seasons, respectively) post-intervention.

Indoor adult mosquito surveys

Adult entomologic surveys were performed in a sub-sample of 32 houses from each cluster. The houses were randomly selected in each cross-sectional survey during each entomologic survey date. Indoor adult mosquitos were collected using modified CDC backpack aspirators (John W. Hock Company, Gainesville, FL, USA) for 15 minutes per house. Collections within each cluster were performed on the same day between 09:00-15:00 hrs. All mosquitoes collected were identified for species and sex and kept in vials for future use.

Larval and pupal surveys

Larval and pupal surveys were conducted in all 2000 houses from the 20 study clusters for each entomologic survey according to a standard protocol.[15] Intradomestic and peridomestic spaces of residential premises were inspected and only water holding containers were examined. Containers were classified according to type, source of water, capacity, presence of a functional lid, proximity to vegetation, and presence of larval control measures. All immatures were collected except in large containers, where a sample of pupae was collected and a correction factor applied.[15] In the laboratory, a sub-sample of 10% of pupae was allowed to develop into adult mosquitoes to identify their species and sex.

Data management and statistical analysis of entomological indicators

From indoor adult collections we recorded: houses positive for female *Aedes*; houses positive for blood-fed female *Aedes*; houses positive for male *Aedes*; number of female *Aedes* per positive house; number of blood-fed female *Aedes* per positive house; and number of male *Aedes* per positive house. From immature collections we recorded: houses positive for immature (larva and pupae) *Aedes*; houses positive for *Aedes* larvae; number of *Aedes* larvae per house; houses positive for *Aedes* pupae; number of *Aedes* pupae per house; and pupae per person: number of *Aedes* pupae/number of inhabitants of a household.

The three classic *Stegomyia* indices: the container index (CI), representing the (number of containers with *Ae. aegypti* immatures/wet containers inspected)×100; the house index (HI), representing the (number of houses with *Ae. aegypti* immatures/houses inspected)×100; the Breteau index (BI), representing the number of containers positive for *Ae. aegypti* immatures/houses inspected)×100; and the pupae per person index (PPI) which is the ratio between pupae and persons living in each cluster were computed at the cluster level (Table 1). The difference between control and treatment clusters across the seven survey dates were evaluated with Mann-Whitney non-parametric tests.

Logistic regression models (for presence-absence data) and negative binomial models (for count data) accounting for each house membership in a given sampling cluster were performed for each cross-sectional entomological evaluation survey as described in Manrique-Saide et al.[11]. Odds ratios (OR) and incidence rate ratios (IRR) with 95% CIs were assessed and significance expressed at the 5% level. A generalized additive mixed model (GAMM) was applied to determine the association between various household-level entomologic indicators and the time (in days) since the installation of the LLIS. Time to intervention (t_i) was calculated by estimating the number of days that elapsed between the installation of the LLIS and the entomologic survey of each treatment house. We excluded the control houses from this analysis because analyses aimed at quantifying the temporal effect of LLIS. The full model had the form: $Y_{Aedes} = \alpha + f(t_i) + Z(cluster_i) + \varepsilon_i$. Where Y_{Aedes} is the entomologic measure and $Z(cluster_i)$, $\varepsilon_i \sim N(0, \sigma^2)$, represents a random effects term associated with observations from the same cluster. We used a negative binomial or binomial link functions depending if Y_{Aedes} was based on counts or binary values, respectively. We quantified the (possibly) non-linear relationship between the response variable and time since LLIS installation by incorporating a smoothing function ($f(t_i)$) representing the

Table 1. Comparison between treated (intervention [I]) and untreated (control [C]) groups on classic Stegomyia indices and pupal indicators at the cluster level in Acapulco, Guerrero

	Dry 2011		Rainy 2011		Dry 2012		Rainy 2012		Dry 2013		Rainy 2013		Dry 2014	
	I	C	I	C	I	C	I	C	I	C	I	C	I	C
CI	0.72	0.64	4.38	4.5	3.56	3.08	1.47	1.65	1.33	1.79	0.86*	1.82*	1.11	1.51
HI	4.40	4.80	20.45*	26.53*	16.21	15.42	7.58	9.38	6.28	9.00	4.36*	9.70*	4.10	5.90
BI	5.50	5.30	31.75	36.5	20.43	19.16	9.18	11.21	7.31	10.80	4.49*	10.40*	5.00	7.00
PPI	0.03	0.03	0.20	0.21	0.17	0.15	0.04	0.05	0.03*	0.10*	0.023*	0.105*	0.018*	0.071*

BI: Breteau index; CI: container index; HI: house index; PPI: pupae per person index.
* Mean of indicators followed by an asterisk symbol indicates significant difference between treated (I) and untreated (C) groups ($p<0.05$, Mann-Whitney non-parametric tests).

additive component [1]. We fitted $f(t_i)$ by applying a penalized cubic spline function to the data [1]. We assessed the importance of time since the installation of LLIS by evaluating the significance of the $f(t_i)$ term. Akaike Information Criterion (AIC) scores were used to compare the full model with a GAMM model without random effects. A model with $\Delta AIC=2$ or more units lower than any other model was considered the best. Once the best model was identified, we plotted each predicted $f(t_i)$ as either a curve (if $f(t_i)$ was significant) or a line (if $f(t_i)$ was not significant). Analyses were performed using STATA 12.0 (Stata Corp, College Station, TX, USA) and the mgcv package from the R statistical software (Foundation for Statistical Computing, Vienna, Austria).

Ethical aspects

This study received clearance from the ethical Committee of the Mexican Ministry of Health of Guerrero and the ERC (Ethical Review Committee) of WHO. Written informed consent was obtained for each participating household.

Results

Impact of house screening with LLIS

During the first 12 months of the study, treatment clusters received only LLIS. The preliminary findings from this part of the study have been reported earlier.[11] At five months post-intervention with LLIS, significantly fewer treated houses were infested with *Ae. aegypti* adult females (OR=0.38, 95% CI 0.21–0.69), blood-fed females (OR=0.36, 95% CI 0.21–0.60) and males (OR=0.39, 95% CI 0.19–0.77). A significant impact was still seen at 12 months post-intervention for adult females (OR=0.41, 95% CI 0.25–0.68) and males (OR=0.41, 95% CI 0.27–0.64) but not for blood-fed females (OR=0.51, 95% CI 0.24–1.05) (Figure 3). Analyses of infestation density showed a similar trend with a significant reduction in mean *Ae. aegypti* abundance in houses with LLIS: adult females at 5 (IRR=0.37, 95% CI 0.27–0.49) and 12 (IRR=0.40, 95% CI 0.23–0.70) months post-intervention; males at 5 (IRR=0.39, 95% CI 0.28–0.54) and 12 (IRR=0.49, 95% CI 0.33–0.72) months; blood-fed females

