BEHAVIOURAL AND PHYSIOLOGICAL INSECTICIDE RESISTANCE OF THE DENGUE VECTOR *AEDES AEGYPTI* IN THE KINGDOM OF SAUDI ARABIA

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

Submitted by

Ashwaq Madani Al Nazawi

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DECLARATION

This work has not been accepted or submitted previously for any degree and is not being currently submitted in candidature for another degree at this or any other university.

The work presented in this dissertation is the result of my own investigations, except where otherwise stated.

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DEDICATION

I dedicate this dissertation to my father, Madani Alnazawi, who dearly accompanied me throughout my long PhD journey in the UK and KSA. He sacrificed his time to support me, but unfortunately, cannot complete this journey with me because he is in a coma. I also dedicate this thesis to my beautiful mum whose encouragement, moral and psychological support gave me the focus and energy to finish my PhD.

ABSTRACT

Background

Dengue has been endemic in the western region of Saudi Arabia since the 1990s. Insecticide-based control of *Aedes aegypti* remains the main dengue control option in Saudi Arabia as currently there is no curative medication for dengue, and the recently-approved vaccine is not yet available in the Middle Eastern region. Accumulating evidence suggests that insecticide resistance can reduce the effectiveness of vector control, but very little information is available on the insecticide resistance profile of *Ae. aegypti* in Saudi Arabia or the Middle Eastern region in general. The present study investigated the prevalence and levels of resistance to commonly used adulticides and larvicides, the mechanisms of physiological resistance and impact of insecticide resistance on host seeking success and behaviours in *Ae. aegypti* populations from the dengue endemic cities of Makkah and Jeddah in western Saudi Arabia.

Methods

Insecticide resistance profiles of the mosquito strains were assessed using WHO tube assays (bendiocarb, fenitrothion, deltamethrin and permethrin with or without PBO), cone assays (PermaNet 2.0 and DuraNet) and larval bioassays (*Bti* and temephos). The impact of mosquito age, duration and frequency of exposure to deltamethrin on mortality was also investigated using WHO tube bioassays. Target site mutations were identified by sequencing and were genotyped using TaqMan quantitative PCR assays, whilst metabolic resistance mechanisms were investigated by qPCR and microarray analysis. The metabolic activity of the leading candidate gene from the microarray study, *CYP9J7*, was characterised via *in vitro* insecticide metabolism assays. The effect of insecticide resistance and an insecticide treated barrier on host seeking behaviour was tested using two laboratory assays: a 'thumb test' of blood feeding behaviour and a wind tunnel test of barrier penetration.

Results

Jeddah and Makkah populations exhibited susceptibility to temephos and *Bti,* suspected resistance to fenitrothion and strong resistance to permethrin, deltamethrin, and bendiocarb. PBO pre-exposure increased pyrethroid mortality significantly in the Jeddah strain, but not in Makkah. Mosquito age and exposure-

duration significantly reduced survival of both strains, but susceptibility decreased after repeated exposures with a proportion of females surviving many successive assays. Three *kdr* mutations (S989P, V1016G, F1534C) were detected for the first time in Saudi Arabia, two of which were previously only identified in Asia. The S989P and V1016G markers were in perfect linkage disequilibrium (LD) and strongly predicted deltamethrin resistance, but were in negative LD with F1534C, which, probably as a consequence, showed negative association with resistance. Enrichment analysis of microarray data showed significant elevation of cytochrome P450s and zinc finger nucleic acid binding proteins in the overexpressed genes. The leading candidate gene *CYP9J7*, did not metabolise deltamethrin and permethrin, but did exhibit moderate metabolism of all three of the organophosphates tested. The Jeddah strain and especially the more resistant Makkah strain were much more successful in penetrating holes in PermaNet 2.0 than the New Orleans susceptible strain in the wind tunnel assay. Analysis of behavioural patterns suggested that changes in behaviour were a result of contact irritancy and differential intoxication of the strains rather than changes in amount of flight, contact with the net barrier and resting on the wind tunnel walls in assays with PermaNet 2.0 compared to the untreated assay.

Conclusion

This study provides the first study on how insecticide resistance mechanisms can impact the behaviour of an *Ae. aegypti* population from the Middle Eastern region. The highly pyrethroid resistant phenotypes are underpinned by a trio of Asian *kdr* mutations, and, to a greater extent in Jeddah, P450-based detoxification mechanisms. However surprisingly, *CYP9J7* was capable of metabolising organophosphates rather than pyrethroids, and is one of very few genes demonstrated to do so in *Aedes*. There was no evidence of major changes in host seeking behaviour in the Saudi strains, but greater barrier penetration suggests diminished protection linked with physiological resistance might be expected if pyrethroid treated materials are used as barriers. The evidence generated by this study has advanced understanding of resistance phenotypes, mechanisms and their possible consequences in a little-studied region, and local control programmes should consider adopting vector control strategies far less reliant on pyrethroids before control failure occurs.

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ABC transporters	ATP-binding cassette transporters
AChE	acetylcholinesterase
ADE	antibody-dependent enhancement
Ae.	*Aedes*
ALA	δ-aminolevulinic acid
ATP	adenosine triphosphate binding
An.	*Anopheles*
Cx.	*Culex*
BLAST	basic local alignment and search tool
Bs	*Bacillus sphaericus*
Bti	*Bacillus thuringiensis israelensis*
BI	Breteau Index
Cas9	CRISPR-associated protein 9
CCE	Carboxylesterases
cDNA	complimentary DNA
CI	Container Index
CIs	confident intervals
COE	carboxylesterases
CYP	cytochrome P450
CPR	cytochrome P450 reductase
E. coli	*Escherichia coli*
DF	dengue fever
DDT	dichlorodiphenyltrichloroethane
DHF	dengue haemorrhagic fever
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EIP	Extrinsic Incubation Period
FC	fold change
GABA	gamma aminobutyric acid
gDNA	genomic DNA
GLiM	Generalized Linear Models
GLM	General Linear Model
GST	glutathione-s-transferases
G6PD	glucose-6-phosphate dehydrogenase
h	hour
HEG	homing endonuclease gene
HCH	hexachlorocyclohexane
HI	House Index
P450	cytochrome P450
IPTG	isopropylβ-D-thiogalactopyranoside
IIP	Intrinsic Incubation Period
ITCs	insecticide-treated curtains
ITM	insecticide treated materials
ITNs	insecticide treated nets
IRAC	Insecticide Resistance Action Committee
IRS	indoor residual spray

ITSs	insecticide-treated window screens
JH	juvenile hormone
kdr	knockdown resistance
KSA	Kingdom of Saudi Arabia
LC_{50}	median lethal concentration
LD	linkage disequilibrium
LSTM	Liverpool School of Tropical Medicine
M	Molar
$MgCl_2$	magnesium chloride
min	minute
nAChR	nicotinic acetylcholine receptor
OP	organophosphate
PBO	piperonyl butoxide
PC	Principal Component
PCA	Principal Components Analysis
PI	Pupa per Person Index
qPCR	quantitative reverse transcription PCR
Rdl	resistance to dieldrin
RNA	ribonucleic acid
RIDL	release of insects with a dominant lethal
s	seconds
SD	standard deviation
SNP	single nucleotide polymorphism
SIT	Sterile Insect Technique
TSE	Tris Sodium EDTA
Tx	*Toxorhynchites*
UGTs	UDP-glycosyltransferases
ULV	ultra-low volume sprays
Vgsc	voltage-gated sodium channel
WHO	World Health Organization

CHAPTER 1
LITERATURE REVIEW

1.0. INTRODUCTION

Arboviruses are a group of viruses transmitted by arthropods, mainly mosquitoes, biting midges, ticks and sand flies (Alatoom and Payne, 2009, Young, 2018). Approximately 534 viruses are registered in the international arbovirus catalogue (Gubler, 2001), and of these, 134 groups have been documented to cause disease in humans. Arboviruses are classified into eight families and 14 genera as follows; Bunyaviridae (5), Flaviviridae (1), Reoviridae (2), Rhabdoviridae (2), Togaviridae (1), Orthomyxoviridae (1), Arenaviridae (1) and Poxiviridae (1) (Gubler, 2001). Arboviruses are distributed across all continents although Africa and South America have the highest diversities with approximately 135 different viruses, while Asia has 78, Australia 60, Europe 35 and North America 91 (Gubler, 2001).

The more clinically-significant arboviral infections for human disease are caused by viruses in the Flaviviridae, Togaviridae, Bunyaviridae and Reoviridae families (Alatoom and Payne, 2009, Beckham and Tyler, 2015). Each family includes diverse viruses **(Table 1.1)** but all share a common feature, an RNA genome. Although 134 arboviruses have been linked to human infections, the most common global outbreaks are caused by Yellow fever, Dengue, Chikungunya, Zika, Rift valley fever and West Nile fever (Gould et al., 2017). Most of the others such as Japanese encephalitis, O'nyongyong and others listed in **Table 1.1** cause localised infections. Of the common arboviral diseases, Dengue is the most important both in Saudi Arabia and globally, because Yellow fever has an effective vaccine hence is already controlled in most countries. Chikungunya is less common, with few annual global episodes, and also less severe symptoms, while Zika also has generally low severity apart from neonatal complications reported in the Americas (Weaver et al., 2018).

Table 1.1 The geographic distribution of arboviruses associated with human infections.

Family/genus	Disease	Vector	Geographic Distribution
Flaviviridae/flavivirus	Dengue	*Aedes sp.*	Southeast Asia, West Africa, Oceania, Australia, South and Central America, Mexico, Caribbean, US
	Yellow fever	*Aedes sp.*	Africa, South America
	Zika	*Aedes sp.*	Central and South America, Mexico, Caribbean, US
	Japanese encephalitis	*Culex sp.*	Japan, Korea, China, India, Nepal, Philippines, Southeast Asia, Russia
	Murray Valley encephalitis	*Culex sp.*	Australia
	Rocio	mosquitoes	South America
	St. Louis encephalitis	*Cx. pipiens*	US, Caribbean, South America
	West Nile	*Cx. pipiens*	Africa, Middle East, southern France, Russia, India, Indonesia, US, southern Canada
	Kyasanur Forest disease	Ticks *Haemaphysalis sp.*	India, Saudi Arabia
	Omsk hemorrhagic fever	Ticks *Dermacentor sp.*	Asia, Russia
	Tick-borne encephalitis	Ticks	India, Saudi Arabia
	Powassan virus	*Ixodes sp.* ticks	Eastern Canada, New York, New England states, Wisconsin
	Murray Valley encephalitis	*Culex sp.*	Australia, New Guinea
Tagaviridae/alphavirus	Chikungunya	*Ae. aegypti* *Ae. albopictus*	Africa, India, Pakistan, Guam, Southeast Asia, Reunion Island, New Guinea, limited areas of Europe, South and Central America, Mexico, US
	Ross River virus	*Aedes sp, Culex sp.*	Australia, New Guinea, Solomon Islands, Samoa, Cook Islands
	Mayaro	Mosquitoes *Haemagogus sp*	Brazil, Bolivia, Trinidad
	O'nyong-nyong	Mosquitoes (primarily *Anopheles*)	Africa
	Sinbis	*Culex sp.*	Africa, Australia, Asia, former Soviet Union, Europe (including Finland and Sweden), Oceania

	Venezuelan equine encephalitis	*Culex sp.*	Argentina, Brazil, northern South America, Panama, Mexico, Florida
	Barmah Forest virus	*Aedes sp, Culex sp.*	Australia
	Eastern equine encephalitis	*Culex sp.*	Atlantic and Gulf coasts of US, Caribbean, upper New York, western Michigan
	Western equine encephalitis	*Culex*	US, Canada, Central and South America
Bunyaviridae/ Bunyavirus	Rift Valley fever	*Aedes sp*. Primary; *Culex* secondary	South Africa, eastern Africa, Egypt, Yemen, Saudi Arabia
	La Crosse encephalitis	*Aedes sp*	North Central States, New York, Appalachian states
	California encephalitis	*Aedes sp.*	North America, Europe, Asia
	Crimean-Congo hemorrhagic fever	*Hyalomma sp* ticks	Africa, southern and eastern Europe, India, China, Turkey, Middle East, former Soviet Union
	Jamestown Canyon virus	*Aedes sp*	US from the Rocky Mountains to the East Coast, SouthEastern Canada
	Severe fever with thrombocytopenia syndrome virus	*Haemaphysalis longicornis*	China, Korea, Japan
	Oropouche virus	*Culicoides paraensis*	South and Central America, Caribbean
	Heartland virus	*Amblyomma americanum* tick	US
	Phlebotomus fever	*Phlebotomus sp* sand flies	Mediterranean basin, Balkans, Middle East, Pakistan, India, China, eastern Africa, Panama, Brazil.
Reoviridae/coltivirus	Colorado tick fever	Ticks *Dermacentor sp*	Western US, western Canada.

Information collected from (Beckham and Tyler, 2015, Alatoom and Payne, 2009)

Cases of dengue fever have been reported worldwide since the 18th century but it was not considered as a major problem until the second half of the 20th Century (Murray et al., 2013). Currently, dengue is one of the most prevalent mosquito-borne diseases, having increased 30-fold in the last five decades (Leta et al., 2018). Autochthonous transmission of dengue has been reported in 111 countries, 36 in Africa, 15 in Asia, 3 in Europe (Croatia, France, and Portugal), 11 in the Pacific islands and Australia and 46 in the Americas (including Central America, North America, the Caribbean, and South America) (Leta et al., 2018). Travel-related cases have been reported in 16 European countries and 40 states in the USA **(Figure 1.1)**.

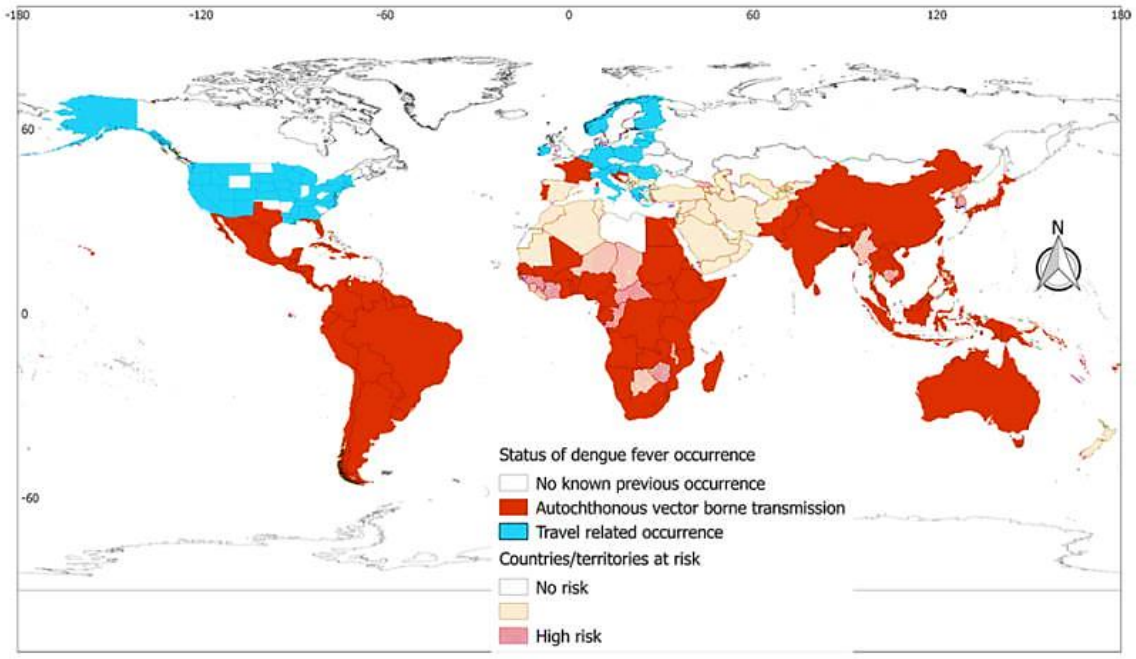


Figure 1.1 Global dengue fever distribution in different countries in the Americas, Asia, Africa, Europe and Oceania. Figure adapted from Leta et al., (2018).

Recent estimates suggest that up to 390 million dengue infections occur in humans annually, and most are asymptomatic (Khetarpal and Khanna, 2016, Freeman et al., 2018). The WHO estimates that approximately 50 million symptomatic dengue fever (DF) cases occur worldwide every year with ~ 500,000 cases of dengue haemorrhagic fever (DHF), which requires hospitalisation and carries a 2.5% mortality rate (World Health Organization, 2018d). The highest dengue incidence occurs in Asia, which contributes to 70% of the global burden followed by Africa (16%) and the Americas (14%) (Bhatt et al., 2013). These estimates may be biased to countries which consider dengue a major concern; for example, in Africa where other diseases manifesting

similar symptoms such as malaria are prioritised, cases of dengue often go unreported or undiagnosed (Weetman et al., 2018b). Severe dengue, which includes dengue haemorrhagic fever and dengue shock syndrome (DSS), were first reported in the Philippines and Thailand in the 1950s and has now been documented in more than 60 countries (World Health Organization, 2000, World Health Organization, 2018a).

Aedes aegypti is the primary vector of multiple arboviruses including Dengue, Zika and Yellow fever **(Table 1.1)**, but *Ae. albopictus* is an equally important vector of Dengue in Asia (World Health Organization, 2012b). In 2005, *Ae. albopictus* was observed to have become equally competent to Chikungunya virus following an outbreak in Reunion island. This was attributed to a mutation E1-A226V on the envelope protein gene of the Chikungunya virus, which increased *Ae. albopictus* competence but resulted in a slight reduction in midgut infectivity to the virus in the previous primary vector, *Ae. aegypti*. The observation in Reunion island highlights the potential of arboviruses to acquire transmission-facilitating mutations and cause outbreaks in regions where the typical primary vector is absent.

Once female mosquitoes are infected with an arbovirus, they remain infected throughout their adult life. Some of the arboviruses such as dengue continue to replicate in the mosquito salivary glands, thus continually replenishing the viral load (Raquin and Lambrechts, 2017).

Dengue has five serotypes (DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5). Serotypes 1-4, which are responsible for the dengue disease in humans (Mustafa et al., 2015) are antigenically distinct and exhibit 65-70% sequence homology (Murrell et al., 2011). These serotypes (DENV1-4) are suggested to have evolved from enzootic viral ancestors maintained in a sylvatic cycle among non-human primates by canopy-dwelling *Aedes* species. Sylvatic cycles remain in the forests of Southeast Asia and West Africa, which occasionally spill over into peri-urban and urban cycles causing major outbreaks in humans (Hanley et al., 2014). The fifth dengue serotype (DENV-5) recently emerged and has only been detected in serum samples from a severe outbreak in Sarawak state of Malaysia in 2013 (Mustafa et al., 2015). The antibody response to this serotype was different from that observed in the other commonly encountered serotypes hence now designated as a new serotype (DENV-5) (Normile,

2013). The serotype is believed to be a spill over from sylvatic transmission cycles since the other four serotypes are found circulating in urban and peri-urban cycles (Mustafa et al., 2015). To date, no dengue fever outbreaks have been associated with DENV-5 serotype.

1.1. Dengue fever and severe dengue

The four dengue (DENV1-4) serotypes can cause a wide spectrum of disease in humans: from mild illness to severe and potentially fatal disease. The classical form of dengue fever is an acute febrile disease characterised by high fever lasting 5 to 6 days, headache, bone or joint and muscular pains, stomach ache, rash, myalgia, nausea and vomiting and arthralgia (Bäck and Lundkvist, 2013, World Health Organization, 1997). Due to lack of specificity of these symptoms, laboratory evidence of dengue virus infection is vital to confirm diagnosis. The severity of dengue fever frequently depends on the age of the patients and rises with repeated infections due to the presence of antibodies against different serotypes which increases symptoms (World Health Organization, 1997).

The increase in the prevalence of four dengue serotypes (DENV1-4) in recent years has led to the rise in dengue haemorrhagic fever (DHF) (Murrell et al., 2011). Severe dengue causes hospitalisation and may lead to death without supportive care. It is characterised by high temperature, ascites, pleural effusion, hypoproteinaemia and haemorrhage signs such as petechiae, ecchymosis, epistaxis and thrombocytopenia (<100,000 platelet count/mm^3) (Guzmán and Kourí, 2002, Bäck and Lundkvist, 2013). Without management, prolonged plasma leakage can lead to dengue shock syndrome (World Health Organization, 1997).

1.2. Treatment of dengue infection

Currently, there are no specific drugs to treat dengue and other arboviruses, but the disease is managed by supportive health care at all stages (World Health Organization, 2018a). It is difficult to determine which dengue cases will develop in severity, but early diagnosis and supportive care reduces the mortality rate to less than 1% (World Health Organization, 2009a, World Health Organization, 2018a). Patients infected with dengue fever can be treated successfully at home by resting

and hydration to compensate for fluids lost through diarrhoea or vomiting (Rigau-Pérez et al., 1998). Although maintenance of hydration has been reported to reduce the number of hospital admissions, hospitalisation is necessary when symptoms progress and a high risk of developing severe DHF and /or DSS is suspected (Harris et al., 2003, Malavige et al., 2004).

1.3. Dengue vaccine development

The challenges that hinder the development of effective vaccines include; complexity in developing a tetravalent vaccine to protect against all virus serotypes, the risks of triggering antibody-dependent enhancement (ADE), lack of an animal model to proliferate human dengue and the difficulty of evaluating candidate vaccines in geographic regions with diverse transmission patterns (Thisyakorn and Thisyakorn, 2014, Guzman et al., 2016).

Despite these challenges, a great advance in dengue vaccine development has occurred in recent years. The most recently advanced vaccine candidate is a recombinant, live attenuated tetravalent dengue vaccine (CYD-TDV, registered as Dengvaxia) developed by Sanofi Pasteur and licensed in December 2015. The vaccine has been evaluated in phase III clinical trials in 20 countries, which include the Philippines, Mexico and Brazil where more than 35,000 children between 2 and 16 years old were enrolled (Hadinegoro et al., 2015). The overall efficacy of the vaccine in Asia was 56.5% (Capeding et al., 2014), while in Latin America the efficacy against confirmed dengue cases was 60.8% and against dengue resulting in hospitalisation was 80.3% (Villar et al., 2015a). The vaccine shows variability in levels of protection depending on serotype. In Latin America, the efficacy (based on intention to treat analysis) for serotype 1, serotype 2, serotype 3 and serotype 4 was 54.8%, 50.2%, 74.2% and 80.2% respectively (Villar et al., 2015a) while in the Asia-Pacific region, the efficacy for serotype 1 was 54.5%, serotype 2 was 34.7%, serotype 3 was 65.2% and serotype 4 was 72.4% (Capeding et al., 2014). When data from the two trial sites was combined, the overall efficacy against asymptomatic infection was 33.5% (Olivera-Botello et al., 2016).

Asymptomatic infections typically go undiagnosed but are thought to be responsible for much of the sustained transmission of dengue. Indeed, asymptomatic individuals

were found to be more infectious to mosquitoes than those with symptomatic infections (Duong et al., 2015). Dengvaxia, which has a 33.5% efficacy against asymptomatic infection, can be used for controlling transmission even in periods when there are no outbreaks that are normally associated with symptomatic cases (Olivera-Botello et al., 2016).

1.4. Transmission of dengue virus

The transmission of dengue can be vertical or horizontal. Horizontal transmission occurs from human to mosquito to human (Gutiérrez-Bugallo et al., 2017). Dengue virus is transmitted horizontally to individuals during feeding or probing of infectious *Aedes sp* (Ruiz-Guzmán et al., 2016). Vertical transmission occurs when an infected *Aedes* female mosquito passes the virus to her progeny (Soni and Sharma, 2017). This is suggested to maintain natural virus circulation in pre-outbreak periods (Soni and Sharma, 2017). The ability of dengue virus to be passed to progeny trans-ovarially has been demonstrated in experimental studies with different dengue strains in *Ae. aegypti* and *Ae. albopictus* (Mourya et al., 2001, Hailin et al., 1996, Castro et al., 2004, Sánchez-Vargas et al., 2018). When each species was infected with dengue serotype 2, 39.1% and 13.6% of *Ae. albopictus* and *Ae. aegypti* offspring were infected respectively (Castro et al., 2004). Transovarial transmission has also been observed in nature in Myanmar (Khin and Than, 1983), Bolivia (Le Goff et al., 2011), India (Thenmozhi et al., 2007, Arunachalam et al., 2008), Brazil, Cuba (Gutiérrez-Bugallo et al., 2017), Indonesia (Hadi and Soviana, 2018) and other (Cruz et al., 2015, Da Costa et al., 2017) regions by RT-PCR or cell culture screening of homogenates of pooled adult *Ae. aegypti* and *Ae. albopictus* collected from the field as eggs or larvae (Sánchez-Vargas et al., 2018). In addition to vertical and horizontal transmission, recently venereal transmission was demonstrated in the lab when infected male mosquitoes were mated with uninfected virgin females. The infection rates resulting from this mode of transmission was 31.6%, with 21% of all females having virus disseminated to their heads (i.e. salivary glands), thus suggesting they were able to infect a host during feeding (Sánchez-Vargas et al., 2018).

The dengue virus transmission cycle includes both extrinsic (EIP) and intrinsic incubation periods (IIP) (Mcbride and Bielefeldt-Ohmann, 2000). The EIP defines the

period from when mosquitoes take an infectious blood meal to the time they become infective (disease transmitters). During this period, the virus invades the mosquito's gut, replicates and propagates throughout the mosquito to reach the salivary glands and reproductive organs. This incubation period depends on environmental conditions (World Health Organization, 1997, Kuno, 1995). It may take an average of 15 days at 25°C or 6.5 days at 30°C for the mosquitoes to be able to transmit the virus to other humans (Chan et al., 2012). The IIP is the period from human receiving an infectious mosquito bite and when they begin to show symptoms owing to the infection, which takes approximately 6 days (Chan et al., 2012).

1.5. Global epidemiology of dengue fever

The incidence of dengue fever has increased in recent decades, expanding to involve more geographic areas with active transmission or travel-related cases of the disease being reported in all continents apart from Antarctica (Leta et al., 2018). The first suspected dengue case in the Americas was reported in Martinique and Guadeloupe in 1635 and in Panama in 1699 (Dick et al., 2012). A severe outbreak occurred in Peru in 1818 with an estimated 50,000 cases. Another outbreak occurred between 1827 and 1828, in the Caribbean, Mexico, Cuba, Jamaica, Colombia, Venezuela and some regions in the United States such as New Orleans, Pensacola, Savannah and Charleston. The clinical characteristics of the cases in this outbreak were near-identical to those of chikungunya and hence this might actually be a first chikungunya outbreak (Kuno, 2015) as a consequence of the African slave trade (Dick et al., 2012). Dengue outbreaks in the 20th century were reported during World War 2 from 1941 to 1946 in Mexico, Panama, Venezuela, Cuba, Puerto Rico and Bermuda (Halstead, 2006). The introduction of the Asian DENV-2 strain in Cuba was linked to the first dengue haemorrhagic fever recorded in the region in 1981 (Wei and Li, 2017). By 1996 more than 1000 cases of DHF were recorded in Colombia and other countries in the Caribbean region (Villar et al., 2015b).

In 1994, severe dengue outbreaks with DENV-3 serotypes were reported in Nicaragua and Panama, which first emerged in 1970s in the Indian sub-continent before spreading (Wilson and Chen, 2015). Rapid urbanisation is a risk factor associated with increased prevalence of dengue and improved public health surveillance systems

captured more health data, which explains why more dengue cases were reported in the 21st century (Bhatt et al., 2013). In 2002, more than 1 million dengue cases were reported in the Americas with Brazil accounting for 75% of the total number in the region. In the same year, 14,374 DHF and 255 deaths associated with dengue were reported (Dick et al., 2012). The annual cases of dengue in the region had risen to 2.35 million. In that year, 10,200 cases of DHF and 1181 deaths were reported (World Health Organization, 2018a). In 2017, the dengue cases recorded were 584,263, which was a 73% reduction compared to the 2,177,171 cases recorded in 2016 (World Health Organization, 2018a).

In the European region, dengue was first reported in 1927 in Greece and later on in 1928 in Turkey (World Health Organization, 2012c). In 2010, other cases were documented in France and Croatia (World Health Organization, 2018a). An outbreak of dengue DENV-1 occurred on the Madeira Islands in 2012 with about 2,100 confirmed cases. During this outbreak, dengue spread into other 13 European countries, mainly facilitated by travellers infected in Madeira. The outbreak is suspected to have been introduced into the regions by returning tourists from dengue-endemic countries like, Venezuela or Brazil (Wilson and Chen, 2015).

In the Pacific region, dengue has been recorded in Australia and most of the Pacific islands such as Vanuatu, New Caledonia, Tahiti, Rarotonga, Fiji, American Samoa, Western Samoa, French Polynesia, Yapa and Palau (Pinheiro and Corber, 1997). As in the Americas, the highest cases of dengue have been reported in the 21st century. For instance, during the 2012–2013 dengue outbreak in Kosrae in the Federated States of Micronesia almost 4% of the residents were hospitalized with suspected dengue infection (Wilson and Chen, 2015). In French Polynesia, between 2013–2014, circulating DENV-1 and DENV-3 caused an estimated 28,000 cases (Wilson and Chen, 2015). Dengue cases have been declining in some countries in the region for the last five years. For example, in Australia, the number of laboratory confirmed dengue cases in 2014 were 1,614 while only 731 cases were documented by 6th December 2018 (World Health Organization, 2015).

Dengue in Africa has been documented in 32 countries since 1960. The most common serotype in the region is DENV-2 (Wilson and Chen, 2015). The annual number of cases reported in the continent are considerably lower compared to Asia that

accounts for 70% of all reported cases (Bhatt et al., 2013). However, some authors have argued, most of the cases in the region fail to be reported, most being treated as malaria or other febrile illnesses hence the significance of dengue in the region being underestimated. This argument is supported by data collected on a household sero-prevalence survey after a dengue outbreak in Mombasa, Kenya in 2013 where only one participant out of the 47% that had been seen by a clinician was not diagnosed with Malaria (Weetman et al., 2018b, Ellis et al., 2015). In the same study, 13% (N=1,500) of all study participants were confirmed to have been infected in the past or had active dengue infection thus suggesting the number of unreported dengue cases in Africa are far more than those estimated.

In Asia, the earliest confirmed dengue cases were reported during World War 2 when the virus was detected in sera of American soldiers in the far east and later in 1944 in India (Pinheiro and Corber, 1997, Wei and Li, 2017). The next outbreaks (Warkentien and Pavlicek, 2016) were reported in Thailand in 1949, Vietnam in 1958, Singapore in 1960, Malaysia in 1962, India in 1963 and 1964, Bangladesh in 1964, Sri Lanka in 1966, Indonesia in 1968, Myanmar in 1970 and in China in 1974–1980s (Messina et al., 2014). Other countries in Asia have also experienced outbreaks with the most recent first outbreaks being reported in Bhutan in 2004 and Nepal in 2006 (World Health Organization, 2018a). Between 1953 to 1954, the first epidemic of DHF was recorded in the in the Philippines, Thailand in 1958 and later in other countries in Southeast Asia where it is now one of the leading causes of morbidity and mortality in children (Gubler, 2011). Currently, most countries in Asia have active transmission of dengue virus (Leta et al., 2018), with the most affected countries (cumulative cases up to August 2018) being Malaysia, which had 36,191 cases and 59 deaths, Vietnam with 32,174 cases and 8 deaths, Philippines with 20,108 cases, Cambodia with 3,868 cases, Lao PDR with 2,832 cases and 12 deaths, Singapore with 1,507 cases and China with 119 cases (World Health Organization, 2015).

In the Arabian Peninsula, dengue was first reported in Yemen and Saudi Arabia in the 1990s (Fakeeh and Zaki, 2001). Genome sequences of DENV-2 isolates from the outbreaks in Saudi Arabia from 1992 to 2014 with DENV-2 strains clustering closely with those from countries providing the highest numbers of pilgrims visiting the country to perform Hajj or Umrah pilgrimages such as Indonesia, Pakistan and India.

Sequence evidence showed involvement of multiple DENV-2 strains suggesting that importation was the driving force that introduced and sustained periodic outbreaks in Saudi Arabia (El-Kafrawy et al., 2016). The largest outbreaks in the Peninsula occurred in 2013 when more than 6,000 cases were reported in Saudi Arabia (Al-Tawfiq and Memish, 2018) and more recently in Yemen when 11,900 suspected cases were recorded between January and April 2016 (World Health Organization, 2018e). This was a 600% increase compared to the recorded cases in the same period in 2015 (World Health Organization, 2018f). The outbreak was associated with the humanitarian crisis in the country (World Health Organization, 2018f). In 2018, the cases recorded in Yemen by end of September were 1,188 and 7 deaths (World Health Organization, 2018e). Compared to 2016, this is a considerable decline facilitated by the scale-up of preparedness and response activities by WHO since 2016 (World Health Organization, 2018f).

