Causes, Origins and Possible Solutions to Insecticide Resistance in *Aedes aegypti* from the Cayman Islands

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

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May 2011

Declaration

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree

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In memory of Nigel Hill who gave me the confidence and encouragement to pursue a career in science

Abstract

Aedes aegypti is the principle vector of dengue and yellow fever. This mosquito species is widely distributed throughout the tropics and international travel of goods and people have helped spread the mosquito and the diseases it transmits. *Ae. aegypti* has been recorded in the Cayman Islands four times in the past 45 years and each time efforts by the Mosquito Research and Control Unit have seemingly served to eliminate it. However, the current population of *Ae. aegypti* in Grand Cayman, believed to have been introduced in 2002, has proved refractory to control with current insecticide based methods. This study was conducted to assess the extent and potential causes of insecticide resistance in *Ae. aegypti* in Grand Cayman, investigate the likely origin of the 2002 introduction, and explore alternative methods of control.

Ae. aegypti from Grand Cayman are highly resistant to DDT and pyrethroid insecticides but show only low level resistance to the organophosphate temephos. Glutathione-S-transferase, cytochrome P450 and esterase activities were elevated in adults of the Cayman Islands strains compared to a susceptible strain although use of the synergist piperonyl butoxide did not implicate increased insecticide detoxification as the major mechanism of resistance. Partial sequencing of the voltage gated sodium channel, the target site of DDT and pyrethroid insecticides, identified two single nucleotide polymorphisms, one within domain II S6 region (V1016I) and the other within domain III S6 (F1534C). A tetraplex PCR assay was designed to detect the latter mutation and its association with the insecticide resistance to permethrin and DDT was confirmed (Fisher's exact test P=0).

Microsatellite analysis was employed to investigate the similarities between Cayman Islands *Ae. aegypti* and other populations collected from around the Caribbean. One hundred and fifty mosquitoes from three sites in the Cayman Islands and a total of 180 mosquitoes from four sites in neighbouring countries were genotyped at 12 loci. The allelic diversity suggests that the Cayman Islands population is well established, most likely receiving new introductions regularly. *Fst* analysis indicates closest comparative similarity to Jamaican populations of *Ae. aegypti* (*Fst* = 0.059-0.083).

The high level of insecticide resistance prompted the search for alternative methods of *Ae. aegypti* control. The first field trial of a genetically modified sterile strain of mosquito (OX513A) was performed in collaboration with the company Oxitec Ltd. This strain carries a dominant lethal and mating with wild mosquitoes induces sterility. In the first field trial, this technology proved capable of significantly suppressing a wild *Ae. aegypti* population in a 16 Ha site in Grand Cayman (reduction in positive ovitraps in relation to control site, P<0.0001).

The work carried out for this thesis has impacted upon decisions made by the department regarding insecticide choice for *Ae. aegypti* control. Knowledge of the mechanisms of resistance will further serve to assist in resistance management, and further studies of genetic control methods are planned.

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List of Abbreviations

ACE	-	Acetylcholine
AChe	-	Acetylcholine esterase
Ae.	-	*Aedes*
An.	-	*Anopheles*
ANOVA	-	Analysis of Variance
Bs.	-	*Bacillus sphaericus*
Bti.	-	*Bacillus thuringiensis israelensis*
CAREC	-	Caribbean Epidemiology Centre
CDC	-	Centers for Disease Control and Prevention
CDNB	-	1-Chloro-2, 4-dinitrobenzene
Cx.	-	*Culex*
D.	-	*Drosophila*
DDT	-	Dichlorodiphenyltrichloroethane
DF	-	Dengue Fever
DHF	-	Dengue Haemorrhagic Fever
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxyribonucleotide triphosphate
EDTA	-	Ethylenediaminetetraacetic acid
Fl	-	Florida
GABA	-	Gamma-Aminobutyric acid
GST	-	Glutathione-S-Transferase
Ha	-	Hectare
HCl	-	Hydrochloric Acid
HOLA	-	Hot Oligonucleotide Ligation Assay
Hr	-	Hour
HWE	-	Hardy Weinberg Equilibrium
IGR	-	Insect Growth Regulator
IPM	-	Integrated Pest Management
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
IRAC	-	Insecticide Resistance Action Committee
Kdr	-	Knock Down Resistance
Km	-	Kilometre
KYR	-	Cayman Resistant Strain of *Ae. aegypti*

LB	-	Luria Bertani
LC	-	Lethal Concentration
LSTM	-	Liverpool School of Tropical Medicine
LT	-	Lethal Time
Min	-	Minute
MRCU	-	The Mosquito Research and Control Unit (Cayman Islands Government)
Ms	-	Mississippi
mtDNA	-	Mitochondrial DNA
NO	-	New Orleans
Pa	-	Pennsylvania
PAHO	-	Pan American Health Organisation
PBO	-	Piperonyl Butoxide
PCR	-	Polymerase Chain Reaction
PNPA	-	Para Nitrophenyl Acetate
Ps.	-	*Psorophora*
Rdl	-	*Resistance to dieldrin*
RIDL	-	Release of Insects with a Dominant Lethal
RNA	-	Ribonucleic Acid
RR	-	Resistance Ratio
SDS	-	Sodium Dodecyl Sulphate
SIT	-	Sterile Insect Technique
SNP	-	Single Nucleotide Polymorphism
SOC	-	Super Optimal broth with Catabolite repression
TE	-	Tris-EDTA
Tet	-	Tetracycline
tTA	-	Tetracycline Repressible Trans-Activator Protein
ULV	-	Ultra Light Volume
USA	-	United States of America
USDA	-	United States Department of Agriculture
W.	-	*Wolbachia*
WHO	-	The World Health Organisation
WT	-	Wild Type
X-Gal	-	5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

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1. Introduction

1.1. Dengue Fever

Dengue Fever (DF) and Dengue Haemorrhagic Fever (DHF) are currently the most rapidly spreading vector borne diseases. It is estimated that around 50 million people worldwide become infected annually with the dengue virus and approximately half of the world's population live at risk of the disease (WHO, 2006). The economic burden comes, not just as a result of mortality, but through morbidity and hospitalisation. In the Americas Dengue is estimated to have cost in the region of US$2.1 billion per year between 2000-2007 (Shepard *et al.*, 2011). In recent years the cases of DF and DHF have increased dramatically; between 2004 and 2006 cases in the Caribbean alone were up by 30% (Jose Luis San Martin, PAHO, Costa Rica, personal communication). Under reporting probably means that figures are in fact much higher than those published by health departments and the World Health Organisation (WHO). With no effective vaccine, useful diagnostic test or drug therapy available the only answer to the disease currently lies in control of the vectors responsible for its spread. *Aedes aegypti* is the major vector globally with other *Aedes* species playing a role in local transmission, notably *Aedes albopictus*.

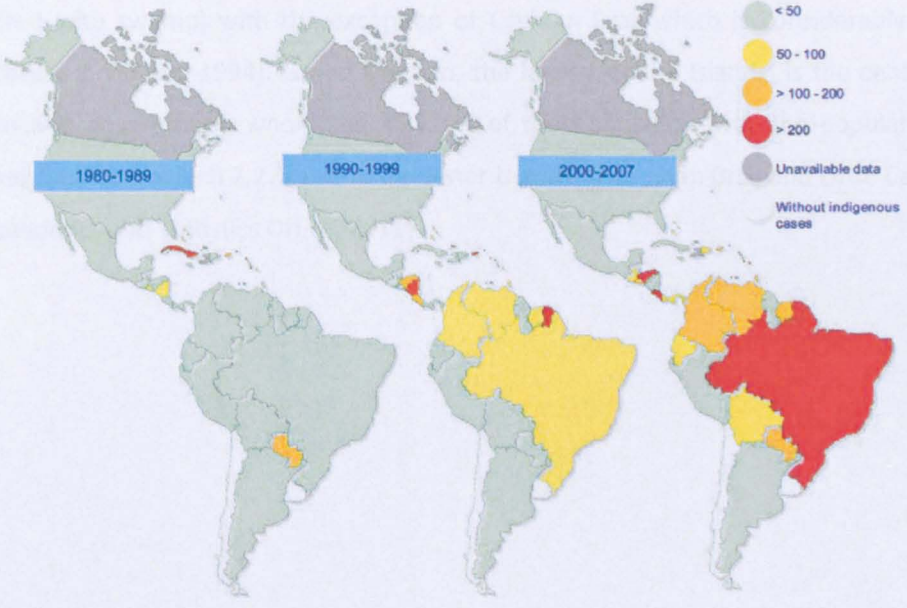


Figure 1.1 Average dengue incidence per 100,000 by country by decade. Taken from San Martin (2010).

Cases of dengue are currently on the increase in the Caribbean. In the 1980s in the non-Hispanic Caribbean there were approximately 29,000 dengue cases. This figure remained

stable in the 1990's, but a dramatic increase has been seen more recently and between 2000-2007 there were over 89,000 cases (San Martin *et al.*, 2010). In 2008 21 out of 28 countries in the Caribbean region reported at least one dengue case to the Pan-American Health Organisation (PAHO), those not reporting include Bermuda, Cuba, Haiti, Turks and Caicos, The US Virgin Islands, Aruba and Curaçao (http://www.paho.org/english/ad/dpc/cd/dengue-cases-2008.htm). It is possible that some of these countries experienced cases of dengue, but failed to report them.

Not only is dengue incidence rising, but geographically it is also spreading. In 2009-2010 28 cases of locally acquired dengue were diagnosed from Key West, representing the first cases of dengue in the mainland USA since 1946 (with the exception of some cases on the Texan border that coincided with outbreaks in Mexico) (Trout *et al.*, 2010).

1.2. The Study Area

The Cayman Islands consist of three islands located in the Caribbean Sea to the south of Cuba and North West of Jamaica (Figure 1.2). These islands are low lying with a total area of approximately 263 sq km. They largely consist of swampland (approx 50% of Grand Cayman is covered with swamp) with the exception of Cayman Brac which is considerably more rocky (Brunt & Davies, 1994). Grand Cayman, the largest of the Islands, is the centre for business and government, where the majority of the population live. The population in 2010 was 54,878 of which 2,277 live in the Sister Islands of Cayman Brac and Little Cayman (The Economics and Statistics Office, 2011).

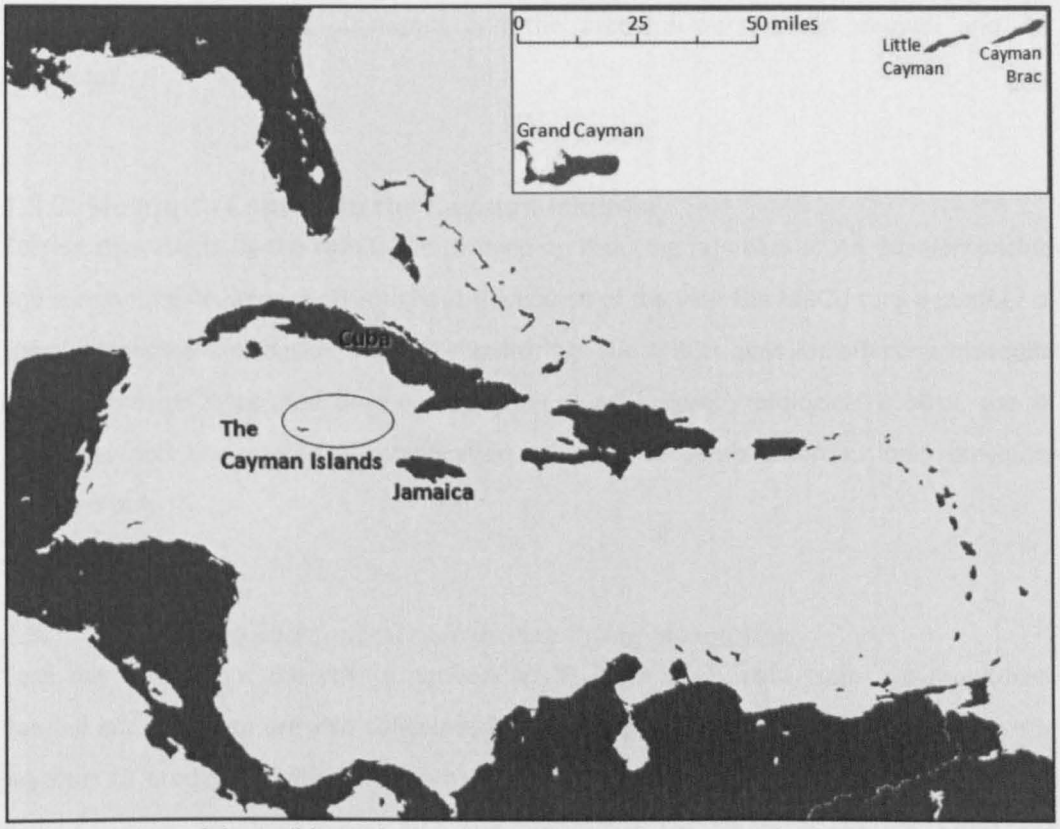


Figure 1.2 A map of the Caribbean showing the position of the Cayman Islands.

1.3. The Mosquito Research and Control Unit

1.3.1. A History of Mosquito Control in the Cayman Islands

Historically the Cayman Islands have been noted for legendary numbers of mosquitoes (Brunt & Davies, 1994). Cattle were reported to have suffocated in the night due to sheer numbers and people were in the habit of keeping a smoke fire lit from dusk onwards to ward off mosquitoes. The first known survey of species was carried out in 1938 by a visiting sanitary inspector, followed the same year by a team from Oxford University. In 1948 and 1965 further surveys of the mosquito fauna of the islands were carried out and finally The Mosquito Research and Control Unit (MRCU) was set up in 1965 (Brunt & Davies, 1994). The department was founded for the purpose of preventing vector borne disease and to reduce nuisance biting.

The Cayman Islands has recorded 32 mosquito species (of these 16 were caught in 2010). The principle concern when the MRCU was first established was the Black Salt Marsh Mosquito *Aedes taeniorhynchus*, the most abundant nuisance biting mosquito in Grand Cayman. A number of potential disease vectors also exist on the island including the

malaria vector *Anopheles albimanus* and the arboviral vectors *Ae. aegypti* and *Ae. albopictus*.

1.3.2. Mosquito Control in the Cayman Islands

Current operations by the MRCU are centred on reducing numbers of *Ae. taeniorhynchus* and eliminating *Ae. aegypti*. Throughout the course of the year the MRCU runs a number of specific campaigns alongside ongoing monitoring. The MRCU aims for effective mosquito control through integrated pest management (IPM) whereby biological control, use of pesticides and environmental modification are used in combination to keep mosquito numbers down.

1.3.2.1. Monitoring and Control of Nuisance Biting Mosquitoes

Each day throughout the year a network of 31 New Jersey light traps are monitored. Rainfall and tide data are also collected. The majority of control efforts are carried out in reaction to predictive indicators (such as a drop in tide or increased rainfall as well as swamp surveys involving larval dips and monitoring for adults in sentinel sites) and increases in trap catches.

Routine adulticide treatment in response to increases in trap numbers for nuisance biting is carried out using ultra light volume (ULV) spraying of permethrin either on the ground via vehicle mounted fogging equipment or aerially from one of two Ayres Turbo Thrush aircraft owned by the department.

Each year during the low tide (February/March) the MRCU carries out its 'Hatch and Strand' operation; large areas of swamp are blocked off using a system of sluice gates and water pumped in from other areas of the swamp to flood level, this is maintained for several days to allow *Ae. taeniorhynchus* eggs laid in the swamp to hatch (Figure 1.3). The gates are then opened to release the trapped water and results in many early season larvae being washed out to sea. This is supported by aerial larviciding campaigns carried out two to three times per year (the first prior to the start of the rainy season). Larvicidal pellets containing temephos or methoprene (in rotation) are dropped into the main swamp areas to reduce the numbers of adult *Ae. taeniorhynchus* emerging after the rains.

Figure 1.3 Photograph showing the MRCU 'Hatch and Strand' operation.
In the foreground a closed sluice gate stops water from draining into channels on the other side of the road. The pumps in the background are pumping water from the channel over the road into the section to be flooded. After three to four days the sluice gates are lifted and the full section of swamp is allowed to drain.

Prior to the first rains, areas of grassland around the island adjacent to habitation are targeted for treatment with temephos. This is applied to pastureland pre-hatch in order to reduce the numbers of *Psorophora* species (*Psorophora ciliata, Psorophora columbiae, Psorophora pygmea and Psorophora johnstoni*) emerging when the rains arrive and these areas flood.

In support of these campaigns staff respond to complaints or requests made by the public, which can range from identifying and managing a source of infestation (for example treating and advising on a compromised septic system) to fogging with permethrin in preparation for an outdoors event.

Aside from the MRCU a number of other companies are in operation to rid and protect people's homes from insect pests such as termites and cockroaches. Routine treatment by these companies is usually recommended to be carried out quarterly and is in the form of a residual spray with bifenthrin. Other chemicals used by these companies for pest control include cypermethrin, fipronil, imidacloprid, permethrin, β-cyfluthrin, disodium octaborate

tetrahydrate and sodium tetraborate decahydrate (Monica Anderson, Truly Nolen Pest Control, Grand Cayman, personal communication).

1.3.3. Mosquitoes as a Public Health Issue in the Cayman Islands and the Caribbean

Whilst the most numerous mosquito in the Cayman Islands, *Ae. taeniorhynchus*, poses no public health threat, others present notably *An. albimanus, Ae. albopictus* and *Ae. aegypti* are potential vectors of malaria, or chikungunya, yellow and dengue fever.

Numbers of *An. albimanus* remain relatively few, breeding in small pockets in the more rural areas of Grand Cayman. Malaria is not endemic in the Cayman Islands, however it is present in a number of other countries in the Caribbean including Belize, Colombia, Costa Rica, Dominican Republic, Guatemala, Haiti, Honduras, Mexico, Nicaragua, Panama and Venezuela (PAHO, 2008). In recent years malaria has re-emerged in countries that have been malaria free for many years such as Jamaica and the Bahamas (CAREC, 2007). In late 2006 Jamaica suffered its first outbreak of malaria for 44 years with 204 cases of falciparum malaria reported between November 2006 and January 2007 (CAREC, 2006). This posed significant risk to the Cayman Islands due to the large number of expatriate workers from Jamaica who returned home over the Christmas period.

Numbers of *Ae. albopictus* remain low compared to *Ae. aegypti* which is currently abundant on Grand Cayman. Dengue is not endemic in the Cayman Islands, but imported cases are regularly seen in travellers returning to the island from overseas. In October 2005 the Cayman Islands saw its first case of locally transmitted dengue and in 2010 there were seven cases of imported dengue reported in the Cayman Islands (The Public Health Department, 2010). Again, the large population of migrant workers, coupled with established populations of potential vectors, means that the Cayman Islands remain at risk of *Aedes* transmitted arboviral diseases such as dengue.

1.3.4. *Aedes aegypti* in the Cayman Islands

Aedes aegypti is a species considered non-endemic to the Cayman Islands however, over the years its presence in the islands has been recorded several times, from the first eradication campaign carried out by a single medical officer in 1950 (prior to the foundation

of the unit) to more recent campaigns in the 1970's and 1990's (Figure 1.4). The latest introduction was recorded in 2002 and the MRCU continues its efforts to control this species. The country's strong transport links with other countries in the region endemic for *Ae. aegypti* and increased urbanisation pose a constant threat of further introductions and establishment of this mosquito.

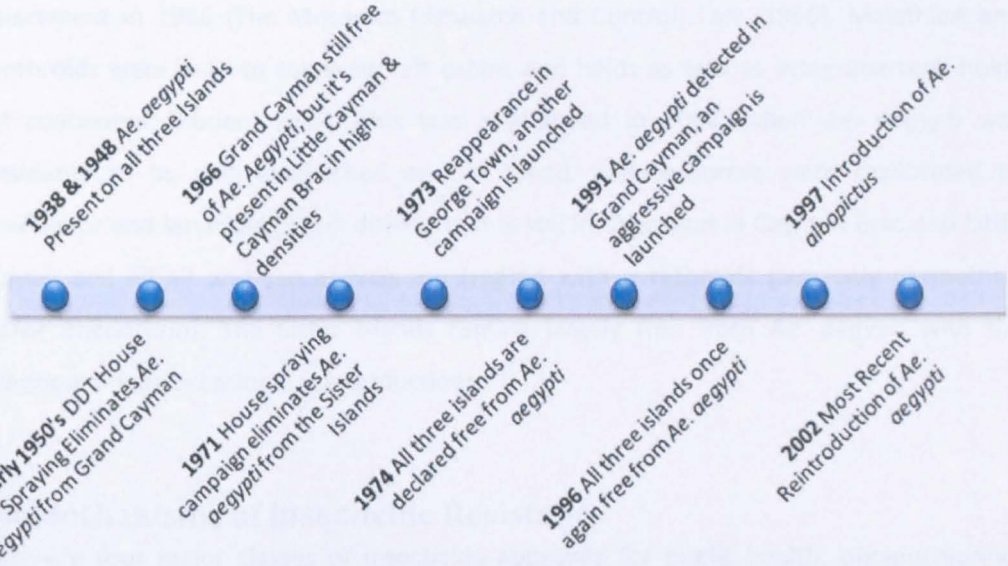


Figure 1.4 A timeline showing the history of *Ae. aegypti* in the Cayman Islands.

1.3.4.1. Control of Aedes aegypti

Surveys using ovitraps and manual inspections of potential larval habitats are carried out in households in the two major urban areas, George Town and West Bay. The target is for weekly collection of ovitraps and monthly inspection of habitats but the actual frequency of surveillance is dependent on resources and infestation levels. Unnecessary water sources are drained and the remainder are treated with larvicide. Until 2006, temephos or methoprene were the insecticides of choice (although the slow mode of action of methoprene often made this unpopular with spray teams). Low level temephos resistance was detected in *Ae. aegypti* populations in 2006 (Harris *et al.*, 2010) and this led to the introduction of *Bacillus thuringiensis israelensis* (*Bti*) as the preferred larvicide. Residual spraying of the external walls of selected buildings in George Town and West Bay with pyrethroid insecticides (commonly bifenthrin) are also carried out in the dry season.

Historically the Dengue Prevention Campaign was restricted to the two main urban areas of Grand Cayman: George Town and West Bay. In times when *Ae. aegypti* was not thought to be present on the island surveillance was largely centred on the air and sea ports, although ovitraps were also distributed island-wide.

In order to control invasion by *Ae. aegypti* a campaign of 'port disinsection' became a legal requirement in 1966 (The Mosquito (Research and Control) Law, 1966). Malathion and pyrethroids were used to spray aircraft cabins and holds as well as living quarters, holds and containers onboard ships. This was abandoned in 2006, when *Ae. aegypti* was considered to be well established on the island, and resources were reallocated to surveillance and larviciding. Port disinsection is still in operation in Cayman Brac and Little Cayman and all air and sea arrivals are treated with pyrethroids (currently permethrin and/or phenothrin). The Sister Islands remain largely free from *Ae. aegypti* with the exception of very occasional reintroductions.

1.4. Mechanisms of Insecticide Resistance

There are four major classes of insecticide approved for public health; organochlorines (most of which are banned for use in Public Health), organophosphates (whose numbers have also been severely restricted), carbamates and pyrethroids. These chemicals have only two modes of action and resistance to all of these groups of insecticides is widespread in mosquitoes.

Juvenile hormone analogues and bacterial insecticides are also used for mosquito control. Methoprene and pyriproxifen are insect growth regulators (IGR) that mimic the action of specific mosquito hormones (Ross *et al.*, 1994). Formulations of these IGRs are used as larvicides by control programmes and work by interfering with metamorphosis during the pupal stage and inhibiting adult emergence (Braga *et al.*, 2005, Ross *et al.*, 1994). Bacterial insecticides used for mosquito control include *Bti*, *Bacillus sphaericus* (*Bs.*) and spinosad (a product from *Saccharopolyspora spinosa*). The use of bacterial based insecticides for mosquito control have become popular more recently due to the perceived environmental benefits. They are relatively specific to particular pest species and have low impact on non-target species. For example, *Bs* is ineffective against *Ae. aegypti* as it is unable to bind to midgut receptors (Nielsen-Leroux & Charles, 1992), but is useful in the control of several

Culex and *Anopheles* species (Mittal, 2003). These *Bacillus* species act as stomach poisons, when ingested by larval mosquitoes they cause the breakdown of the midgut and eventual death (Gill *et al.*, 1992). Spinosad works on the insect nervous system on the nicotinic acetylcholine and γ-aminobutyric acid (GABA) receptors (Hertlein *et al.*, 2010).

Insecticide resistance can be defined as the survival of an insect to a dose of insecticide that would otherwise have killed it (Hemingway *et al.*, 2002). There are four main mechanisms by which insecticide resistance can occur; metabolic resistance, target site resistance, reduced penetration and behavioural resistance; the first two types of resistance are currently the better understood. It is possible that any particular insect may possess resistance to multiple insecticides via more than one mechanism.

1.4.1. Metabolic Resistance

This can occur either by the increased production of metabolic enzymes or by a change in the enzyme active site. In either case the insect's ability to metabolise or sequester toxic compounds is increased and the amount of insecticide reaching the target site is reduced.

There are three enzyme families implicated in metabolic resistance: esterases, cytochrome P450s or mono-oxygenases and glutathione-s-transferases. *Aedes aegypti* have an abundance of detoxification genes in comparison with *Anopheles gambiae* or *Drosophila melanogaster* (Table 1.1) (Strode *et al.*, 2008).

	D. melanogaster	***An. gambiae***	***Ae. aegypti***
Esterases	26	40	49
P450s	86	105	160
GSTs	37	28	26

Table 1.1 Number of detoxification genes relating to insecticide resistance found in *D. melanogaster*, *An. gambiae* and *Ae. aegypti*.
Adapted from Strode (2008).

Esterases are capable of detoxifying a wide range of insecticides; elevated levels of esterases may account for organophosphate, carbamate and pyrethroid resistance (Hemingway *et al.*, 2004, Montella *et al.*, 2007, Mourya *et al.*, 1993). Several studies have

implicated increased levels of esterases in temephos resistance in *Ae. aegypti* (Marcombe *et al.*, 2009b, Melo-Santos *et al.*, 2010, Montella *et al.*, 2007, Mourya *et al.*, 1993). Esterases have been found up regulated in both adults and larvae (Marcombe *et al.*, 2009b), in *Ae. aegypti* in Martinique.

Cytochrome P450s are an important family of enzymes responsible for many metabolic reactions within an organism. In insects they are involved in the metabolism of virtually all insecticides (Hemingway & Ranson, 2000) and often the oxidase reaction catalysed by P450s is the first step in a detoxification cascade involving other enzyme families (Hemingway *et al.*, 2004). Elevated P450 activity is associated with resistance to pyrethroids, organophosphates and carbamates (Hemingway & Ranson, 2000, Marcombe *et al.*, 2009b, Strode *et al.*, 2008). *Aedes aegypti* has 160 P450 genes representing a 52% increase over *An. gambiae*. The number of CYP9 genes is particularly large in *Ae. aegypti* compared to *An. gambiae*. Comparison to other Diptera (Waterhouse *et al.*, 2008) suggest that this is an expansion of the CYP9 family in *Ae. aegypti* rather than gene loss in *Anopheles*. Genes from both the CYP6 and CYP9 families are upregulated in pyrethroid resistant *Ae. aegypti* from Mexico and Thailand (Strode *et al.*, 2008).

Cytochrome P450s have also been implicated in temephos resistance in *Ae. aegypti*. A study by Marcombe (2009b) revealed upregulation of a number of the CYP enzymes mostly from the CYP6 and CYP9 families. Four P450s (CYP6M6, CYP6Z6, CYP9J23 and CYP9J22) were over expressed in the resistant compared to the susceptible strain at both the larval and adult stage.

Piperonyl butoxide (PBO) is a cytochrome P450 inhibitor and can be used to synergise insecticides that are metabolised primarily by P450s. Combination of synergist into insecticide formulations are often found in formulations used by control programs to reduce present and future resistance; the two main adulticide products currently in use by the MRCU (Aqua-Reslin® and Aqualuer®) each contain 20% permethrin (the active ingredient) and 20% piperonyl butoxide.

Glutathione-S-Transferases (GSTs) are involved in detoxification and in the protection of tissues against oxidative damage and stress (Vontas *et al.*, 2001). Within insects six classes

of GSTs have been identified (Ranson *et al.*, 2002) and of these the two largest classes (Delta and Epsilon) are specific to insects and may play a role in insecticide resistance (Hemingway & Ranson, 2000, Lumjuan *et al.*, 2005, Strode *et al.*, 2008).

Delta and Epsilon GSTs have been implicated in resistance to all the major classes of insecticides (Hemingway *et al.*, 2004). In *Ae. aegypti* there are 26 GST genes (Lumjuan *et al.*, 2007). Over expression of GSTe2, GSTe5 and GSTe7 has been found in DDT and pyrethroid resistant *Ae. aegypti* (Lumjuan *et al.*, 2005, Lumjuan *et al.*, 2011, Marcombe *et al.*, 2009b, Strode *et al.*, 2008). GSTe2 and GSTe5 both metabolise DDT but no direct metabolism of pyrethroid insecticides by GSTs has been reported. However, silencing expression of GSTe7 by RNA interference increased the susceptibility of *Ae. aegypti* to pyrethroids; suggesting a role for this enzyme in pyrethroid resistance (Lumjuan *et al.*, 2011).

1.4.2. Target Site Resistance

Target site resistance typically results from an amino acid substitution in the target site receptor that reduces the binding of the insecticide. This type of resistance will often affect all chemicals that have a similar mode of action on the insect. The target sites of the insecticides most commonly used in public health are the voltage-gated sodium channel and acetylcholinesterase. The phenyl pyrazole fipronil and cyclodienes target the GABA receptors.

Mutations in the voltage-gated sodium channel have been associated with resistance to DDT and pyrethroids in many insect species. This resistance is often referred to as 'kdr' to describe the 'knockdown resistant' phenotype caused by these mutations. The kdr mutations found in *Ae. aegypti* are discussed in detail in Chapter 3.

The mode of action of organophosphates and carbamates is through the inhibition of acetylcholinesterase (AChE) (Hemingway *et al.*, 2002). AChE is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACE). Under normal circumstances insecticides phosphorylate or carbamylate the active site serine in AChE, preventing it detoxifying ACE. The build up of neurotransmitter at the nerve synapse causes repetitive firing and paralysis. In resistant insects a mutation in AChE results in a modified or insensitive version to which the insecticide cannot bind (Hemingway & Ranson, 2000). A glycine to serine mutation at

position 119 has been identified in the AChE enzymes in *Culex pipiens, An. gambiae* and *An. albimanus* (Weill *et al.*, 2003, Weill *et al.*, 2004), but are yet to be identified in *Ae. aegypti*, despite widespread reports of the insensitive AChE phenotype (Alout *et al.*, 2007, Bisset *et al.*, 2006, Polson *et al.*, 2011, Vaughan *et al.*, 1998).

GABA receptors are a site of action for cyclodienes and phenylpyrazole insecticides. The GABA receptor consists of five subunits around a central transmitter-gated ion channel (Hemingway & Ranson, 2000). A single base pair substitution in the membrane spanning domain occurs in which alanine is replaced by serine at position 302 this is known as the *Resistance to Dieldrin* (*Rdl*) gene (Ffrench-Constant *et al.*, 2000, Ffrench-Constant *et al.*, 1994, Severson *et al.*, 1997, Thompson *et al.*, 1993). While laboratory studies have shown the presence of this mutation in *Ae. aegypti* (Severson *et al.*, 1997, Thompson *et al.*, 1993) there are no reports of it within wild populations.

1.4.3. Reduced Penetration

Changes in the cuticle of an insect or its digestive tract linings can increase tolerance to certain types of insecticide that rely on these routes to reach their target site (IRAC, 2006). This mechanism of resistance is not specific to any particular class of insecticide; cuticular thickening can cause the reduced rate of uptake of all types of xenobiotics.

The cuticle is a major route of insecticide penetration in insects (Vontas *et al.*, 2007). In *An. gambiae* the over-expression of two cuticular genes (CPLCG3 and CPLCG4) are associated with resistance to permethrin (Awolola *et al.*, 2009, Cornman & Willis, 2009, Willis, 2010). Similarly, in *Anopheles stephensi* the CPLCG3 gene is over-expressed in pyrethroid resistant populations (Vontas *et al.*, 2007) and over-expression of cuticular protein genes has been reported in deltamethrin resistant strains of *Ae. aegypti* from Thailand (Lertkiatmongkol *et al.*, 2010). Further work is required to see if these increases in expression of cuticular genes are translated into reduced insecticide penetration.

1.4.4. Behavioural Resistance

Behavioural resistance occurs when an insect is able to avoid a lethal dose of insecticide through changes in behaviour. This can be due to reduced contact due to increased contact irritancy or complete avoidance of treated areas due to sensing the insecticide (excito-

repellancy). Other changes in the behaviour such as changes in the time of biting may have the effect of reducing insecticide exposure and thus appear as a method of behavioural resistance. (Kongmee *et al.*, 2004, Roberts *et al.*, 1997).

Behavioural responses of insects exposed to insecticide are difficult to quantify and thus the extent and impact of this type of mechanism on the efficacy of insecticides is poorly understood. Avoidance of insecticides by mosquitoes could be detrimental to vector control programmes, however repellent effects could result in reduced vector human contact and therefore reduce disease transmission (Kongmee *et al.*, 2004).

1.5. Insecticide Resistance in *Aedes aegypti*

Insecticide resistance reported is mostly to those chemicals used for control notably DDT, pyrethroids and organophosphates. The most commonly reported mechanisms of insecticide resistance in *Ae.aegypti* are *kdr* mutations and metabolic resistance due to increases in esterases.

Insecticide resistance in *Ae. aegypti* is very widespread (Figure 1.5) and has been reported in populations of this mosquito in the Americas (including the Caribbean), Asia and Australia and to all four classes of insecticide (Ranson *et al.*, 2010). While many groups globally are investigating resistance there are also many areas where active control is ongoing without having a resistance monitoring programme in place. Indeed a lot of data on insecticide resistance are not released into the public domain by countries that have very good monitoring systems, making it hard to determine the overall prevalence of insecticide resistance.

Bias towards only publishing data where resistance is present also means it is very difficult to determine where populations of *Ae. aegypti* remain susceptible to insecticides. However, populations in Brazil, South East Asia, India, French Polynesia, Peru and Ivory Coast have been reported as remaining susceptible to temephos and pyrethroids (Morou *et al.*, 2010, Ranson *et al.*, 2010). Limitations also exist in determining the mechanisms of resistance. Information may be missing due to laboratories (especially those in the field) not having access to the technology required to identify molecular methods of resistance.

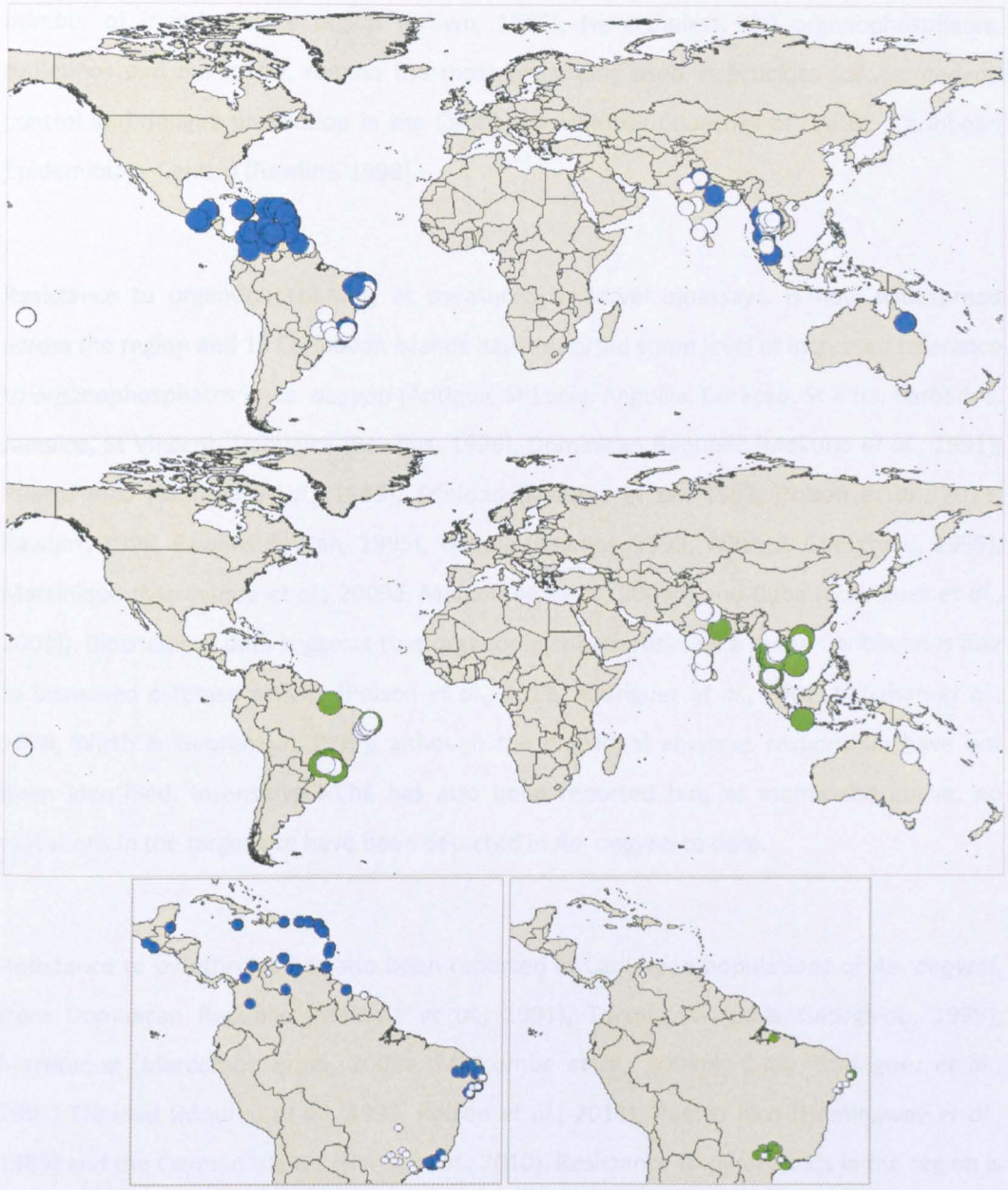


Figure 1.5 Distribution of published reports of insecticide resistance and susceptibility in *Ae. aegypti* including emphasis on Latin America.
Blue dots represent temephos resistance, green dots represent pyrethroid resistance, white spots represent susceptibility to either temephos (top) or pyrethroids (bottom). Taken from Ranson (2010).

1.5.1. Insecticide Resistance in the Caribbean

DDT resistance in Caribbean populations of *Ae. aegypti* developed as early as 1955, at which time malathion and fenthion were employed as alternatives. By 1976 the Cayman Islands were, according to PAHO figures, reporting confirmed resistance to DDT and dieldrin, therefore temephos became the insecticide of choice for control (Pal, 1976). Within 20 years of their introduction, resistance to these two chemicals was present in a

number of islands in the region (Brown, 1986). Nevertheless, the organophosphates, temephos and malathion, remain the most commonly used insecticides for *Ae. aegypti* control and dengue prevention in the Caribbean Member Countries of CAREC (Caribbean Epidemiology Centre) (Rawlins, 1998).

Resistance to organophosphates, as measured by larval bioassays, is now widespread across the region and 15 Caribbean Islands have reported some level of increased tolerance to organophosphates in *Ae. aegypti* (Antigua, St Lucia, Anguilla, Curaçao, St Kitts, Barbados, Jamaica, St Vincent, Dominica (Rawlins, 1998), Dominican Republic (Mekuria *et al.*, 1991), Puerto Rico (Mourya *et al.*, 1993), Trinidad (Mourya *et al.*, 1993, Polson *et al.*, 2011, Rawlins, 1998, Rawlins & Wan, 1995), Tortola (Rawlins, 1998, Wirth & Georghiou, 1999), Martinique (Marcombe *et al.*, 2009a, Marcombe *et al.*, 2009b) and Cuba (Rodriguez *et al.*, 2001)). Biochemical data suggests that organophosphate resistance in the Caribbean is due to increased esterase activity (Polson *et al.*, 2011, Rodriguez *et al.*, 2001, Vaughan *et al.*, 1998, Wirth & Georghiou, 1999); although the individual enzymes responsible have not been identified. Insensitive AChE has also been reported but, as mentioned above, no mutations in the target site have been detected in *Ae. aegypti* to date.

Resistance to pyrethroids has also been reported in Caribbean populations of *Ae. aegypti*, from Dominican Republic (Mekuria *et al.*, 1991), Tortola (Wirth & Georghiou, 1999), Martinique (Marcombe *et al.*, 2009a, Marcombe *et al.*, 2009b), Cuba (Rodriguez *et al.*, 2001) Trinidad (Mourya *et al.*, 1993, Polson *et al.*, 2011), Puerto Rico (Hemingway *et al.*, 1989) and the Cayman Islands (Harris *et al.*, 2010). Resistance to pyrethroids in the region is associated with kdr and increased expression of cytochrome P450s (Harris *et al.*, 2010, Marcombe *et al.*, 2009b).

1.5.2. Insecticide Susceptibility Status of Other Mosquitoes in the Cayman Islands

Despite insecticidal pressures being maintained on the swamps of Grand Cayman throughout the year *Ae. taeniorhynchus* populations remain susceptible to the two main insecticides used against them; temephos and permethrin. Bioassays were carried out as a prelude to this study in early 2008. Adult bioassays resulted in 100% mortality after a one hour exposure to 0.75% permethrin in WHO susceptibility assays and hence the population would be defined as susceptible according to WHO standards. For temephos, a comparison

of the LC_{50} of the Grand Cayman *Ae. taeniorhynchus* with the USDA susceptible strain showed a 1.1 fold elevation in LC_{50} in the local strain, but this was not significant (P = 0.63 unpaired t test, data not shown). It was therefore decided not to pursue this work any further.

1.6. Population Genetics of *Aedes aegypti*

Increases in global transport in recent years has aided the spread of a number of vectors, not least *Ae. aegypti* which travels easily due to the characteristic of its eggs to be able to withstand desiccation. In this way *Ae. aegypti* has been able to rapidly disperse worldwide on timber, tyres and various other commercial goods.

It is possible that as *Ae. aegypti* can disperse as eggs it can serve as an effective method of spreading insecticide resistance genes. Brengues (2003) noted mutations in the sodium channel gene of *Ae. aegypti* from Martinique, French Guiana and Brazil that were tightly linked and therefore possibly due to a common origin of resistance. Similarly, studies on the flanking regions of esterase genes related to organophosphate resistance in *Cx. pipiens* showed similarities in areas as distant as Africa, Asia and North America which was thought to be due to mass migration following a single mutation event (Raymond *et al.*, 1991).

The movement of shipping traffic through the Caribbean and indeed the world has increased drastically in recent years due to globalisation (worldwide container throughput has increased from 36 million units in 1980 to 266 million units in 2002 (Notteboom, 2004)) and this may have aided the movement of *Ae. aegypti*. It is believed by the MRCU that the current Grand Cayman population of *Ae. aegypti* was imported sometime in early 2002 as following the elimination of this species in 1996 no further samples were detected by adult or ovitrapping until the summer of 2002. However, this is very difficult to verify as by 2005, the mosquito species was firmly established and found throughout the island. The presence of multiple forms of insecticide resistance in the region led the MRCU to question whether this mosquito arrived on island bringing with it resistance genes or whether resistance has occurred as a direct result of insecticide campaigns by the department. Such information could prove valuable for the future design of effective control strategies (Bracco *et al.*, 2007). Analysis of resistance alleles as well as neutral markers such as microsatellites

provide information on the migration dynamics of the population and may help determine the origin of the Cayman Islands *Ae. aegypti* population.

Microsatellites are non-coding regions of simple repetitive DNA, generally consisting of repeats of two to six bases which can be found throughout the eukaryote genome (Lovin *et al.*, 2009). The length of a microsatellite region and the number of repeats can vary greatly from individual to individual due to the high rate of mutations that occur in these regions due to slippage during replication (Marquardt *et al.*, 2005). The greater the variation within a population generally the older the population is considered to be, as more mutational events have been able to occur over time.

The fact that microsatellites are highly polymorphic and of mendelian inheritance make them a powerful tool for population studies (Ravel *et al.*, 2001) an individual can possess two different length microsatellites as inherited from each separate parent thus enabling population structure to be closely studied (Marquardt *et al.*, 2005). The *Ae. aegypti* genome is well populated with microsatellite loci (Lovin *et al.*, 2009). Several of these microsatellites have been shown to be polymorphic and thus suitable for population genetic studies (Huber *et al.*, 2001, Ravel *et al.*, 2001, Urdaneta-Marquez *et al.*, 2008).

Microsatellite data can be used to determine genetic variability within populations, F-statistics (*Fis*, *Fst* and *Fit*) can be calculated to measure genetic divergence between and within populations. F-statistics describe the amount of inbreeding effects within populations (*Fis*), between subpopulations (*Fst*) and within entire populations (*Fit*) (Wright, 1951).

Previous studies have used population genetics to trace American and Caribbean populations of *Ae. aegypti* back to Africa (Bracco *et al.*, 2007, Brown *et al.*, 2011, Powell *et al.*, 1980). Studies by Bracco (2007) support the hypothesis that *Ae. aegypti* were introduced into the Americas in the 17th and 18th century and experienced a genetic bottleneck due to control operations in the 1950-60s. Some are believed to have remained and repopulated the region as control measures collapsed. This study also suggests that additional haplotypes were introduced into the Americas in the 1980s from Asia due to increases in globalisation. Additionally a study in Haiti (Lovin *et al.*, 2009) looked at levels of

polymorphism and found evidence for population differentiation which was observed even across relatively short geographical distances.

1.7. Non-Insecticidal Control for *Aedes aegypti*

Increasing levels of insecticide resistance combined with the lack of insecticides with new modes of action mean that it is important to consider non-insecticidal control for *Ae. aegypti*. Historically the MRCU have investigated different methods of control mainly for the abatement of *Ae. taeniorhynchus* including the various methods described above (Section 1.3.2.1). In the 1950s biological control via the treatment of wells and cisterns with mosquito fish (*Gambusia* species) was employed as part of an integrated pest management programme (Brunt & Davies, 1994), but was not sustained as a method of control when the MRCU was founded in 1965.

Genetic approaches to *Ae. aegypti* control are described below.

1.7.1. Genetic Approaches

1.7.1.1. Sterile Insect Technique (SIT)

Knipling (1955) proposed the concept of sterile insect technique in the 1950's to control agricultural pests. This method involves mass rearing of large numbers of pest insects that are then sterilised by chemical means or radiation and released into the environment. The released insects compete to mate with the wild ones and thus the population declines due to these non-viable matings. Often it is the case that only one sex is released, both to reduce mating between released insects, but also to avoid biting and disease transmission in cases where only females are blood feeders (Alphey & Andreasen, 2002).

Historically SIT has been successful for a number of species, an example of which is the New World Screw Worm (*Cochliomyia hominivorax*). This pest causes massive economic losses in sheep and cattle farming as the females burrow into the flesh of fresh wounds to lay their eggs. From the late 1950's a programme was launched in which insects sterilised by gamma radiation were released in the southern United States and by 1966 this region was declared free from this pest (Klassen & Curtis, 2005). The programme continued south through Central America by means of a rolling front moving steadily forward eliminating the screw worm as it went. Operations continued throughout the 1980's and 1990's and by 1999 all

countries to the north of Panama were free from the New World Screw Worm (Figure 1.6) (Wyss, 2000). Releases continue at a rate of approximately 40-50 million sterile New World Screw Worm per week across a barrier area in Southern Panama and Northern Columbia in order to prevent reinvasion from South America (Alphey *et al.*, 2010).