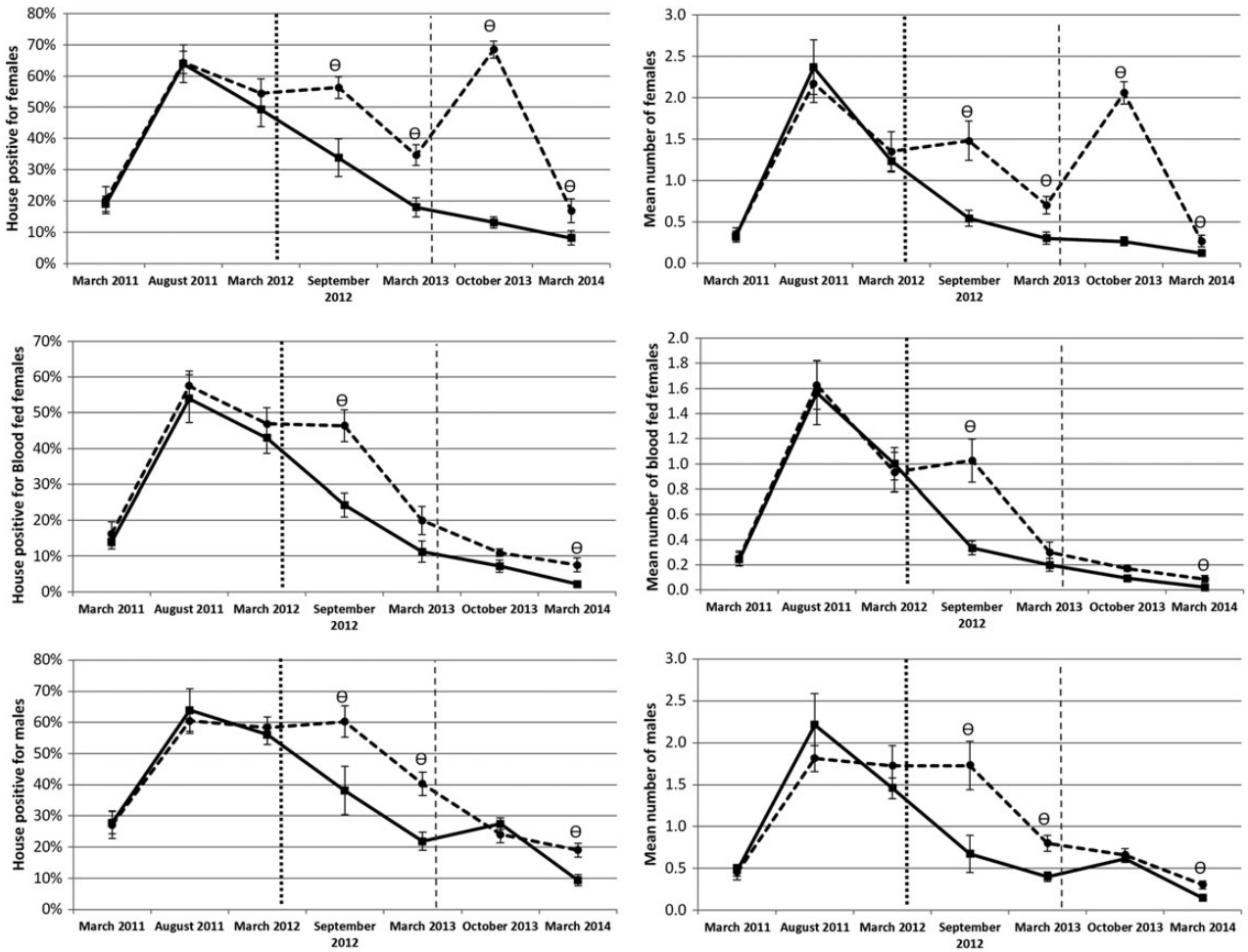


Figure 3. Comparison between treated (solid line) and untreated (broken line) groups of percentage of infested houses (left) and infestation density (right) for *Aedes aegypti* in Acapulco, Guerrero. The vertical dotted and dashed lines represent the start of long-lasting insecticidal net screens (LLIS) and targeted treatment (TT) interventions, respectively. The symbol denotes dates when the index was significantly different between treated and control groups on that date. Error bars show the standard error of the mean.

at 5 (IRR=0.32, 95% CI 0.23–0.45) but not at 12 (IRR=0.49, 95% CI 0.23–1.05) months (Figure 3).

At 5 months post-intervention with LLIS only, no significant differences between treated and untreated houses were observed in the immature-based indicators (Figure 4). However, a significant impact was seen at 12 months post-intervention with LLIS for all pupae-based indicators (as a proxy for adult vectors): i.e., houses positive to *Aedes* pupae (OR=0.56, 95% CI 0.33–0.96), number of *Aedes* pupae per house (IRR=0.29, 95% CI 0.12–0.70) and pupae per person (IRR=0.31, 95% CI 0.11–0.86). When analysing larval-based indicators these were lower in intervention clusters after the intervention compared to control clusters, but the differences were not statistically significant (Figure 4).

At baseline, all the *Stegomyia* indices and PPI were similar between both intervention and control groups (Figure 5). Five months after the installation of LLIS, indices showed a slight decrease in the intervention clusters (Figure 5). At 12 months, only water-holding containers and containers positive for pupae were significantly different between intervention and control clusters (Figure 5).

Impact of the combination of LLIS and TT

The impact of both approaches was assessed at 18 and 24 months, following introduction of TT at 14 months post-intervention. At 18 months post-intervention, significantly fewer treated houses were infested with *Ae. aegypti* adult females (OR=0.07, 95% CI 0.05–0.10), but not with blood-fed females (OR=0.63, 95% CI 0.36–1.09) or males (OR=1.19, 95% CI 0.84–1.7) (Figure 3). At 24 months post-intervention, significantly fewer adult females (OR=0.44, 95% CI 0.20–0.95), blood-fed females (OR=0.28, 95% CI 0.10–0.74) and males (OR=0.44, 95% CI 0.27–0.71) were found in treated houses.

Analyses of infestation density based on adult catches showed a similar trend with a significant reduction in adult females (IRR=0.12, 95% CI 0.08–0.19) at 18 months post-intervention; but not for blood-fed females (IRR=0.54, 95% CI 0.29–1.0 or males (IRR=0.93, 95% CI 0.72–1.22) (Figure 3). At 24 months post-intervention, significantly lower numbers of indoor adult females (IRR=0.04, 95% CI 0.21–0.98); blood-fed females

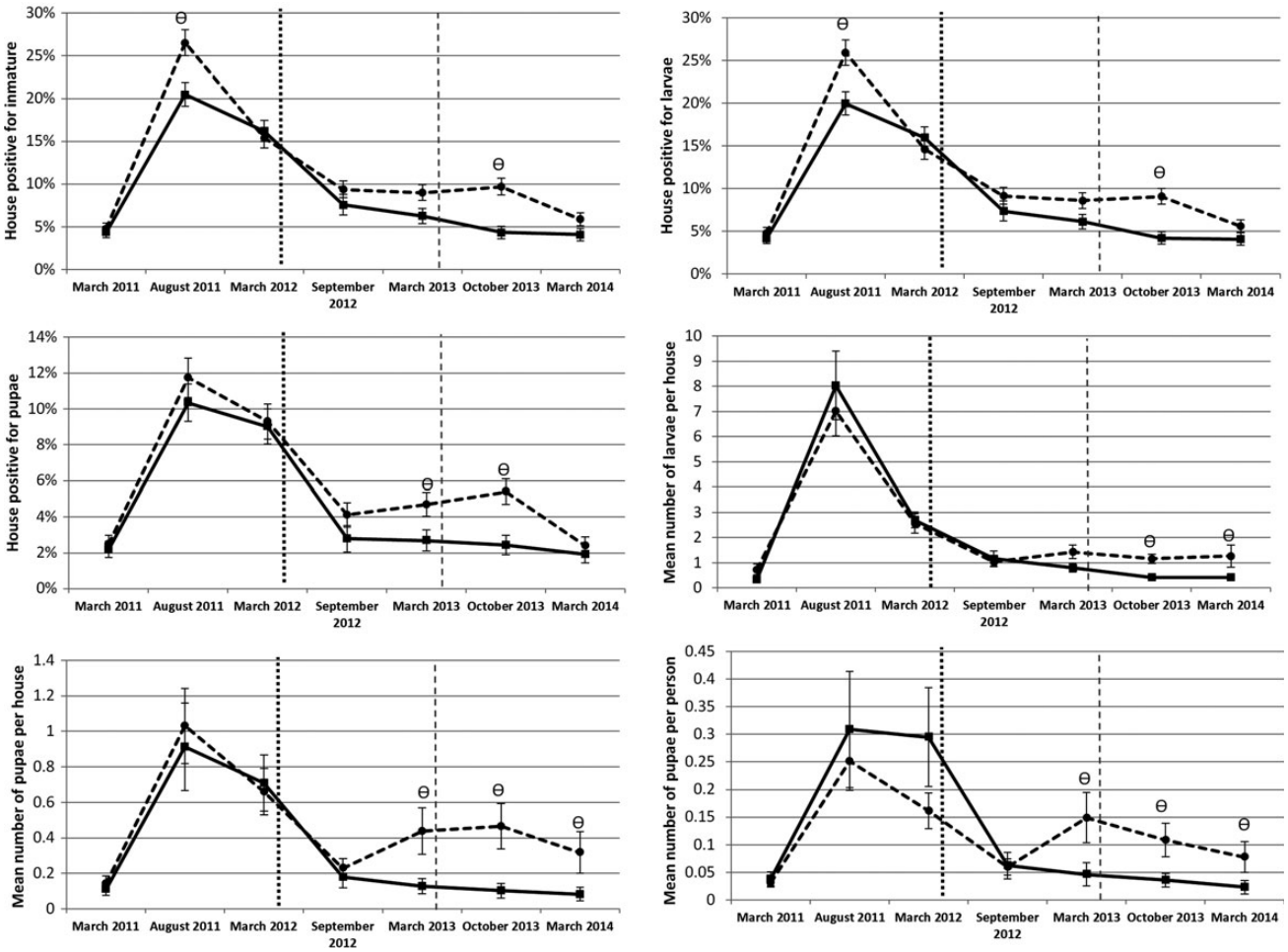


Figure 4. Comparison between treated (solid line) and untreated (broken line) groups of *Aedes aegypti* immature-based indicators for in Acapulco, Guerrero. The vertical dotted and dashed lines represent the start of long-lasting insecticidal net screens (LLIS) and targeted treatment (TT) interventions, respectively. The symbol denotes dates when the index was significantly different between treated and control groups on that date. Error bars show the standard error of the mean.