1.6. Epidemiology of dengue fever in the Kingdom of Saudi Arabia

The Kingdom of Saudi Arabia (KSA) occupies most of the Middle East region bordering Jordan, Israel and Iraq to the north, Kuwait, Bahrain, Qatar and UAE to the east, Yemen and Oman to the south while the west is mostly the shoreline of the Red Sea. Apart from the south-western region, which has a semi-desert climatic conditions, the rest of the country is a desert (Gosling et al., 2011, Tarawneh and Chowdhury, 2018). Dengue cases are reported in the country throughout the year (Ministry of Health, 2018) with higher incidences occurring in late winter, spring and early summer which coincide with months with the highest densities of *Ae. aegypti* (Al-Ghamdi et al., 2009, Al-Tawfiq and Memish, 2018, Al-Zubyani et al., 2010, Aziz et al., 2014, Khormi and Kumar, 2012). The common circulating dengue serotypes in KSA are dengue 1, 2 and 3 (during 1994-2013) with a few instances of dengue 4 being found in patient samples (Al-Tawfiq and Memish, 2018).

Apart from Jazan and Najran, dengue episodes in KSA are mainly reported in the Province of Makkah (which includes Makkah and Jeddah) (Al-Tawfiq and Memish, 2018). In 2017, more than 75% of all reported dengue cases were in Jeddah (the second biggest city in KSA); Makkah, Taif and Madinah, accounted for 14% of the reported dengue cases while Jazan and Najran accounted for 9.2% and <1%

respectively. No dengue cases were reported in other parts of the country (Ministry of Health, 2018).

Climatic differences have been suggested to play a role in limiting dengue episodes to the western region, which experiences hot and humid weather conditions throughout the year even in winters where daytime temperatures exceed 20°C and are accompanied by relatively high humidity of >60% (Al-Ghamdi et al., 2009). These climatic conditions are ideal both for rapid development of *Aedes* larvae and a short extrinsic incubation period of dengue in mosquitoes (Ebi and Nealon, 2016). A short development window for both the vector and the virus thus facilitate sustained transmission of dengue throughout the year in the cities in the western region of KSA (Al-Tawfiq and Memish, 2018). The first case of dengue hemorrhagic fever in KSA was reported in Jeddah in 1994, which was caused by DENV-2 serotype (Fakeeh and Zaki, 2001), and triggered multiple outbreaks in the other cities in the province of Makkah and the south-west regions (Alshammari et al., 2018). From 1994 to 2002, the total number of suspected cases documented in Jeddah was 1,020 (Fakeeh and Zaki, 2003). Between 2004-2005, 300 cases were reported in the country. By 2006, the annual cases of dengue in KSA had increased to over 1000 cases (personal communication with Ministry of Municipal and Rural Affairs team) with the highest number of cases to date in 2013 **(Figure 1.2; Appendix1:8.1)** (Aziz et al., 2014). Another major reason for higher cases of dengue in the province of Makkah compared to other regions in Saudi Arabia could be due to the growing levels of urbanisation, international trade and travel. Rapid urbanization in the region has resulted in an increase in construction projects with incomplete sewage networks, and water storage on construction sites: all of which provide many breeding sites for *Ae. aegypti*.

Several risk factors have been identified as predictors of dengue infection. These include age, low socioeconomic status, household factors such as scarce water supply and unscreened houses, environmental factors such as inappropriate waste disposal and biological factors such as immune status and genetic background (Kholedi et al., 2012, Al-Raddadi et al., 2018). The risk factors associated with dengue sero-positivity in KSA include, stagnant water in indoor drainage holes, construction sites, and older age of patients (Kholedi et al., 2012, Jamjoom et al., 2016). Al-Raddadi et al. (2018)

categorised the risk factors associated with dengue sero-prevalence in Jeddah, Makkah, Jizan and Madinah into demographic and environmental factors. The demographic factors were older age, type of house and number of occupants/households. The environmental risk factors included the lack of vector control in residential areas and existence of mosquitoes in the home (Al-Raddadi et al., 2018). Some of the risk factors associated with dengue infection or sero-prevalence in KSA are related to those identified in other countries. For example, in Colombia, a sero-prevalence survey among children aged 5-19 years showed that age and socio-economic status were significant risk factors for dengue sero-prevalence (Piedrahita et al., 2018). In Pakistan, a systematic review of all studies published between 1980 and 2014 found age was a major risk factor (Khan et al., 2018) of dengue infection. Ages between 20-45 were reported to be at highest risk of contracting dengue. Other factors included socioeconomic status, urbanisation and population growth. In Venezuela, the risk factors linked to dengue sero-prevalence were poverty-related socioeconomic factors such as place and duration of residence, crowding, household size, living in a poor housing (shacks) and environmental factors such as potential mosquito breeding sites (storing water and used tires) (Velasco-Salas et al., 2014).

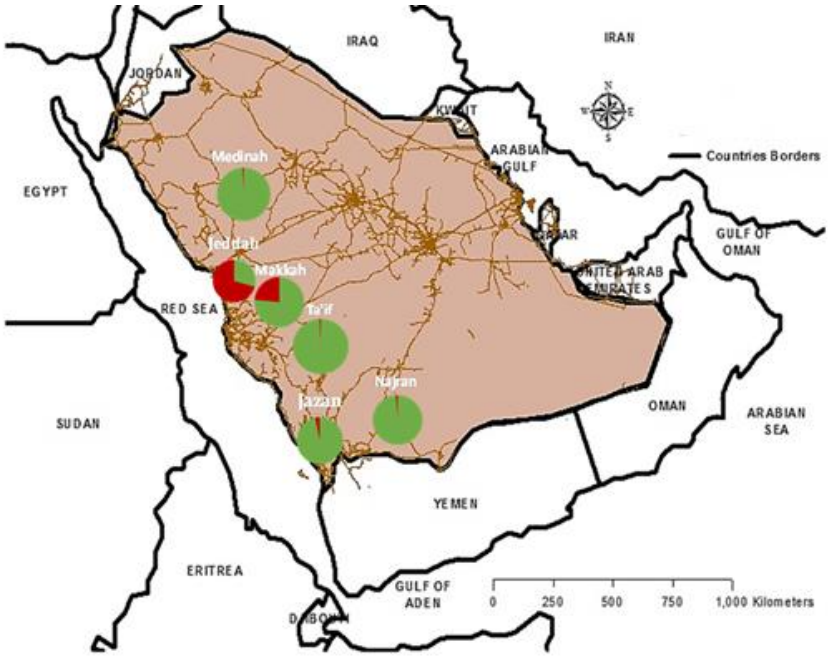


Figure 1.2 Dengue fever cases in cities of Saudi Arabia from 2013–2015. Red colour in pie charts indicate numbers of dengue cases in each city: Jeddah (9,096), Makkah (3,035), Madinah (93),Ta'if (92), Jazan (429) and Najran (147), as a proportion of the total number of reported dengue cases (12,892) across all cities combined (Ministry of Health, 2018).

1.7. Dengue vectors

Aedes aegypti and *Ae. albopictus* are vectors of dengue and other arboviral diseases including Zika, Chikungunya and Yellow fever (Hochedez et al., 2006, Phillips, 2008). These mosquitoes belong to the family *Culicidae*, subfamily *Culicinae*, tribe *Aedini*, subgenus *Stegomyia*; genus *Aedes* (Ward, 1992).

1.7.1. *Aedes aegypti* (Linnaeus)

Aedes aegypti also known as the 'yellow fever mosquito' (Powell and Tabachnick, 2013), was first described by Linnaeus (1762). *Ae. aegypti* is distributed in the tropical and subtropical regions between 35°N and 35°S around the world (World Health Organization, 2009a). *Aedes aegypti* has two subspecies; *Ae. aegypti aegypti*, which is lighter in colour and anthropophilic and *Ae. aegypti formosus,* which is dark in colour, lives in forested habitats and is less human associated. It is believed that *Ae. aegypti* originated in the African subcontinent and arrived in the Americas by water barrels in the ships of explorers, colonists and slave traders (Powell, 2018). A study investigating the origin of the domestic species, *Ae. aegypti aegypti* found the domestic population in Senegal in West Africa was more closely related to other domestic populations in different parts of the world than to other local *Ae. aegypti*

populations in Africa. This suggested the domestic population descended from an ancestral population in Africa and spread to other continents (Crawford et al., 2017). *Aedes aegypti* are container-breeders that preferentially breed in close proximity to human settlements. The immature stages can develop in a wide range of man-made containers from discarded bottles, cans, birds baths, discarded tyres, tree holes, domestic water tanks to underground standing water or pools (Powell and Tabachnick, 2013). Eggs have the ability to withstand desiccation for up to one year, which enables dispersal over large distances (Faull and Williams, 2015). The larval stages normally takes 7-8 (males) or 8-9 (females) days to develop into pupa, and then an additional 2-3 days to emerge as adults (Clemons et al., 2010). Biotic factors such as food, competition, predation and abiotic factors such as temperature, evaporation and rainfall influence egg hatching, larval performance and survival of progeny (Farjana and Tuno, 2013). The life cycle can occur in 9 (males) or 10 (females) days in ideal climatic and environmental conditions (Clemons et al., 2010). The lifespan for adult *Ae. aegypti* ranges between 15 days to a month (Silveira et al., 2018).

Dispersal occurs to find mates, food or oviposition sites and to seek hosts (Honório et al., 2003). Dipersal ranges reported for *Ae. aegypti* range from 20m to 1km (Bergero et al., 2013). Honório et al,.(2003) using radiolabelled eggs found that *Ae. aegypti* and *Ae. albopictus* in Brazil were able to spread up to 800 m in 6 days from the release point where the eggs deposited but on average the flight distance was 100-500m (Honório et al., 2003). The variation in dispersal patterns has been suggested to be due to unavailable oviposition places and environmental differences such as wind that might decrease dispersal (Bergero et al., 2013).

In general, *Ae. aegypti* are considered endophagic and endophilic species, i.e. they prefer to feed and rest in or around houses (Harrington et al., 2001b, Scott et al., 2000). However, feeding and resting preference can vary depending on locality. In Kenya, larvae collected from domestic, semi-domestic and forest, reared in lab, marked and adults released revealed *Ae. aegypti* resting and feeding behaviour to be distinct depending on where the larvae were collected. The authors reported, out of the 407 mosquitoes they recaptured indoors by the landing method, 83% were those

originally collected in domestic habitats, 15.5% in peri-domestic habitats and the rest from forest habitats (Trpis and Hausermann, 1975).

The females are highly anthropophilic and exhibit a bi-diurnal feeding habit, feeding mainly in the morning and before dusk (Jahangir et al., 2008). Although *Ae. aegypti* are highly anthropophilic sometimes they feed on multiple hosts during a single gonotrophic cycle (Huber et al., 2008). Sivan and colleagues found, apart from humans who were the main host (88 %), *Ae. aegypti* in India also fed on cows (5%), goats (3%), fowl (0.7%) and unidentified sources (4%) (Sivan et al., 2015). In Thailand, *Ae. aegypti* fed overwhelmingly on human hosts (99%) (Ponlawat and Harrington, 2005). In Providencia and Recio, Rural Puerto Rico, *Ae. aegypti* was also reported to primarily feed on humans (76–79%) (Barrera et al., 2012).

1.7.1.1. *Aedes aegypti* life cycle

As for other holometabolous insects, mosquitoes' lifecycle progress from egg, larva, pupa and into an adult **(Figure 1.3)**. The immature stages are restricted to aquatic environments. *Aedes* and other culicine larvae have a short siphon containing a single hair tuft that is located on the terminal abdominal scales **(Figure 1.4)** while *Anopheline* larvae breath through spiracles located on the lateral sides of the larvae (Service, 2015).

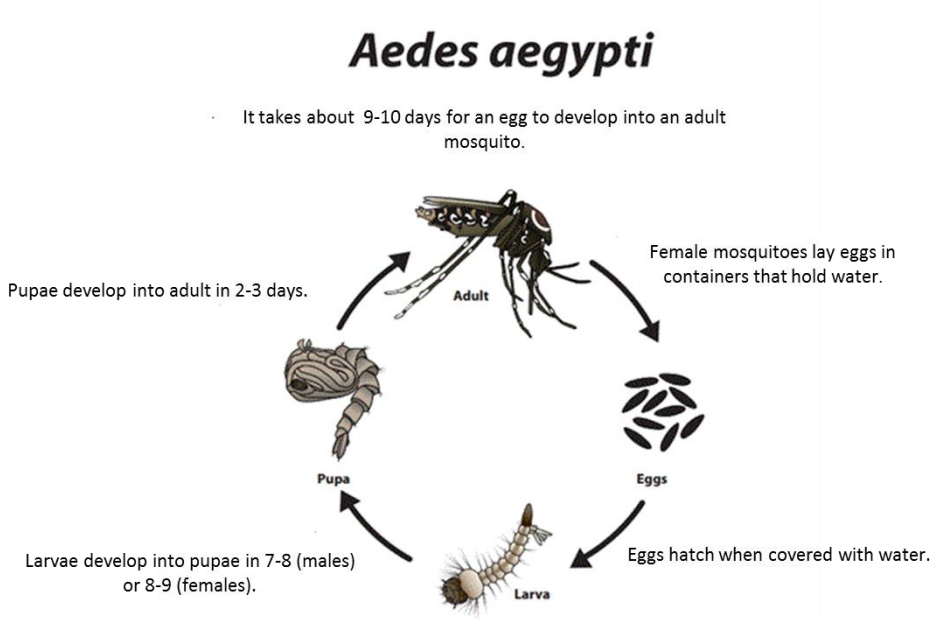


Figure 1.3 Life cycle of *Ae. aegypti* (Envis Centre On Climate Change And Public Health, 2016).

The larvae live at or beneath the surface of stagnant water, filter feeding on fungi, bacteria or algae (Clemons et al., 2010, Levi et al., 2014). The pupal stage is comma-shaped with a siphon located on the dorsal side of the head-thorax end **(Figure 1.5)**. Unlike other insects, mosquito pupae are active but do not feed. Males tend to develop faster than females and emerge before females (Elzinga, 1961). However, at higher temperatures, it has been reported females emerge earlier than males. At 33°C, the male to female emerging ratio was 0.9 and 0.79 at 35°C while at 24°C the M/F ratios was between (1.1–1.14) (Mohammed and Chadee, 2011, Farjana et al., 2012).

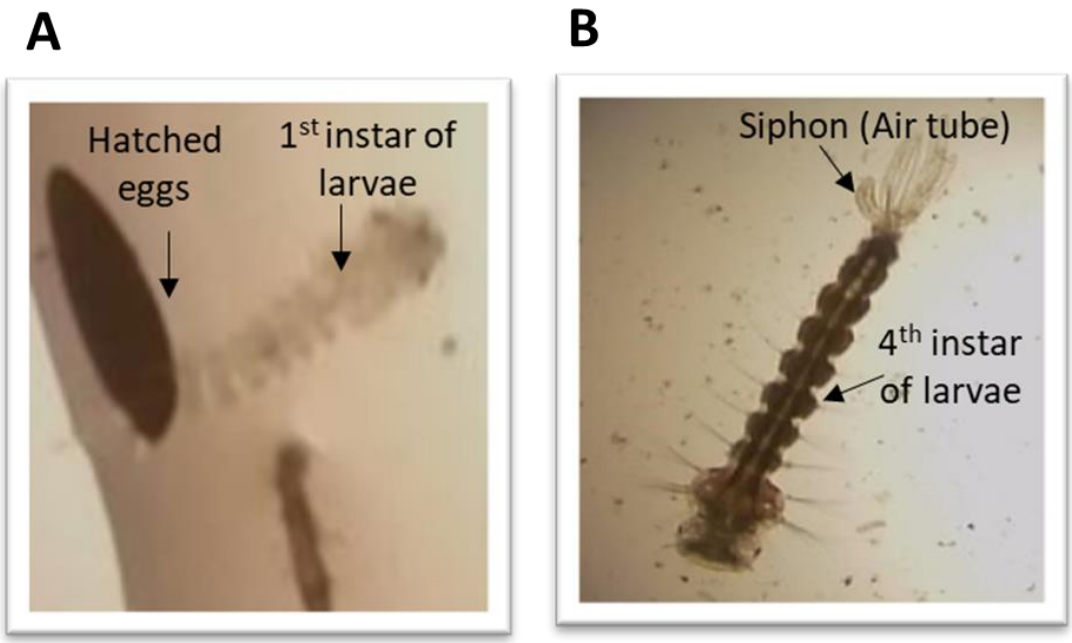


Figure 1.4 *Aedes aegypti* larval stages. A) First instar larva emerging from the eggshell, B) Fourth instar larva. Photo by Ashwaq Al Nazawi.

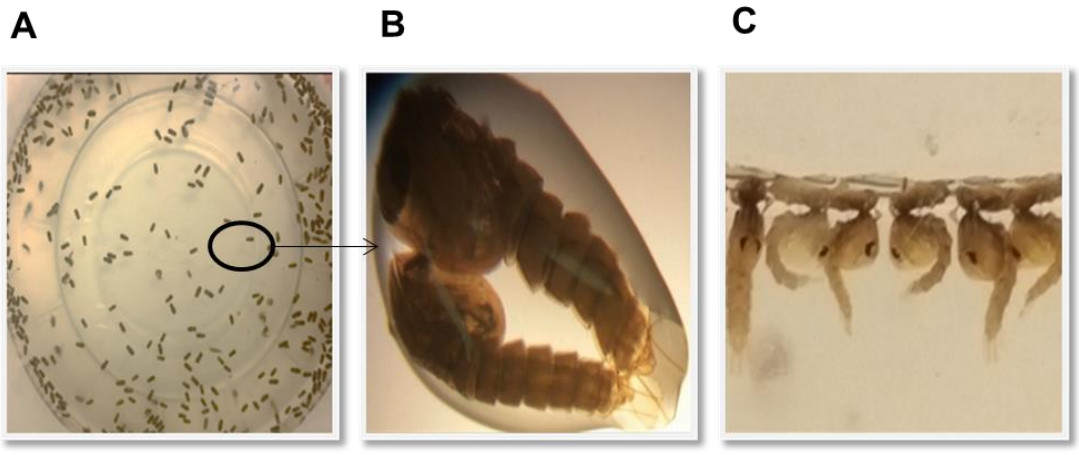


Figure 1.5 *Aedes aegypti* pupae. A) *Aedes aegypti* pupae in larval pot, B) Enlargement of two pupae in drop of water and, C) pupae at the water surface. Photo by Ashwaq Al Nazawi.

A similar study on both laboratory *Ae. aegypti and Ae. albopictus* strains found that males develop faster than females between 20–30°C, but at 35°C, the developmental growth in males was much slower (Farjana et al., 2012). The adult stage is terrestrial. Adult males are smaller than females (Briegel, 1990) and have a proboscis that is adapted to feed on plant juices or sugars (Nikbakhtzadeh et al., 2016). The males only feed on plant sugars while females feed on both plant sugars and blood.

1.7.2. *Aedes albopictus (Skuse)*

Aedes albopictus also known as the "Asian tiger mosquito" was first described by Skuse (1894) in India (Gatt et al., 2009). Both *Ae. aegypti* and *Ae. albopictus* have black and white markings on their bodies but can be distinguished easily by the patterns on the dorsal thorax **(Figure 1.6)**.

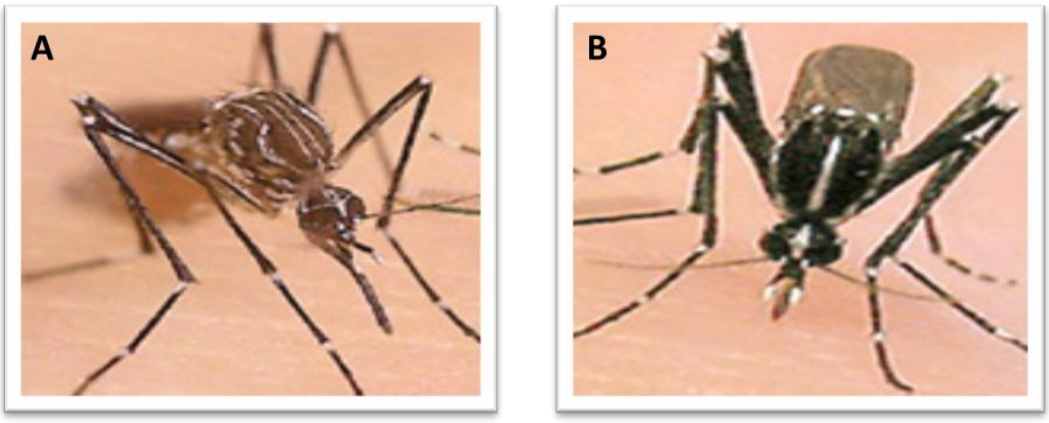


Figure 1.6 Images illustrating the morphological differences between *Aedes* species. A) *Ae. aegypti* with black scales and a silver-white lyre-shaped marking on its thorax. B) *Ae. albopictus* has a single white stripe on its thorax.
Source: https://www.cdc.gov/dengue/entomologyecology/

Although previously considered as a secondary vector owing to its more zoophilic behaviour (Sivan et al., 2015), it is an important arbovirus vector in Asia (Li et al., 2014) and has now spread to North and South America, Europe and Africa (Schaffner and Mathis, 2014) dispersed as eggs aided by the international trade of used tires (Armistead et al., 2008). The species originated in Asia and then began spreading to the western islands of the Indian Ocean and the eastern islands of Pacific Ocean. The spread occurred during the 19th century and early 20th century. In the 1980s, this species rapidly expanded across temperate regions, in Europe, the Americas and Africa (Manni et al., 2017). Climate change and increased global maritime transportation are some of the factors attributed to the species geographical

expansion (Roche et al., 2015). The expansion has raised public health concerns in the countries it has invaded owing to its vector competence for many arboviruses, notably chikungunya, dengue and Zika (Manni et al., 2017). In Western Europe, it facilitated local transmission of imported chikungunya cases that led to outbreak in Italy in 2007 with 200 cases confirmed, which then spread to France and Croatia (Manni et al., 2017, Roche et al., 2015).

Like *Ae. aegypti*, the immature stages can survive in artificial containers containing stagnant water such as discarded cans, buckets, tyres, flower pots, plates under potted plants and birdbaths. They can also be found in natural habitats such as tree holes and leaf axils. *Aedes albopictus* prefer rural areas and was believed to be restricted to vegetated areas and forests before it adapted to human environments (Rey and O'connell, 2014). The development times of the immature stages range between 24 days at 20°C and 12 days at 30°C while the average lifespan of adult stage mosquitoes is 3 weeks (Estrada-Franco and Craig, 1995). The adults rarely disperse more than 300m from the larval habitat they emerged from (Medeiros et al., 2017).

Aedes albopictus is an aggressive biter and daytime feeder. It feeds early in the morning and late afternoon, indoors and outdoors but mainly outdoors (Chaves et al., 2010, Ponlawat et al., 2005, Valerio et al., 2010). It is less anthropophagic than *Ae. aegypti*, and more opportunistic with a wider host range including humans and other mammals (Tandon and Ray, 2000, Hawley, 1988). A study assessing the feeding preference of *Aedes* in India found 88% of fed *Ae. aegypti* had obtained the blood from humans while only 49% of *Ae. albopictus* had fed on humans, with other preferred host being cows (18%), goats (10%), fowl (5%), pigs (4%), Rats (2%), and unknown sources (12.5%) (Sivan et al., 2015).

1.7.3. Behaviour of *Aedes* mosquitoes

1.7.3.1. Mating behaviour

Unlike most Anopheline species, *Ae. aegypti* males do not form large swarms but tend to aggregate and mate near their potential hosts in nature. The males recognise females by visual cues, female flight tones and contact pheromones (Cabrera and Jaffe, 2007, Nijhout and Craig, 1971).

Mating in female mosquitoes can begin immediately after emergence but females are rarely inseminated before 2-3 days, potentially allowing time for dispersal and

mixing in a population, and avoiding inbreeding (Hartberg, 1971). Male mosquitos upon emergence are also sexually immature until their genitalia rotate 180^{o} 15-24 h post-emergence (Gwadz and Craig Jr, 1968). Upon mating, sperm and a number of proteins are transferred from the male to female's bursa copulatrix and eventually stored in the spermathecae (Villarreal et al., 2018, Clements, 1963). These sperm will be used by the female to fertilise all eggs in her lifetime without further mating. Mating success in *Ae. aegypti* is impacted by factors such as age, body size, and swarm density (Ponlawat and Harrington, 2009, Alongkot and Laura, 2009). For example, older *Ae. aegypti* males (10 days old) transfer higher amounts of sperm to females compared to younger ones during mating, resulting in a competitive advantage (Ponlawat and Harrington, 2007).

1.7.3.2. Sugar-feeding behaviour

Sugar-feeding is an essential characteristic of mosquito life; they drink mostly nectar, but sometimes also fruits, honeydew or nectar (Spitzen and Takken, 2018). Nectar provides energy for flight, fertility and cell metabolism (Nayar and Sauerman Jr, 1975, Magnarelli, 1978, Stone and Foster, 2013, Foster, 1995, Manda et al., 2007). Deprivation of sugar sources affects the flight capacity and can consequently affect mosquito dispersal, mating success and/or host-finding (Spitzen and Takken, 2018). A study by Klowden et al., (1986) observed that only 33% of females that were sugar-deprived before blood feeding developed eggs compared to pre-sugar fed females before a blood meal where 90% developed eggs. He also observed higher host seeking responses post blood meal in the sugar deprived group compared to the group fed on sugar before a blood meal (Klowden, 1986).

Both males and females start sugar-feeding within hours of emergence (Foster, 1995). Most female mosquitoes take a sugar meal before they acquire their first blood meal (Hancock & Foster, 1993) because their sensory organs are initially insensitive to hosts (Haramis and Foster, 1990, Jahangir et al., 2008). In the absence of host stimuli, sugar-feeding is frequently observed in *Ae. aegypti* and *Ae. albopictus* under laboratory conditions. Sugar-feeding normally ceases temporarily if females are exposed to host stimuli, and also after blood-feeding in gravid stages when nutrients are obtained from the blood meal (Edman et al., 1992, Yee et al., 1992). In the field, it has been reported that the proportion of sugar-fed *Ae. aegypti* collected

at or near human residents was between 1-27% for females and 9-65% for males, but away from humans, the proportion of sugar-fed females increased to 74% (Scott and Takken, 2012).

1.7.3.3. Host-seeking behaviour

Host-seeking behaviour is defined as a sequence of behavioural events that progress from random flight, activation, orientation to host cues and landing on a host to obtain a blood meal (Bowen, 1991). Mosquitoes use many cues to locate and select their hosts for blood-feeding, including visual, odour and thermal cues (Takken and Knols, 1999). The cues mosquitoes respond to depend on their preferred host seeking time. As diurnal feeders, *Aedes* are more attracted to visual cues such as colour and movement, in contrast to nocturnal mosquitoes, which rely more on odour cues (Allan et al., 1987). Host-seeking behaviour commences with random dispersal flight. When the mosquito detects host stimuli, they orient their flight towards the source of the stimuli until they land on the host (Sutcliffe, 1987).

Host-seeking behaviour can be classified into three stages; long-range, middle-range and short-range activation events. Long-range orientation comprises reception and appraisal of olfactory and visual cues (Gibson and Torr, 1999). Olfactory receptors on the antennae, maxillary palpi and labellum respond to exhaled carbon dioxide and host odour, which is normally a cocktail of organic compounds such as lactic acid, and butanol (Kellogg, 1970, Kwon et al., 2006, Pitts and Zwiebel, 2006). Carbon dioxide exhaled by the host is a cue detected by mosquitoes and it triggers long range activation and attraction (Gillies, 1980, Smallegange et al., 2010). Visual cues at long range for day-feeders, such as *Ae. aegypti* and *Ae. albopictus,* include movement (Day, 2016, Obenauer et al., 2009), shape of the host and low intensity colours around the blue-green spectrum (Allan et al., 1987). Middle range orientation also relies on olfactory and visual cues, which at this stage facilitates host identification and recognition (Allan et al., 1987). Mosquitoes in short-range orientation utilise thermal cues including body heat and moisture to pinpoint and land on the host (Daykin et al., 1965). When fully fed, the females are not attracted to host stimuli until the eggs are fully developed and laid (Klowden and Lea, 1979). Oocyte-induced host seeking inhibition begins from the egg development until after oviposition. It has been proposed that ovaries release ecdysteroid hormone 6-12h post-blood feeding, which

stimulates the fat body to generate compounds that reduces sensitivity of the peripheral olfactory receptors to lactic acid (Bowen, 1991). The reduction in sensitivity to lactic acid, which is one of the odour cues that activate host seeking behaviour, explains why host-seeking behaviour is inhibited during egg development (Klowden, 1994).

1.7.3.4. Blood-feeding and oviposition behaviour

When a female *Aedes* mosquito lands on the host skin, it probes a few times to locate a blood capillary and punctures the skin to take a blood meal (Walker and Edman, 1985). Mosquito saliva contains anticoagulants transferred to the host's skin tissue to prevent blood clot formation, which could interrupt feeding. In a single feed, *Ae. aegypti* mosquitoes may ingest about 5 µl of blood (Klowden, 1990). *Aedes aegypti* females continue active blood-seeking and-feeding behaviours as long as the volume of blood in the abdomen is beneath the required amount (Klowden, 1994) - either from a single feeding or an accumulation of multiple feedings (Klowden, 1994). Once fully fed, *Ae. aegypti* females rest in a safe place for blood digestion and egg maturation (Klowden, 1990).

Egg development of mosquitoes takes 28-36h after bloodmeal digestion, but in *Ae. aegypti* it takes 48-72h after the completion of embryonic development (Day, 2016). Gravid *Ae. aegypti* normally lay small batches of eggs in multiple breeding sites, a phenomenon termed "skip oviposition" (Corbet and Chadee, 1993). It has been estimated, on average *Ae. aegypti* deposits 52.7 eggs per female while *Ae. albopictus* deposits 52.4 eggs per female per batch (Rey and O'connell, 2014). Many factors affect *Aedes* mosquito oviposition behaviour and oviposition site selection including humidity, diet, larval density and resource quantity and quality (Canyon et al., 1999, Fader and Juliano, 2014). For example, maintaining low humidity and providing highly concentrated sugar meals was reported to delay oviposition for 1-4 days in a laboratory *Ae. aegypti* strain from Cairns, Australia (Canyon et al., 1999).

Body size also influences *Ae. aegypti* oviposition behaviour whereby larger females store more energy enabling wider dispersion of eggs among habitats (Tsunoda et al., 2010). Experimental choice studies of oviposition sites in *Ae. aegypti* and *Ae. albopictus* have shown that whilst resource quantity and quality are the main influencing factors, larval densities also alter choice for oviposition site (Fader and

Juliano, 2014). Resource quality and quantity in a container corresponded to the level of detritus material in the habitat which the mosquitoes detect through odour cues. The type of detritus present in water can alter interspecific competition between *Ae. aegypti* and *Ae. albopictus* larvae, with generally greater competitive ability of *Ae. albopictus* when both species share a habitat, except with grass detritus (Murrell and Juliano, 2008, Fader and Juliano, 2014).

1.8. Dengue vector control

Vector control strategies have been promoted as the most sustainable and effective way of reducing transmission of arboviruses, malaria and other vector borne diseases (Achee et al., 2015, World Health Organization, 2018b). Methods for control include; environmental management, chemical control, biological control and genetic control (World Health Organization, 2018b, Alphey, 2014). Integration of various control strategies or interventions instead of using one method is necessary for effective control of vector borne diseases (Hemingway, 2018). The aims of vector control interventions are to provide individual, household and/or community protection by reducing human-mosquito contact and mosquito densities, thus interrupting transmission of viral infections (World Health Organization, 2018b).

1.8.1. Quantitative measures for assessing outcomes of vector control

The impact of control interventions on *Ae. aegypti* populations are mainly assessed by measures of immature indices, and less frequently by adult indices and disease outcomes (Bowman et al., 2014, Connor and Monroe, 1923, Breteau, 1954). Disease outcomes mostly involve testing for sero-positivity rate, which is the proportion of people who have antibodies against the disease of interest. The measure was used to assess the effectiveness of indoor residual spraying in controlling dengue transmission in South Taiwan and Queensland, Australia (Lien et al., 1994, Vazquez-Prokopec et al., 2010). The adult and immature indices are widely used to assess vector densities after implementation of control interventions. Examples of studies where they have been used are discussed in sections (1.8.4.2 and 1.8.4.3) and are reviewed in (Bowman et al., 2014, Alvarado-Castro et al., 2017). The commonly used indices include;

-House Index (HI): the percentage of houses infested with larvae and/or pupae.