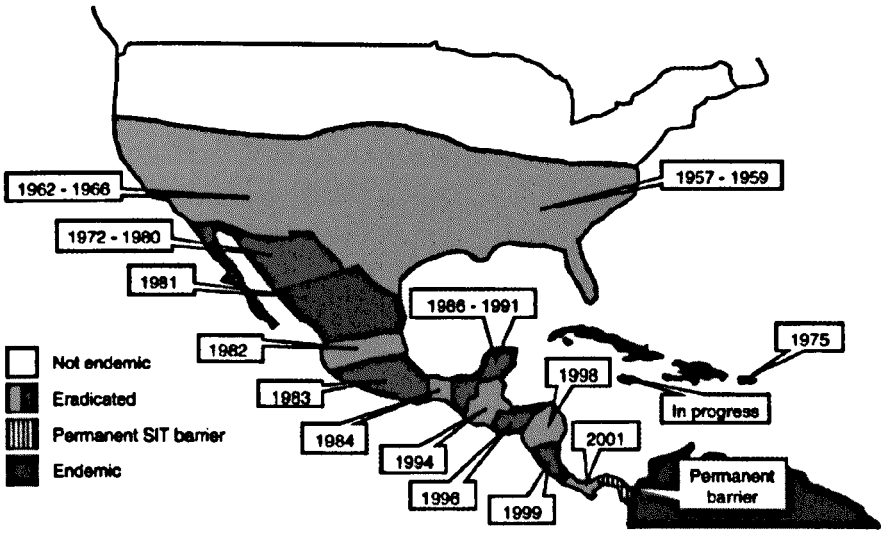


Figure 1.6 Map showing the eradication of the screw-worm *Cochliomyia hominivorax* from the Southern States of the USA and countries in Central America.
Reproduced from Robinson (2002). Successful eradication in each zone is indicated by date (although parameters for eradication are not defined); in 2001 the screw-worm programme was still in progress in Panama.

Other successes for SIT can be found in the case of the Mediterranean fruit fly (*Ceratitis capitata*). Flies are released in several countries in Latin America as well as Israel, South Africa, Thailand and some countries in Europe. The objective in these cases being towards reduced pesticide use and suppression rather than eradication. Control of this pest improves international trade without the need for quarantine restrictions. SIT for Mediterranean fruit fly is carried out in several US cities to protect against invasion as a result of smuggled produce (Klassen & Curtis, 2005).

SIT has also been successfully applied on a small scale to control medically important insect pests. The tsetse fly (*Glossina* species) found in Sub-Saharan Africa transmits trypanosomiasis causing sleeping sickness in humans and nagana in cattle. A small scale program carried out on Zanzibar in the 1990's successfully eradicated *Glossina austeni* from the island (Vreysen *et al.*, 2000).

Mosquitoes have also been the subject of studies involving SIT, perhaps the most well known of these studies was carried out in India. The Indian study was directed against *Culex quinquefasciatus*, *An. stephensi* and *Ae. aegypti*. Despite successful reductions in the mosquito numbers, adverse publicity resulted in premature termination of the trial (Unknown, 1975, WHO, 1976). Many other studies have been carried out with varying levels of success, those pertaining to *Ae. aegypti* can be seen in Table 1.2, details of other species can be seen in Benedict (2003).

Year	Location	No. Released	Objective	Outcome
1960-1961	Pensacola, Fl, USA	4.6 million over 43 weeks	Population Reduction	Despite extremely overwhelming ratios of release to wild material, no effect could be concluded
1967	Meridian, MS, USA	17,000 fertile males over 2 weeks	Morphological allele introgression	Out of 1084 eggs, two matings were to marked individuals
1971	Model Basti, India	30,000 Translocation males over 4 weeks	Persistence of translocation in wild population	Males were competitive and persistence of translocation was observed
1971	Shastri Nagar, India	~50,000 marked males over 4 weeks	Allele introgression in wild population	Males were competitive and introgression of marker allele was observed
1974	Delhi, India	40,500 in 3 experiments of 6 days each	Male mating competitiveness	Males were competitive
1974	Mombassa, Kenya	57,000 over 10 weeks	Population reduction and semi-sterility	Semi sterility, but there was no long term persistence of translocations nor a great effect on pupal and adult populations
1975	Mombassa, Kenya	31,500 over 9 weeks	Population reduction and dynamics	Released males mated with wild females, but eggs were not deposited in ovitraps and hybrid progeny did not survive to pupa

Table 1.2 *Ae. aegypti* releases related to sterile insect technique.
Reproduced from Benedict (2003).

One of the major barriers to SIT in mosquitoes is reduced male competitive mating ability and longevity following sterilisation (Alphey & Andreasen, 2002). For this reason, genetic methods for obtaining sterilisation are being pursued.

1.7.1.2. Release of Insects Carrying a Dominant Lethal (RIDL)

The release of insects carrying a dominant lethal (RIDL) replaces the need for damaging irradiation used in conventional SIT by genetic modification. The inclusion of a specific construct causes the demise of insects in the absence of an antidote. It is important

however, that the antidote is not present or will not become present in the natural environment of the insect (Alphey & Andreasen, 2002) which would lead to the failure of the lethal system. RIDL is therefore an autocidal system that will rapidly eliminate itself from the environment unless deliberately maintained by constant reintroduction (Phuc *et al.*, 2007).

The inserted genetic construct is dominant and therefore still able to cause death even in the heterozygous state, released insects are all homozygous, they mate with the wild types producing progeny heterozygous for the dominant lethal and so die (Alphey & Andreasen, 2002). Lethality in this system is late acting with progeny from RIDL insects dying before sexual maturity usually in the late larval or pupal stage. This late acting system means larvae that hatch in the wild compete with the wild type larvae for limited resources. In an early acting system the reduction of competition for the wild type larvae could in fact result in stronger or larger wild mosquitoes due to greater nutrition in the immature stages (Phuc *et al.*, 2007) thus giving them an overall greater fitness advantage.

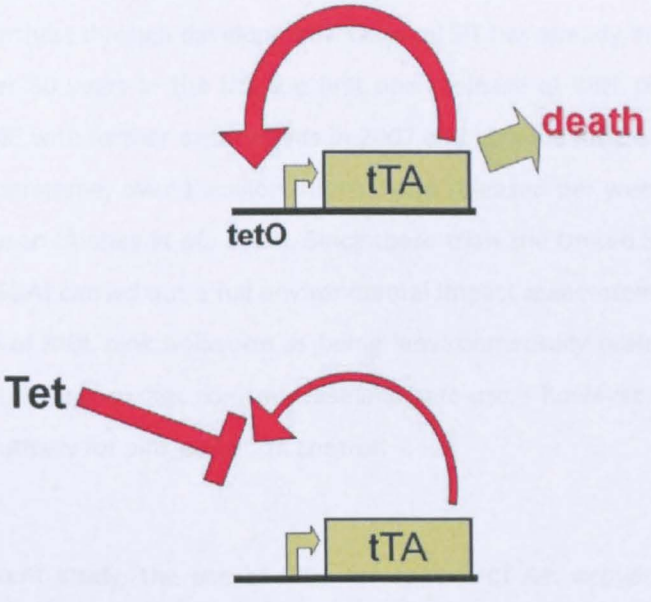


Figure 1.7 The tetracycline-repressible lethal system.
In the absence of tetracycline basal expression of tTA leads to the synthesis of more tTA, which accumulates to a high level, at the highest levels expression is lethal. In the presence of tetracycline, tTA is inactivated and therefore only expressed at basal (non-lethal) levels. Taken from Gong (2005).

In order to mass rear RIDL insects for release it is important that the lethal system is repressible; the antidote in this case is a dietary supplement of tetracycline, which is added to the larval rearing water. The genetic modification results in the overproduction of a specific protein (tetracycline repressible trans-activator protein or tTA) in a positive feedback loop, production of tTA stimulates further production of tTA which when expressed at high levels is lethal to the immature insect (Figure 1.7). The addition of tetracycline switches off this feedback, tTA is inactivated and does not stimulate further production, but remains benign at low levels and development of the insect is normal. Additionally, this construct is attached to a DSRed fluorescent marker (clontech) to allow for easy screening of the construct in individuals whose parentage is unknown (Alphey & Andreasen, 2002), this provides a faster alternative to more labour intensive PCR methods.

The RIDL construct has been transformed into a number of different strains of insects including the Pink bollworm (*Pectinophora gossypiella*), The Mediterranean fruit fly (*Ceratitis capitata*), The Mexican fruit fly (*Anastrepha ludens*), The olive fruit fly (*Bactrocera oleae*), *Ae. albopictus* and *Ae. aegypti* (Phuc *et al.*, 2007), most of these species are still in development and undergoing contained trials except for the pink bollworm that has progressed the furthest through development. Classical SIT has already been in use for pink bollworm for over 30 years in the US, the first open release of RIDL pink bollworm was carried out in 2006 with further experiments in 2007 and by 2008 RIDL was integrated into the bollworm programme; over 1 million insects were released per week until the end of the bollworm season (Alphey *et al.*, 2010). Since these trials the United States Department of Agriculture (USDA) carried out a full environmental impact assessment and in May 2009 declared the use of RIDL pink bollworm as being 'environmentally preferable to all other alternatives.' (http://www.oxitec.com/our-research/safe-use/) however this technology is yet to be used routinely for pink bollworm control.

Prior to the current study, the use of RIDL for control of *Ae. aegypti* had never been investigated before. Various strains had shown promise within the laboratory (Phuc *et al.*, 2007). The OX513A strain was created in the insecticide susceptible Rockefeller strain, but has been back crossed into a Latin American background strain (from Mexico) and contains the late acting dominant lethal gene described above, it is produced and supplied by Oxitec Ltd. This study represents the first open release of a transgenic strain of *Ae. aegypti*. It is unclear whether genetic engineering compromises mosquito fitness in the way that

classical sterile insect technology does and currently evidence from laboratory studies involving different genetic modifications is mixed (Catteruccia *et al.*, 2003, Irvin *et al.*, 2004, Marrelli *et al.*, 2007, Marrelli *et al.*, 2006) one objective of this study was to assess the specific competitiveness of the OX513A transgenic strain in the field.

1.7.1.3. Alternative Methods of Genetic Control

Other genetic methods of controlling mosquito disease vectors and especially *Ae. aegypti* are still under development. These methods include attempts to make the mosquito refractory to infection from parasites and viruses, gene driver systems to push novel genes into populations and selfish genetic elements that ensure the spread of these genes by killing individuals that don't contain them (Chen *et al.*, 2007, James, 2005, Sinkins & Gould, 2006, Windbichler *et al.*, 2011). Of these, the method that is probably closest to application for control within the field is *Wolbachia*.

1.7.1.4. Wolbachia

Wolbachia are naturally occurring symbiotic intracellular bacteria that are maternally inherited, found in a range of different insects and can cause cytoplasmic incompatibility (Min & Benzer, 1997, Werren, 1997). In mosquitoes, if a *Wolbachia* infected mosquito mates with one that is not infected this results in failure of correct fusion of gametes during fertilisation and arrested development of embryos (Brelsfoard *et al.*, 2008). One control strategy using this bacterium relies on repeated releases of *Wolbachia* infected males causing sterile mating. However, not all eggs fail to hatch as a result of cytoplasmic incompatibility and *Wolbachia* can be passed transovarially from female to offspring; in cage studies 100% infection frequency was present after just seven generations (Xi *et al.*, 2005). Egg hatch rate is normal between *Wolbachia* infected males and *Wolbachia* infected females meaning that once it becomes fixed within a population *Wolbachia* is unable to cause cytoplasmic incompatibility. Use of *Wolbachia* alone as a method of genetic control relies upon the target population having no naturally occurring *Wolbachia* (Xi *et al.*, 2005).

Virulent strains of *Wolbachia* (*wMelPop* or *popcorn* (Min & Benzer, 1997)) have been isolated from *Drosophila* species and introduced into *Ae. aegypti* resulting in a shortened life span (by up to half) in comparison with uninfected mosquitoes. This may have implications for disease transmission; for example incubation time of Dengue is

approximately two weeks within *Ae. aegypti* (McMeniman *et al.*, 2009) reduced lifespan of the mosquito population may result in an epidemiological impact.

Some strains of the bacterium also provide protection from dengue virus (Bian *et al.*, 2010, Frentiu *et al.*, 2010, Moreira *et al.*, 2009). A higher cellular load of *Wolbachia pipientis* causes greater protection from Dengue infection in *Ae. aegypti* as a result of viral inhibition (Frentiu *et al.*, 2010). Currently studies are underway in Australia to test the effects of *W. pipientis* infected *Ae. aegypti* released into the wild. Early data shows that after approximately three months of releasing 6,000 infected male mosquitoes per week 25% of wild larvae are *Wolbachia* infected (Coffey, 2011) which is a promising start for this technology.

1.8. Aims and Objectives of this Study.

The aim of this study is to support vector control activities at the MRCU through research. The main objectives are:-

- To determine the level of susceptibility of local *Ae. aegypti* populations to insecticides used by the MRCU in Grand Cayman.
- To identify the mechanisms responsible for insecticide resistance.
- To study the population genetics of the Grand Cayman population of *Ae. aegypti.*
    - To determine if the Grand Cayman population of *Ae. aegypti* is a single panmictic population.
    - To determine if the potential source of *Ae. aegypti* to the island can be identified using phylogenetic analysis and resistance genes as markers.
- To determine if insecticide resistance in the Cayman Island population of *Ae. aegypti* arose from migration or de novo mutation.
- To determine whether a wild population of *Ae. aegypti* can be suppressed by releasing genetically sterile males.

2. Characterisation of Insecticide Resistance in *Aedes aegypti* from Grand Cayman

2.1. Introduction

Since 1965 the MRCU has employed many different insecticides for the control of *Ae. aegypti.* These include adulticiding with permethrin, external wall treatments on houses with deltamethrin and lambdacyhalothrin, larvaciding with temephos, methoprene and more recently *Bti.* In addition to insecticidal treatment, source reduction during the course of yard to yard surveys is carried out by trained personnel. Insecticides are widely used in the Cayman Islands to control other mosquito species mainly *Ae. taeniorhynchus* and by householders to target domestic pests such as cockroaches, ants and termites.

In October 2006 a small scale study revealed low level resistance of Cayman *Ae. aegypti* to the organophosphate temephos. Larval bioassays were carried out as per WHO guidelines and F_1 generation *Ae. aegypti* had a LC_{50} of 0.017mg/L compared to the Rockefeller susceptible strain (0.0059mg/L). The Rockefeller strain is an insecticide susceptible strain of Caribbean origin that was colonised in the 1930's (Rodriguez *et al.*, 2001), it was supplied by Dr. George O'Meara at the Florida Medical Entomology Lab, Vero Beach, Florida. The detection of approximately 3-fold resistance to temephos resulted in a change in larviciding policy and from this time *Bti* has been the larvicide of choice.

In this chapter, the impact of this intensive insecticidal pressure on the local *Ae. aegypti* population was investigated. Adult and larval bioassays were used to test the susceptibility to a range of insecticides and biochemical assays employed to assess the activity of a range of enzymes potentially involved in insecticide resistance.

2.2. Methods

2.2.1. Origins of Strains

The Cayman Islands strain of *Ae. aegypti* originated from larvae collected in the George Town and West Bay areas of Grand Cayman in January 2008. Larvae were collected from multiple water containers, pooled and reared to adulthood in the insectaries at the MRCU. Rearing conditions were not climate controlled, temperatures typically range from 23°C - 30°C and outside humidity averaging 77% although this was increased using a desktop humidifier (Lasko 1115). Larval diet comprised of a mix of liver powder and guinea pig

pellets. These mosquitoes were then blood-fed on a live sedated guinea pig and the F_1 generation used in the experiments described in this chapter.

The New Orleans (NO) strain is an insecticide susceptible strain that was supplied by the Liverpool School of Tropical Medicine (LSTM), but was originally colonised by the Centers for Disease Control and Prevention (CDC).

The Cayman Islands strain was selected with temephos over the course of three generations resulting in the Cayman Islands Resistant strain (KYR). Batches of fifty L_4 Cayman Islands strain (F_1 generation, n=3,300) larvae were added to 100ml distilled water containing 0.035mg/L temephos. Survivors were reared to adults and the L_4 progeny (n=4,900) were exposed to 0.05mg/L temephos. In the final generation of selection, 6,400 F_3 generation L_4 larvae were exposed to 0.08mg/L temephos. On each occasion larvae were exposed for 24 hours, survivors were then rinsed three times in fresh distilled water and reared to adulthood.

2.2.2. Larval Bioassays

Larval bioassays were carried out according to World Health Organization (WHO) guidelines; 1 ml of temephos (Chemservice, PA) dissolved in ethanol was added to 249 ml distilled water containing 25 third to fourth instar larvae. Five different final concentrations between 0.0015 and 0.06 mg/L temephos and an ethanol only control were tested in triplicate on different days. Mortality was scored in each group over a 24 hour test period. Mosquitoes that were unable to swim to the surface were counted as dead. Any larvae that had pupated during the course of the experiment were disregarded from the totals. The lethal concentration that kills 50 percent (LC_{50}) of larvae was calculated using Log dose Probit (LdP) Line® software (Ehabsoft).

2.2.3. Adult Bioassays

Adult bioassays were carried out on 1-3 day old mosquitoes using WHO insecticide susceptibility test kits and insecticide treated papers supplied by WHO: 4% DDT, 0.75% permethrin, 0.05% deltamethrin, and 0.05% lambda-cyhalothrin. Etofenprox papers (0.5%) were made using Whatman number 1 filter paper with etofenprox standard (Sigma-Aldrich) dissolved in acetone and Dow Corning 556 Silicon Fluid as a carrier.

The exposure time was varied to determine the lethal time that kills 50 per cent of the population (LT_{50}). Control assays, in which mosquitoes were exposed to papers impregnated with carrier oil only were conducted in parallel. After exposure mosquitoes were transferred to a holding tube and supplied 10% sugar solution on a cotton pad. Mortality was scored 24 hours after exposure; LT_{50} values were determined from log time versus probit mortality lines generated using the Ldp Line® software.

The effect of pre exposure to the synergist, piperonyl butoxide (PBO) on permethrin induced mortality was also assessed. Adult 1-3 day old females were exposed to papers impregnated with 4% PBO or to control papers for one hour and then immediately exposed to 0.75% permethrin for a further two hours using WHO susceptibility test kits. Mortality was scored after 24 hours.

2.2.4. Biochemical Assays

Fifty individual three-day-old females from each strain were used in each assay. Each mosquito was homogenized in 200µl distilled water and kept on ice. Crude homogenate was used in the acetylcholinesterase assay, while for the other assays the homogenate was centrifuged at maximum speed for 30 seconds and the supernatant used.

Protein content for each mosquito was quantified using the Quantipro™ BCA Assay Kit (Sigma-Aldrich) so that enzyme activities per mg of protein could be calculated. All other assays were carried out as per the methods of Penilla (1998).

2.2.4.1. Acetylcholinesterase Activity

Two replicates of 25µl of crude mosquito homogenate were added to adjacent wells of a microtitre plate and 145µl of 1% Triton X-100 in 0.1M phosphate buffer pH7.8 was added to each followed by 10µl 0.01M dithiobis 2-nitrobenzoic acid in 0.1M phosphate buffer pH7.0. Twenty five µl of 0.01M acetylthiocholine iodide containing 0.1M propoxur was added to one replicate; the other well received the acetylthiocholine alone. The assay was left at room temperature for an hour before being read at 405nm. Blank wells were set up using distilled water in place of mosquito homogenate.

2.2.4.2. Esterase Activity

Esterase activities were measured using the model substrates α- and β-naphthyl acetate and para-nitrophenyl acetate (PNPA). To test esterase activity using naphthyl acetate, 20µl of homogenate supernatant was added to duplicate wells. One well received 200µl 0.3mM α-naphthyl acetate in 0.02M phosphate buffer pH7.2 and the other 200µl 0.3mM β-naphthyl acetate in 0.02M phosphate buffer pH7.2. The assay was left for 30 minutes and then the reaction terminated by the addition of 50µl Fast blue solution (0.023g of Fast Blue Salt in 2.25ml $_dH_2O$ + 5.25ml of 5% SDS). The assay was left five more minutes before being read at 570nm. A blank was set up with 20µl distilled water instead of homogenate.

For the PNPA assay duplicates of 10µl of mosquito homogenate were added to the wells of a microtitre plate followed by 200µl 1mM para-nitrophenyl acetate in 50mM sodium phosphate buffer pH7.4. A blank was set up using 10µl distilled water instead of mosquito homogenate. The assay was then read at 405nm continuously for 6 minutes.

2.2.4.3. Glutathione Transferase Activity

Glutathione transferase (GST) activity was measured using chlorodinitrobenzene (CDNB). Duplicates of 10µl mosquito homogenate (or distilled water for the blanks) were set up and 200µl 0.1M phosphate buffer pH6.5 containing 3mM chlorodinitrobenzene and 10mM reduced glutathione added to each well. The assay was allowed to stand for 1 minute and then read at 340nm continuously for five minutes.

2.2.4.4. Cytochrome P450 Activity

Cytochrome P450 levels were determined using haem peroxidase. Duplicates of 20µl mosquito homogenate or distilled water as a blank were added to wells of the microtitre plate and 200µl 8mM tetramethylbenzidine in 0.25M sodium acetate buffer pH5.0 added. To each replicate 25µl 3% hydrogen peroxide was added and the mixture left at room temperature for 2 hours before absorbance was read at 650nm.

2.3. Results

2.3.1. Larval Bioassays

Three strains were tested for resistance to the larvicide temephos; the New Orleans susceptible strain, the Cayman Islands wild type and the Cayman Resistant strain (KYR).

Experiments were carried out in parallel with untreated control batches and no mortality was observed in any controls during the course of these tests (n=173) (Appendix 1). The dose response curves are shown in Figure 2.1. The parental Cayman Strain had a resistance ratio (RR) of 1.6 at the LC_{50} compared to the susceptible New Orleans strain. The RR increased to 2.7 fold in the selected KYR strain vs NO (Table 2.1). No significant difference was observed in the LC_{90} between New Orleans and the parental Cayman strain. The KYR : Cayman resistance level ranged from 1.6 to 1.9 at LT_{50} and LT_{90} respectively.

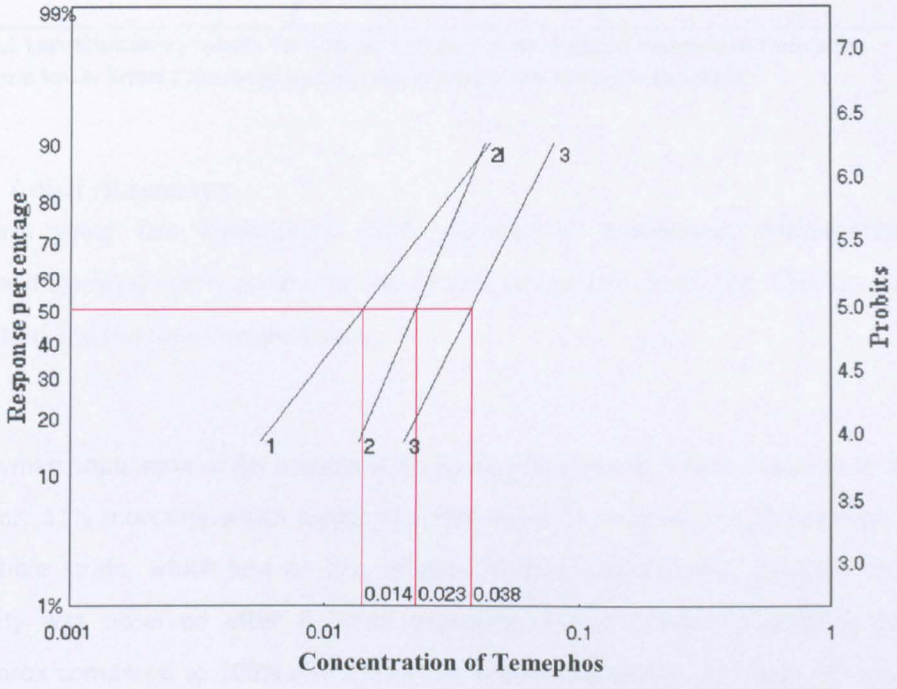


Figure 2.1 Probit analysis of dose response of different strains of *Ae. aegypti* exposed to temephos.
New Orleans (1), Cayman Parental (2) and KYR (3). Red lines indicate LC_{50} values.

Strain	N	LC_{50} mg/L	RR	LC_{90} mg/L	RR
New Orleans	315	0.014 (0.012-0.017)	1	0.045 (0.035-0.064)	1
Cayman	427	0.023 (0.021-0.025)	1.64	0.043 (0.039-0.049)	0.96
KYR	180	0.038 (0.032-0.043)	2.71	0.081 (0.069-0.102)	1.8

Table 2.1 Larval bioassay results for different strains of *Ae. aegypti* exposed to temephos. Upper and lower limits calculated by Ldp Line software are shown in brackets.

2.3.2. Adult Bioassays

Bioassays using five insecticides (DDT, permethrin, etofenprox, deltamethrin and lambdacyhalothrin) were conducted on adult mosquitoes from the Cayman parental population and the New Orleans strain.

The Cayman population of *Ae. aegypti* was able to withstand an 8 hour exposure to 4% DDT with only 11% mortality which suggests a high level of resistance in comparison to the susceptible strain, which had an LT_{50} of approximately 34 minutes. Similarly only 17% mortality was observed after 8 hours exposure of the Cayman population to 0.5% etofenprox compared to 100% mortality after one hour exposure for New Orleans strain (Appendix 2). Exposures longer than 8 hours were not performed and therefore it was not possible to determine RR for these two insecticides.

The Cayman Islands *Ae. aegypti* are resistant to the three pyrethroids with resistance ratios at the LT_{50} ranging from 30-fold to 128 fold (Table 2.2). However, it should be noted that the sample size was low for some insecticides due to limited availability of mosquitoes. Furthermore accurate estimates of the RR for lambdacyhalothrin were difficult as the New Orleans strain is extremely sensitive to this insecticide.

Insecticide	Strain	n	LT_{50} (minutes)	RR	LT_{90} (minutes)	RR
Permethrin (0.75%)	New Orleans	265	1.2 (0.05-2.6)	1	7.1 (4.0-10.1)	1
	Cayman	331	127.8	108.8	3077.2	434.3
Deltamethrin (0.05%)	New Orleans	88	1.8	1	6.1	1
	Cayman	106	53.5 (37.3-71.7)	29.9	176.9 (110.9-897.1)	29.1
Lambda-cyhalothrin (0.05%)	New Orleans*	100	-	-	<5.0	1
	Cayman	143	100.2 (86.7-114.2)	-	206.3 (170.3-285.7)	>41.2

Table 2.2 Bioassay results for New Orleans and Cayman populations of *Ae. aegypti* exposed to pyrethroids.
Upper and lower limits calculated by the Ldp Line software are shown in brackets. *The New Orleans strain was killed very rapidly by Lambda-Cyhalothrin making it difficult to calculate an accurate resistance ratio for this insecticide.

2.3.2.1. Effect of Synergist

Cayman strain mosquitoes were pre-exposed to piperonyl butoxide before being held for two hours (the approximate LT_{50}) on permethrin impregnated papers. A control group pre-exposed to papers containing carrier oil only were also exposed to permethrin for two hours. On average the group pre-exposed to PBO resulted in 37% mortality after the 24 hour holding period (n=110), while the group exposed to permethrin only had a 26% mortality (n=104); a chi squared test showed that this difference is not significant (P=0.08).

2.3.3. Biochemical Assays

In the insensitive acetylcholine assay remaining AChE activity after addition of propoxur was less than 30% for all individuals, suggesting that this is not a major resistance mechanism in the Cayman Islands population (Figure 2.2). A one way ANOVA test determined there was no significant difference in the percentage of remaining AChE activity in the three strains (New Orleans and Cayman strain n=49, KYR n=50, F=1.386, P=0.2535).

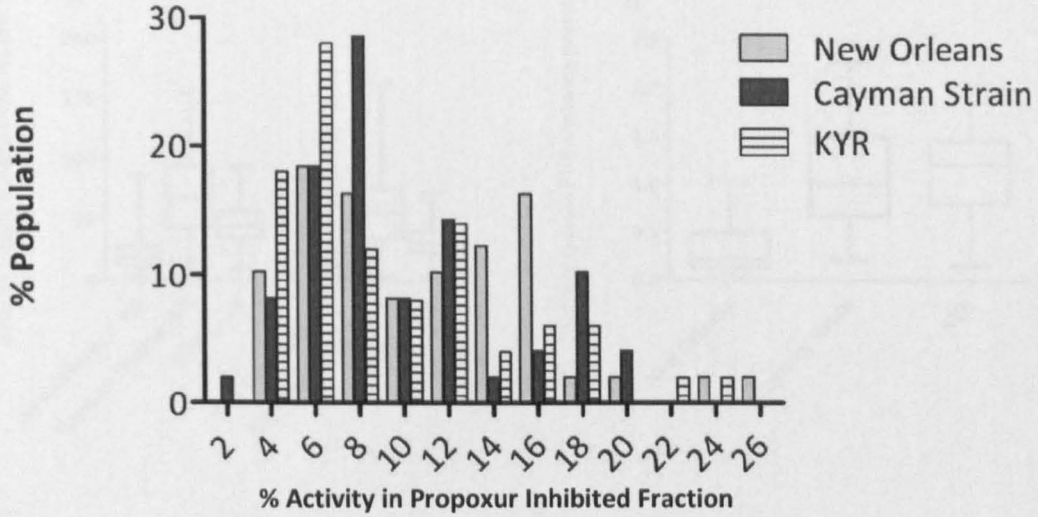


Figure 2.2 Histogram of acetylcholinesterase activity in the presence of propoxur.
In all populations remaining AChE activity was less than 30% for all individuals, suggesting that insensitive acetlycholinesterase is not a major resistance mechanism in the Cayman Islands.

Elevated levels of esterases (with all three substrates; α and β naphthyl acetate and PNPA), cytochrome P450s and GSTs were found in the Cayman population compared with the susceptible New Orleans strain (Figure 2.3). The greatest difference was observed in PNPA activity with a 3.3 fold difference in mean activity between New Orleans and Cayman strain (Appendix 3). GST activity and esterase activity with PNPA were elevated in the KYR temephos selected strain compared to the parental Cayman strain. Esterase activity as measured using naphthyl acetate and total P450 activity were lower in the KYR strain compared to the Cayman strain. For all substrates the difference in activity between strains was significantly different when analysed by One Way ANOVA ($P<0.0001$) with the exception of PNPA (Appendix 4). Tukey's Multiple Comparison test confirmed significant difference between Cayman strain and New Orleans and New Orleans and KYR, however there was no significant difference between Cayman strain and KYR using this test.

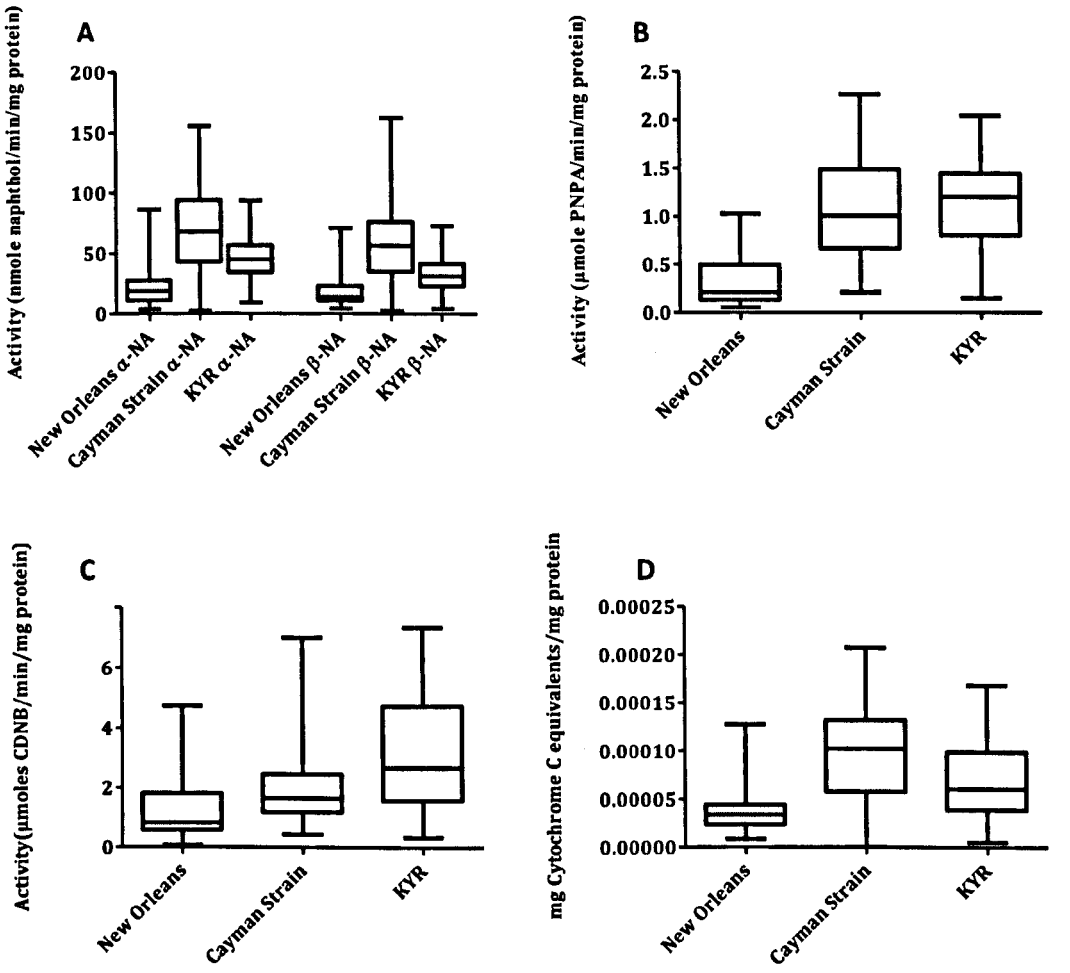


Figure 2.3 Box plots of results from biochemical assays.
Median activity is shown by a horizontal bar, the box denotes upper and lower quartiles. Vertical lines show the full range of the data set.
Panel A = Esterase assay using α and β naphthol Panel B = Esterase assay using PNPA
Panel C = GST assay using CDNB Panel D = P450 assay using haem peroxidase
Results are expressed as µmole/min/mg protein with the exception of the P450 Assay, which is expressed as mg of Cytochrome C equivalents/mg protein.

2.4. Discussion

The *Ae. aegypti* population in the Cayman Islands is highly resistant to DDT and pyrethroid insecticides. DDT resistance was first reported in the Caribbean in the 1950s and contributed to the failure of the *Ae. aegypti* eradication campaign (Brown & Pal, 1971, Brown, 1986). Resistance to DDT has persisted in the region despite the fact that the use of this insecticide for *Aedes* control was largely phased out in the 1960s when organophosphate insecticides became available. It is possible that DDT resistance is being maintained in the population by selection with pyrethroid insecticides as both share the same target site. The presence of etofenprox resistance in Grand Cayman may also be attributed to cross resistance from sharing of the target site. This compound has not been

used in the Cayman Islands for control of *Ae. aegypti*, but high levels of resistance were found.

The level of resistance to pyrethroids in the Cayman Islands population is particularly high. The discriminating doses for adult *Ae. aegypti* set by the WHO are a 1 hour exposure to 0.25% permethrin or 0.03% lambda-cyhalothrin (no discriminating doses have been established for deltamethrin or etofenprox for *Ae. aegypti*) (http://www.who.int/whopes/resistance/en/discriminating_concentrations.pdf). In this study less than 80% mortality was observed after a 1 hour exposure to higher concentrations of insecticide (0.75% permethrin and 0.05% lambda-cyhalothrin) and hence the Cayman Islands population would clearly be defined as pyrethroid resistant by WHO standards. When compared with the susceptible New Orleans strain, the resistance ratios of the Cayman Islands population are 29- to 434-fold at the LT_{90} and these resistance levels are higher than reported in neighbouring islands in the Caribbean. For example, resistance ratios of 4.7-fold to deltamethrin were reported in *Ae. aegypti* from Cuba in 2001 (Rodriguez *et al.*, 2001) and 35-fold resistance to permethrin was recorded in a population from Martinique in 2003 (Brengues *et al.*, 2003). However, care should be taken when comparing resistance ratios between different studies as the value obtained will be dependent on the susceptible strain used.

The Cayman Islands population of *Ae. aegypti* is not as resistant to temephos (LC_{50} 0.023 mg/L) as populations from Cuba (LC_{50} 0.0713mg/L) (Rodriguez *et al.*, 2001), and British Virgin Islands (LC_{50} 0.0603mg/L) (Wirth & Georghiou, 1999). The WHO discriminating dose for temephos for *Ae. aegypti* is 0.012 mg/L so the Cayman population would be classified as 'resistant to temephos'. However, in this study, the New Orleans laboratory susceptible strain had an LC_{50} of 0.014 and so would also be classified as resistant. The field dose of temephos is 1mg/L (WHO, 2009) and, at this concentration, 100% mortality would be expected in the Cayman population and hence temephos resistance is unlikely to be of operational significance at present.

It is common in resistance studies for resistance ratios to be quoted. Care must be taken in this case as the values can vary dependant on the susceptible strain used. Even within strains >2-fold variations in LC_{50} have been reported (Table 2.3). The high LC_{50} seen in the

New Orleans strain could be due to strain contamination although this is unlikely to have occurred during the course of this study as Cayman and New Orleans individuals were not reared alongside each other, eggs were hatched separately and bioassays carried out several weeks apart.

Strain	LC_{50}	Reference
Rockefeller	0.0023	da Grao Macoris (2007)
Rockefeller	0.003	Beserra (2007)
Rockefeller	0.0054	Ponlawat (2005)
Rockefeller	0.0059	Harris (2010)
Rockefeller	0.00364	Rodriguez (2002)
Bora	0.0063	Jirakanjanakit (2007)
Bora	0.0037	Marcombe (2009b)

Table 2.3 Temephos LC_{50} values for laboratory susceptible Rockefeller and Bora *Ae. aegypti* strains as determined in different studies.

No evidence for insensitive acetylcholinesterase was detected in the Grand Cayman *Ae. aegypti* population therefore this mechanism was not investigated further. Biochemical assays indicate elevated levels of all three of the major detoxification enzyme families in the Cayman Islands population relative to the New Orleans strain. However, pre-exposure to the synergist PBO, which acts as a general inhibitor of cytochrome P450s and esterases, (Khot *et al.*, 2008, Sun & Johnson, 1960) did not significantly increase the level of permethrin-induced mortality. PBO was the only synergist used in this study, it was chosen as preliminary microarray data identified an upregulation of P450s (from the CYP9 and CYP6 families) in *Ae. aegypti* from Grand Cayman compared to the New Orleans strain (Vassia Bariami, University of Crete, unpublished). Other synergists such a DMC (a GST inhibitor) were not tested.

The synergist data obtained suggests that enhanced metabolism due to expression of P450s is not a major cause of permethrin resistance in this population and it is possible that the elevated levels of P450 observed may be caused by differences between the Cayman and New Orleans strains that are unrelated to their resistance status. However, recent studies using the *Ae. aegypti* Detox chip have identified elevated expression of CYP9, P450s and Epsilon GSTs in multiple pyrethroid-resistant strains (Rajatileka, unpublished, Strode *et al.*, 2008). Further transcriptomic and metabolism studies are needed to determine whether

metabolic resistance is contributing to the resistance phenotype in the Cayman Islands population.

3. Detection of *Kdr* in the Caribbean

3.1. Introduction

Amino acid substitutions at five residues have been previously reported in the *Ae. aegypti* sodium channel (Figure 3.1). Four of these variable sites are found in the domain II S6 region (residues 923, 982, 1011 and 1016) and the fifth is located in the linker between the S5 and S6 region of domain IV at residue 1763 (Brengues *et al.*, 2003, Chang *et al.*, 2009, Saavedra-Rodriguez *et al.*, 2007) (numbering of residues is based on the reference sequence from *Musca domestica* (Williamson *et al.*, 1996)). The evidence linking some of these mutations with resistance to insecticides is circumstantial; G923V, L982W and D1763Y have been identified in resistant strains but a causal link between the substitutions and resistance has not been shown (Brengues *et al.*, 2003, Chang *et al.*, 2009). However, the V1016I and I1011M substitutions have been linked to resistance to pyrethroids through comparing allele frequencies in susceptible and resistant populations (Martins *et al.*, 2009b, Saavedra-Rodriguez *et al.*, 2007).

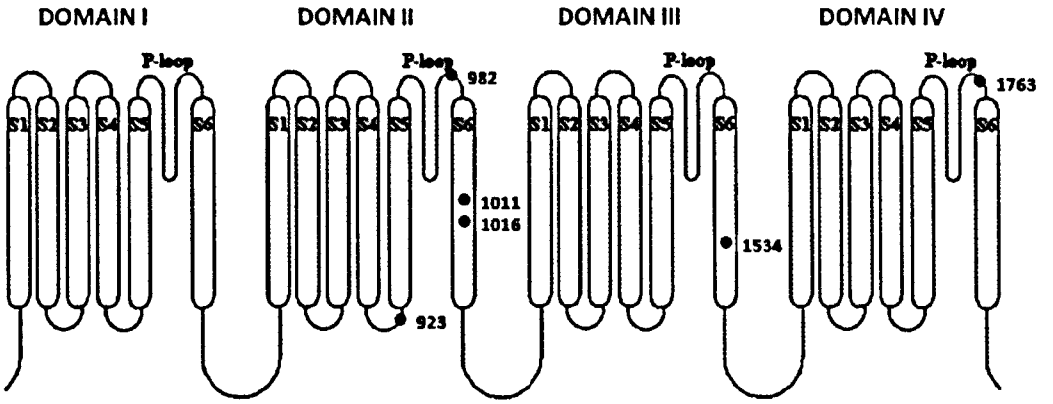


Figure 3.1 Schematic of the transmembrane voltage-gated sodium channel.
The unit consists of four homologous domains, each comprising six transmembrane helices (S1-S6). The positions of those residues where substitutions have been reported are shown. Adapted from O'Reilly (2006).

Pyrethroid resistance is widespread in *Ae. aegypti* in the Caribbean having been reported in Puerto Rico, The Dominican Republic, The British Virgin Islands, Cuba (Hemingway *et al.*, 1989, Mekuria *et al.*, 1991, Rodriguez *et al.*, 2001, Wirth & Georghiou, 1999) and Martinique for which the V1016I substitution has been implicated (Brengues *et al.*, 2003, Marcombe *et al.*, 2009b). The V1016I substitution also has a widespread distribution across Latin America and has been identified in *Ae. aegypti* populations in Brazil, Nicaragua, Costa

Rica, Panama, Venezuela and Mexico (Garcia *et al.*, 2009, Martins *et al.*, 2009a, Saavedra-Rodriguez *et al.*, 2007). An alternative mutation at this residue, V1016G, has previously only been reported in Asian populations of *Ae aegypti.* The two alternative substitutions at I1011; I1011M and I1011V, have both been reported in Latin America (Rajatileka *et al.*, 2008, Saavedra-Rodriguez *et al.*, 2007).

Models of the insect sodium channel have facilitated predictions of residues involved in insecticide binding and hence possible sites for resistance mutations (Figure 3.2). The 3-dimensional model predicts that substitutions at residues in the S4-S5 linker in domain II (Residue 918) and the domain II S5 (Residues 922, 925, 929, 932, 933, 936) and domain III S6 region (Residues 1530, 1534, 1538) are most likely sites to affect insecticide binding. Note that neither the 1011 or 1016 residues, implicated in resistance in *Ae. aegypti*, or the 1014 residue which is one of the most commonly mutated residues in other pyrethroid resistant insects (O'Reilly *et al.*, 2006) lie within this putative binding site.

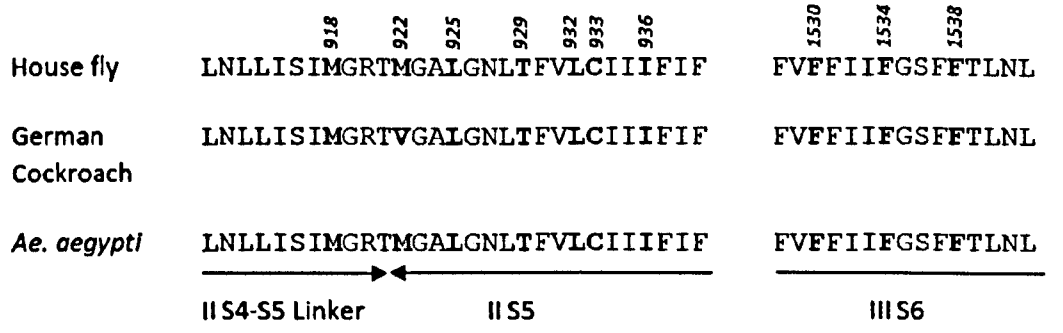


Figure 3.2 Sequence alignments of the predicted residues thought to be involved in insecticide binding. Adapted from O'Reilly (2006).

In order to identify mutations in the *Ae. aegypti* sodium channel putatively associated with resistance in the Cayman Islands population, residues within regions of the gene encompassing the putative insecticide binding channel were amplified and sequenced. Genotyping assays were then used to determine association between genotype and phenotype.

The spread of *Ae. aegypti* is often attributed to growing international traffic. With populations readily traversing the globe it is possible that they carry with them any number of resistance alleles that may be present at their point of origin. Whether mutant alleles

arise as a result of local selection pressures or are a result of migration of resistant insects deserves further scrutiny. This information could be used in combination with monitoring tools to make informed decisions on how to create appropriate strategies for control. This is particularly pertinent to *Ae. aegypti* control in the Cayman Islands as there is no significant farming or industry; therefore all supplies, construction materials, vehicles and household goods must be shipped in from overseas. It is undoubtedly through one of these routes that *Ae. aegypti* arrived on the island. A number of populations collected from different locations within the Caribbean were screened for mutant alleles in the voltage-gated sodium channel. Intron 21 (Chang *et al.*, 2009) of the voltage-gated sodium channel was sequenced to compare genetic backgrounds in different populations to determine whether mutations associated with resistance have occurred many times concurrently or whether they arise just once and spread.

3.2. Methods

3.2.1. Origin of Strains

Several collections of *Ae. aegypti* were made in Grand Cayman (Figure 3.3). For the majority of collections, larval samples were collected from the field, pooled and reared to adulthood in the insectary at the MRCU. The exception was the Seven Mile Beach collection where samples were collected as adults using a BG Sentinel Trap (Biogents, Germany). Details of these collections can be found in Table 3.1. *Ae. aegypti* eggs were supplied from additional sites within the Caribbean (Figure 3.4) with the exception of Martinique where adults were supplied. All egg samples were reared to adulthood in the MRCU facility before use in this study.

Historical samples from Grand Cayman came from a collection by David Malone (MRCU); larvae were collected from Diaz Lane (19°18'02"N 81°22'50"W) in George Town on 21st August 2003; a little over a year after *Ae. aegypti* is thought to have been reintroduced to Grand Cayman. The larvae were preserved in ethanol until their use in this study.

Sample Set	From	Approximate GPS	Collection Date	Provided by
East End	16 containers from 10 yards within Sea View Road and John Maclean Drive	19°17'53"N, 81°06'28"W	March 2008	
George Town	A Drainage System on the Industrial Estate and The Centre of 'Town'	19°17'52"N, 81°21'46"W and 19°17'32"N, 81°22'52"W	February 2008 and January 2009	
West Bay	17 Containers from 6 yards, in North West, West Bay	19°23'11"N, 81°23'41"W	February 2008	
Seven Mile Beach	The Cayman Beach Suites, Dive Shop	19°19'39"N, 81°22'56"W	October 2007 and March 2008	
Rackleys	6 containers from 5 yards within the North Sound Estates	19°17'53"N, 81°17'49"W	February 2008	
Havana	11 Neighbourhoods of the City of Havana		2008 (supplied March 2009)	Dr Magdelena Rodriguez, Instituto "Pedro Kourí", Havana Cuba.
Jamaica	19 Locations in the Parish of St James		July 2009	Sherine Huntley and Syddonna Brown Grizzle, Vector Control Program, Ministry of Health, Jamaica
Tampa Bay	East Bradenton, Bradenton	27°29'08"N 82°33'16"W	May 2008 and July 2009	Mark Latham and Gail Stout, Manatee County Mosquito District
Miami	Olympia Heights, Miami	25°44'13"N 80°22'59"W	July 2008	Sandra Fisher, Chalmers Vasquez and Mario Porcelli, The Public Works Division, Miami-Dade County
Florida Keys	Stock Island	24°34'02"N, 81°44'18"W	March 2008	Andrea Leal, The Florida Keys Mosquito District, Key West
Puerto Rico	Reparto Metropolitano, San Juan	18°23'44"N, 66°04'39"W	April 2008	Roberto Barrera and Manuel Amador, Dengue Branch CDC, Puerto Rico
Honduras	La Ceiba airport and El Confite Siete, La Ceiba	15°44'30"N, 86°51'22"W 15°44'56"N, 86°51'34"W	July and September 2008	Oscar Urrutia, Ministry of Health, Honduras, Zoila Ebanks (MRCU) and Nora Andrade
Martinique	Lamentin and Forte-de-France	14°36'01"N, 60°59'59"W and 14°36'33"N, 61°04'22"W	June 2009	Vincent Corbel, Institut de Recherche pour le Développement, France

Table 3.1 Origins of *Ae. aegypti* strains used in this study.