(IRR=0.25, 95% CI 0.09–0.70) and males (IRR=0.48, 95% CI 0.27–0.86) were found in treated houses.

Houses in treated clusters also had significantly lower immature infestation levels and densities in comparison with untreated houses at 18 months post-intervention (Figure 4): numbers of houses positive for any developing stage (OR=0.44, 95% CI 0.26–0.75), number of houses with larvae (OR=0.44, 95% CI 0.26–0.75), number of larvae per house (IRR=0.36, 95% CI 0.20–0.66), houses with pupae (OR=0.44, 95% CI 0.23–0.82), number of pupae per house (IRR=0.22, 95% CI 0.08–0.57) and numbers of pupae per person (IRR=0.33, 95% CI 0.13–0.82). At 24 months, post-intervention significant reductions were found in immature density (i.e., number of larvae per house (IRR=0.33, 95% CI 0.13–0.83), number of pupae per house (IRR=0.26, 95% CI 0.10–0.68), and number of pupae per person (IRR=0.30, 95% CI 0.10–0.88).

Right after the implementation of the TT intervention, all the *Stegomyia* indices and PPI showed a significant difference between intervention and control clusters (Figure 5). However, significance was transient over time, with water-holding

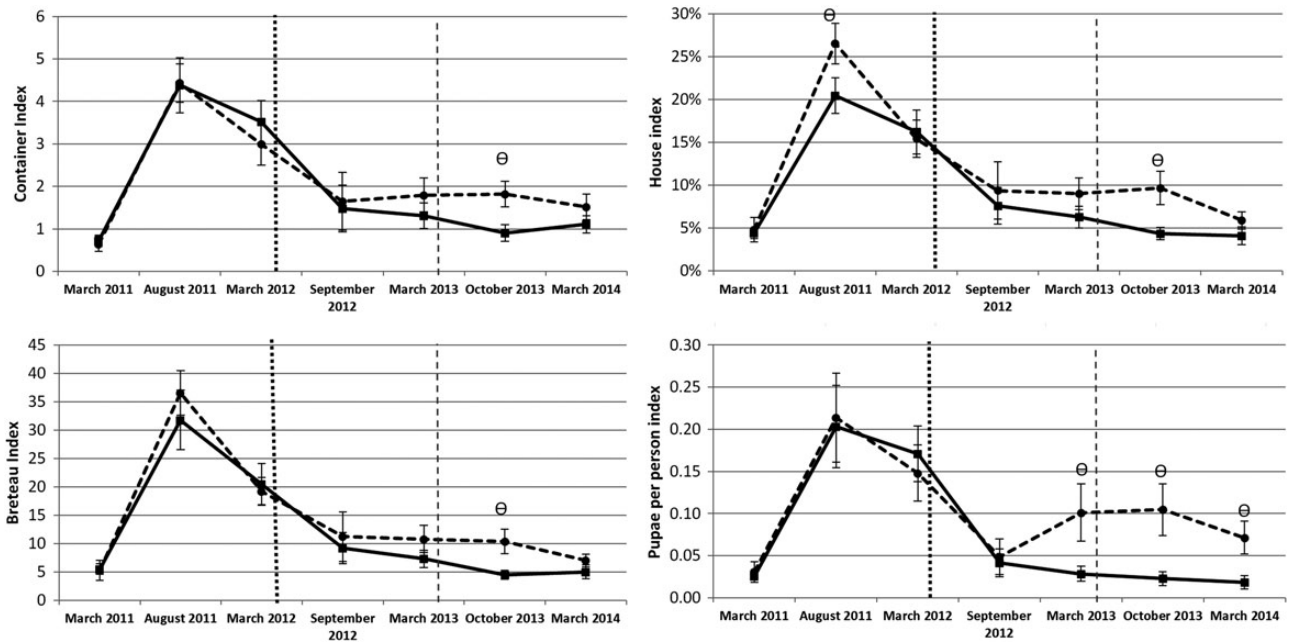


Figure 5. Comparison between treated (intervention) and untreated (control) groups on classic *Stegomyia* indices and pupal indicators at the cluster level in Acapulco, Guerrero The vertical dotted and dashed lines represent the start of long-lasting insecticidal net screens (LLIS) and targeted treatment (TT) interventions, respectively.

Table 2. Parameter value and significance of non-linear parameter ($f(t_i)$) on GAMM models estimating the association between entomologic indices and the time since LLIS installation. ΔAIC represents the difference between AIC values of a model excluding (AIC_{GAM}) and including (AIC_{GAMM}) a random effect associated with each cluster

Life stage	Indicator	Estimated df	*F*	p	ΔAIC (AIC_{GAM} - AIC_{GAMM})
Immature	No. immatures	5.32	26.1	<0.0001	105
	No. pupae	3.33	9.5	<0.0001	60
	Positive houses	5.35	23.0	<0.0001	956
	Pupae presence	4.55	15.8	<0.0001	1026
Adult	No. females	5.13	39.5	<0.0001	25
	No. bloodfed females	6.49	43.7	<0.0001	4
	No. adults	6.49	43.7	<0.0001	23
	Presence adults	5.62	40.1	<0.0001	21
	Presence females	5.29	39.2	<0.0001	2

AIC: Akaike Information Criterion; GAM: generalized additive model; GAMM: generalized additive mixed model; LLIS: long-lasting insecticidal net screens