-Container Index (CI): the percentage of water-holding containers infested with active immatures.

-Breteau Index (BI): the number of positive containers per 100 houses.

-Pupae per Person index (PI): the total number of pupae in water containers in a house divided by number of inhabitants.

-Ovitrap index (OI): the number of ovitraps positive for egg laying.

-Indoor resting densities: the total number of mosquitoes collected in a house for standard period of time divided by the total number of collection days or houses.

-Abundance total: number of adults or immature mosquitoes collected per site.

1.8.2. Environmental control

Environmental management can be divided in three definitions: modification, manipulation and altering human habitation (World Health Organization, 2018c). Modification is any long-term physical transformation aimed at reducing or preventing formation of larval habitats such as installation of drainage water supply, filling, land levelling and transformation of impoundment margins (World Health Organization, 2018c, World Health Organization, 1982).

Manipulation involves altering the habitats of immature *Ae. aegypti* vectors such as continual emptying and cleaning of water-storage containers, discarding or recycling containers, covering water storage containers, cleaning of gutters and sheltering stored tyres from rainfall. Altering human habitation involves modification to homes such as installing eaves and door and window screens, which reduces the human-vector contact (World Health Organization, 2018c, World Health Organization, 1982, Sommerfeld and Kroeger, 2015). The effectiveness of environmental control on reduction of *Aedes sp.* populations has been demonstrated in several studies. For example, in Indonesia, covering water pots, emptying, cleaning and refilling water pots and recycling or disposing containers resulted in a reduction in container index by 2.71% and Breteau index by 80% six months post implementation of the program (Suroso, 1990). In Cuba, educating the community on the importance of eliminating breeding sites through proper disposal of containers, covering tanks and cleaning public and inhabited areas was associated with a reduction in house index from 3.72% to 0.6% after a year of implementing the program, but no change in house index was

recorded in control sites where no communication and social mobilisation on eliminating breeding sites was implemented (Sanchez et al., 2005).

As an alternative to insecticide-based methods, this control method should be highly considered especially in areas where high levels of insecticide resistance in the local population has been observed. But as demonstrated in the study conducted in Cuba, for environmental control to be successful, it should involve regular public educational campaigns through the media on the importance of environmental management as a tool for control of dengue disease (Sanchez et al., 2005).

1.8.3. Biological control

Biological control is the use of natural enemies such as predators, parasites or pathogens to decrease a target species population (Woodring and Davidson, 1996, Rozendaal, 1997). For instance, *Bacillus sphaericus (Bs)* and *Bacillus thuringiensis israelensis (Bti*) produce toxins capable of killing mosquito larvae (Boyce et al., 2013) and have been used widely for mosquito larval control (Lima et al., 2005). *Toxorhynchites spp*. actively feed on larvae of other mosquitoes and thus have been exploited as tools of *Aedes* control. In the Caribbean, the introduction of first instar *Toxorhynchites moctezuma* into potential *Ae. aegypti* oviposition sites resulted in a 80% reduction of females collected indoors compared to the number collected before introduction (Rawlins et al., 1991). Although these results were promising, the effectiveness of *Toxorhynchites* to control *Aedes* depends on mode of introduction, periodicity and dependence on the correspondence of preferred oviposition sites for the control and target species (Collins and Blackwell, 2000). In Fiji, where this control method has been widely tested, introduction of *Toxorhynchites splendens* and *Tx. inornatus* did not reduce the densities of *Ae. polynesiensis,* which the authors suggested was due to differences in breeding sites of the predator and target *Aedes* species (Toohey et al., 1985). However, when adult *Tx. amboinensis* were released in the same area, this was far more effective, with a reduction in *Aedes* species breeding in containers and tyres over a 10 month period of 61.5% and 90%, respectively. This greater effectiveness of *Aedes* control was suggested to be linked to breeding site preferences of *Tx. amboinensis* (Toohey et al., 1985).

Another predator of mosquito larvae is the cycloid copepod of the genus *Mesocyclops*, which was evaluated in Vietnam for its ability to control *Aedes*. In areas

where it was used for vector control in conjunction with clearing containers where the *Mesocyclops* could not be introduced, complete or near complete eradication of *Ae. aegypti* and *Ae. albopictus* was achieved (Kay et al., 2002, Vu et al., 1998). During the same period, no cases of dengue were reported in those regions (Kay and Nam, 2005). Larvivorous fish have also been used for control of *Aedes*. Introduction of *Oreochromis mossambicus* in water storage tanks in Jaffna, Sri Lanka eliminated *Aedes* larvae within a month (Surendran et al., 2008). Introduction of *Betta splendens* in tanks in Brazil reduced *Ae. aegypti* larval densities in tanks from 70.4% to 0.2% (Pamplona et al., 2004).

Apart from predators, mosquito pathogens such as entomopathogenic fungi have also been evaluated for their effectiveness in *Aedes* control. Laboratory and semi-field experiments have demonstrated that entomopathogenic fungi such as *Metarhizium anisopliae* and *Beaveria bassiana* are pathogenic to *Aedes* species, with some strains being able to achieve LT_{50} in three days (Luz et al., 2007, Darbro et al., 2011, De Paula et al., 2008, Scholte et al., 2007).

To date, the main challenge has been developing techniques to deploy the tool in field conditions but several methods developed recently like the "PET trap", a fungus-impregnated black cloths covered in adhesive film that attracts *Aedes sp.* may facilitate deployment of these pathogenic fungi in mosquito control (Silva et al., 2018).

1.8.4. Chemical control

1.8.4.1. Classification of Insecticides

In vector control programmes, the four main classes of insecticides include organochlorines, organophosphates, carbamates and pyrethroids. These insecticides are classified based on their chemical composition and their modes of action (Nauen, 2007).

1.8.4.1.1. Organochlorines

This family is comprised of dichlorodiphenyltrichloroethane(DDT); hexachlorocyclohexane (HCH); cyclodienes such as aldrin, dieldrin, endrin; toxaphene; mirex and chlordecone (Sparling, 2016). Organochlorine pesticides were used extensively in the past in agriculture and mosquito control until they were generally banned from use due to their long term residual toxic effect on all insects,

and bioaccumulation in food chains (Heckel, 2012, Rozendaal, 1997). Currently only DDT remains in (limited) use for mosquito control mainly against *Anopheles*. DDT targets the voltage gated sodium channel, while most organochlorines target gamma-aminobutyric acid receptors.

1.8.4.1.2. Carbamates

Carbamates inhibit acetylcholinesterase, the enzyme which breaks down the neurotransmitter acetylcholine in nerve synapses. This causes accumulation of acetylcholine at the receptors and produces repetitive impulses leading to paralysis owing to energy exhaustion (Fukuto, 1990). Carbamates in general, degrade rapidly in the environment, and have relatively low mammalian toxicity but are highly toxic to many insects including pollinators (Nauen, 2007). Currently, propoxur and bendiocarb are the only insecticides within the class approved by the WHO for use in public health (Nauen, 2007). This class of insecticide is mainly used for indoor residual spraying in malaria control. However, indoor feeding and resting is also observed in *Ae. aegypti*, thus, carbamates may be useful for IRS against *Aedes* populations, with success in a recent experimental trial in Mexico (Vazquez-Prokopec et al., 2017).

1.8.4.1.3. Organophosphates

Organophosphates (OP) share the same mode of action as carbamates but inhibit acetylcholinesterase through phosphorylation rather than carbamylation (Becker et al., 2003). In *Aedes* control programmes, the most widely used organophosphates are malathion and fenitrothion for space spraying, and temephos as a larvicide (Zaim et al., 2007, Viana-Medeiros et al., 2017). Like carbamates this class is chemically unstable and non-persistent in the environment, but OPs are generally more toxic to mammals (World Health Organization, 2006).

1.8.4.1.4. Pyrethroids

Like DDT, pyrethroids target the voltage-gated sodium channel (VGSC) in the nervous system where they bind causing a prolonged opening of the channel thus allowing an influx of sodium ions. This leads to hyperpolarisation of the membrane, repeated nerve firing and insect knockdown due to muscle overstimulation and paralysis (Du et al., 2016a). Pyrethroids can be divided into two main groups (Type I and II) based on their structure. Type I pyrethroids such as allethrin, bifenthrin, d-phenothrin and permethrin differ from type II compounds, such as cyhalothrin, cypermethrin,

cyfluthrin, deltamethrin and lambda-cyhalothrin by the presence of an alpha-cyano moiety in the latter, which typically increases toxicity (Miller and Adams 1982, Palmquist et al., 2012).

Pyrethroids account for around one third of currently used insecticides for dengue vector control (Martin-Reina et al., 2017). For dengue vectors, pyrethroids are used in thermal fogging or ultra-low volume sprays (ULV) and insecticide treated materials such as curtains, window screens and eaves. Pyrethroids remain the primary compound for insecticide treated nets, though combination nets are becoming increasingly important for malaria control (Protopopoff et al., 2018), though their utility for dengue control remains unclear.

1.8.4.2. Adulticides used for *Aedes* control

Adult *Aedes* are mainy targeted through space spraying methods such as thermal fogging and ultra-low volume sprays, delivered outside or inside the home (Aponte et al., 2013). Space spraying involves spreading droplets of insecticide in the air, which are intended to spread widely but not persist, and is used especially during arbovirus outbreaks. Space spraying has two forms; thermal fogging and ultra-low volume (ULV) aerosols also known as cold fogs. In thermal fog, the insecticides are mixed with oil and vaporised through injection into a high-velocity stream of hot gas. ULV spraying creates aerosols using large volumes of air at low pressures to transform concentrated insecticides into extremely small droplets (1-150μm) that are dispersed into the atmosphere. Space spraying can be applied using vehicle-mounted generators, helicopters or fixed-wing aircraft, and in small areas using portable backpack equipment (World Health Organization, 1997). Space spraying are mainly used during outbreaks to slowdown spread of dengue fever (Esu et al., 2010). Although, space spraying is recommended by WHO for control of dengue during outbreaks, in the past 35 years, no randomised control trials have been conducted to evaluate the effectiveness of space spraying or fogging on dengue control (Bowman et al., 2016). However, space spraying through outdoor fogging could reduce dengue cases if applied early in transmission season as shown in Peru (Stoddard et al., 2014). In Honduras, combined ULV and thermal fogging using lambda-cyhalothrin led to a significant decline in *Ae. aegypti* densities with fewer adults being collected in blocks of houses where insecticdes were applied compared to control blocks after a month

of treatment (Perich et al., 2001). Combined application of lambda-cyhalothrin by ULV and thermal fogging in Puntarenas, Costa Rica also led to a significant reduction of *Ae. aegypti* densities in houses that received ULV or thermal fogging on the front door or each room after 7 weeks compared to untreated houses (Perich et al., 2003). A similar outcome was reported in Mexico, where ULV application of permethrin combined with piperonyl butoxide (PBO), an inhibitor of cytochrome P450s and other metabolic genes reduced the incidence of dengue to 14.4% compared to 30.2% in control sites (Mendoza-Cano et al., 2017). In some areas, space spraying has been notably less effective in reducing *Ae. aegypti* populations (Gubler and Clark, 1996). In Trinidad, there was no difference in the number of *Ae. aegypti* eggs that were collected in areas where malathion was applied by ULV compared to control sites; a failure attributed to lack of access to people's housing compounds for spraying (Dave, 1985).

The use of insecticide treated materials (ITMs) such as insecticide-treated nets (ITNs), insecticide-treated window screens or curtains and water storage jar covers has also been found effective in controlling *Ae. aegypti* (Kroeger et al., 2006, Lenhart et al., 2008). The effectiveness of ITNs was evaluated in a cluster-randomised trial in Haiti where one-month post-distribution of ITNs, a significant reduction in three larval and a pupal index (BI, CI, HI, PPI) and oviposition positivity was observed in bednet clusters compared to baseline and control indices. Evaluation at 5 months and 12 months post-net distribution revealed a positive spill-over of bednet effect on *Aedes* population in houses (without ITNs) 50-100 meters away. A significant reduction in dengue sero-prevalence in bednet clusters was also observed (Lenhart et al., 2008). In a study in Trujillo, Venezuela, the Breteau Index (BI) in sites that received curtains and water jar covers reduced by 55%. This reduction in *Ae. aegypti* infestation was significantly associated with curtain coverage but not water covers (Vanlerberghe et al., 2011). DuraNet window and door screens were reported in Acapulco, Mexico to have significantly reduced infestations of *Aedes* assessed by adult and pupal-based vector indices in intervention sites compared to control clusters at 5 and 12 months after installation (Che-Mendoza et al., 2015).

In some studies, ITMs were not effective in controlling *Aedes* populations. In a cluster randomised community trial in Poptun, Guatemala, water covers, windows and door

screens made from PermaNet 2.0 nets did not significantly reduce the total number of pupae, HI, BI and PPI after 6 weeks in the intervention arm compared to the control arm. The authors argued the lack of significant reduction of *Aedes* infestation could be attributed to heavy tropical rain that followed shortly after the trial began, which increased outdoor breeding sites resulting in high densities that could not be effectively controlled by the ITMs. On their second intervention trial, which combined larval source management and ITMs, a significant reduction in the total number of pupae and HI was observed in the intervention arm but PPI and BI differences were marginal. Therefore, it appears that ITMs alone were not effective in controlling the population, which was not tested for pyrethroid resistance. This could be a key factor explaining the lower efficacies compared to those reported in other studies (Rizzo et al., 2012). The impact of resistance on effectiveness of ITMs was demonstrated in a study conducted in Mexico. In urban centres of the Yucatan region in Mexico where *Ae. aegypti* populations are highly resistant to pyrethroid insecticides, treated curtains failed to control the population but in the rural area where resistance was lower, a reduction in indoor adult abundance and dengue-infected mosquitoes was observed (Loroño-Pino et al., 2018).

Indoor residual spraying (IRS) is widely used in malaria control to target indoor resting mosquito species (World Health Organization, 2013b, Hemingway et al., 2013). The effectiveness of IRS in interrupting dengue transmission during outbreaks as an emergency control measure for dengue control was first investigated in Southern Taiwan where over 50,000 houses were sprayed with alphacypermethrin between 1991-1992. The BI fell from over 35 before the spraying to under 5 after spraying. The cases of dengue also reduced from more than 3000 to less than 1000 (Lien et al., 1994). Indoor residual spraying was also demonstrated to significantly reduce dengue during outbreaks in North Queensland, Australia where fewer infections were reported in houses sprayed with lambda-cyhalothrin compared to unsprayed houses (Vazquez-Prokopec et al., 2010). In the Peruvian Amazon, IRS with deltamethrin significantly reduced adult index, BI, CI and HI in sprayed houses compared to control and baseline measurements (Paredes-Esquivel et al., 2016). Although, IRS has proved to be effective in controlling *Aedes* and dengue transmission, pyrethroid resistance

in *Ae. aegypti* in Mexico led to failed IRS, when compared to carbamate IRS, to which the population was susceptible (Vazquez-Prokopec et al., 2017, Wilson et al., 2014).

1.8.4.3. Larvicides

The organophosphate, temephos and various insect growth regulating hormones such as diflubenzuron, methoprene, triflumuron, pyriproxyfen and methoprene are the most commonly used insecticides for larval control (Zaim et al., 2007). Temephos is mainly applied in breeding sites that cannot be eliminated such as large water storage containers for construction sites, industrial use and domestic use such as laundry and washrooms. Temephos is inexpensive and of low toxicity to humans and can be used safely to treat drinking water although acceptance levels are often low (World Health Organization, 2009b) and has been widely used in Latin America and Asia (Thongwat and Bunchu, 2015, Del Rio-Galvan et al., 2016, George et al., 2015, Mohiddin et al., 2016).

Insect Growth Regulators were first used in insect control in 1956 when juvenile hormone (JH) was isolated from the moth *Hyalophora cecropia*. Synthetic Juvenile Hormone Analogues (JHA) affect insects by inhibiting metamorphosis of the juvenile stage to adult stage (Tunaz and Uygun, 2004, Maoz et al., 2017). They have been used for control of larval populations in different countries in the world. In a field trial in Colombia, monthly application of pyriproxyfen from February to August 2009 to larval habitats significantly reduced their positivity rate for immature stages of *Aedes* (Ocampo et al., 2014). In laboratory studies, 100% inhibition of emergence of adults in larvae collected in the region was observed thus confirming the reduction in immature positivity could be attributed to application of pyriproxyfen (Ocampo et al., 2014). In Martinique, pyriproxyfen alone was active in reducing adult emergence for five months while spinosad remained active for three months. When combined, pyriproxyfen and spinosad diminished the rate of emerging adults to 20% for more than four months post-application but remained active for up to 8 months (Darriet et al., 2010). Instead of adding pyriproxyfen directly to the breeding sites, Caputo et al., (2012) employed an auto dissemination technique, whereby, pyriproxyfen powder was applied to dissemination sites targeting resting *Ae. albopictus,* which later contaminated sentinel breeding sites with the compound bound to their bodies while ovipositioning. The authors observed 50-70% pupal mortality in sentinel sites

compared to less than 2% in control sites that were covered to prevent introduction of pyriproxyfen in the breeding sites by oviposition seeking contaminated females (Caputo et al., 2012). In an earlier study employing the same technique using pyriproxyfen in Iquitos, Peru, 49 to 84% mortality of the juvenile *Ae. aegypti* stages was observed in contaminated sites compared to 7 to 8% in control sites (Devine et al., 2009).

1.8.5. Release of modified mosquitoes for vector control

This control strategy aims to suppress or replace populations through release of modified mosquitoes. Modifications aimed at population suppression include the sterile insect technique (SIT), release of insect carrying a dominant lethal gene (RIDL) and *Wolbachia*-induced cytoplasmic incompatibility. The SIT involves release of very large numbers of males sterilised by radiation or chemicals (Oliva et al., 2014). If a female mates with a sterile male, then all her egg clutches will be inviable, and if enough sterilised males mate successfully, the population will decline (de Valdez et al 2011, Strugarek et al 2018). The SIT has been used successfully to control agricultural pests such as the Mediterranean fruit fly *Ceratitis capitata,* which was stopped from spreading from Central America to Southern Mexico (Hendrichs et al., 1995), to eradicate *Cochliomyia hominivorax* (sheep blowfly) in the Americas (Vargas-Terán et al., 2005) and to locally eradicate *Glossina austeni* in Zanzibar in 1997 (Vreysen et al., 2000). The release of irradiated males in Italy between 2005 and 2009 was reported to have been effective in suppressing *Ae. albopictus* population in the release sites (Bellini et al., 2013). Adult density was estimated by weekly monitoring egg density found in ovitraps while induced sterility was estimated by measuring the hatching percentage of weekly collected eggs in SIT and control areas. Results showed that sterile males released at the rate of 896-1,590 males/ha/wk induced a significant sterility level in the local population and reduction of eggs densities in the ovitraps (Bellini et al., 2013).

Although the SIT strategy is effective in suppressing target populations, it requires periodic release of a very large number of males, which makes it very expensive and probably unsustainable in developing countries. Another challenge results from the effect of the sterilising technique on male fitness. In areas with high densities of wild

males, the irradiated males are likely to be outcompeted. Immigration of fertilised females from non-control is also a major challenge for this technique (Alphey, 2014)

Release of insects carrying a dominant lethal gene is designed to suppress target populations based on transgenes carried by the males that are lethal in offspring (Alphey, 2014). During rearing, colonies are maintained on a diet supplemented with the antibiotic tetracycline to repress expression of the lethal gene, thus the population can be increased in captivity without the deleterious effect of the gene manifesting in the progeny. However, when the males carrying the gene are released into the field where the repressor is absent at sufficient concentrations, the progeny of females that mate with the engineered males will not survive to reproductive age (Bouyer and Marois, 2018). The RIDL technique was initially developed specifically for *Ae. aegypti*, with a first field trial in the Cayman Islands demonstrating that RIDL males (OX513A strain) can effectively compete for mating and suppress a wild population (Harris et al., 2011). In addition, a study in Malaysia showed RIDL OX513A males had similar longevity to their unmodified counterparts suggesting good competitive ability (Lacroix et al., 2012, Miller, 2011, Shelly and Mcinnis, 2011). As with SIT, RIDL faces the challenge of the need for periodic release of large numbers of males for it to be effective. The technique relies on sexing the mosquitoes based on size, males considered to be smaller than females. The probability of incorrect sexing maybe small but with released mosquitoes averaging millions, the number of introduced females in the control site will be considerable over several releases (Shelly and Mcinnis, 2011, Slade and Morrison, 2014, Miller, 2011).

An alternative to using irradiated or chemically-sterilised males, or genetically-modified males, is to release of males infected with *Wolbachia,* an endosymbiont that induces cytoplasmic incompatibility when infected males mate with uninfected females resulting in unviable eggs (Strugarek et al., 2018). This method overcomes the damaging effect of irradiation on males but still requires periodic release of massive number of males to be effective (Ritchie et al., 2018). This technique has been effective in suppressing *Ae. polynesiensis* in French Polynesia and *Ae. aegypti* in Australia (Coffey 2011, Iturbe-Ormaetxe et al 2011).

Apart from suppressing populations through cytoplasmic incompatibility, some *Wolbachia* strains can confer parasite or pathogen resistance to the host, which is

being exploited in population replacement programmes (Iturbe Ormaetxe et al., 2011). In population replacement strategy, infected females or both males and females are released in the target area for several rounds until the desired frequency of the infected population is achieved. Females carrying *Wolbachia* will contribute more offspring in the target population since their eggs are viable when they mate either with infected or uninfected males (Strugarek et al., 2018). Uninfected females will have viable eggs only when they mate with uninfected males. The reproductive advantage of infected females thus allows the bacteria to spread rapidly in the target population (Jiggins, 2017). For example, in Australia, after 3 months of releasing 6000 infected male *Ae. aegypti* per week, approximately 20% of the mosquito population was already infected with the *Wolbachia* bacterium (Coffey, 2011, Iturbe Ormaetxe et al., 2011).

The latest population replacement method is gene drive technology, which like *Wolbachia*-based population replacement, has the potential to invade the target population from release of relatively few individuals over a limited timescale, which depend on super-Mendelian inheritance of a homing endonuclease gene (HEGs) (Esvelt et al., 2014). The most promising method uses the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, which is being developed to spread anti-*Plasmodium* or antiviral genes depending on target species. In *Ae. aegypti*, CRISPR-CAS9 technology for this purpose is still in its early stages of development. The most promising outcomes of the current research includes the optimised site-specific introduction of mutations (Kistler et al., 2015) that can be aimed at disrupting arbovirus receptors to increase pathogen refractoriness in target populations and virus-inducible CRISPR-CAS9 systems which cleaves virus genomes thus an antiviral mechanism to be exploited in population replacement (Savidis et al., 2016, Dong et al., 2016).

1.9. Insecticide resistance in mosquitoes

Insecticide resistance in mosquitoes is a global concern that threatens the ability to control disease vectors. Resistance to insecticides as described by the World Health Organisation (WHO), is the capacity of an insect species to tolerate doses of a toxicant that have been proved lethal to most individuals in a normal population of the same

species (World Health Organization, 2013c). The Insecticide Resistance Action Committee (IRAC) use a different definition to capture operational failure resulting from resistance. They define insecticide resistance as a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species (IRAC, 2018).

1.10. Mechanisms of insecticide resistance in mosquitoes

The four main mechanisms that cause resistance to insecticides in insects include target-site insensitivity, metabolic resistance, behavioural avoidance and reduced cuticle penetration (Singh et al., 2013, Chareonviriyaphap et al., 2013)

1.10.1. Behavioural Avoidance

Behavioural resistance has been defined as any modification to mosquito behaviour that facilitates avoidance or contact with insecticide-treated surfaces or limit the period of this contact (Sokhna et al., 2013). However, Zalucki and Furlong (2017), proposed a more rigid definition which emphasises genetic changes in behaviour to be considered as the resistance mechanism, to fit the IRAC definition of insecticide resistance. The changes should be heritable and associated with repeated failure to achieve targeted control levels. According to them, most of the reported cases of behavioural resistance could be explained by aversion driven by learning, avoidance or the repellent effect of insecticides (Zalucki and Furlong, 2017).

Most of the available evidence on behaviour change is based on before and after studies (Gatton et al., 2013). The commonly reported behaviour changes include changes in feeding times and location, resting location and host choice (Pates and Curtis, 2005). A shift in feeding time in *An. gambiae* from late in the night to early in the morning and evening has been suggested to be driven by increased bed net use which limit feeding opportunities on its normal feeding time (Wamae et al., 2015). In the same species, a switch to outdoor feeding has been reported despite formally being highly endophagic (Reddy et al., 2011). Outdoor feeding and a switch to feeding during the day, early in the evenings and in the morning in *An. funestus,* which primarily feed indoors late in the night, has also been reported after introduction of insecticide treated bednets in Senegal and Tanzania (Sougoufara et al., 2014, Russell

et al., 2011). These examples of a switch in feeding behaviour in response to introduction of bed nets are most likely due to opportunistic feeding or behavioural plasticity (Thomsen et al., 2016, Gatton et al., 2013). Behavioural changes in response to interventions have not been reported in *Ae. aegypti* but deviations from the expected behaviour when mosquitoes encounter contact irritants and spatial repellents have been reported and are discussed in section 1.10.1.1

1.10.1.1 Effect of insecticide exposure on mosquito behaviour

Insecticides combine different properties such as toxicity, irritation and excito-repellency, which in addition to selecting for physiological resistance, can have an impact on vector behaviour (Gatton et al., 2013). When insects encounter insecticides, their response to the insecticide can be stimulus-dependent or stimulus-independent. Stimulus-dependent responses such as contact irritancy occur when an insect leaves an insecticide-treated surface after physical contact with the pesticide, whereas stimulus-independent response (such as spatial repellency) are when the insect actively avoids the insecticide treated surfaces without contact. Contact irritancy on exposure to pyrethroids has been reported in many studies of different mosquito species (Chareonviriyaphap et al., 2013).

In *Ae. aegypti*, contact irritancy to alphacypermethrin, bifenthrin, deltamethrin, lambda-cyhalothrin, cyphenothrin, D-tetramethrin, and tetramethrin has been demonstrated in experimental studies (Thanispong et al., 2009, Mongkalangoon et al., 2009, Cooperband and Allan, 2009). In one study, contact irritancy was recorded as the difference in landing frequencies and the duration *Ae. aegypti* rested on the treated surface compared to an untreated surface, with less resting and landings observed on the treated surface (Cooperband and Allan, 2009). A similar effect has been reported in *Anopheles spp*. in the laboratory (Hughes, 2018) and in a semi-field setting (Parker et al., 2015) when they encounter insecticide treated bed nets while host seeking. In the semi-field setting, the researchers observed rapid bouncing on insecticide treated bed nets, with mosquitoes on average spending less time contacting the treated nets compared to untreated bed nets (Parker et al., 2015).

DDT, essential oils and some pyrethroids such as transfluthrin and cyphenothrin have been demonstrated to have spatial repellent effects on mosquitoes (Thanispong et al., 2009, Lee, 2007). Mosquitoes actively avoid contact with the surface sprayed with

the compound or when they encounter the volatiles in air (Achee et al., 2012). In a laboratory set-up, spatial repellency of DDT and alphacypermethrin to *Ae. aegypti* was investigated by introducing field and lab strains into an excito-repellency test box containing either of the insecticides (Thanispong et al., 2009). The lab strain was susceptible to DDT, one field strain from Chiang Mai (CM) Province, northern Thailand had reduced susceptibility while the other field strain from Kanchanaburi (KAN) Province, western Thailand was highly resistant to DDT (Thanispong et al., 2009). In all strains, higher escape responses were observed to DDT than alphacypermethrin in the non-contact trials, but an equal level of escape responses was observed to both insecticides in contact trials. From these findings the authors concluded DDT has both spatial and contact irritancy effect on *Ae. aegypti* while alphacypermethrin only had a strong contact irritancy effect with minimal repellence. Apart from the responses induced by contact irritancy and spatial repellence effect of insecticides, exposure to sub-lethal insecticide concentrations has been associated with modification of host seeking behaviour such as activation and orientation (Cooperband and Allan, 2009).

In a wind tunnel assay, flight patterns corresponding to activation of host seeking and orientation to host odours were investigated in *Ae. aegypti*, *Culex quinquefasciatus* and *Anopheles quadrimaculatus* before and 24h after exposure to sublethal (LD_{25}) concentrations of either permethrin or deltamethrin (Cooperband and Allan, 2009). Sub lethal exposures to both pyrethroids significantly reduced host seeking responses in *Ae. aegypti* and *Cx. quinquefasciatus,* but had no effect on *An. quadrimaculatus* which also failed to exhibit a significant response to carbon dioxide, 1-octen-3-ol and 1-hexen-3-ol (attractants). This study demonstrated insecticides even at sub-lethal doses may still be protective by interfering with host seeking behaviour of some species. However, the authors did not investigate how long it took for the species to recover from the alteration in host seeking behaviour. They also failed to investigate whether a similar effect would be extended to pyrethroid resistant strains.

The long-term impact of sublethal exposure of pyrethroids on behaviour beyond 24h was investigated by Bibbs et al., (2018). They exposed *Ae. aegypti* and *Ae. albopictus* to LC_{10}, LC_{20} and LC_{30} sublethal concentrations of the spatial repellent, transfluthrin. At all concentrations, a significant reduction in skip oviposition behaviour was

observed in both strains compared to unexposed controls. Apart from oviposition behaviour, fecundity was also significantly reduced post exposure. As with the study on the sub-lethal effect of permethrin and deltamethrin on host seeking, they failed to demonstrate the effect of spatial repellents on resistant population (Bibbs et al., 2018).

As mentioned at the end of section 1.10.1, deviation from typical behaviour when mosquitoes encounter contact irritants and spatial repellents has been shown in *Ae. aegypti* and other mosquitoes. For example, insensitivity to DEET, a spatial repellent which interferes with host odour recognition and elicits avoidance behaviour has been linked to genetic polymorphism in odour receptor genes in *Ae. aegypti* (Pellegrino et al., 2011). Strains carrying the polymorphism no longer responded to DEET and thus this was the first insecticide-related behaviour change in mosquitoes confirmed to have a genetic basis (Stanczyk et al., 2010). Reduction in contact irritancy effect on mosquitoes can also be inferred from reports of extended resting on insecticide treated surfaces. For instance, in Kenya, pyrethroid resistant *An. gambiae* were observed resting on pyrethroid treated nets (Ochomo et al., 2013) despite the widely reported contact irritancy effect of permethrin and deltamethrin on *Anopheles* and other species (Thanispong et al., 2009, Mongkalangoon et al., 2009, Cooperband and Allan, 2009, Hughes, 2018).

The reduction in contact irritancy and sensitivity to spatial repellents thus indicates that resistance, or at least non-genetic behavioural plasticity (resilience) may modify behaviours normally elicited when mosquitoes are exposed to insecticides (Govella et al., 2013). The impact of behavioural change in vector control is an important area that should be evaluated alongside studies characterising physiological resistance in a population (Gatton et al., 2013).

1.10.2. Reduced cuticular penetration resistance

The cuticle (exoskeleton) is the outer part of a mosquito's body. It provides protection from desiccation, penetration of external compounds and sensory perception of the environment (Balabanidou et al., 2018). The cuticle structure consists of different layers **(Figure 1.7)**. Epicuticle is the outermost layer that is covered by a film of wax and cement. It is made up of hydrocarbons, proteins and lipids. Procuticle is located underneath the epicuticle and is comprised of chitin fibres and proteins. It can be

partitioned into the exo-cuticle which is the upper harder part and the endo-cuticle which is the lower softer part. A layer of epidermal cells that secrete the cuticular components lies at the base of the cuticle (Balabanidou et al., 2018) **(Figure 1.7)**.