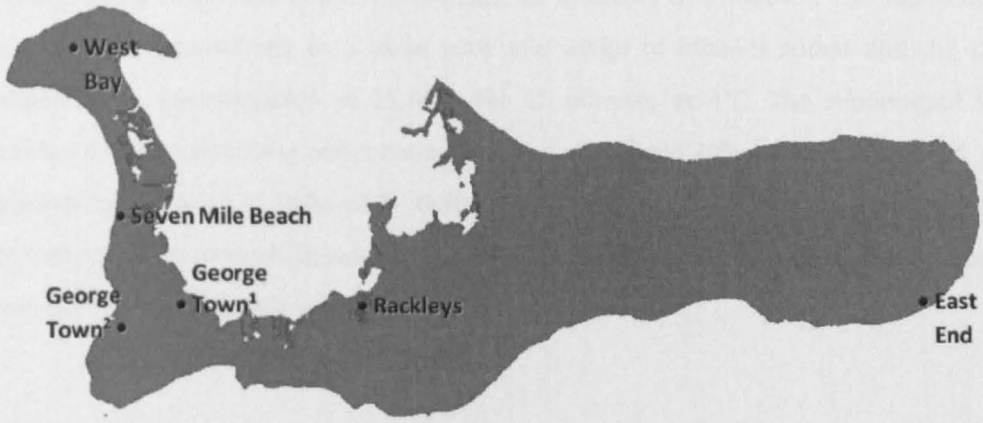


Figure 3.3 A map of collection sites in Grand Cayman.
Two collections were made in George Town denoted by the numbers in superscript.

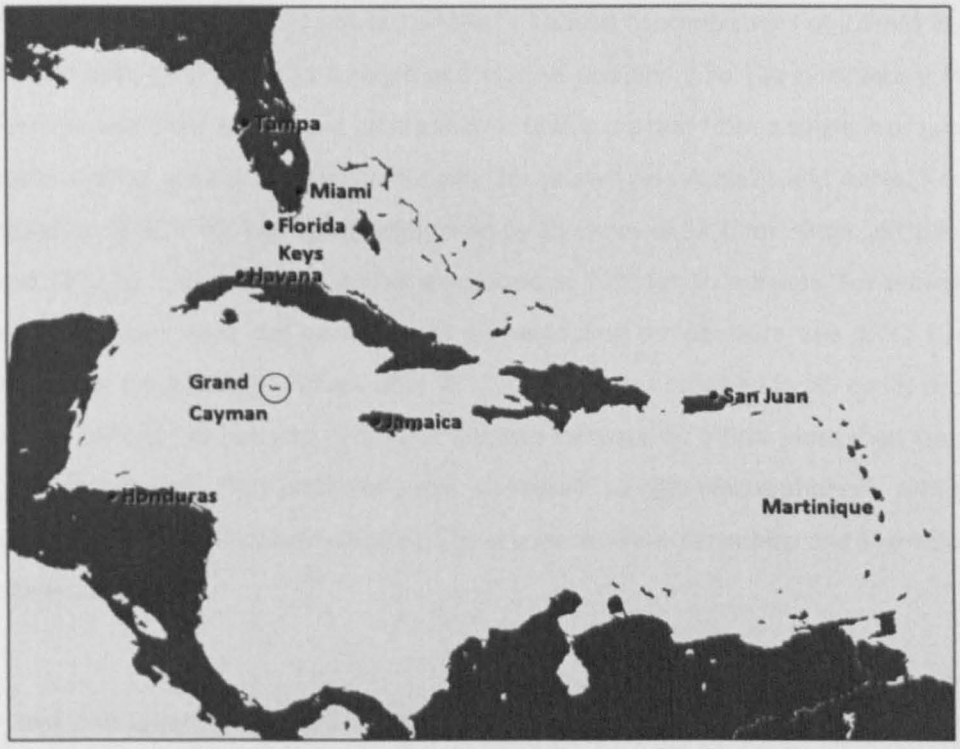


Figure 3.4 A map of collection sites for Caribbean populations used in this study.

3.2.2. Extraction of DNA

DNA extraction was carried out as per the methods of Livak (1984). Single mosquitoes were ground in 100µl of preheated Livak buffer (80mM NaCl, 0.16M Sucrose, 100mM Tris-HCl, 50mM EDTA and 0.5% SDS) and incubated at 65°C for 30 minutes. Potassium acetate (8M) was added to a final concentration of 1M and incubated on ice for a further 30 minutes. The contents were centrifuged at 15,800g for 20 minutes at 4°C. The supernatant was

transferred to a clean tube and re-centrifuged for a further five minutes. The supernatant was once again transferred to a clean tube and 200µl of ethanol added and the DNA precipitated by centrifugation at 15,800g for 15 minutes at 4°C. The supernatant was discarded and the remaining pellet rinsed in 100µl of ice cold 70% ethanol. The pellet was dried and resuspended in 100µl of TE buffer containing 10µg/ml RNAse A (Sigma-Aldrich). Fifty individuals from each population were selected for analysis, except in the case of Miami where only 30 individuals were available.

3.2.3. Identification of *kdr* Alleles

A section of exons 21, 22 and 31 which encode domain II S4, S5 and S6 as well as domain III S6 (Figure 3.5) were sequenced from 10 individuals of known resistance phenotype from the Cayman population to identify mutations that may be associated with resistance. PCR reactions were carried out in a volume of 25µl with final concentrations of 2.5mM $MgCl_2$, 0.2-0.4mM each dNTPs, 0.5µM forward and reverse primers, 2.5u *Taq* polymerase (Kapa Biosystems) and 1 per cent of the total genomic DNA extracted from a single mosquito as template. Cycling conditions were as follows: for primer sets AaNa21 and AaNa22 initial denaturation of 95°C for five minutes followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 minute, then a final elongation at 72°C for 10 minutes. For primer set AaNa31 conditions were the same except the annealing temperature was 59°C. Cycling conditions for the Ae2122a primers were 95°C for 5 minutes followed by 35 cycles of 94°C for 30 sec, 60°C for 45 sec and 72°C for 2 minutes followed by a final elongation stage of 72°C for 7 minutes. PCR products were visualised by gel electrophoresis and then sequenced directly by Macrogen, (Korea). The sequences were assembled and aligned using Lasergene (DNAstar).

Only two non-synonymous single nucleotide polymorphisms were identified, a G to A substitution in exon 22 resulting in V1016I and a T to G substitution in exon 31 resulting in F1534C.

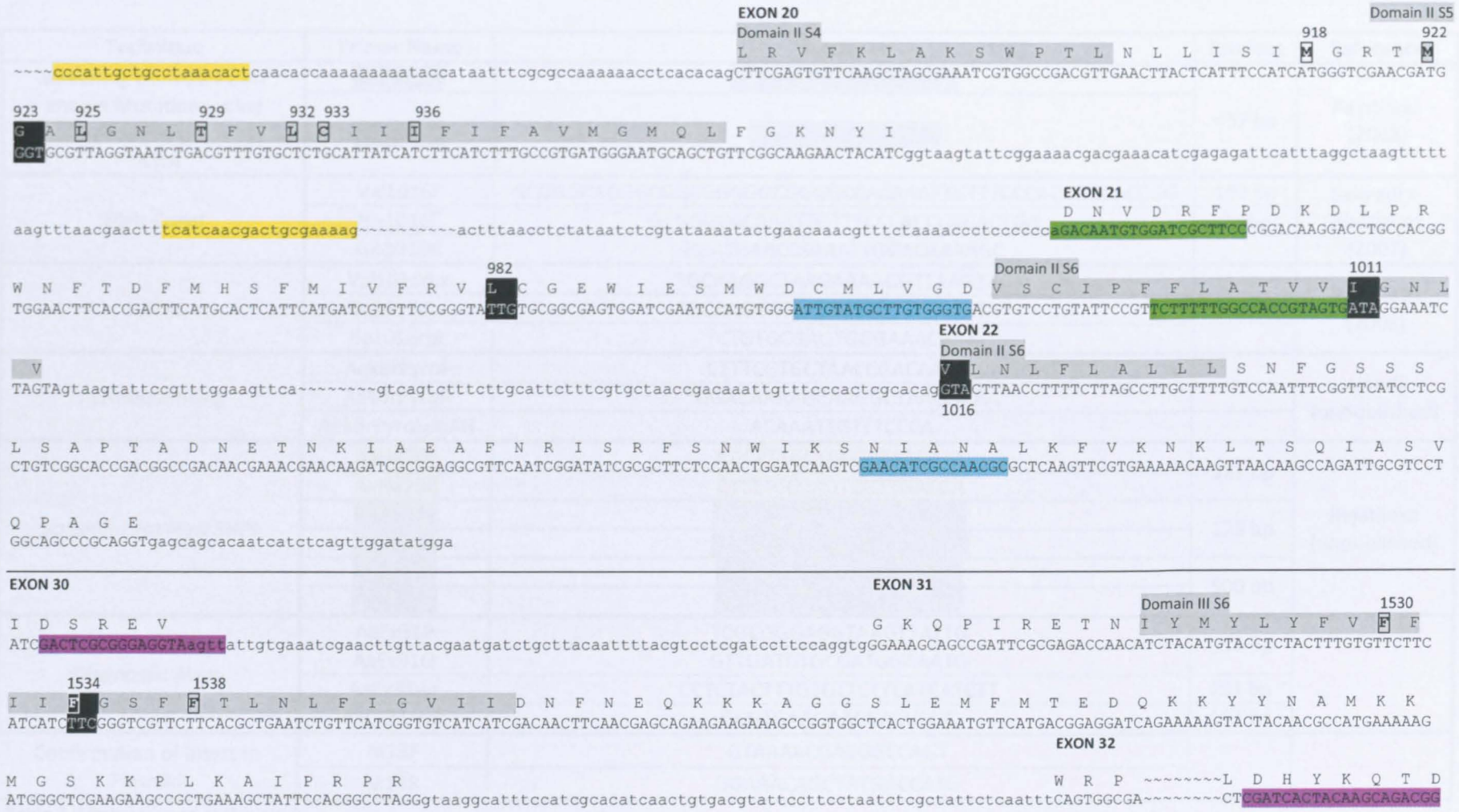


Figure 3.5 Partial sequencing of the voltage-gated sodium channel, showing exons 20-22 (domain II S4-S6) and exon 31-32 (domain III, S6).
Exons are shown in capital letters, introns in lower case. Transmembrane domains are highlighted in grey and amino acids putatively associated with resistance are highlighted in black. Residues predicted to play a role in insecticide binding are bold in boxes. Primer sequences are colour coded as in Table 3.2

Technique	Primer Name	Sequence	Product	Reference
Screening Exon 21-22 for known Mutations using Automated Sequencing/ HOLA	Ae2122aF	ATTGTATGCTTGTGGGTG	457 bp	Rajatileka (2008)
	Ae2122aR	GCGTTGGCGATGTTC		
Melt Curve	Val1016F	GCGGGCAGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCCACCCGCACCGG	102 bp	Saavedra-Rodriguez (2007)
	Iso1016F	GCGGGCACAAATTGTTTCCCACCCGCACTGA	82 bp	
	Iso1016R	GGATGAACCSAAATTGGACAAAAGC		
HOLA	Val1016dtc	*GCAAGGCTAAGAAAAGGTTAAGTAC		Rajatileka (2008)
	Ile1016dtc	*GCAAGGCTAAGAAAAGGTTAAGTAT		
	Ile1016rpt	§CTGTGCGAGTGGGAAACAAT		
Pyrosequencing	AekdrPyroF	CTTTCGTGCTAACCGACAAATT		Wondji (unpublished)
	AekdrPyroR	*GGACAAAAGCAAGGCTAAGAAAA		
	AekdrPyroSeqAH	ACAAATTGTTTCCCA		
Screening for New SNPs	AaNa20F	CCCATTGCTGCCTAAACACT	321 bp	Rajatileka (unpublished)
	AaNa20R	CTTTTCGCAGTCGTTGATGA		
	AaNa21F	AGACAATGTGGATCGCTTCC	175 bp	
	AaNa21R	CACTACGGTGGCCAAAAAGA		
	AaNa31F	GACTCGCGGGAGGTAAGTT	500 bp	
	AaNa31R	CCGTCTGCTTGTAGTGATCG		
Diagnostic Assay	AaEx31P	TCGCGGGAGGTAAGTTATTG	350 bp	
	AaEx31Q	GTTGATGTGCGATGGAAATG		
	AaEx31wt	CCTCTACTTTGTGTTCTTCATCATCTT	231 bp	
	AaEx31mut	GCGTGAAGAACGACCCGC	163 bp	
Confirmation of Insert in Plasmid	M13F	GTAAAACGACGGCCAGT		
	M13R	GGAAACAGCTATGACCATG		

Table 3.2 Primers used in this study.
*** denotes 5' Biotin label § denotes 5' Phosphorylation, 3' Fluoroscein. Positions of shaded primer sets can be seen on Figure 3.5.**

3.2.4. Genotyping for Codon 1016

Several different methods have been described to genotype the 1016 codon in *Ae. aegypti*. The following methods were tried in the current study.

3.2.4.1. Melt curve

The melt curve method (Saavedra-Rodriguez *et al.*, 2007) uses two forward primers, one with a long tail extension and one with a short tail extension, and a common reverse primer. The forward primers (Table 3.2) differ at the mutation point which falls at the 3' end. The primer for the wild type allele has a gc rich 26 base pair tail at the 5' end while the primer for the mutant allele has a short 6 base pair tail in order that the difference between wild type and mutant alleles can be resolved on an electrophoresis gel. PCR reactions were carried out in 25µl volumes each containing 3mM $MgCl_2$, 0.2mM each dNTPs, 1µM each primer, 0.625 units of *Taq* polymerase and 1% of the total extracted DNA from each single mosquito. Cycling conditions were 95°C for 7 minutes followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec followed by a final elongation stage of 72°C for 7 minutes. Products were resolved on a 4% agarose gel and a 20 bp DNA ladder (Sigma-Aldrich) was used for sizing.

The same primers were also used in a real-time fluorescent assay. IQ SYBR Green Supermix (BIO-RAD) was used at 1x concentration with 1µM each primer and 0.5% of the total extracted DNA from one mosquito to a total volume of 25µl. Conditions for cycling were 95°C for 12 minutes followed by 40 cycles of 95°C for 20 seconds, 60°C for 1 minute and 72°C for 30 seconds followed by an extension step of 72°C for 5 minutes. Results were visualised using MJ Opticom Monitor Version 3.1.

3.2.4.2. HOLA- Hot Oligonucleotide Ligation Assay

The Hot Oligonucleotide Ligation Assay (Rajatileka *et al.*, 2008) is a colorimetric method able to ascertain allele type by a colour change as a result of binding by a specifically labelled probe. Template DNA was first amplified using primers Ae2122aF and Ae2122aR conditions as described previously (Chapter 3.2.3). This product was then used as template in the hot ligation step.

A separate reaction was set up for each allele comprising 1x Ampligase buffer (Epicentre® Biotechnologies), 1μM each detector (dtc) and reporter (rpt) probes as described in Table 3.2 (the reporter is common to both wild type and mutant alleles), 1 unit of Ampligase and 3μl of product from the Ae2122a PCR in a 20μl reaction. Ligation conditions were 95°C followed by 25 cycles of 94°C for 1 minute and 62°C for 2 minutes.

Ligated product was hybridised to Even Coat™ Streptavidin Microplates (R&D Systems). Samples positive for a particular allele were detected using anti-fluorescein antibody and 100μl TMB Blue Pod Substrate (Roche). Colour change was scored after five minutes.

3.2.4.3. Pyrosequencing

PCR was carried out in 15μl volumes containing 2.75mM $MgCl_2$, 0.2mM each dNTPs, 0.3mM each primer (Table 3.2), 1.25u hot start *Taq* polymerase (Qiagen) and 1 percent of the total genomic DNA extracted from a single mosquito as template. Cycling conditions were 95°C for 15 minutes followed by 30 cycles of 94°C for 25 seconds, 60°C for 25 seconds and 72°C for 30 seconds and a final elongation step of 72°C for 10 minutes.

PCR products were immobilised onto streptavidin coated sepharose beads and 0.4mM sequencing primer added. Strand separation was carried out by heating to 80°C for 3 minutes. Qiagen PyroMark Gold reagents were set up as per instructions from the SQA Software and run on the Pyromark PSQ™ 96MA system.

3.2.5. Development of a Genotyping Assay for the F1534C Mutation

A tetra primer PCR assay was designed to genotype mosquitoes at the 1534 codon (Figure 3.6). In this assay, the flanking primers amplify a control band of 350 bp. Two internal allele specific primers were designed to give PCR products of either 231 bp ('wild type' phenylalanine allele) or 167 bp ('mutant' cysteine allele) by forming PCR primer pairs with the flanking primers. Each PCR reaction (25μl) contained 2.5mM $MgCl_2$, 0.4mM each dNTPs, 0.5μM each primer, 2.5u *Taq* polymerase, and 1 percent of the total genomic DNA extracted from a single mosquito as template and the cycling conditions were 95°C for five minutes followed by 35 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec, and a final elongation at 72°C for 10 minutes. PCR products were resolved on a 2% agarose gel and a 100 bp ladder (Bioline Hyperladder IV) was used for sizing.

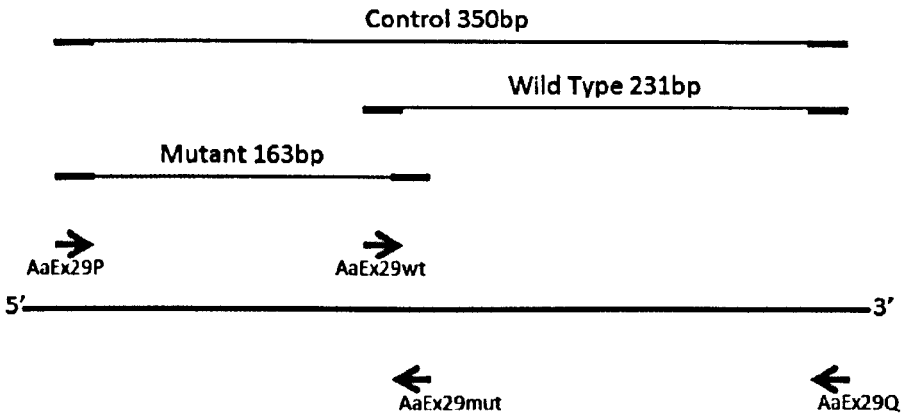


Figure 3.6 Diagnostic PCR for identifying the F1534C mutation.
Flanking primers amplify a 350bp control band. Internal allele specific primers amplify 'wild type' (231bp) and/or 'mutant' (163bp) bands by pairing with the flanking primers.

After validating this test on samples of known sequence it was used to genotype 145 mosquitoes collected from distinct areas around Grand Cayman, 6 historical samples collected in 2003 and 215 samples from different parts of the Caribbean.

3.2.6. Genotyping of Phenotyped Material

An additional 200 mosquitoes that had been exposed to permethrin (2 hours) or DDT (24 hours) were also genotyped for both V1016I and F1534C mutations to test for genotype : phenotype association. Tests for Hardy Weinberg Equilibrium and linkage disequilibrium of the two alleles were performed using Genepop Version 4.0 (Raymond & Rousset, 1995).

3.2.7. Intron Sequencing

The intron between exons 21 and 22 was amplified from 24 Cayman Islands and 18 Caribbean samples using the Ae2122a primers and conditions as described previously (Chapter 3.2.3). Direct sequencing of the PCR product was performed by McLab, (San Francisco, CA) and Macrogen USA (Rockville, MD).

3.2.8. Cloning

Cloning was carried out on samples where direct sequencing did not yield good quality data in both directions. The PCR product from amplification of the intron was quantified using a nanodrop (Thermo Scientific) and ligated into pGem®-T Easy Vector using the pGem®-T

Easy Vector Ligation System (Promega). This system requires a 1:3 molar ratio of vector to insert. In order to calculate this, the following equation is used.

$$\frac{\text{Length of Insert (Kb)}}{\text{Length of Vector (Kb)}} \times \text{ng of Vector} = \text{ng of Insert required for a 1:1 Molar Ratio}$$

$$\frac{0.457}{3.015} \times 50 = 7.6\text{ng}$$

Ligation reactions were set up containing 50ng pGem® Vector, 1x Rapid Ligation Buffer, 3 units T4 DNA ligase, and 22.8ng of insert in a final volume of 10µl. Reactions were carried out overnight at 4°C.

Plasmid containing the insert was transformed into competent *E. coli* JM109 cells (Promega), 50µl of cells was added to 2µl ligation reaction and incubated on ice for 20 minutes, cells were heat shocked for 45 seconds at 42°C and returned to ice for 2 minutes. Following this 950µl SOC medium (2% bacto-tryptone, 0.5% yeast, 0.05% NaCl and 20mM glucose) was added and tubes were incubated for 1.5 hours at 37°C with shaking (150 rpm). An aliquot (100µl) of each culture was then plated out onto duplicate LB plates (0.5% Bacto yeast extract, 1% Bacto Tryptone, 1% NaCl and 1.5% Bacto agar) containing 50µg/ml ampicillin and coated with 100µl 50mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in dimethyl-formamide and 10µl 0.1M IPTG (Isopropyl β-D-1 thiogalactopyranoside) and incubated overnight at 37°C. Using this system colonies containing insert are coloured white, whilst those that do not contain insert are dark blue. A maximum of three white colonies from each plate were selected using a sterile pipette tip and spread onto additional LB/ampicillin plates and incubated for 37°C overnight.

Samples were then taken from each colony for use as template in a PCR reaction to confirm the presence of the insert. PCR reactions were carried out in volumes of 15µl containing 3.5mM $MgCl_2$, 0.2mM each dNTPs, 0.3mM M13 Forward and Reverse Primer and 0.4u *Taq* polymerase as well as 1µl of $_dH_2O$ taken from 50µl into which a colony had been inoculated. Cycling conditions were as follows; 95°C for five minutes, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by a final elongation stage of 72°C for five minutes. Product was visualised by electrophoresis on a 2% agarose gel before being sent for sequencing.

3.2.9. Phylogenetic Analysis

Sequences from PCR products and clones were aligned using ClustalW. Phylogenetic trees were generated using MEGA Version 4 (Tamura *et al.*, 2007) Evolutionary distances were calculated by the neighbour-joining method using the Jukes-Cantor algorithm and support determined by 500 bootstrap replicates (Jukes & Cantor, 1969). Maximum parsimony trees were also generated using MEGA Version 4 (500 bootstrap replicates).

3.3. Results

3.3.1. Sequence Analysis and Genotyping

Partial DNA sequencing of the voltage-gated sodium channel identified two amino acid substitutions in the Cayman population compared to the susceptible New Orleans strain. The first, a valine to isoleucine substitution found at codon 1016, domain II, subunit 6, has been reported elsewhere in Latin America (Saavedra-Rodriguez *et al.*, 2007) and shown to be associated with resistance to pyrethroids. The second substitution was at codon 1534 where a single base pair substitution changes the codon from TTC to TGC resulting in a phenylalanine to cysteine substitution in domain III, subunit 6. Substitution at codon 1011 was not detected in the Cayman samples (n=28), however one sample each from Havana (n=5) and Honduras (n=3) were found to be heterozygous for isoleucine and methionine at this locus. Exon assignment is based upon the annotation of the sodium channel gene in Chang (2009).

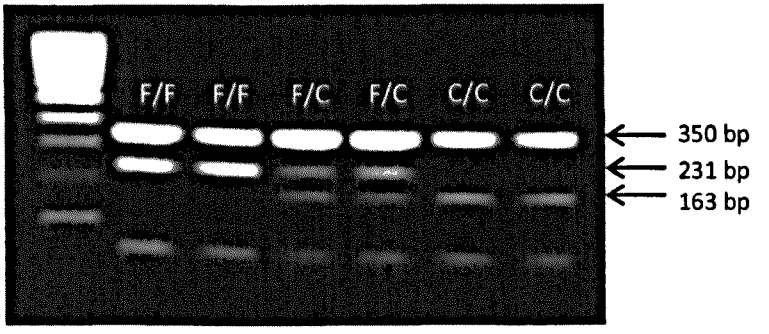


Figure 3.7 An example of the diagnostic assay for identifying genotypes associated with the F1534C mutation.
Lane 1 contains a 100bp ladder, lanes 2-7 contain PCR product from individual mosquitoes displaying the three different genotypes.

Given the importance of S6 in domain III in the binding of pyrethroid insecticides (O'Reilly *et al.*, 2006) it was predicted that this amino acid substitution may be associated with

insecticide resistance. Hence a new, simple, allele specific PCR assay to screen for this mutation in *Ae. aegypti* was developed. The assay works on the same principles as the assay developed by Martinez-Torres (1998) for detecting the L1014F *kdr* mutations in *An. gambiae* and can readily distinguish all three genotypes (SS, RS and RR) (Figure 3.7).

To determine the association between the genotypes at codons 1016 and 1534 and resistance to insecticides, the offspring of adults reared from wild caught *Ae. aegypti* larvae were exposed to either 4% DDT for 24 hours or 0.75% permethrin for 2 hours and 50 surviving and 50 dead mosquitoes (for codon 1016) or 100 surviving and dead (for codon 1534) were genotyped (Table 3.3). Using Fisher's exact test the 1016I mutation was positively associated with permethrin survival (p=0, n=26) but not survival to DDT (p=0.145, n=19). The 1534C mutation was very strongly associated with survival to both insecticides (p=0, permethrin n=50, DDT n=49). Individuals homozygous for both resistance alleles (1016I and 1534C) survived permethrin exposure (P=0.008) but this double homozygous genotype was not associated with DDT survival (P=1.0). In the current study only a very small number of New Orleans samples were genotyped. A single individual was confirmed to be homozygous for the wild type allele at codon 1016 (1016V) and 3 individuals were found to be homozygous for 1534F. However, further work at LSTM genotyped 50 individuals from the New Orleans strain and confirmed the absence of the putative resistance alleles (Hilary Ranson, personal communication).

Pop	1016			Freq of I	P	1534			Freq of C	P	Double Homozygotes	
	V/V	I/V	I/I			F/F	F/C	C/C			V/V F/F	I/I C/C
Permethrin Dead	2	22	0	0.46	0.15	4	35	11	0.57	0.0	2	0
Permethrin Survived	0	12	14	0.77		0	0	50	1.00		0	14
DDT Dead	0	16	7	0.65	0.0	3	20	27	0.74	0.0	0	6
DDT Survived	0	9	10	0.76		0	3	46	0.97		0	9

Table 3.3 Genotypes and allele frequencies for the V1016I and F1534C mutations from *Ae. aegypti* from Grand Cayman that survived or died after 24 hours exposure to DDT or 2 hours exposure to permethrin.

The new tetraplex PCR to detect F1534C and various different assays to detect V1016I were used to determine the frequency of these two substitutions in Grand Cayman and across

the Caribbean. Fifty mosquitoes from three areas of the Island (East End, George Town and West Bay) 50 each from Havana, Jamaica, Tampa Bay and 30 from Miami as well as a small selection from other Caribbean sites were genotyped at both loci. The overall frequency of the 1016I allele in Grand Cayman was 0.79 (Table 3.4). The East End and West Bay population were in Hardy Weinberg Equilibrium (using Genepop 4.0 option 1.2 to test for an excess of heterozygotes) but the George Town population had an excess of heterozygotes. The overall frequency of the 1534C allele was 0.68. Significant deviations from Hardy Weinberg Equilibrium were observed in West Bay only, which also had an excess of heterozygotes at this locus.

Pop	1016			Freq of I	P	1534			Freq of C	P
	V/V	I/V	I/I			F/F	F/C	C/C		
East End	3	18	28	0.76	1.0	6	21	22	0.66	0.76
George Town	0	25	24	0.74	0.02	1	19	30	0.79	0.67
West Bay	0	9	32	0.89	1.0	1	36	9	0.59	0.0
Grand Cayman	**3**	**52**	**84**	**0.79**		**8**	**76**	**61**	**0.68**	
Havana	1	20	25	0.76	0.41	0	11	39	0.89	1.0
Jamaica	7	26	17	0.60	0.77	0	0	50	1.00	-
Tampa Bay	31	17	1	0.19	0.66	31	16	3	0.22	0.68
Miami	24	5	0	0.09	1.00	11	10	9	0.47	0.07
Martinique	0	0	6	1.00		0	0	20	1.00	
Honduras	10	1	0	0.05		2	2	1	0.40	
Florida Keys	12	0	0	0.00		4	1	0	0.10	
Puerto Rico	0	0	10*	1.00		0	1	4	0.90	

Table 3.4 Genotypes and allele frequencies for the V1016I and F1534C mutations in Caribbean populations of *Ae. aegypti*.
Tests for Hardy Weinberg were carried out and P calculated. There were not sufficient numbers in some populations to carry out this analysis. *An extra sample was genotyped from the Puerto Rico population that was heterozygous for isoleucine and glycine at codon 1016.

At least one of the two *kdr* mutations was found in all the Caribbean populations (Table 3.4). The allele frequency of the 1016I mutation ranged from 0 in the Florida Keys (n=12) to 1.0 in Martinique (n=6) and from 0.1 to 1.0 for the 1534C mutation in the Florida Keys (n=5) and Jamaica respectively. All populations studied are in Hardy Weinberg Equilibrium for both alleles except for Jamaica which has reached fixation for the 1534 mutation. In some cases the sample size was not large enough to carry out Hardy Weinberg analysis.

Of the 3 samples sequenced from Puerto Rico, one individual was heterozygote at both the 1st and 2nd position of codon 1016 (Figure 3.8). This PCR product was cloned and 3 clones sequenced, all of which had the sequence GGA encoding glycine. It was concluded from analysis of the direct sequencing that this individual was heterozygous for Ile and Gly at 1016.

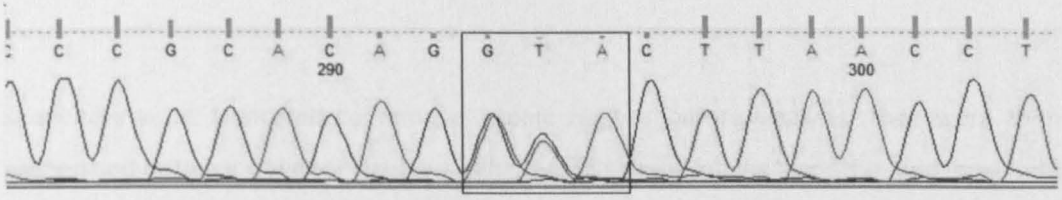


Figure 3.8 Sequence trace of a sample from San Juan, Puerto Rico.
The boxed area indicates codon 1016, at this position this individual has copies of two different *kdr* mutations – isoleucine (ATA) and glycine (GGA).

In historical samples from 2003 the wild type allele only was found at the 1534 (n=6) position while all samples except for one heterozygote were wild type at position 1016 (n=25).

Analysis of linkage disequilibrium (carried out using Genepop 4.0 option 2.1) determined that the two *kdr* alleles are linked in the combined Cayman and Caribbean populations (P=0). However, analysis of sample sets from different locations (Table 3.5) found that in West Bay the two SNPs were independent.

Sample Set	P
East End	0
George Town	0
West Bay	1
Cayman	0
Havana	0.02
Jamaica	-
Tampa Bay	0
Miami	0.03
Caribbean	0

Table 3.5 Linkage disequilibrium P values of the two *kdr* alleles in different Caribbean samples.
No P value is available for Jamaica as the 1534 position is fixed for the mutant allele.

3.3.2. Intron Analysis

PCR products encompassing part of exons 21 and 22 and intron 21 in the domain II S6 region were sequenced from 57 individuals. Indels in the intron sequence resulted in truncated sequences for PCR products from some individuals and these products were therefore cloned into a plasmid vector to enable individual alleles to be sequenced. A subset of 26 clones from 10 individuals were sequenced and individual haplotypes were studied.

Sequences were truncated to remove exonic regions before analysis; they were then aligned and distance trees constructed using the neighbour joining algorithm and maximum parsimony. Representative sequences from Asia, Mexico and from the 3 intron types described in Saavedra-Rodriguez (2007) were included in the alignment. Within the 260 bp intron there were 121 segregating sites (Figure 3.9). Phylogenetic trees are shown in Figure 3.10 and Figure 3.11.

```
Clade 1         GTAAGTATTCCGTTTGGAAGTTCATCTGTAAGGCTGACTGAAAGTAAATTGGAGCGCACA
Havana 1b       GTAAGTATTCCGTTTGGAAGTTCATCTGTAAGGCTGACTGAAAGTAAATTGGAGCGCACA
East End 2      GTAAGTATTCCGTTTGGGAGTTCATCTATAAGGCTGACTGGAAGTAAATTGGAGTGCACA
East End 1      GTAAGTATTCCGTTTGGGAGTTCTTCTATAAGGCTGACTGAAAGTAAATTGGAGCGCACA
West Bay        GTAAGTATTCCGTTTGGGAGTTCTTCTATAAGGCTGACTGAAAGTAAATTGGAGCGCACA
Tampa Bay 2     GTAAGTATTCCGTTTGGGAGTTCTTCTATAAGGCTGACTGAAAGTAAATTGGAGCGCACA
Clade 2         GTAAGTATTCCGTTTGGGAGTTCTTCTATAAGGCTGACTGAAAGTAAATTGGAGCGCACA
Clade 3         GTAAGTATTCCGTTTGGAAGTTCATCTGTAAGGCTGACTGAAAGTAAATTGGAGCGCACA
                ********************* ***** *** ************ ************* *****

Clade 1         ACA-GACCTATTAAGCTGTAA-TTCGTG-ATTCAA---CT-ATTTACAAAATACCGTTGA
Havana 1b       ACA-GACCTATTATGCTGTAA-TTCGTG-ATTCAA---CT-AGTTACAAAAGACCGTTGA
East End 2      AC-AGACGTATTATGCTGTAA-TTCGTG-ATTCAA---CT-A-GTTAAAATGACCGTTGA
East End 1      ACAAGACCTGTTATGCTGTAAGTTCCAGCACTAAATTTCTCAGGTTGAATTG-CAGTAGT
West Bay        ACAAGACCTGTTATGCTGTAAGTTCCAGCACTAAATTTCTCAGGTTGAATTG-CAGTAGT
Tampa Bay 2     ACAAGACCTGTTATGCTGTAAGTTCCAGCACTAAATTTCTCAGGTTGAATTG-CAGTAGT
Clade 2         ACAAAACCTGTAATGCTGTAAGTTCCAGCACTAAATTTCTCAGGTAGAATTGA-ACTAGT
Clade 3         ACAAAACCAGTAAGGCGGAAATCTCG---ATTCAA----CAATTTAAAAAGGAGAATTGA
                **   **   * * ** * **  **    * * **      *  *  **       * *

Clade 1         TC--TTGATAGCATC-AA---T---ATTA-GAGGCG-TGCTAT-C-A-AC--GA-GCG-A
Havana 1b       TC-TT-GATAGCATC-AA---T--A-TTA-GAGGCG-TGCTA-GC-AG-C--GA-GCG-A
East End 2      TC-TT-GATAGCATC-AAC------ACTA-GAGGCG-TGCTA-GC-A-GC--GA-GCG-A
East End 1      TCAATCGAAATC-TCGAAC-------TTTCATTTTGATAACA-GCAATACTAGACGCGCA
West Bay        TCAATCGAAATC-TCGAAC-------TTTCATTTTGATAACA-GCAATACTAGACGCGCA
Tampa Bay 2     TCAATCGAAATC-TCGAAC-------TTTCATTTTGATAACA-GCAATACTAGACGCGCA
Clade 2         TCAATCCAAATC-TCGAAC-------TTTGATTTTGATAACA-G-AATACTAGACGCGCA
Clade 3         TC--TTGATAAC-TCCAAC-------TCTCAAAGTT-TGCTT--------TTGACAACCG
                **  *  * * * ** **                   *              **

Clade 1         GGG-GCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCTTGCTAACCGACAA
Havana 1b       GGG-GCGTACCAATTTACTTTTAGTCAGTCTTTCTTGCATTCTTTCGTGCTAACCGACAA
East End 2      GGG-GCGTACCAATTTACTTTTGGTCAGCCTTTCTTGCATTCTATCGTGCTAACCGACAA
East End 1      TAGAACATACAAATTTACATATAGTCAGCCTTTCATGCATTCTATCGTGCTAACCGACAA
West Bay        TAGAACATACAAATTTACATATAGTCAGCCTTTCATGCATTCTATCGTGCTAACCGACAA
Tampa Bay 2     TAGAACATACAAATTTACATATAGTCAGCCTTTCATGCATTCTATCGTGCTAACCGACAA
Clade 2         TATAACATACAAATTTACATATATTCAGCCTTTCATGCATTCTATCTGGCTAACCGACAA
Clade 3         AGGGGCGTACCACTTTAATTATATACAACCTTTC-TATATTCTATCTTGCTAGCCTACTA
                     * *** * ****  * *   **  ***** *  ***** **  **** ** ** *

Clade 1         ATTGGTTCCCACTCGCACAG
Havana 1b       ATTGTTTCCCACTCGCACAG
East End 2      ATTGTTTCCCACCCGCACAG
East End 1      ATTGTTTCCCACCCGCACAG
West Bay        ATTGTTTCCCACCCGCACAG
Tampa Bay 2     ATTGTTTCCCACCCGCACAG
Clade 2         ATTGGTTCCCACCCGCACAG
Clade 3         ACTGGTTCCCCCACGAATTG
                * ** ***** * ** *  *
```

Figure 3.9 Sequence alignment of intron 21 of representative samples from Grand Cayman, the Caribbean and the consensus sequences from Saavedra-Rodriguez (2007) showing segregating sites.

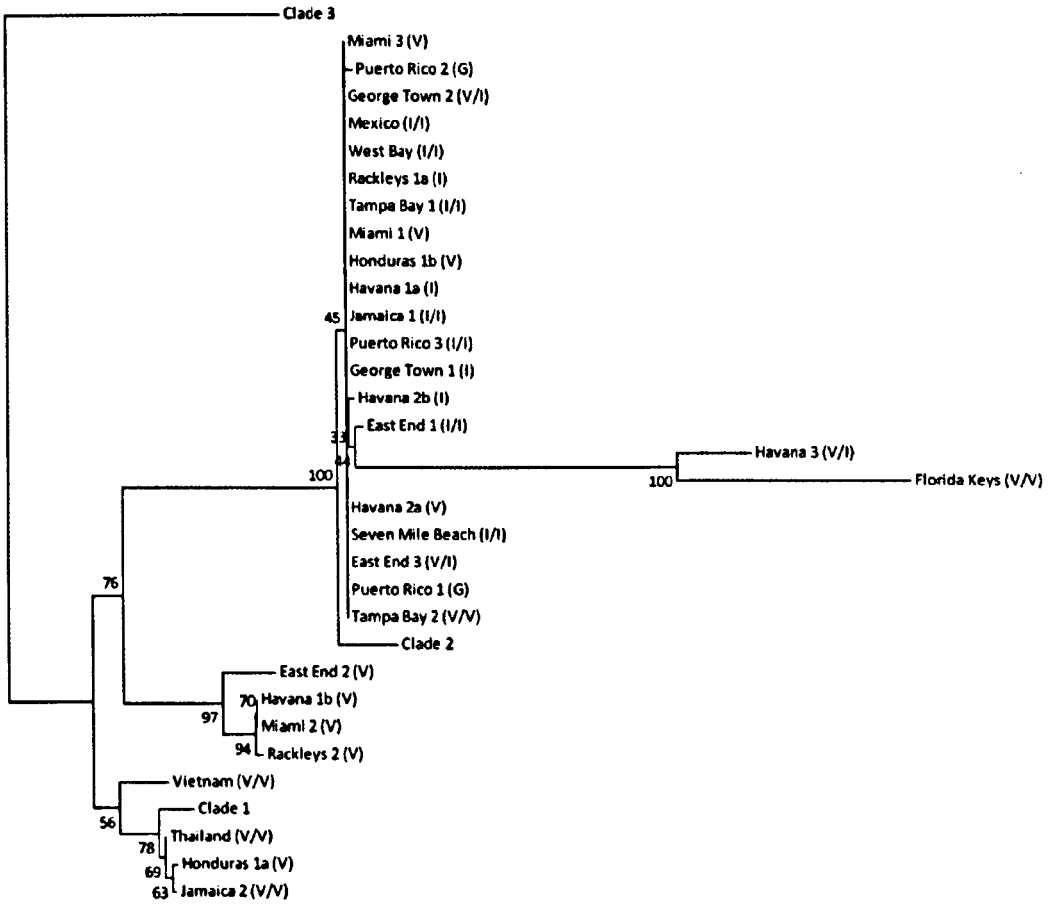


Figure 3.10 Neighbour joining phylogenetic tree of intron 21 of the sodium channel in *Ae. aegypti* sampled from the Caribbean, Mexico, Thailand and Vietnam.
Consensus sequences from populations studied in Saavedra-Rodriguez, (2007) are also included for comparison. The tree was constructed using the neighbour joining method and the Jukes-Cantor algorithm (Jukes & Cantor, 1969). Values indicate percentage support of node position after 500 bootstrap replicates. Letters in brackets indicate the amino acid at residue 1016 which immediately succeeds the intron. For PCR products the genotype is given, while for sequenced clones, the allele is given.

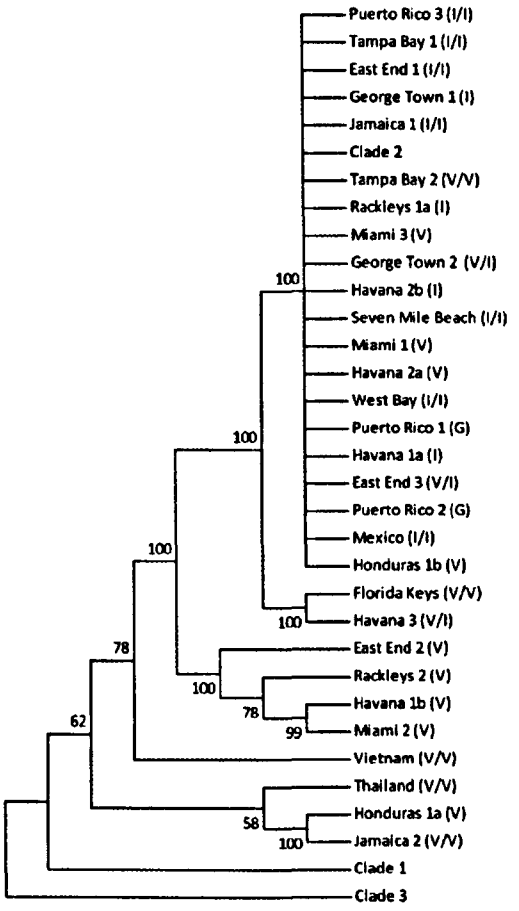


Figure 3.11 Maximum parsimony phylogenetic tree of intron 21 of the sodium channel in *Ae. aegypti* sampled from the Caribbean, Mexico, Thailand and Vietnam.
Consensus sequences from populations studied in Saavedra-Rodriguez, (2007) are also included for comparison. The tree was constructed using maximum parsimony. Values indicate percentage support of node position after 500 bootstrap replicates. Letters in brackets indicate the amino acid at residue 1016 which immediately succeeds the intron for PCR products the genotype is given while for sequenced clones, the allele is given.

In general there is good agreement between the two trees with the majority of the sequences falling into one of two well supported clades. The first is similar to the clade 2 intron reported from Saavedra-Rodriguez (2007). (Note that the Havana 3 and Florida Keys sequences were truncated PCR products as cloning was unsuccessful. This may explain the long branch lengths, analysis was carried out using the 'complete deletion' option on the Mega 4 software, in which sites containing missing data are removed before analysis begins). The second clade contains two Cayman, one Havana and one Miami individual. The remaining samples including those from Asia and clade 1 from Saavedra-Rodriguez (2007) have fairly low support in both models and their similarity to other samples is not clear. Clade 3 is far removed from the other samples in both models. Some heterozygous individuals have haplotypes in two different clades (e.g the haplotypes from Havana (1a

and 1b) and Honduras (1a and 1b) came from single individuals) suggesting that some sample sets may have multiple historical origins.

3.4. Discussion

Partial sequencing of the sodium channel identified two amino acid substitutions in the Cayman population compared to the susceptible New Orleans strain. The Cayman population was 'wild type' at residues 923, 928 and 1011 (Figure 3.2) but showed variation at residues 1016 and 1534. The V1016I mutation has previously been reported in Mexico and the Caribbean (Marcombe *et al.*, 2009b, Saavedra-Rodriguez *et al.*, 2007) at frequencies ranging from 0 to 0.71. Although this allele has not yet reached fixation it is found in high frequencies within the Cayman population (0.79), similar to that reported in Martinique (0.71) (Marcombe *et al.*, 2009b). The F1534C mutation is not restricted to the Cayman Islands. This mutation was recently reported in Thailand (Yanola *et al.*, 2010) and Vietnam (Kawada *et al.*, 2009) and screening of additional *Ae. aegypti* populations in this study has identified this mutation in Cuba, Jamaica, Florida, Martinique, Honduras, Puerto Rico and Mexico.

Both mutations were present in the majority of the Caribbean populations studied, the exception to this being the Florida Keys in which the 1016I allele was not detected (n=12) and the 1534C allele (n=5) was at very low frequency. Overall the Florida populations (Florida Keys, Tampa Bay and Miami) had lower frequencies of the *kdr* mutations than the rest of the Caribbean with the exception of Honduras. In Jamaica the population is fixed for the 1534C mutation (n=50).

Glycine at position 1016, reported in populations from Indonesia and Thailand (Brengues *et al.*, 2003, Rajatileka *et al.*, 2008), was not detected in the Cayman Islands. However, one sample from Puerto Rico was heterozygous at this locus for both isoleucine and glycine. This is the first time this glycine substitution has been recorded in the Caribbean; previously it has been considered to be geographically isolated to Asia. The appearance of this 1016G mutation along with the high frequency of 1016I mean that pyrethroid resistance in Puerto Rico is likely to be high. As both 1016 and 1534 mutations in this population appear to be close to fixation it is important that control personnel look to other methods of control.

Substitutions at the 1011 codon have been identified in populations from Brazil, French Guyana, Martinique (Brengues *et al.*, 2003), Mexico, Nicaragua, Costa Rica, Panama, Cuba, Venezuela (Saavedra-Rodriguez *et al.*, 2007) and Thailand (Rajatileka *et al.*, 2008). In Grand Cayman the population is fixed for isoleucine at this position and neither methionine nor the valine mutations have been found (n=37). One sample each from Havana and Honduras were heterozygous for isoleucine and methionine at this locus.

The 1016I allele was associated with survival after permethrin exposure (Fisher's exact test p=0). Earlier selection studies also showed an increase in frequency of this allele after permethrin selection (Saavedra-Rodriguez *et al.*, 2007). Surprisingly, there was no significant correlation between the 1016I allele and resistance to DDT (Fisher's exact test P=0.145). This site has not been predicted to play a role in DDT binding; it is believed that three sites in the Domain II S5 (933C, 936I) and Domain III S6 (1530F) regions are key in DDT binding (O'Reilly *et al.*, 2006). In pyrethroid binding the same residues play a role hence cross resistance between the two insecticides is often seen, however, as pyrethroids are larger molecules than DDT, more residues are predicted to be involved in pyrethroid binding than that of DDT (O'Reilly *et al.*, 2006).