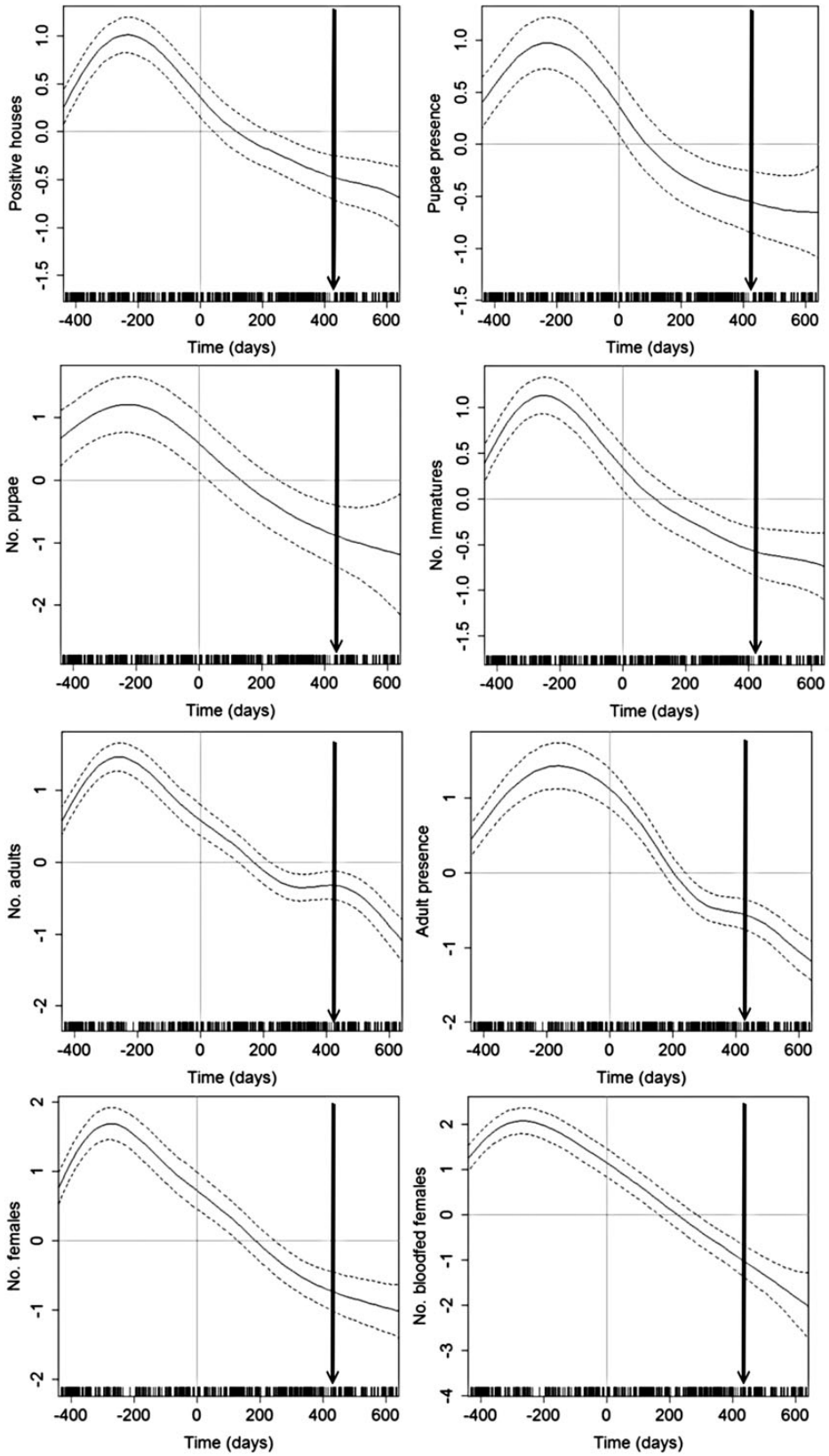


Figure 6. Predicted values for the best generalized additive mixed model (GAMM) showing the association between the time since long-lasting insecticidal net screens (LLIS) installation ($f(t_i)$) and each entomologic indicator. Horizontal line shows the area of no difference and vertical line the time when LLIS were installed.

container immatures per house and immatures per container being the only statistically significant value in the following survey (Figure 5).

Temporal persistence of interventions

Table 2 shows the parameter value and significance of non-linear parameter (f(ti)) on GAMM models estimating the association between entomologic indices and the time since LLIS installation. For all variables, a non-linear model explained better the data than a model with a linear term (ΔAIC>2). Figure 6 shows the plot of $f(t_i)$ for each entomologic indicator (immatures and adults). The y-axis can be interpreted as the effect of time since LLIS installation on each entomologic measure. When the predicted value and its 95% credible interval are negative, it means that there is a protective effect of LLIS for that factor. In all cases, LLIS achieved a protective effect for at least 600 days post installation. Adult indices (presence and abundance) showed a second reduction at 500 days post intervention, coincidentally with the introduction of the TT strategy (Figure 6).

Discussion

The study showed a significant and persistent impact on *Ae. aegypti* adult and immature vector populations for up to 2 years after deployment. LLIS fixed on doors and windows should provide a mechanical as well as a chemical barrier for mosquitoes. Insecticide-treated materials have been field-evaluated in numerous different settings worldwide with some degree of success against dengue vectors, when used as a physical barrier to oviposition[3,16,17] or to reduce human-contact and provide personal protection in the home as bednets[18] or as window/door curtains.[3,5,6,19,20]

'Mosquito-proofing' houses has been employed historically in places where mosquito nuisance and disease transmission are a problem.[21] However, few studies have evaluated simple house screening/netting for dengue vectors. Manrique-Saide et al.[11] reported in Merida, Mexico, that the presence of untreated window screening significantly decreased both the odds of having *Aedes* adult mosquitoes inside the house and of the number of females found indoors. The pyrethroid insecticide reduces the number of vectors entering the house and potentially reduces the survival of those attempting to exit.[22,23]

The combination of LLIS with TT in the most productive container types in Acapulco was successful in further reducing the number of *Aedes* pupae and consequently adult dengue vectors. Control of breeding sites, even if applied in a TT strategy, is heavily affected by the coverage, residuality and water availability by rainfall or human practices.[24,25] Nevertheless, the effect in Acapulco was achieved because TT was applied in the largest coverage possible and at least every two months.

The effect of controlling containers that are productive all the year round such as water tanks and metal drums, has alone a long-term effect in vector density, both as immatures and adults.[9] Indeed in Acapulco, after treating the most productive containers, we observed a cumulative effect of the combined intervention particularly pronounced during the rainy season.

The house is an important place for human–vector contact. The prevention of human–vector contact is necessary to interrupt the dengue transmission cycle.[26] Protection against mosquito bites and disease transmission with mosquito netting in houses has been historically observed as a fundamental technique of malaria control in the early 1900s.[21] Protecting houses with screens has been shown to be effective in reducing malaria transmission[27–29] and also to be a well-appreciated and sustainable vector control measure.[30] Our study has shown that this is also true for dengue.

Control of dengue vector density at the household level and cluster level in Acapulco was notable, but it has still to be shown that this measure reduces dengue transmission and incidence. Mexican authorities have shown their interest in this approach to dengue control and offered their support by implementing a large scale study to show the impact of the measure on dengue incidence. This is now in preparation.

Conclusions

The combination of long-lasting insecticidal screens fitted to external windows and doors and targeted treatment of the most productive *Ae. aegypti* breeding sites can impact significantly on dengue vector populations and sustain that impact for up to 24 months.

Authors' disclaimer: The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the Institutions involved.

Authors' contributions: ACM, PMS, MBP conceived the study; ACM, PMS designed the study protocol; GGM, JHB, FDM, CGC, JAJ, GST directed and participated in the interventions; GVP carried out the analysis of the data. ACM, PMS, PJM drafted the manuscript; AK, JS, AL, HR, PJM critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. ACM and PMS are guarantors of the paper.

Acknowledgements: Our special thanks goes to the field team and to the population in Acapulco, who were exceptionally helpful and supportive throughout the study. Regrettably, Professor Mario Barrera-Pérez passed away prior to the completion of this manuscript, and we wish to dedicate this study as a small memorial to his significant contribution to tropical medicine research in Mexico.

Funding: This investigation received financial support from the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) with the aid of a grant from the International Development Research Centre (IDRC), Ottawa, Canada. We also thank FOMIX CONACYT-Guerrero for the financial support of the Project 'Control Integrado de Aedes aegypti con estrategias y herramientas innovadoras en una zona de alto riesgo para la transmisión del dengue en Guerrero, México' (Project GUE-2008-02-108686).

Competing interests: None declared.

Ethical approval: This study received clearance from the Ethical Committee of the Mexican Ministry of Health of Guerrero and WHO's Research Ethics Review Committee (ERC).

References

1 Tun-Lin W, Lenhart A, Nam VS et al. Reducing costs and operational constraints of dengue vector control by targeting productive breeding places: a multicountry non-inferiority cluster randomized trial. Trop Med Int Health 2009;14:1143–53.

2 Morrison AC, Zielinski-Gutierrez E, Scott TW et al. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. PLoS Med 2008;5(3):e68.

3 Kroeger A, Lenhart A, Ochoa M et al. Effective control of dengue vectors with curtains and water container covers treated with insecticide in Mexico and Venezuela: cluster randomised trials. BMJ 2006; 332:1247–52.

4 McCall PJ, Kittayapong P. Control of Dengue Vectors: Tools and Strategies. In: Report of the Scientific Working Group Meeting on Dengue, Geneva, 15 October 2006. Geneva: WHO/TDR 2007; p. 110–9.

5 Vanlerberghe V, Villegas E, Oviedo M et al. Evaluation of the effectiveness of insecticide treated materials for household level dengue vector control. PLoS Negl Trop Dis 2011; 5(3):e994.