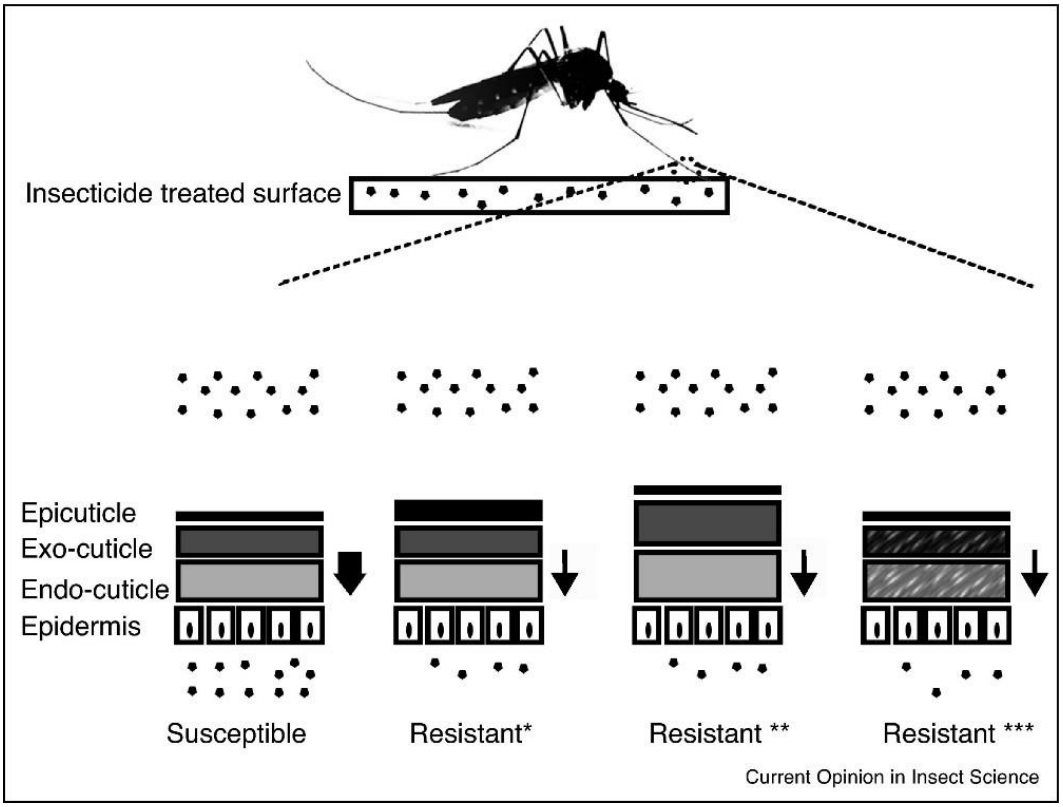


Figure 1.7 Cuticular modification associated with reduced insecticide uptake in mosquitoes. *Resistant thickening of the epicuticle, **Resistant thickening of the procuticle, ***Resistant altered cuticle composition (Balabanidou et al., 2018).

Cuticular resistance refers to alterations to the cuticle of insects or their digestive tract linings that inhibit the penetration of insecticide molecules within insects' bodies. This delay allows detoxification mechanisms to reduce the quantity of insecticide reaching the target site. Reduced cuticular penetration mechanism can affect a broad range of insecticides (McCaffery and Nauen, 2006). There are two mechanisms of penetration resistance that have been characterised: cuticle thickening and changes in cuticle composition.

The evidence for cuticle resistance is limited in mosquitoes (Balabanidou et al., 2016, Yahouédo et al., 2017, Wood et al., 2010, Fang et al., 2015). The first report of cuticular resistance in mosquitoes was in *Culex* species (Stone and Brown, 1969, Apperson and Georghiou, 1975). Since then, it has been reported in different species across the world. For example, in India and Pakistan, an altered hydrocarbon

structure in the cuticular lipids was detected in *An. stephensi* resistant to DDT and malathion which was absent in susceptible strains from Iraq and Russia (Anyanwu et al., 1997). A similar finding was observed in malathion resistant *An. gambiae* from Nigeria compared to susceptible strains from Tanzania (Anyanwu et al., 2000). Another study measured the mean cuticle thickness in *An. funestus* from Johannesburg, resistant to permethrin using a scanning electron microscopy (SEM). The resistant population had a 9.5-10% increased cuticle thickness compared to susceptible mosquitoes from southern Angola (Wood et al., 2010). Some of the genes found overexpressed in insecticide resistant mosquitoes that have been linked to cuticular modification include CYP4G16, which was experimentally confirmed to increased hydrocarbon production and subsequent cuticle thickening in *An. gambiae* (Balabanidou et al., 2016), CPLCG5 in deltamethrin resistant *Cx. pipiens* pallens was suggested to increase cuticle thickness (Huang et al., 2018) and cuticular proteins CpCPR63 and CpCPR47 that are overexpressed in pyrethroid-resistant *Culex* indicate involvement in insecticide resistance through cuticle modifications (Sun et al., 2017).

1.10.3. Target site insensitivity

Target site resistance results from amino acid substitutions in the target receptor, which interferes with the binding affinity of the insecticide (Hemingway and Ranson, 2000). In mosquitoes, target site mutations have been reported in the voltage gated sodium channel (*Vgsc*), acetylcholinesterase (AChE) and GABA receptors which are the target sites of the different major classes of insecticides used in vector control but none have yet been reported in nicotinic acetylcholine receptors (nAChR), the target of neonicotinoids, recently approved for IRS (Sathantriphop et al., 2006, Hemingway and Ranson, 2000, Ihara et al., 2017).

1.10.3.1. Knockdown resistance (*kdr*)

Mutations in the *Vgsc* which decrease nerve sensitivity to pyrethroids and DDT, results in knockdown resistance (*kdr*) (Hemingway and Ranson, 2000, Dong, 2007). The voltage gated sodium channel is comprised of four domains. Each of the four domains (DI-DIV) contains six hydrophobic subunits (SI–SVI) (Usherwood et al., 2005) **(Figure 1.8)**.

L1014F is most common *kdr*-mutation and has been reported in numerous insects of agricultural importance such as potato tuber moth (*Tecia solanivora*) (Bacca et al.,

2017), tomato leaf miner (*Tuta absoluta*) (Haddi et al., 2012), house flies (*Musca domestica*) (Rinkevich et al., 2012) as well as medically important species *Anopheles gambiae* (Ranson et al., 2000) and *Phlebotomus argentipes*, in both of which 1014S is also found. Mutations at 1014 have not been found in *Ae. aegypti* probably because two nucleotide substitutions within the coding triplet are required to convert L1014 to 1014F (Kushwah et al., 2015, Davies et al., 2007).

In *Ae. aegypti*, thirteen amino acid substitutions have been identified in different domains in the VGSC **(Figure 1.9)**. These substitutions vary in geographical spread, frequency, and impacts on resistance (Moyes et al., 2017). Out of these substitutions, S989P, I1011 M, V1016 G, F1534C and V410L (housefly nomenclature) have been functionally validated using *Xenopus* oocysts to reduce the sensitivity of the VGSC to pyrethroids (Haddi et al., 2017, Du et al., 2013, Hirata et al., 2014). Among the functionally confirmed substitutions, the most widespread is 1534C, which has been discovered in the Americas, Africa, Asia (Moyes et al., 2017) and now the Middle Eastern region (see Chapter 3). This substitution has been associated with resistance to the type 1 pyrethroids (permethrin)(Hirata et al., 2014), type 2 (deltamethrin) when in combination with other mutations (Plernsub et al., 2016a) and DDT when alone (Du et al., 2016b). Two geographically distinct substitutions at position 1016 have been identified. In Asia and the Middle East (Chapter 3), a substitution from valine to glycine occurs, whereas in the Americas and Africa, a substitution from valine to isoleucine is found (Moyes et al., 2017). The effect of the V1016G substitution on pyrethroid binding has been investigated in *Xenopus* oocysts where it was found to reduce sensitivity to pyrethroids with the magnitude of reduction in sensitivity increasing when combined with S989P. The S989P was suggested to have a synergistic effect on reduction of sensitivity to deltamethrin and is likely to have occurred on a V1016G background because it is very rarely found independently and confers no resistance alone (Sayono et al., 2016). Extreme resistance to deltamethrin has been reported in the presence of a triple mutation 989P+1016G+1534C haplotype, which was up regulated by 90 fold compared to 10 fold in the double mutation V1016G+S989P when compared to wildtype haplotype. The results demonstrate acquisition of additional mutations in *Vgsc* is important for development of extreme resistance phenotypes (Hirata et al., 2014).

Unlike V1016G, V1016I alone has no effect on either type I or type II pyrethroid binding (Du et al., 2013), but when in combination with F1534C, it has been associated with a strong resistance phenotype (Brito et al., 2018). This may be the explanation why the 1016I+1534C haplotype is more common and has been rising in frequency in pyrethroid resistant *Aedes* populations in the Americas compared to the rare 1016I+1534C haplotype (Vera-Maloof et al., 2015, Brito et al., 2018). A recent study investigating the history of 1016I, 1534C and V410L in Mexico found V1016I and V410L were more strongly linked than 1016I+1534C suggesting the former had a stronger effect size on pyrethroid resistance (Saavedra-Rodriguez et al., 2018). In contrast, the V410L and F1534C haplotype in *Ae. aegypti* strain from Rio de Janeiro was more common than V1016I and V410L (Haddi et al., 2017).

Expressing *Vgsc* with the 410L mutation was found to confer resistance to permethrin and deltamethrin alone or in combination with 1534C in *Xenopus* oocytes, but unfortunately combination with 1016I was not investigated (Haddi et al., 2017). Recently, two new mutations in the *Vgsc* have been found in Vietnam A1007G and F1558C (Lien et al., 2018). The effect of these mutations on the resistance phenotype has not been demonstrated.

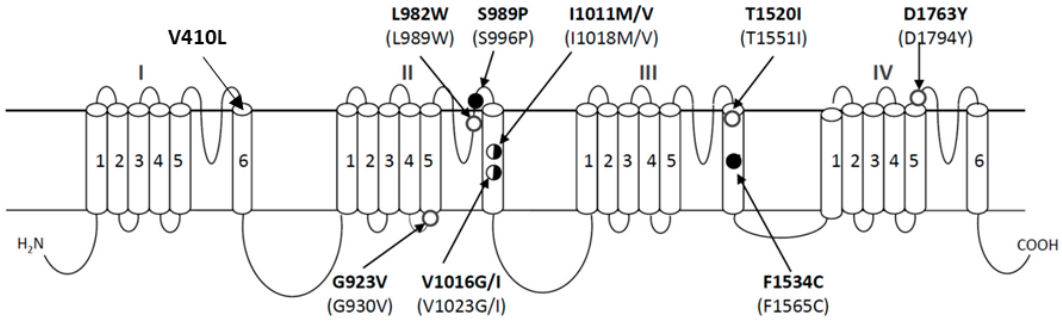


Figure 1.8 Schematic of the voltage gated sodium channel (*Vgsc*). The location of mutations in *Ae. aegypti* are represented by different coloured dots and are numbered according to the sequence of the housefly *Vgsc*. Black circles indicate the mutations that have been functionally confirmed in *Xenopus* oocytes, half-black/white circles indicate one of the amino substitutions has been confirmed in oocytes, and white circles indicate the mutations that have not been confirmed in oocytes The picture was adapted with modification from Du et al., (2016a).

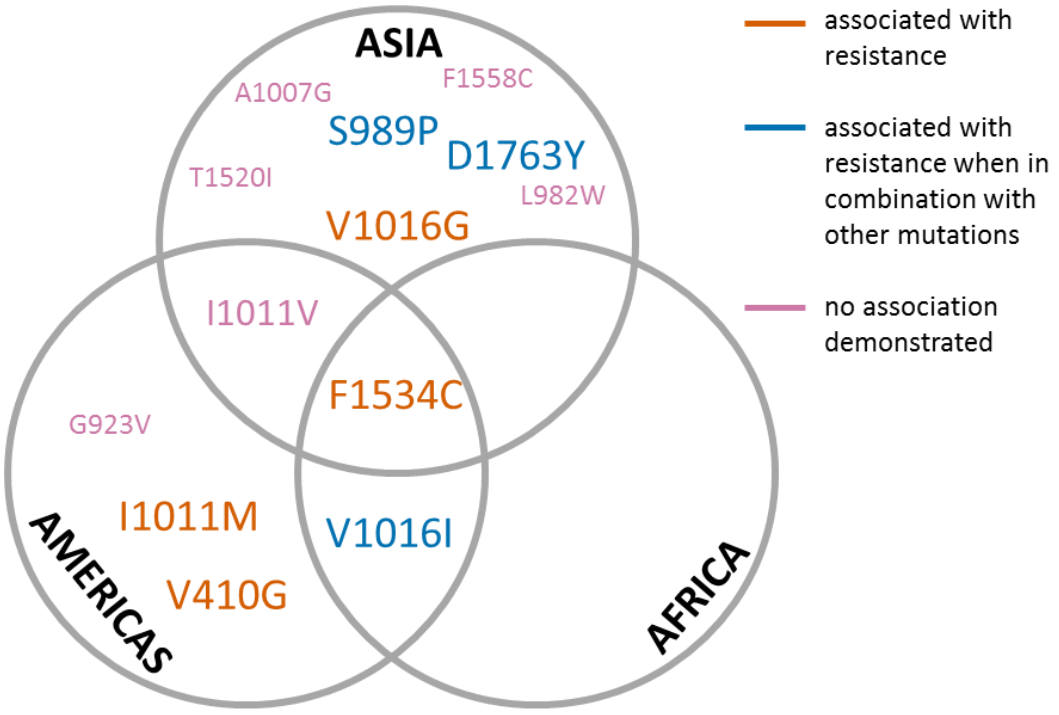


Figure 1.9 Geographical distribution of knockdown resistance mutations in the voltage gated sodium channel gene. Image adapted with modification from Moyes et al., (2017).

1.10.3.2. Insensitivity of Acetylcholinesterase (AChE) and GABA receptor

Mutation on the AchE has been detected in *Cx. pipiens* and *Anopheles* species, where an amino acid substitution from glycine to serine at codon 119 leads to increased tolerance to carbamates and organophosphates (Weill et al., 2004).The mutation in *Aedes* is considered unlikely to occur because two mutations are required to substitute glycine with serine (Weill et al. 2003). To date, G119S has only been reported in *Ae. aegypti* from India (Muthusamy and Shivakumar, 2015). Another mutation F290V, has been observed in *Cx. pipiens,* which is responsible for resistance to organophosphate and carbamate (Alout et al., 2009).

GABA receptors are comprised of five subunits, which arrange around the central ion channel. When GABA binds to the receptors, chloride ion selective channel are activated and conduct a nervous impulse (Hemingway et al., 2004). Gamma-aminobutyric acid (GABA) receptors are the main target of dieldrin, phenylpyrazoles and fipronil. Although, most of the organochlorides targeting the gamma aminobutyric acid (GABA) receptor have been withdrawn, resistance to dieldrin (Rdl) persists in some species (Du et al., 2005, Wondji et al., 2011). Rdl is mainly attributed to mutations in the GABA receptor. The A302S mutation is the only one linked to,

dieldrin resistance in *Ae. aegypti* and *Ae. albopictus* strains from La Reunion and Java (Moyes et al., 2017).

1.10.4. Metabolic resistance

Metabolic resistance involves alterations in the activity of a complex array of enzymes involved in detoxification pathways, which remain incompletely understood (Perry et al., 2011). Metabolic resistance is facilitated by overexpression or an altered structure (enhancing activity) of enzymes like esterases, cytochrome P450s, ABC transport proteins and glutathione-s-transferases (GST) (Hemingway and Ranson, 2000). Metabolic resistance is common in *Ae. aegypti* and *Ae. albopictus* and has been reported against pyrethroids, temephos and carbamates (Moyes et al., 2017).

The number of detoxification genes in *Ae. aegypti* is higher than in *Drosophila melanogaster and An. gambiae* (**Table 1.2**), but most of their functions are unknown.

Table 1.2 Numbers of annotated members of detoxification gene families found across five insect species. Adapted from Chena et al., (2015) with data for ABC transporters in *Culex* (*) from Lu et al.,(2016).

Detoxification gene	*Ae. aegypti*	*Ae. albopictus*	*D. melanogaster*	*An. gambiae*	*Cx. quinquefasciatus*
Monooxygenases (P450)	168	186	87	104	196
Carboxy/cholinesterases Esterases	59	64	34	46	71
Glutathione-S-Transferases	26	32	37	28	35
ABC transporters	58	71	56	52	70*

1.10.4.1. Cytochrome P450s

Cytochrome P450s are a family of hemoprotein (2015) enzymes that use heme as a co-factor to catalyze reactions (Bergé et al., 1998). The P450s act as terminal oxidases in monooxygenase systems in the presence of NADPH and cytb5. P450s catalyse metabolism of endogenous compounds and numerous xenobiotics (Feyereisen, 1999, Bergé et al., 1998). Raised levels of P450 activity have been associated with pyrethroid resistance in *Ae. aegypti* (Smith et al., 2016, Moyes et al., 2017). Being a family composed of multiple genes, identifying the specific P450 genes

involved in resistance can be challenging owing to inability to infer substrates for each P450 based on classification, the lack of clear orthologs of many P450s between species and different populations of the same species evolving resistance via different P450s the resistance (Smith et al., 2016). However, some P450s have often been found overexpressed in pyrethroid resistant adult *Ae. aegypti* populations resistant to deltamethrin. Mosquitoes from French Overseas are found to overexpress C*YP12F7*, *CYP9J10*, *CYP9J27* and *CYP6BB2* compared to New Orleans susceptible strains (Dusfour et al., 2015). On Madeira Island in Portugal, microarray data showed a significant upregulation of CYP*9J32*, CYP*9J28*, CYP*6BB2*, *CYP9J27*, *CYP9M5*, *CYP9M6*, *CYP6N12 and CYP6M9* in pyrethroid resistant *Ae. aegypti* (Seixas et al., 2017). Marcombe et al., (2009b), found *CYP6BB2*, *CYP6M6*, *CYP6Y3*, *CYP6Z6*, *CYP6M10*, *CYP6AA5*, *CYP9J23*, *CYP9J22* and *CYP9J9* were differentially transcribed in larvae while *CYP6CB2*, *CYP6M11*, *CYP6Z6*, *CYP6M6, CYP9J22*, *CYP9M9* and *CYP9J6* were differentially expressed in adults of the Vauclin permethrin resistant strain when compared to the susceptible BORA strain (Marcombe et al., 2009b). Among these and other studies, the commonly overexpressed P450s, which have also been validated *in vitro* or *in vivo* to have the ability to metabolise pyrethroids includes *CYP6BB2*, *CYP9J24*, *CYP9J26*, *CYP9J28*, *CYP9J32* and *CYP9M6* (Stevenson et al., 2012, Kasai et al., 2014, Pavlidi et al., 2012).

Overexpression of P450s has been attributed to mutations in cis regulatory elements and increases in copy number (Weetman et al., 2018a). In *Cx. quinquefasciatus*, the duplication of the *CYP9M10* gene, an insertion of a transposable element upstream and a subsequent non-synonymous change in its core promoter region was associated with 5.9 fold change in expression level of the gene compared to the wildtype haplotype (Itokawa et al., 2015). In *Aedes*, overexpression of P450s has been linked more to gene amplification than mutation in cis regulatory elements (Faucon et al., 2015, Weetman et al., 2018a).

1.10.4.2. Glutathione-s-transferases (GST)

Glutathione-s-transferases (GSTs) detoxify compounds through binding, sequestration, and protection against oxidative stress induced by insecticide exposure (Enayati et al., 2005). The GST family is divided into three major groups; cytosolic, microsomal and mitochondrial. Most GSTs are cytosolic which have further

been divided into six groups: Delta, Epsilon, Omega, Sigma, Theta and Zeta (Lumjuan et al., 2007, Lumjuan et al., 2005). The first two play a key role in insecticide resistance and are specific to insects (Strode et al., 2008, Lumjuan et al., 2005). For example, GSTe2, GSTe5 and GSTe7 are overexpressed in *Ae. aegypti* mosquitoes resistant to DDT and pyrethroids (Lumjuan et al., 2005). GST activity has been associated with resistance to several classes of insecticides. It confers resistance via direct metabolism (DDT), or sequestration of chemicals, providing protection against oxidative stress induced by insecticide exposure in directly (Pavlidi et al., 2018). For examples, increased levels of GSTe2 have been associated with resistance to DDT, pyrethroids and organophosphate in *Ae. aegypti* and *Ae. albopictus* (Moyes et al., 2017, Harris et al., 2010).

1.10.4.3. Carboxylesterases (CCE)

Carboxylesterases are a diverse family of enzymes that hydrolyse esters. The molecular mechanism involved in CCE-related insecticide resistance includes, up-regulation, gene amplification and coding sequence mutations which facilitate increased hydrolysis or sequestration of insecticide molecules (Li et al., 2007). One of the earliest reports of CCE involvement in resistance was in *Culex* where two common esterase loci, Esterase A (Est-3) and Esterase A (Est-2) were suggested to be involved alone or in combination in detoxifying insecticides containing carboxylic esters (Raymond et al., 1998). Since then, many studies have reported association between elevated CCE activity and insecticide resistance.

Apart from the three metabolic families discussed above, the other gene families possibly important in insecticide resistance in *Aedes* are UDP-glycosyltransferases (UGTs) and adenosine triphosphate (ATP)-binding cassette (ABC) transporters (Moyes et al., 2017).

UDP-glycosyltransferases (UGTs) enzymes participate in Phase II detoxification of xenobiotics and can conjugate glycosyl groups to electrophilic substrates. Overexpression of UDP-glycosyltransferases has been reported in pyrethroid and temephos resistant *Ae. aegypti* (Moyes et al., 2017, Grigoraki et al., 2015). ATP-binding cassette (ABC) transporters act as ATP-dependent efflux pumps for transferring drugs and xenobiotics including insecticides outside the cells thus reducing their intracellular concentration below the lethal dose (Lima et al., 2014).

Overexpression of ABC transporters which is attributed to gene amplification has been linked to pyrethroid resistance in *Ae. aegypti* (Bariami et al., 2012, Vontas et al., 2012).

1.11. Sources of selection for insecticide resistance in mosquitoes

The widespread use of insecticides by vector control programmes is thought to be an important factor responsible for selection of increased insecticide resistance in mosquito populations globally (Ranson et al., 2010, Vontas et al., 2012). Resistance to DDT, the first insecticide introduced for mosquito control in 1946 was reported the following year in *Ae. tritaeniorhynchus* and *Ae. solicitans* thus signifying resistance can develop rapidly when mosquito populations are exposed to strong selection pressure (Hemingway and Ranson, 2000).

Apart from insecticides used for vector control, pesticides used in agriculture, households, and possibly industrial waste might also facilitate selection of insecticide resistance in mosquitoes (Nkya et al., 2013). Exposure of susceptible field collected *An. gambiae* larvae to a mixture of commonly used pesticides, herbicides and pyrethroid metabolites for 24h every generation resulted in a gradual increase in insecticide resistance (Nkya et al., 2014). By the 20th generation, the frequency of L1014S knockdown resistance mutation had risen from 1.6% to 12.5%. In addition, the authors observed significant differential expression of cuticle proteins, detoxification enzymes, proteins linked to neurotransmitter activity and transcription regulators when they compared expression patterns of selected vs unselected mosquitoes. The experiment demonstrated resistance in adult stages can be selected through periodic exposure of larval stages to pesticide and herbicide that contaminate their habitats in regions that heavily use them for agricultural production.

In field studies, high resistance to different classes of pesticides commonly used in cotton production in Pakistan was observed in *Ae. albopictus* larvae. The authors did not expose the adult stage to the same insecticides hence it is not clear if resistance to these compounds persisted to adult stages (Khan et al., 2011).

Heavy metals, and car tyre leachate compounds such as benzothiazole and other pollutants found in urban drainage systems have also been linked to increased

tolerance to insecticides. For example, *An. arabiensis* larvae exposed to salts of lead, copper and cadmium had a 2.2 fold, 4.0 fold and 5.5 fold increase in the time required to kill 50% (LT_{50}) of adults with malathion (respectively). The same increased LT_{50} was observed with deltamethrin (Oliver and Brooke, 2018). *Aedes albopictus* exposed to benzothiazole were found to be more tolerant to carbaryl, rotenone, and temephos (Suwanchaichinda and Brattsten, 2002).

1.12. Impacts of insecticide resistance on control

Although insecticide resistance to pyrethroids, carbamates, organochlorines and organophosphates has been reported in *Ae. aegypti* in multiple countries, no study has linked resistance to failure in control of dengue or other arboviruses (Moyes et al., 2017). However, the evidence of entomological control failure has been accumulating since 1956 when *Ae. aegypti* resistant to DDT failed to be eliminated from the Cayman Islands despite the intense DDT application campaigns (Brown, 1986). Entomological control failure in the commonly used adulticide, deltamethrin and the larvicide, temephos has been reported in various countries. When the efficacy of thermal fogging using deltamethrin, natural pyrethrins or an organophosphate (Naled) was evaluated in pyrethroid resistant strains in Brazil, pyrethroids had the lowest effect on knockdown and mortality on field strains compared to the organophosphate (Marcombe et al., 2009a). Approximately 48% recovery in pyrethrum knockdown after 24h compared to 7% in organophosphate was observed thus indicating metabolic resistance contributed to pyrethroid resistance. In Thailand, target site insensitivity was demonstrated to have an impact on mortality from thermal spraying of *Ae. aegypti* with Damthrin-SP (deltamethrin + S-bioallethrin + piperonyl butoxide). Partial mortality was observed in outdoor caged *Ae. aegypti* carrying the double heterozygote *kdr* mutations V1016G and F1534C, but no mortality was recorded in homozygous mutants 1016G while high mortality was observed in homozygous wildtype F1534 (Plernsub et al., 2016a). Recently in a randomised control trial done in Merida, southern Mexico, no difference in indoor abundance was observed in houses where deltamethrin was used for IRS compared to controls but a 60% reduction in abundance was observed in houses treated with bendiocarb (Vazquez-Prokopec et al., 2017). The study

demonstrated deltamethrin failed to control indoor resting populations but bendiocarb was still effective in that region. The control failure was linked to the high levels of pyrethroid resistance in the population, which remained susceptible to bendiocarb.

One of earliest studies that demonstrated loss efficacy of temephos in resistant populations was a trial carried out in Brazil in early 2000s. In the experimental sites, temephos was applied every three months alongside source reduction, while in controls, only source reduction was carried out. Monthly data on larval indices was recorded to evaluate the impact of the control interventions. There was no difference in larval infestation in experimental and control sites thus indicating temephos had lost its efficacy against that population (Camargo Donalisio et al., 2002). A loss in efficacy to the same compound was reported in a different population in Brazil where less than 70% mortality was observed by the fourth week after application of temephos while complete control was achieved in the susceptible stain for more than 7weeks (Montella et al., 2007).

No studies have been done to link entomological control failure arising from insecticide resistance to failure in control of diseases spread by *Ae. aegypti*. In *Anopheles* species, a loss in protection provided by pyrethroid-based IRS and LLIN has been reported in different sites in Africa (Toé et al., 2014, Glunt et al., 2015, Omondi et al., 2017, Asidi et al., 2012, Protopopoff et al., 2018). The evidence of operational failure in malaria control is demonstrated by correlating the prevalence or incidence of malaria with vector control events such as switching or addition of insecticides with different chemistries (Katureebe et al., 2016, Raouf et al., 2017, Hargreaves et al., 2000). For examples, in South Africa, increased malaria cases and the reappearance of *An. funestus* previously eliminated in the region was linked to a switch from using DDT to pyrethroids for IRS (Hargreaves et al., 2000).

The evidence of control failure in pyrethroid resistant *Aedes* and *Anopheles* emphasises the importance of integrating monitoring of insecticide resistance as a core element of vector-borne diseases control programs. Implementation of insecticide-based vector control strategies should always be guided by insecticide resistance data collected periodically in the target population (World Health Organization, 2012a).

1.13. Insecticide resistance of *Aedes* mosquitoes in Saudi Arabia

Control programmes in Saudi Arabia rely extensively on insecticides to reduce the prevalence of dengue in the disease-foci cities. The insecticides currently used for controlling adults are pyrethroids (permethrin, deltamethrin, cyfluthrin, lambda-cyhalothrin), organophosphate (fenitrothion) and carbamate (bendiocarb). Temephos, *Bti*, Spinosad and insect growth regulatory hormones such as pyriproxyfen are used as larvicides in breeding sites, but *Bti* and Spinosad are more common in Jeddah and Makkah (Aziz et al., 2014, Alsheikh et al., 2016, Farooqui et al., 2012).

The available published data on insecticide use in Saudi Arabia suggest increasing usage since the first dengue outbreak in 1994. The usage of insecticides in 2013 was 3,130 tonnes of active ingredients in comparison with 2012, which was 2,889 tonnes. Organophosphates were heavily used in 2007 (1,787 tonnes of active ingredients) in comparison to 1996 and 1997. In the same year, carbamate consumption was 426 tonnes and pyrethroids 1,112 tonnes (Saggu et al., 2016).

Despite potential impacts on control, little is known about insecticide resistance in *Ae. aegypti* in the Middle Eastern Region including Saudi Arabia, and furthermore, nothing is known about underlying resistance mechanisms. Before the present study, insecticide resistance in *Ae. aegypti* in Saudi Arabia had only been screened in populations from Makkah (Aziz et al., 2011) and more recently in Jazan (Alsheikh et al 2016). No information on insecticide resistance was available in Jeddah, which has the highest prevalence of dengue fever in the Kingdom (Al-Raddadi et al., 2018, Al-Tawfiq and Memish, 2018). *Aedes aegypti* strains from Makkah were found to be resistant to lambda-cyhalothrin, deltamethrin and cyfluthrin but still susceptible to pirimiphos-methyl (actellic) and *Bacillus thuringiensis israelensis Bti* (Bacilod) (Aziz et al., 2011).

In Jazan, the population was resistant to lambda-cyhalothrin, DDT, bendiocarb and showed moderate resistance to permethrin, deltamethrin and fenitrothion (yet remained susceptible to cyfluthrin). The larvae were highly resistant to temephos with an LC_{50} of 61.8 mg/L, which needs to be confirmed as this extreme LC_{50} concentration has not been reported in other temephos resistant populations in the world (Biber et al., 2006, Dos Santos Dias et al., 2017). The larvae were susceptible to

methoprene and diflubenzuron (Alsheikh et al., 2016). Aziz et al., (2011) suggested an over-reliance on a single class of insecticides (pyrethroids) to control adults and the sale of adulterated insecticides for personal use in the cities that experience periodic dengue outbreaks, as the major causes of resistance in *Ae. aegypti* in Saudi Arabia (Aziz et al., 2011).

Despite the commitment of the government to increase funding for dengue control in the Kingdom, the increased budget has remained ineffective for decreasing the number of dengue cases (Aziz et al., 2014). Factors such as hiring private companies that lack experience and knowledge on insecticide dosage and proper application techniques may be contributing to this failure in Saudi Arabia (Farooqui et al., 2012, Aziz et al., 2014). The vector control coverage in most cities is also hindered by poor compliance where residents decline access to their homes for indoor residual spraying and larval control (Aziz et al., 2014).

1.14. Rationale of research project

This thesis will address these knowledge gaps by investigating the physiological resistance status, levels and mechanisms of insecticide resistance in *Ae. aegypti* at key sites for dengue transmission in the Kingdom of Saudi Arabia (KSA). Mosquitoes colonised from the field were used for investigations of behavioural patterns relevant to tools designed to control adult mosquitoes, e.g. screens and other insecticide-treated materials (ITM), and involved comparative testing with mosquitoes differing in their resistance status. It is anticipated that the information gained may feed into tests of field interventions and provide guidance on potential efficacy in the context of dengue control in KSA, for which such relevant entomological data are currently limited.

1.14.1. Research aims and objectives

1. To characterise the prevalence and level of insecticide resistance in *Ae. aegypti* from different areas in Saudi Arabia during the period of highest dengue fever transmission.
2. To investigate the insecticide resistance mechanisms: alteration of target sites and metabolic resistance in dengue vectors from Jeddah and Makkah of Saudi Arabia.
3. To conduct behavioural studies in *Ae. aegypti* populations from Jeddah and Makkah to investigate entry, host-seeking and resting behaviour (using video recording technology) and to determine the effectiveness of available insecticide-treated materials against *Ae. aegypti* populations in KSA.

CHAPTER 2
EVALUATING THE SUSCEPTIBILITY STATUS OF *AEDES AEGYPTI* IN SAUDI ARABIA

2.0. ABSTRACT

Background Vector control programs worldwide are facing the challenge of mosquitoes becoming resistant to available insecticides. For the two most important dengue foci in Saudi Arabia, Jeddah and Makkah, data on insecticide resistance of *Ae. aegypti* is either limited or absent. This chapter aims to determine the susceptibility status of larval and adult *Ae. aegypti* from each city to different insecticides, with a preliminary investigation of mechanisms using synergist assays. In addition, the impact of mosquito age and repeated exposure on insecticide susceptibility was investigated.

Methods *Ae. aegypti* were sampled in Jeddah and Makkah. Larvae were assayed for resistance to common larvicides (temephos and *Bacillus thuringiensis israelensis Bti*) and adults for resistance to permethrin, fenitrothion, bendiocarb and deltamethrin - with and without the synergist piperonyl butoxide (PBO).

Results Jeddah and Makkah populations exhibited susceptibility to temephos and *Bti* but resistance to multiple adulticides. Age and exposure duration had a significant effect on survival of both strains whereby susceptibility to insecticides increased with age and duration of exposure but decreased after repeated exposures. Piperonyl butoxide (PBO) pre-exposure increased pyrethroid mortality significantly in the Jeddah, but not the Makkah strain.