All survivors to permethrin and most to DDT were homozygous for the cysteine mutation at codon 1534 while all individuals homozygous for the wild type phenylalanine died in bioassays to either insecticide. In total, there was a strong correlation between 1534 genotype and resistance to both insecticides (Fisher's exact test p=0) Not all individuals homozygous for this mutation survived the bioassay. However it must be noted that the DDT bioassay comprised of 24 hours insecticide exposure followed by a 24 hour holding period so it is therefore possible that some mortality may not be explained by insecticide exposure alone.

Substitutions in an alternative phenylalanine residue in domain III S6, F1538, have been associated with pyrethroid resistance in the southern cattle tick, *Boophilus microplus* (He *et al.*, 1999) and the two-spotted spider mite, *Tetranychus urticae* (Tsagkarakou *et al.*, 2009). Recently site directed mutagenesis has been used in an attempt to delineate the role of residues in this helix in pyrethroid binding (Du *et al.*, 2009). In initial experiments the replacement of the F1538 residue (referred to as F1518 in the Du study) with alanine

almost completely abolished pyrethroid binding. However an alanine replacement of F1534 had no effect. The substitution observed at residue 1534 in the Caribbean *Ae. aegypti* populations replaces phenylalanine with a polar, hydrophilic cysteine. A recent study using *Xenopus* oocytes has confirmed that this F1534C substitution confers resistance to type I, but not type II pyrethroids (Hu *et al.*, 2011).

Whether the two mutations confer different levels of resistance is also of interest. If strains could be identified (or back crossed) to create lines with only one of the two mutations present, the importance of each could be identified separately, however this could not be distinguished in samples used in the current study as the two mutations are tightly linked. Studies have been carried out using *Xenopus* expression to determine the comparative resistance of three mutations found at position 1014 (1014F, 1014S and 1014H) in *Kdr* resistant *An. gambiae* (using a modified *Drosophila* sequence) (Burton *et al.*, 2011) similar studies looking at the comparative resistance of the 1016 and 1534 mutations in *Ae. aegypti* could prove interesting.

Hardy Weinberg analysis revealed that the East End population was in Hardy Weinberg Equilibrium at both loci whereas the George Town and West Bay populations had significant deviations from Hardy Weinberg Equilibrium at the 1016 and 1534 mutation respectively. Despite high frequencies of both mutations neither has yet reached fixation within the Cayman population. Both George Town and West Bay receive regular insecticide treatment aimed at *Ae. aegypti* and both the air and sea ports are located in George Town. The selection caused by insecticide pressure, combined with possibility for immigration may disrupt Hardy Weinberg equilibrium of these populations. Meanwhile the population in East End is discreet and fairly isolated; it receives no chemical intervention from the MRCU which has perhaps allowed it to reach equilibrium.

Other Caribbean populations were mostly in Hardy Weinberg equilibrium for these alleles with the exception of Havana (at residue 1016) and Miami (at residue 1534). Jamaica was fixed for the mutant allele at the 1534 residue. Equilibrium in these populations reflects that these populations fit expected values and while they are unlikely to be completely free from immigration, migration or mutation that which is experienced is not enough to affect

equilibrium. Lack of equilibrium in Havana or Miami may suggest a comparatively greater level of gene flow or selection pressure.

Mutations associated with insecticide resistance may arise from one or few original mutations which have been spread around the world or the same mutation may occur independently in different locations. In *Cx. pipiens* it is believed that mutations occurred within genes encoding specific esterases at some stage historically possibly in Africa or Asia and then spread globally with the migration of the mosquito (Raymond *et al.*, 1991). The data in this chapter also supports a single origin for the 1016I mutation. The 1016I allele is found in a single clade. The 1016G allele found in Puerto Rico was found on the same genetic background and as a heterozygote with the 1016I allele. This may suggest that this is a recent mutation in Puerto Rico although unfortunately this study did not include sequence data from other individuals with the 1016G allele.

Intron analysis in *An. gambiae* have shown that introgression may play a part in the spread of *Kdr* genes between M and S forms (Weill *et al.*, 2000). Non-synonymous mutations within a stable West African haplotype are commonly found in wild type S forms, but only in the mutant (1014S) M form; suggesting that the resistance gene along with the intron came from the S taxon through introgression, thus supporting the hypothesis of a single original origin. However subsequent analysis of the S form found seven distinct intron haplotypes in East and West African *An. gambiae* and evidence to suggest that at least four mutation events have originated two kdr alleles (L1014F and L1014S) within this taxon (Pinto *et al.*, 2007). The chromosomal positioning of the sodium channel gene may be leading to higher than expected rates of recombination (Lynd *et al.*, 2010).

With such intensive insecticide use on Grand Cayman it is interesting to know whether *kdr* mutations found in the present population have been there since its introduction to the island or whether they have arisen as a result of insecticidal pressures. Unfortunately very few samples remain from 2003 and all that can be concluded from this analysis is that the 1016I mutation was present in Grand Cayman at this time.

Screening for *kdr* is now an important component of resistance management programmes in some countries (Garcia *et al.*, 2009, Harris *et al.*, 2010, Kawada *et al.*, 2009, Marcombe *et*

al., 2009b, Martins *et al.*, 2009a, Yanola *et al.*, 2010). This requires robust, low cost assays. A number of diagnostic assays are available to detect *Kdr* mutations in both *Ae. aegypti* and *An. gambiae*. A study carried out to compare *Kdr* assays for *An. gambiae* determined that allele specific PCR is the most widely used method, as it is low cost, however it is not as sensitive or high throughput as the Taqman assay which was determined to be the assay of choice (Bass *et al.*, 2007).

The tetraplex PCR developed in this thesis does not require any specialised equipment and has proven to be highly reproducible by other users. The 1016 mutation has not proved so amenable to the development of genotyping assays. The melt curve assay designed by Saavedra-Rodriguez (2007), requires real time PCR which is not within the realms of many labs. The more low tech version can be carried out on agarose gel which may be accessible to more labs and comes at a lower cost. However resolution of the small products can be difficult as well as the possibility of primer dimers resulting from hairpin formation of the val1016f primer that may cause false positive readings or seeming contamination in the negative control. This was experienced in both the real time and the agarose gel versions of this assay, therefore the use of this assay was not pursued, and data is not shown.

Heated Oligonucleotide Ligation Assay (HOLA) is a mid-priced assay for the detection of single nucleotide polymorphisms. It relies on a simple enzyme-linked colour change and can easily be carried out in field laboratories assuming access to a PCR machine. It is fairly labour intensive and although a number of samples can be screened simultaneously it is not suited to rapid screening of large numbers of samples. Experience of this assay using self coated and commercially coated streptavidin plates proved this assay can, on occasion be prone to false positivity. Studies by others using this method for genotyping *kdr* mutations in *An. gambiae* determined the HOLA assay had a lower failure rate than other methods including allele specific PCR, but an 11% rate of incorrect scoring (Bass *et al.*, 2007). In the current study, difficulties in reproducibility were encountered.

Pyrosequencing was the final method used for detection of this mutation, it allows for high throughput screening of large numbers of samples with great accuracy, but when compared to other methods it is considerably more expensive both in reagents and equipment upon which to run the assay. A taqman assay has recently become available for

the detection of the F1534C mutation in *Ae. aegypti* (Yanola *et al.*, 2011), but this was not evaluated in the current study.

With different methods available for screening the different *kdr* loci, work is ongoing to develop a single robust platform that would enable simultaneous detection of all the known *kdr* alleles in *Ae. aegypti* (WC Black, personal communication). This would facilitate the inclusion of *kdr* genotyping as part of insecticide resistance monitoring and evaluation.

4. Genetic Structure in *Aedes aegypti* Populations from across the Caribbean

4.1. Introduction

The Cayman Islands has very little industry or farming, therefore all goods are shipped in from overseas and it is through this route that it is believed *Ae. aegypti* first arrived on Grand Cayman. In the past, the MRCU carried out a campaign of port disinsection whereby container ships, private vessels and aircraft entering the islands were treated with insecticides (malathion and more recently pyrethroids) in order to keep new introductions of this mosquito from the island. This was abandoned in 2006 and resources were reallocated to focus on yard to yard surveys as the principle means of control of *Ae. aegypti* on Grand Cayman.

In this chapter, the degree of genetic differentiation within *Ae. aegypti* populations collected from within Grand Cayman and from neighbouring regions was examined in an attempt to determine the origin of the introductions. Locations with the strongest transport links to Grand Cayman, including neighbouring Caribbean islands and mainland locations in Florida were analysed using twelve microsatellite markers.

Microsatellites are a useful tool for studying genetic structure within populations and several polymorphic microsatellites have been identified in *Ae. aegypti* (Chambers *et al.*, 2007, Huber *et al.*, 2001, Lovin *et al.*, 2009, Slotman *et al.*, 2007). Studies in Mexico (Ravel *et al.*, 2001), Ivory Coast (Ravel *et al.*, 2002), Vietnam (Huber *et al.*, 2002a, Huber *et al.*, 2002b), Cambodia and Thailand (Huber *et al.*, 2004) have shown that there are significant levels of genetic differentiation between populations of this species both geographically as well as temporally. On a more global scale microsatellites have been used to differentiate between African populations and those from elsewhere providing evidence for the existence of different subspecies within *Ae. aegypti* (Brown *et al.*, 2011).

The aims of this chapter were to:-

- Identify whether the *Ae. aegypti* on Grand Cayman fall into one population or are comprised of several subpopulations.
- Determine the origin or origins of the Cayman Islands *Ae. aegypti* population.
- Assess the likelihood that *Ae. aegypti* were actually eradicated from the island in 1996.

- Determine the effect of shipping on migration of populations and whether MRCU has grounds to reinstate the port disinsection campaign.

4.2. Methods

4.2.1. Origin of Samples

The origin of the samples from the Cayman Islands and other parts of the region used in this chapter have been described previously (Section 3.2.1). Fifty individuals were collected from each of three different locations in Grand Cayman (East End, George Town and West Bay) as larvae from a number of containers in different yards (See Table 3.1) and 50 individuals from Jamaica, Havana, Tampa Bay and 30 from Miami. Eggs were collected from ovitraps set in the field and reared to adulthood in the MRCU facility. Havana samples were collected from the field in 2008 and maintained in colonies at the Instituto "Pedro Kourí" until they were supplied for this study. Two collections took place in Tampa Bay the first in May 2008 and the second in July 2009. Effort was made to ensure minimal sampling of siblings.

4.2.2. Microsatellite Amplification

Microsatellite markers were selected from previously published work (Chambers *et al.*, 2007, Slotman *et al.*, 2007). Twenty one sets of primer pairs were synthesised with a linker added to the end of the 5' primer. The tailed primer system, first described by Oetting (1998) and now used routinely at LSTM, was used to label the PCR products with a fluorescent dye to reduce the genotyping costs. Amplification reactions were set up in 15µl volumes containing 0.08mM each dNTPs, 0.3mM each primer, 0.6u *Taq* polymerase (Kapa biosystems) and 1 per cent of the total genomic DNA extracted from a single mosquito as template. Cycling conditions were as follows; 95°C for 7 minutes followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds then a final elongation step of 72°C for 7 minutes. Products were visualised on a 2% agarose gel alongside a 100bp DNA ladder to confirm amplification. Twenty primer sets gave good amplification products and were evaluated further. For each sample 1µl of PCR product was added to 30µl CEQ Sample Loading Solution (Beckman Coulter) and 0.5µl CEQ DNA Size Standard 400. Products were sized using the Beckman CEQ8000 fragment analysis software. Primer pairs that amplified consistently and generated products of different sizes were selected for inclusion in this study and the forward primers were re-synthesised with a 5' fluorescent label.

Primer Name	Sequence	Product (bp)	Repeat	Super contig	Multiplexed with	Linker
*AG7F	CGTGCGAGTGAATGAGAGAC	137-191	AG	1.48	H08, M201	D2
*AG7R	CATCCTCTCATCAGCTTCTAATAAA					
†H08F	AAAAACCACGATCACCGAAG	184-223	TCG_7	1.1217	AG7, M201	D4
†H08R	ACGCGATCACACACTGAAAATG					
†M201F	GGAGCATTCATAGAGAATTGTCA	107-116	ATA_{36}	1.28	AG7, H08	D3
†M201R	GAGATGAACCAGTCATAGGGC					
*AT1F	CGTCGACGTTATCTCCTTGTT	140-164	AT	1.88	M313, AC2	D2
*AT1R	GGACCGGAAAGACACAGACA					
†M313F	CACCTCGTGACATACAAACACC	108-126	$ATG_5(ATA)ATG$	1.98	AT1, AC2	D4
†M313R	ACGTACCCAAGCCACGTACA					
*AC2F	GAATACAACGCGATCGACTCC	168-174	AC	1.65	AT1, M313	D3
*AC2R	AACGATTAGCTGCTCCGAAA					
*AC7F	TCGGCAAATTACCACAAACA	116-134	AC	1.127	AG3, A10	D2
*AC7R	CATTGGACTCGCTATAACACACA					
*AG3F	CGCCAAAACTGAAAACTGAA	148-162	AG	1.73	AC7, A10	D4
*AG3R	AAGGGCGGTGATGACTTTCT					
†A10F	GAATCGTGACGCGTCTTTTG	230-238	$CT_{10}(TT)CT$	1.998	AC7, AG3	D3
†A10R	TAACTGCATCGAGGGAAACC					
*AG5F	GATCTTGAGAAGGCATCCA	149-162	AG	1.210	AG1, AG5	D2
*AG5R	CGTTATCCTTTCATCACTTGTTTG					
*AG1F	AATCCCCACACAAACACACC	102-112	AG	1.73	AG5, AC5	D4
*AG1R	GGCCGTGGTGTTACTCTCTC					
*AC5F	GTGGATTGTTCTTAACAAACACGAT	138-152	AC	1.118	AG5, AG1	D3
*AC5R	CGATCTCACTACGGGTTTCG					

Table 4.1 Primers used for amplification of microsatellite regions within *Ae. aegypti*.
Primers were labelled using WELLRED fluorescent dyes and combined into multiplex reactions denoted by shading. Primer names preceded by an asterisk (*) are taken from Slotman (2007) Primer names preceded by a dagger symbol (†) are taken from Chambers (2007). Super contig refers to the position on the *Ae. aegypti* genome (Vectorbase.org)

A set of twelve loci were amplified for each sample in multiplex reactions as indicated in Table 4.1. Loci with differing product size ranges and labelled with different dyes (D2, D3 or D4) were multiplexed in order to increase the throughput of the genotyping. PCR conditions and sizing were performed as described above.

4.2.3. Analysis

For the purpose of analysis all mosquitoes collected from one geographical area were treated as a single sample set. Tests for deviation from Hardy Weinberg of individual loci were carried out to determine if each sample set was a single panmictic population as well as locus suitability for use within this study (i.e. free from locus specific constraints such as null alleles or preferential amplification of one allele over another in heterozygotes). Analysis was carried out assuming an alternative hypothesis (H1) of heterozygote deficit using Genepop option 1.1 (Raymond & Rousset, 1995). *Fis* was calculated according to Weir (1984) and also according to Robertson (1984) and sequential Bonferroni correction (Holm, 1979) made at the 0.05 significance level. *Fis* is a measure of inbreeding within a population with high *Fis* values indicative of a considerable degree of inbreeding. Negative *Fis* values represent an excess of heterozygotes whilst positive values represent a deficit. These figures are only of interest if they are calculated to be significant at the 0.05 level after correction. Bonferroni correction is carried out in order to take into consideration the effect of multiple testing while determining the statistical significance of the probability in order to reduce the possibility of significance due to chance. If there are a low number of heterozygotes within a population this may be due to subpopulation structure known as the Wahlund effect.

Fst is a measure of genetic divergence between populations and can be used to estimate the level of gene flow between individuals from different populations, greater *Fst* values (closer to 1) are indicative of greater divergence (less similarity) between populations. Therefore *Fst* estimations were performed in this study to estimate genetic differentiation between Caribbean sample sets; analysis was performed using GenePop option 6.2 and significance of differentiation of genotype frequencies by option 3.4. Critical significance of differences between populations was corrected using the Bonferroni method (Holm, 1979).

A phylogenetic tree was constructed to assess how closely related the different populations are; Phylip 3.68 (Felsenstein, 1989) was used to calculate Cavalli-Sforza and Edward's Chord Distance for each pair of populations, the resulting matrix was used to construct a neighbour joining tree in MEGA4 (Tamura *et al.*, 2007).

To determine the relationship between the genetic differentiation of different sample sets and their geographical distance, Xlstat (Addinsoft) was used to analyse the isolation by distance; the relationship between *Fst*/(1-*Fst*) values and the logarithm of geographic distances in Km was plotted to determine the correlation between *Fst* and distance. R^2 was calculated in order to determine the strength of the association between genetic similarity (*Fst*) and distance. R^2 is a form of regression analysis; an R^2 of 1 indicates that a regression line fits the data exactly therefore the greater the value the stronger the association. A mantel test was carried out (using Spearman's correlation) to determine the statistical significance of the correlation.

Assignment tests use multilocus genotype data to study the origin of individual samples within perceived populations. They can also be used to determine immigration between different populations (Rannala & Mountain, 1997). Unlike *Fst* analysis in which genetic differentiation between populations is calculated by a single combined figure, this method assigns individuals probabilistically to populations using multiple loci (Pritchard *et al.*, 2000). Briefly each sample set is assessed as a whole and a representative sample created, each individual sample is then assigned to a population on the basis of their genotype at all the different loci. Assignment was used in this study to assess how many samples could be correctly assigned to their collection site and to determine what frequency of Grand Cayman/Caribbean samples were assigned within Grand Cayman and what frequency were assigned to elsewhere in the Caribbean. Tests were carried out using Gene Class 2 (Piry *et al.*, 2004) and a probability approach using the Bayesian method according to Rannala (1997) was carried out for analysis.

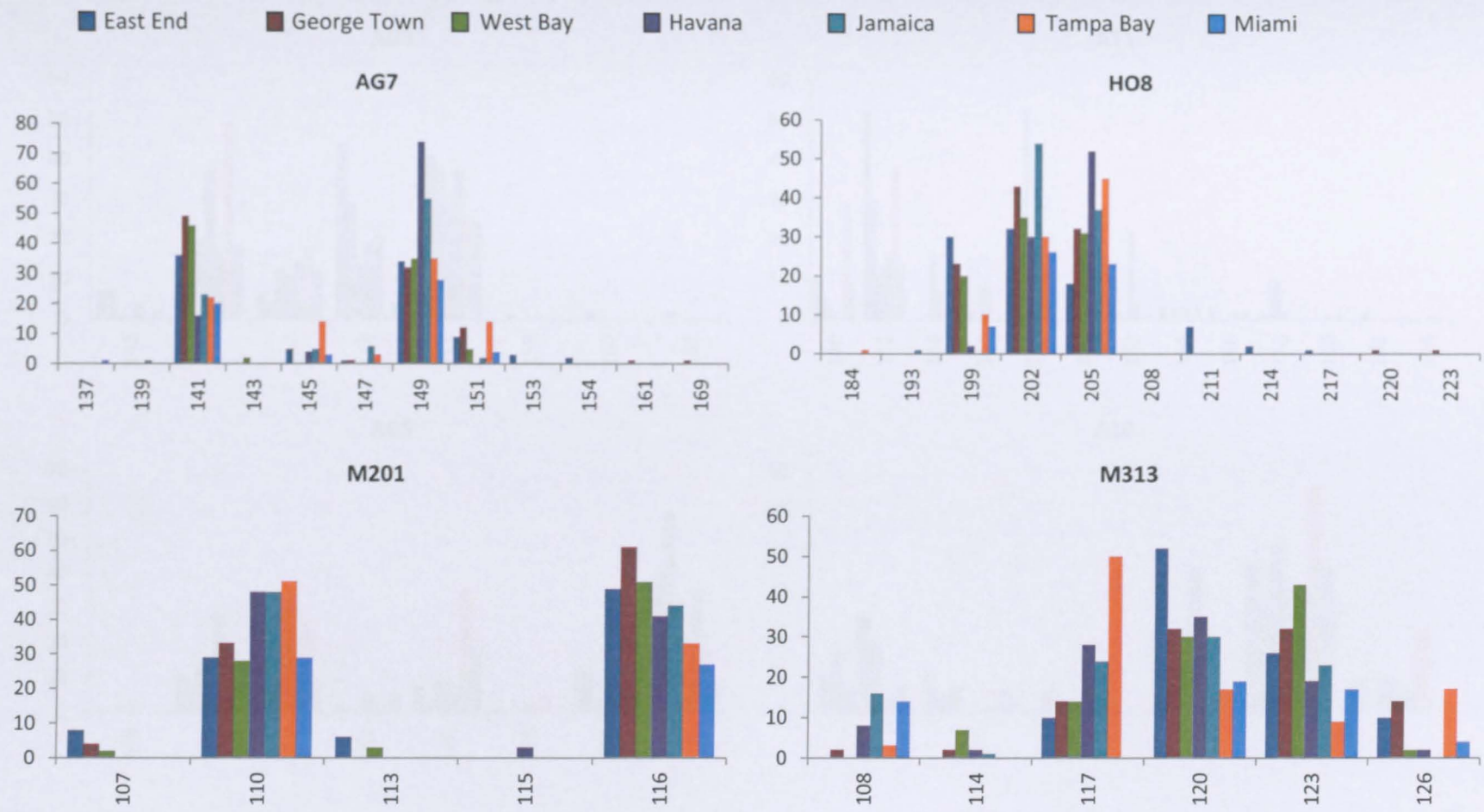
4.2.4. DNA Sequence Analysis of an Intronic Portion of the Sodium Channel Gene

The partial sequencing of exons 21 and 22 and the intervening intron of the sodium channel gene was described in Chapter 3. The polymorphism of this fragment was analysed for 36 individuals (n=72 chromosomes). All sequence traces for this region were checked by eye

using Bioedit (Hall, 1999). An unphased sequence was generated for each individual where heterozygote positions were represented with respective degenerate letters (Y for C/T etc.). Samples were truncated to a common 400bp length which included intronic (259bp) and exonic (141bp) regions. The determination of the haplotype phases and the analysis of the polymorphism of the fragment were carried out using DnaSP 5.0 (Rozas & Rozas, 1995). A cladogram of all samples was constructed in TCS 1.21 (Clement *et al.*, 2000).

4.3. Results

Samples from Grand Cayman and four locations across the Caribbean were genotyped at 12 microsatellite loci. All 12 loci amplified successfully and are highly polymorphic; the number of alleles per locus ranging from 4 (AC2) to 13 (AT1). Most alleles are distributed across the geographical range (Figure 4.1), while some alleles are rare; there is little evidence of private alleles (alleles that are present in a single population and not found in others).


East End
George Town
West Bay
Havana
Jamaica
Tampa Bay
Miami
AG7
80
70
60
50
40
30
20
10
0
137
139
141
143
145
147
149
151
153
154
161
169
HO8
60
50
40
30
20
10
0
184
193
199
202
205
208
211
214
217
220
223
M201
70
60
50
40
30
20
10
0
107
110
113
115
116
M313
60
50
40
30
20
10
0
108
114
117
120
123
126

AG3
0
10
20
30
40
50
60
148
150
152
154
156
158
160
162
AG5
0
10
20
30
40
50
60
70
149
150
152
154
156
158
160
162
AT1
0
10
20
30
40
50
60
140
142
144
146
148
150
152
154
156
158
160
162
164
A10
0
10
20
30
40
50
60
230
232
233
234
236
238

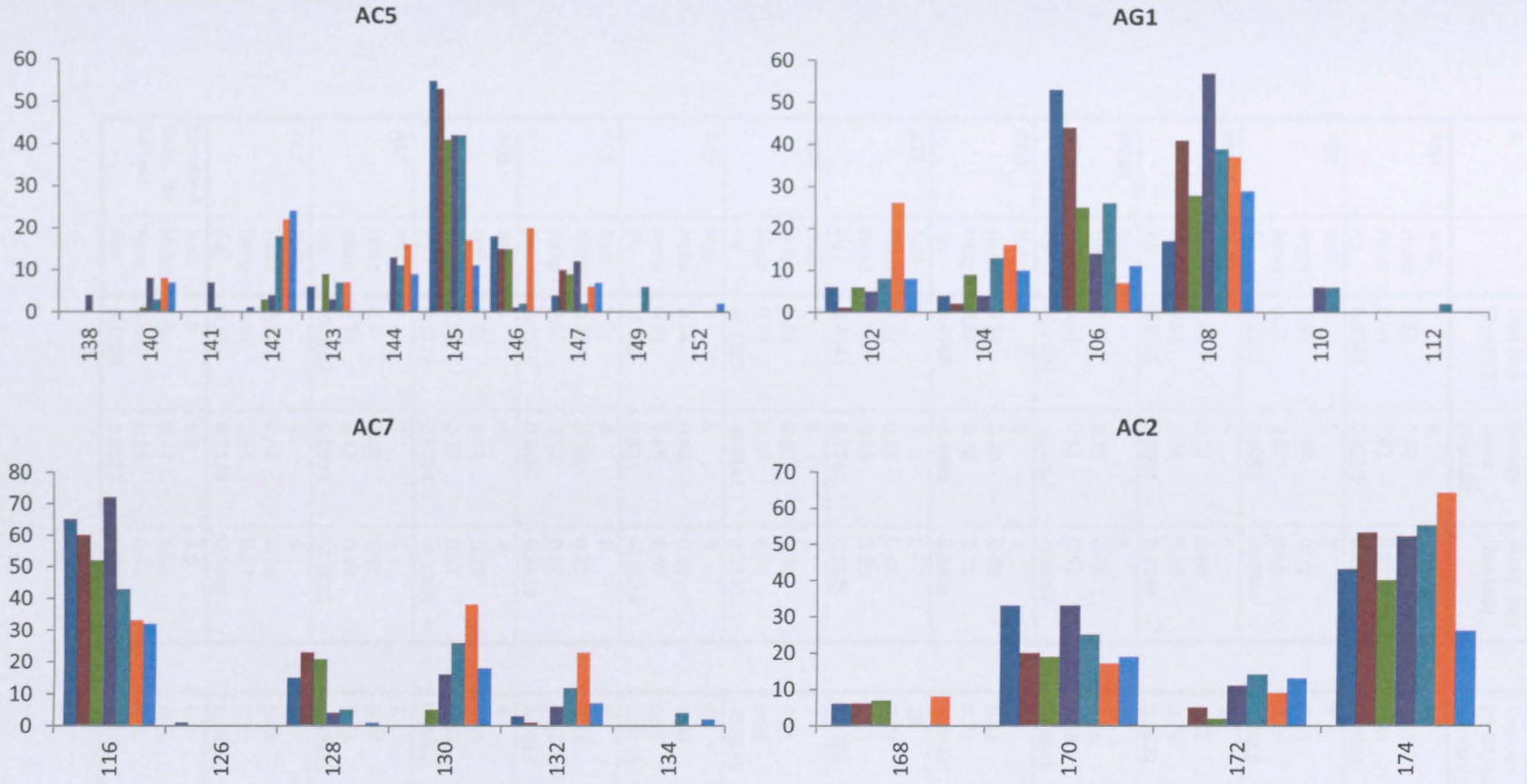


Figure 4.1 Histograms of the allelic diversity of 12 markers across seven sample sets. Allele sizes are represented on the x axis, frequencies on the y axis.

4.3.1. Hardy Weinberg Tests

A.		East End (2n=100)	George Town (2n=100)	West Bay (2n=100)	Cayman EE,GT,WB (2n=300)
AG7	Nall	7	6	4	10
	Hobs	0.67	0.56	0.50	0.58
	Hexp	0.69	0.62	0.57	0.63
	Fis	**0.0344**	0.0919	**0.1261**	**0.0823**
H08	Nall	6	5	3	8
	Hobs	0.55	0.66	0.72	0.64
	Hexp	0.71	0.67	0.65	0.69
	Fis	0.2345	0.0095	-0.0969	**0.0612**
M313	Nall	4	6	5	6
	Hobs	0.45	0.52	0.60	0.52
	Hexp	0.63	0.74	0.68	0.70
	Fis	**0.2936**	0.3004	0.1149	**0.2523**
M201	Nall	4	3	4	4
	Hobs	0.74	0.75	0.78	0.76
	Hexp	0.61	0.51	0.52	0.55
	Fis	-0.2109	-0.5102	-0.5067	**-0.3945**
AG3	Nall	5	8	5	8
	Hobs	0.34	0.49	0.46	0.43
	Hexp	0.67	0.70	0.71	0.73
	Fis	**0.4969**	**0.3096**	**0.3471**	**0.4044**
AT1	Nall	6	7	6	10
	Hobs	0.32	0.14	0.46	0.32
	Hexp	0.59	0.63	0.55	0.67
	Fis	**0.4543**	**0.7742**	0.1625	**0.5233**
AG5	Nall	7	6	5	7
	Hobs	0.83	0.60	0.70	0.71
	Hexp	0.74	0.59	0.71	0.69
	Fis	-0.1223	-0.0141	0.0142	-0.0359
A10	Nall	5	5	6	6
	Hobs	0.64	0.46	0.52	0.60
	Hexp	0.63	0.69	0.49	0.66
	Fis	-0.003	0.334	-0.0613	0.0923
AC5	Nall	5	3	6	6
	Hobs	0.28	0.08	0.31	0.23
	Hexp	0.52	0.52	0.67	0.56
	Fis	**0.4579**	**0.8451**	**0.5419**	**0.6017**
AG1	Nall	4	4	4	4
	Hobs	0.35	0.52	0.79	0.54
	Hexp	0.51	0.38	0.67	0.60
	Fis	0.3222	0.0294	-0.1708	0.0894
AC7	Nall	4	3	3	5
	Hobs	0.4	0.38	0.56	0.45
	Hexp	0.37	0.42	0.49	0.43
	Fis	-0.0882	0.0933	-0.1652	-0.0503
AC2	Nall	3	4	4	4
	Hobs	0.46	0.42	0.56	0.48
	Hexp	0.56	0.54	0.57	0.56
	Fis	0.181	**0.2128**	0.0249	**0.1496**
Combined Data for All Loci	Nall	5.0	5.0	4.6	6.5
	Hobs	0.5	0.47	0.58	0.52
	Hexp	0.6	0.58	0.61	0.62
	Fis	**0.1709**	**0.2063**	**0.0276**	**0.148**

B.		Havana (2n=100)	Jamaica (2n=100)	Tampa (2n=100)	Miami (2n=60)	All (2n=660)	Mean of All (2n=660)*
AG7	Nall	3	6	5	5	12	5.1
	Hobs	0.30	0.54	0.82	0.68	0.57	0.58
	Hexp	0.35	0.58	0.74	0.63	0.63	0.60
	Fis	0.1582	0.0613	-0.1133	-0.0869	**0.0962**	**0.0388**
H08	Nall	3	3	4	3	10	3.9
	Hobs	0.48	0.54	0.74	0.71	0.63	0.63
	Hexp	0.49	0.50	0.60	0.61	0.64	0.60
	Fis	0.0376	-0.0901	-0.2485	-0.1726	**0.0203**	-0.0466
M313	Nall	6	5	5	4	6	5.0
	Hobs	0.15	0.55	0.46	0.52	0.46	0.46
	Hexp	0.73	0.75	0.66	0.72	0.75	0.70
	Fis	**0.7981**	**0.2665**	**0.3111**	0.2813	**0.3853**	**0.338**
M201	Nall	3	2	2	2	5	2.9
	Hobs	0.78	0.78	0.74	0.89	0.78	0.78
	Hexp	0.53	0.50	0.48	0.51	0.54	0.52
	Fis	-0.4734	-0.5607	-0.5387	-0.781	-0.4369	-0.5117
AG3	Nall	4	5	4	5	8	5.1
	Hobs	0.63	0.70	0.40	0.7	0.52	0.53
	Hexp	0.72	0.74	0.62	0.72	0.75	0.70
	Fis	0.1275	0.0591	**0.3521**	0.0233	**0.2953**	**0.2451**
AT1	Nall	9	10	6	6	13	7.1
	Hobs	0.58	0.58	0.67	0.57	0.48	0.47
	Hexp	0.83	0.83	0.69	0.77	0.79	0.70
	Fis	**0.3134**	**0.3056**	0.0315	**0.2604**	**0.3969**	**0.3288**
AG5	Nall	8	6	7	6	8	6.4
	Hobs	0.73	0.78	0.71	0.93	0.75	0.75
	Hexp	0.79	0.73	0.75	0.81	0.79	0.73
	Fis	**0.0817**	-0.0693	0.0617	-0.1592	**0.0505**	-0.0296
A10	Nall	4	4	6	5	6	5.0
	Hobs	0.59	0.52	0.46	0.52	0.55	0.53
	Hexp	0.69	0.69	0.58	0.58	0.68	0.62
	Fis	0.1442	0.2427	0.2095	0.1102	**0.1936**	0.1395
AC5	Nall	9	9	7	6	11	6.4
	Hobs	0.38	0.46	0.69	0.83	0.43	0.43
	Hexp	0.76	0.73	0.84	0.77	0.75	0.69
	Fis	**0.4957**	**0.3813**	0.1807	-0.0861	**0.4323**	**0.4024**
AG1	Nall	5	6	4	5	6	4.6
	Hobs	0.47	0.49	0.53	0.79	0.55	0.56
	Hexp	0.53	0.73	0.69	0.68	0.68	0.60
	Fis	0.1236	**0.3304**	0.2272	-0.1646	**0.1956**	**0.0996**
AC7	Nall	4	5	3	5	6	3.9
	Hobs	0.41	0.78	0.70	0.57	0.54	0.54
	Hexp	0.43	0.67	0.66	0.62	0.58	0.52
	Fis	0.057	-0.1579	-0.0638	0.0887	0.0618	-0.0337
AC2	Nall	3	3	4	3	4	3.4
	Hobs	0.48	0.66	0.53	0.75	0.54	0.55
	Hexp	0.58	0.57	0.53	0.65	0.58	0.57
	Fis	0.1773	-0.1575	0.006	-0.1656	0.0585	0.0398
Combined Data for All Loci	Nall	5.1	5.3	4.8	4.6	7.9	4.9
	Hobs	0.50	0.62	0.62	0.71	0.57	0.51
	Hexp	0.62	0.67	0.65	0.67	0.68	0.55
	Fis	**0.1701**	**0.051**	**0.0346**	-0.071	**0.1458**	**0.0842**

Table 4.2 Genetic variability and deviation from Hardy Weinberg at twelve microsatellite loci of samples collected from Grand Cayman (A) and the Caribbean (B).
Nall = number of alleles, Hobs = heterozygotes observed, Hexp = heterozygotes expected, deviation from Hardy Weinberg is shown by *Fis*; significance after Bonferroni correction is represented by bold (P<0.05). The final row in each table shows the combined analysis for all loci in the population. The final two columns in Table B include all populations from Grand Cayman and the Caribbean. * Mean of all *Fis* values is calculated by the sum of values for each locus divided by the total number of sites (7).

Locus	Samples Deviated from HWE at 0.05 Significance		
	Cayman	Caribbean	Total
AG7	2	0	2
H08	0	0	0
M313	1	3	4
M201	0	0	0
AG3	3	1	4
AT1	2	3	5
AG5	0	1	1
A10	0	0	0
AC5	3	2	5
AG1	0	1	1
AC7	0	0	0
AC2	1	0	1

Table 4.3 The number of sample sets that deviate significantly from Hardy Weinberg Equilibrium for each marker after sequential Bonferroni correction at 0.05 significance.
For Cayman there are 3 sample sets in total and the Caribbean 4 sample sets.

Sample Set	No. of Markers Deviating from HWE at 0.05 Significance
East End	5
George Town	4
West Bay	3
Cayman Combined	8
Havana	4
Jamaica	4
Tampa Bay	2
Miami	1
All Samples Combined	9

Table 4.4 The number of markers (out of 12) that deviate significantly from Hardy Weinberg for each sample set after sequential Bonferroni correction at 0.05 significance.

Each locus showed varying degrees of polymorphism across the different populations with some markers identifying greater numbers of alleles e.g. AT1 (nall=13) than others e.g.

M201 (nall=5) (Table 4.2). In all sample sets at least one marker deviated from Hardy Weinberg. In total 34 out of 84 tests showed significant deviation from Hardy Weinberg Equilibrium (HWE) before Bonferroni correction and 23 out of 84 showed significant deviation after Bonferroni correction and all except one (Cayman combined locus M201) revealed a deficit of heterozygotes. Four markers (H08, M201, A10 and AC7) showed no deviation from HWE in any of the sample sets (Table 4.3), while three deviated in both Cayman and Caribbean sample sets (AG3, AT1 and AC5). The number of markers deviating from HWE increased in the pooled analysis (Table 4.4). When all Grand Cayman samples were treated as one population two thirds of the loci showed significant deviation from HWE with all except one of the eight loci (M201) showing a deficit of heterozygotes. When all the samples were pooled and treated as one Caribbean population nine out of twelve markers deviated significantly from Hardy Weinberg all showing a heterozygote deficit. The deficit of heterozygotes is likely due to the existence of subpopulations (the Wahlund effect). However the presence of null alleles, preferential amplification of one allele in heterozygotes or other locus specific constraints (Wondji *et al.*, 2002) cannot be ruled out.

In all sample sets markers AG3 and AG1 were consistently significantly linked (data not shown); this was subsequently confirmed by searching the *Ae. aegypti* genome (Nene *et al.*, 2007) which verified that these two markers amplify the same genomic region. Subsequent analysis was therefore carried out with the AG1 marker removed. Overall no major linkage was detected between any other pairs of loci.

4.3.2. Genetic Differentiation between Populations

Levels of genetic differentiation between populations were estimated using *Fst* (Weir & Cockerham, 1984) (Table 4.5). *Fst* is a statistic that can be used to estimate the level of gene flow between individuals from different populations.

		No. Of Significant Loci (P<0.05)	AG7	H08	M313	M201	AG3	AT1	AG5	A10	AC5	AC7	AC2	*Fst* All Loci
George Town	West Bay	4	0	0	**0.001**	0	0.027	**0.093**	**0.032**	**0.04**	0.012	0	0	**0.017**
East End	George Town	2	0.004	0.015	0.021	0.007	0.036	**0.069**	0.017	**0.023**	0	0.005	0.023	**0.02**
Havana	Jamaica	5	0.038	**0.083**	0	0	0.028	**0.071**	**0.085**	0.039	**0.018**	**0.067**	0	**0.039**
Jamaica	Miami	6	0.009	**0.016**	**0.037**	0	0.016	**0.054**	**0.107**	**0.101**	**0.074**	0	0.012	**0.042**
Tampa Bay	Miami	6	0.017	0.01	**0.22**	0.011	0.034	0.009	**0.037**	**0.046**	**0.035**	**0.033**	**0.061**	**0.052**
East End	West Bay	4	0.006	0.02	**0.056**	0.002	**0.101**	**0.277**	0.003	0	0.002	**0.019**	0.004	**0.053**
West Bay	Jamaica	9	**0.087**	**0.062**	**0.046**	**0.045**	0	**0.124**	0.009	**0.113**	**0.043**	**0.107**	**0.010**	**0.059**
Havana	Miami	5	0.108	0.044	**0.065**	0	0.015	**0.133**	**0.073**	**0.059**	**0.106**	0.047	0.003	**0.062**
George Town	Jamaica	9	**0.105**	**0.059**	**0.028**	**0.049**	0.008	**0.071**	**0.054**	**0.09**	**0.095**	**0.149**	0.003	**0.064**
West Bay	Miami	6	0.02	0.001	**0.062**	0.043	0	**0.132**	**0.11**	0.002	**0.177**	**0.112**	**0.041**	**0.067**
Jamaica	Tampa Bay	9	0.042	**0.064**	**0.093**	0.01	**0.111**	**0.112**	**0.13**	**0.157**	**0.062**	**0.025**	**0.011**	**0.08**
East End	Jamaica	8	0.05	**0.139**	**0.061**	**0.043**	0.06	**0.137**	0.020	**0.14**	**0.08**	**0.151**	**0.032**	**0.083**
Havana	Tampa Bay	9	**0.148**	0.006	**0.069**	0.005	**0.031**	**0.17**	**0.052**	**0.097**	**0.08**	**0.174**	**0.031**	**0.083**
East End	Miami	9	0	**0.059**	**0.062**	**0.039**	**0.149**	0.049	**0.09**	**0.016**	**0.262**	**0.15**	**0.039**	**0.09**
West Bay	Havana	10	**0.227**	**0.087**	**0.05**	**0.053**	0.018	**0.271**	**0.076**	**0.059**	**0.035**	**0.062**	**0.007**	**0.091**
George Town	Miami	6	0.031	0.003	**0.037**	0.046	**0.072**	0.029	**0.188**	0.03	**0.271**	**0.155**	**0.045**	**0.091**
East End	Havana	11	**0.175**	**0.176**	**0.042**	**0.046**	**0.105**	**0.199**	**0.076**	**0.056**	**0.075**	**0.046**	**0.006**	**0.096**
George Town	Havana	10	**0.252**	**0.096**	**0.025**	**0.057**	**0.074**	**0.192**	**0.176**	**0.012**	**0.072**	**0.079**	0.011	**0.101**
West Bay	Tampa Bay	8	**0.069**	0.025	**0.181**	**0.105**	**0.08**	**0.228**	**0.091**	0.004	**0.101**	**0.224**	0.005	**0.11**
East End	Tampa Bay	10	0.017	**0.098**	**0.197**	**0.09**	**0.243**	**0.046**	**0.095**	**0.026**	**0.173**	**0.28**	**0.055**	**0.129**
George Town	Tampa Bay	9	**0.059**	0.037	**0.127**	**0.111**	**0.191**	**0.091**	**0.194**	**0.089**	**0.193**	**0.274**	0	**0.132**

Table 4.5 Differentiation of *Fst* for microsatellite loci between sample sets and across all loci.
Significance after Bonferroni correction is represented by bold (P<0.05). Shaded areas indicate groupings of Grand Cayman sample sets with other Caribbean locations. The pair-wise combinations are listed in order of increasing overall *Fst* values.

As expected, the three populations from Grand Cayman show the greatest similarity with overall significant *Fst* values of 0.017, 0.02 and 0.053. When comparing Grand Cayman populations to others from the Caribbean region, the lowest *Fst* values were obtained for Jamaica followed by Miami, Havana and finally Tampa. Geographically Grand Cayman is close to Jamaica although the major shipping routes are through Miami. Of the Grand Cayman populations, West Bay is the most similar to the other Caribbean populations despite this not being the likely entry point of *Ae. aegypti* into the Island; both air and sea ports are located in George Town. East End is less similar to the other Caribbean populations than the other Grand Cayman samples, likely due to its distance from both ports combined with its relative isolation. Outside the Cayman Islands, the greatest similarities between sample sets are between Jamaica and Havana, Jamaica and Miami and Miami and Tampa Bay.

The *Fst* values were used to construct a neighbour joining tree (Figure 4.2) to determine the evolutionary distance between the different populations. Three clades were resolved, one encompassing the three Grand Cayman populations, another for the two Florida populations and the third for Havana and Jamaica. However it is important to note that bootstrap support has not been calculated, so it is not possible to determine the confidence behind these three clades.

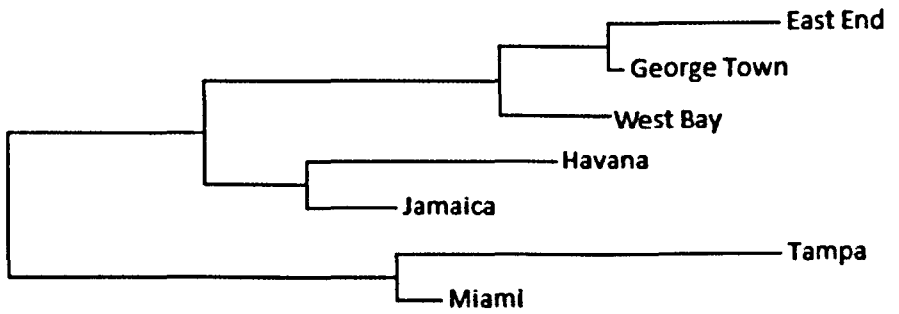


Figure 4.2 Neighbour joining phylogenetic distance tree of linearised *Fst* (*Fst*/(1-*Fst*)) of Caribbean sample sets.

4.3.3. Isolation by Distance

	East End	George Town	West Bay	Havana	Jamaica	Tampa Bay
George Town	28.8 Km					
West Bay	31.7 Km	10.8 Km				
Havana	441 Km	438 Km	425 Km			
Jamaica	359 Km	386 Km	389 Km	718 Km		
Tampa Bay	966 Km	962 Km	953 Km	534 Km	1,172 Km	
Miami	723 Km	729 Km	718 Km	367 Km	869 Km	327 Km

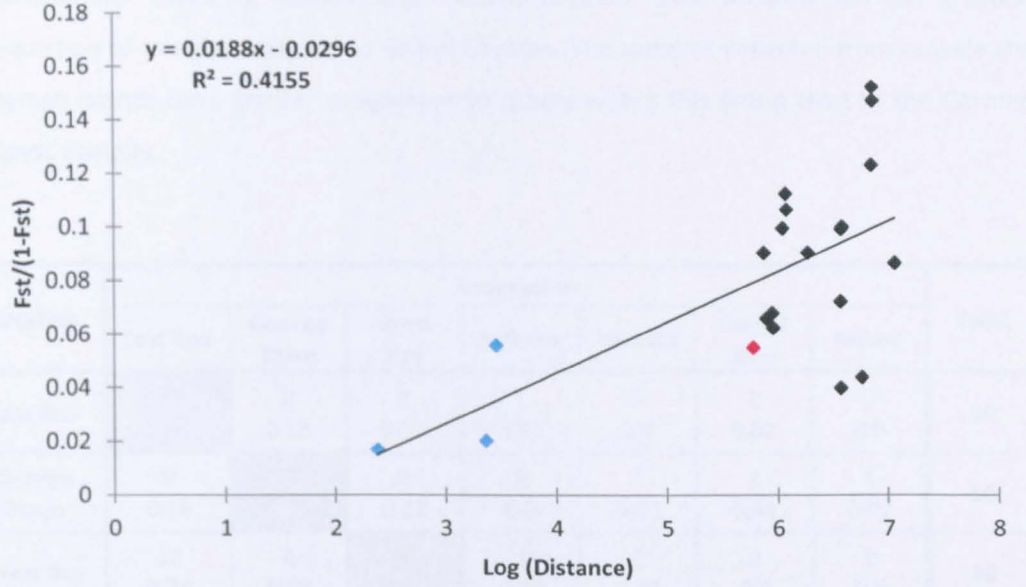


Figure 4.3 Effect of geographical distance on genetic differentiation between Caribbean sample sets.
The grid shows the approximate distance in Km between all sampling sites. The graph shows the relationship between genetic differentiation between different sample sets (*Fst*) and geographical distance. Pairs of Grand Cayman sample sets are represented by pale blue points, Miami and Tampa are represented by a red point, all other pairs of locations are represented by black points.

To test whether the genetic similarity between the populations was correlated with geographical distance, isolation by distance analysis was completed (Figure 4.3). Significant isolation by distance was observed when a Mantel test was carried out (P=0.001). The strength of the association is shown by the R^2 value in this case $R^2 = 0.4155$ which shows a significant association between geographical distance and genetic differentiation.

4.3.4. Assignment Tests

Population structure can be further studied using multilocus genotype data to assign individuals to different populations, these assignment tests were therefore carried out to

determine the potential origins of individual samples. Between 56 and 87% of individual mosquitoes were correctly assigned to the population from which they were derived (Table 4.6). In Figure 4.4, samples are grouped according to whether they were assigned to the correct population, to one of the three Grand Cayman populations or to one of the four other Caribbean populations, 94% of samples from Grand Cayman were either assigned to their original population or to other Grand Cayman populations. Of the three Grand Cayman populations, George Town had the greatest number of samples assigned to populations outside of the Cayman Islands, possibly indicating a number of recent introductions. Excluding samples from Grand Cayman itself, Jamaica had the greatest proportion of samples assigned to Grand Cayman. The samples collected from outside the Cayman Islands have greater assignment to others within this group than to the Cayman Islands samples.