6 Rizzo N, Gramajo R, Cabrera-Escobar M et al. Dengue vector management using insecticide treated materials and targeted interventions on productive breeding-sites in Guatemala. BMC Public Health 2012;2:931.

7 WHO. Guidelines for Laboratory and Field Testing of Long-Lasting Insecticidal Nets. WHO/HTM/NTD/WHOPES/2013.1. Geneva: World Health Organization; 2013.

8 Focks D, Alexander N. Multicountry study of *Aedes aegypti* pupal productivity survey methodology: findings and recommendations. (TDR/IDE/Den/06.1). Geneva: World Health Organization; 2006.

9 Maciel-de-Freitas R, Lourenço-de-Oliveira R. Does targeting key-containers effectively reduce *Aedes aegypti* population density? Trop Med Int Health 2011;16:965–73.

10 Sommerfeld J, Kroeger A. Eco-bio-social research on dengue in Asia: a multicountry study on ecosystem and community-based approaches for the control of dengue vectors in urban and peri-urban Asia. Pathog Glob Health 2012;106:428–35.

11 Manrique-Saide P, Che-Mendoza A, Barrera-Pérez M et al. Long-lasting insecticide treated screens significantly reduce indoor *Aedes aegypti* presence and abundance in Acapulco, Mexico. Emerg Infect Diseases [Forthcoming 2015].

12 Quintero J, Brochero H, Manrique-Saide P et al. Ecological, biological and social dimensions of dengue vector breeding in five urban settings of Latin America: a multi-country study. BMC Infect Dis 2014;14:38.

13 SINAVE. Sistema Nacional de Vigilancia Epidemiológica. http://www.sinave.gob.mx/ [accessed 12 December 2013].

14 Hernández-Ávila JE, Rodríguez MH, Santos-Luna R et al. Nation-wide, web-based, geographic information system for the integrated surveillance and control of dengue fever in Mexico. PLoS One 2013;6;8(8):e70231.

15 Manrique-Saide P, Che-Mendoza A, Rizzo N et al. Operational guide for assessing the productivity of *Aedes aegypti* breeding sites. Geneva: TDR/World Health Organization; 2011.

16 Seng CM, Setha T, Nealon J et al. The effect of long-lasting insecticidal water container covers on field populations of *Aedes aegypti* (L.) mosquitoes in Cambodia. J Vector Ecol 2008;33:333–41.

17 Tsunoda T, Kawada H, Huynh TT et al. Field trial on a novel control method for the dengue vector, *Aedes aegypti* by the systematic use of Olyset® Net and pyriproxyfen in Southern Vietnam. Parasit Vectors 2013;6:6.

18 Lenhart A, Orelus N, Maskill R et al. Insecticide-treated bednets to control dengue vectors: preliminary evidence from a controlled trial in Haiti. Trop Med Int Health 2008;13:56–67.

19 Lenhart A, Trongtokit Y, Alexander N et al. A cluster-randomized trial of insecticide-treated curtains for dengue vector control in Thailand. Am J Trop Med Hyg 2013;88:254–9.

20 Vanlerberghe V, Trongtokit Y, Jirarojwatana S et al. Coverage-dependent effect of insecticide-treated curtains for dengue control in Thailand. Am J Trop Med Hyg 2013;89:93–8.

21 Lindsay SW, Emerson PM, Charlwood JD. Reducing malaria by mosquito-proofing houses. Trends Parasitol 2002;18:510–4.

22 Kirby MJ, Ameh D, Bottomley C et al. Effect of two different house screening interventions on exposure to malaria vectors and on anaemia in children in The Gambia: a randomised controlled trial. Lancet 2009;374:998–1009.

23 Ogoma SB, Lwetoijera DW, Ngonyani H et al. Screening mosquito house entry points as a potential method for integrated control of endophagic filariasis, arbovirus and malaria vectors. PLoS Negl Trop Dis 2010;4(8):e773.

24 Horstick O, Runge-Ranzinger S, Nathan MB et al. Dengue vector-control services: how do they work? A systematic literature review and country case studies. Trans R Soc Trop Med Hyg 2010;104:379–86.

25 Garelli FM, Espinosa MO, Weinberg D et al. Water use practices limit the effectiveness of a temephos-based *Aedes aegypti* larval control program in northern Argentina. PLoS Negl Trop Dis 2011;5(3):e991.

26 Beaty B, Bernhardt S, Black W et al. Novel Strategies to Control *Aedes aegypti* and Dengue. In: Atkinson PW (editor). Vector Biology, Ecology and Control. Springer: The Netherlands; 2009; p. 99–111.

27 Lindsay SW, Jawara M, Paine K et al. Changes in house design reduce exposure to malaria mosquitoes. Trop Med Int Health 2003;8:512–7.

28 Kirby M, Green C, Milligan P et al. Risk factors for house-entry by malaria vectors in a rural town and satellite villages in The Gambia. Malar J 2008;7:2.

29 Walker N. The hygienic house: mosquito-proofing with screens Am J Trop Med Hyg 2010;83:963–4.

30 Kirby MJ, Bah P, Jones CO et al. Social acceptability and durability of two different house screening interventions against exposure to malaria vectors, *Plasmodium falciparum* infection, and anemia in children in the Gambia, West Africa. Am J Trop Med Hyg 2010; 83:965–72.

Use of Insecticide-Treated House Screens to Reduce Infestations of Dengue Virus Vectors, Mexico

Pablo Manrique-Saide, Azael Che-Mendoza, Mario Barrera-Perez, Guillermo Guillermo-May, Josue Herrera-Bojorquez, Felipe Dzul-Manzanilla, Cipriano Gutierrez-Castro, Audrey Lenhart, Gonzalo Vazquez-Prokopec, Johannes Sommerfeld, Philip J. McCall, Axel Kroeger, Juan I. Arredondo-Jimenez[1]

Dengue prevention efforts rely on control of virus vectors. We investigated use of insecticide-treated screens permanently affixed to windows and doors in Mexico and found that the screens significantly reduced infestations of *Aedes aegypti* mosquitoes in treated houses. Our findings demonstrate the value of this method for dengue virus vector control.

Vector control is the primary method for prevention and control of the increasingly frequent dengue outbreaks that threaten more than half the global human population (*1*). Existing approaches target breeding sites or attack adult mosquitoes by insecticide space-spraying, but these methods, at best, offer only immediate solutions and are rarely effective or sustainable for the long term (*2*). Methods that target the largely endophilic adult female *Aedes aegypti* mosquito vectors within buildings where they rest and bloodfeed have greater potential for sustained results and acceptance at the community level. One such method, long-lasting insecticidal-net (LLIN) curtains hung at windows or doors, can greatly reduce vector populations at high coverage rates (*3*–*5*), but efforts are compromised when curtains remain open during daytime or when all house entry points cannot be protected (*6*,*7*). Fixed or permanent screens covering doors and windows could eliminate this problem. Mosquito-proofing of houses is effective in malaria control (*8*), and reduced risk for dengue has been associated with the use of untreated (*9*) and insecticide-treated (*3*,*10*) screens.

Author affiliations: Universidad Autónoma de Yucatan, Merida, Mexico (P. Manrique-Saide, M. Barrera-Perez, G. Guillermo-May, J. Herrera-Bojorquez); Servicios de Salud de Yucatán, Gobierno del Estado de Yucatan, Merida (A. Che-Mendoza); Servicios Estatales de Salud de Guerrero, Chilpancingo, Mexico (F. Dzul-Manzanilla, C. Gutierrez-Castro); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A. Lenhart); Emory University, Atlanta (G. Vazquez-Prokopec); World Health Organization, Geneva, Switzerland (J. Sommerfeld, A. Kroeger; Liverpool School of Tropical Medicine, Liverpool, UK (P.J. McCall); Centro Nacional de Programas Preventivos y Control de Enfermedades, Mexico City, Mexico (J.I. Arredondo-Jimenez)

DOI: http://dx.doi.org/10.3201/eid2102.140533

The Study

During 2011–2013, in the city of Acapulco in Guerrero state, Mexico (Figure 1), an area of consistently high dengue transmission (http://www.epidemiologia.salud.gob.mx/dgae/panodengue/intd_dengue.html), we investigated the effect on vector infestations of permanently mounted, insecticide-treated screens fitted to door and windows of residential houses. The screens (Duranet, Clarke Mosquito Control, Roselle, IL, USA) were made of 0.55% wt/wt α-cypermethrin–treated nonflammable polyethylene netting (145 denier; mesh = 132 holes/in^2); the design is approved by the World Health Organization (WHO) Pesticide Evaluation Scheme (http://www.who.int/whopes/en/).