Conclusion The study provides the first insecticide resistance phenotype data in Jeddah and updates existing data in Makkah. The study outcome provides tentative evidence that can be considered by vector control programs in management of insecticide resistance and implementation of successful control intervention in the region.

2.1. INTRODUCTION

In Saudi Arabia, insecticides are extensively used by the Ministry of Health, Ministry of Municipal and Rural affairs and private companies to combat mosquito-borne diseases and other household pests, as well as in agriculture (Aziz et al., 2014). *Aedes aegypti* is primarily controlled by larvicides such as Spinosad (Natular®), *Bacillus thuringiensis israelensis* (*Bti*) toxin (VectoBac®), pyriproxyfen and diflubenzuron. Adulticides such as deltamethrin, permethrin, cyfluthrin and fenitrothion are also used for fogging and indoor residual spraying. A major limitation of the control program in the region is the inadequate surveillance efforts to monitor the effectiveness of the control intervention, or changes in the resistance of populations that may undermine the control efforts. Despite the potential impact on control, little is known about insecticide resistance in *Ae. aegypti* in Saudi Arabia or the Middle Eastern Region generally. For instance, information on the insecticide resistance status of Saudi Arabian populations of *Ae. aegypti* remains limited to two studies, one from Makkah (Aziz et al., 2011) and one from Jazan (Alsheikh et al., 2016); the resistance profile of *Ae. aegypti* in Jeddah has not been investigated.

To monitor insecticide resistance the WHO recommends the use of 2-5 day old, non-blood fed female mosquitoes in the standard WHO bioassays. This standardisation facilitates comparison among different tests, and this is important for surveillance of spatial or longitudinal variations in resistance in the field. However, females of this age do not transmit disease to humans (Rajatileka et al., 2011) and thus it is important to study the bionomics of old mosquitoes, including their insecticide resistance profile for effective control of disease transmission. Individual mosquitoes that are 14 days post-emergence are able to transmit Dengue (Harrington et al., 2001a). This period is when the mosquitoes need to take an infectious blood meal and survive the extrinsic incubation period (EIP) during which the virus replicates and migrates to the salivary glands. The EIP is influenced by biological and environmental conditions as described in chapter 1 section 1.4. Several studies on *Aedes* and *Anopheles* have shown that susceptibility to insecticides increases with chronological age (Rajatileka et al., 2011, Chouaibou et al., 2012, Sikulu et al., 2014, Knecht et al., 2018, Mbepera et al., 2017). One of these studies observed young *Ae. aegypti* females (3 day old, sugar fed) were significantly more tolerant to 4% DDT (mortality <10%) and 0.05%

deltamethrin (mortality ~60%) compared to (14 day old sugar fed) mosquitoes to DDT (mortality ~40%) and deltamethrin (mortality <80%) (Rajatileka et al., 2011). But most of these studies evaluating the impact of age on insecticide-induced mortality usually record mortality only 24h post exposure, which can be misleading since they do not account for the effect of delayed mortality or multiple exposure to the same insecticide. Delayed mortality has been demonstrated to have a significant impact on survival of resistant *An. gambaie* strains where up to 50% mortality was observed in mosquitoes that were alive when mortality was recorded 24h post exposure to pyrethroids (Viana et al., 2016). However, it is not known if repeated exposure or longer exposure duration will have a greater effect on mortality in addition to the delayed mortality effect.

The aim of the study presented in this chapter was to characterise the resistance status of *Ae. aegypti* populations from Jeddah and Makkah to the different compounds used to control them in these cities. Larval bioassays were performed to assess resistance towards temephos and *Bti,* while standard and synergistic adult bioassays were conducted to determine the susceptibility to the four main classes of insecticides and the possible involvement of metabolic resistance. In addition, the effect of single exposure at different ages to measure age effect alone or repeated exposure, which measured both the effect of age and multiple encounters with insecticide over time, was investigated. The outcome of this study will provide additional and updated data on the resistance profile of *Ae. aegypti* populations from Saudi Arabia and may provide indication of which insecticides may be more effective.

2.1.1. Objectives

1- To assess the susceptibility status of *Ae. aegypti* collected from Jeddah and Makkah to larvicides (temephos and *Bti*) using larval bioassays and adulticides (pyrethroids, organophosphates and carbamates) tested with WHO tube bioassays.

2- To investigate age-dependent pyrethroid-induced mortality.

3- To investigate whether extended exposure duration or repeated exposure to pyrethroids increases mortality.

4- To investigate the potential role of metabolic enzymes inhibited by PBO in pyrethroid resistance.

2.2. MATERIALS AND METHODS

2.2.1. Mosquito strains, collection and rearing

The first collections in Jeddah (21°63'1.24"N, 39°19'8.71"W) and Makkah (21°45'8.36"N, 39°78'6.98"E) were performed from June to August 2015 and in December 2014 to April 2015 respectively **(Table 2.1)**. In both areas, 50 ovitraps were set to target sites where dengue cases have been reported. In Makkah, collections were made from private farms, brick factories, car tyre shops, fuel stations and residential houses **(Figure 2.1)**. In Jeddah, traps were set in buildings under construction, in shops and houses. All traps were checked every two days and collected after five days.

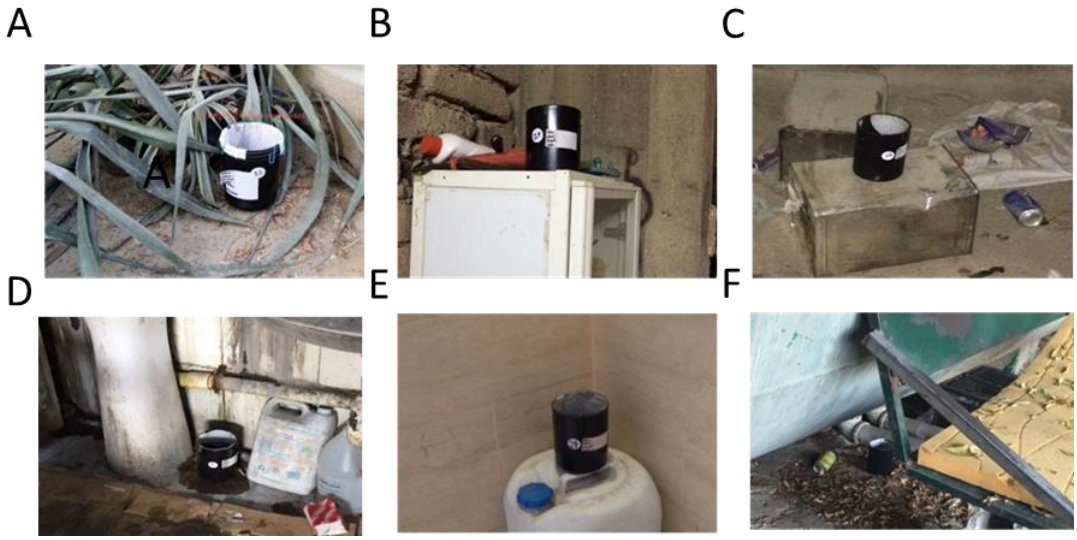


Figure 2.1 Ovitraps were designed by Ashwaq Alnazawi and placed in selected field sites to collect *Ae. aegypti* eggs in Makkah and Jeddah. Panel A) Ground floor of a private lounge next to patio plants with pooled water at the container base. Panel B) Kitchen of a first floor dwelling above the cupboard. Panel C) Ground floor of a building under construction with nearby containers filled with water. Panel D) Ground floor bathroom of domestic workers next to a sink. Panel E) Basement of a building under construction next to the containers filled of water. Panel F) Ground floor of dwelling next to a large water container where stagnant water has pooled on the floor.

The eggs were dried, preserved at room temperature in a sealed plastic bag and shipped to the Liverpool School of Tropical Medicine (LSTM) **(Figure 2.2)**. Egg batches were hatched, and larvae reared to adults on a diet of chinchilla pellets under insectary conditions of 27°C and 70% relative humidity with a constant photoperiod (12h of light and 12h of darkness). Adult females, 3-5 days old, from the first laboratory generation (i.e. the offspring of the adults from the egg collections,

hereafter referred to as 'F1 females'), which had been fed *ad libitum* on 10% sugar solution, were used for bioassays. The Cayman strain (Harris et al., 2010) was used as a reference resistant strain and raised under the same conditions.

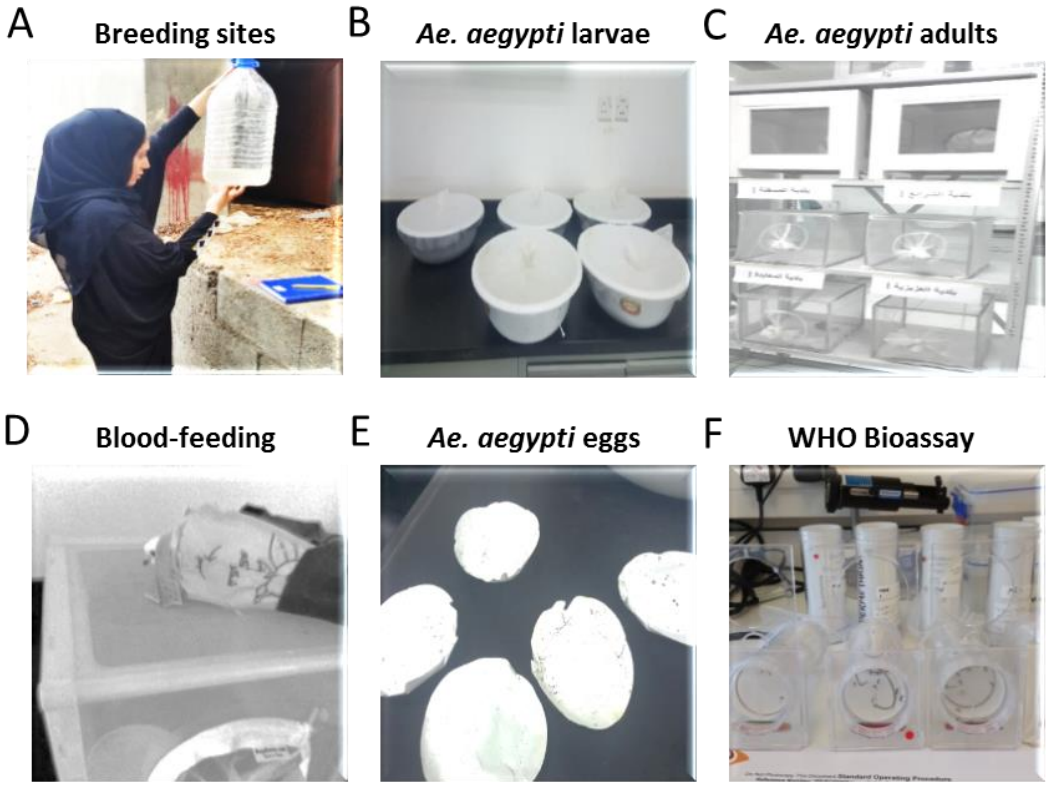


Figure 2.2 Collection and handling protocols utilised in the Makkah and Jeddah insectaries. Panel A) Ashwaq examining discarded water bottle for *Aedes* larvae. Panel B) Larval pots used in the Makkah insectary. Panel C) Placing mosquito pupae pot into cages for rearing the adult mosquitoes. Panel D) Blood feeding adult mosquitoes on an immobilised pigeon. Panel E) Dried egg papers under insectary conditions, Panel F) WHO tube bioassay to test for insecticide resistance in adult mosquitoes.

A second field collection from a wide area within Jeddah and Makkah in March-April 2016 targeted larvae present in water coolers, barrels, buckets and water containers such as under air conditioners and buildings under construction **(Figure 2.3)**.

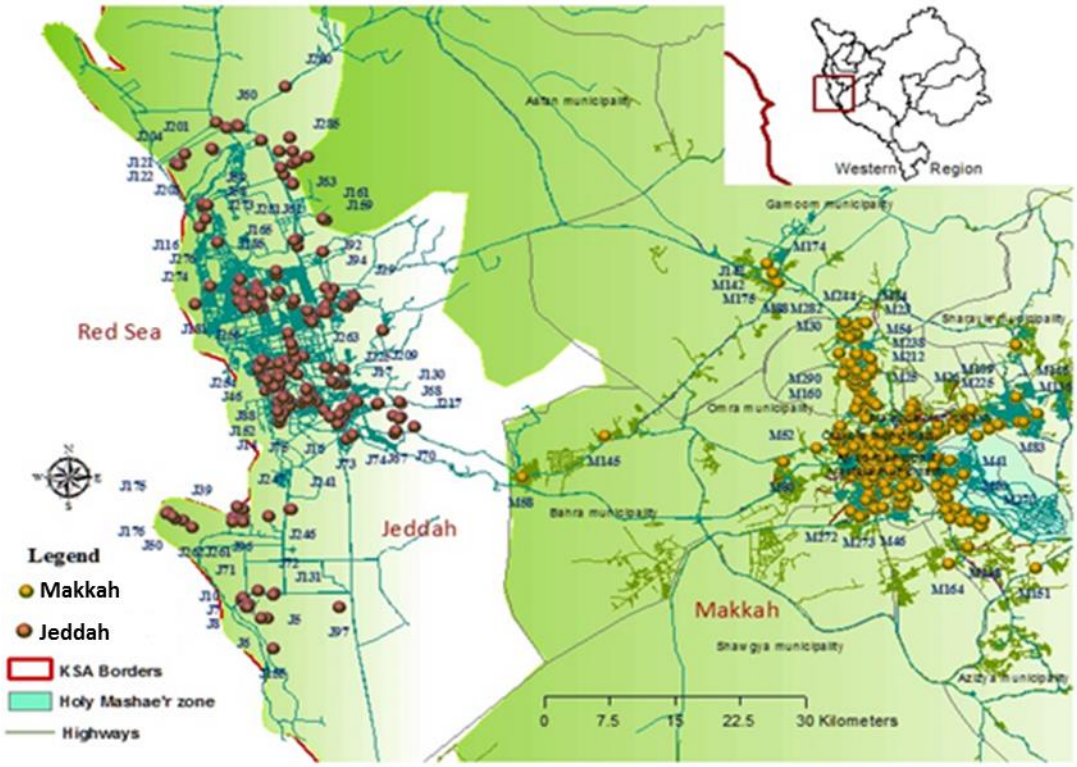


Figure 2.3 Map of where *Ae. aegypti* larval collections in Jeddah and Makkah were sampled in March-April 2016. The yellow circles show the Makkah sampling sites and the red shows the Jeddah sampling sites.

The larvae were reared at the local Municipal insectaries on a diet of yeast under insectary conditions of 27°C and 75% relative humidity with a constant photoperiod (12h of light and 12h of darkness). The emerged females (hereafter referred to as 'field females') were fed *ad libitum* on 10% sugar solution and were phenotyped for insecticide resistance genes when 3-5 days old. The last collection in January-February 2018 targeted larvae from Jeddah **(Table 2.1)** isolated from water tanks, water coolers, stored water in construction sites and animal watering points. The first generation were used for the larval assays (*Bti* only) in section 2.2.2 and adult bioassays (for Jeddah only) in section 2.2.3.2 and 2.2.3.3

Table 2.1 Field collection performed in different locations of Jeddah and Makkah.

Number of collections	Date	strain	Generation	Coordinates	Type of collection	test	Number of Chapter
First collection in Jeddah7	06-08/2015	Jeddah	F0	21°63'1.24"N 39°19'8.71"W	Eggs	-DNA sequencing	Chapter 3
		Jeddah	F1		Eggs	-WHO bioassay 60 min exposure performed at LSTM -Long duration exposure of young and old mosquitoes -Deltamethrin survival with the synergist piperonyl butoxide (PBO)	This chapter
First collection in Makkah	12/2014 04/2015	Makkah	F0	21°45'8.36"N 39°78'6.98"E	Eggs	DNA sequencing	Chapter 3
		Makkah	F1		Eggs	WHO bioassay 60 min exposure performed at LSTM -Long duration exposure of young and old mosquitoes -Deltamethrin survival with the synergist piperonyl butoxide (PBO)	This chapter
Second collection in Jeddah		Jeddah	F0	21°35'2.13"N 39°13'9.42"E	Larvae	-WHO bioassay 60 min exposure performed in Jeddah.	This chapter
	03-04/2016		F0-F3-F5		Larvae (F0), eggs (F3)	-Microarray technique	Chapter 4
			F2		Eggs	-Cone bioassay -Thumb test	Chapter 5
			F4		Eggs	-Wind tunnel assay	Chapter 6

			F5			-Larval bioassay (temephos) at LSTM in cooperation With Msc student.	This chapter
Second collection in Makkah	03-04/2016	Makkah	F0	21°45'2.13"N 39°92'1.96"E		-WHO bioassay 60 min exposure performed in Makkah -Larval bioassay (temephos) at LSTM in cooperation With Msc student.	This chapter
			F0		Larvae	Microarray technique	Chapter 4
			F2			-Cone bioassay - Thumb test	Chapter 5
			F4			-Wind tunnel assay	Chapter 6
			F5			-Larval bioassay (temephos) at LSTM in cooperation With Msc student.	This chapter
Third collection in Jeddah	01-02/2018		F0	21°60'3.97"N, 39°27'2.49"E	Larvae	-Larval bioassay (Bti). -Age dependent deltamethrin-induced mortality -Multiple exposure effects	This chapter
Third collection in Makkah	01-02/2018		F0	21°40'7.70"N, 39°86'3.19"E	Larvae	-Larval bioassay (*Bti*)	This chapter

2.2.2. Larval Bioassays

Larval bioassays were carried out on *Aedes* strains shown in **Table 2.2** according to the WHO protocol (World Health Organization, 2005) to determine the lethal concentrations (LC_{50}) and the resistance ratio (RR_{50}).

Table 2.2 Number of *Ae. aegypti* larvae from Jeddah and Makkah used in each bioassay at different times.

Temephos assay		*Bacillus thuringiensis israelensis* assay	
Strain	Sample size	Strain	Sample size
New Orleans	900	New Orleans	650
Makkah lab F5 (second collection)	900	Makkah lab F5 (second collection)	590
Jeddah lab F5 (second collection)	900	Jeddah lab F5 (second collection)	650
		Makkah field F0 (third collection)	546
		Jeddah field F0 (third collection)	567
		Cayman	605

The bioassays were performed using temephos (Sigma-Aldrich, Dorset, UK), or *Bti* (Vectobac®12AS 1.2%,1200 ITU/mg; supplied by Dr Craig Wilding, Liverpool John Moores University). A total of eight different concentrations of *Bti* and nine of temephos were used for each strain. *Bacillus thuringiensis israelensis* (*Bti*) concentrations were 0.006 ppm, 0.0036 ppm, 0.002 ppm, 0.0012 ppm,0.00089 ppm, 0.0006 ppm, 0.00024 ppm, 0.00012 ppm. Temephos concentrations were 0.08 mg/L, 0.07 mg/L, 0.06 mg/L, 0.04 mg/L, 0.03 mg/L, 0.02 mg/L, 0.01 mg/L, 0.005 mg/L, 0.0025 mg/L and 0.0025 mg/L. The concentrations were selected as they have been reported to result in larval mortality between 10% and 95% (World Health Organization, 2005). The data was used to calculate the lethal dose that can kill 50% (LC_{50}) in each population. Dilutions of temephos (stock dissolved in absolute ethanol) with distilled water up to a total volume of 200mL are detailed in **Appendix1: Table 8.1**. For each concentration of each insecticide, three or four replicates of a pool of approximately 25 late third or early fourth instar larvae were tested along with a negative control pool (1mL absolute ethanol mixed into 199 mL of distilled water for temephos or into 100ml of distilled water for *Bti* assays.

All larval bioassays were performed in small plastic bowls; 6 cm in diameter. Mortality was recorded after 24h of exposure. Any larvae failing or unable to swim up to the

surface independently were counted as dead. Any larvae that had pupated during exposure were omitted from the total count. The same bowls were reused for bioassays of the same concentration and mosquito strain on the following day after washing.

2.2.3. Adult bioassays

2.2.3.1. WHO Bioassay 60 min exposure

WHO tube bioassays were performed with approximately 25 females per tube for a minimum of four replicates plus control impregnated with carrier oils **(Figure 2.4)**. I used the standard WHO protocol (World Health Organization, 2006), and the recommended concentration used for phenotyping *Anopheles spp.* (World Health Organization 1998, World Health Organization, 2016b) because the correct concentration of 0.25% for permethrin is rarely used (Moyes et al., 2017) while for other insecticides used in the assay such as deltamethrin, there is no agreed diagnostic dose for *Ae. aegypti* at present.

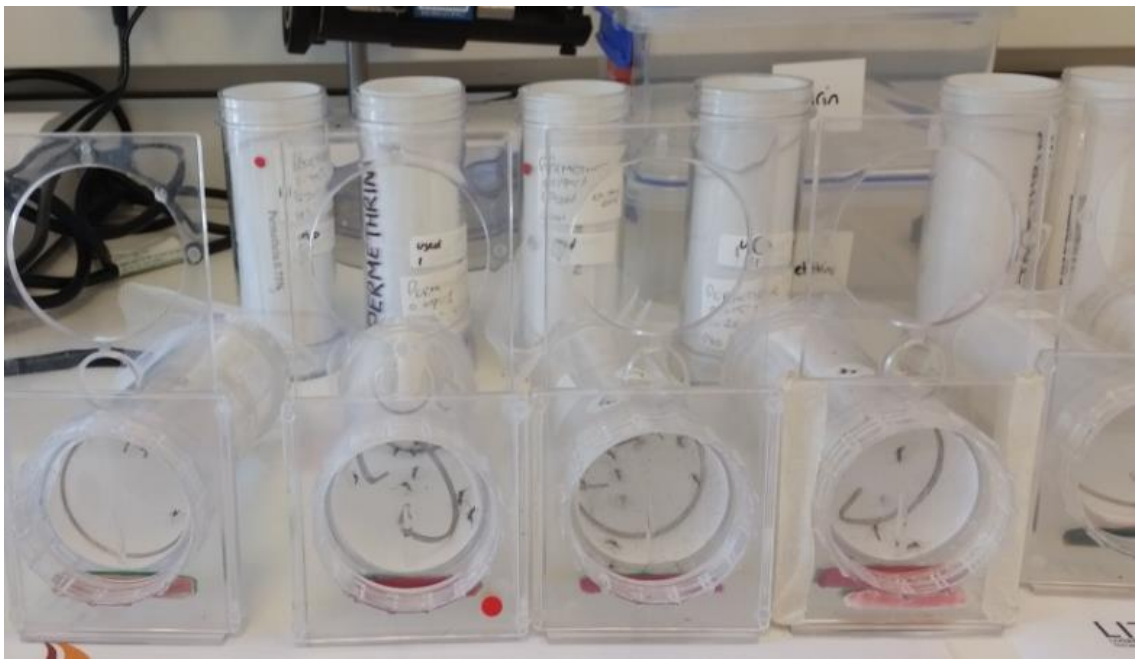


Figure 2.4 WHO tube bioassay experimental set up.

The insecticide-resistant Cayman strain was used as the reference strain to compare with the Saudi strains. Three to five day old field-collected F1 (first collection exposed in LSTM) and F0 (second collection exposed in Saudi) adult mosquitoes **(Table 2.1)** were exposed to permethrin (0.75%), deltamethrin (0.05%), fenitrothion (1.0%),

bendiocarb (0.1%) for 60 min as per the standard WHO protocol and then transferred to recovery tubes with access to 10% sugar solution. The mortality rate was recorded 24h after exposure. Owing to logistical limitations (correctly impregnated WHO bendiocarb papers not available at the time the experiment was conducted), bendiocarb was not tested in the field F0 females.

2.2.3.2. Age dependence of single-exposure deltamethrin-induced mortality

Female F0 (third collection) mosquitoes **(Table 2.1)** of different ages were exposed to WHO deltamethrin papers to test the hypothesis that susceptibility increases with age. In this assay, exposed pools of 25 mosquitoes per replicate of ages between 5 and 14 days (separately) to deltamethrin or control paper for 1h. After the exposure, females were transferred to recovery tubes and provided with 10% sucrose. After a 24h recovery period, the final mortality was recorded, and all survivors were preserved in RNA later and stored at -20°C for later conducting qRT-PCR analysis (See Chapter 4, section 4.3.4.1).

2.2.3.3. Age and multiple exposure effects

Unfed female mosquitoes (5day old) Cayman and F0 Jeddah (third collection) mosquitoes **(Table 2.1)** were exposed to 0.05% deltamethrin for 1h. After the 24h recovery period, all survivors were re-exposed to the same deltamethrin dosage. The exposure was continued every day until the remaining mosquitoes were 14 days old to test the hypothesis that repeated exposure would increase mortality rate. Mortality was recorded every day. The surviving mosquitoes were preserved in RNA Later and stored at -20°C at the end of the assay for later analysis (Chapter 4, section 4.3.4.2).

2.2.3.4. Exposure duration and age

I hypothesised that the duration of insecticide exposure increases the mortality rate. The interaction between the level of resistance to deltamethrin and age was investigated using bioassays with longer durations of exposure with either 3-5 or 10-day old F1 adult females from the Jeddah, Makkah (First collection) mosquitoes **(Table 2.1**) and Cayman strains exposed for 1h, 6h or 8h. Mortality was recorded 24h after the bioassay.

2.2.3.5. Deltamethrin survival with the synergist piperonyl butoxide (PBO)

To test the hypothesis that PBO (an inhibitor of CYP450 and some esterase enzymes) increases susceptibility to pyrethroids, 3-5 day old F1 females (first collection) **(Table 2.1)** were exposed to deltamethrin after pre-exposure to 4% PBO. The following procedure was used; 1h control paper + 1h deltamethrin or 1h PBO + 1h deltamethrin or 1h PBO + 1h control paper or 1h + 1h control paper. Mortality was recorded after 24h as before.

2.2.4. Statistical analysis

The mortality (%) was calculated for the number of mosquitoes or larvae that were dead post 24h exposure. The LC_{50} value for the larval bioassay was calculated using probit regression analysis (SPSS version 24). The resistance ratio (RR) was calculated by comparison of the resistant Makkah and Jeddah strains against the susceptible New Orleans strain using the formula below to monitor the level of insecticide resistance in a field population.

$$\text{Resistance ratio (RR)} = \frac{LC_{50} \text{ of resistant strain}}{LC_{50} \text{ of susceptible strain}}$$

Effects of strains, age, exposure duration and synergist exposure were analysed using generalised linear models with binomial link functions in SPSS version 24. Error bars represent 95%confidence intervals. The cumulative mortality analysis was performed in GraphPad prism7.

2.3. RESULTS

2.3.1. Larval bioassays

Mortality was not observed in any strain in the control assays. Based on the mortality rate across different concentrations of temephos and *Bti*, resistance to the larvicides was higher in field strains when compared to the New Orleans strain (**Table 2.3 and Table 2.4**).

Table 2.3 Average percentage mortality of *Ae. aegypti* larvae from Makkah and Jeddah and the susceptible strain, New Orleans exposed to nine concentrations of temephos.

Strain	Temephos Concentration (mg/L)								
	0.0025 (%)	0.005 (%)	0.01 (%)	0.02 (%)	0.03 (%)	0.04 (%)	0.06 (%)	0.07 (%)	0.08 (%)
Makkah	0	0	9.18	70	89.6	100	99	99	100
Jeddah	1	0	1.02	32	79	73	94.8	98	100
New Orleans	5.2	9.9	52.6	81	96	99	100	100	100

Table 2.4 Average percentage mortality of *Ae. aegypti* larvae from Makkah, Jeddah (field and lab strains), New Orleans and Cayman strains exposed to eight concentrations of *Bti*.

	Bacillus thuringiensis israelensis (*Bti)* Concentration (ppm)							
Strain	0.00012 (%)	0.00024 (%)	0.0006 (%)	0.00089 (%)	0.0012 (%)	0.002 (%)	0.0036 (%)	0.006 (%)
Makkah field	10	19.3	26.2	58.1	63.2	98.8	98.4	100
Jeddah field	0	0	16.4	48.6	59.2	94.9	97.5	100
Jeddah lab	0	5	12.7	21.9	32.2	72.9	93.6	100
New Orleans	20.5	22.9	59.5	70	98.9	100	100	100
Cayman	5.3	12.5	21.6	43.1	52.2	95	100	100

In temephos bioassays, the LC_{50} confidence intervals were not overlapping in comparisons of any strain, therefore the difference in mortality between the strains is significant (**Table 2.5)**. LC_{50} estimates to *Bti* was slightly different among the Saudi strains tested but all were significantly higher than New Orleans **(Table 2.5)**. Current guidelines (Mazzarri and Georghiou, 1995), suggest that a resistance ratio <5 indicates limited/no resistance; 5-10 moderate resistance, and >10 is substantial

resistance. Based on this classification, no definitive resistance to temephos and *Bti* was identified in any of the strains tested.

Table 2.5 Lethal concentrations of temephos and *Bti* that kills 50% of *Ae. aegypti* strains.

	Temephos assay				*Bti* assay	
Strain	LC50, mg/L (95% C.I.)	RR	Chi-squared test (χ^2)	LC_{50}, ppm (95% C.I.)	RR	Chi-squared test (χ^2)
New Orleans	0.010[a] (0.009-0.011)	1	23.3 df=33, *P*= 0.894	0.000407[a] (0.000276-0.000537)	1	13.53 df=30, *P*= 0.996
Makkah lab	0.017[b] (0.014-0.019)	1.7	122.4 df=34, *P* <<0.05	n/c	n/c	n/c
Jeddah lab	0.029[c] (0.025-0.034)	2.9	202.6 df=34, *P* <<0.05	0.001483[b] (0.001341-0.001629)	3.6	13.5 df=30, *P*= 0.996
Makkah field	n/a			0.000834[c] (0.000688-0.000982)	2.1	41.13 df=26, *P*= 0.03
Jeddah field	n/a			0.00098[b,c] (0.000882-0.001076)	2.4	18.7 df=27, *P*= 0.88
Cayman	n/a			0.001018[b,c] (0.000882-0.001157)	2.5	38.4 df=30, *P*= 0.14

Shared letters within a column indicate no significant difference based on overlapping confidence limits. Chi-square tests measure the fit of the model to the data; where *P* <0.05, a correction factor is applied to the confidence intervals. The Makkah lab *Bti* assay was not calculated (n/c) because a very poor fit of the probit model meant that LC_{50} confidence intervals could not be reliably estimated even with a correction factor. Makkah and Jeddah field strains were not assessed (n/a) with temephos because the strains were not available at LSTM. The RR shown in the table indicates the resistance ratio.

2.3.2. Adult bioassays

2.3.2.1. WHO Bioassay 60 min exposure

Bioassays on field (F0-second collection) and laboratory adapted (F1-first collection) Jeddah and Makkah strains indicated a high prevalence of resistance to permethrin, deltamethrin, and bendiocarb. Lab strain and field strain mortality were generally comparable but, in some cases, mortalities were significantly higher in the field than lab strains **(Figure 2.5)**. A high prevalence of resistance to the tested pyrethroids was found in both Jeddah and Makkah and was especially pronounced in permethrin assays in which fewer than 10% of the exposed females died. Similarly, mortality from bendiocarb exposure (tested in lab-adapted females only) was negligible in both Makkah and Jeddah strains. Fenitrothion assays revealed suspected resistance (mortality 90–97%) in Makkah field, confirmed resistance in the Makkah lab-adapted strain, borderline suspected resistance (98%) in Jeddah field and suspected resistance

(92%) in the lab-adapted Jeddah strain **(Figure 2.5)**. Although very similar for permethrin and bendiocarb, and equivocal for fenitrothion (owing to lab adapted vs field variation in Makkah), the prevalence of deltamethrin resistance was higher in Makkah than Jeddah females, whether tested on F1 (χ^2=12.26, df=1, *P*=0.0005) or field females (χ^2= 35.94, df=1, *P*<0.0001).