Location	Assigned to							Total
	East End	George Town	West Bay	Havana	Jamaica	Tampa Bay	Miami	
East End	31 0.62	9 0.18	8 0.16	1 0.02	0 0.0	1 0.02	0 0.0	50
George Town	9 0.18	28 0.56	9 0.18	0 0.0	1 0.02	2 0.04	1 0.02	50
West Bay	12 0.24	4 0.08	31 0.62	1 0.02	2 0.04	0 0.0	0 0.0	50
Havana	0 0.0	0 0.0	0 0.0	41 0.82	6 0.12	1 0.02	2 0.04	50
Jamaica	1 0.02	3 0.06	1 0.02	4 0.08	36 0.72	0 0.0	5 0.1	50
Tampa Bay	0 0.0	0 0.0	1 0.02	1 0.02	2 0.04	40 0.8	6 0.12	50
Miami	0 0.0	0 0.0	2 0.07	1 0.03	1 0.03	0 0.0	26 0.87	30

Table 4.6 Assignment of individuals to different population groups.
The top figure in each grouping indicates the number of individuals assigned to that group; the bottom figure shows the proportion of the total.

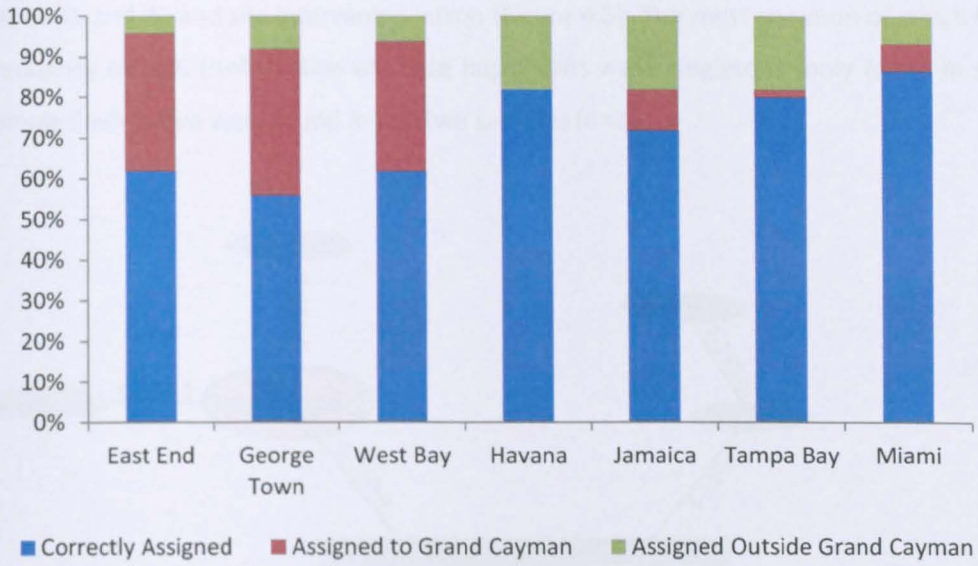


Figure 4.4 Assignment of individuals from different sample sets to populations within Grand Cayman and the Caribbean.

4.3.5. Kdr Haplotype Analysis

```
                  0000000000000011111111111111111111111111111111111111112222222222233
                  2333467799999001112222223344445555555666666677788888990001223566 59
                  5015825724568033692345780116780234568012456906901346080284395201 40

Hap 01 (43)       GCTAACCGCAGCCATTAGGT-ATTAACCTTCTTTTGTACACAACTCATAAAAAAAACAACGCATAT
Hap 02 (8)        ...............................................................G...
Hap 03 (1)        ..............-............................................T.....
Hap 04 (8)        A.AG...ATGTGTCC-GAAA..ACGTTGCA.A.A.TGGGCTGCGCAGAGGGGCTT.TTT..TG...
Hap 05 (1)        ...............................................C..............G...
Hap 06 (1)        ...............................................................GG..
Hap 07 (2)        ..................T.....................................G.......
Hap 08 (2)        ..A.GTGATGTGTCC-GAAA.TACGTTGCA.ACACTGGGCTGCGCAGAGGGGCTTG.T....G...
Hap 09 (1)        ............................G..................................
Hap 10 (1)        .T..........................G..................................
Hap 11 (1)        ...............................................................G.
Hap 12 (1)        A.AG...ATGTGTCC-GAAA..ACGTTGCA.A.A.TGGGCTGCGCAGAGGGGCTT.TTT..TG..A
Hap 13 (1)        ..............-..............................................
Hap 14 (1)        ..................T-..........................................
```

Figure 4.5 Haplotypes of a 400 bp region of the voltage-gated sodium channel in samples from Grand Cayman and across the Caribbean.
Total number of haplotypes = 72; from 36 individuals (27 PCR and 9 clones); two cloned individuals were assumed homozygote due to a lack of an alternative haplotype. Number in brackets = total number of haplotypes observed. Positions 260-262 represent the 1016 amino acid residue; single nucleotide polymorphisms within this residue can result in kdr type insecticide resistance.

Sequence data from samples that were previously analysed (Chapter 3) were used to analyse the genetic variability, notably the haplotype diversity among populations. These samples are from more locations than were used for microsatellite analysis; including five from Grand Cayman and six from the Caribbean region (information on sample locations can be found in Table 3.1, Figure 3.3 and Figure 3.4). Fourteen different haplotypes were identified within a 400bp region of the voltage-gated sodium channel spanning partial

exons 21 and 22 and the intervening intron (Figure 4.5). The most common of which had a frequency of 60% (n=43). Nine of these haplotypes were singletons (only found in single samples) while two were found in just two samples (n=2).

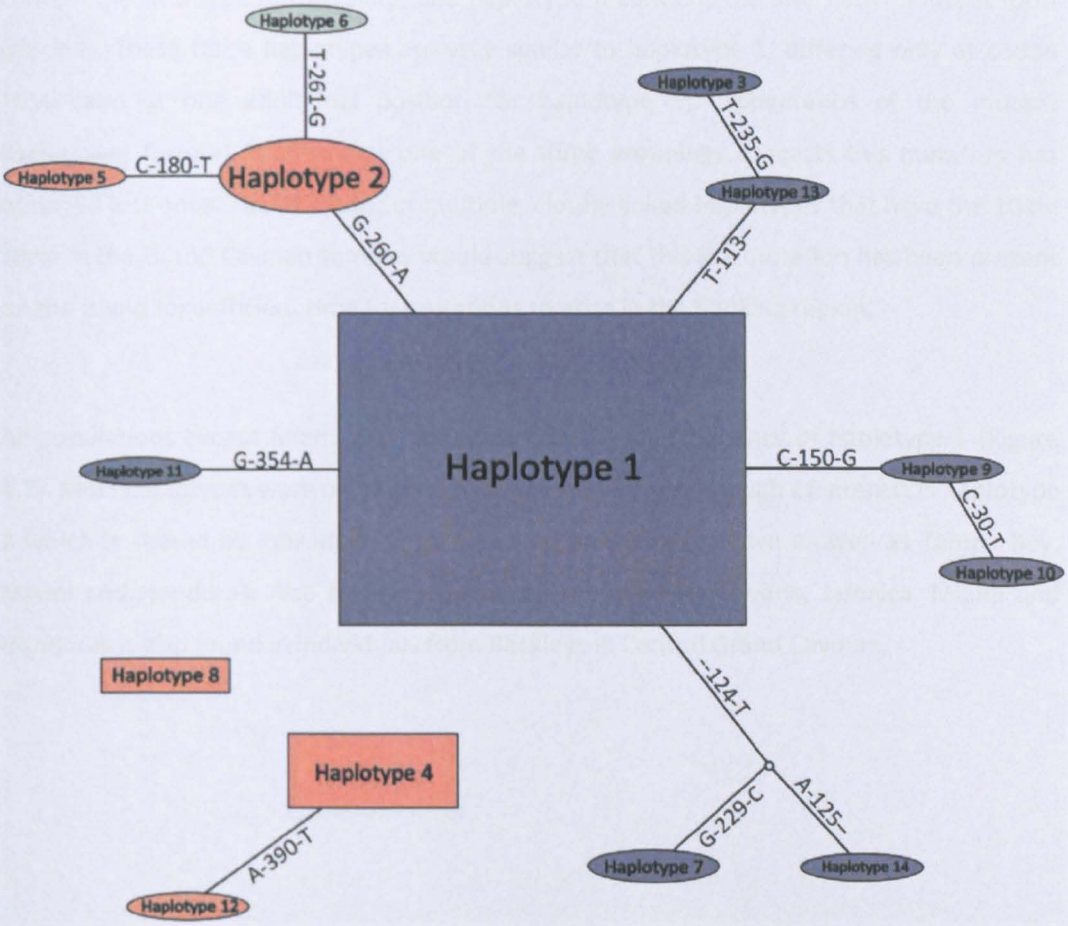


Figure 4.6 Haplotype network of haplotypes of a 400 bp region of the voltage-gated sodium channel showing the genealogical relationships between haplotypes.
Each haplotype is represented by a square (for the most predominant haplotype in each grouping) or an oval proportional to its frequency. Mutational steps are represented by lines upon which are shown the position and base changes between haplotypes. Colouring relates to amino acid residue 1016 that is linked to kdr type resistance. Blue nodes are linked to the mutant (isoleucine) form, pink nodes are linked to the wild type (valine) whilst the green node is linked to the alternative mutant form (glycine).

Haplotype network analysis resolves three different groupings, each of which contain haplotypes with the wild type (valine codon) at position 1016. Different groupings are resolved when haplotypes are beyond the limits of statistical parsimony to join i.e. there are more than 8 mutational steps. One of the groupings contains just one haplotype (haplotype 8), and indeed represents just one sample from East End, with a very different genealogy to all others analysed. A second grouping contains only 1016V alleles and had

two haplotypes, consisting of sequences from Rackleys (central Grand Cayman), Jamaica, Havana, Miami and Honduras. The majority of samples fall into haplotype 1, this haplotype contains the mutant form of the 1016 residue linked with kdr type resistance from which seven other haplotypes are linked that contain this mutant residue. Haplotypes 2 and 5 contain the wild type form (valine) and haplotype 6 contains the alternative mutant form (glycine). These three haplotypes are very similar to haplotype 1, differing only at codon 1016 (and at one additional position for haplotype 5). Appearance of the mutant (isoleucine) form at 1016 in just one of the three groupings suggests this mutation has occurred just once. The existence of multiple, closely linked haplotypes that have the 1016I allele in the Grand Cayman samples would suggest that this kdr mutation has been present on the island for sufficient time for mutations to arise in the flanking region.

All populations except Miami and Honduras have a high frequency of haplotype 1 (Figure 4.7). Most haplotypes were only found in single sequences although of interest is haplotype 2 which is shared by individuals from East End and George Town as well as Tampa Bay, Miami and Honduras. Also haplotype 4 which is shared by Havana, Jamaica, Miami and Honduras is also found in individuals from Rackleys in Central Grand Cayman.

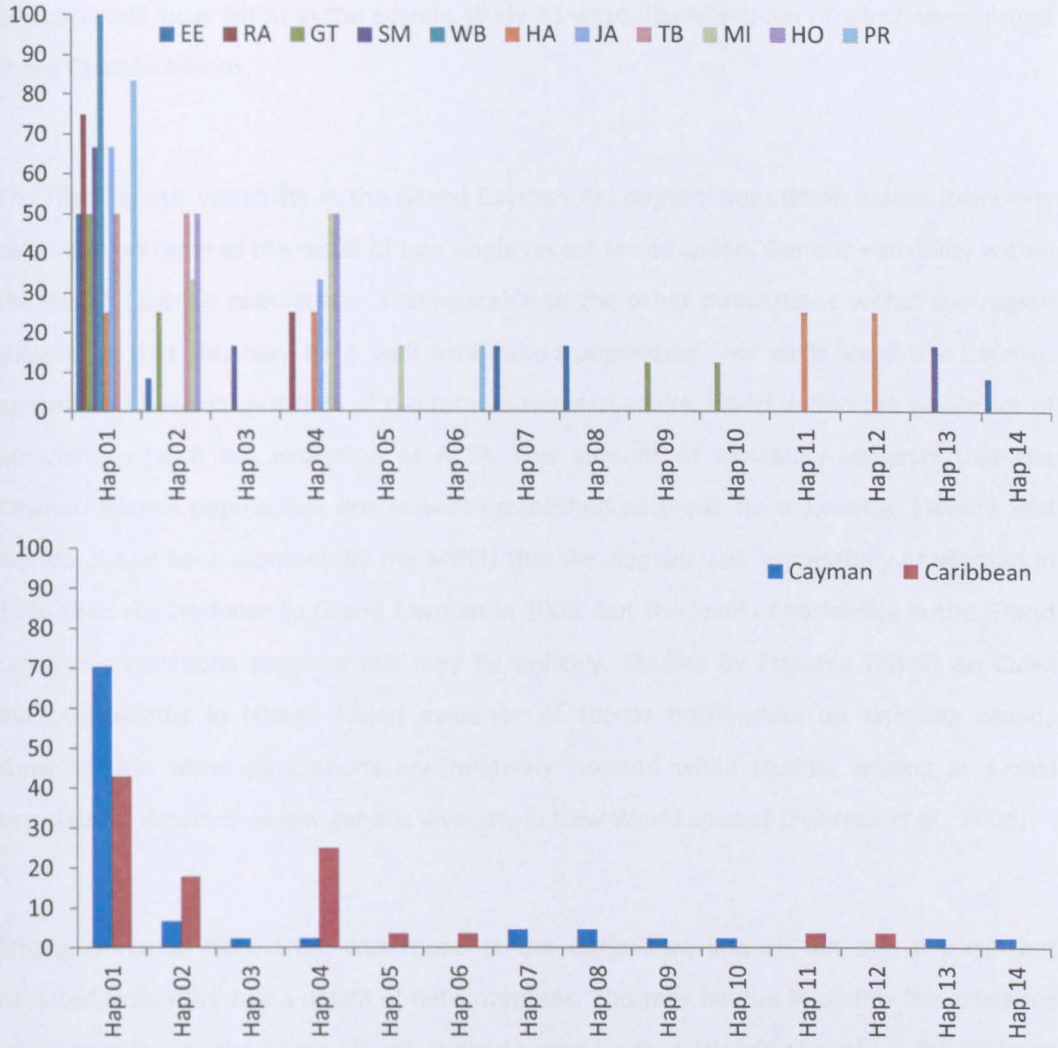


Figure 4.7 Distribution of haplotypes across different Caribbean locations.
EE = East End, RA = Rackleys, GT = George Town, SM = Seven Mile Beach, WB = West Bay, HA = Havana, JA = Jamaica, TB = Tampa Bay, MI = Miami, HO = Honduras, PR = Puerto Rico.

4.4. Discussion

The microsatellite markers chosen were well suited for this type of study; all gave good amplification and were easily combined into multiplex reactions. The markers were not physically linked with the exception of AG3 and AG1. On discovery that they mapped to the same chromosome region, AG1 was removed from further analysis. Each of the markers were polymorphic with loci having an average of 4.9 alleles. This is lower than the diversity found by Lovin (2009) who tested 20 microsatellite loci and found an average of 8.75 alleles per locus, one locus having as many as 24 alleles. One marker (88AT1) used by Lovin (2009) was the same as the AT1 marker used in the present study. Lovin (2009) identified 14

alleles at this locus whilst in the current study 13 were identified, ten of which were found in the Cayman Islands.

The high genetic variability in the Grand Cayman *Ae. aegypti* population makes it unlikely that invasion came as the result of one single recent introduction. Genetic variability within the Grand Cayman populations is comparable to the other populations within the region suggesting that this may be a well established population. For each locus the Cayman populations have at least 66% of the total number of alleles found within the whole set of populations (with the exception of ACS). This amount of variability suggests that the Cayman Islands populations are as well established as those from Jamaica, Havana and Florida. It had been assumed by the MRCU that *Ae. aegypti* was successfully eradicated in 1996 then reintroduced to Grand Cayman in 2002, but the level of variability in the Grand Cayman populations suggests this may be unlikely. Studies by Fonseca (2000) on *Culex quinquefasciatus* in Hawaii found evidence of recent bottlenecks on differing islands showing that some populations are relatively isolated while studies looking at global populations determined low genetic diversity in New World species (Fonseca *et al.*, 2006).

Strong deviation from HWE was found in the Caribbean, and all but one marker that deviated from HWE had a deficit of heterozygotes. This may be due in part to the existence of subpopulations (Wahlund effect). Indeed when Cayman Islands samples and Caribbean samples were combined as single populations greater deviation from HWE was observed, presumably reflecting the fact that these are not single panmictic populations. The high *Fis* values in the Cayman Islands samples, particularly in George Town and East End, are also indicative of considerable levels of inbreeding. Hardy Weinberg assumes an infinitely large population size (Marquardt *et al.*, 2005) hence restrictions to gene flow or small population size may contribute towards the HWE deviation. However the deficit of heterozygotes is also indicative of population expansion (Pinto *et al.*, 2003) so the samples in this study may represent recently founded, growing populations. Other studies on island populations of mosquitoes have also noted high *Fis* values (Bataille *et al.*, 2009) and a deficit of heterozygotes (Pinto *et al.*, 2003, Ravel *et al.*, 2002). Ravel (2002) attributes this to population substructure and the possibility of cryptic species of *Ae. aegypti* a hypothesis that may not be ruled out in the current study.

Prior to 2005 control efforts for *Ae. aegypti* were limited to George Town and West Bay. When eradication was previously announced in 1996 it seems probable that a small population remained on the island and went undiscovered until their eventual reappearance in George Town in 2002. Conversely variation may be due to multiple introductions to the island from elsewhere, each introduction bringing with it new alleles. It would be expected however that these introductions would be through the ports of George Town and spread from there, as a result genetic variability would be expected to be greater in George Town populations than those further from the perceived entry point. Assignment tests used to determine immigration patterns of *Ae. aegypti* around the Caribbean revealed that the population with the most immigration was George Town (only 56% of this population was correctly assigned to itself). However, although the three Grand Cayman populations show similar diversity, the greatest variability of the Cayman populations is found in East End. This may suggest that this is the most established population or alternatively that which is experiencing the most new introductions. However the latter seems unlikely due to the geographical position of this population on the island as East End is furthest from the likely point of entry into Grand Cayman. Overall, the data suggest that eradication was not fully achieved in 1996 and multiple introductions have been occurring until the present time.

Effort was made when sampling the different populations to avoid the possibility of siblings within sample groups; eggs collected from a single ovitrap might be assumed to have all come from the same mother and therefore samples were collected from multiple locations or in the case of Tampa Bay on different collection dates. Unlike the other populations, individuals from Tampa Bay were temporally separated; the first collection took place in May 2008 and the second in July 2009. Studies by Ravel (2002) used temporal analysis of HWE to show a dramatic change in population structuring over time, however preliminary analysis suggests this was not the case for the Tampa Bay samples (data not shown).

Analysis of patterns of genetic differentiation using *Fst* values confirm the three Cayman populations to be the most similar out of the seven populations studied, with George Town and East End the closest followed by George Town and West Bay. Outside Grand Cayman analysis of *Fst* values suggest a high degree of similarity between Jamaica and the Grand Cayman populations, Havana and Jamaica are also very similar (*Fst* =0.0401). Predictably Miami and Tampa Bay have a low genetic differentiation (*Fst* = 0.0554) being both

geographically close and not separated by the sea unlike the other samples. Studies in Hawaii have determined multiple introductions of *Cx. quinquefasciatus* to the islands, it is believed that the rate of introductions from outside Hawaii is slow and exchange between the islands is low. However, low *Fst* values may be due to common ancestry and reduced diversity of the founder event (Fonseca *et al.*, 2000, Fonseca *et al.*, 2006). In the Galapagos, populations are also as a result of multiple introductions and there is evidence of mosquitoes entering the islands on aircraft as well as ships (Bataille *et al.*, 2009).

The high level of similarity between the Jamaica population and all others within the study is interesting and suggests frequent interchange between Jamaica and the other locations either through import or export. Additional to this the collection of samples in Jamaica was not from areas close to either air or sea ports, which suggests that similarity is not due to sampling bias or recent exchange.

Surprisingly there is little evidence of genetic exchange between Grand Cayman and Florida populations. Tampa Bay, a hub for shipping in the region shows the greatest level of difference from all other populations. Also interesting is the similarity between populations from Havana and Miami. Despite the US embargo blocking trade between the US and Cuba *Ae. aegypti* from Havana can be considered to be as similar to those from Miami as the Grand Cayman *Ae. aegypti* are. This is despite much stronger trade links between Miami and Grand Cayman, including up to six direct flights daily as well as more than one cargo ship per week (Julette Wright, Records Office, Port Authority of the Cayman Islands, personal communication). It is possible that although recently trade between the USA and Cuba has been restricted similarities seen through genetic analysis are due to ancestral polymorphisms that mixed between the two countries when trade was still active prior to the 1950s and these populations have maintained a similar diversity over time. Similarities between Grand Cayman and Jamaican *Ae. aegypti* may be due to founder effects as a result of populations having been founded by the same ancestral population rather than current active gene flow between the two islands.

This study found evidence for isolation by distance. Other studies looking at the influence of geographical proximities on genetic differentiation have found less strongly associated isolation by distance in *Ae. aegypti*. Huber (2004) in South East Asia found a correlation by

distance (P=0.026) however, Lovin (2009) found no evidence for genetic isolation by distance (P=0.41) in Haiti despite the relatively small geographical distances, ranging from just 1.4 – 44.5km and Failloux (2002) in French Guiana also found no positive correlation with distance (P=0.31) sample collection sites in this study were also close in comparison to the current study (3 – 275km).

The correlation of isolation by distance within this study should be viewed with caution. The separation of different populations by the sea adds a confounding factor since it is not possible that discrete populations may gradually spread over distance or mix more easily with closer populations. Furthermore, the distances between the populations within the study are not feasible distances to be flown by individual mosquitoes (although mosquitoes can travel large distances on the wind (Ritchie & Rochester, 2001)). It might therefore be assumed that this correlation is as a result of air and sea traffic. International traffic is clearly of key importance in the mixing of the *Ae. aegypti* populations within the Caribbean. However shipping to the Cayman Islands does not correlate with distance, with more air and sea traffic arriving in Grand Cayman originating from Miami than from Jamaica, so the strength of association of isolation by distance in this case is very interesting. Many cargo ships from Miami stop in Jamaica on their way to the Cayman Islands however, it is unlikely that this would provide a feasible source of infestation as this would rely on mosquitoes in Jamaica laying their eggs on cargo whilst the ship is in port. Interestingly, a good correlation of isolation by distance was found in *Culex quinquefasciatus* between Hawaiian Islands (R^2=0.89) in which movement by mosquitoes is also hampered by stretches of water in between individual populations (Fonseca *et al.*, 2000).

Havana, Jamaica and Tampa Bay showed greater assignment to the other Caribbean populations than those from Grand Cayman, which may suggest the Cayman Islands population of *Ae. aegypti* is more isolated from mixing with other populations than others within the Caribbean. This may be due in part to shipping to the Cayman Islands being one way, goods are delivered to the island yet very little is exported. However exchange between Grand Cayman populations and others within the region may be masked due to having three geographically very close populations from one country while the other locations only have one and are comparatively distant from all others. A study of allelic richness in Atlantic coast populations of *Cx. quinquefasciatus* supports the hypothesis that extensive boat traffic across the Caribbean and Atlantic may have led to extensive mixing of

mosquito populations (Fonseca *et al.*, 2006). Also studies in the Galapagos implicate ongoing introductions and movement of *Cx. quinquefasciatus* aided by tourism and inter-island traffic in the increased risk of avian malaria and West Nile Virus (Bataille *et al.*, 2009).

The Miami population shows the greatest assignment to self, suggestive that little genetic exchange is occurring there. The port of Miami is considered the 'Cargo Gateway of the Americas' ships travelling to and from Miami serve over 250 ports worldwide from the Americas, Asia, the Caribbean, Europe and the Middle East (http://www.co.miami-dade.fl.us/portofmiami/information.asp). However, it must be noted that assignment analysis is limited by the populations available for analysis and similarities can only be drawn on those samples genotyped, therefore any population with origins outside those studied will show greater similarity to self. In this case genotypes that are recognised as different from the four Caribbean populations studied here would be incorporated into the representative genotype for Miami and assumed to be most similar to Miami, yet it is very possible that some of these samples have origins outside of the region which were not included within this study.

The majority of the samples on which haplotype analysis was carried out have the same few haplotypes and in all sample sets except Miami and Honduras haplotype 1 is predominant, indeed these populations have no samples that fall into this haplotype (in the case of the Honduras population only two samples were sequenced). The presence of one dominant haplotype is a sign of reduced diversity within this region of the genome. Sample size is a limiting factor for this data set as only a total of 36 samples were analysed. The region of DNA used for haplotype analysis contains amino acid residue 1016 that is linked to kdr type resistance (Saavedra-Rodriguez *et al.*, 2007). Haplotypes 1, 3, 7, 9, 10, 11, 13 and 14 are all linked to the mutant (isoleucine) form, haplotypes 2, 4, 5, 8 and 12 are linked to the wild type (valine), whilst haplotype 6 is linked to the alternative mutant form (glycine). Haplotype analysis of mtDNA of *Cx. quinquefasciatus* in Hawaii determined five distinct haplotypes leading authors to hypothesise the possibility of five separate introductions despite these haplotypes only having one or two base pair differences from the most predominant form (Fonseca *et al.*, 2000). Using the same rationale would lead to the conclusion that the Cayman Islands may have seen up to ten separate introductions of *Ae. aegypti*. An alternative, and perhaps more probable explanation is that the presence of multiple closely related haplotypes reflects an established population, containing the 1016I

allele, that has since diversified in Grand Cayman. The presence of three distinct groupings, two of which contain only the wild type 1016V allele suggests at least three different introductions of *Ae. aegypti* have occurred in Grand Cayman, only one of which contained the resistance allele.

Using the data from this study it would be very difficult to draw concise conclusions regarding the origins of the Grand Cayman *Ae. aegypti*. However the data indicate a certain amount of genetic exchange occurring throughout the islands of the Caribbean and it is probable that the Cayman Islands has received a number of introductions in recent years and/or that complete eradication was not achieved in 1996. This may provide the MRCU with some evidence to support re-establishment of port disinsection. Although a lack of similarity between Cayman Islands populations and that of Miami where most of the goods landing in Grand Cayman originate mean that port disinsection alone will not be enough. Appropriate methods need to be investigated regarding the best insecticidal treatment for eggs on all manner of shipped goods. Despite the Cayman Islands population being most similar to the Jamaican population this cannot confidently be identified as the only source of introduction, neither is it wise to look towards a targeted approach if the port disinsection campaign was reinstated. Although, as Jamaica may represent an important source of re-infestation research on contact points with Jamaica and the ability to block any source of *Ae. aegypti* coming from there may help the control program to some extent.

5. A Pilot Study of Genetic Control of *Aedes aegypti* in Grand Cayman

5.1. Introduction

The high levels of insecticide resistance in *Ae. aegypti* in Grand Cayman and neighbouring regions (Chapters 2 and 3) prompted the MRCU to look for an alternative non-insecticidal method of control.

The RIDL technique, described in Chapter 1, has been evaluated for control of *Ae. aegypti* in caged experiments but prior to this study there had been no field evaluations of the ability of this method to suppress mosquito populations. In this chapter the RIDL OX513A strain (Phuc *et al.*, 2007), developed by Oxitec Ltd., was evaluated in the laboratory and in field releases in Grand Cayman. There were four sequential stages to this trial.

1. The survival of OX513A hybrids with Cayman females was tested with and without tetracycline, to ensure the lethal phenotype was still observed in the Cayman Islands genetic background.
2. A cage study was then carried out to determine the ability of the OX513A males to compete with wild type Cayman males and mate with Cayman females.
3. Two pilot studies were carried out to determine the feasibility of RIDL for control of *Ae. aegypti* in Grand Cayman.
    a. The first was a small study, carried out over six weeks, to determine the ability of lab-reared, engineered male mosquitoes to find and mate wild females, in competition with wild males. Estimates were also made on 'overflooding' ratios of wild to OX513A males to enable an approximation to be made on the numbers of OX513A males required for the suppression trial. Overflooding was calculated assuming a 1:1 ratio of wild males to wild females. The number of trapped females was subtracted from the number of trapped males and the remainder used to calculate the ratio.
    b. The second, larger trial aimed to suppress the target population within a determined treatment site.

The following targets were set to monitor the success of the second trial:

1. Increase the male : female ratio to approximately 10:1 (this is the ratio determined by Oxitec modellers that should result in suppression of the target population (Andrew McKemey, Oxitec Ltd., personal communication)).
2. Demonstrate repeated mating events between OX513A males and wild females by detection of fluorescence in larvae emerging from eggs collected from the field.
3. Suppression of the target population demonstrated by a reduction in the numbers of adult females caught in traps and the ovitrap index within the treatment site compared with the control site.

5.2. Methods

5.2.1. Mosquito Strains

The strain used throughout this trial (OX513A) is a strain in which males and females rely on the addition of tetracycline to larval rearing water for healthy development. In the absence of tetracycline all mosquitoes will die at the L4 - pupal stage (Phuc *et al.*, 2007). The transgenic line has a DsRed fluorescent marker attached to allow for screening of progeny to determine the presence or absence of the transgene. Mosquitoes were bred at the Oxitec Ltd. facility and shipped to Grand Cayman as batches of egg papers. Importation of mosquitoes was conducted under licence from the Cayman Islands Government Department of Agriculture (Appendices 5 and 6).

5.2.2. Site Selection

Two sites were selected on the basis of the following criteria;

1. They are not already a part of an active *Ae. aegypti* control programme.
2. They already support an *Ae. aegypti* population.
3. The human population is dense (small plots of land) and living in fairly open housing as opposed to screened air conditioned 'high quality' housing found in many parts of the island.
4. Both sites are comparable to each other in terms of the above criteria as well as approximate size and isolation from other settlements.

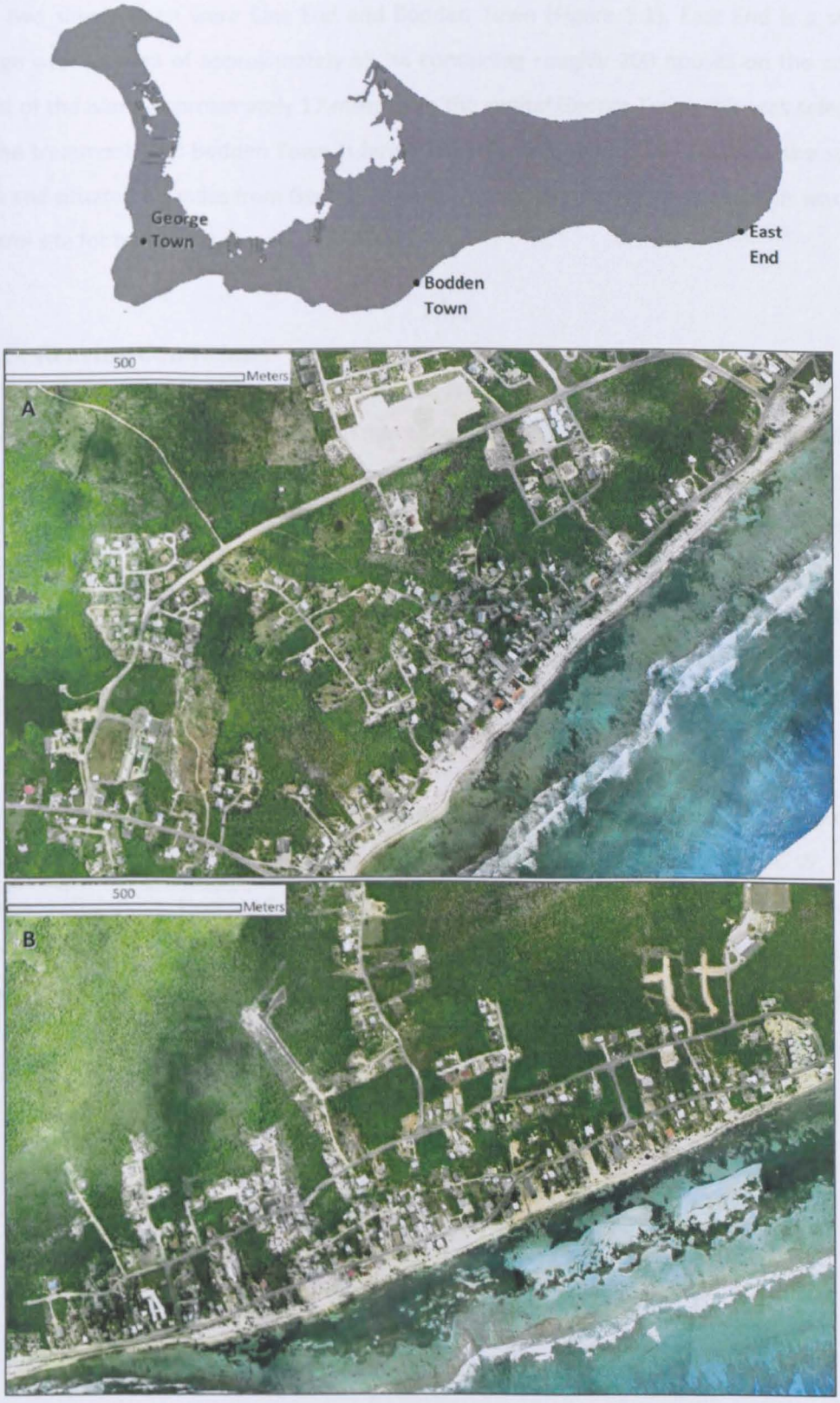


Figure 5.1 Maps showing the study sites for the RIDL field trial.
The schematic shows the locations of the two sites on island as well as the capital George Town. Panel A shows the area of Bodden Town used as the control site. Panel B shows the village of East End used as the treatment site.

The two sites chosen were East End and Bodden Town (Figure 5.1). East End is a small village with an area of approximately 55 Ha containing roughly 200 houses on the south coast of the island approximately 17 miles from the capital George Town; this was selected as the treatment site. Bodden Town is larger (90 Ha) with about 250 houses in the study area and situated 6½ miles from George Town on the south coast of the island; this was the control site for both trials.

5.2.3. Rearing Conditions

Rearing of *Ae. aegypti* was carried out as per protocols developed by Oxitec Ltd. Briefly OX513A eggs provided on seed germination paper were hatched under vacuum maintained for one to three hours; L1 larvae were aliquoted into trays (25.4 x 35.6 x 8.9cm) containing 1.5L tap water at approximately 2 larvae per ml. Tetracycline (30µg/ml) was added to enable eclosion and larvae were fed tetramin fish food until pupation. Initially, the temperature and humidity of the insectary was maintained at ambient. For the suppression trial the insectary facility was climate controlled to maintain the temperature between 26 – 31°C.

In the pilot study, pupae were separated from larvae and sexed using the 'Improved Separator for the Developmental Stages, Sexes and Species of Mosquito' (John. W. Hock Company, Gainsville, Florida - Figure 5.2). However it was feared that this may have caused some mortality in the pupae. For the suppression trial larvae were separated from pupae using tall jugs of cold water; in this case larvae sink and pupae float so can be gently poured off, leaving remaining pupae or larvae to be picked by hand using a 7ml Pasteur pipette.

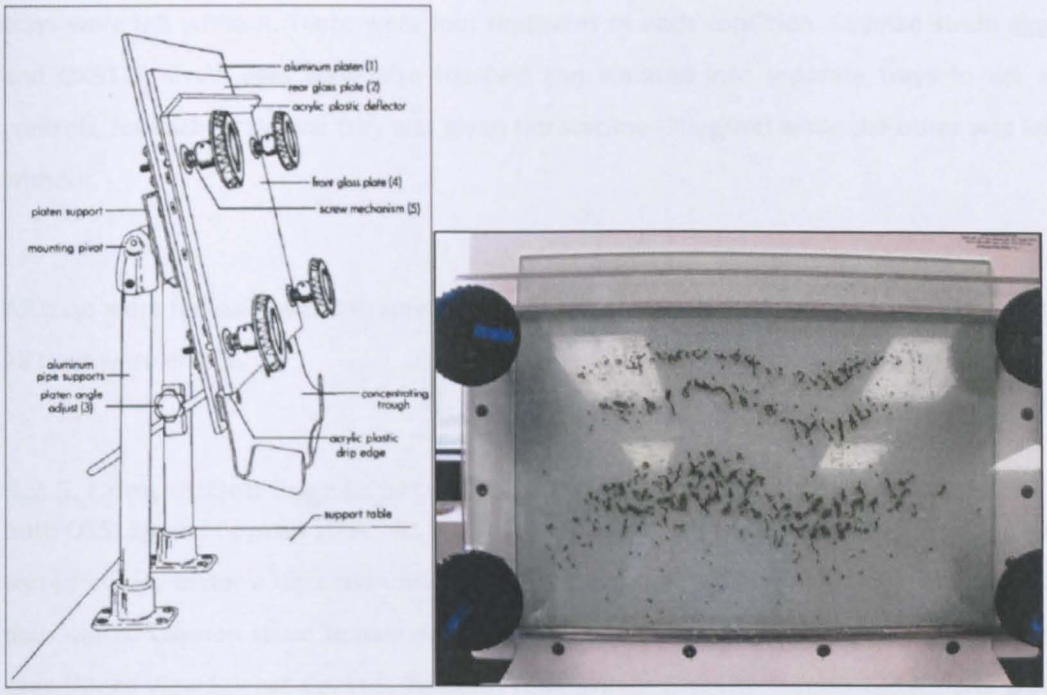


Figure 5.2 The improved separator for the developmental stages, sexes and species of mosquito (John. W. Hock).
Larvae and pupae are sorted by size by adjusting the distance between the two glass plates. Water is flushed through the machine and the different life stages/sexes caught at separate levels. Larvae being the smallest are removed first, followed by male pupae then female pupae last.

Pupae obtained from these initial procedures were put through an additional sorting process using the Oxitec Wire Sorter, a device that enables separation of pupae by size. Female pupae, which are larger than males, were removed and killed and males aliquoted into release devices either by weight or by volume.

5.2.4. Testing the RIDL Construct

Both OX513A and Cayman strain (Section 2.2.1) *Ae. aegypti* were reared separately to pupal stage and sex sorted by eye under a dissection microscope. OX513A males and Cayman strain females were selected and allowed to emerge into separate 30 x 30 x 30cm cages (Mega View Science Co. Ltd., Taiwan) and maintained on 10% sugar water on cotton wool (OX513A females and Cayman strain males were discarded at the pupal stage). At 3 days old 100 Cayman strain females were added to a cage of 50 OX513A males and allowed to mate for two days before being offered a blood meal.

The resulting heterozygous eggs were hatched and L1 larvae counted into batches of 300 in larval rearing trays, half the trays were given tetracycline at 30µg/ml while the remaining

trays were left without. There were four replicates of each condition. Cayman strain eggs and OX513A strain eggs were also hatched and counted into separate trays to act as controls, for each strain one tray was given tetracycline (30μg/ml) while the other was left without.

All trays were fed daily with tetramin fish food and mortality and emergence counted until all trays were empty.

5.2.5. Competition Cage Experiments

Both OX513A and Cayman strain *Ae. aegypti* were reared separately to pupal stage and sex sorted by eye under a dissection microscope into individual 30ml universal tubes. At four days old 10 Cayman strain female *Ae. aegypti* were released into a 30cm x 30cm x 30cm cage (Mega View Science Co. Ltd., Taiwan). Ten Cayman strain and ten OX513A males were removed from their tubes into a plastic cup to allow them to mix before being released into the cage along with the females. Five replicates were carried out. Each cage was maintained for 48 hours and 10% sugar water supplied on cotton wool.

After 48 hours females were removed to individual tubes supplied with sugar and blood meals as well as a damp substrate on which to lay eggs. The resulting eggs were then hatched and the larvae screened for presence of the fluorescent marker indicative of an OX513A father.

5.2.6. Monitoring (Trapping)

Eight weeks prior to the first release in November 2009 a network of 23 ovitraps was set up in both sites. Each ovitrap comprised a one pint mason jar painted black and two thirds filled with tap water containing a 'paddle,' a strip of hardboard (approx 3cm x 15cm) placed rough side up in order to provide a substrate for oviposition (Figure 5.3). Once a week paddles were replaced with fresh strips of hardboard and the water in the pot topped up. The paddles were brought back to the laboratory and scored as positive or negative for eggs and the number of eggs counted. During the release periods all egg positive paddles from East End were hatched in the laboratory, and L1 larvae counted and screened for fluorescence one to two days post hatching. Screening for fluorescence continued after cessation of releases until no fluorescent larvae were detected for at least three weeks.

Due to a small number of *Ae. albopictus* on Grand Cayman all eggs retrieved from all sites during the study were reared through to adulthood to be sure data pertained to *Ae. aegypti* only.

Figure 5.3 Ovitraps and BG sentinel traps used for monitoring during the RIDL field trials. The BG sentinel trap is powered by a battery charged by a solar panel.

BG Sentinel traps (Biogents, Germany. Figure 5.3) were placed outside houses for adult trapping; for the pilot study there were five traps in Bodden Town and 15 in East End, while for the suppression trial there were 10 traps in Bodden Town and 23 in East End (Figure 5.4). Each trap bag was replaced once a week; bags were placed in sealed ziplock bags, frozen for several hours and the numbers of male and female *Ae. aegypti* recorded. All mosquitoes were kept for later analysis.

The BG traps are designed to run on a 12 volt (18 Amp hour) motorbike battery which requires changing and recharging on a daily basis. In order to reduce the labour requirement traps were connected to a small solar panel (17.2 V) which provided power to charge the batteries that run the traps. This enabled traps and batteries to be left in the field for the full six months with minimal maintenance.

Figure 5.4 Maps showing the positioning of BG Sentinel traps during the trials.
Panel A shows trap positions in East End. Note due to changes in the trial site throughout the course of the suppression trial, trap positions varied during the early months of the trial, positions shown are final positions from 17th August onwards. Panel B shows trap positions in Bodden Town. Red = pilot study only, Yellow = both studies, Blue = suppression trial only.

5.2.7. Quality Control

For the pilot study, to ensure that all mosquitoes released into the wild were male and no female contamination occurred, all pupae sorted as males were double-checked by eye under a dissection microscope. This was feasible in the pilot study where approximately 4000 males were being released each time and was considered to be an essential step to

verify that fluorescent eggs/larvae recovered from the field were a result of OX513A males mating wild type females and not the progeny of OX513A females.

Individual screening of the entire release population was not feasible in the suppression trial. Instead, for each batch of pupae sorted, a sample was checked for sex under the microscope by eye. As well as checking for female contamination, the number of males per aliquot were counted so that an estimate could be made of the numbers released. Any females found during inspection under the microscope were removed. A contamination threshold was set at 0.5%; if more than 5 females were found per thousand pupae the batch was re-sorted until quality control came within this limit.

5.2.8. The Pilot Study

In the initial study pupae were released from devices comprising of a number of chambers, a sugar meal (Oasis® Floral Foam soaked in 10% sugar solution) and an emergence cone to protect from the rain through which eclosed males could escape (Figure 5.5).

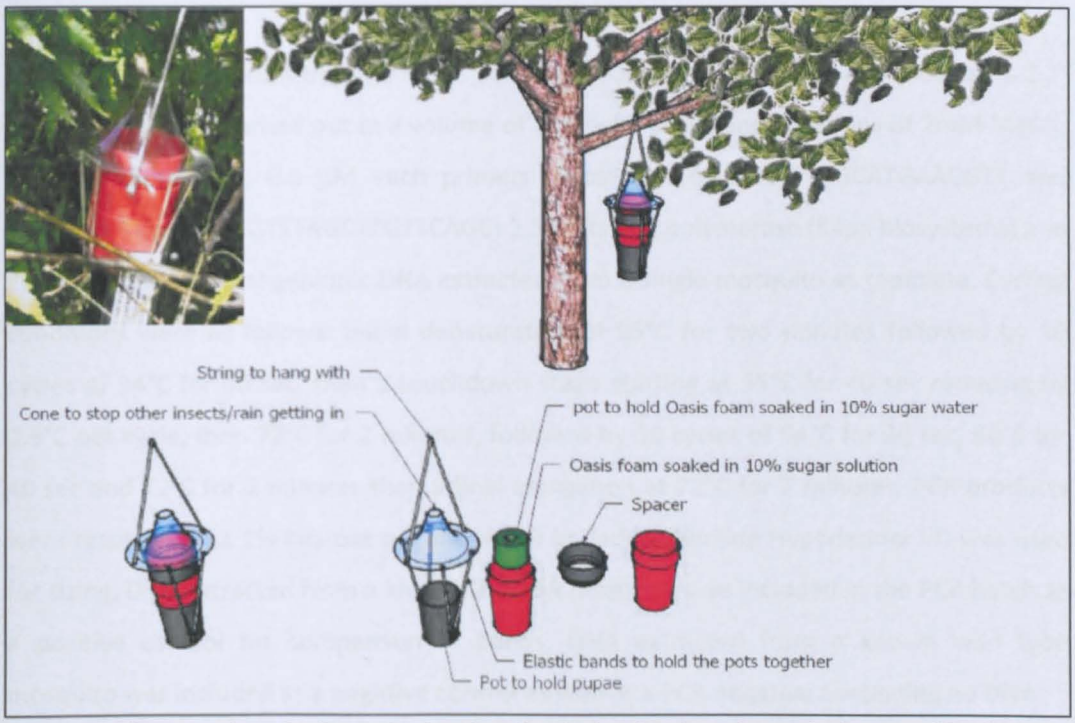


Figure 5.5 Photograph and schematic showing the design for the release devices used in the first RIDL pilot study.
Schematic by D. Nimmo (Oxitec Ltd.)

Pupae were sorted into batches of approximately 80-100 pupae by weight after being dried on kitchen towel, then added to the release devices.

Releases took place three times a week over a ten hectare area (Figure 5.6) at a density of four release devices per hectare for a period of four weeks (approximately 18,600 adult male mosquitoes exited the release devices in total). When new release devices were deployed those from the previous release were collected and returned to the laboratory for analysis. Each device was checked for evidence of ants. Dead pupae, live pupae, dead and live adults as well as empty pupal cases were counted in order to get an estimation of the numbers of adult males successfully released.

5.2.8.1. PCR Analysis of Trap Catches

PCR was used to estimate the over-flooding ratio (the ratio of OX513A : wild males). Primers were used that amplify a region of DNA only found in mosquitoes carrying the OX513A construct, therefore it was possible to differentiate between released and wild mosquitoes by scoring PCR positive or negative respectively.

DNA was extracted from trapped males individually as described previously (Section 3.2.2) and PCR reactions carried out in a volume of 25µl with final concentrations of 2mM $MgCl_2$, 0.2mM each dNTPs, 0.5 µM each primers (DrosF - ATGAGCAATTAGCATGAACGTT and HspdiagR - GCAGATTGTTTAGCTTGTTCAGC) 2.5 units *Taq* polymerase (Kapa biosystems) and 2 per cent of the total genomic DNA extracted from a single mosquito as template. Cycling conditions were as follows: initial denaturation of 95°C for two minutes followed by 10 cycles of 94°C for 30 sec, then a touchdown stage starting at 55°C for 40 sec reducing by 0.5°C per cycle, then 72°C for 2 minutes, followed by 30 cycles of 94°C for 30 sec, 50°C for 40 sec and 72°C for 2 minutes then a final elongation at 72°C for 7 minutes. PCR products were resolved on a 1% agarose gel and a 500 bp ladder (Bioline Hyperladder III) was used for sizing, DNA extracted from a known OX513A mosquito was included in the PCR batch as a positive control for comparison of bands, DNA extracted from a known wild type mosquito was included as a negative control as well as a PCR negative containing no DNA.

5.2.9. The Suppression Study

In the pilot study, survival and eclosion of the pupae in the release devices was variable, likely due to a number of environmental factors including predation by ants. To overcome these problems, in the suppression trial adult males were released directly. Pupae were allowed to eclose in containers in the laboratory; a sugar meal was provided on emergence and time was allowed for the rotation of the genitalia approximately 24 hours in most cases (Roth, 1948).