We used a cluster-randomized sampling design constructed on the basis of earlier studies (*4*–*6*,*11*) to select 20 clusters (10 treatment, 10 control; 100 households/cluster) from a possible 30 clusters by using digital maps (Google Earth software; Google Inc., Mountain View, CA, USA) (Figure 1). Sample size was determined by using a 2-level hierarchical model to achieve 80% power at a 5% level of significance. Thus, for a negative binomial distribution with a dispersion coefficient of 0.02 and intracluster coefficient of 0.05, a minimum of 8.9 clusters/arm were required. Written informed consent was obtained from participating households; the WHO Ethical Review Committee (WHO reference no. 2010/82951-0, unit reference no. A90297) and Guerrero State Ministry of Health granted ethical permission for the study.

Participating households in the treatment arm were instructed on LLIS maintenance during installation (April–December 2012). Control houses received no treatment. Five entomologic surveys of randomly selected houses were conducted: before intervention (March 2011, September 2011, March 2012) and at 5 and 12 months after intervention (September 2012, March 2013; wet and dry seasons, respectively). Before intervention, 32 houses per cluster were sampled at each survey; after intervention, 210 houses from treated clusters and 302 from control clusters

[1]Current affiliation: Universidad Autonoma de Nuevo Leon, Monterrey, Mexico.

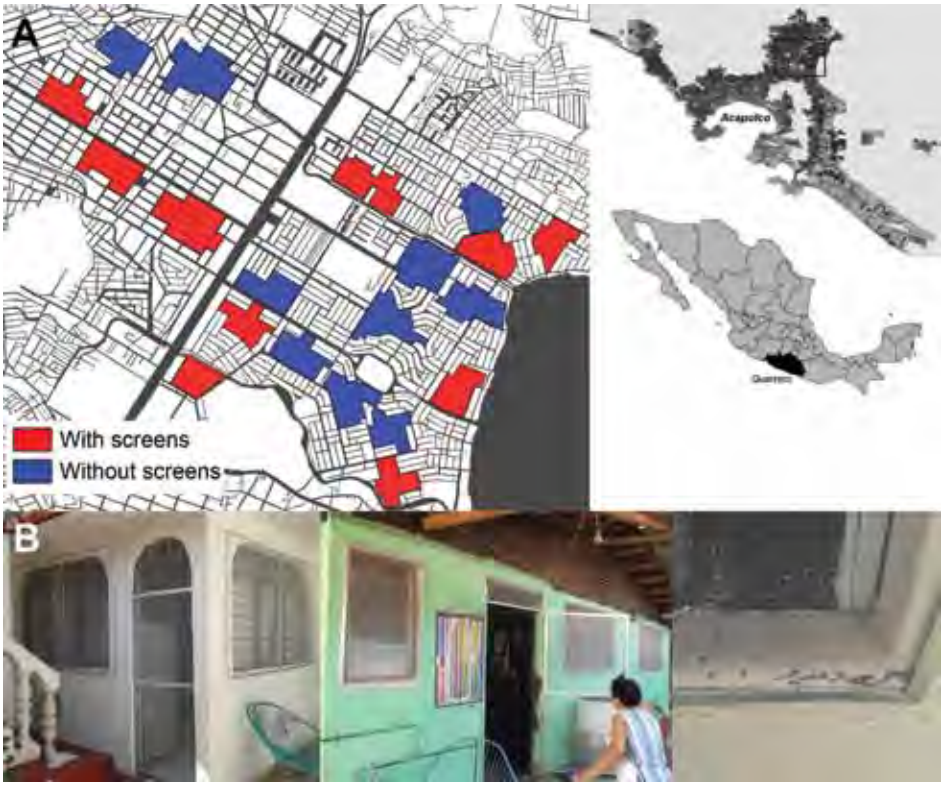


Figure 1. Area of study of long-lasting insecticide-treated screens in Acapulco, Mexico, March 2011–March 2013. A) Locations of clusters in the neighborhoods of Ciudad Renacimiento and Zapata, showing areas with (red) and without (blue) screens. Insets show location of study area (black box) in Acapulco and Guerrero state (black shading) in Mexico. B) Photographs of screens mounted on aluminum frames and fixed to windows and external doors of treated houses in 2012. The insects visible in the right photograph are dead house flies.

were sampled in September 2012 and 311 houses from treated and 320 from control clusters in March 2013.

Indoor resting adult mosquitoes were collected by using modified CDC backpack aspirators (John W. Hock Co., Gainesville, FL, USA) from all houses in a cluster on the same day during 9 AM–3 PM. Indices for *Ae. aegypti* mosquitoes (the only *Aedes* species found) were calculated to quantify house infestation (percent of all houses positive) and infestation density (numbers per infested house) for all mosquitoes, all females, all blood-fed females, and males.

For presence–absence data, we performed logistic regression models with a single predictor variable identifying houses with LLIS and control houses (coded as 1 and 0, respectively) and accounting for each house membership in a given sampling cluster (cluster-robust SE calculation). Odds ratios (ORs) and 95% CIs indicating the effect of LLIS on each entomologic indicator were calculated. Overdispersed index data were compared between arms by using the Mann-Whitney U test. The effect of treatment on each metric was analyzed by negative-binomial regression using, as with the logistic models, treatment as the sole predictor variable (1 and 0 coding). Negative binomial models also accounted for membership of a house in a sampling cluster (cluster-robust SE calculation). ORs and incidence rate ratios (IRRs) were calculated with 95% CIs; significance was set at $p<0.05$. Analyses were performed by using Stata 12.0 (StataCorp, College Station, TX, USA).

Before intervention, indices were similar for both study arms on all sampling dates. House infestation rates (Figure 2, panels A–D) and mosquito densities (Figure 2, panels E–H) followed seasonal patterns (2-sample Wilcoxon rank-sum test for all treatment–control comparisons, $|z|<1.0$; $p>0.1$). At 5 months postintervention, significantly fewer treated than control houses were infested with *Ae. aegypti* adult female mosquitoes (OR 0.38, 95% CI 0.21–0.69), blood-fed females (OR 0.36, 95% CI 0.21–0.60), and males (OR 0.39, 95% CI 0.19–0.77). A significant effect was still seen at 12 months for adult females (OR 0.41, 95% CI 0.25–0.68) and males (OR 0.41, 95% CI 0.27–0.64) but not for blood-fed females (OR 0.51, 95% CI 0.24–1.05). Analyses of infestation density showed similar trends, with significantly fewer *Ae. aegypti* mosquitoes found in treated than in control houses: adult females at 5 (IRR 0.37, 95% CI 0.27–0.49) and 12 (IRR 0.40, 95% CI 0.23–0.70) months postintervention, males at 5 (IRR 0.39, 95% CI 0.28–0.54) and 12 (IRR = 0.49, 95%CI 0.33–0.72) months postintervention, and blood-fed females at 5 (IRR 0.32, 95% CI 0.23–0.45) but not 12 (IRR 0.49, 95% CI 0.23–1.05) months postintervention.

A comparison of wet season data from treatment houses before (August 2011) and after (September 2012) intervention showed that significantly fewer females and blood-fed females were found postintervention (Wilcoxon matched pairs W = 30706, $z = 3.717$, and W = 20706,

z = 3.146; p<0.05 for both comparisons). However, the number of male mosquitoes did not change significantly (W = 20706, z = 1.385; p>0.05).

At 5 months postintervention, fewer LLIS-treated houses (33%) than control houses (56%) remained infested with female *Ae. aegypti* mosquitoes. Lower numbers of female mosquitoes were also found per infested house (0.54 ± 0.9) than per control house (1.39 ± 2.0); this effect was still detectable at 12 months postintervention (18%, 0.3 ± 0.8, vs. 35%, 0.7 ± 1.4).