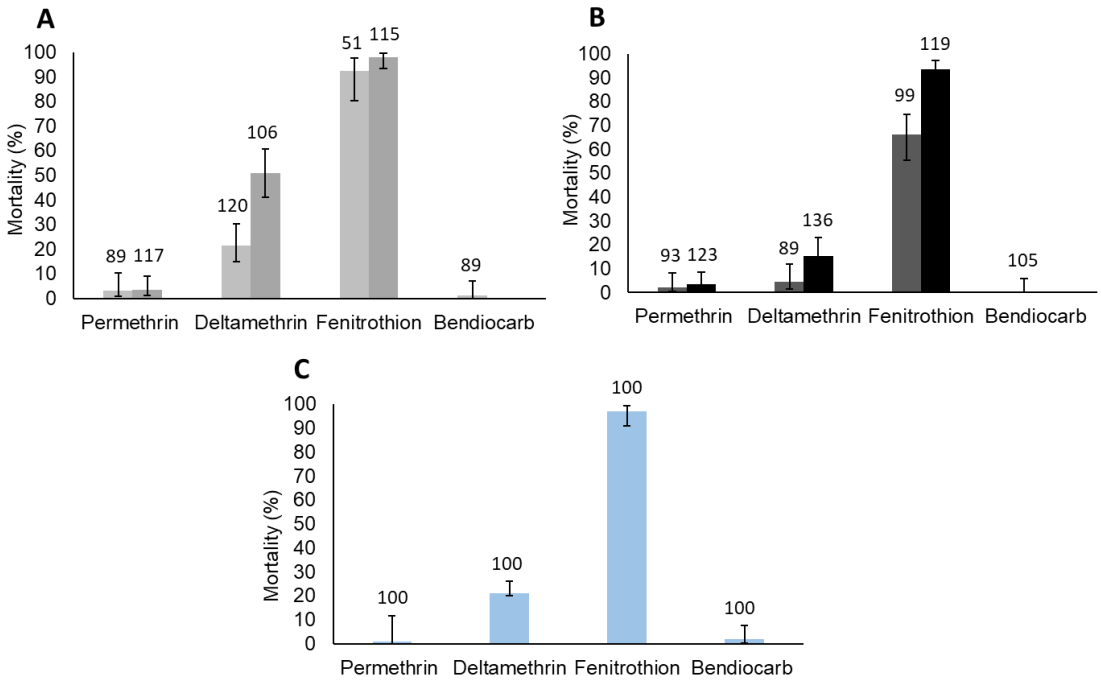


Figure 2.5 Susceptibility status of female *Ae. aegypti* to insecticides in 60 min bioassays with exposure to permethrin, deltamethrin, fenitrothion and bendiocarb. A) Jeddah laboratory strain (light grey) and field strain (dark grey), B) Makkah laboratory strain (dark grey) and field strain (black), C) Cayman Laboratory strain (light blue). Error bars are 95% confidence intervals. The number of *Ae. aegypti* mosquitoes assayed is presented above each bar.

2.3.2.2. Age dependence of deltamethrin-induced mortality (single exposure)

Age had no significant effect on mortality for the Cayman strain (χ^2=2.76, df=1, *P*=0.097) but significantly increased mortality in the Jeddah strain (χ^2=5.46, df=1, *P*=0.02) **(Figure 2.6)**. The GLiM analysis **(Table 2.6)** also showed a significant association of mortality with age in Jeddah (χ^2=14.66, df=1, *P*=0.000129) but not in Cayman (χ^2=1,619, df=1, *P*=0.203). Although there was a significant difference in mortality versus day of exposure in Jeddah strain, the impact of age on mortality was not different until the oldest age (14 days). Therefore, mortality-age association was not a simple linear relationship.

Table 2.6 Generalised Linear Model for the effects of strain and age on deltamethrin-induced mortality of *Ae. aegypti* females.

Source	Wald χ2	df	Probability
(Intercept)	15.4	1	0.000
strain	0.423	1	0.516
day	14.54	1	0.000
strain * day	5.14	1	0.023

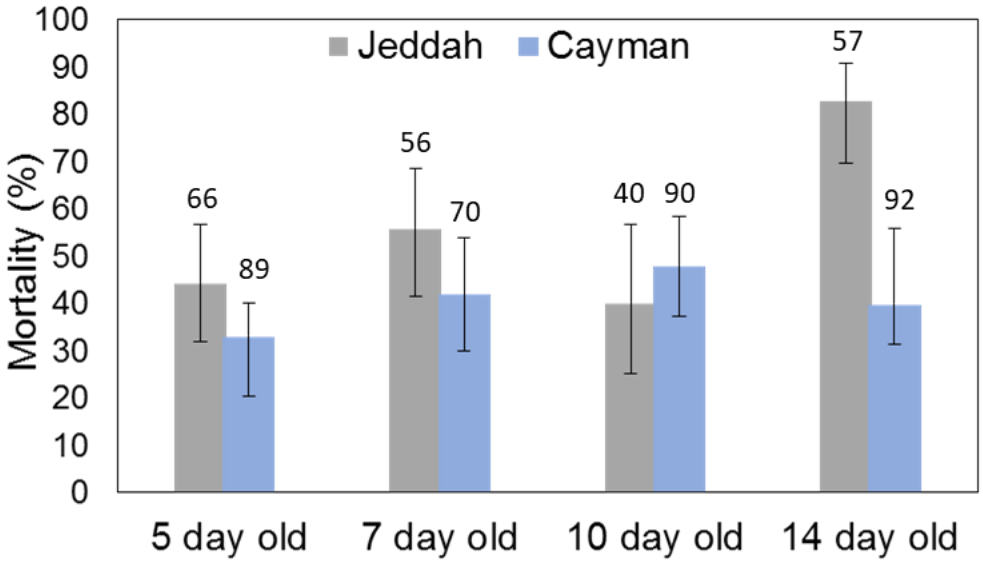


Figure 2.6 Single exposure of Cayman and Jeddah mosquitoes to deltamethrin for 1h at age 5, 7, 10- and 14. The number of *Ae. aegypti* mosquitoes assayed is presented above each bar.

2.3.2.3. Repeated exposure effects

High mortality was observed at the beginning of insecticide exposure which progressively declined as demonstrated by the flattening of the cumulative mortality curve **(Figure 2.7)**. The mortality rate in Jeddah and Cayman strains reduced from 43.7% and 15.1% in day 1 to 0% and 6.3% in day 10 respectively. Mortality was significantly negatively correlated with the number of days of exposure in the Cayman (Spearman rank correlation ρ=-0.77, *P*=0.01) but in the Jeddah strain it was not significant (ρ= -0.42, *P*=0.23). However, there was a highly significant association of survival with repeated exposures in Jeddah (χ2=43.6, df=1, $P=4.1X10^{E-11}$) and Cayman (χ2=12.5, df=1, *P*=0.0004). GLiM analysis showed a significant strain, day and strain*day interaction indicating mortality rate is influenced by the strain or day (corresponding to age) **(Table 2.7)**.

Table 2.7 Generalised Linear Model for effects of multiple exposure to deltamethrin and strain on mortality of *Ae. aegypti* females.

Source	Wald χ2	df	Probability
(Intercept)	36.929	1	1.2252E-9
strain	10.274	1	0.001349
Day	16.383	1	0.000052
strain * day	5.572	1	0.018

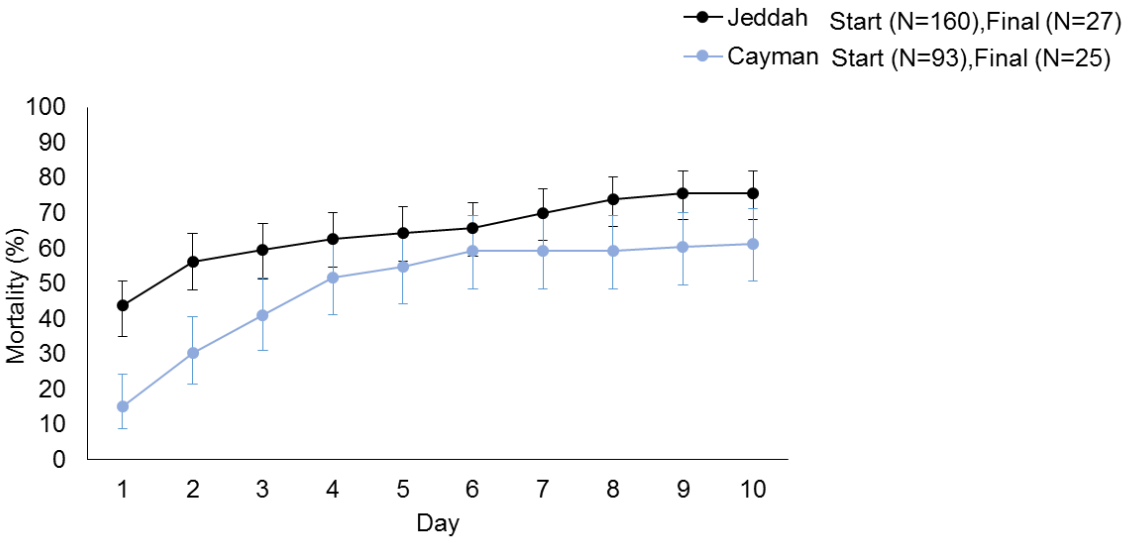


Figure 2.7 Cumulative mortality for each strain at different days. The x-axis represents the number of mosquitoes at the beginning of the experiment and the y-axis is the number of mosquitoes alive at the end of the experiment.

2.3.2.4. Resistance level in young and old mosquitoes

The level of mortality and its dependence on female age was assessed in each strain by exposing either 3-5 day-old or 10 day-old to deltamethrin for progressively longer periods of time: 1h, 6h or 8h. Strain, age, and exposure were all significant in the GLiM, but effects on mortality were not straightforward, as evident from the significance of all two-way interaction terms **(Figure 2.8; Table 2.8)**. In both Saudi strains, the mortality was low following short exposure, though somewhat higher in older females, but mortality was much higher after longer exposures, irrespective of age (70-100%). In contrast, young females of the reference resistant strain Cayman, exhibited no difference in mortality across exposure durations but in older females longer exposures induced greater mortality **(Figure 2.8)**.

Table 2.8 Generalised Linear Model for the effects of strain, age and duration of deltamethrin exposure on mortality of *Ae. aegypti* females.

Source	Wald χ2	df	Probability
(Intercept)	90.28	1	0.000
strain	97.45	2	0.000
age	105.04	1	0.000
exposure	372.62	2	0.000
strain * age	9.16	2	0.010
strain * exposure	163.61	4	0.000
age * exposure	6.92	2	0.032

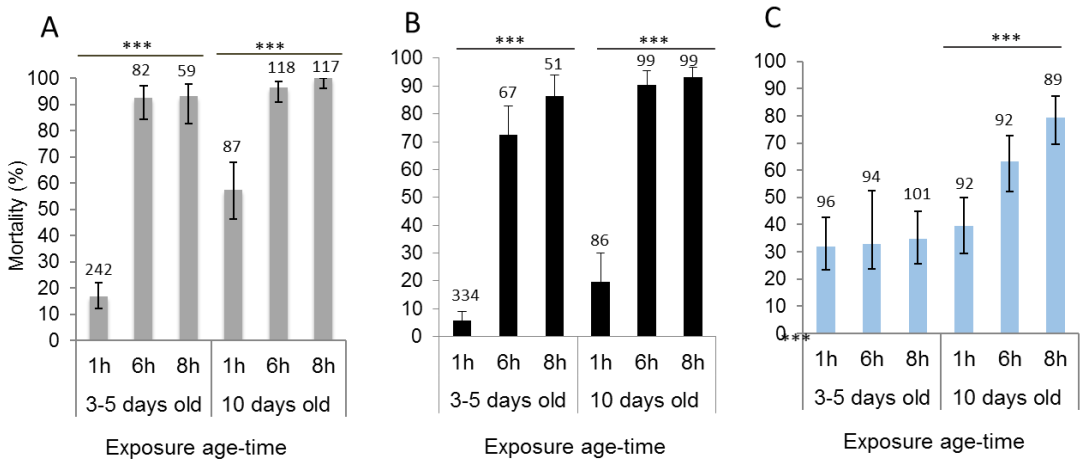


Figure 2.8 Impacts of age and the duration of deltamethrin exposure on survivorship in A) Jeddah, B) Makkah, C) Cayman strains. Statistically significant variation among exposure times (ANOVA) is indicated by***P<0.001. Error bars represent the 95% confidence intervals. The number of *Ae. aegypti* mosquitoes assayed is presented above each bar.

2.3.2.5. Effect of single and repeated deltamethrin exposure on mortality of 10 old mosquitoes

Ten-day old Cayman and Jeddah strains had lower mortality in the group that had been repeatedly exposed (6 times/1h) to deltamethrin compared to the group exposed only once to deltamethrin for either 1h, 6h or 8h as illustrated in **Figure 2.9** because of the diminishing rate across exposures.

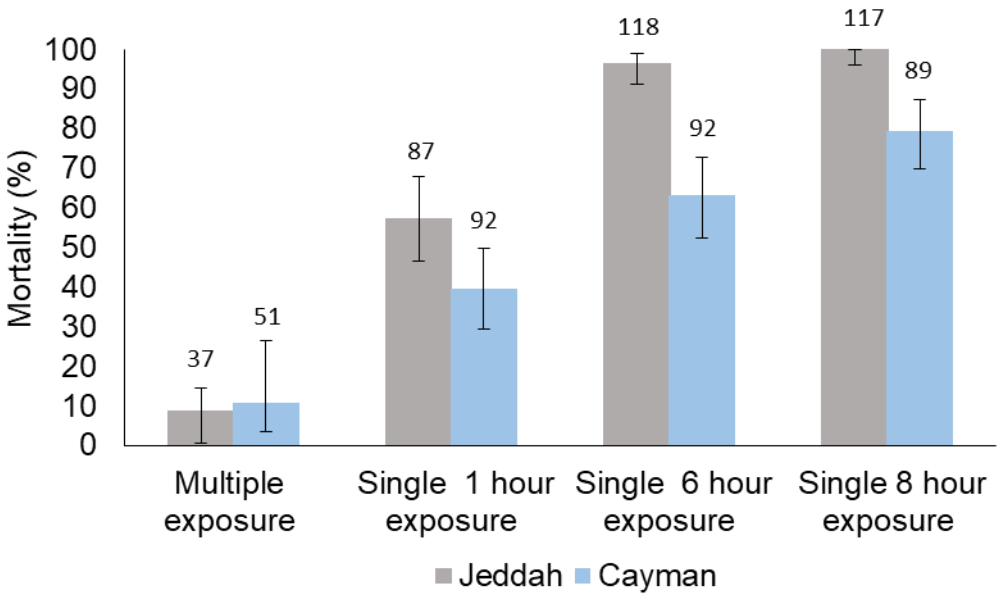


Figure 2.9 A composite figure comparing the effect of different deltamethrin exposure durations on mortality of ten-day old females. In grey and blue is 10 day old Jeddah and Cayman strain, respectively. Error bars represent 95% confidence intervals. The number of *Ae. aegypti* mosquitoes assayed is presented above each bar.

2.3.2.6. PBO synergist bioassay

PBO bioassays significantly increased susceptibility of the Jeddah strain to deltamethrin (χ^2=12.17, df=1, *P*=0.0005) **(Figure 2.10)** but the change was not significant in the Cayman and Makkah strains (Makkah F1 strain, χ^2=2.26, df=1, *P*= 0.13; Cayman strain; χ^2=3.41, df=1, *P*=0.065). The effect of strain and PBO on mortality was significant but the strain-PBO interaction wasn't significant **(Table 2.9)**.

Table 2.9 Generalised Linear Model for the effects of strain and the addition of PBO synergist before deltamethrin exposure on mortality of *Ae. aegypti* females.

Source	Wald χ2	df	Probability
(Intercept)	76.11	1	0.000
strain	18.87	2	0.000
PBO	13.78	1	0.000
strain * PBO	1.83	2	0.401

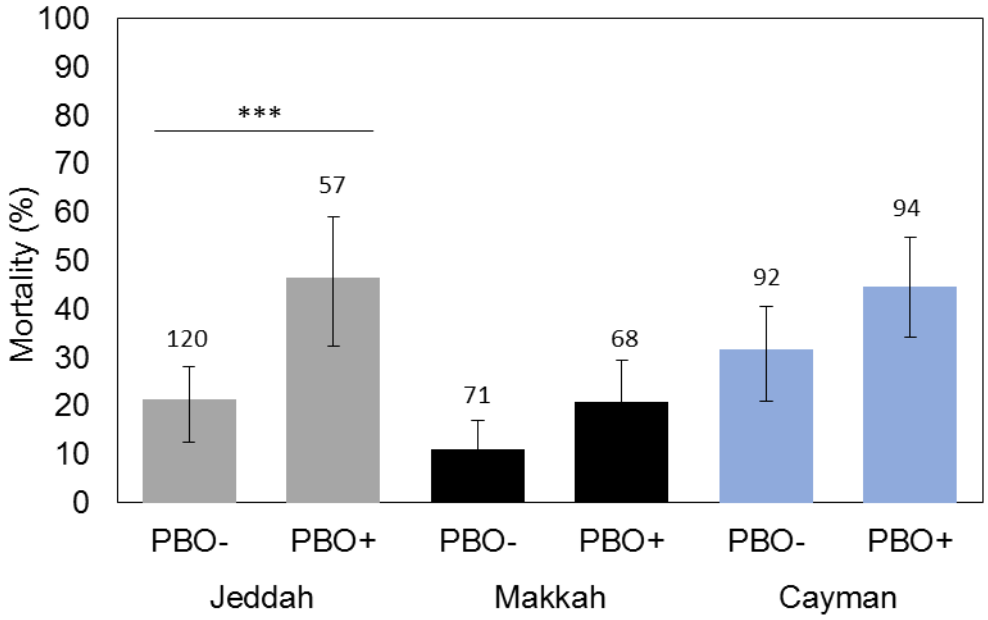


Figure 2.10 Deltamethrin 60 min bioassays against field and lab-reared mosquitoes, with and without a 60 min pre-exposure to the synergist PBO (PBO+, PBO- respectively). Statistical significance is indicated by ****P*<0.001. Error bars are 95% confidence intervals. The number of *Ae. aegypti* mosquitoes assayed is presented above each bar.

2.4. DISCUSSION

The current study was conducted to assess the susceptibility of *Ae. aegypti* to commonly used insecticides in the cities of Jeddah and Makkah. Larval bioassays did not detect resistance in either Makkah or Jeddah to temephos or *Bti* (all resistance ratios <5 suggest a lack of resistance; (World Health Organization, 2016a). In contrast, extreme temephos resistance in *Ae. aegypti* larvae from Jazan (LC_{50}=61.8 mg/L) was reported in 2016 (Alsheikh et al., 2016). When compared to the average LC_{50} of multiple separate studies of the susceptible reference strains Rockefeller, New Orleans and Bora Bora (Moyes et al., 2017), this equates to a resistance ratio above 10,000, far exceeding the highest ratio of 224 previously recorded (in Brazil; Moyes et al. 2017). This estimate from Jazan thus appears unlikely to be correct, and in the absence of additional data, a provisional assessment of temephos susceptibility in Saudi Arabia seems appropriate. Temephos resistance in *Ae. aegypti* larvae has been recorded globally including British Virgin Islands (Wirth and Georghiou, 1999), Thailand (Ponlawat et al., 2005), Brazil (Melo-Santos et al., 2010), Cuba (Bisset et al., 2011), Colombia (Grisales et al., 2013), Martinique (Marcombe et al., 2012) and Santiago island (Rocha et al., 2015). Resistance to organophosphates including temephos in *Aedes* has been attributed to target site insensitivity (Muthusamy and Shivakumar, 2015) and differential expression of metabolic, transport, cuticle and other genes (Grigoraki et al., 2015, Grisales et al., 2013). Target site insensitivity is associated with mutations on the acetylcholinesterase (*Ace-1*) gene. The G119S mutation on this gene has been linked to organophosphate resistance in *Culex* and *Anopheles* species, but rarely reported in *Ae. aegypti* (Grisales et al., 2013, Marcombe et al., 2012) until recently (Muthusamy and Shivakumar, 2015). The mutation was also absent in the Jeddah and Makkah populations (Chapter 3, section 3.3.1.2). Despite this, they were highly resistant as adults to bendiocarb (a carbamate) and slightly resistant to fenitrothion (an organophosphate). The absence of this mutation in these strains suggests target-site insensitivity and does not contribute to carbamate and organophosphate resistance but detoxification enzymes or another mechanism may be involved (Grisales et al., 2013, Marcombe et al., 2012).

Bacillus thuringiensis israelensis (*Bti*) is a bacterial derived toxin that has been widely used for vector control. The populations from Jeddah and Makkah were susceptible

to this compound in comparison with the New Orleans strain and hence, this represents an effective tool for controlling them despite being highly resistant to pyrethroids and bendiocarb. Other studies have reported similar findings including Martinique populations that were susceptible to *Bti* compared to the Bora-Bora strain (Marcombe et al., 2012), Santiago island (Rocha et al., 2015), Cameroon (Kamgang et al., 2011) and Malaysia (Loke et al., 2010). Although rare in field populations, resistance was detected in *Cx. pipiens,* from Syracuse, New York which had a resistance ratio of 33 fold when compared to the S-Lab susceptible strain (Paul et al., 2005). Resistance to *Bti* has also been demonstrated in *Aedes rusticus Rossi* mosquitoes, selected for resistance through annual *Bti* treatment in larval sites in the Rhône-Alpes region. The mosquitoes collected in the treatment area had a low resistance ratio up to 7.9 fold compared to the untreated area (Boyer et al., 2012). The attained resistance levels were still relatively low compared to when mosquitoes are selected for resistance to other insecticides (Boyer et al., 2012). The multiple active toxins-Cry4A, Cry4B, Cry11A and Cyt1A- produced by *Bti* might act at different receptors, making evolution of resistance to *Bti* very difficult (Wirth, 2013). Although the *Ae. aegypti* population in Jeddah and Makkah are still susceptible to temephos, rotational application of *Bti* and temephos, or another larvicide to which there is full susceptibility, would be advisable to slow down evolution of resistance to either of them thus retaining their efficacy over extended periods of use in vector control. Our finding indicates the population from the two cities had a significantly higher LC_{50} to *Bti* compared to the New Orleans strain. This suggests that they might have the potential to develop resistance to *Bti* and hence continued monitoring is advisable, along with consideration of other options such as insecticide growth regulators. Previous studies using WHO susceptibility tests conducted on wild *Ae. aegypti* from Makkah found resistance to 0.05% lambda-cyhalothrin (mortality 77%) and deltamethrin (mortality 86%) and suspected resistance to 0.15% cyfluthrin (mortality 90%) (Aziz et al., 2011). In the present study, the populations from Jeddah and Makkah were highly resistant to deltamethrin (mortality F1=4.4%;field F0=15.4%) with higher prevalence than that reported in Makkah (Aziz et al., 2011). The continued use of pyrethroids for control of adult populations in Makkah is possibly selecting for extreme levels of resistance in this population, thus calling for

management of insecticide resistance before control failure is experienced, a situation previously reported in *An. funestus* species in South Africa (Hargreaves et al., 2000). A worrying observation is the high level of resistance to bendiocarb and at least reduced susceptibility to fenitrothion, which belong to the only other classes of insecticides fully approved for adult *Aedes* control. The existence of multi-insecticide class resistance limits the options available for management of resistance in these populations, and testing of new insecticidal products involving neonicotinoid clothianidin and the pyrrole chlorfenapyr for *Aedes* control should be a priority.

Longevity is a key factor for disease transmission in disease vectors. Prolonged survival of vectors in the wild increases their chances of them becoming infected, and of a successful completion of the extrinsic incubation period of the pathogens allowing subsequent transmission in later feeding events. In previous studies conducted on *Aedes* and other species, age has been negatively associated with insecticide exposure survival even in resistant populations (Rajatileka et al., 2011, Chouaibou et al., 2012, Hunt et al., 2005, Hodjati and Curtis, 1999, Lines and Nassor, 1991, Rowland and Hemingway, 1987, Harrington et al., 2001a). For example unfed 4 day old *An. funestus* survival was significantly higher than older (10day) mosquitoes after 24h post exposure to 0.1% lambda-cyhalothrin (Hunt et al., 2005). Rajatileka et al., (2011) observed unfed young (3 day) *An. gambiae* lab strains from Zanzibar-Tanzania, Kisumu-Kenya, and Akron-Benin and *Ae. aegypti* from Merida-Mexico and Ho Chi Minh-Vietnam survived significantly more 24h post-exposure to DDT, bendiocarb and deltamethrin compared to their 14 days old unfed counterparts (Rajatileka et al., 2011).

In *Ae. aegypti* from Makkah and Jeddah, ten-day-old females were significantly more susceptible to deltamethrin than 3–5 days old in a standard WHO susceptibility test. Increased susceptibility to pyrethroids at older age in resistant field populations is one of the explanations why IRS and LLIN remain effective in reducing malaria transmission even in highly resistant populations. Although the barrier effect of the nets may be equally important (Strode et al., 2014). LLINs in the context of resistant *An. gambiae* population may no longer be toxic to young mosquitoes but they are still partially effective in killing old mosquitoes especially infected mosquitoes which have been reported to be more susceptible compared to uninfected mosquitoes

(Alout et al., 2017). However, increased susceptibility to deltamethrin with age was not observed in the Cayman strain thus indicating the trait is not universal across all mosquito populations. Data on efficacy of control intervention against *Aedes* is limited hence it is difficult to ascertain whether insecticides will remain effective in controlling transmission of arboviruses by *Aedes* populations highly resistant to insecticides (Moyes et al., 2017).

With long exposure times of six or eight hours, up to 30% survival was observed, demonstrating that not only are most of the population classed as resistant (from 60 min bioassay data), but at least a small proportion should be regarded as highly resistant, even when tested as older females. Surprisingly, given the slightly lower resistance at 60 min, Cayman females of either age class survived long exposures better, suggesting a dissociation between the incidence and level of resistance, as documented in *An. gambiae* (Toé et al., 2014). Multiple or repeated exposure to pyrethroids diminished the mortality rate, suggesting that there are indeed resistant extremes in the population that can survive either high level exposure or multiple exposure. If these extreme individuals possess a heritable element absent in others, if they reproduce successfully, extreme resistance could evolve.

Females from Jeddah suffered a significant increase in deltamethrin mortality, (more than double) following pre-exposure to PBO. For Makkah (and Cayman) the increase was slighter and not significant in either case, although the difference among populations was not large enough for detection of statistically significant heterogeneity. Similarly, PBO caused little change in mortality suggesting only a minor role for the PBO-targeted metabolic enzymes in permethrin resistance in the Cayman population (Harris et al., 2010). This contrasts with the almost complete synergy of deltamethrin by PBO (from approximately 5 to 98% mortality) reported by Bingham et al., in the Nha Trang *Ae. aegypti* strain from Vietnam (Bingham et al., 2011), which, together with overexpression of the strongly pyrethroid-metabolising gene *CYP9J32* (Stevenson et al., 2012) relative to the susceptible Bora Bora strain, was interpreted as evidence for a dominant role of CYP450 enzymes in deltamethrin resistance. While the impact of PBO was clear, some caution is required in causal links with CYP450s because, while the action of P450s can be blocked, as demonstrated

directly *in vivo* in *Ae. aegypti* (Kasai et al., 2014) other effects such as increased cuticular penetration may occur (Sanchez-Arroyo et al., 2001).

2.5. CONCLUSION

Aedes aegypti from Makkah and Jeddah were found to be resistant to three classes of insecticides recommended for public health use, and very strongly resistant to pyrethroids and a carbamate. Comparing the results in this study, and from previous work conducted in the region, suggests that the prevalence of resistance to pyrethroids may be rising and an assessment of pyrethroid use for vector control is warranted. Insecticide resistance management is recommended in these cities before the populations fail to respond to the insecticides being used. The populations remain susceptible to the larvicides assessed in this study and thus larval source management and larviciding could remain an effective tool in control, given appropriate formulations and implementation.

CHAPTER 3
TARGET SITE (KDR) MUTATIONS AND CYTOCHROME P450-BASED METABOLIC MECHANISMS PLAY A PRIMARY ROLE IN PYRETHROID RESISTANT PHENOTYPES OF *AEDES AEGYPTI* FROM SAUDI ARABIA

3.0. ABSTRACT

Background Pyrethroid resistance is a threat to effective vector control of *Ae. aegypti*, the vector of dengue, Zika and other arboviruses, but there are major knowledge gaps on the mechanisms of resistance. In Saudi Arabia and the Middle Eastern region, pyrethroids are used widely for *Ae. aegypti* control but the underlying genetic basis of resistance is unknown.

Methods Two fragments of the voltage-gated sodium channel (*Vgsc*), encompassing four previously identified mutation sites, and part of the Acetylcholinesterase (*Ace*-1) gene were sequenced and subsequently genotyped to determine associations with resistance. Expression of five candidate genes (*CYP9J10*, *CYP9J28*, *CYP9J32*, *CYP9M6*, *ABCB4*) identified from published studies as associated with pyrethroid resistance was compared between assay survivors and controls.

Results Three potentially-interacting *Vgsc* mutations were detected, V1016G and S989P were in perfect linkage disequilibrium in each strain and strongly predicted survival, especially in the Makkah strain, but were in negative linkage disequilibrium with 1534C, though a few females with the *Vgsc* triple mutation were detected. No mutations in the *Ace-1* gene was observed in Jeddah and Makkah strains. The candidate gene *CYP9J28* was significantly over-expressed in Jeddah compared to two susceptible reference strains, but none of the candidate genes were consistently up-regulated in the Makkah strain.

Conclusions Despite their proximity, Makkah and Jeddah exhibit some differences in pyrethroid resistance phenotypes, with results suggesting a different balance of mechanisms. For example, and consistent with previous PBO results, there may be more impact associated with CYP450s in the Jeddah strain than in the more resistant Makkah strain where the dual *kdr* mutations 989P and 1016G are present at a higher frequency. The results overall demonstrate a major role for the paired target site

mutations in pyrethroid resistance and highlight their utility for diagnostic monitoring.

3.1. INTRODUCTION

Aedes control using insecticides will remain a key intervention for dengue prevention, especially given the added benefit of simultaneously targeting other *Aedes* transmitted arboviruses including chikungunya and Zika. In Saudi Arabia, several areas are endemic regions for the dengue virus of which Jeddah and Makkah are by far the most important (Chapter 1, section 1.6). Multiple insecticides are applied to target immature vector stages in Makkah and Jeddah, but pyrethroids are the most commonly used to control adult *Ae. aegypti* by indoor and outdoor space spraying. The efficacy of pyrethroid based adult control has been found to be impacted by insecticide resistance in *Ae. aegypti* from diverse locations (Marcombe et al., 2012, Plernsub et al., 2016a). Information on the insecticide resistance status of Saudi Arabian populations of *Ae. aegypti* remains limited (Aziz et al., 2011, Alsheikh et al., 2016), and is absent for Jeddah, the primary national centre for dengue. The most common mechanisms of insecticide resistance in *Ae. aegypti* involve target site alterations and metabolic resistance, comprising altered activity of enzymes from three superfamilies, esterases (CCEs), glutathione-s-transferases (GSTs) and cytochrome P450s (CYP450s), although several other enzyme families are likely to be involved (Faucon et al., 2015).

Overexpression of *Ae. aegypti* CYP450s, especially from the CYP9 and CYP6 subfamilies has frequently been implicated in pyrethroid resistant phenotypes (David et al., 2013), and several overexpressed P450s have been shown to metabolise pyrethroids *in vitro* (Stevenson et al., 2012, Chandor-Proust et al., 2013, Kasai et al., 2014). Pyrethroid and DDT target site resistance results from amino acid substitutions in the voltage-gated sodium channel (*Vgsc*) that usually reduce insecticide binding, although other mechanisms unrelated to binding are known (Hemingway and Ranson, 2000, Dong, 2007). Some mutations in the insect *Vgsc* can prevent the normal action of pyrethroids and DDT (repetitive nerve firing, paralysis and death) leading to knockdown resistance (*kdr*) (Dong, 2007).

Multiple *kdr* mutations have been recorded in *Ae. aegypti* worldwide including G923V, L982W, I1011M/V, S989P, V1016G/I, F1534C, V410L and D1763Y (Vontas et al., 2012, Chang et al., 2009, Haddi et al., 2017). Of these, I1011M, V1016G/I

(Saavedra-Rodriguez et al., 2007), S989P (Wuliandari et al., 2015) and F1534C (Harris et al., 2010, Yanola et al., 2010), either individually or in combination, have been associated directly with pyrethroid resistance. The vast majority of studies of resistance mechanisms in *Ae. aegypti* are from the Americas and Asia (Smith et al., 2016, Vontas et al., 2012, Ranson et al., 2010), though a study from West Africa identified the presence of target site resistance shared with American populations (Kawada et al., 2016). Knowledge of resistance mechanisms in the Middle East and their relationship with other regions is currently lacking.

3.1.1. Objectives

1- To investigate the resistance profiles of *Ae. aegypti* strains from Jeddah and Makkah, focusing on pyrethroid resistance and its underlying mechanisms (in comparison with established resistant and susceptible strains).

3.2. MATERIALS AND METHOD

3.2.1. Mosquito strains, collection and rearing

Adult females from the first collection as described in **Table 2.1** (see Chapter 2, section 2.2.1) were used for phenotypic bioassays and characterisation of molecular resistance mechanisms. Cayman, a resistant lab strain (Harris et al., 2010) and the standard susceptible strains New Orleans and Rockefeller were used a reference in qPCR. All strains were raised under the same conditions (see Chapter 2, section 2.2.1).