Male pupae were measured out volumetrically using specially manufactured measuring spoons. Initially they were measured out into disposable coffee cup release devices. Before release the coffee cups were drained by puncturing a hole in the bottom and sealing it back up once water had drained. However, survival rates were low in the cups and therefore part way through the trial the protocol was altered so that adult males were released directly from 30cm x 30cm x 30cm cages (Mega View Science Co. Ltd., Taiwan). Pupae were then aliquoted into weigh boats which were removed from the cages before they were transported to the field. In each case a diet of 10% sugar and 0.2% (w/v) methylparaben was provided either in oasis blocks or in standard cotton wool sugar feeders (Benedict *et al.*, 2009). To monitor survival, 10%, or no less than 8 individual units (release devices or weigh boats), were sampled and mortality counted prior to release. In addition, a further 10%, or no less than 5 units, were returned from the field intact after release so adult mortality could be counted. Mortality data combined with the quality control meant an estimate could be made for each batch on the number of males released. Adults were initially released 6 days after pupae were aliquoted, but during the course of the trial this was reduced to 3 days.

Releases were carried out three times a week across the trial area (Figure 5.6) from May until October 2010. Initially the trial area consisted of the entire village of East End, but as the male : female ratio in the traps did not increase sufficiently the area was reduced to 32 Ha in mid June and finally to 16 Ha at the end of July. Reduction of the field site meant that an internal control site could be evaluated. The first area to be removed from the release site (known as area C) at the far west of the village became the control site. Areas A (the treatment site) and C are separated by a central buffer zone of a few hundred metres (Figure 5.6).

Figure 5.6 Map of release areas for field trials carried out in 2009 and 2010.
Purple = release area for the pilot study (10 Ha), Green = release area 7th May- 21st June (55 Ha), Yellow = release area 24th June-30th July (32 Ha), Red (area A) = release area 2nd August-15th October (16 Ha). Area C = the westerly most portion of the trial site (the green area up to the border with the yellow area).

Ten BG traps were deployed in the control site (Bodden Town) and 23 in East End. In addition 20 and 80 ovitraps were deployed in the control and release sites respectively.

5.3. Results

5.3.1. Testing the RIDL Construct

As expected the absence of tetracycline led to mortality in larvae containing the OX513A construct either in the homozygous or heterozygous state (Table 5.1). Addition of tetracycline resulted in increased survival and was not deleterious to the Cayman strain. This proved the OX513A construct will function correctly in the Cayman background and was therefore suitable for further study. Two percent of heterozygous individuals survived to adulthood, this is to be expected due to incomplete penetrance of the lethal phenotype (Phuc *et al.*, 2007). Survivors are weak due to sub-lethal effects of the genetic lethality and it is anticipated that this figure will be much less in the environment where conditions are harsh and these individuals will therefore die out more rapidly. Indeed trap catches were tested by PCR for 12 days following the suppression trial and no heterozygotes were detected (n=279) (Luke Alphey, Oxitec Ltd., personal communication).

Strain +/- Tetracycline	Mortality (95% Confidence Interval)	(n)
Cayman Strain - tetracycline	2% (0.8-6.9)	(123)
Cayman Strain + tetracycline	1% (0.3-2.9)	(300)
OX513A - tetracycline	100% (99.3-100)	(524)
OX513A + tetracycline	2% (1.0-4.4)	(292)
Heterozygous Hybrids - tetracycline	96% (95.1-97.5)	(913)
Heterozygous Hybrids + tetracycline	2% (1.2-3.0)	(932)

Table 5.1 Mortality, with 95% confidence intervals, of immature *Ae. aegypti* of three different strains reared in the presence and absence of tetracycline.

5.3.2. Competition Cage Experiments

Overall the OX513A male *Ae. aegypti* competed well with the Cayman strain. Fifty five percent (n=31) of the matings that could be determined had an OX513A male as the father (Table 5.2). This provided a preliminary indication that the strain should compete well in the field. However, it should be noted that, although the different strains were reared under identical conditions (with the exception of the addition of tetracycline to the OX513A strain), the size of the males was not determined. It is therefore possible that differential response to the rearing conditions used, e.g. adult size on eclosion, might have influenced the outcome of the mating competition.

Mosquito Number	Cage Number				
	1	2	3	4	5
1	WT	OX513A	WT	OX513A	WT
2	OX513A	OX513A	♀ Died	OX513A	OX513A
3	WT	OX513A	WT	♀ Died	OX513A
4	WT	♀ Died	WT	Didn't hatch	♀ Died
5	Didn't hatch	OX513A	WT	OX513A	♀ Died
6	♀ Died	WT	OX513A	OX513A	♀ Died
7	♀ Died	OX513A	♀ Died	WT	♀ Died
8	WT	WT	OX513A	Didn't hatch	♀ Died
9	WT	OX513A	♀ Died	WT	♀ Died
10	♀ Died	OX513A	♀ Died	OX513A	♀ Died
Frequency RIDL	**0.17**	**0.78**	**0.33**	**0.71**	**0.67**

Table 5.2 Cage experiment of mating competition between OX513A and wild type *Ae. aegypti*. For each mated female that produced eggs the genotype of the offspring (WT=Wild type or OX513A) was determined. Unexpectedly high levels of mortality were observed in cage 5.

5.3.3. The Pilot Study

Approximately 465 RIDL males/Ha/week were released totalling roughly 18,600 adults from about 25,500 pupae. From trap catch numbers it was possible to calculate an approximate over-flooding ratio of about 0.2 : 1 OX513A to wild type males. This was calculated assuming a 1:1 ratio of wild type males : wild type females. PCR was carried out to confirm this (Figure 5.7); during a period of 18 days 143 male *Ae. aegypti* were caught in the BG traps of which 22 (15%) were confirmed as OX513A by PCR. Calculation of over-flooding achieved in the pilot study enabled an estimation to be made on the likely number of OX513A males required to achieve the target of 10:1 OX513A to wild type in the suppression trial.

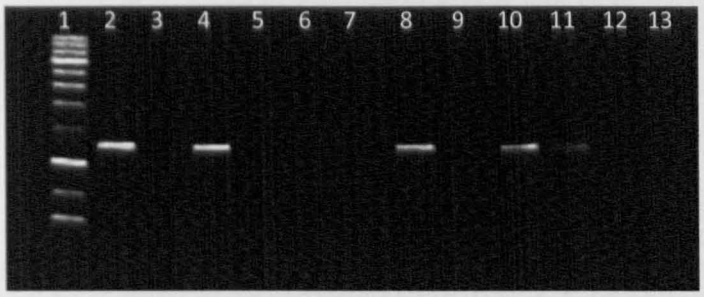


Figure 5.7 An example of PCR of OX513A and wild type *Ae. aegypti* collected from traps during the pilot study.
Lane 1 contains a 100bp DNA ladder, Lanes 2, 4, 8 and 10 contain PCR product from OX513A males, lanes 3, 5-7 and 9 do not contain PCR product (these samples were wild type males), lane 11 contains a positive control (DNA from confirmed OX513A) lane 12 contains a mosquito negative control (DNA from wild type *Ae. aegypti*) and lane 13 contains a PCR negative control. A ribosomal PCR was carried out on all samples prior to detection of the RIDL construct to confirm the success of the DNA extraction. All samples were positive for DNA using this protocol, confirming that DNA quality was not a limiting factor in the above PCR.

Ovitrap index remained at approximately 60% throughout the course of the release period (Figure 5.8). Fluorescent larvae were detected in ovitraps on the 7th, 14th and 21st of December (Figure 5.9) providing evidence that the OX513A males showed some success at competing with the wild type males despite only small numbers being released. This success led to further investigation into using RIDL as a control method and a suppression study was therefore carried out.

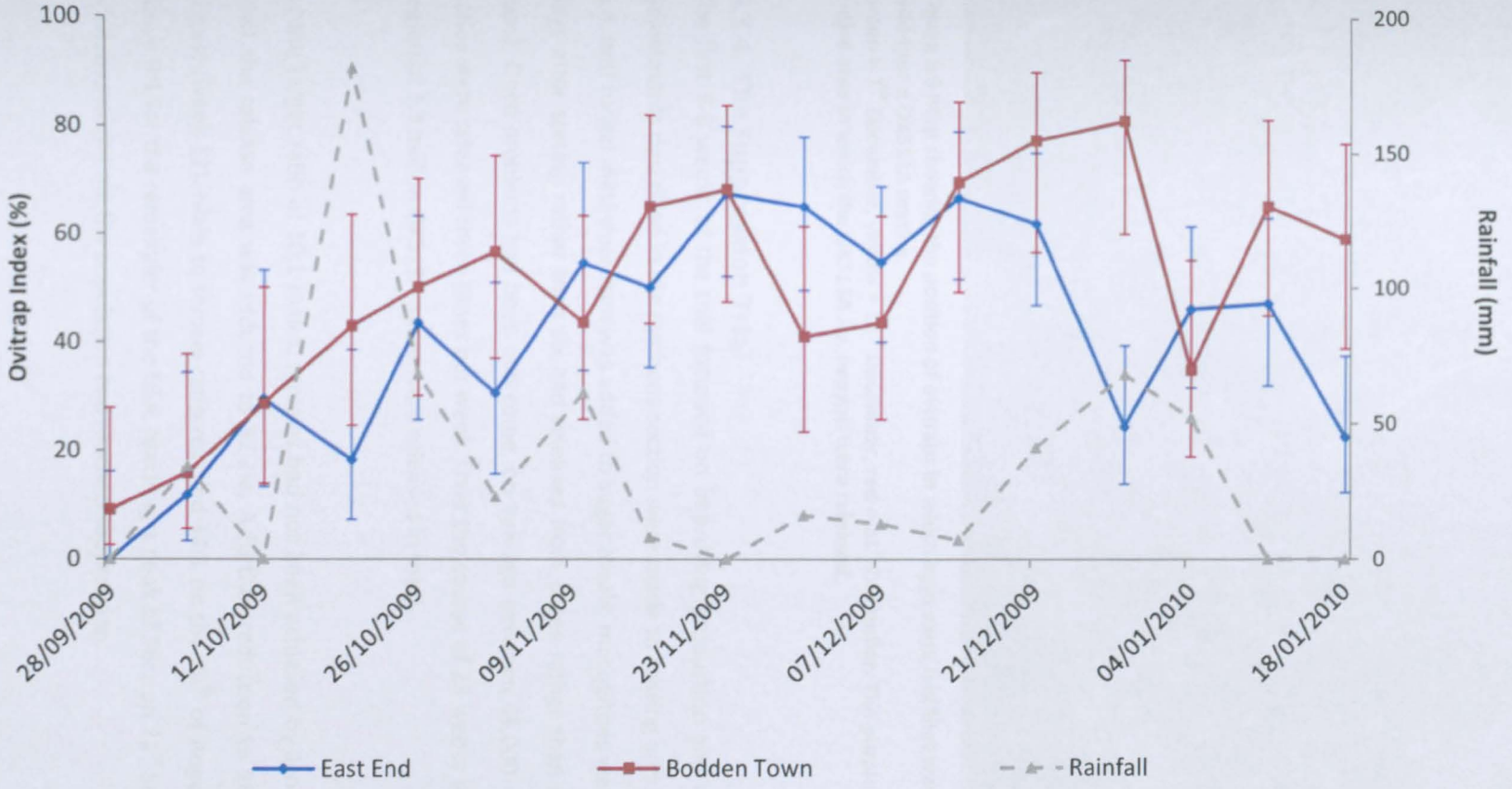


Figure 5.8 Ovitrap positivity in East End (treatment site) and Bodden Town (control site) and East End rainfall during the course of the pilot study and eight weeks preceding it.
Error bars show 95% confidence intervals

Figure 5.9 Map showing the position of ovitraps in which eggs were laid that were the product of a wild type x OX513A mating.
Green = 7th December, yellow = 14th December, red = 21st December. The purple outline shows the entire area in which the OX513A *Ae. aegypti* were released.

5.3.4. The Suppression Trial

The first 6-8 weeks of the trial focussed on improving production and release methods. Adjustments described in the methods section were made to rearing and release protocols (i.e. anti fungal methylparaben was added to sugar meals, mosquitoes were released three days after sorting rather than six and released from cages rather than disposable paper cups). Once problems had been overcome, on average between 45,000 – 85,000 OX513A males were released three times per week. Over the course of 23 weeks somewhere in the region of 3.3 million OX513A males were released in total.

As the target ratio of 10:1 males: females had not been achieved by June (week 7 of the trial) the release area was reduced to 32 Ha. A further reduction to 16 Ha occurred in August (week 12). Male to female ratio reached 14:1 on the 10th of August and remained above 9:1 for the remainder of the trial, reaching a peak of 96:1 on 21st September (Figure 5.10), by which time the population had reached suppression.

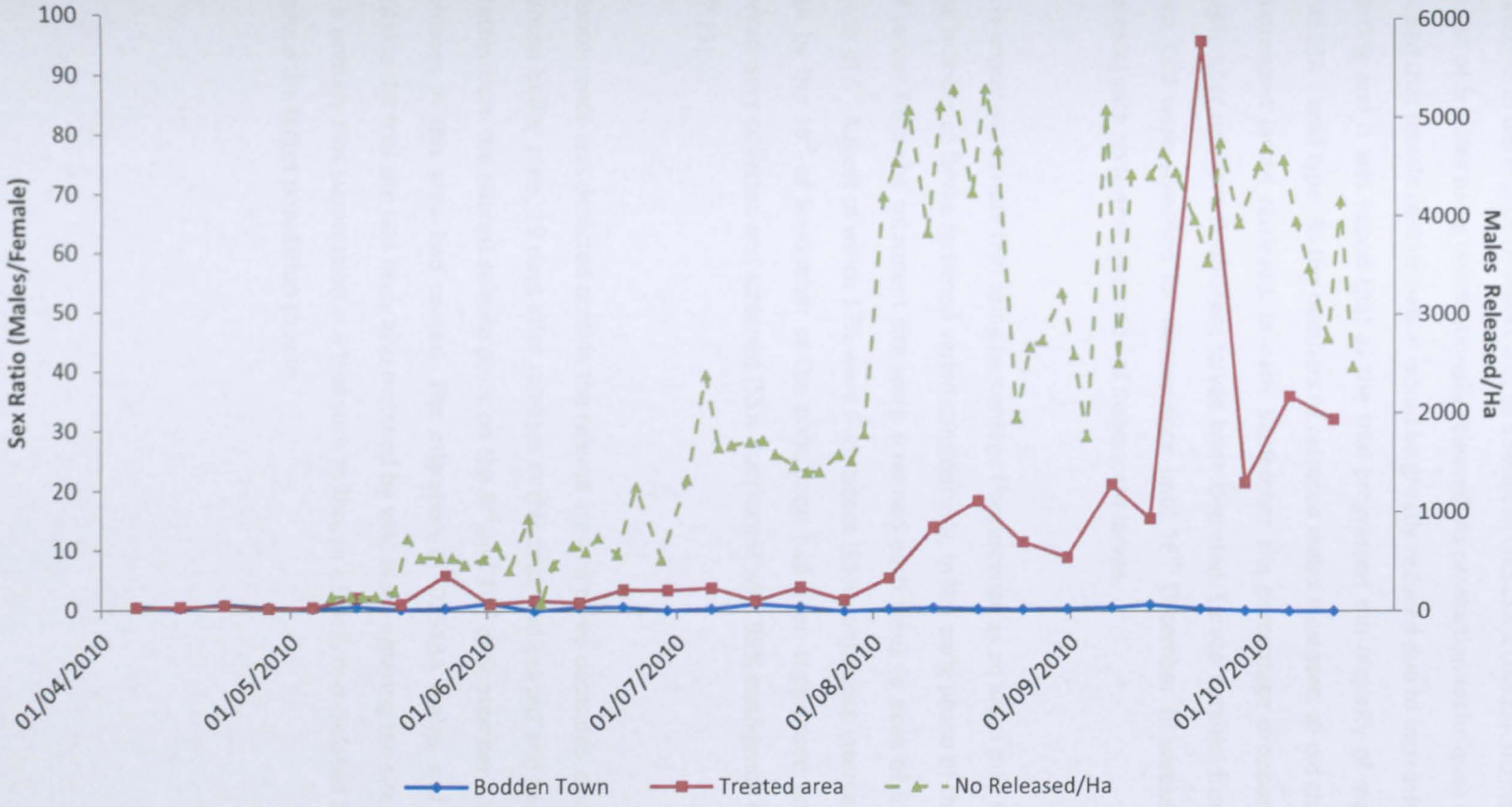


Figure 5.10 Sex ratio of male to female *Ae. aegypti* in both the treatment (East End) and control (Bodden Town) sites. Release numbers per hectare per release are also included.

Fluorescence was detected in wild caught larvae within one week of releases (Figure 5.11); however maternity tests carried out at Oxitec Ltd. indicate that 88% of the fluorescence detected in the first three weeks was as a result of OX513A x OX513A matings probably as a result of females being unintentionally released. As production techniques improved it was hoped that female contamination would be greatly reduced due to increasing experience in sorting and it was hoped that as the trial progressed the majority of matings would be OX513A x wild type. As the numbers of released males increased so did the percentage of fluorescent larvae retrieved. In early September the percentage dropped and after 16th September no more fluorescent larvae were detected. Larvae hatched from all ovitraps in East End were screened for fluorescence until 16th December (9 weeks after the final release) with no further detection of fluorescent larvae.

It is important to note that using percentage fluorescence as an index may be misleading as the number of larvae screened varied considerably. In the early phase of the trial hundreds of larvae from the treatment site were screened each week (a peak of 636 larvae in the week of 5th August of which 17% were fluorescent (95% confidence intervals 13.97-19.76)), but by the 16th of September as the population had been suppressed only a total of 51 larvae were collected and screened (55% fluorescent with 95% confidence intervals 41.38 – 67.73).

Fluorescence was detected outside the release area on three occasions, once in a pot in the central buffer zone, 19 days after releases in this area had ceased and twice in a pot 600 metres from the nearest release point on the 9th and 16th of September 11-12 weeks after releases in this area had ceased. The migration of OX513A males and mated females outside the trial site was likely also matched by wild types entering the site. Because of this it is unlikely that suppression in a trial such as this, in a small, non-isolated area, would ever reduce the target population to zero.

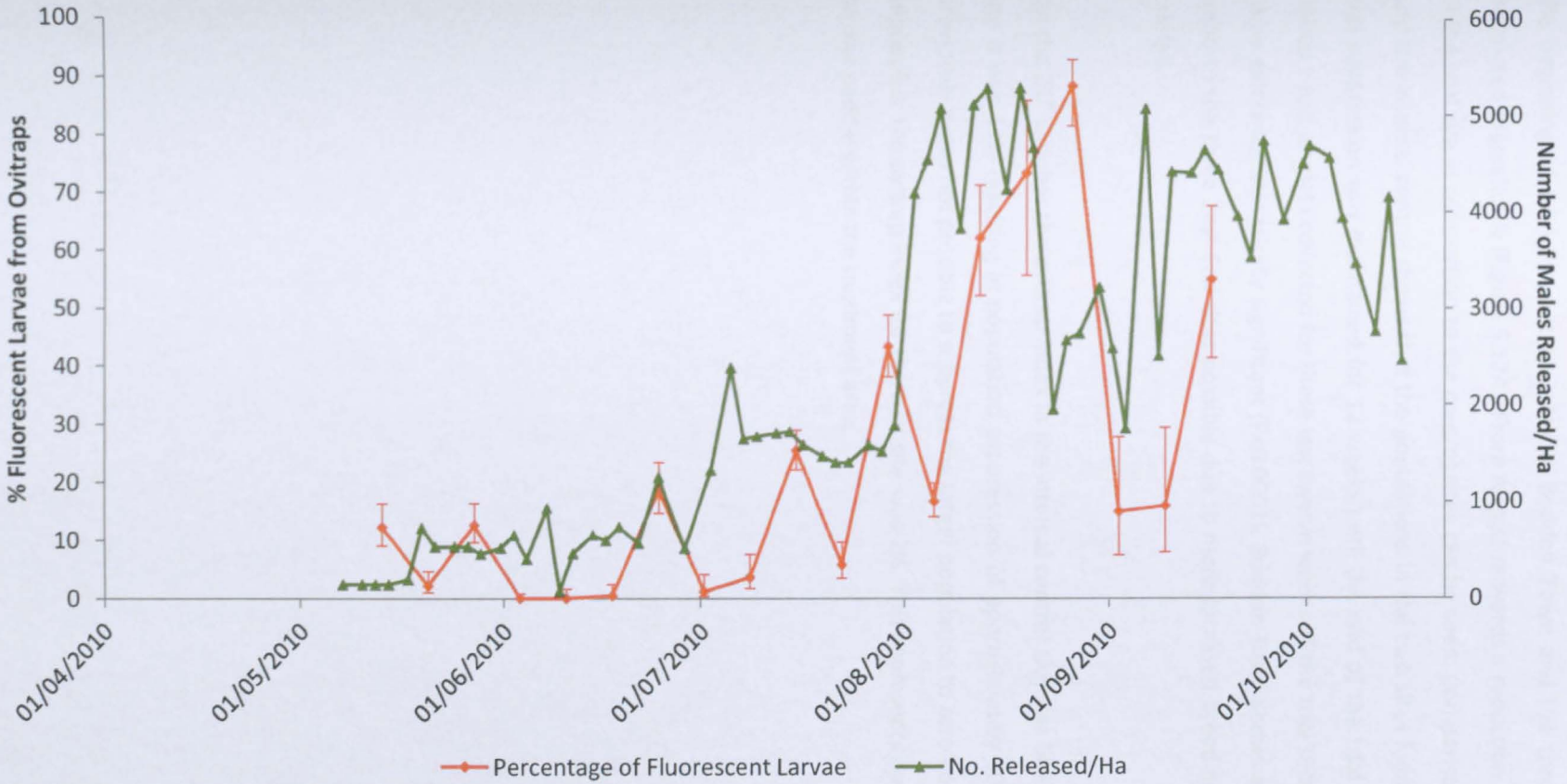


Figure 5.11 Percentage of fluorescent larvae hatched from ovitraps in relation to number of OX513A males released per hectare in the treatment site. Error bars show 95% confidence intervals.

Until numbers of released *Ae. aegypti* males were consistently >1500 males/Ha/week (in the beginning of July) the ovitrap index in Bodden Town and the three East End sites fluctuated dramatically (Figure 5.12A). From August onwards a reduction in the population in the trial site in comparison to the control sites can be seen. Comparison of the trial site and the internal control shows that the populations in the two sites follow a similar trend and suppression was maintained for 12 weeks until the end of the trial (Figure 5.12B). A paired t-test on data collected for these last twelve weeks of the trial shows the difference to be extremely statistically significant ($P<0.0001$). Bodden Town shows a slightly different trend to the three East End sites possibly due to more localised breeding factors, such as rainfall.

On the 21st October the ovitrap index in the internal control site was 60% while in the trial site it was 12% resulting in population suppression of approximately 80%. As mentioned previously it was not possible to suppress the target population to zero however on the 30th September the ovitrap index for the trial site was 2%. This represents one *Ae. aegypti* egg on one paddle within the treatment area.

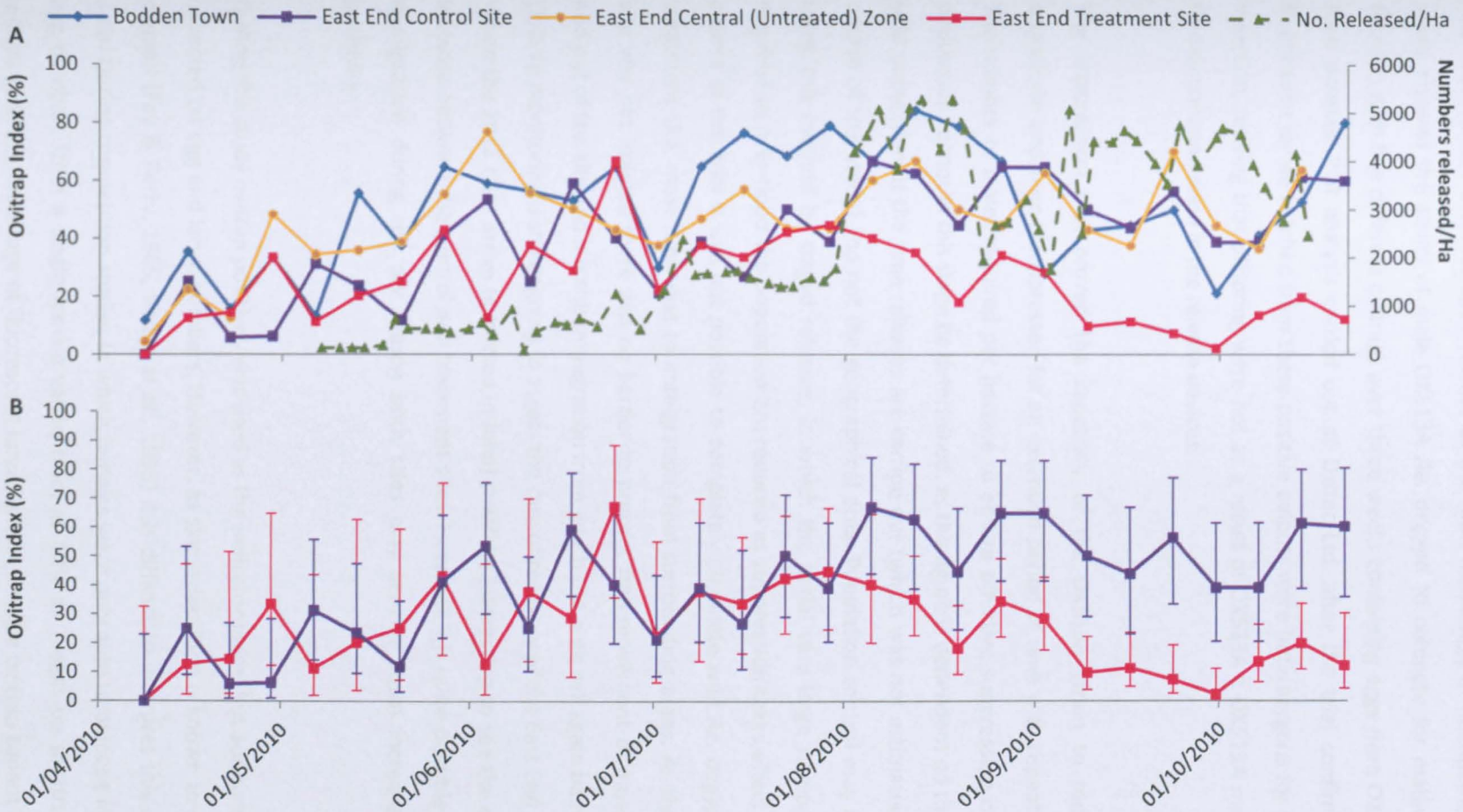


Figure 5.12 Ovitrap index throughout the course of the suppression trial.
Panel A shows all areas being monitored as well as release numbers per hectare. Panel B is focussing on the treatment site and internal control site only. Error bars show 95% confidence intervals.

5.4. Discussion

This trial marked the first open release of the RIDL technology in mosquitoes. The pilot study showed the ability of male OX513A *Ae. aegypti* to compete for mating with wild females with five different ovitraps over three weeks containing eggs from OX513A x wild type parents. PCR analysis carried out at Oxitec Ltd. after the trial confirmed that all fluorescent larvae hatched from these positive ovipots were heterozygous for the OX513A insertion, proving that offspring were not as a result of OX513A x OX513A mating due to female contamination in the release devices.

The suppression trial proved the capability of the OX513A strain to reduce a wild population and keep it suppressed for an extended period of time with repeated releases. The number of males required per hectare to ensure effective suppression of the whole population cannot at this stage be determined, as this figure is dependent on the size of the wild population at the time releases are carried out (which was not estimated during the course of this study) and not the geographical area. Population control may be achieved using this method by staged releases, in which the initial very large numbers that are required to over-flood the population are reduced as suppression takes effect. During the course of this trial it was not possible to completely eliminate wild *Ae. aegypti* from the treatment site, most likely due to immigration from surrounding areas. As the treatment site was not isolated there was no barrier to prevent free movement of mosquitoes into and out of the site and it is this immigration from which the area will again be repopulated. Ovitrap monitoring is still ongoing to assess the rate of recovery of the East End *Ae. aegypti*. When the 2011 rains arrive (expected in June) it will be interesting to see the comparative increase between the control and treatment sites. However it is quite possible that due to immigration during the dry season both sites may see an equal increase in ovitrap positivity.

During this study ovitrap positivity was used as the indicator index, but additional data was collected on egg and larval numbers. However, as skip oviposition is known to occur in *Ae. aegypti* (Fay & Perry, 1965, Reiter *et al.*, 1995) it is difficult to interpret this quantitative data. During competition studies in which females were only able to oviposit in one place, egg numbers from a single female varied from as little as 7 eggs up to 101. Therefore indices such as percentage of fluorescent larvae within a single ovitrap cannot be used for the purposes of estimating population size or the number of OX513A mated females in the

wild at any time. It is also unknown at this time whether the OX513A construct has an effect on relevant post-copulatory processes such as female fecundity or egg viability (hatch rate).

During the course of the field trials many observations were made regarding mass rearing and transportation of healthy male *Ae. aegypti* into the field. Pupal releases in the pilot study were somewhat inefficient. Only approximately 73% of pupae placed in the field eclosed as adults and predator insects e.g. ants probably further reduced the numbers of successful adults emerging. Additionally the effects on adult morbidity due to sorting and aliquoting procedures and temperature in the release devices once they were left in the field remain unquantified. Despite these limitations, and the relatively low release numbers, the pilot demonstrated successful mating between transgenic and wild types.

Early observations in the suppression trial led to an improved regime for insectary hygiene. Mould growing on feeders, dead mosquitoes and other surfaces had a noticeable effect on mosquito survival and health. Mosquitoes released in the early phases seemed lethargic and did not fly vigorously when released. Improvements in laboratory hygiene as well as the addition of an anti-fungal, methylparaben, to sugar feeders (Benedict *et al.*, 2009) resulted in noticeable improvements in mosquito health.

Space also appears to be a factor in mosquito wellbeing. Initially OX513A males were aliquoted into 500ml paper coffee cups at a rate of approximately 600 mosquitoes per cup. Small cage studies determined that sub-lethal effects of overcrowding resulted in reduced longevity of the mosquitoes (Isaac Black, Oxitec Ltd., personal communication). It is also possible that volatiles within the fabric of the cups further reduced the fitness or longevity. Observations were also made on different numbers of mosquitoes transported to the field in 30cm x 30cm x 30cm cages. When large numbers (>4,000 mosquitoes per cage) were moved the overcrowding resulted in many cases of leg loss and frayed wings. This was substantially reduced when the density was decreased to approximately 1,500 per cage.

Increasing the number of males released at each point during the trial met with some objections from the local community. Although male mosquitoes do not bite they are still considered a nuisance. The short flight range of *Ae. aegypti* (typically less than 100m)

would require releases in close proximity to much of the population in more urban areas and this might meet with objections. Potential mitigations might include: reducing the number of mosquitoes released at each point by increasing the spatial or temporal density of releases (though this might have cost implications); reverting to pupal release, which leads to the males emerging over an extended period, rather than all at once; adjusting the time of day at which the mosquitoes are released, e.g. to allow dispersal before more sensitive times, such as the morning and evening rush hours.

The use of genetically engineered mosquitoes to reduce the risk of disease transmission is a contentious issue and historically trials have been halted due to public misconception (Unknown, 1975, WHO, 1976). As yet no guidelines have been developed to address ethical, legal and cultural issues (Lavery *et al.*, 2008). It is therefore vital that all relevant stakeholders are consulted prior to any release. In the current study, a press release announcing the plans was published in the national newspaper as per usual Government protocol (Appendix 7), in addition, a short video documentary was produced to address some of the questions raised whilst working with the local community (http://www.youtube.com/watch?v=_nY_AlWe5kM). Members of the MRCU discussed the trial with key political figures including the current Minister for the district of East End and the opposition party. Further discussions with interested members of the public took place during field work. All staff working on the project wore instantly recognisable uniforms and travelled in a Government identifiable vehicle. In general the public were not shy in approaching staff with questions and ideas and over a short time were aware of who the key members of the project were. On the whole most people were receptive to the idea once they understood that males cannot bite. The main concern surrounded the annoyance of released insects entering houses and in each case it was possible to adjust nearby release point positions to reduce problems. For every incident where a member of the team spoke to the public, the topic, questions, answers provided and any follow up discussions were recorded (Isaac Black, Oxitec Ltd., personal communication).

Currently *Ae. albopictus* is only present in small numbers in the Cayman Islands. Conceivably, the success of a species specific control method such as RIDL could enable other potential species to thrive as new ecological niches become available. This in turn could have implications for dengue transmission. Whilst *Ae. albopictus* serves as a maintenance vector in much of South East Asia where it originates, it is an important vector

of dengue in areas where *Ae. aegypti* is absent (Gratz, 2004). Comparatively *Ae. albopictus* from South East Asia have a much lower oral receptivity for dengue virus (type 2) and are less susceptible to infection than *Ae. aegypti* (Vazeille *et al.*, 2003), *Ae. albopictus* are also capable of transmitting dengue from male to female experimentally during copulation as well as vertically from female to offspring, which is seen to a much lesser extent in *Ae. aegypti* (Rosen, 1987, Rosen *et al.*, 1983). Whilst many studies have been carried out regarding the vector status of *Ae. albopictus* in South East Asia no clear indication has as yet been seen incriminating this species as an arbovirus vector in the Americas (Gratz, 2004). Although local transmission of dengue has only been recorded once on Grand Cayman in recent times it may nevertheless be of importance in the future.

The overall aim of these studies was to appraise RIDL as a potential method of control for *Ae. aegypti* in the Cayman Islands. While data shows that it is possible to impact the mosquito population significantly using this method the logistics supporting this deserve further scrutiny particularly with regard to cost effectiveness. For an area of 16 Ha this study required that 350,000 eggs were produced and available for hatching as well as >360 litres of clean water for rearing and sorting, per week. Staffing, laboratory overheads and equipment requirements are much greater than would be required for conventional insecticide based control (albeit that conventional control is not generally considered very effective) carrying with it greater expense. Overall this technology may not be considered by some to be as cost effective as conventional methods due to increased requirements. With improvements in rearing efficiency it might be possible to scale up production in order to aim control in larger areas however the likely cost of this may be prohibitive to many developing countries where this type of technology could potentially have the widest application.

6. Final Discussion

Resistance to insecticides in *Ae. aegypti* is not unusual within the Caribbean region and reports of DDT resistance date back to the 1950's. Metabolic based resistance to organophosphates such as temephos and malathion, that have been the mainstay for control for over 30 years, is widespread.

Comparisons of resistance levels between different populations is complicated by the wide variation in methodologies used (Ranson *et al.*, 2010) but it appears as if pyrethroid resistance is much greater in Grand Cayman than neighbouring countries. The resistance ratio of 643 to permethrin in adults (at the LC_{95}) from Grand Cayman compares to 35 fold in larvae from Martinique (Brengues *et al.*, 2003). Similarly the RR for deltamethrin in adults from Grand Cayman is approximately 6-fold higher than observed in larvae in Cuba (Rodriguez *et al.*, 2001). Studies into resistance mechanisms in the Caribbean have previously been limited to biochemical assays to detect metabolic mechanisms. In order to confirm kdr type resistance, molecular techniques are required; technology that may not be easily accessible to some research and control groups within the region.

Kdr resistance was confirmed in Grand Cayman as well as a number of other locations within the region (Cuba, Jamaica, Florida, Martinique, Honduras and Puerto Rico). Two kdr mutations were identified in the domain II and domain III regions of the voltage-gated sodium channel of samples from Grand Cayman. The first, a V1016I mutation, has been correlated with pyrethroid resistance (Saavedra-Rodriguez *et al.*, 2007). The second mutation F1534C was previously unreported; this study confirmed this substitution is important in conferring resistance to DDT and pyrethroids. Additional to these a third (V1016G) mutation was identified in a single mosquito sample from Puerto Rico.

The detection of three kdr mutations in the Caribbean, including two (1016G and 1534C) previously only reported in Asia, suggests that these alleles are spreading. This implies that the future for insecticide based control of *Ae. aegypti* in the Caribbean is bleak and continuous selection pressures in the presence of these mutations could lead to eventual fixation. It is unknown at this time whether alternative substitutions at each site confer different levels of resistance, for example whether 1016G results in greater resistance to pyrethroids than 1016I. In addition the combined effect of mutations at residues 1016 and

1534 is unknown. It may be possible that insecticide binding is affected differently depending on the conformation of amino acids within the binding site.

Elevated esterases, cytochrome P450s and GSTs also play a potential role in insecticide resistance in *Ae. aegypti* from the Cayman Islands. Microarray studies have indicated up-regulation in the CYP6 and CYP9 families of cytochrome P450s in Grand Cayman *Ae. aegypti* when compared to the susceptible strain (Vassia Bariami, University of Crete, unpublished) and these enzyme families have already been implicated in pyrethroid resistance (Strode *et al.*, 2008) in this species.

Understanding the molecular basis of resistance is important to improve monitoring, but too little attention has been paid to how resistance impacts upon actual control. Resistance in *Ae. aegypti* in Grand Cayman pertains to the use of pyrethroids classically used in adult control. Larviciding remains the main *Ae. aegypti* control activity in the Cayman Islands and the current insecticide of choice is *Bti* to which resistance in *Ae. aegypti* has not yet been reported. Therefore the operational impact of the pyrethroid resistance on the MRCU control programme is expected to be limited.

No recent historical data on the levels of insecticide resistance in Grand Cayman *Ae. aegypti* is available, from limited samples from 2003 it is only possible to tell that the 1016I mutation was present on island at this time. In September 2004 Hurricane Ivan, a category 5 storm, hit Grand Cayman after which time *Ae. aegypti* numbers soared. This was presumed due to a massive increase in available breeding sites created by storm wreckage. It would be interesting to know the resistance status of the population at this time and whether individuals with resistance alleles had a competitive advantage over others allowing the frequency of these alleles to increase. With a break down in infrastructure (electricity to run air conditioning and piped water) at this time numbers of domestic pests also increased possibly leading to greater use of insecticides by householders.

Operationally the use of insecticides and especially larvicides by the MRCU is poorly quantified; while all mosquito control products are labelled with specific dosage for use, the amount of insecticide required is something often massively overestimated by control personnel. A couple of pellets of the temephos formulation Abate may not seem enough to

treat, for example, a 50 gallon drum so more is frequently applied to be certain it will have the desired effect. Fortunately this does not appear to have caused a problem in Grand Cayman *Ae. aegypti* as they remain largely susceptible to temephos. However this could be a contributory factor in resistance in other Caribbean populations. For example in Tortola the public are issued packets of larvicide on request which could lead to intensive use hastening the development of resistance (Georghiou *et al.*, 1987). In order to apply pyrethroid formulations by ULV spraying, a more measured application is required. At the MRCU specific dosages are measured into fogging equipment which is calibrated to deliver a regular quantity of insecticide. This means that application is not due to estimation in the way that larvicidal treatments are, therefore pyrethroid formulations are at less risk of overdose which might lead to the further development of resistance.

As a result of this study, pyrethroid insecticides are no longer used for control of *Ae. aegypti* in the Cayman Islands. However, selection pressure has only been removed from the dengue campaign and pyrethroids are still routinely aerially sprayed in order to control *Ae. taeniorhynchus* as well as used by pest control agencies around houses. Whether this non-direct use of these chemicals will maintain sufficient insecticidal pressure and result in further increases in the frequency of these mutant alleles remains to be seen.

Movement of goods around the Caribbean and increased globalisation as a whole have no doubt greatly contributed to the migration of *Ae. aegypti*. *Fst* analysis identified the Jamaican population as that with which Cayman Islands *Ae. aegypti* are the most similar and similarity between Jamaica and all other populations supports the notion that there are high levels of genetic interchange between populations in the Caribbean. This is further supported by assignment analysis in which each sample had individuals assigned to at least three other populations besides itself. This widespread movement of alleles means that the appearance of new insecticide resistance mutations becomes a concern not just for individual countries, but for entire regions.

At the outset of this project concerns surrounded the origins of the Grand Cayman *Ae. aegypti* and whether they arrived on the island carrying resistance alleles. With the discovery of high levels of pyrethroid resistance in the Cayman Islands attention should perhaps now be diverted to where this resistance could ultimately be exported to. The

irresponsible use of pesticides by 'rich' countries could impact on poorer countries where resources for public health and vector control are very limited. In this sense the Cayman Islands is fortunate. The existence of a Government department solely concerned with the control of mosquitoes is quite unusual and compared to other countries the MRCU enjoys a generous budget that enables the use of a wide range of tools and products for mosquito control. Insecticides can be and historically have been used liberally for many years.

The principle goal of the dengue prevention campaign is eradication of *Ae. aegypti* from the Cayman Islands. It was thought this had been achieved in 1996 but microsatellite analysis suggests the current population is well established. Mosquito samples that were collected just six years after it is believed to have been introduced to the island showed similar genetic diversity to established populations from Florida, Jamaica and Cuba meaning it may be unlikely that total eradication was achieved in 1996. An alternative explanation is that repeated introductions over a period of years could lead to greater levels of genetic diversity. If this was the case it would be expected that greater diversity would be concentrated in the entry points and this was not observed. The results from this study have led the MRCU to question the feasibility of eradicating this species using existing methods.

With such high levels of insecticide resistance in the local *Ae. aegypti* population it was decided to seek out a new method of control. Therefore the genetic RIDL technique was appraised for its utility for control. The specificity of the RIDL technology compared with conventional insecticidal control is one of the key benefits as it is considered to be more environmentally friendly. The potential to hunt out the last few individuals is also beneficial in the case of an eradication campaign where cryptic breeding sites may remain hidden from survey crews. This method is also considered reversible: unless eradication is achieved its effects are no longer seen once releases of sterile males cease. The stability of the construct also means that there are no undesired effects to non-target organisms further up the food chain. This may not be the case for sterile insect techniques using chemosterilisation. A study was carried out on spiders fed a diet of chemosterilised mosquitoes that resulted in reduced fertility of the spiders (Bracken & Dondale, 1972). Studies in which OX513A strain larvae were fed to *Toxorhynchites splendens* larvae, (this species will consume other mosquito larvae at the larval stage) showed no adverse effects

to the development and survival of the *Toxorhynchites* mosquitoes (Derric Nimmo, Oxitec Ltd., personal communication).

Since the success of the suppression trial in this study, a small scale trial has been carried out in an unpopulated area of Malaysia with plans to carry out further studies to assess the potential of RIDL for control of Dengue. Other RIDL strains have also been produced for testing, including a female specific, flightless strain (Fu *et al.*, 2010). The dominant gene is once again repressible using the tetracycline system, but this time is specific to females and acts on indirect flight muscles incapacitating the adult female making it impossible to feed or reproduce. This version of RIDL, unlike the OX513A strain used in the Grand Cayman study, is not self limiting and can be passed on through the male line to future generations of mosquitoes.

While the RIDL suppression trial proved the principle that this method of SIT can have a significant effect on wild *Ae. aegypti* populations, experience with this technology warrants a thorough economic evaluation. In order to release approximately 150,000 to 250,000 OX513A male mosquitoes per week in Grand Cayman over a 16 Ha area required at least 4-5 full time staff working seven days a week. Economies of scale may reduce marginal costs once a certain threshold production level is reached, but this is still a huge requirement in terms of staffing. For many control districts or public health departments staffing requirements for a 'mosquito factory' would be far greater than for conventional survey staff, these staff would need to be semi skilled rather than labourers that are usually employed for *Ae. aegypti* control and therefore more expensive in terms of salary. The production unit would also need to be supported by field staff for the collection and maintenance of traps as well as the release of the insects. During the course of the suppression trial and undoubtedly future trials a lot of time and effort was invested in running experiments. It is likely that the labour required for an operational rather than an experimental program will to some extent be reduced, but still additional to requirements for a conventional program.

Despite these concerns, this technology may have utility in developing countries where dengue is endemic. Labour costs in these places are far lower than the developed world making RIDL seem an affordable option. However, while labour costs may be less it is also

likely that the budget for vector control will also be greatly reduced. When faced with the decision of using a staffing budget for survey crews or lab technicians it is likely that most management of control departments, constrained by budget would rather have men on the ground, even though this method may not be incredibly effective. Additional to this the initial investment required to set up a facility would likely stretch far beyond the budget of most control efforts in the developing world requiring external investment.

Another consideration for many developing countries is water supply, for this relatively small trial something in the region of 400 litres of water per week was required for the rearing and sorting of larvae and pupae. In the planning of a rearing factory it might be wise to include a water treatment facility. This would add to the initial outlay and ongoing maintenance costs of the plant and, in countries where fresh, clean drinking water is not easily available to the public, the morals and ethics of using precious water for the purposes of raising and sorting pupae are open to question.

It is still early days for this technology and many more improvements need to be made regarding rearing protocols and release devices. The decision to whether adult or pupal release is most effective, how to overcome the seemingly large amounts of space required by adult mosquitoes reared in the laboratory and how to transport them to the field are all areas in which developments must be made that may result in this technology becoming more feasible for use in the field. More trials are planned both in the Cayman Islands and a number of locations globally that may improve upon current logistical problems.

In conclusion, intense use of insecticides has led to high levels of insecticide resistance in the Caribbean region and especially in the Cayman Islands. Extensive migration of *Ae. aegypti* resulting in the movement of resistance alleles globally may mean it could be too late in many locations to introduce effective resistance management programmes, as strategies such as rotations or mosaics cannot protect from resistance once is has been imported. Constant introductions to Grand Cayman combined with the high levels of insecticide resistance already present will continue to make control of this species challenging. With a lack of new insecticides in development, in combination with the declassification of others means new alternative control methods need to be investigated.

Whilst early studies into the release of genetically modified insects show some promise at this time they may not hold the definitive answer.

References

- Alout, H., Berthomieu, A., Hadjivassilis, A. & Weill, M. (2007). A New Amino-Acid Substitution in Acetylcholinesterase 1 Confers Insecticide Resistance to *Culex pipiens* Mosquitoes from Cyprus. *Insect Biochemistry and Molecular Biology* **37**, 41-47.

- Alphey, L. & Andreasen, M. (2002). Dominant Lethality and Insect Population Control. *Molecular and Biochemical Parasitology* **121**, 173-178.

- Alphey, L., Benedict, M., Bellini, R., Clark, G. G., Dame, D. A., Service, M. W. & Dobson, S. L. (2010). Sterile-Insect Methods for Control of Mosquito-Borne Diseases: An Analysis. *Vector-Borne and Zoonotic Diseases* **10**, 295-311.

- Awolola, T. S., Oduola, O. A., Strode, C., Koekemoer, L. L., Brooke, B. & Ranson, H. (2009). Evidence of Multiple Pyrethroid Resistance Mechanisms in the Malaria Vector *Anopheles gambiae* sensu stricto from Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **103**, 1139-1145.

- Bass, C., Nikou, D., Donnelly, M. J., Williamson, M. S., Ranson, H., Ball, A., Vontas, J. & Field, L. M. (2007). Detection of Knockdown Resistance (kdr) Mutations in *Anopheles gambiae*: a Comparison of Two New High-Throughput Assays with Existing Methods. *Malaria Journal* **6**, 111.

- Bataille, A., Cunningham, A. A., Cedeno, V., Cruz, M., Eastwood, G., Fonseca, D. M., Causton, C. E., Azuero, R., Loayza, J., Cruz Martinez, J. D. & Goodman, S. J. (2009). Evidence for Regular Ongoing Introductions of Mosquito Disease Vectors into the Galapagos Islands. *Proceedings of the Royal Society B-Biological Sciences* **276**, 3769-3775.

- Benedict, M. Q., Hood-Nowotny, R. C., Howell, P. I. & Wilkins, E. E. (2009). Methylparaben in *Anopheles gambiae* s.l. Sugar Meals Increases Longevity and Malaria Oocyst Abundance but is not a Preferred Diet. *Journal of Insect Physiology* **55**, 197-204.

- Benedict, M. Q. & Robinson, A. S. (2003). The First Releases of Transgenic Mosquitoes: An Argument for the Sterile Insect Technique. *Trends in Parasitology* **19**, 349-355.