Conclusions

In our study, the entomologic effect of LLIS was greater than that detected in a recent study of deltamethrin-treated window curtains (*12*), in which a 27% reduction of adult *Ae. aegypti* mosquitoes was only sustained for a short time after curtain installation. Other studies of insecticide-treated curtains in Latin America have reported entomologic effects by using immature stage indicators alone (*4*,*5*,*7*). Whether these reductions were sufficient to affect dengue transmission is unknown, and the overall effect on dengue infections remains to be evaluated.

Our results are encouraging in view of high levels of insecticide resistance in *Ae. aegypti* mosquitoes in Acapulco. Although resistance to α-cypermethrin has yet to be reported in Guerrero, high frequencies of mutations in the voltage-gated sodium channel gene, which is associated with pyrethroid resistance in *Ae. aegypti* mosquitoes, have been reported (*13*). If insecticide resistance began to reduce the efficacy of the method we describe, the screens could be treated with different insecticide classes.

We found the use of LLIS was a popular intervention, and perceived efficacy was reinforced by a reduction in other domestic pests (Figure 1) (*14*). The likely effects on

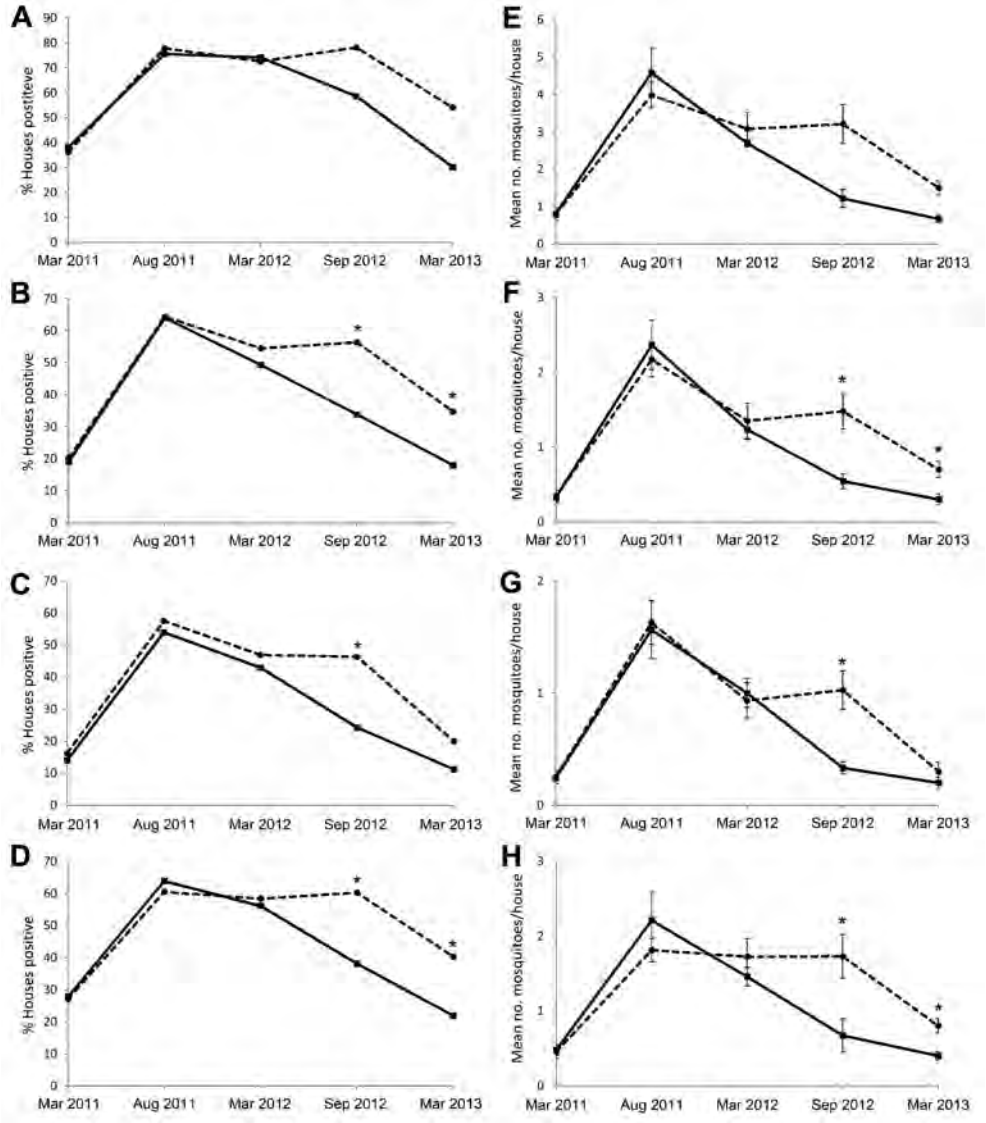


Figure 2. Infestation indices for adult *Aedes aegypti* mosquitoes in intervention (solid lines) and control (dashed lines) households before and after intervention in Acapulco, Mexico, as measured during dry (March) and wet (August–September) season cross-sectional surveys, 2011–2013. A–D) Vector prevalence: percentage of houses positive for A) all adults; B) all females; C) blood-fed females; D) males. E–H) Vector density: mean number per infested house for E) all adults; F) all females; G) blood-fed females; H) males. Error bars indicate SEs. Fitting of insecticide-treated window and door screens commenced during April 2012. Asterisks (*) denote dates when the index was significantly different between treated and control groups.

other peridomestic disease vectors could promote increased adoption of the intervention with additional cost benefits. The polyethylene netting was durable on windows; it was often damaged on the lower sections of doors (*14*) but readily repaired by reinforcement with metal mesh.

Dengue vector control programs using house screens are ongoing in selected cities in Mexico and Brazil. These results were obtained during an exploratory phase of that initiative. Stakeholders in other countries may also consider evaluating this novel approach for dengue vector control.

Acknowledgments

We acknowledge and thank the field team for their work and the study site populations in Ciudad Renacimiento and Zapata, Acapulco, for their cooperation throughout the study.

Financial support was provided by the United Nations Development Programme/World Bank/WHO Special Program for Research and Training in Tropical Diseases/International Development Research Center (project no. A70585) and FOMIX CONACYT-Guerrero (project GUE-2008-02-108686).