3.2.2. Target site mutations

3.2.2.1. Sequencing of Voltage gated sodium channel (*Vgsc*)

Genomic DNA was extracted from F0 females (first collection; **Table 2.1**) from the Jeddah (N=26), and Makkah (N=15) strains using Nexttec kits (Nexttec™, Biotechnologie GmbH, Hilgertshausen, Germany) according to the manufacturer's instructions. PCR primers (IIS5-6 (3) F 5'-ATC GCT TCC CGG ACA AAG AC-3'; IIS5-6 (3) R 5'-GTT GGC GAT GTT CGA CTT GA-3') were designed using Primer 3 (Koressaar and Remm, 2007, Untergasser et al., 2012) to amplify *kdr* mutations in the sixth transmembrane segment of domain II of the *Vgsc*, which includes the resistance-associated codons 989, 1011, 1016. A segment of domain III that includes codon 1534 from exon 30 was amplified using the primers AaNa31F and AaNa31R(Harris et al., 2010). PCR reactions were carried out in a 25 µl reaction volume containing 12.5 µl TaqRed Mix (Bioline, London, UK), 0.5 µl forward and reverse primers (10µM) and 2 µl of DNA extract. Cycling conditions for the domain II primers were 95°C for 5 min, 35 cycles of (94°C for 30s, 65°C for 30s, 72°C for 1 min) and a 10 min final elongation step at 72°C. For the domain III primers, conditions were the same except that the annealing temperature was 62°C for 30s. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Manchester, UK) and sequenced commercially (Source Bioscience, Rochdale, UK). For detection of the mutation in codon 410, the primers and method were from the study which first discovered the mutation (Haddi et al., 2017).

Sequence data were assembled and aligned using Codon Code Aligner version 4.2.7. A TaqMan SNP genotyping assay for F1534C was designed **(Figure 3.1)** and compared

to an existing PCR tetraplex assay (Harris et al., 2010)and validated by comparison with DNA sequencing of field-caught Jeddah and Makkah mosquitoes **(Figure 3.2)**.

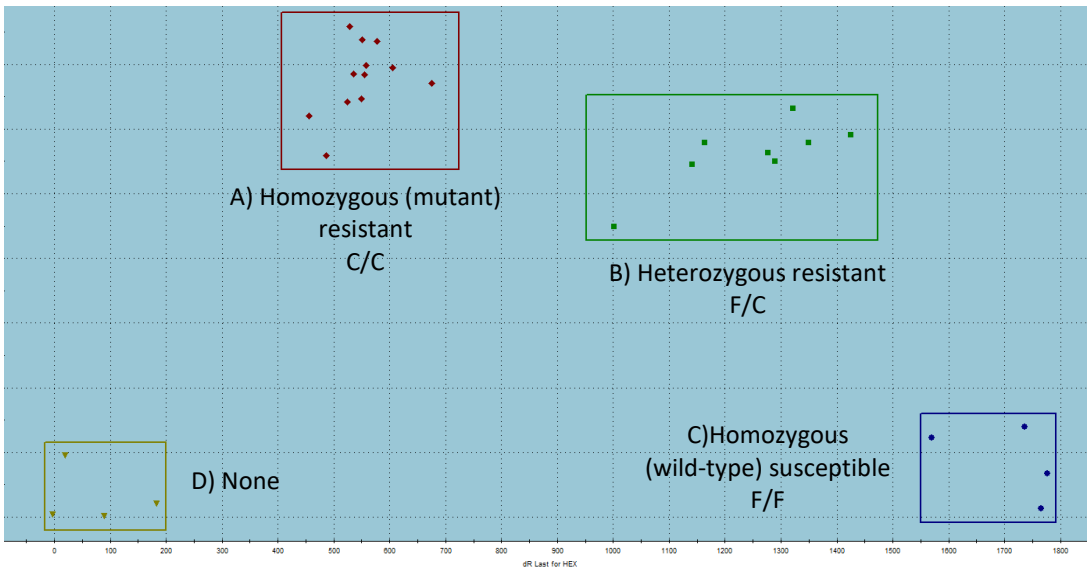


Figure 3.1 Example of a scatter plot for F1534C genotype. A) 12 Homozygous resistant mosquitoes B) 8 Heterozygous resistant (C) 4 Homozygous susceptible, D) 4 None (failed not scored).

TaqMan reactions were performed in a 10 µl final volume containing 5 µl of TaqMan® Gene Expression SensiMix (Applied Biosystem, Foster city, USA), 0.125µl primer/probe, 3.875 µl sterile water (Sigma, Gillingham, Dorset, UK) and 1 µl of DNA. Assays were run on an Agilent MX3000P qPCR thermal cycler under the following conditions a 10 min cycle at 95°C, and 40 cycles of 92°C for 15 min and 60 °C for 1min. Based on 21 samples, the concordance between the TaqMan SNP screening results and DNA sequencing analysis was 100%. Results were concordant between assays in 19 of 21 samples with remaining two genotypes giving an unclear score using the tetraplex method.

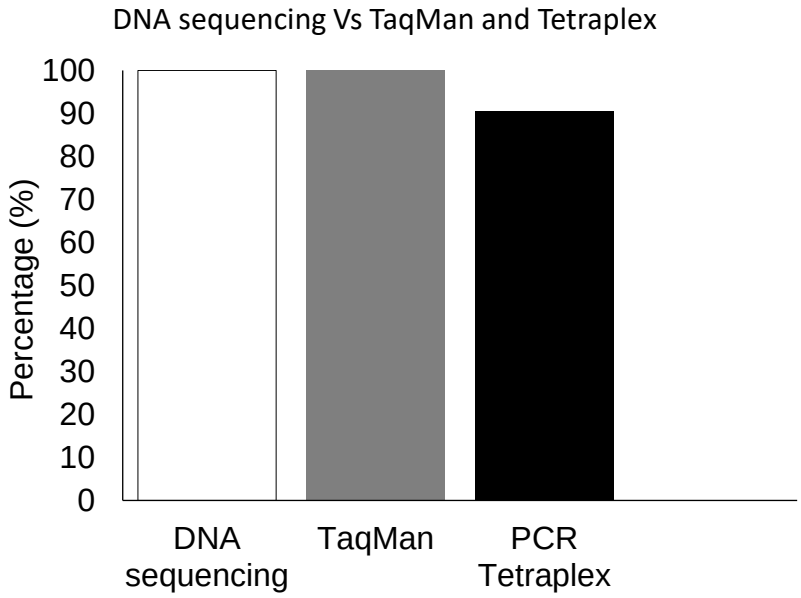


Figure 3.2 The concordance percentage (%) for genotyping results between DNA sequencing, TaqMan and tetraplex technologies. DNA sequencing and TaqMan presented the same genotyping results (100%) but the genotyping PCR tetraplex gave 85% so was less efficacious than DNA sequencing or TaqMan.

To investigate the association between target site mutations and survival, F1 females from the Jeddah and Makkah strains were exposed to deltamethrin in WHO bioassays for either a standard 1h duration or for longer periods of 4–6h. Genotypes of females killed by 1h of deltamethrin exposure (susceptible), were compared with survivors of the longer exposures to produce strongly distinct resistance phenotypes.

3.2.2.2. Sequencing of Acetylcholinesterase 1 (*Ace-1*)

Results for resistance of carbamate (bendiocarb) and organophosphate (fenitrothion) in Jeddah and Makkah was presented in Chapter 2, section 2.3.2 (**Figure 2.5)**. To investigate any presence of resistance mutations associated with the above insecticide resistance, *Ace-1* cDNA from F0 Makkah and Jeddah (10 individual or 10 pools of 10 mosquitoes) was amplified for the *Ace-1* gene using the primers from (Muthusamy and Shivakumar, 2015). The reaction was performed in a 15µl final volume containing 1.5µl of kapa Taq buffer, 0.75µl $MgCl_2$, 0.12µl DNTP, 0.51µl primer F/R, 0.12µl KAPA Taq (KAPA Taq PCR Kit, US), 10.5µl sterile water and 1µl of gDNA template. Cycling conditions for the primers were 95°C for 5 min, 35 cycles of (94°C for 30 s, 62°C for 30 s, 72°C for 1 min) and a 10 min final elongation step at 72°C. The

PCR product was purified, sent for direct sequencing and data analysed as described in section 3.2.2.1.

3.2.3. Gene expression

Expression profiles of four CYP450 genes previously associated with *Ae. aegypti* pyrethroid resistance (*CYP9J10*, *CYP9J28, CYP9J32*, *CYP9M6*) and an ABC transporter (*ABCB4*) (Kasai et al., 2014, Bariami et al., 2012, Strode et al., 2008) **(Appendix 1: Table 8.4)** were assessed using quantitative reverse transcription PCR (qRT-PCR), in relation to two susceptible strains, New Orleans and Rockefeller. Total RNA was extracted from three pools of five female mosquitoes in each strain, which had not been exposed to insecticide, using the Ambion RNAqueous® Kit (LifeTechnologies, Paisley, UK), with the quantity of RNA yields assessed using a Nanodrop ND-1000 (Thermo Scientific, Delaware, USA). Synthesis of cDNA used Superscript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines, and cDNA was purified using a QIAquick spin column (QIAuick PCR Purification Kit, Qiagen). The qRT PCR reactions were performed in a volume of 10µl with 5µl of SYBR® Green (Applied Biosystems, Texas, USA), 0.4µl forward and reverse primer (10µM), 3.2µl ddH_2O, and 1µl of cDNA (approximately 2ng), under the following conditions 95°C for 3 min, followed by 40 cycles of 95°C for 10s and 60°C for 10s. The relative expression level and fold change (FC) of each candidate gene relative to the susceptible strains was calculated using the ΔΔcT method (Schmittgen and Livak, 2008) after normalisation with two housekeeping genes, RPS3 (ribosomal protein S3) and the 60S ribosomal protein L8. All primer sequences and their origins are shown in **Appendix 1: Table 8.4.**

3.2.4. Statistical analyses

Pearson chi-square tests were used to assess associations between *kdr* mutations and phenotypes in genotypic and allelic tests, with odds ratios used to measure effect size. Error bars represent 95% confidence intervals calculated using the method of Wilson, with continuity correction (Newcombe, 1998). Haploview (Barrett et al., 2005) was used to estimate *kdr* haplotype frequencies and perform haplotype association tests. Two-tailed t-tests were used to compare candidate gene expression

levels between strains; significance was accepted only when detected between a resistant strain and both susceptible strains.

3.3. RESULTS

3.3.1. Target site mutations

3.3.1.1. Sequencing of voltage gated sodium channel (*Vgsc*)

DNA sequencing from wild (F0) mosquitoes of Jeddah and Makkah populations revealed three *kdr* substitutions in the *Vgsc* S989P, V1016G and F1534C but neither of the I1011M or I1011V mutations, nor any others in this area (GenBank Accession Nos. S989P_V1016G KY626180–KY626197, GenBank Accession Nos F1534C KY046222–KY046237). In addition, the V410L mutation was not found. The V1016G and S989P mutations were in perfect linkage disequilibrium (LD) in the Jeddah (N=26) and Makkah (N=15) F0 collections (r^2=1), with allele frequencies for each of 0.46 (95% CI0.33–0.59) and 0.67 (95%CI 0.49–0.81), respectively. The allele frequency of 1534C was 0.5 (95% CI 0.37– 0.63) in Jeddah and 0.4 (95% CI 0.25–0.58) in Makkah and F1534C was in strong, but imperfect, repulsive LD with S989P/V1016G (r^2=0.59 in Jeddah and 0.87 in Makkah); such that resistance alleles rarely co-occurred. However, five Jeddah females and one Makkah female possessed a triple mutant (989P+1016G+1534C) haplotype **(Appendix 1: Table 8.5)**. To evaluate impacts of the mutations on deltamethrin survival, haplotypes (estimated from genotypic data) of mosquitoes killed by standard duration WHO susceptibility bioassays (<20% mortality in each strain) were compared with survivors of long 4-6h bioassays (≥ 60% mortality in each strain). Four haplotypes were estimated in the two populations **(Table 3.1)** of which 989P+1016G+F1534 was strongly associated with resistance and S989+V1016+1534C with susceptibility. The triple mutant 989P+1016G+1534C was somewhat more common in susceptible females, but the estimated frequencies were very low. Six distinct and one ambiguous triple-locus genotype could be discerned though not all were present in both Makkah and Jeddah **(Table 3.1)**.

In concordance with the haplotypic tests, the double locus mutant genotype 989P/P+1016G/G+ 1534 F/F and single locus mutant genotype 989S/S+1016 V/V+ 1534C/C (numbers 1 and 2 in **Table 3.2**) differed strongly in the effect on resistance (χ^2=27.7, df=1, *P*<0.0001; OR=79.2, 95%CI 12–522), with the latter more common in susceptible females. Though relatively rare, none of the six individuals possessing genotypes which must have contained a triple mutant allele (i.e. 989P+1016G+1534C; genotype numbers 6 and 7) survived exposure, suggesting a

lack of resistance conferred by this allele when heterozygous. Interestingly, the triple heterozygote genotype (number 5 in **Table 3.2**) showed significant association with resistance (χ2=17.4,df=1,*P*<0.0001; OR=29.2, 95%CI 5-164), which was approximately 2.7 times lower than the most resistant genotype (number 1), though not significantly so (*P*=0.36) **(Table3.2)**.

Table 3.1 Estimated haplotype frequencies and their association with deltamethrin resistance in *Ae. aegypti* from each strain.

		Jeddah				Makkah		
Haplotype	Number	Susceptible	Resistant	*P*-value	Number	Susceptible	Resistant	*P*-value
989P/1016G/F1534	10	0.187	0.628	6×10^{-6}	12	0.143	0.853	2×10^{-8}
S989/V1016/1534C	18	0.655	0.338	0.002	9	0.786	0.147	4×10^{-7}
S989/V1016/F1534	4	0.079	0.030	0.319	0	0.036	0.000	0.269
989P/1016G/153C	2	0.079	0.004	0.096	0	0.036	0.000	0.269

Table 3.2 Triple-locus *kdr* genotypes, shown as amino acids at codons 989, 1016 and 1534, and their frequencies in mosquitoes surviving either a long (4–6h) deltamethrin exposure or killed by a 1h exposure.

		Jeddah		Makkah		Combined				
Genotype		Alive	Dead	Alive	Dead	Alive	Dead	Mortality	LCL	UCL
1	**PG**F/**PG**F	7	3	12	0	19	3	0.14	0.04	0.36
2	SV**C**/SV**C**	2	16	0	9	2	25	0.93	0.74	0.99
3	SVF/SV**C**	0	4	0	0	0	4	1.00	0.40	1.00
4	SVF/**PG**F	1	1	0	1	1	2	0.67	0.13	0.98
5	SV, SV**C**/**PG**F, **PGC**	9	3	5	3	14	6	0.30	0.13	0.54
6	SV**C**/**PGC**	0	3	0	1	0	4	1.00	0.40	1.00
7	**PG**F/**PGC**	0	2	0	0	0	2	1.00	0.20	1.00

Mutant amino acids are shown in bold type. LCL and UCL are 95% binomial confidence limits. Note that for genotype 5, alleles could not be determined unambiguously and alternates are shown.

3.3.1.2. Sequencing of the Acetylcholinesterase 1 (*Ace-1*) gene

The *Ace-1* gene was amplified in 10 individuals and 5 pools of 10 mosquitoes from the Jeddah and Makkah field populations studied. Neither the G119S, nor any other mutation was identified in the *Ace-1* gene fragments sequenced from the Saudi strains **(Figure 3.3)**.

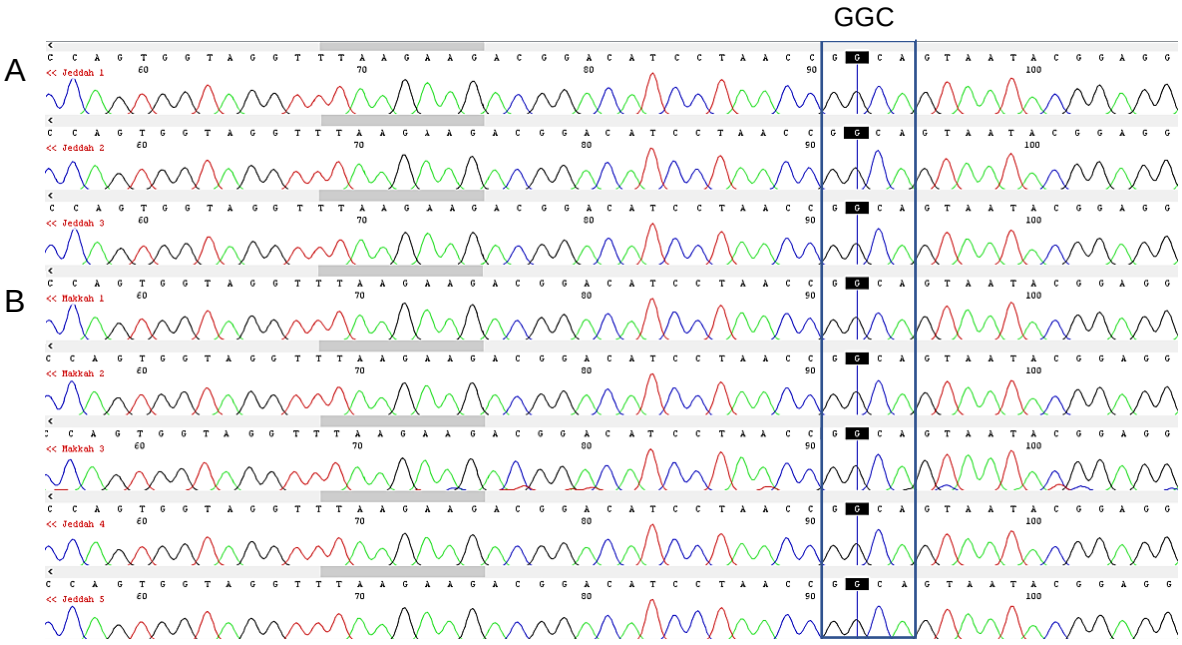


Figure 3.3 Chromatograms of nucleotide sequence from Codon code aligner software illustrating no mutation at position 119. Example of samples A) Jeddah, B) Makkah. GGC is a wild-type codon.

3.3.1.3. Gene expression and resistance

Quantitative real-time PCR in all strains (each unexposed to insecticide) showed several genes to be more highly expressed than in the New Orleans colony. However, when considering comparisons with Rockefeller, fewer of the candidate genes were significantly upregulated **(Figure 3.4)**. In the Makkah strain only *ABCB4* neared significance; in the Jeddah strain, *CYP9J28* and *CYP9J10* were significantly and consistently overexpressed to high levels; and in the Cayman strain, the *CYP9M6* gene was over-expressed compared to both susceptible colonies. In each case, expression levels of the four P450 genes were lower in Makkah than in Jeddah.

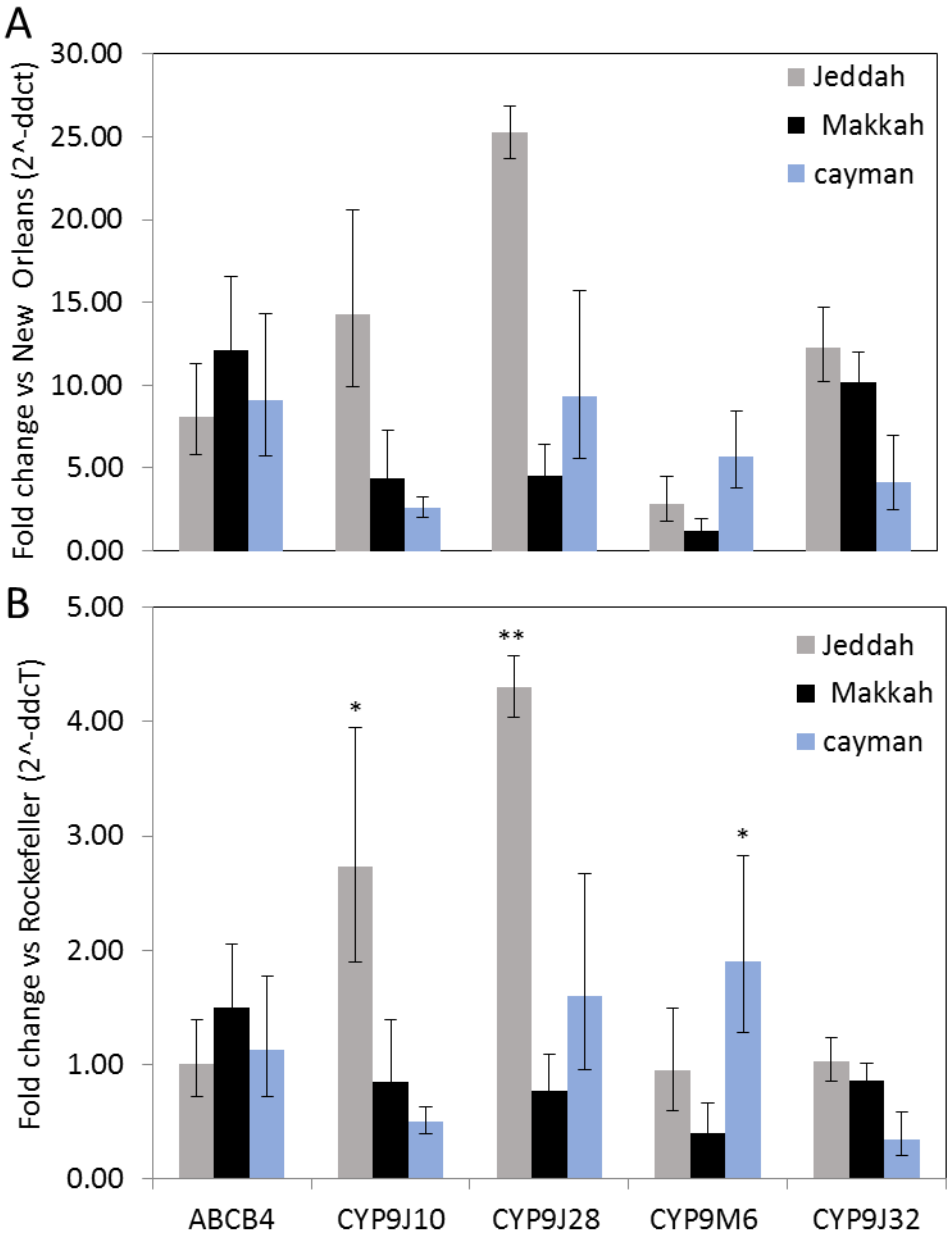


Figure 3.4 Quantitative PCR analysis of candidate genes. Relative-fold changes compared to two susceptible strains. A) New Orleans, B) Rockefeller are shown following normalisation to two endogenous reference genes. Error bars represent 95% confidence intervals. Significance is indicated for Rockefeller only where New Orleans is also significant (*P<0.05, **P<0.01).

3.4. DISCUSSION

Physiological resistance to pyrethroids in adult *Ae. aegypti* has now been detected worldwide with reports from Brazil, Mexico, Thailand, China, Grand Cayman, Latin America, Indonesia and Malaysia (Smith et al., 2016). Reports of resistance from the Middle-Eastern region involve studies from the Ibb region of Yemen (Haq et al., 2013) , from Jazan in Saudi Arabia (Alsheikh et al., 2016) approximately 500 km north of Yemen, and from Makkah (Aziz et al., 2011), a further 700 km north. WHO bioassay results in each case were similar, with mortalities ranging from 75-93% to different pyrethroids. The previous study from Makkah (Aziz et al., 2011) performed collections in 2008, and our data, which was gathered using the same methodology, suggests that in the intervening years, resistance to both permethrin and deltamethrin (< 20% mortality in our results, see Chapter 2, section 2.3.2.1) appears to have increased substantially, though we note that slight differences in methodologies may have affected results. Interestingly, despite the proximity of the sites (Jeddah is less than 100km from Makkah), deltamethrin resistance was significantly lower in Jeddah, although was still higher than in the Cayman reference resistant strain, at least with 60 min exposure (see Chapter 2, section 2.3.2.1). This observation suggests that resistance may vary over a relatively small spatial scale, suggesting the involvement of local pressures. In this chapter, resistance target site and candidate metabolic gene resistance mechanisms were identified and their differences between strains examined to determine whether a mechanistic basis for the phenotypic variation could be identified.

Comparison of gene expression of field strains with a single susceptible strain may be problematic, because variation in expression levels may be unrelated to resistance, even for candidate genes. In our gene expression results, most candidate genes including *CYP9J32*, were overexpressed relative to New Orleans, but far fewer in comparison with the Rockefeller strain, although both are fully susceptible to pyrethroids. It is, however, interesting to note that in Jeddah, which showed the highest PBO synergism (see Chapter 2, section 2.3.2.6), two candidate CYP450s were overexpressed, of which both *CYP9J10* and *CYP9J28* are each amongst the most frequently upregulated in resistant strains (Moyes et al., 2017), and the latter metabolises pyrethroids (Stevenson et al., 2012), suggesting some involvement in

resistance. In Cayman, which showed more limited (and marginally non-significant) synergism, *CYP9M6*, also a known pyrethroid metaboliser (Kasai et al., 2014) was consistently overexpressed, though at a moderate level, whereas in Makkah, which shows the highest prevalence of deltamethrin resistance but no PBO synergy, no candidate genes were significant. Taken together, these results suggest that, despite an understandable concentration on the link between CYP450s and pyrethroid resistance, upregulation of genes with proven metabolic capacity may not translate into higher resistance, emphasising the need to consider alternatives.

Three *kdr* substitutions were detected at high frequency in the *Vgsc* (S989P, V1016G, and F1534C) in wild females from Jeddah and Makkah. Each of these has been linked to pyrethroid resistance (Srisawat et al., 2010, Li et al., 2015, Sayono et al., 2016), but none have previously been identified in the Middle Eastern region. The 1011 M/V mutation described in Latin America, Mexico and French Guiana (Saavedra-Rodriguez et al., 2007, Dusfour et al., 2015, Vera-Maloof et al., 2015) and V410 detected in Brazil (Haddi et al., 2017), Mexico (Saavedra-Rodriguez et al., 2018) and Columbia (Granada et al., 2018) were absent although we only sequenced a few individuals per study site. The S989P mutation was in perfect linkage disequilibrium with V1016G as observed in previous research (Plernsub et al., 2016a, Srisawat et al., 2010, Kawada et al., 2014), though this is the first record of either mutation outside of Asia. In both Makkah and Jeddah the 1016G+989P haplotype was very strongly associated with survival of long deltamethrin exposures, supporting results from *in vitro* expression of the *Vgsc* mutations in *Xenopus* oocytes (Hirata et al., 2014) and field demonstration in Thailand (Plernsub et al., 2016a) that this combination of mutations works additively to generate high-level resistance. I detected strong repulsive linkage disequilibrium between 989S+1016V/989P+1016G and F1534C, which has also been observed in Thai populations of *Ae. aegypti* (Plernsub et al., 2016a), and is likely to at least partially explain the lack of association of the F1534C mutation with deltamethrin resistance. The 1534C mutation alone can confer permethrin resistance (Hirata et al., 2014, Du et al., 2013) and while in combination with 989P and 1016G *in vitro* confers strong deltamethrin insensitivity, this has yet to be demonstrated in the field. Indeed, none of the six individuals possessing the triple mutant haplotype (989P+1016G+1534C) survived deltamethrin exposure.

However, as also demonstrated in *Ae. aegypti* from Thailand, the triple heterozygous genotype S/P989+V/G1016+F/C1534 significantly improved deltamethrin survival and was relatively common, suggesting an important intermediate step for otherwise recessive mutations (Plernsub et al., 2016b). Triple mutant haplotypes have now been detected at low frequency in Myanmar (Kawada et al., 2014), Java (Sayono et al., 2016), and now in Saudi Arabia, and the potential for combined impact of the three *Vgsc* mutations must remain a cause for concern.

TaqMan qPCR assays provide useful tools for high throughput screening for the presence of F1534C in addition to the other mutations. The Cayman strain we used has a high frequency of the 1534C mutation in addition to the V1016I substitution (Harris et al., 2010), both of which are common in Latin America (Smith et al., 2016), and when combined have been shown to increase the survival rate of deltamethrin in Brazilian and Mexican populations (Vera-Maloof et al., 2015, Kawada et al., 2014, Hirata et al., 2014, Du et al., 2013, Plernsub et al., 2016b, Linss et al., 2014). While the 1534C mutation is widespread, our study contributes to knowledge of the boundaries for each of the V1016 mutations, with the most westerly report of the 1016G mutation to date. Interestingly, the 1016I mutation has recently been detected in Ghana alongside the F1534C substitution, suggesting a possible contact zone between the 1016G and 1016I mutations located between Saudi Arabia and West Africa.

3.5. CONCLUSION

The moderate or very limited impact of PBO, especially in the more resistant Makkah population that also lacked significant P450 overexpression, suggests that *kdr* mutations, perhaps in combination with other as yet unknown mechanisms, are more significant here than CYP450 based metabolic resistance. Possible differences in the contribution of contrasting resistance mechanisms between populations may arise from different histories of insecticide usage for vector control, in addition to informing future control options.

CHAPTER 4
TRANSCRIPTOMIC ANALYSIS OF METABOLIC RESISTANCE IN *AEDES AEGYPTI* POPULATIONS FROM SAUDI ARABIA

4.0. ABSTRACT

Background Metabolic mechanisms are thought to be an important contributor to resistance phenotypes in *Ae. aegypti*, but to date studies identifying genes involved are lacking for the Middle Eastern Region.

Methods Microarrays were used to identify genes differentially expressed between two susceptible strains and Saudi Arabian strains from Makkah and Jeddah.

Results The results showed enrichment of P450s, some previously identified as pyrethroid metabolisers. Several candidate genes were tested using qPCR for their association with resistance in samples selected for enhanced resistance, and in samples from repeated and age-dependent exposure assays from earlier experiments. The metabolic activity of the lead candidate gene from the microarray, *CYP9J7*, against pyrethroids was investigated via *in vitro* insecticide metabolism assays. No depletion of either deltamethrin or permethrin was observed, but surprisingly *CYP9J7* metabolised the organophosphate malathion, pirimiphos-methyl and fenitrothion, a rare example of a gene capable of metabolising organophosphates in *Aedes*.

Conclusions The P450 gene-based metabolic mechanism plays an important role in pyrethroid detoxification, but the leading candidate P450 gene *CYP9J7* metabolizes organophosphates rather than pyrethroids. Further work will clarify whether metabolism represents detoxification or activation of the organophosphates, but in either case the gene may have value as a marker for management of insecticide resistance.

4.1. INTRODUCTION

Metabolic resistance arises due to elevated activity of genes involved in detoxification, sequestration and excretion of insecticides (Nkya et al., 2013). The increase in detoxification activity is mainly due to overexpression of these genes(Hemingway et al., 2004), sometimes associated with increased copy number (Faucon et al., 2015, Faucon et al., 2017), but allelic variations have also been linked to enhanced detoxification process (Ibrahim et al., 2015, Itokawa et al., 2013, Wondji et al., 2009). Three major classes of enzymes are engaged in insecticide metabolism in mosquitoes: monooxygenases (Cytochromes P450), glutathione S-transferases (GST) and carboxylesterases (COE) (Faucon et al., 2015). Among these, P450s often play a primary role in pyrethroid resistance; their overexpression permitting resistant mosquitoes to metabolise insecticide more rapidly (Hemingway and Ranson, 2000). Cytochromes P450 (CYPs) are haem-containing enzymes mainly bound to endoplasmic reticulum in eukaryotes but also occur in soluble form in mitochondria and prokaryotes. CYPs actively catalyse metabolism of diverse xenobiotics and endogenous compounds (Chandor-Proust et al., 2013). The CYPs are often a large gene family and in *Ae. aegypti,* there are around 160 CYP genes (Strode et al., 2008). The large number of genes encoding this class of detoxification genes in addition to GSTs, COE and also other gene families which may be involved in transport of products such as adenosine triphosphate (ATP) binding cassette (ABC) transporters, UDP-glycosyltransferase (UGTs), sulfotransferases, alcohol dehydrogenases, short-chain dehydrogenases and aldehyde oxidases makes detection of metabolic resistance more complicated than screening target site resistance which mostly involves a single gene (Moyes et al., 2017). Microarrays, RNA sequencing and reverse transcriptase quantitative PCR have facilitated characterisation of genes differentially transcribed between susceptible and resistant mosquitoes, with in-*vitro* and in *vivo* metabolism assays used to validate the potential involvement of top candidates identified in insecticide resistance.