- Beserra, E. B., Fernandes, C. R. M., De Queiroga, M. D. F. C. & De Castro, F. P., Jr. (2007). Resistance of *Aedes aegypti* (L.) (Diptera : Culicidae) Populations to Organophosphate Temephos in the Paraiba State, Brazil. *Neotropical Entomology* **36**, 303-307.

- Bian, G. W., Xu, Y., Lu, P., Xie, Y. & Xi, Z. Y. (2010). The Endosymbiotic Bacterium *Wolbachia* Induces Resistance to Dengue Virus in *Aedes aegypti*. *Plos Pathogens* **6**.

- Bisset, J., Rodriguez, M. M. & Fernandez, D. (2006). Selection of Insensitive Acetylcholinesterase as a Resistance Mechanism in *Aedes aegypti* (Diptera : Culicidae) from Santiago de Cuba. *Journal of Medical Entomology* **43**, 1185-1189.

- Bracco, J. E., Capurro, M. L., Lourenco-de-Oliveira, R. & Sallum, M. A. M. (2007). Genetic Variability of *Aedes aegypti* in the Americas Using a Mitochondrial Gene: Evidence of Multiple Introductions. *Memorias Do Instituto Oswaldo Cruz* **102**, 573-580.

- Bracken, G. K. & Dondale, C. D. (1972). Fertility and Survival of *Achaearanea tepidariorum* (Araneida : Theridiidae) on a Diet of Chemosterilized Mosquitoes. *Canadian Entomologist* **104**, 1709-&.

- Braga, I., Mello, C. B., Peixoto, A. A. & Valle, D. (2005). Evaluation of Methoprene Effect on *Aedes aegypti* (Diptera : Culicidae) Development in Laboratory Conditions. *Memorias Do Instituto Oswaldo Cruz* **100**, 435-440.

- Brelsfoard, C. L., Sechan, Y. & Dobson, S. L. (2008). Interspecific Hybridization Yields Strategy for South Pacific Filariasis Vector Elimination. *PLOS Neglected Tropical Diseases* **2**.

- Brengues, C., Hawkes, N. J., Chandre, F., McCarroll, L., Duchon, S., Guillet, P., Manguin, S., Morgan, J. C. & Hemingway, J. (2003). Pyrethroid and DDT Cross-Resistance in *Aedes aegypti* is Correlated with Novel Mutations in the Voltage-Gated Sodium Channel Gene. *Medical and Veterinary Entomology* **17**, 87-94.

- Brown, A. W. & Pal, R. (1971). Insecticide Resistance in Arthropods. *Public Health Pap* **38**, 1-491.

- Brown, A. W. A. (1986). Insecticide Resistance in Mosquitoes - a Pragmatic Review. *Journal of the American Mosquito Control Association* **2**, 123-140.

- Brown, J. E., McBride, C. S., Johnson, P., Ritchie, S., Paupy, C., Bossin, H., Lutomiah, J., Fernandez-Salas, I., Ponlawat, A., Cornel, A. J., Black, W. C., Gorrochotegui-Escalante, N., Urdaneta-Marquez, L., Sylla, M., Slotman, M., Murray, K. O., Walker, C. & Powell, J. R. (2011). Worldwide Patterns of Genetic Differentiation Imply Multiple 'Domestications' of *Aedes aegypti*, a Major Vector of Human Diseases. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 2446-2454.

- Brunt, M. A. & Davies, J. E. (1994). The Cayman Islands Natural History and Biogeography: Kluwer Academic Publishers.

- Burton, M. J., Mellor, I. R., Duce, I. R., Davies, T. G. E., Field, L. M. & Williamson, M. S. (2011). Differential Resistance of Insect Sodium Channels with kdr Mutations to Deltamethrin, Permethrin and DDT. *Insect Biochemistry and Molecular Biology* **41**, 723-732.

- CAREC. (2006). CAREC Annual Report 2006.

- CAREC. (2007). CAREC Annual Report 2007.

- Catteruccia, F., Godfray, H. C. J. & Crisanti, A. (2003). Impact of Genetic Manipulation on the Fitness of *Anopheles stephensi* Mosquitoes. *Science* **299**, 1225-1227.

- Chambers, E. W., Meece, J. K., McGowan, J. A., Lovin, D. D., Hemme, R. R., Chadee, D. D., McAbee, K., Brown, S. E., Knudson, D. L. & Severson, D. W. (2007). Microsatellite Isolation and Linkage Group Identification in the Yellow Fever Mosquito *Aedes aegypti*. *Journal of Heredity* **98**, 202-210.

- Chang, C., Shen, W. K., Wang, T. T., Lin, Y. H., Hsu, E. L. & Dai, S. M. (2009). A Novel Amino Acid Substitution in a Voltage-Gated Sodium Channel is Associated with Knockdown Resistance to Permethrin in *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* **39**, 272-278.

- Chen, C. H., Huang, H. X., Ward, C. M., Su, J. T., Schaeffer, L. V., Guo, M. & Hay, B. A. (2007). A Synthetic Maternal-Effect Selfish Genetic Element Drives Population Replacement in *Drosophila*. *Science* **316**, 597-600.

- Clement, M., Posada, D. & Crandall, K. A. (2000). TCS: A Computer Program to Estimate Gene Genealogies. *Molecular Ecology* **9**, 1657-1659.

- Coffey, R. (2011). Outsmarting Dengue Fever. *Scientific American* **304**, 16.

- Cornman, R. S. & Willis, J. H. (2009). Annotation and Analysis of Low-Complexity Protein Families of *Anopheles gambiae* that are Associated with Cuticle. *Insect Molecular Biology* **18**, 607-622.

- da Grao Macoris, M. d. L., Macoris Andrighetti, M. T., Garbeloto Otrera, V. C., de Carvalho, L. R., Caldas Junior, A. L. & Brogdon, W. G. (2007). Association of Insecticide Use and Alteration on *Aedes aegypti* Susceptibility Status. *Memorias Do Instituto Oswaldo Cruz* **102**, 895-900.

- Du, Y., Lee, J. E., Nomura, Y., Zhang, T. X., Zhorov, B. S. & Dong, K. (2009). Identification of a Cluster of Residues in Transmembrane Segment 6 of Domain III of the Cockroach Sodium Channel Essential for the Action of Pyrethroid Insecticides. *Biochemical Journal* **419**, 377-385.

- The Economics and Statistics Office (2011). The Cayman Islands 2010 Population and Housing Census: Preliminary Report. The Economics and Statistics Office, The Cayman Islands Government.

- Failloux, A. B., Fouque, F., Vazeille, M. & Rodhain, F. (2002). Isoenzyme Differentiation of *Aedes aegypti* Populations in French Guiana. *Medical and Veterinary Entomology* **16**, 456-460.

- Fay, R. W. & Perry, A. S. (1965). Laboratory Studies of Ovipositional Preferences of *Aedes aegypti*. *Mosquito News* **25**, 276-281.

- Felsenstein, J. (1989). PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164-166.

- Ffrench-Constant, R. H., Anthony, N., Aronstein, K., Rocheleau, T. & Stilwell, G. (2000). Cyclodiene Insecticide Resistance: From Molecular to Population Genetics. *Annual Review of Entomology* **45**, 449-466.

- Ffrench-Constant, R. H., Steichen, J. C. & Shotkoski, F. (1994). Polymerase Chain-Reaction Diagnostic for Cyclodiene Insecticide Resistance in the Mosquito *Aedes aegypti*. *Medical and Veterinary Entomology* **8**, 99-100.

- Fonseca, D. M., LaPointe, D. A. & Fleischer, R. C. (2000). Bottlenecks and Multiple Introductions: Population Genetics of the Vector of Avian Malaria in Hawaii. *Molecular Ecology* **9**, 1803-1814.

- Fonseca, D. M., Smith, J. L., Wilkerson, R. C. & Fleischer, R. C. (2006). Pathways of Expansion and Multiple Introductions Illustrated by Large Genetic Differentiation among Worldwide Populations of the Southern House Mosquito. *American Journal of Tropical Medicine and Hygiene* **74**, 284-289.

- Frentiu, F. D., Robinson, J., Young, P. R., McGraw, E. A. & O'Neill, S. L. (2010). *Wolbachia*-Mediated Resistance to Dengue Virus Infection and Death at the Cellular Level. *Plos One* **5**.

- Fu, G. L., Lees, R. S., Nimmo, D., Aw, D., Jin, L., Gray, P., Berendonk, T. U., White-Cooper, H., Scaife, S., Phuc, H. K., Marinotti, O., Jasinskiene, N., James, A. A. & Alphey, L. (2010). Female-Specific Flightless Phenotype for Mosquito Control. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 4550-4554.

- Garcia, G. P., Flores, A. E., Fernandez-Salas, I., Saavedra-Rodriguez, K., Reyes-Solis, G., Lozano-Fuentes, S., Bond, J. G., Casas-Martinez, M., Ramsey, J. M., Garcia-Rejon, J., Dominguez-Galera, M., Ranson, H., Hemingway, J., Eisen, L. & Black, W. C. (2009). Recent Rapid Rise of a Permethrin Knock Down Resistance Allele in *Aedes aegypti* in Mexico. *PLOS Neglected Tropical Diseases* **3**.

- Georghiou, G. P., Wirth, M., Tran, H., Saume, F. & Knudsen, A. B. (1987). Potential for Organophosphate Resistance in *Aedes aegypti* (Diptera, Culicidae) in the Caribbean Area and Neighboring Countries. *Journal of Medical Entomology* **24**, 290-294.

- Gill, S. S., Cowles, E. A. & Pietrantonio, P. V. (1992). The Mode of Action of *Bacillus thuringiensis* Endotoxins. *Annual Review of Entomology* **37**, 615-636.

- Gong, P., Epton, M. J., Fu, G. L., Scaife, S., Hiscox, A., Condon, K. C., Condon, G. C., Morrison, N. I., Kelly, D. W., Dafa'alla, T., Coleman, P. G. & Alphey, L. (2005). A Dominant Lethal Genetic System for Autocidal Control of the Mediterranean Fruitfly. *Nature Biotechnology* **23**, 453-456.

- Gratz, N. G. (2004). Critical review of the vector status of *Aedes albopictus*. *Medical and Veterinary Entomology* **18**, 215-227.

- Hall, T. A. (1999). Bioedit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95-98.

- Harris, A. F., Rajatileka, S. & Ranson, H. (2010). Pyrethroid Resistance in *Aedes aegypti* from Grand Cayman. *American Journal of Tropical Medicine and Hygiene* **83**, 277-284.

- He, H. Q., Chen, A. C., Davey, R. B., Ivie, G. W. & George, J. E. (1999). Identification of a Point Mutation in the Para-Type Sodium Channel Gene from a Pyrethroid Resistant Cattle Tick. *Biochemical and Biophysical Research Communications* **261**, 558-561.

- Hemingway, J., Boddington, R. G., Harris, J. & Dunbar, S. J. (1989). Mechanisms of Insecticide Resistance in *Aedes aegypti* (L) (Diptera, Culicidae) from Puerto Rico. *Bulletin of Entomological Research* **79**, 123-130.

- Hemingway, J., Field, L. & Vontas, J. (2002). An Overview of Insecticide Resistance. *Science* **298**, 96-97.

- Hemingway, J., Hawkes, N. J., McCarroll, L. & Ranson, H. (2004). The Molecular Basis of Insecticide Resistance in Mosquitoes. *Insect Biochemistry and Molecular Biology* **34**, 653-665.

- Hemingway, J. & Ranson, H. (2000). Insecticide Resistance in Insect Vectors of Human Disease. *Annual Review of Entomology* **45**, 371-391.

- Hertlein, M. B., Mavrotas, C., Jousseaume, C., Lysandrou, M., Thompson, G. D., Jany, W. & Ritchie, S. A. (2010). A Review of Spinosad as a Natural Product for Larval Mosquito Control. *Journal of the American Mosquito Control Association* **26**, 67-87.

- Holm, S. (1979). A Simple Sequentially Rejective Multiple Test Procedure. *Scandinavian Journal of Statistics* **6**, 65-70.

- 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 Biochemistry and Molecular Biology* **41**, 9-13.

- Huber, K., Le Loan, L., Chantha, N. & Failloux, A. B. (2004). Human Transportation Influences *Aedes aegypti* Gene Flow in Southeast Asia. *Acta Tropica* **90**, 23-29.

- Huber, K., Le Loan, L., Hoang, T. H., Ravel, S., Rodhain, F. & Failloux, A. B. (2002a). Genetic Differentiation of the Dengue Vector, *Aedes aegypti* (Ho Chi Minh City, Vietnam) Using Microsatellite Markers. *Molecular Ecology* **11**, 1629-1635.

- Huber, K., Le Loan, L., Hoang, T. H., Tien, T. K., Rodhain, F. & Failloux, A. B. (2002b). Temporal Genetic Variation in *Aedes aegypti* Populations in Ho Chi Minh City (Vietnam). *Heredity* **89**, 7-14.

- Huber, K., Mousson, L., Rodhain, F. & Failloux, A. B. (2001). Isolation and Variability of Polymorphic Microsatellite Loci in *Aedes aegypti*, the Vector of Dengue Viruses. *Molecular Ecology Notes* **1**, 219-222.

- IRAC. (2006). Prevention and Management of Insecticide Resistance in Vectors and Pests of Public Health Importance. Insecticide Resistance Action Committee (IRAC).

- Irvin, N., Hoddle, M. S., O'Brochta, D. A., Carey, B. & Atkinson, P. W. (2004). Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 891-896.

- James, A. A. (2005). Gene Drive Systems in Mosquitoes: Rules of the Road. *Trends in Parasitology* **21**, 64-67.

- Jirakanjanakit, N., Saengtharatip, S., Rongnoparut, P., Duchon, S., Bellec, C. & Yoksan, S. (2007). Trend of Temephos Resistance in *Aedes* (Stegomyia) Mosquitoes in Thailand During 2003-2005. *Environmental Entomology* **36**, 506-511.

- Jukes, T. H. & Cantor, C. R. (1969). Evolution of Protein Molecules. Munro, H. N. (Edited by). Mammalian Protein Metabolism. Vol. III. Academic Press: New York, N.Y., U.S.A, 21-132.

- Kawada, H., Higa, Y., Komagata, O., Kasai, S., Tomita, T., Nguyen, T. Y., Luu, L. L., Sanchez, R. A. P. & 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 Neglected Tropical Diseases* **3**.

- Khot, A. C., Bingham, G., Field, L. M. & Moores, G. D. (2008). A Novel Assay Reveals the Blockade of Esterases by Piperonyl Butoxide. *Pest Management Science* **64**, 1139-1142.

- Klassen, W. & Curtis, C. F. (2005). History of the Sterile Insect Technique. Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, 3-36.

- Knipling, E. F. (1955). Possibilities of Insect Control or Eradication through the Use of Sexually Sterile Males. *Journal of Economic Entomology* **48**, 459-462.

- Kongmee, M., Prabaripai, A., Akratanakul, P., Bangs, M. J. & Chareonviriyaphap, T. (2004). Behavioral Responses of *Aedes aegypti* (Diptera : Culicidae) Exposed to Deltamethrin and Possible Implications for Disease Control. *Journal of Medical Entomology* **41**, 1055-1063.

- Lavery, J. V., Harrington, L. C. & Scott, T. W. (2008). Ethical, Social, and Cultural Considerations for Site Selection for Research with Genetically Modified Mosquitoes. *American Journal of Tropical Medicine and Hygiene* **79**, 312-318.

- Lertkiatmongkol, P., Pethuan, S., Jirakanjanakit, N. & Rongnoparut, P. (2010). Transcription Analysis of Differentially Expressed Genes in Insecticide Resistant *Aedes aegypti* Mosquitoes after Deltamethrin Exposure. *Journal of Vector Ecology* **35**, 197-203.

- Livak, K. J. (1984). Organization and Mapping of a Sequence on the *Drosophila melanogaster* X Chromosome and Y Chromosome that is Transcribed During Spermatogenesis. *Genetics* **107**, 611-634.

- Lovin, D. D., Washington, K. O., deBruyn, B., Hemme, R. R., Mori, A., Epstein, S. R., Harker, B. W., Streit, T. G. & Severson, D. W. (2009). Genome-Based Polymorphic Microsatellite Development and Validation in the Mosquito *Aedes aegypti* and Application to Population Genetics in Haiti. *Bmc Genomics* **10**.

- Lumjuan, N., McCarroll, L., Prapanthadara, L. A., Hemingway, J. & Ranson, H. (2005). Elevated Activity of an Epsilon Class Glutathione Transferase Confers DDT Resistance in the Dengue Vector, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* **35**, 861-871.

- Lumjuan, N., Rajatileka, S., Changsom, D., Wicheer, J., Leelapat, P., Prapanthadara, L. A., 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 Biochemistry and Molecular Biology* **41**, 203-209.

- Lumjuan, N., Stevenson, B. J., Prapanthadara, L. A., Somboon, P., Brophy, P. M., Loftus, B. J., Severson, D. W. & Ranson, H. (2007). The *Aedes aegypti* Glutathione Transferase Family. *Insect Biochemistry and Molecular Biology* **37**, 1026-1035.

- Lynd, A., Weetman, D., Barbosa, S., Yawson, A. E., Mitchell, S., Pinto, J., Hastings, I. & Donnelly, M. J. (2010). Field, Genetic, and Modeling Approaches Show Strong Positive Selection Acting upon an Insecticide Resistance Mutation in *Anopheles gambiae* s.s. *Molecular Biology and Evolution* **27**, 1117-1125.

- Marcombe, S., Carron, A., Darriet, F., Etienne, M., Agnew, P., Tolosa, M., Yp-Tcha, M. M., Lagneau, C., Yebakima, A. & Corbel, V. (2009a). Reduced Efficacy of Pyrethroid Space Sprays for Dengue Control in an Area of Martinique with Pyrethroid Resistance. *American Journal of Tropical Medicine and Hygiene* **80**, 745-751.

- Marcombe, S., Poupardin, R., Darriet, F., Reynaud, S., Bonnet, J., Strode, C., Brengues, C., Yebakima, A., Ranson, H., Corbel, V. & David, J. P. (2009b). 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**.

- Marquardt, W. C., Black, W. C., Higgs, S., Freier, J. E., James, A. A., Hagedorn, H. H., Kondratieff, B., Hemingway, J. & Moore, C. G. (2005). Biology of Disease Vectors: Elsevier Academic Press.

- Marrelli, M. T., Li, C., Rasgon, J. L. & Jacobs-Lorena, M. (2007). Transgenic Malaria Resistant Mosquitoes have a Fitness Advantage when Feeding on Plasmodium Infected Blood. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 5580-5583.

- Marrelli, M. T., Moreira, C. K., Kelly, D., Alphey, L. & Jacobs-Lorena, M. (2006). Mosquito Transgenesis: What is the Fitness Cost? *Trends in Parasitology* **22**, 197-202.

- Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur, N. & Pauron, D. (1998). Molecular Characterization of Pyrethroid Knockdown Resistance (kdr) in the Major Malaria Vector *Anopheles gambiae* S.S. *Insect Molecular Biology* **7**, 179-184.

- Martins, A. J., Lima, J. B. P., Peixoto, A. A. & Valle, D. (2009a). Frequency of Val1016Ile Mutation in the Voltage-Gated Sodium Channel Gene of *Aedes aegypti* Brazilian Populations. *Tropical Medicine & International Health* **14**, 1351-1355.

- Martins, A. J., Lins, R., Linss, J. G. B., Peixoto, A. A. & Valle, D. (2009b). Voltage-Gated Sodium Channel Polymorphism and Metabolic Resistance in Pyrethroid Resistant *Aedes aegypti* from Brazil. *American Journal of Tropical Medicine and Hygiene* **81**, 108-115.

- McMeniman, C. J., Lane, R. V., Cass, B. N., Fong, A. W. C., Sidhu, M., Wang, Y. F. & O'Neill, S. L. (2009). Stable Introduction of a Life Shortening *Wolbachia* Infection into the Mosquito *Aedes aegypti*. *Science* **323**, 141-144.

- Mekuria, Y., Gwinn, T. A., Williams, D. C. & Tidwell, M. A. (1991). Insecticide Susceptibility of *Aedes aegypti* from Santo Domingo, Dominican Republic. *Journal of the American Mosquito Control Association* **7**, 69-72.

- Melo-Santos, M. A. V., Varjal-Melo, J. J. M., Araujo, A. P., Gomes, T. C. S., Paiva, M. H. S., Regis, L. N., Furtado, A. F., Magalhaes, T., Macoris, M. L. G., Andrighetti, M. T. M. & Ayres, C. F. J. (2010). Resistance to the Organophosphate Temephos: Mechanisms, Evolution and Reversion in an *Aedes aegypti* Laboratory Strain from Brazil. *Acta Tropica* **113**, 180-189.

- Min, K. T. & Benzer, S. (1997). *Wolbachia*, Normally a Symbiont of *Drosophila*, can be Virulent, Causing Degeneration and Early Death. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10792-10796.

- Mittal, P. K. (2003). Biolarvicides in Vector Control: Challenges and Prospects. *Journal of Vector Borne Diseases* **40**, 20-32.

- Montella, I. R., Martins, A. J., Viana-Medeiros, P. F., Lima, J. B. P., Braga, I. A. & Valle, D. (2007). Insecticide Resistance Mechanisms of Brazilian *Aedes aegypti* Populations from 2001 to 2004. *American Journal of Tropical Medicine and Hygiene* **77**, 467-477.

- Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G. J., Pyke, A. T., Hedges, L. M., Rocha, B. C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L. E., Johnson, K. N., Kay, B. H., McGraw, E. A., van den Hurk, A. F., Ryan, P. A. & O'Neill, S. L. (2009). A *Wolbachia* Symbiont in *Aedes aegypti* Limits Infection with Dengue, Chikungunya, and Plasmodium. *Cell* **139**, 1268-1278.

- Morou, E., Dowd, A. J., Rajatileka, S., Steven, A., Hemingway, J., Ranson, H., Paine, M. & Vontas, J. (2010). A Simple Colorimetric Assay for Specific Detection of Glutathione S Transferase Activity Associated with DDT Resistance in Mosquitoes. *PLOS Neglected Tropical Diseases* **4**.

- Mourya, D. T., Hemingway, J. & Leake, C. J. (1993). Changes in Enzyme Titers with Age in 4 Geographical Strains of *Aedes aegypti* and their Association with Insecticide Resistance. *Medical and Veterinary Entomology* **7**, 11-16.

- Nene, V., Wortman, J. R., Lawson, D., Haas, B., Kodira, C., Tu, Z. J., Loftus, B., Xi, Z. Y., Megy, K., Grabherr, M., Ren, Q. H., Zdobnov, E. M., Lobo, N. F., Campbell, K. S., Brown, S. E., Bonaldo, M. F., Zhu, J. S., Sinkins, S. P., Hogenkamp, D. G., Amedeo, P., Arensburger, P., Atkinson, P. W., Bidwell, S., Biedler, J., Birney, E., Bruggner, R. V., Costas, J., Coy, M. R., Crabtree, J., Crawford, M., deBruyn, B., DeCaprio, D., Eiglmeier, K., Eisenstadt, E., El-Dorry, H., Gelbart, W. M., Gomes, S. L., Hammond, M., Hannick, L. I., Hogan, J. R., Holmes, M. H., Jaffe, D., Johnston, J. S., Kennedy, R. C., Koo, H., Kravitz, S., Kriventseva, E. V., Kulp, D., LaButti, K., Lee, E., Li, S., Lovin, D. D., Mao, C. H., Mauceli, E., Menck, C. F. M., Miller, J. R., Montgomery, P., Mori, A., Nascimento, A. L., Naveira, H. F., Nusbaum, C., O'Leary, S., Orvis, J., Pertea, M., Quesneville, H., Reidenbach, K. R., Rogers, Y. H., Roth, C. W., Schneider, J. R., Schatz, M., Shumway, M., Stanke, M., Stinson, E. O., Tubio, J. M. C., VanZee, J. P., Verjovski-Almeida, S., Werner, D., White, O., Wyder, S., Zeng, Q. D., Zhao, Q., Zhao, Y. M., Hill, C. A., Raikhel, A. S., Soares, M. B., Knudson, D. L., Lee, N.

H., Galagan, J., Salzberg, S. L., Paulsen, I. T., Dimopoulos, G., Collins, F. H., Birren, B., Fraser-Liggett, C. M. & Severson, D. W. (2007). Genome Sequence of *Aedes aegypti*, a Major Arbovirus Vector. *Science* **316**, 1718-1723.

- Nielsen-Leroux, C. & Charles, J. F. (1992). Binding of *Bacillus sphaericus* Binary Toxin to a Specific Receptor on Midgut Brush-Border Membranes from Mosquito Larvae. *European Journal of Biochemistry* **210**, 585-590.

- Notteboom, T. E. (2004). Container Shipping and Ports: An Overview. *Review of Network Economics* **3**, 86-106.

- O'Reilly, A. O., Khambay, B. P. S., Williamson, M. S., Field, L. M., Wallace, B. A. & Davies, T. G. E. (2006). Modelling Insecticide Binding Sites in the Voltage-Gated Sodium Channel. *Biochemical Journal* **396**, 255-263.

- Oetting, W. S., Armstrong, C. M., Ronan, S. M., Young, T. L., Sellers, T. A. & King, R. A. (1998). Multiplexed Short Tandem Repeat Polymorphisms of the Weber 8A Set of Markers Using Tailed Primers and Infrared Fluorescence Detection. *Electrophoresis* **19**, 3079-3083.

- PAHO. (2008). Malaria in the Americas: Time Series Epidemiological Data from 2000 to 2007.

- Pal, R. (1976). Problems of Insecticide Resistance in Insect Vectors of Human Disease. *XV International Congress of Entomology*. Washington D.C.: Entomological Society of America.

- Penilla, R. P., Rodriguez, A. D., Hemingway, J., Torres, J. L., Arredondo-Jimenez, J. I. & Rodriguez, M. H. (1998). Resistance Management Strategies in Malaria Vector Mosquito Control. Baseline Data for a Large-Scale Field Trial Against *Anopheles albimanus* in Mexico. *Medical and Veterinary Entomology* **12**, 217-233.

- Phuc, H. K., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., Fu, G. L., Condon, K. C., Scaife, S., Donnelly, C. A., Coleman, P. G., White-Cooper, H. & Alphey, L. (2007). Late Acting Dominant Lethal Genetic Systems and Mosquito Control. *Bmc Biology* **5**.

- Pinto, J., Donnelly, M. J., Sousa, C. A., Malta-Vacas, J., Gil, V., Ferreira, C., Petrarca, V., do Rosario, V. E. & Charlwood, J. D. (2003). An Island within an Island: Genetic Differentiation of *Anopheles gambiae* in Sao Tome, West Africa, and its Relevance to Malaria Vector Control. *Heredity* **91**, 407-414.

- Pinto, J., Lynd, A., Vicente, J. L., Santolamazza, F., Randle, N. P., Gentile, G., Moreno, M., Simard, F., Charlwood, J. D., do Rosario, V. E., Caccone, A., della Torre, A. & Donnelly, M. J. (2007). Multiple Origins of Knockdown Resistance Mutations in the Afrotropical Mosquito Vector *Anopheles gambiae*. *Plos One* **2**.

- Piry, S., Alapetite, A., Cornuet, J. M., Paetkau, D., Baudouin, L. & Estoup, A. (2004). GENECLASS2: A Software for Genetic Assignment and First Generation Migrant Detection. *Journal of Heredity* **95**, 536-539.

- Polson, K. A., Brogdon, W. G., Rawlins, S. C. & Chadee, D. D. (2011). Characterization of Insecticide Resistance in Trinidadian Strains of *Aedes aegypti* Mosquitoes. *Acta Tropica* **117**, 31-38.

- Ponlawat, A., Scott, J. G. & Harrington, L. C. (2005). Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* across Thailand. *Journal of Medical Entomology* **42**, 821-825.

- Powell, J. R., Tabachnick, W. J. & Arnold, J. (1980). Genetics and the Origin of a Vector Population - *Aedes aegypti*, A Case Study. *Science* **208**, 1385-1387.

- Pritchard, J. K., Stephens, M. & Donnelly, P. (2000). Inference of Population Structure Using Multilocus Genotype Data. *Genetics* **155**, 945-959.

- The Public Health Department (2010). Communicable Disease Surveillance Report December 2010. Office of the Medical Officer of Health, The Public Health Department, Cayman Islands Health Services Authority, Grand Cayman.

- Rajatileka, S., Black IV, W. C., Saavedra-Rodriguez, K., Trongtokit, Y., Apiwathnasorn, C., McCall, P. J. & 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 Tropica* **108**, 54-57.

- Rannala, B. & Mountain, J. L. (1997). Detecting Immigration by Using Multilocus Genotypes. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9197-9201.

- Ranson, H., Burhani, J., Lumjuan, N. & Black IV, W. C. (2010). Insecticide Resistance in Dengue Vectors. *TropIKA.net Journal* **1**.

- Ranson, H., Nikou, D., Hutchinson, M., Wang, X., Roth, C. W., Hemingway, J. & Collins, F. H. (2002). Molecular Analysis of Multiple Cytochrome P450 Genes from the Malaria Vector, *Anopheles gambiae*. *Insect Molecular Biology* **11**, 409-418.

- Ravel, S., Herve, J. P., Diarrassouba, S., Kone, A. & Cuny, G. (2002). Microsatellite Markers for Population Genetic Studies in *Aedes aegypti* (Diptera : Culicidae) from Cote d'Ivoire: Evidence for a Microgeographic Genetic Differentiation of Mosquitoes from Bouake. *Acta Tropica* **82**, 39-49.

- Ravel, S., Monteny, N., Olmos, D. V., Verdugo, J. E. & Cuny, G. (2001). A Preliminary Study of the Population Genetics of *Aedes aegypti* (Diptera : Culicidae) from Mexico Using Microsatellite and AFLP Markers. *Acta Tropica* **78**, 241-250.

- Rawlins, S. C. (1998). Spatial Distribution of Insecticide Resistance in Caribbean Populations of *Aedes aegypti* and its Significance. *Rev Panam Salud Publica* **4**, 243-251.

- Rawlins, S. C. & Wan, J. O. H. (1995). Resistance in Some Caribbean Populations of *Aedes aegypti* to Several Insecticides. *Journal of the American Mosquito Control Association* **11**, 59-65.

- Raymond, M., Callaghan, A., Fort, P. & Pasteur, N. (1991). Worldwide Migration of Amplified Insecticide Resistance Genes in Mosquitoes. *Nature* **350**, 151-153.

- Raymond, M. & Rousset, F. (1995). Genepop (Version 1.2) Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity* **86**, 248-249.

- Reiter, P., Amador, M. A., Anderson, R. A. & Clark, G. G. (1995). Dispersal of *Aedes aegypti* in an Urban Area after Blood Feeding as Demonstrated by Rubidium Marked Eggs. *American Journal of Tropical Medicine and Hygiene* **52**, 177-179.

- Ritchie, S. A. & Rochester, W. (2001). Wind-Blown Mosquitoes and Introduction of Japanese Encephalitis into Australia. *Emerging Infectious Diseases* **7**, 900-903.

- Roberts, D. R., Chareonviriyaphap, T., Harlan, H. H. & Hshieh, P. (1997). Methods of Testing and Analyzing Excito-Repellency Responses of Malaria Vectors to Insecticides. *Journal of the American Mosquito Control Association* **13**, 13-17.

- Robertson, A. & Hill, W. G. (1984). Deviations from Hardy Weinberg Proportions - Sampling Variances and Use in Estimation of Inbreeding Coefficients. *Genetics* **107**, 703-718.

- Robinson, A. S. (2002). Mutations and Their Use in Insect Control. *Mutation Research-Reviews in Mutation Research* **511**, 113-132.

- Rodriguez, M. M., Bisset, J., De Fernandez, D. M., Lauzan, L. & Soca, A. (2001). Detection of Insecticide Resistance in *Aedes aegypti* (Diptera : Culicidae) from Cuba and Venezuela. *Journal of Medical Entomology* **38**, 623-628.

- Rodriguez, M. M., Bisset, J., Ruiz, M. & Soca, A. (2002). Cross Resistance to Pyrethroid and Organophosphorus Insecticides Induced by Selection with Temephos in *Aedes aegypti* (Diptera : Culicidae) from Cuba. *Journal of Medical Entomology* **39**, 882-888.

- Rosen, L. (1987). Sexual Transmission of Dengue Viruses by *Aedes albopictus*. *American Journal of Tropical Medicine and Hygiene* **37**, 398-402.

- Rosen, L., Shroyer, D. A., Tesh, R. B., Freier, J. E. & Lien, J. C. (1983). Transovarial Transmission of Dengue Viruses by Mosquitoes - *Aedes albopictus* and *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene* **32**, 1108-1119.

- Ross, D. H., Cohle, P., Blase, P. R., Bussard, J. B. & Neufeld, K. (1994). Effects of the Insect Growth Regulator (S)-Methoprene on the Early Life Stages of the Fathead Minnow *Pimephales promelas* in a Flow Through Laboratory System. *Journal of the American Mosquito Control Association* **10**, 211-221.

- Roth, L. M. (1948). A Study of Mosquito Behaviour - An Experimental Laboratory Study of the Sexual Behaviour of *Aedes aegypti* (Linnaeus). *American Midland Naturalist* **40**, 265-352.

- Rozas, J. & Rozas, R. (1995). DnaSP, DNA Sequence Polymorphism: An Interactive Program for Estimating Population Genetics Parameters from DNA Sequence Data. *Computer Applications in the Biosciences* **11**, 621-625.

- Saavedra-Rodriguez, K., Urdaneta-Marquez, L., Rajatileka, S., Moulton, M., Flores, A. E., Fernandez-Salas, I., Bisset, J., Rodriguez, M., McCall, P. J., Donnelly, M. J., Ranson, H., Hemingway, J. & Black, W. C. (2007). A Mutation in the Voltage-Gated Sodium Channel

Gene Associated with Pyrethroid Resistance in Latin American *Aedes aegypti*. *Insect Molecular Biology* **16**, 785-798.

- San Martin, J. L., Brathwaite, O., Zambrano, B., Solorzano, J. O., Bouckenooghe, A., Dayan, G. H. & Guzman, M. G. (2010). The Epidemiology of Dengue in the Americas Over the Last Three Decades: A Worrisome Reality. *American Journal of Tropical Medicine and Hygiene* **82**, 128-135.

- Severson, D. W., Anthony, N. M., Andreev, O. & Ffrench-Constant, R. H. (1997). Molecular Mapping of Insecticide Resistance Genes in the Yellow Fever Mosquito (*Aedes aegypti*). *Journal of Heredity* **88**, 520-524.

- Shepard, D. S., Coudeville, L., Halasa, Y. A., Zambrano, B. & Dayan, G. H. (2011). Economic Impact of Dengue Illness in the Americas. *American Journal of Tropical Medicine and Hygiene* **84**, 200-207.

- Sinkins, S. P. & Gould, F. (2006). Gene Drive Systems for Insect Disease Vectors. *Nature Reviews Genetics* **7**, 427-435.

- Slotman, M. A., Kelly, N. B., Harrington, L. C., Kitthawee, S., Jones, J. W., Scott, T. W., Caccone, A. & Powell, J. R. (2007). Polymorphic Microsatellite Markers for Studies of *Aedes aegypti* (Diptera : Culicidae), the Vector of Dengue and Yellow Fever. *Molecular Ecology Notes* **7**, 168-171.

- Strode, C., Wondji, C. S., David, J. P., Hawkes, N. J., Lumjuan, N., Nelson, D. R., Drane, D. R., Karunaratne, S., Hemingway, J., Black, W. C. & Ranson, H. (2008). Genomic Analysis of Detoxification Genes in the Mosquito *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* **38**, 113-123.

- Sun, Y. P. & Johnson, E. R. (1960). Synergistic and Antagonistic Actions of Insecticide Synergist Combinations and Their Mode of Action. *Journal of Agricultural and Food Chemistry* **8**, 261-266.

- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

- Thompson, M., Shotkoski, F. & Ffrench-Constant, R. (1993). Cloning and Sequencing of the Cyclodiene Insecticide Resistance Gene from the Yellow Fever Mosquito *Aedes aegypti* - Conservation of the Gene and Resistance Associated Mutation with *Drosophila*. *Febs Letters* **325**, 187-190.

- Trout, A., Baracco, G., Rodriguez, M., Barber, J., Leal, A., Radke, E., Weis, K., Stanek, D., Stark, L., Blackmore, C., Gallagher, G., Hunsperger, E., Tomashek, K., Gregory, C. & Sauber-Schatz, E. (2010). Locally Acquired Dengue - Key West, Florida, 2009-2010. *Morbidity and Mortality Weekly Report* **59**, 577-581.

- Tsagkarakou, A., Van Leeuwen, T., Khajehali, J., Ilias, A., Grispou, M., Williamson, M. S., Tirry, L. & Vontas, J. (2009). Identification of Pyrethroid Resistance Associated Mutations in the Para Sodium Channel of the Two Spotted Spider Mite *Tetranychus urticae* (Acari: Tetranychidae). *Insect Molecular Biology* **18**, 583-593.

- Unknown. (1975). Oh, New Delhi; oh, Geneva. *Nature* **256**, 355-357.

- Urdaneta-Marquez, L., Bosio, C., Herrera, F., Rubio-Palis, Y., Salasek, M. & Black, W. C. (2008). Genetic Relationships Among *Aedes aegypti* Collections in Venezuela as Determined by Mitochondrial DNA Variation and Nuclear Single Nucleotide Polymorphisms. *American Journal of Tropical Medicine and Hygiene* **78**, 479-491.

- Vaughan, A., Chadee, D. D. & Ffrench-Constant, R. (1998). Biochemical Monitoring of Organophosphorus and Carbamate Insecticide Resistance in *Aedes aegypti* Mosquitoes from Trinidad. *Medical and Veterinary Entomology* **12**, 318-321.

- Vazeille, M., Rosen, L., Mousson, L. & Failloux, A. B. (2003). Low Oral Receptivity for Dengue Type 2 Viruses of *Aedes albopictus* from Southeast Asia Compared with that of *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene* **68**, 203-208.

- Vontas, J., David, J. P., Nikou, D., Hemingway, J., Christophides, G. K., Louis, C. & Ranson, H. (2007). Transcriptional Analysis of Insecticide Resistance in *Anopheles stephensi* Using Cross Species Microarray Hybridization. *Insect Molecular Biology* **16**, 315-324.

- Vontas, J. G., Small, G. J. & Hemingway, J. (2001). Glutathione S-Transferases as Antioxidant Defence Agents Confer Pyrethroid Resistance in *Nilaparvata lugens*. *Biochemical Journal* **357**, 65-72.

- Vreysen, M. J. B., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., Dyck, V. A., Msangi, A. R., Mkonyi, P. A. & Feldmann, H. U. (2000). *Glossina austeni* (Diptera : Glossinidae) Eradicated on the Island of Unguja, Zanzibar, Using the Sterile Insect Technique. *Journal of Economic Entomology* **93**, 123-135.

- Waterhouse, R. M., Wyder, S. & Zdobnov, E. M. (2008). The *Aedes aegypti* Genome: a Comparative Perspective. *Insect Molecular Biology* **17**, 1-8.

- Weill, M., Chandre, F., Brengues, C., Manguin, S., Akogbeto, M., Pasteur, N., Guillet, P. & Raymond, M. (2000). The kdr Mutation Occurs in the Mopti Form of *Anopheles gambiae* s.s. through Introgression. *Insect Molecular Biology* **9**, 451-455.

- Weill, M., Lutfalla, G., Mogensen, K., Chandre, F., Berthomieu, A., Berticat, C., Pasteur, N., Philips, A., Fort, P. & Raymond, M. (2003). Insecticide Resistance in Mosquito Vectors. *Nature* **423**, 136-137.

- Weill, M., Malcolm, C., Chandre, F., Mogensen, K., Berthomieu, A., Marquine, M. & Raymond, M. (2004). The Unique Mutation in ace-1 giving High Insecticide Resistance is Easily Detectable in Mosquito Vectors. *Insect Molecular Biology* **13**, 1-7.

- Weir, B. S. & Cockerham, C. C. (1984). Estimating F-Statistics for the Analysis of Population Structure. *Evolution* **38**, 1358-1370.

- Werren, J. H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology* **42**, 587-609.

- WHO. (1976). WHO Supported Collaborative Research Projects in India - Facts. *Who Chronicle* **30**, 131-139.

- WHO. (2006). Report on Dengue (1955-2005). *Scientific Working Group*. Geneva, Switzerland: Special Programme for Research & Training in Tropical Diseases (TDR) Sponsored by UNICEF/UNDP/World Bank/WHO, World Health Organisation, 6, 120-122.

- WHO. (2009). Temephos in Drinking Water: Use for Vector Control in Drinking Water Sources and Containers.

- Williamson, M. S., MartinezTorres, D., Hick, C. A. & Devonshire, A. L. (1996). Identification of Mutations in the Housefly Para Type Sodium Channel Gene Associated with Knockdown Resistance (kdr) to Pyrethroid Insecticides. *Molecular and General Genetics* **252**, 51-60.

- Willis, J. H. (2010). Structural Cuticular Proteins from Arthropods: Annotation, Nomenclature, and Sequence Characteristics in the Genomics Era. *Insect Biochemistry and Molecular Biology* **40**, 189-204.

- Windbichler, N., Menichelli, M., Papathanos, P. A., Thyme, S. B., Li, H., Ulge, U. Y., Hovde, B. T., Baker, D., Monnat, R. J., Burt, A. & Crisanti, A. (2011). A Synthetic Homing

Endonuclease Based Gene Drive System in the Human Malaria Mosquito. *Nature*, **473**, 212-215.

- Wirth, M. C. & Georghiou, G. P. (1999). Selection and Characterization of Temephos Resistance in a Population of *Aedes aegypti* from Tortola, British Virgin Islands. *Journal of the American Mosquito Control Association* **15**, 315-320.

- Wondji, C., Simard, F. & Fontenille, D. (2002). Evidence for Genetic Differentiation Between the Molecular Forms M and S within the Forest Chromosomal Form of *Anopheles gambiae* in an Area of Sympatry. *Insect Molecular Biology* **11**, 11-19.

- Wright, S. (1951). The Genetical Structure of Populations. *Annals of Eugenics* **15**, 323-354.

- Wyss, J. H. (2000). Screw Worm Eradication in the Americas. *Tropical Veterinary Diseases* **916**, 186-193.

- Xi, Z. Y., Khoo, C. C. H. & Dobson, S. L. (2005). *Wolbachia* Establishment and Invasion in an *Aedes aegypti* Laboratory Population. *Science* **310**, 326-328.

- Yanola, J., Somboon, P., Walton, C., Nachaiwieng, W. & Prapanthadara, L. A. (2010). A Novel F1552/C1552 Point Mutation in the *Aedes aegypti* Voltage-Gated Sodium Channel Gene Associated with Permethrin Resistance. *Pesticide Biochemistry and Physiology* **96**, 127-131.

- Yanola, J., Somboon, P., Walton, C., Nachaiwieng, W., Somwang, P. & Prapanthadara, L.-a. (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. *Tropical Medicine & International Health* **16**, 501-509.

- http://www.co.miami-dade.fl.us/portofmiami/information.asp - accessed 6th April 2011
- http://www.oxitec.com/our-research/safe-use/ - accessed 4th May 2011
- http://www.paho.org/english/ad/dpc/cd/dengue-cases-2008.htm - accessed 1st April 2011
- http://www.who.int/whopes/resistance/en/discriminating_concentrations.pdf - accessed 11th May 2011
- http://www.youtube.com/watch?v=_nY_AlWe5kM - accessed 19th August 2011

Appendix 1 - Temephos Bioassay Data for New Orleans, Cayman Parental and KYR Strain *Aedes aegypti*

New Orleans			
Concentration (µg/L)	**Responded**	**Total**	**% Response**
0 (Control)	0	57	0
0.002	1	65	1.5
0.0035	1	10	10
0.005	8	66	12.1
0.01	21	56	37.5
0.02	32	57	56.1
0.05	58	61	95.1

Cayman Parental Strain			
Concentration (µg/L)	**Responded**	**Total**	**% Response**
0 (Control)	0	83	0
0.01	3	76	3.9
0.02	39	96	40.6
0.03	39	59	66.1
0.04	86	97	88.7
0.06	96	99	97.0

KYR			
Concentration (µg/L)	**Responded**	**Total**	**% Response**
0 (Control)	0	33	0
0.02	4	33	12.1
0.04	19	35	54.3
0.06	31	37	83.8
0.08	33	38	86.8
0.09	34	37	91.9

Appendix 2 - Adult Bioassay Data for New Orleans and Cayman Parental Strain *Aedes aegypti*

New Orleans						
Exposure Time	4% DDT			Control		
	Responded	Total	% Response	Responded	Total	% Response
15 min	0	22	0	0	22	0
30 min	7	22	32	0	22	0
45 min	17	18	94	0	23	0
1 hr	19	22	86	0	19	0
1 hr 15 min	24	24	100	0	22	0
Cayman Parental						
1 hr	0	21	0	2	17	12
2 hr	0	19	0	0	12	0
8 hr	3	27	11	3	19	16

New Orleans						
Exposure Time	0.75% Permethrin			Control		
	Responded	Total	% Response (corrected)	Responded	Total	% Response
5 min	59	69	86 (84)	5	64	8
10 min	64	66	97 (97)	3	66	5
15 min	63	67	94 (94)	4	60	7
30 min	43	43	100 (100)	10	48	21
1 hr	20	20	100 (100)	0	20	0
Cayman Parental						
30 min	8	22	36 (35)	1	38	3
1 hr	28	59	47 (47)	1	71	1
2 hr	27	69	39 (34)	4	51	8
3 hr	31	60	52 (51)	1	62	2
4 hr	46	76	61 (61)	0	69	0
5 hr	18	22	82 (81)	2	32	6
8 hr	20	23	87 (80)	9	25	36

New Orleans						
Exposure Time	0.5% Etofenprox			Control		
	Responded	Total	% Response	Responded	Total	% Response
1 hr	17	17	100	0	21	0
Cayman Parental						
1 hr	0	24	0	0	20	0
3 hr	0	23	0	0	18	0
8 hr	4	23	17	0	16	0

New Orleans						
Exposure Time	0.05% Deltamethrin			Control		
	Responded	Total	% Response (corrected)	Responded	Total	% Response
5 min	26	27	96 (96)	1	20	5
10 min	19	20	95 (95)	0	22	0
15 min	15	15	100 (100)	1	15	7
30 min	26	26	100 (100)	2	23	9
Cayman Parental						
30 min	5	22	23 (20)	1	25	4
1 hr	28	44	64 (63)	1	35	3
2 hr	13	18	72 (72)	0	23	0
3 hr	22	22	100 (100)	3	24	13

New Orleans						
Exposure Time	0.05% Lambdacyhalothrin			Control		
	Responded	Total	% Response (corrected)	Responded	Total	% Response
5 min	20	21	95 (95)	1	20	5
15 min	15	15	100 (100)	1	15	7
30 min	23	23	100 (100)	2	23	9
1 hr	23	23	100 (100)	0	10	0
Cayman Parental						
1 hr	11	44	25 (20)	3	44	7
1 hr 30 min	9	19	47 (47)	0	20	0
2 hr	8	20	40 (40)	0	23	0
2 hr 30 min	17	19	89 (89)	0	20	0
3 hr	36	41	88 (86)	6	42	14

Appendix 3 - Column Statistics of Biochemical Data

Data from the biochemical assays was analysed using the GraphPad Prism 5 software (GraphPad Software, La Jolla, Ca, USA). The mean and confidence intervals for each strain and assay are given below.