Dr. Manrique-Saide is a professor and Responsible of the Collaborative Unit for Entomological Bioassays of the Universidad Autonoma de Yucatan. His main research interest within public health entomology is the improvement of surveillance and control of vector-borne diseases with particular emphasis on the vectors of dengue and chinkungunya viruses, *Ae. aegypti* mosquitoes.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7. http://dx.doi.org/10.1038/nature12060
2. World Health Organization. Dengue: guidelines for diagnosis, treatment, prevention and control. Geneva: The Organization; 2009.
3. Igarashi A. Impact of dengue virus infection and its control. FEMS Immunol Med Microbiol. 1997;18:291–300. http://dx.doi.org/10.1111/j.1574-695X.1997.tb01058.x
4. Kroeger A, Lenhart A, Ochoa M, Villegas E, Levy M, Alexander N, et al. Effective control of dengue vectors with curtains and water container covers treated with insecticide in Mexico and Venezuela: cluster randomised trials. BMJ. 2006;332:1247–52. http://dx.doi.org/10.1136/bmj.332.7552.1247
5. Vanlerberghe V, Trongtokit Y, Jirarojwatana S, Jirarojwatana R, Lenhart A, Apiwathnasorn C, et al. Coverage-dependent effect of insecticide-treated curtains for dengue control in Thailand. Am J Trop Med Hyg. 2013;89:93–8. http://dx.doi.org/10.4269/ajtmh.13-0015
6. Lenhart A, Trongtokit Y, Alexander N, Apiwathnasorn C, Satimai W, Vanlerberghe V, et al. A cluster-randomized trial of insecticide-treated curtains for dengue vector control in Thailand. Am J Trop Med Hyg. 2013;88:254–9. http://dx.doi.org/10.4269/ajtmh.2012.12-0423
7. Rizzo N, Gramajo R, Cabrera-Escobar M, Arana B, Kroeger A, Manrique-Saide P, et al. Dengue vector management using insecticide treated materials and targeted interventions on productive breeding-sites in Guatemala. BMC Public Health. 2012;12:931. http://dx.doi.org/10.1186/1471-2458-12-931
8. Gimnig JE, Slutsker L. House screening for malaria control. Lancet. 2009;374:954–5. http://dx.doi.org/10.1016/S0140-6736(09)61078-3
9. Ko YC, Chen M, Yen S. The predisposing and protective factors against dengue virus transmission by mosquito vector. Am J Epidemiol. 1992;136:214–20.
10. Nguyen HT, Tien TV, Tien NC, Ninh TU, Hoa NT. The effect of Olyset net screen to control the vector of dengue fever in Vietnam. Dengue Bull. 1996;20:87–92.
11. Quintero J, Brochero H, Manrique-Saide P, Barrera-Pérez M, Basso C, Romero S, et al. Ecological, biological and social dimensions of dengue vector breeding in five urban settings of Latin America: a multi-country study. BMC Infect Dis. 2014;14:38. http://dx.doi.org/10.1186/1471-2334-14-38
12. Loroño-Pino MA, García-Rejón JE, Machain-Williams C, Gomez-Carro S, Nuñez-Ayala G, Nájera-Vázquez Mdel R, et al. Towards a casa segura: a consumer product study of the effect of insecticide-treated curtains on *Aedes aegypti* and dengue virus infections in the home. Am J Trop Med Hyg. 2013;89:385–97. http://dx.doi.org/10.4269/ajtmh.12-0772
13. Aponte-Hincapie A, Penilla-Navarro P, Dzul-Manzanilla F, Che-Mendoza A, Lopez AD, Solis F, et al. The pyrethroid resistance status and mechanisms in *Aedes aegypti* from Guerrero state, Mexico. Pestic Biochem Physiol. 2013;107:226–34. http://dx.doi.org/10.1016/j.pestbp.2013.07.005
14. Huw-Jones CJ, Benítez-Valladares D, Guillermo-May G, Dzul-Manzanilla F, Che-Mendoza A, Barrera-Pérez M, et al. Use and acceptance of long lasting insecticidal net screens for dengue prevention in Acapulco, Guerrero, Mexico. BMC Public Health. 2014;14:846.

Address for correspondence: Juan I. Arredondo-Jimenez, Universidad Autonoma de Nuevo Leon, Pedro de Alba S/n, Ciudad Universitaria, 66450 San Nicolas de Los Garza, Nuevo Leon, Mexico; email: jiarre1@gmail.co

List of commercial household insecticide products availables in local markets in Mexico

Commercial name (formulation)	Target insects (according to the label)	Ingredients and chemical group (according to the label)
H24 domestico (liquid)	**Mosquitoes**, flies, moths, bugs, cockroaches and ants	Permethrin, propoxur prallethrin
Baygon liquido verde (liquid)	**Mosquitoes**, moths, bugs, cockroaches, spiders, ants and cochineals	Imiprothrin
H24 verdugo (aerosol)	Fleas, ants, cockroaches, bugs, biting midges	Propoxur, deltamethrin, prallethrin
H24 Lavanda (aerosol)	**Mosquitoes**, flies, biting midges	Tetramethrin, cyphenothrin
H24 mata cucarachas (aerosol)	Cockroaches, ants, scorpions, spiders, bugs	Propoxur, deltamethrin, prallethrin
H24 casa y jardín (aerosol)	**Mosquitoes**, aphids, beetles, spiders, ants	Tetramethrin, cyphenothrin
H24 mata moscas y mosquitos (aerosol)	**Mosquitoes**, flies, biting midges	Tetramethrin, cyphenothrin
H24 Poder fulminante (aerosol)	Fleas, ants, spiders, bugs, cockroaches, scorpions	Propoxur, tetramethrin, fenvalerate
H24 domestico (aerosol)	**Mosquitoes**, aphids, spiders, beetles, ants	Tetramethrin, cyphenothrin
H24 citronox (aerosol)	**Mosquitoes**, aphids, spiders, beetles, ants	Sumithrin, tetramethrin
H24 poder total (aerosol)	Bugs, termites, ticks, moths, cockroaches, scorpions, ants, spiders	Sumithrin, imiprothrin
Baygon ultra (aerosol)	Scorpions, cockroaches, ants	**Cypermethrin** (RS)-α-cyano-3-phenoxybenzyl (1RS,3RS; 1RS,3RS-3- (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate. (0,1%/1,0g/kg). Imiprothrin 2,5-dioxo-3-(prop-2-ynyl)imidazolidin-1-ylmethyl (1R)-cis, trans-chrysanthemate (0,05% 0,5g/kg)
Raid automatic (aerosol)	**Mosquitoes**, flies, cockroaches, ants	Tetramethrin ciclohex-1eno-1,2 dicarboximidmetil (1RS,3RS,1SR)-2,2-dimetil-3-(2-metilprop-1enil) ciclopropanocarboxilato (0,35/3,5g/kg). Allethrin (RS)-3alil-2metil-4oxciclopent-2 enil(1RS)-cis, chrysanthemate (0,44%/4,4kg).

		Pyrethrins: (z)-(s)-2-metil-4oxo-3-(penta-2-4-diezil) ciclopent-2,2-dimetil-3-(2-metilprop-1-enil) ciclopropanocarboxilato (0,11%/1,1g/kg).
Raid max (aerosol)	Scorpions, cockroaches, ants	**Cypermethrin** (RS)-α-cyano-3-phenoxybenzyl (1RS,3RS; 1RS,3RS)-3- (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate. (0,1%/1,0g/kg). Imiprothrin Mix of 20% 2,5-dioxo-3-(prop-2-ynyl)imidazolidin-1-ylmethyl (1R)-cis, trans-chrysanthemate (0,05% 0,5g/kg).
Raid acción total (aerosol)	Scorpions, cockroaches, ants	Cyflutrin (SR)-alpha-Cyano-4-fluoro-3-phenoxybenzyl(1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate Imiprothrin 2,5-dioxo-3-(prop-2-ynyl)imidazolidin-1-ylmethyl (1R)-cis, trans-chrysanthemate.
Raid mata bichos (aerosol)	**Mosquitoes**, flies, wasps, cockroaches, ants, spiders, fleas, bugs	Tetramethrin, allethrin, suminithrin
Raid mata cucarachas, mosquitos y cucarachas (aerosol)	**Mosquitoes**, cockroaches, flies, ants, spiders, fleas	**Cypermethrin** (RS)- α-cyano-4-fluroro- phenoxybenzyl (1RS,3RS; 1RS,3RS)-3- (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (0,15%/1,5g/kg). Imiprothrin Mix of 2,5-dioxo-3-(prop-2-ynyl)imidazolidin-1-ylmethyl (1R)-cis, trans-chrysanthemate (0,05% 0,5g/kg).
Raid casa y jardín base aceite (aerosol)	**Mosquitoes,** flies, ants, spiders, cockroaches	Tetramethrin, allethrin, suminithrin
Raid casa y jardín baseagua (aerosol)	**Mosquitoes,** flies, ants, spiders, cockroaches	Tetramethrin, allethrin, suminithrin
Baygon casa y jardín esencia natural eucalipto) (aerosol)	**Mosquitoes,** flies, ants, spiders, cockroaches	Tetramethrin, cyclopropanecarboxylate, allethrin, suminithrin
Baygon poder mortal (aerosol)	**Mosquitoes**, scorpions, cockroaches, spiders, fleas, flies	**Cypermethrin** (RS)-α-cyano-3-phenoxybenzyl (1RS,3RS; 1RS,3RS)-3- (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate. Imiprothrin 2,5-dioxo-3-(prop-2-ynyl)imidazolidin-1-ylmethyl (1R)-cis, trans-chrysanthemate (0,05% 0,5g/kg).
Raidolitos anti mosquitos -lavanda (coils)	**Mosquitoes**	Transfluthrin
Raidolitos anti mosquitos (coils)	**Mosquitoes**	Transferina

Raid plaquitas (tablets)	**Mosquitoes**	Allethrin, piperonyl
H24 Mats (tablets)	**Mosquitoes**	Allethrin