GSTs	New Orleans	Cayman Strain	KYR
Number of values	50	50	50
Mean	1.181	1.966	3.148
Lower 95% CI of mean	0.9119	1.591	2.607
Upper 95% CI of mean	1.449	2.341	3.689

P450s	New Orleans	Cayman Strain	KYR
Number of values	50	50	50
Mean	3.764×10^{-5}	9.881×10^{-5}	7.046×10^{-5}
Lower 95% CI of mean	3.131×10^{-5}	8.295×10^{-5}	5.828×10^{-5}
Upper 95% CI of mean	4.396×10^{-5}	0.0001147	8.265×10^{-5}

Naphthyl Acetate	New Orleans α-NA	New Orleans β-NA	Cayman Strain α-NA	Cayman Strain β-NA	KYR α-NA	KYR β-NA
Number of values	50	50	50	50	50	50
Mean	24.23	20.25	67.93	57.92	45.15	33.05
Lower 95% CI of mean	18.91	15.75	58.26	48.60	39.87	28.57
Upper 95% CI of mean	29.55	24.75	77.59	67.24	50.43	37.54

PNPA	New Orleans	Cayman Strain	KYR
Number of values	50	50	50
Mean	0.3284	1.084	1.104
Lower 95% CI of mean	0.2520	0.9370	0.9732
Upper 95% CI of mean	0.4047	1.230	1.234

Appendix 4 – ANOVA and Tukey's Multiple Comparison Test of Biochemical Data

One way ANOVA and Tukey's multiple comparison tests carried out using GraphPad Prism 5 software (GraphPad Software, La Jolla, Ca, USA).

GSTs

One-Way Analysis of Variance

P value = <0.0001

F = 23.49

R^2 = 0.2422

Tukey's Multiple Comparison Test

Comparison	Mean Difference (95% Confidence interval of difference)	q	Significant? (P<0.05)
New Orleans vs Cayman	-0.7858 (-1.471 to -0.1001)	3.846	Yes
New Orleans vs KYR	-1.968 (-2.653 to -1.282)	9.629	Yes
Cayman vs KYR	-1.182 (-1.867 to -0.4961)	5.784	Yes

P450s

One-Way Analysis of Variance

P value = <0.0001

F = 25.82

R^2 = 0.26

Tukey's Multiple Comparison Test

Comparison	Mean Difference (95% Confidence interval of difference)	q	Significant? (P<0.05)
New Orleans vs Cayman	-6.117×10^{-5} (-8.139×10^{-5} to -4.095×10^{-5})	10.15	Yes
New Orleans vs KYR	-3.283×10^{-5} (-5.304×10^{-5} to -1.261×10^{-5})	5.449	Yes
Cayman vs KYR	2.834×10^{-5} (8.128×10^{-6} to 4.856×10^{-5})	4.705	Yes

α-Napthyl Acetate

One-Way Analysis of Variance

P value = <0.0001

F = 38.71

R^2 = 0.345

Tukey's Multiple Comparison Test

Comparison	Mean Difference (95% Confidence interval of difference)	q	Significant? (P<0.05)
New Orleans vs Cayman	-43.7 (-55.48 to -31.91)	12.44	Yes
New Orleans vs KYR	-20.92 (-32.71 to -9.133)	5.956	Yes
Cayman vs KYR	22.78 (10.99 to 34.56)	6.484	Yes

β-Napthyl Acetate

One-Way Analysis of Variance

P value = <0.0001

F = 34.92

R^2 = 0.3221

Tukey's Multiple Comparison Test

Comparison	Mean Difference (95% Confidence interval of difference)	q	Significant? (P<0.05)
New Orleans vs Cayman	-37.67 (-48.55 to -26.8)	11.62	Yes
New Orleans vs KYR	-12.81 (-23.68 to -1.929)	3.951	Yes
Cayman vs KYR	24.87 (13.99 to 35.74)	7.672	Yes

PNPA

One-Way Analysis of Variance

P value = <0.0001

F = 53.37

R^2 = 0.4207

Tukey's Multiple Comparison Test

Comparison	Mean Difference (95% Confidence interval of difference)	q	Significant? (P<0.05)
New Orleans vs Cayman	-0.7552 (-0.9582 to -0.5522)	12.48	Yes
New Orleans vs KYR	-0.7754 (-0.9784 to -0.5723)	12.82	Yes
Cayman vs KYR	-0.02014 (-0.2231 to 0.1829)	0.3329	No

Appendix 5 – Cayman Islands Government Import Permit for OX513A Eggs 2009

Please Address all Correspondence
To: Director of Agriculture
Tel: (345) 947-3090
Fax: (345) 947-6501

P. O. Box 459
KY1-1106
Grand Cayman
Cayman Islands

Cayman Islands

DEPARTMENT OF AGRICULTURE

REF: OF-17 Living Organisms/Aede aegypti-MRCU08.09
OUTPUT #:3100

Permit for the Importation of Living Organisms

26 August 2009

Species:	Aedes aegypti strain 513A (mosquitoes)
Sex:	Genetically modified sterilised males.
Number:	Approximately 350,000.
Stage of development:	Eggs
Consignor:	Oxitec Limited 71 Milton Park Abingdon OX14 4RX United Kingdom
Consignee:	Miss Angela Harris Mosquito research and Control unit Street name Grand Cayman, Cayman Islands

Permission is granted for the importation of the above described organisms.

These insects may be imported in a number of separate shipments to make up the total number.

Each shipment shall be accompanied by:

1. A schedule describing of the contents and the approximate number if insects being imported, and

2. A signed letter from Oxitec Limited, attesting to the disease freedom of the mosquitoes in the shipment.

Signed:

Date and Official Stamp:

Dr. Colin Wakelin
Veterinary Officer
For Director of Agriculture

Appendix 6 – Cayman Islands Government Import Permit for OX513A Eggs 2010

Please Address all Correspondence
To: Director of Agriculture
Tel: (345) 947-3090
Fax: (345) 947-6501

P. O. Box 459
KY1-1106
Grand Cayman
Cayman Islands

Cayman Islands

DEPARTMENT OF AGRICULTURE

REF: OF-17 Living Organisms/Aedes aegypti - MRCU 03.10
OUTPUT #:3100

Permit for the Importation of Living Organisms

31 March 2010

Species:	Aedes aegypti strain 513A (mosquitoes).
Sex:	Genetically modified sterilised male eggs.
Number:	Up to a total of 500 grams of mosquito eggs.
Stage of development:	Eggs.
Consignor:	Oxitec Limited 71 Milton Park Abingdon OX14 4RX United Kingdom.
Consignee:	Miss Angela Harris Mosquito Research and Control Unit (MRCU). 99 Red Gate Road Grand Cayman, Cayman Islands.

Permission is granted for the importation of the above described organisms.

These insects may be imported in any number of separate shipments to make up, but not exceed, the total number.

A schedule shall be maintained by MRCU, describing, the total weight of eggs, and the approximate number of insects imported.

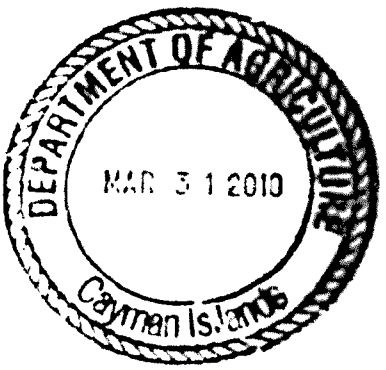
Each shipment shall be accompanied by a signed schedule from Oxitec Limited which:

1. describes of the contents, the total weight of eggs, and the approximate number of insects being imported, and

2. attests to the disease freedom of the mosquitoes in the shipment.

Signed:

Date and Official Stamp:



Dr. Colin Wakelin
Veterinary Officer
For Director of Agriculture

Appendix 7 – Article from the Caymanian Compass 2nd October 2009 on Plans to Release Genetically Modified Mosquitoes

MRCU looks to modify mozzies

BY STUART WILSON
stuart@cfp.ky

The Mosquito Research and Control Unit is considering using genetically-modified Aedes Aegypti mosquitoes to prevent dengue fever in the Cayman Islands.

Director of MRCU Bill Petrie said a final decision had not been made on the project, but it is essentially a new spin on an old technique, where males alone are released. He said the difference here is that the male mosquitoes are made sterile by genetically modifying them so that they cannot successfully breed with a female.

"We already have genetically-modified livestock and crops in the Cayman Islands and whereas we consume these products, this will not be the case with the mosquitoes and as such, it is quite a different situation," said the director.

Mr. Petrie said the male Aedes Aegypti mosquito cannot bite and lives a very short lifespan, adding that since their eggs will not survive using this new method of control, it is thought that the population of the species would decrease, as would the probability of dengue fever transmission.

A feeding Aedes Aegypti mosquito. - PHOTO: SUBMITTED

"This will be great if we can get it to work, but we are still in discussions and a pilot study to determine the feasibility of the project would have to be done first."

Mr. Petrie cautioned however, that any decision would be subject to budget concerns, logistics and priorities.

He said a permit would also have to be obtained from the Department of Agriculture before the treated mosquitoes are released and admitted he did not know how much the undertaking would cost at this point in time.

"There is no risk that I can see in this exercise," said Mr. Petrie, who elaborated: "This is actually a technique that is 30 to 40 years old, only now we are using the science of genetics as opposed to radiation and/or chemicals."

The director also remarked that several other countries were looking at this approach for controlling the Aedes Aegypti mosquito and predicted that it could become a conventional method for keeping their numbers down in the future.

It was pointed out by the director that the introduction of genetically-modified mosquitoes would not detrimentally affect the environment as the species are already found in the Cayman Islands.

Currently the MRCU is also studying the DNA of different types of mosquitoes to observe their resistance to pesticides in an effort to determine what products are most effective.

Appendix 8

Am. J. Trop. Med. Hyg., 83(2), 2010, pp. 277–284
doi:10.4269/ajtmh.2010.09-0623

Pyrethroid Resistance in *Aedes aegypti* from Grand Cayman

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Abstract. The Grand Cayman population of *Aedes aegypti* is highly resistant to DDT and pyrethroid insecticides. Glutathione transferase, cytochrome P450, and esterase levels were increased in the Grand Cayman population relative to a susceptible laboratory strain, but synergist studies did not implicate elevated insecticide detoxification as a major cause of resistance. The role of target site resistance was therefore investigated. Two substitutions in the voltage-gated sodium channel were identified, V1016I in domain II, segment 6 (IIS6) (allele frequency = 0.79) and F1534C in IIIS6 (allele frequency = 0.68). The role of the F1534C mutation in conferring resistance to insecticides has not been previously established and so a tetraplex polymerase chain reaction assay was designed and used to genotype mosquitoes that had been exposed to insecticides. The F1534C mutation was strongly correlated with resistance to DDT and permethrin.

INTRODUCTION

Aedes aegypti is a vector of several human pathogens including the viruses responsible for dengue, yellow fever, and chikungunya. This mosquito species has a cosmotropical distribution and is established in the majority, if not all, of the countries in the Americas.[1] The Cayman Islands are located in the western Caribbean, south of Cuba. The country consists of three islands, Grand Cayman, Cayman Brac, and Little Cayman with the majority of the population living in Grand Cayman. Although *Ae. aegypti* is not considered endemic to the Cayman Islands, this species has been continually present in Grand Cayman since 2002, and occasional specimens have been collected from Cayman Brac. There have been several cases of imported dengue, but local transmission is very rare with the only recorded case occurring in 2005. However, with the vector established, the climatic conditions favorable, and with frequent travel between the Cayman Islands and dengue endemic areas, there is an ever present risk of a dengue outbreak. Therefore, like past introductions of this species, the discovery of *Ae. aegypti* in 2002 stimulated an aggressive eradication campaign by the Mosquito Research and Control Unit (MRCU), an agency of the Cayman Islands Government. This campaign has not achieved the level of success expected and the reasons for this need to be explored.

The Dengue Prevention Campaign in Grand Cayman focuses on monitoring the urban centers of George Town and West Bay. Data are collected from a network of 670 ovipots, which are supplemented by yard-to-yard surveys carried out by crews who collect larval samples for identification. Crews eliminate breeding sites by emptying any unnecessary sources of standing water and treat those that remain with larvicide. The organophosphate temephos, and insect growth regulator methoprene, were used in rotation until late 2006 when temephos was replaced with *Bacillus thuringiensis israelensis* (*Bti*). In addition, yards with the greatest number of larval finds over the course of the previous year are targeted for external residual wall treatment with lambda-cyhalothrin or bifenthrin used in rotation. In cases of imported dengue fever, areas surrounding the homes of the patient are thermally fogged using permethrin to reduce adult numbers within the risk area.

In addition to the Dengue Prevention Campaign the MRCU use an array of insecticides and control methods to reduce nuisance biting mosquitoes notably *Ochlerotatus taeniorhynchus*, the Salt Marsh Mosquito, that plagues the swamps that cover over 50% of the islands. This currently involves three prehatch campaigns annually in which temephos or methoprene are applied aerially in rotation to large swamp areas to reduce numbers of larvae when swamp levels rise caused by rain or high tide. This is supplemented by aerial adulticiding with permethrin if unexpectedly high numbers of adult mosquitoes are observed. There is also extensive private sector use of insecticides with many homes employing pest control services or using aerosols to control cockroaches, ants, termites, centipedes, and scorpions.

Resistance to insecticides is common in *Ae. aegypti*. In the Caribbean, resistance to DDT developed as early as 1955.[2] Organophosphate resistance is also widespread in the region[3–7] and pyrethroid resistance has been reported in Puerto Rico,[8] Dominican Republic,[3] British Virgin Islands,[6] Cuba,[7] and Martinique.[9] Two major mechanisms are thought to be largely responsible for insecticide resistance: changes in the target site or increases in the rates of insecticide detoxification. Both of these mechanisms have been implicated in conferring resistance to insecticides in *Ae. aegypti*. For example, elevated levels of esterases have been associated with temephos resistance in Trinidad,[10] British Virgin Islands,[6] and Cuba[7] and several cytochrome P450 genes have been found over-expressed in pyrethroid-resistant populations of *Ae. aegypti*.[11,12] Multiple substitutions in the target site of DDT and the pyrethroid insecticides, the voltage-gated sodium channel on the insects' neurones, have also been described,[9,13,14] often referred to as *kdr* mutations (describing the knockdown resistance phenotype). However, only one of these, a valine to isoleucine substitution at codon 1016, has been clearly linked to insecticide resistance.[13]

Rising levels of insecticide resistance in the region combined with strong Caribbean transport links, increased urbanization, and heavy pesticide usage on the island make it imperative that the MRCU take a proactive approach to insecticide resistance monitoring and management. A pilot study in November 2006 found low levels of resistance to the organophosphate temephos in *Ae. aegypti* in Grand Cayman and prompted a change in larviciding policy to introduce *Bti*. Here, we report the results of a larger survey of the insecticide resistance status of the local *Ae. aegypti* population and describe the underlying mechanisms responsible for this resistance.

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MATERIALS AND METHODS

Mosquito strains. *Aedes aegypti* larvae were collected from field surveillance sites in George Town and West Bay, Grand Cayman in January 2008. The collections were pooled and reared to adults in the insectary at the MCRU. The F_1 generation was used for insecticide bioassays and the F_2 generation for the biochemical assays. Two insecticide susceptible strains were used in the study: the Rockefeller strain, an insecticide susceptible strain of Caribbean origin that has been in colony since the early 1930s,[15] and the New Orleans strain, originally colonized by the Centers for Disease Control and Prevention (CDC).

Further larval field collections were made from West Bay, George Town, and East End in February and March 2008. These were reared to adults and then frozen for later molecular analysis.

Insecticide bioassays. Larval bioassays were performed according to World Health Organization (WHO) guidelines[16]; briefly, 1 mL of temephos (Chemservice, West Chester, PA) dissolved in ethanol was added to 249 mL distilled water containing 25 third- to fourth-instar larvae. Five different concentrations between 0.0015 and 0.06 mg/L temephos and an ethanol-only control were tested in triplicate on different days. Mortality was scored in each group over a 24-hour test period. Mosquitoes with abnormal appearance or that were unable to swim to the surface were counted as dead. Any larvae that had pupated during the course of the experiment were disregarded from the totals. The lethal concentration that kills 50% (LC_{50}) values was calculated using Log dose Probit (LdP) Line software (Ehabsoft, Cairo, Egypt).

Adult bioassays were carried out on 1–3-day-old mosquitoes using WHO insecticide susceptibility test kits using papers supplied by WHO: 4% DDT, 0.75% permethrin, 0.05% deltamethrin, and 0.05% lambda-cyhalothrin. The exposure time was varied to determine the Lethal Time that kills 50% of the population (LT_{50}). Control assays, in which mosquitoes were exposed to papers impregnated with carrier oil only, were conducted in parallel. After exposure mosquitoes were transferred to a holding tube and supplied 10% sugar solution on a cotton pad. Mortality was scored over a 24-hour test period; LT_{50} values were from log time versus probit mortality lines generated using Log dose Probit (Ldp) line software.

The effect of pre-exposure to the synergist, piperonyl butoxide (PBO) on permethrin-induced mortality was also assessed. Adult 1–3-day-old females were exposed to papers impregnated with 4% PBO or to control papers and then immediately exposed to 0.75% permethrin for a further 2 hours using WHO susceptibility test kits. Mortality was scored after 24 hours. Over 100 mosquitoes were used in each assay.

Biochemical assays. Esterase activities were measured using the model substrates α- and β-naphthyl acetate and para-nitrophenyl acetate (PNPA). Glutathione transferase (GST) activity was measured using chlorodinitrobenzine (CDNB). Cytochrome P450 levels were determined using heme peroxidase and acetylcholinesterase activities were determined, according to the methods described by Penilla.[17] Fifty individual, 3-day-old females from both the Cayman strain and the New Orleans strain were used in each assay. Protein levels were quantified using the QuantiPro BCA Assay Kit (Sigma-Aldrich, St. Louis, MO) and the enzyme activities/mg protein were calculated as in Penilla.[17] One-tailed Mann-Whitney tests were used to compare the enzyme activities in the Cayman and New Orleans strains.

Partial sequencing of the *Ae. aegypti* sodium channel. DNA was extracted from individual mosquitoes using the method of Livak.[18] The polymerase chain reaction (PCR) primer pairs shown in Table 1 were designed to amplify four exons of the voltage-gated sodium channel, exons 20, 21, 22, and 31, which encode domain II subunit 4, 5, and 6, and domain III, subunit 6.[14]

The PCR reactions were carried out in a volume of 25 μL with final concentrations of 2.5 mM $MgCl_2$, 0.2–0.4 mM each dNTPs, 0.5 μM forward and reverse primers, 2.5 U *Taq* polymerase, and 1% of the total genomic DNA extracted from a single mosquito as template. Cycling conditions were as follows: for primer sets AaNa20 and AaNa21 initial denaturation of 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min, then a final elongation at 72°C for 10 min. For primer set AaNa31 conditions were the same except the annealing temperature was 59°C. Cycling conditions for the Ae2021a primers were 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 2 min followed by a final elongation stage of 72°C for 7 min. The PCR products were visualized by gel electrophoresis and then sequenced directly by Macrogen, (Seoul, Korea). The sequences were assembled and aligned using Lasergene (DNAstar, Madison, WI).

***Kdr* genotyping.** The hot oligonucleotide ligation assay (HOLA) method described in Rajatileka[19] was used to genotype the Cayman Islands populations for the V1016I mutation. A second amino acid substitution, F1534C, was detected in the sequenced regions of the sodium channel of *Ae. aegypti* from Grand Cayman and a tetra primer PCR assay was designed to genotype mosquitoes at this locus (Figure 1).

TABLE 1

Sequences of primers used for partial amplification of the *Aedes aegypti* sodium channel gene in the current study

Region amplified	Primer name	Sequence (5'–3')	Product size (bp)
Exon 20	AaNa20F	CCCATTGCTGCCTAAACACT	321
	AaNa20R	CTTTTCGCAGTCGTTGATGA	
Exon 21	AaNa21F	AGACAATGTGGATCGCTTCC	175
	AaNa21R	CACTACGGTGGCCAAAAAGA	
Exon 21 22 (including Intron)	Ae2021aF[19]	ATTGTATGCTTGTGGGTG	457
	Ae2021aR[19]	GCGTTGGCGATGTTC	
Exon 31	AaNa31F	GACTCGCGGGAGGTAAGTT	500
	AaNa31R	CCGTCTGCTTGTAGTGATCG	
	AaEx31P	TCGCGGGAGGTAAGTTATTG	350
	AaEx31Q	GTTGATGTGCGATGGAAATG	
	AaEx31wt	CCTCTACTTTGTGTTCTTCATCATCTT	231
	AaEx31mut	GCGTGAAGAACGACCCGC	163

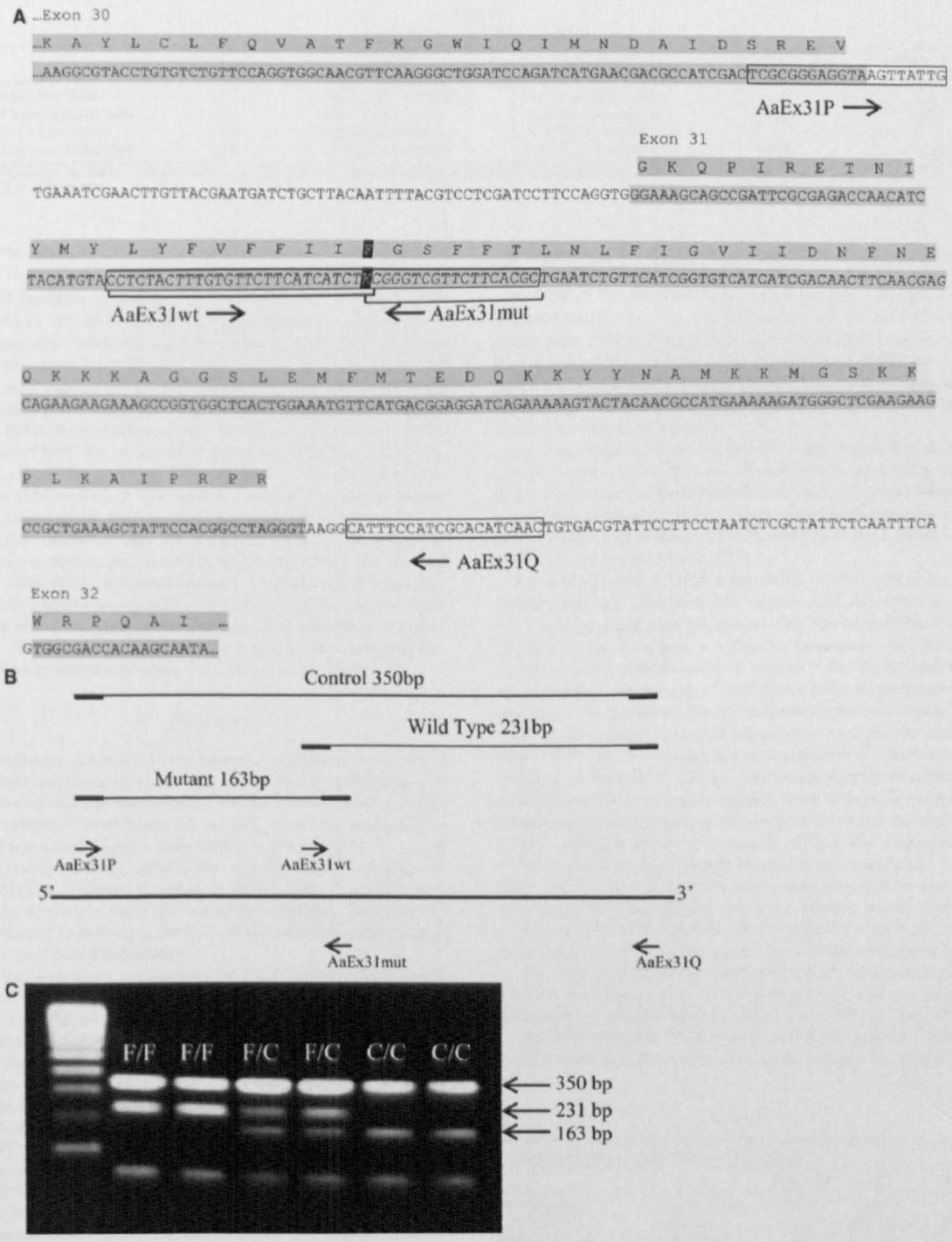

FIGURE 1. Diagnostic polymerase chain reaction (PCR) for F534C sodium channel mutation. Panel A: shows the partial sequence of the *Aedes aegypti* sodium channel with the position of the primers used in the assay marked. Exonic regions are shown in grey with the amino acid translation above the sequence data, boxed text indicates the position of the primers and the mutation detected in the Cayman population is indicated in black. Panel B: shows a schematic of the tetraplex PCR assay indicating the expected product sizes. Panel C: provides an example of the results obtained. Lane 1: contains a 100-bp ladder and lanes 2–7: contain PCR products obtained using template from a single mosquito. The amino acid sequence at position 1534, as deduced by the results of this tetraplex assay and confirmed by sequencing, is indicated above each lane.

TABLE 2
Larval bioassays with temephos*

	Sample size	LC_{50} mg/L (95% upper and lower limits)	LC_{95} mg/L (95% upper and lower limits)	RR at the LC_{50} vs. Rockefeller strain	RR at the LC_{50} vs. NO strain
Rockefeller 2006	341	0.0059 (0.0054–0.0065)	0.011 (0.0099–0.013)	–	–
F_1 Cayman strain 2006	262	0.017 (0.015–0.02)	0.037 (0.031–0.045)	2.88	1.21
New Orleans 2008	315	0.014 (0.012–0.017)	0.045 (0.035–0.064)		
F_1 Cayman strain 2008	427	0.023 (0.021–0.025)	0.043 (0.039–0.049)	3.89	1.64

*The Rockefeller and New Orleans strains are two long established laboratory insecticide susceptible strains that were used as controls in 2006 and 2008, respectively.
RR = resistance ratio.

In this assay, the flanking primers amplify a control band of 350 bp. Two internal allele-specific primers were designed to give PCR products of either 231 bp ("wild-type" phenylalanine allele) or 167 bp ("mutant" cysteine allele) by forming PCR primer pairs with the flanking primers. Each PCR reaction (25 µL) contained 2.5 mM $MgCl_2$, 0.4 mM each dNTPs, 0.5 µM each primer, 2.5 U *Taq* polymerase, and 1% of the total genomic DNA extracted from a single mosquito as template and the cycling conditions were 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec, and a final elongation at 72°C for 10 min. The PCR products were resolved on a 2% agarose gel and a 100-bp ladder (Hyperladder IV, Bioline, MA) was used for sizing.

After validating this allele-specific PCR on templates of known sequence, the assay was used to genotype 150 mosquitoes collected from Grand Cayman. An additional 200 mosquitoes that had been exposed to the LT_{50} for permethrin or DDT were also genotyped to test for genotype:phenotype association (Fisher's exact test). Tests for Hardy Weinberg equilibrium were performed using Genepop version 4.0.[20]

RESULTS

Bioassays. A low level of resistance to temephos was detected in field populations of *Ae. aegypti* from Grand Cayman in 2006 and this was the stimulus for the current study. In 2008, the resistance level based on the LC_{50} of the local population had increased slightly from 0.017 to 0.023 mg/L, a 1.3-fold increase ($P < 0.01$), despite the withdrawal of temephos for larviciding in Grand Cayman in 2006 (Table 2). Calculations of the resistance ratios for temephos are complicated by the significant variations in the LC_{50} of the two susceptible strains ($P < 0.01$) (see Discussion).

Very high levels of resistance to DDT and pyrethroid insecticides are present in Cayman *Ae. aegypti*. All of the Cayman *Ae. aegypti* population survived 1 hour exposure to the WHO pyrethroid impregnated papers. When comparing LT_{50} times for the Cayman versus the New Orleans strain the resistance ratios (RR), are 434, 29, and > 41.2 for permethrin, deltamethrin, and lambda-cyhalothrin, respectively (Table 3). The New Orleans strain showed 86% mortality after 1 hour exposure to DDT (100% after 75 min), whereas the Cayman strain was able to withstand exposure in excess of 8 hours at which point only 11% mortality was observed (data not shown). The very low levels of mortality induced by DDT exposure precluded an accurate determination of the RR for this insecticide.

Pre-exposure to the synergist piperonyl butoxide had no significant effect on permethrin mortality ($P = 0.16$) (data not shown). The effect of PBO on DDT mortality was not assessed.

Biochemical assays. Elevated levels of esterases (with all three substrates), cytochrome P450s, and GSTs were found in the Cayman population compared with the susceptible New Orleans strain (Figure 2). The greatest increase was observed in the esterase assays with median activity in the Cayman strain 4.74, 3.57, and 3.97 times than the New Orleans strain with PNPA, α-naphthol, and β-naphthol, respectively. The corresponding fold changes for GST and, P450, are 1.98 and 2.63. A one-tailed Mann-Whitney test to determine the significance of the increase in activity in each of these enzymes results in P values of < 0.0001.

For the insensitive acetylcholine assay remaining AchE activity was less than 30% for all individuals, suggesting that this is not a major resistance mechanism in the Cayman Islands population (Figure 3). There was no significant difference in the percentage of remaining AchE activity in the Cayman or New Orleans strains ($P = 0.2453$).

***Kdr* alleles.** Partial DNA sequencing of the voltage-gated sodium channel identified two amino acid substitutions in the Cayman population compared with the susceptible New Orleans strain. The first, a valine to isoleucine substitution found at codon 1016, domain II, subunit 5, has been reported elsewhere in Latin America[13] and shown to be associated with resistance to pyrethroids. The second substitution was at codon 1534 where a single base pair substitution changes the codon from TTC to TGC resulting in a phenylalanine to cysteine substitution in domain III, subunit 6 (note numbering of residues is based on the reference sequence from *Musca domestica*,[21] exon assignment is based on the annotation of the *Ae. aegypti* sodium channel gene in Chang[14]). Given the importance of this subunit in the binding of pyrethroid insecticides (see below) we predicted that this amino acid substitution may be associated with insecticide resistance. Hence, we developed a new, simple, allele-specific PCR assay to screen for this mutation in *Ae. aegypti*. The assay works on the same principles as the assay developed by Martinez-Torres[22] for detecting the L1014F *kdr* mutations in *Anopheles gambiae* and can readily distinguish all three genotypes (SS, RS, and RR) (Figure 1B).

The new tetraplex PCR to detect F1534C and the HOLA assay to detect V1016I were used to determine the frequency

TABLE 3
Bioassay results for New Orleans and Cayman population of *Aedes aegypti* exposed to pyrethroid insecticides

		Sample size	LT_{50}	RR
Permethrin (0.75%)	New Orleans	265	7 min	
	Cayman	331	3077 min	434
Deltamethrin (0.05%)	New Orleans	88	6 min	
	Cayman	106	177 min	29
Lambda-cyhalothrin (0.05%)	New Orleans	100	< 5 min*	> 41.2
	Cayman	143	206 min	

*The New Orleans strain was killed very rapidly by lambda cyhalothrin making it difficult to calculate an accurate resistance ratio for this insecticide.
RR = resistance ratio.

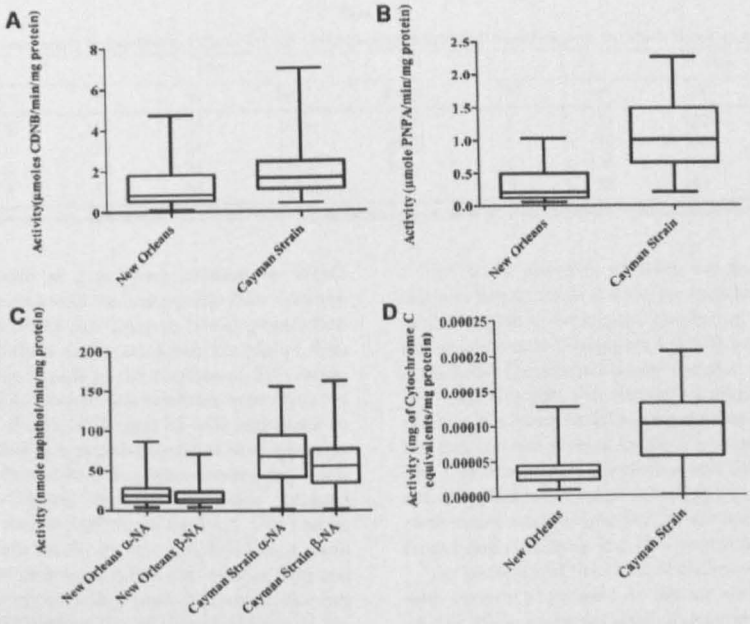

FIGURE 2. Boxplots of results from biochemical assays. The median activity is shown by a horizontal bar; the box denotes the upper and lower quartiles. The vertical lines show the full range of the data set. Panel A = GST assay using CDNB; B = esterase assay using PNPA; C = esterase assay using α and β naphthol and D = P450 assay using heme peroxidase. Results are expressed as µmole/min/mg protein with the exception of the P450 assay, which is expressed as mg of cytochrome C equivalents/mg protein.

of these two substitutions in Grand Cayman. Fifty mosquitoes from three areas of the Island (East End, George Town, and West Bay) were genotyped at both loci. The two loci were in genotypic equilibrium. The overall frequency of the 1016I allele was 0.79 (Table 4). The East End and West Bay population were in Hardy Weinberg equilibrium but the George Town population had an excess of heterozygotes. The overall frequency of the 1534C allele was 0.68. Significant deviations from Hardy Weinberg equilibrium were observed in West Bay only, which also had an excess of heterozygotes at this locus (Table 4).

To determine the correlation between the genotypes at codons 1016 and 1534 and resistance to insecticides, the offspring of adults reared from wild caught *Ae. aegypti* larvae were exposed to either 4% DDT for 24 hours or 0.75% permethrin for 2 hours and 50 surviving and 50 dead mosquitoes (for codon 1016) or 100 surviving and dead (for codon 1534) were genotyped (Table 5). The 1016I mutation was positively associated with permethrin survival ($P = 0$) but not survival to DDT ($P = 0.145$). The 1534C mutation was strongly associated with survival to both insecticides ($P = 0$). Individuals homozygous for both resistance alleles (1015I and 1534C) survived permethrin exposure, but this double homozygous genotype was not associated with DDT survival.

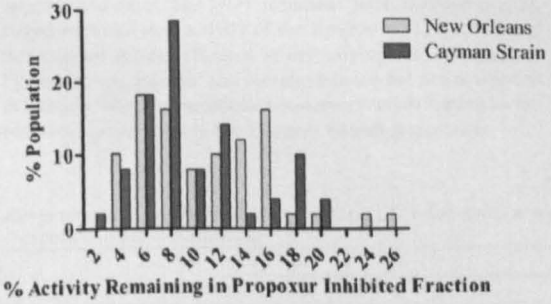

FIGURE 3. Histogram showing acetylcholinesterase activity in the presence of propoxur. In both the New Orleans and Grand Cayman populations, remaining AchE activity was less than 30% for all individuals, suggesting that insensitive acetylcholinesterase is not a major resistance mechanism in the Cayman Islands population.

DISCUSSION

The *Ae. aegypti* population in the Cayman Islands is highly resistant to DDT and pyrethroid insecticides. The DDT resistance was first reported in the Caribbean in the 1950s and contributed to the failure of the *Ae. aegypti* eradication campaign.[2,23] Resistance to DDT persists in the region despite the fact that the use of this insecticide for *Aedes* control was largely phased out in the 1960s when organophosphate insecticides became available. As discussed below, it is possible that DDT resistance is being maintained in the population by selection with pyrethroid insecticides as both shares the same target site. The level of resistance to pyrethroids in the Cayman Islands population is particularly high. The discriminating doses for adult *Ae. aegypti* set by the WHO (http://www.who.int/whopes/resistance/en/) are a 1 hour exposure to 0.25% permethrin or 0.03% lambda-cyhalothrin (no discriminating dose is set for deltamethrin for *Ae. aegypti*). In this study, less than 80% mortality was observed after a 1 hour exposure to higher concentrations of insecticide (0.75% permethrin and 0.05% lambda-cyhalothrin) and hence the Cayman Islands population

TABLE 4

Genotypes and resistance allele frequencies of three Grand Cayman populations of *Aedes aegypti* for the V1016I and F1534C mutations*

Population	1016					1534				
	V/V	V/I	I/I	Freq I	*P* value	F/F	F/C	C/C	Freq C	*P* value
East End	3	18	28	0.76	1.00	6	21	22	0.66	0.758
George Town	0	25	24	0.74	0.021	1	19	30	0.79	0.667
West Bay	0	9	32	0.89	1.0	1	36	9	0.59	0.000
Grand Cayman	**3**	**52**	**84**	**0.79**		**8**	**76**	**61**	**0.68**	

* Tests for Hardy Weinberg Equilibrium were applied to the data and the *P* values are shown. The final row shows the combined analysis for all three populations.

would clearly be defined as pyrethroid resistant by WHO standards. When compared with the susceptible New Orleans strain, the resistance ratios of the Cayman Islands population are 29- to 434-fold and these resistance levels are higher than reported in neighboring islands in the Caribbean. For example, resistance ratios of 4.7-fold to deltamethrin were reported in *Ae. aegypti* from Cuba in 2001[7] and 35-fold resistance to permethrin was recorded in a population from Martinique in 2003.[9] However, care should be taken when comparing resistance ratios between different studies as the value obtained will be dependent on the susceptible strain used. This can be clearly seen in the results for the larval temephos bioassays in the current study. If the resistance ratios obtained in 2006 and 2008 are compared, it appears that temephos resistance has decreased after the cessation of use of this insecticide in the Dengue Prevention Campaign. However, the actual LC_{50} for temephos increased in the Cayman Islands population between 2006 and 2008. Nevertheless, the Cayman Islands population of *Ae. aegypti* is considerably more susceptible to temephos (LC_{50} 0.023 mg/L) than populations from Cuba (LC_{50} 0.0713 mg/L[7]), and British Virgin Islands (LC_{50} 0.0603 mg/L[6]).

The biochemical assays indicate elevated levels of all three of the major detoxification enzyme families in the Cayman Islands population relative to the New Orleans strain. However, pre-exposure to the synergist PBO, which acts as a general inhibitor of cytochrome P450s and esterases,[24,25] did not significantly increase the level of permethrin-induced mortality. This synergist data suggest that enhanced metabolism is not a major cause of permethrin resistance in this population and it is possible that the elevated levels of P450 observed may be caused by differences between the Cayman and New Orleans strains that are unrelated to their resistance status. Several recent studies using the *Ae. aegypti* Detox chip have identified elevated expression of CYP9 P450s and Epsilon GSTs in multiple pyrethroid-resistant strains[11] (Rajatileka and others, unpublished data). The DDT resistance in *Ae. aegypti* is associated with elevated activity of the Epsilon GST, GSTE2, and this enzyme is very efficient at detoxifying this insecticide.[26] Further transcriptomic and metabolism studies are needed to determine whether metabolic resistance is contributing to the resistance phenotype in the Cayman Islands population.

This study provides evidence for the role of two sodium channel mutations in conferring resistance to both DDT and/or permethrin in *Ae. aegypti*. The first of these, a V1016I substitution, in domain II, segment 6 (IIS6), has been reported previously in the Caribbean and was found at a high frequency (0.79) in Grand Cayman. An alternative glycine substitution at this position has been found in populations from South East Asia but this was not present in the Cayman Islands population.[9,19] The Cayman Islands population was fixed for the ATA codon encoding isoleucine, at position 1011 and neither the valine or methionine substitutions that have been detected in *Ae. aegypti* from Latin American and Thai populations[9,13,19] were found.

The presence of the 1016I allele was significantly correlated with survival to permethrin but not with DDT. The frequency of this allele increases dramatically in response to selection with pyrethroids in the laboratory[13] and a recent field study in Mexico identified a rapid increase in frequency of this allele in the last decade.[27] Models of the interaction of pyrethroid and DDT insecticides with the sodium channel predict that residues in the helices IIS5 and IIIS6 play a key role in binding of insecticides.[28] These regions of the sodium channel were therefore amplified and sequenced from bioassay survivors to search for any additional mutations that may be associated with resistance to these insecticide classes. A substitution in codon 1534 within IIIS6 from TTC to TGC, resulting in the replacement of phenylalanine with cysteine, was detected and a tetraplex PCR reaction was developed and used to assess the correlation of this mutation with the resistance phenotype. All of the permethrin survivors and 46/49 DDT survivors were homozygous for the cysteine allele. This allele is present at a high frequency in the Cayman Islands population (allele frequency = 0.68) and so the numbers of "wild-type" phenylalanine homozygotes in the bioassayed individuals were low ($N = 7$), but all of these were killed by insecticide exposure.

The 1534C allele is largely recessive with heterozygotes being overwhelmingly found within the dead subset of the bioassayed mosquitoes. Not all cysteine homozygote individuals survived insecticide exposure but it should be noted that for DDT, mosquitoes were exposed to insecticide for 24 hours and then held for a further 24 hours and hence some of this mortality may not be induced by insecticide exposure alone.

TABLE 5

Kdr genotypes and allele frequencies for Grand Cayman *Aedes aegypti* that survived or died after a 24-hour exposure to 4% DDT or a 2-hour exposure to 0.75% permethrin*

		1016					1534					Double homozygotes	
		V/V	V/I	I/I	Freq I		F/F	F/C	C/Cs	Freq C		V/V & F/F	I/I & C/C
DDT	Alive	0	9	10	0.76	$P = 0.145$	0	3	46	0.97	$P = 0$	0	9
	Dead	0	16	7	0.65		3	20	27	0.74		0	6
Permethrin	Alive	0	12	14	0.77	$P = 0$	0	0	50	1.0	$P = 0$	0	14
	Dead	2	22	0	0.46		4	35	11	0.57		2	0

* Fisher's exact test was used to test for correlation between genotype and phenotype.

Several additional amino acid substitutions have been identified in the voltage-gated sodium channel of *Ae. aegypti* but for the majority of these (G923V, L982W, I1011M, V1016G,[9] and D1763Y[14]), there is little evidence associating these mutations with resistance. Hence, to date the only two sodium channel mutations with a clear association with resistance to insecticides are the 1016I and 1534C substitutions described in this study. Preliminary screening of *Ae. aegypti* populations from South East Asia indicate that the 1534C mutation has a widespread geographical distribution (Rajatileka S, unpublished data). Substitutions in an alternative phenylalanine residue in IIIS6, F1538, have been associated with pyrethroid resistance in the southern cattle tick, *Boophilus microplus*[29] and the two-spotted spider mite, *Tetranychus urticae*.[30] Recently, site directed mutagenesis has been used in an attempt to delineate the role of residues in this helix in pyrethroid binding.[31] This study found that replacement of the F1538 residue (referred to as F1518 in the Du study[31]) with alanine almost completely abolished pyrethroid binding. However, an alanine replacement of F1534 had no effect. The substitution observed at residue 1534 in the Cayman Islands *Ae. aegypti* population replaces phenylalanine with a polar, hydrophilic cysteine, and this may potentially have a more profound effect on the properties of the channel than an alanine substitution. In any case the results from this study strongly suggest that this F1534C substitution is very important in conferring resistance to pyrethroid and DDT insecticides.

The high level of resistance in *Ae. aegypti* poses a significant threat to the MRCUs Dengue Prevention Campaign. It is not yet known whether the *Ae. aegypti* population that arrived on the island in 2002 already contained the resistance alleles detected in the current study or whether resistance has arisen as a result of the intensive use of pyrethroid insecticides by both the control program and householders on the island. However, the high frequency of the *kdr* alleles suggests that alternatives to pyrethroid insecticides should be considered to control *Ae. aegypti* in the Cayman Islands.

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REFERENCES

1. Gubler DJ, 2002. The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res 33:* 330–342.
2. Brown AW, 1986. Insecticide resistance in mosquitoes: a pragmatic review. *J Am Mosq Control Assoc 2:* 123–140.
3. Mekuria Y, Gwinn TA, Williams DC, Tidwell MA, 1991. Insecticide susceptibility of *Aedes aegypti* from Santo-Domingo, Dominican-Republic. *J Am Mosq Control Assoc 7:* 69–72.
4. Rawlins SC, Wan JO, 1995. Resistance in some Caribbean populations of *Aedes aegypti* to several insecticides. *J Am Mosq Control Assoc 11:* 59–65.
5. Rawlins SC, 1998. Spatial distribution of insecticide resistance in Caribbean populations of *Aedes aegypti* and its significance. *Rev Panam Salud Publica 4:* 243–251.
6. 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:* 315–320.
7. Rodriguez MM, Bisset J, De Fernandez DM, Lauzan L, Soca A, 2001. Detection of insecticide resistance in *Aedes aegypti* (Diptera: Culicidae) from Cuba and Venezuela. *J Med Entomol 38:* 623–628.
8. Hemingway J, Boddington RG, Harris J, Dunbar SJ, 1989. Mechanisms of insecticide resistance in *Aedes aegypti* (*L.*) (Diptera, Culicidae) from Puerto-Rico. *Bull Entomol Res 79:* 123–130.
9. 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.
10. Vaughan A, Chadee DD, Ffrench-Constant R, 1998. Biochemical monitoring of organophosphorus and carbamate insecticide resistance in *Aedes aegypti* mosquitoes from Trinidad. *Med Vet Entomol 12:* 318–321.
11. Strode C, Wondji CS, David JP, Hawkes NJ, Lumjuan N, Nelson DR, Drane DR, Karunaratne S, Hemingway J, Black WC, Ranson H, 2008. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. *Insect Biochem Mol Biol 38:* 113–123.
12. Marcombe S, Poupardin R, Darriet F, Reynaud S, Bonnet J, Strode C, Brengues C, Yebakima 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.
13. Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas I, Bisset J, Rodriguez M, McCall PJ, Donnelly MJ, Ranson H, Hemingway J, Black WC, 2007. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. *Insect Mol Biol 16:* 785–798.
14. 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:* 272–278.
15. Coto MM, Lazcano JA, De Fernandez DM, Soca A, 2000. Malathion resistance in *Aedes aegypti* and *Culex quinquefasciatus* after its use in *Aedes aegypti* control programs. *J Am Mosq Control Assoc 16:* 324–330.
16. WHO, 2005. *Guidelines for Laboratory and Field Testing of Mosquito Larvicides*. WHO/CDS/WHOPES/GCDPP/2005.13.
17. Penilla RP, Rodriguez AD, Hemingway J, Torres JL, Arredondo-Jimenez JI, Rodriguez MH, 1998. Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale field trial against *Anopheles albimanus* in Mexico. *Med Vet Entomol 12:* 217–233.
18. Livak KJ, 1984. Organization and mapping of a sequence on the drosophila-melanogaster X-chromosome and Y-chromosome that is transcribed during spermatogenesis. *Genetics 107:* 611–634.
19. 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.
20. Rousset F, 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources 8:* 103–106.
21. 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.
22. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge 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:* 179–184.

23. Brown AW, Pal R, 1971. Insecticide resistance in arthropods. *Public Health Pap 38:* 1–491.
24. Khot AC, Bingham G, Field LM, Moores GD, 2008. A novel assay reveals the blockade of esterases by piperonyl butoxide. *Pest Manag Sci 64:* 1139–1142.
25. Sun YP, Johnson ER, 1960. Synergistic and antagonistic actions of insecticide-synergist combinations and their mode of action. *J Agric Food Chem 8:* 261–266.
26. Lumjuan N, McCarroll L, Prapanthadara LA, 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:* 861–871.
27. Ponce García G, Flores AE, Fernandez-Salas I, Saavedra-Rodriguez K, Reyes-Solis G, Lozano-Fuentes S, Bond JG, Casas-Martinez M, Ramsay JM, García-Rejón J, Domínguez-Galera M, Ranson H, Hemingway J, Eisen L, Black WC IV, 2009. Recent rapid rise of a permethrin knock down resistance allele in *Aedes aegypti* in Mexico. *PLoS Negl Trop Dis 3:* e531.
28. O'Reilly AO, Khambay BP, Williamson MS, Field LM, Wallace BA, Davies TG, 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochem J 396:* 255–263.
29. He HQ, Chen AC, Davey RB, Ivie GW, George JE, 1999. Identification of a point mutation in the para-type sodium channel gene from a pyrethroid-resistant cattle tick. *Biochem Biophys Res Commun 261:* 558–561.
30. Tsagkarakou A, Van Leeuwen T, Khajehali J, Ilias A, Grispou M, Williamson MS, Tirry L, Vontas J, 2009. Identification of pyrethroid resistance associated mutations in the para sodium channel of the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae). *Insect Mol Biol 18:* 583–593.
31. Du Y, Lee JE, Nomura Y, Zhang TX, Zhorov BS, Dong K, 2009. Identification of a cluster of residues in transmembrane segment 6 of domain III of the cockroach sodium channel essential for the action of pyrethroid insecticides. *Biochem J 419:* 377–385.