In multiple gene expression studies, cytochromes P450 have consistently been found upregulated in pyrethroid resistant *Ae. aegypti* populations from different regions. In Singapore, Kasai et al,.2014 reported overexpression of *CYP6BB2*, *CYP6Z7*, *CYP6Z8*, *CYP6F2*, *CYP6F3*, *CYP9M4*, *CYP9M5*, *CYP9M6* and *CYP4C50* in the pyrethroid-resistant

Ae. aegypti population compared to a susceptible SMK strain obtained from Sumitomo Chemical Co.(Kasai et al., 2014). Bariami et al., (2012) found that Cayman and Cuba deltamethrin resistant strains upregulated *CYP9J9, CYP9J10, CYP9J26, CYP9J27*, *CYP9J28*, *CYP6BB2* and *CYP329B1* relative to the susceptible New Orleans strain (Bariami et al., 2012). Additional P450s found overexpressed in pyrethroid resistant *Ae. aegypti* include *CYP9J22* in a pyrethroid resistance population from Martinique (Marcombe et al., 2012) and *CYP9J7* and *CYP305A6* in a population from French Guiana (Moyes et al., 2017, Smith et al., 2016). Among these overexpressed genes, *CYP9J10*, *CYP6BB2*, *CYP9J26*, *CYP6J28* and *CYP9J32* have been validated in vitro (Kasai et al., 2014, Stevenson et al., 2012) and *CYP9J28* in vivo (Pavlidi et al., 2012) as pyrethroid metabolisers.

In *Ae. aegypti* from Jeddah and Makkah V1016G and S989P *kdr* mutations appear to play an important role in pyrethroid resistance (Chapter 3, section 3.3.1.1). Bioassays with the synergist piperonyl butoxide (PBO) suggested that metabolic resistance plays an additional role in conferring pyrethroid resistance, more so in Jeddah (Chapter 2, section 2.3.2.6). Quantitative PCR of candidate genes (Chapter 3 section, 3.3.1.3) showed two candidate CYP genes *CYP9J28* and *CYP9J10* were significantly overexpressed in the Jeddah strain compared to the New Orleans and Rockefeller susceptible strains, providing preliminary evidence of specific CYPs that may be linked to pyrethroid resistance. Using a genome-wide microarray-based transcriptional analysis, the current study seeks to comprehensively characterise genes potentially involved in metabolic resistance in resistant populations from Jeddah and Makkah.

4.1.1. Objectives

1-To characterise gene expression profiles of pyrethroid resistant *Ae. aegypti* from Jeddah and Makkah using whole genome microarrays.

2-To investigate cross resistance mechanisms in *Ae. aegypti* to different classes of insecticides by analysing gene expression profiles of pyrethroid resistant Saudi Arabian strains selected for resistance to malathion and bendiocarb.

3-To perform *in vitro* insecticide metabolism assays of a leading candidate gene identified in (1) to investigate its role in resistance.

4.2. MATERIALS AND METHODS

4.2.1. Mosquito strains, collection and rearing

The following mosquito strains were used in this study (i) Fully pyrethroid-susceptible New Orleans, Liverpool and Rockefeller laboratory strains; (ii) Pyrethroid resistant field F0 (second collection, Chapter 2:**Table 2.1**) strains collected in Jeddah (21°35'2.13"N,39°13'9.42"E) and Makkah (21°45'2.13"N, 39°92'1.96"E), Saudi Arabia, in March and April 2016; (iii) Jeddah strains F3 (second collection, Chapter 2: **Table 2.1**) that were selected for deltamethrin, bendiocarb or malathion resistance or (iv) unselected but maintained in the lab for the same duration as the selected strains. Field collected mosquitoes were sampled in water coolers, barrels, buckets and water containers such as under air conditioners and in buildings under construction. The larvae collection and rearing methods are explained in Chapter 2 section 2.2.1.

4.2.2. Selection of insecticide resistance

To select for extreme deltamethrin resistance (<20% mortality) and resistance to bendiocarb and malathion within a few generations, different selection protocols described in section 4.2.2.1 and 4.2.2.2 were tested. Starting with the F3 generation, both females and males were exposed to insecticides before mating. The surviving males and females were mated. To avoid mating before insecticide exposure, pupae were sexed based on structural differences on the genital lobe **(Figure 4.1)**. The different sexes were maintained separately until emergence.

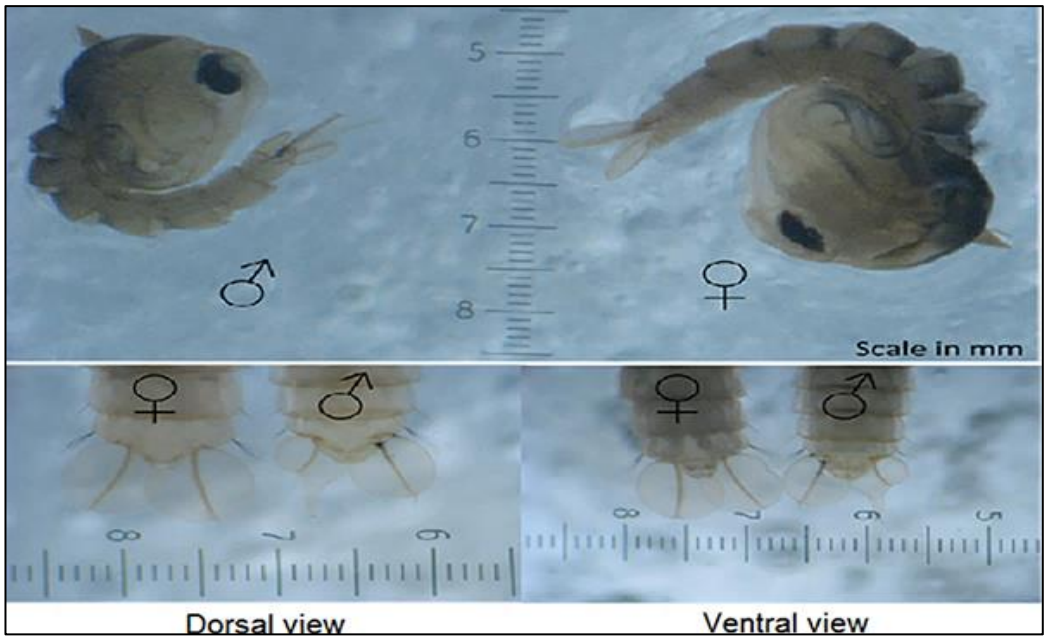


Figure 4.1 The differences between male and female *Ae. aegypti* pupae. Pupae can be reliably sexed by the structure on the end of the pupal abdominal segments just above the paddles. Males also tend to be much smaller than females (Carvalho et al., 2014).

4.2.2.1. Single exposure

In this protocol, F3 Jeddah strain females and males were exposed to deltamethrin papers in WHO tubes for 4h and 2h respectively. Surviving males and females were mated, the females blood fed and prepared for oviposition. The same procedures were repeated for two generations as illustrated in **Figure 4.2.**

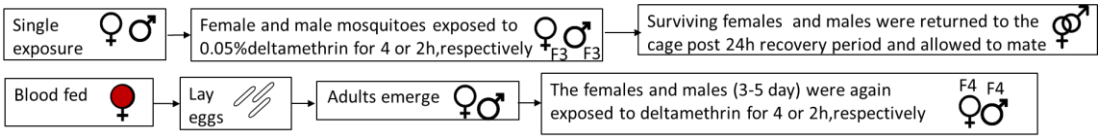


Figure 4.2 A diagram showing single exposure insecticide resistance selection process for males and female mosquitoes.

4.2.2.2. Multiple exposure

The desired level of deltamethrin resistance was not achieved using the previous protocol and it was suspected that some individuals might be avoiding full exposure during the single bioassay. Therefore, the protocol was modified to include multiple shorter deltamethrin exposures (2h each) of both males and females (of the Jeddah strain). Each short exposure was separated by a 24h recovery period as illustrated in **Figure 4.3.** The multiple short exposures were carried out after two rounds of single-

exposure selection as described in 4.2.2.1. After the second short exposure, the surviving females and males were mated, the females blood fed and prepared for oviposition. Their offspring were exposed to the final short exposure and all survivors preserved in RNA Later **(Figure 4.4).**

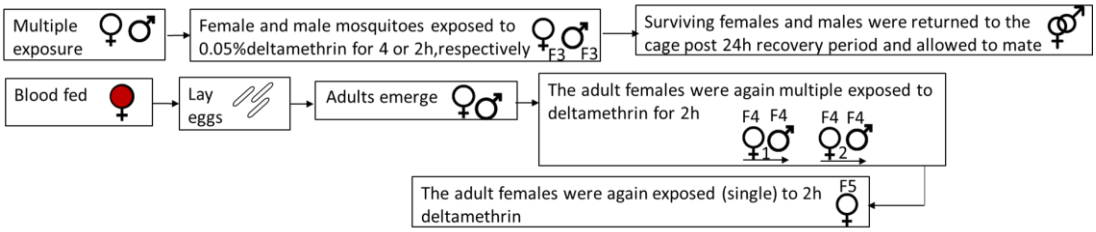


Figure 4.3 A diagram outlining the multiple exposure insecticide resistance selection process for female and male *Ae. aegypti*.

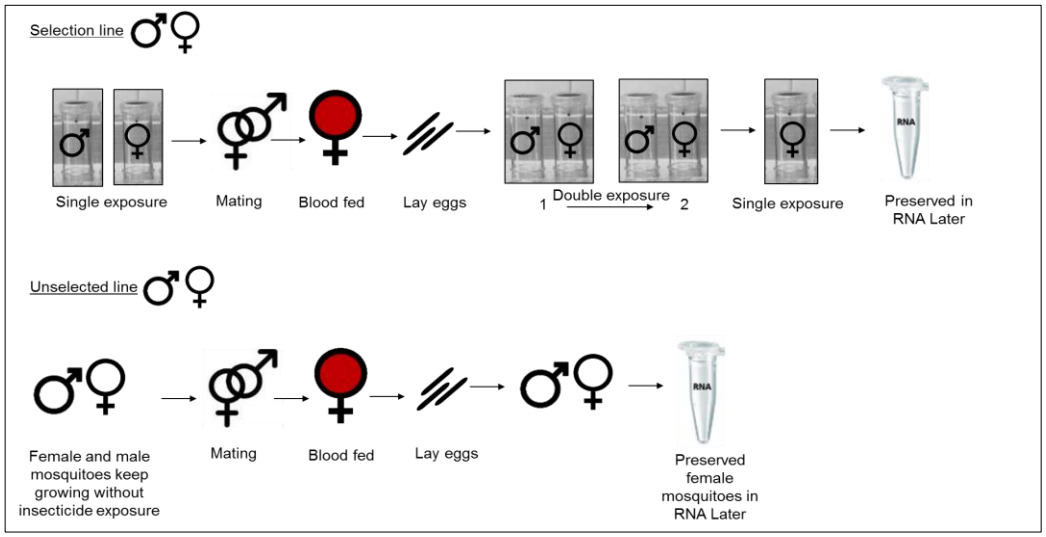


Figure 4.4 Insecticide selection process for Jeddah strain.

4.2.3. Genome-wide transcriptome profiling using microarrays

Genome-wide transcription profiling was performed to detect genes that are differentially expressed in Makkah and Jeddah populations in comparisons with the susceptible strains, New Orleans and Rockefeller.

4.2.3.1. Microarray plan

A microarray experimental design (**Figure 4.5**) with dye balancing was adopted to compare the gene expression profiles of the following experimental groups;

- Field collected Makkah and Jeddah strains unexposed to any insecticide
- Fully insecticide susceptible New Orleans and Rockefeller laboratory strains.

- Laboratory Jeddah strain (F5) without exposure to insecticides
- Laboratory Jeddah strains selected for deltamethrin resistance (F5).

For each comparison (field, lab selected or unselected vs susceptible), three biological replicates were used. The two susceptible strains, New Orleans and Rockefeller served as reference.

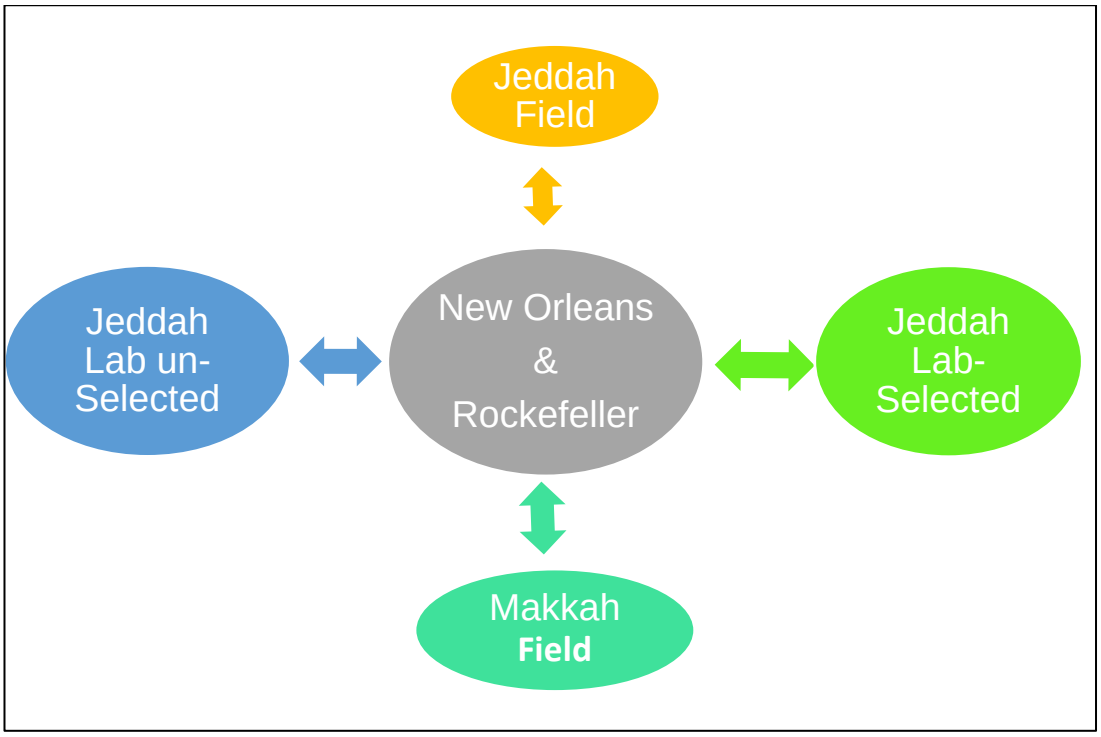


Figure 4.5 Microarray experimental design for studying differential gene expression of *Ae. aegypti* from Makkah and Jeddah strains in comparison to New Orleans and Rockefeller susceptible strains.

4.2.3.2. RNA extraction

In each experimental group, total RNA was isolated from 3-5-day old, non-blood-fed adult female mosquitoes in pools of 10 individuals using the Ambion RNAqueous® Kit (Life Technologies, USA). Three pools per group were extracted to serve as biological replicates in the microarray experiment. The quantity and quality of RNA yield was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Delaware, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). Good quality RNA was expected to have 1.8-2 and 1.8 or greater ratios of 260/230 and 260/280 respectively obtained using the Nanodrop. A band size of between 1000-2000 nucleotides (18S and 28S bands) as illustrated on the bioanalyzer

output in **Figure 4.6** was also used to assess RNA quality.

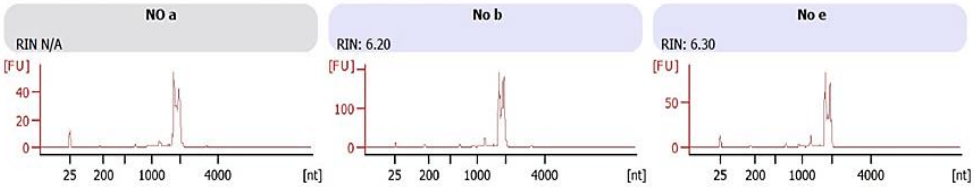


Figure 4.6 Example of RNA analysis extracted from New Orleans mosquitoes using the Agilent 2100 Bioanalyzer.

4.2.3.3. Complementary RNA Labelling

100 ng of total RNA from the three biological replicates of each experimental group was amplified and labelled with Cy-3 and Cy-5 fluorescent dyes using the Two-Colour Low Input Quick Amp Labelling Kit (Agilent technologies) as per the manufacturer's protocol. Labelled cRNAs were cleaned and purified using Qiagen RNeasy spin columns (Qiagen, Hilden, Germany). The quantity and quality of the purified, labelled cRNA was assessed using the Nanodrop and the Agilent 2100 Bioanalyzer (Agilent Technologies) representative profiles are shown in **Figure 4.7.**

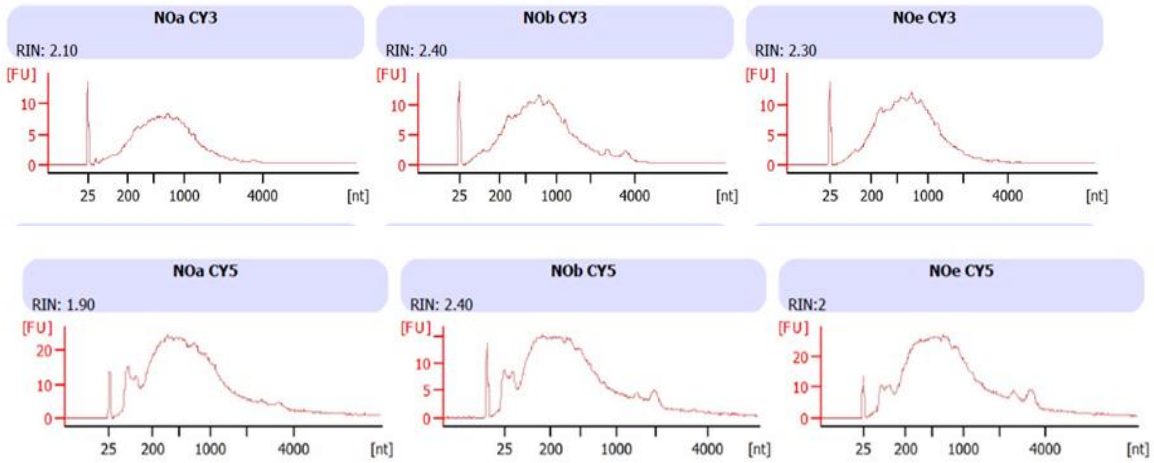


Figure 4.7 Example of Bioanalyzer traces from Cy3 and Cy5 labelled cRNA samples from New Orleans mosquitoes.

4.2.3.4. Hybridization

The 15k Agilent *Ae. aegypti* chip with eight arrays (ArrayExpress accession number A-MEXP-1966) was used for hybridisation. Dyes were balanced across the design by alternating the CY3 and CY5 dyes among biological replicates. Labelled cRNA was hybridised to the arrays for 17h at 65°C using a rotation speed of 10 rpm. After hybridisation, the slides were washed to get rid of unhybridized RNAs and scanned

on an Agilent G2565AA/G2565BA Microarray Scanner System using Agilent Feature extraction software v12 (Agilent technologies).

4.2.3.5. Microarray data analysis and enrichment analysis

Microarray data were analysed using the MAANOVA and LIMMA packages in R version 3.5.1 and a custom script for input file processing (provided by M. Donnelly). To evaluate the total number of genes differentially expressed the ANOVA F-test results were used with P values corrected using the Beniamini-Hochberg procedure implemented within the software Q-value (Dabney et al., 2010). Genes that were considered as significantly differentially expressed overall (i.e. using the ANOVA F-test) were used for Gene Ontology (GO) enrichment analysis in DAVID bioinformatics resources 6.8 (Huang et al., 2008b, Huang et al., 2008a). Descriptions and GO-terms of transcript-IDs generated in DAVID were extracted from VectorBase. However, to identify specific probes overexpressed an approach based on consistency was applied with a probe required to have a P value < 0.05 and fold change (FC) value of ≥2 in all comparisons with the susceptible strains. An additional rule, that FC (Jeddah lab deltamethrin selected) > FC (Jeddah lab unselected) was applied to emphasise genes more likely involved with deltamethrin resistance.

4.2.3.6. Quantitative Real-time PCR

Overexpression of six of the top genes from the microarray analyses were validated by qRT-PCR using primers designed using NCBI Primerblast **(Appendix 1: Table 8.6)** except for *CYP9J27* for which primers were available (Ishak et al., 2017). RNA of the different experimental groups was used to synthesise cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. The cDNA was purified by a QIAquick PCR Purification Kit spin column (Qiagen, Germany) as described in Chapter 3 section 3.2.3. Standard curves were performed by running qRT-PCR on serially diluted cDNA to assess PCR efficiency. The quantitative RT-PCR amplification and relative expression level were conducted as described after normalisation with the housekeeping genes (Schmittgen and Livak, 2008). The relative expression level and fold change (FC) of each candidate gene relative to the susceptible strains was calculated using the ΔΔcT method (Schmittgen and Livak, 2008). T test (two tailed) was used for comparison of gene expression levels of the

different strains quantified by qRT-PCR.

4.2.4. In vitro functional characterization of the *CYP9J7* candidate resistance gene

4.2.4.1. Amplification of full length *CYP9J7* alleles

Different variants of full-length cDNA sequences of the *CYP9J7* gene were obtained by amplifying three replicates of cDNA of each experimental group in section 4.2.3 using the *Ae*CYP9J7_F ATGGACACCATATTCGTTCTG and *Ae*CYP9J7_R TTATTTCGGCACCATCTTCA primers.

One microliter of cDNA was added to 14µl of PCR mix made up of 3µl Phusion 5xHF Buffer (with 1.5 mM $MgCl_2$), 0.12 µl dNTP mix (25 mM), 0.51 µl forward and 0.51 µl reverse primers (10 µM), 0.15 µl of Phusion High-Fidelity DNA Polymerase (Thermo Fisher scientific, UK) and 10.71 µl of distilled water (Sigma, UK). Amplification was conducted using the following conditions one cycle at 98°C for 1min (polymerase activation); 35 cycles of 98°C for 30s (denaturation), 58°C for 30s (annealing), and elongation at 72°C for 2 min 30s; and one cycle at 72°C for 10 min (final elongation). Three microliters of PCR product were separated on a 1.5% agarose gel stained with PeqGREEN (PeQlab,UK) and visualised using a transilluminator to confirm size of products.

4.2.4.2. Cloning of the full length of the *CYP9J7* resistance gene

PCR products were purified with QIAquick® PCR Purification Kit (Qiagen, UK) and cloned into pJET1.2 blunt cloning vector using the CloneJET PCR Cloning Kit (Thermo Scientific, UK) as explained below

- <u>blunting reaction</u> 5µl of 2x Reaction Buffer, 3.5µl Purified PCR Product and 0.5µl blunting enzyme were mixed briefly by vortexing and centrifuging (5s). The blunting reaction mix was incubated in a thermocycler for 5min at 70°C and chilled on ice for 3min.
- <u>ligation reaction</u> at room temperature, 0.5µl pJET1.2/Blunt Cloning Vector (50ng/µl), 0.5µl T4 ligase and blunting reaction product was incubated for 30 min.

4.2.4.3. Transformation and selection

A heat shock transformation method was used to transform competent *E. coli* cells with the ligation product to amplify and select plasmids containing the *CYP9J7* gene

sequence. To 20µl of sub-cloning high efficiency™ DH5α Competent *E. coli* cells (Invitrogen) in a 1.5ml microcentrifuge tube, 2µl of ligation product was added. The cells were gently mixed by flicking the bottom of the tube to avoid disrupting their fragile membranes. The cells and the ligation product were incubated on ice for 30 min, heat shocked for 45s at 42°C in a water bath and further incubated on ice for 2 min. To the bacteria, 900µl of pre-warmed SOC medium was added and incubated in a shaking incubator set at 37°C and 200rpm for 1h to allow the cells to recover before selection with antibiotics.

Lysogeny Broth (LB) agar supplemented with ampicillin was used to select transformed bacteria. A hundred microlitres of cells was spread using a blue stick spreader (Sarstedt, Germany) onto on the LB plates supplemented with 100mg/ml ampicillin. The plates were incubated overnight at 37°C. The following day, five colonies from each plate were suspended separately in 15µl distilled water (Sigma, UK). A colony PCR with KAPPA Taq DNA Polymerase reaction mix (Kapa biosystems, UK), 1.5 (10x) Taq buffer A, 0.75µl of $MgCl_2$ (25mM), 0.12µl of dNTP mix (25mM), 0.4µl of pJET1.2 (10mM) primers pJET_1.2FCGACTCACTATAGGGAGAGCGGC, pJET_1.2RAAGAACATCGATTTTCCATGGCAG, 0.12µl of KAPA Taq DNA Polymerase, 10.71µl distilled water (Sigma, UK) and 1µl of colony) was carried out to screen colonies containing the cloning vector. The cycling conditions were one cycle of 95°C for 3 min; 25 cycles each of initial denaturation (94°C for 30s), annealing (60°C for 30s) and extension (72°C for 2.5 min); one cycle of final extension for 5 min at 72°C. The PCR product was run on 1.5% agarose gel stained with PeqGREEN (PeQlab, UK) to confirm the presence of the gene sequence.

Positive colonies were cultured overnight at 37°C in 5ml LB medium (containing 5µl ampicillin 100mg/ml) in a shaking incubator set at 200rpm. Plasmids were isolated from the cultures using the QIAprep® Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer' guidelines. Quantity and quality of plasmid DNA was determined using NanoDrop ND1000 Spectrophotometer (Thermo Scientific, Delaware, USA). The isolated plasmids were commercially sequenced (Source Bioscience, Rochdale, UK) to confirm they had the full-length cDNA sequence. Sequence data were assembled and aligned using Codon Code Aligner version 4.2.7.

One clone from the deltamethrin selected Jeddah experimental group was selected for further analysis.

4.2.4.4. Cloning *CYP9J7* cDNA into the expression vector

To prepare the *CYP9J7* gene variant of interest for expression, the full-length coding sequence of *CYP9J7* was modified using the ompA+2 strategy described in Pritchard et al., (1997). This involves addition of a leader sequence containing the coding sequence of a bacterial outer membrane protein A (ompA), a signal peptide that facilitates translocation of the expressed P450 to the bacterial membranes and two extra spacer amino acid residues (alanine and proline that act as cleavage site to free the native P450) on the 5' end of the P450. In addition, restriction sites *Nde*I and *Xba*I were introduced by the forward and reverse primers on the 5' and 3' ends respectively. These restriction sites facilitated transfer the fusion protein from the cloning vector into the expression vector PCWri+ **(Figure 4.8)**. The ompA+2 modification is achieved through a two-step fusion PCR.

The first step is to link the ompA+2 leader sequence to a short fragment on the 5' end of the P450 to produce a linker fragment. The forward primer (ompA+2F) GGAATTCCATATGAAAAAGACAGCTATCGCG is specific for the ompA 5' end while the reverse primer (linker primer) ompA+2_CYP9J7F *AGAACGAATA TGGTGTCCAT***CGGACGGCCTGCGCTACGGTAGCGAA** has bases complementary to the 3' end of ompA+2 (in bold) and a few bases complementary to the 5' end of the P450 (in italics). The PCR reaction mix contained 0.5 µl *E. coli* JM109 gDNA (50 ng) and 0.5 µl pJET1.2 containing *CYP9J7* cDNA as a template, 3 µl (10x) Phusion high fidelity buffer (containing 5mM $MgCl_2$), 0.12 µl dNTP mix (25mM), 0.15 µl Phusion Taq Polymerase, 10.2 µl distilled water (Sigma, UK), 0.51 µl (10µM) of each primer. The PCR cycle conditions were 1 cycle at 98 °C for 1 min, 35 cycles of 98°C for 30s, 60°C for 30s and 72°C for 10s; and 1 cycle of final extension at 72°C for 5min. The PCR product, an intermediate fragment (linker) which containing the ompA+2 signal sequence and the first 24 nucleotides of *CYP9J7* was confirmed by gel electrophoresis and cleaned with QIAquick®PCR Purification Kit (Qiagen, Germany).

In the second PCR step, the first reaction product and pJET1.2 containing *CYP9J7* cDNA were the templates. In this step, the intermediate product is fused to the full-

length cDNA of *CYP9J7* using the forward primer used in the first reaction and a reverse primer specific to the 3' end of *CYP9J7* ompA+2_CYP9J7R GTCGACTCTAGATTATTTCGG CACCATCTTCA. The PCR conditions were the same as in the first step. The PCR product was a chimera containing ompA+2 signal sequence joined to the full-length cDNA was approximately 1600bp.

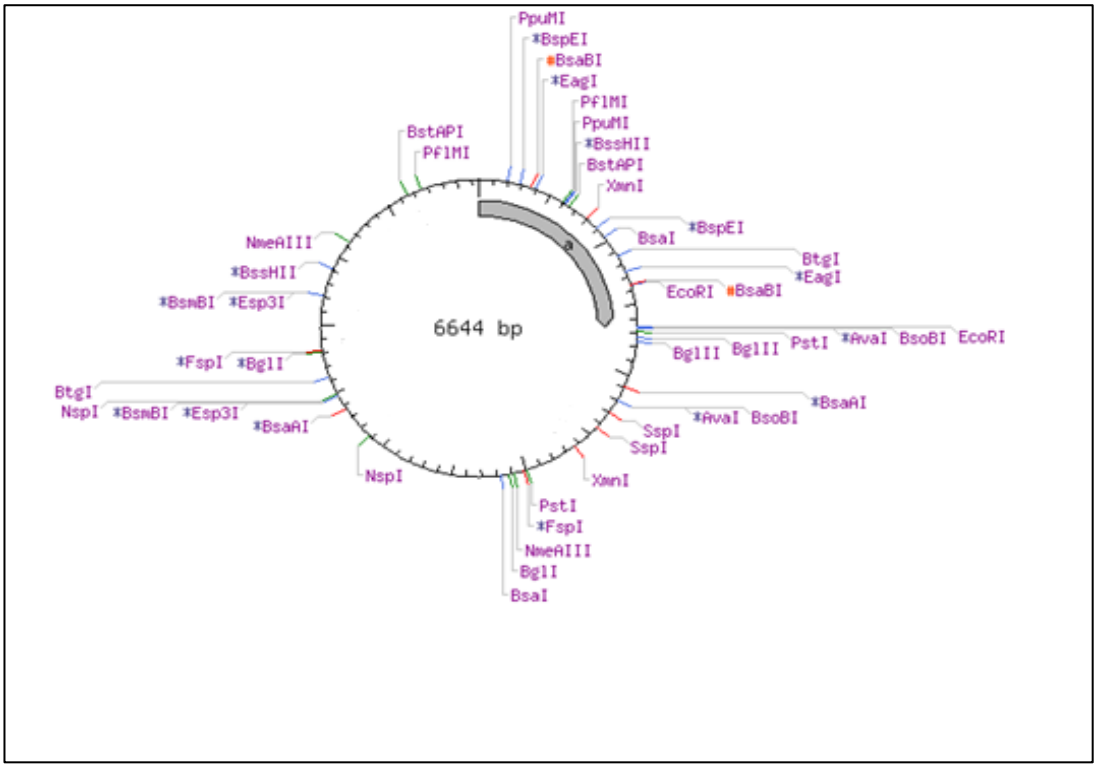


Figure 4.8 Map of pCWOri+ompA+2*CYP9J7* construct. A cloning site for *CYP9J7*-ompA+2 flanked by restriction sites *Nde*I and *Xba*I. Prepared using the NEB Cutter v2.0 (http//tools.neb.com/NEBcutter2/index). The grey section (a) indicates the *CYP9J7* insert. Restriction endonuclease cut sites are indicated in purple.

4.2.4.5. Restriction Digestion of pJET1.2-ompA+2-*CYP9J7* construct and ligation into PCWOri+ plasmid

To clone the gene chimera into the expression vector, pJET1.2ompA+2-*CYP9J7* was double-digested with two restriction enzymes, *Nde*I and *Xba*I (Fermentas). The reaction included 5µl 10x Fast Digest Green Buffer, 2µl Fast Digest *Nde*I, 2µl Fast Digest *Xba*I, 28µl Plasmid/PCR product and 13µl distilled water (Sigma, UK). Digestion at 37°C for 2h was followed by enzyme deactivation at 65°C for 15min. The products were separated by gel electrophoresis and the band with the estimated gene size was sliced out and purified using the QIAquick®Gel Extraction Kit (Qiagen, Germany). The ompA+2-*CYP9J7* digest was ligated overnight at 16°C into PCWOri+ expression

plasmid already linearized with *Nde*I and *Xba*I restriction enzymes. The reaction mix contained 1µl 10x Ligase buffer, 6µl linearized PCWOri+, 20 µl linearized *CYP9J7*, 22µl distilled water (Sigma, UK) and 1µl T4 ligase. 4-6µl of the ligation product was used to transform high efficiency DH5α using the protocol outlined in section 4.2.4.3.

Positive colonies were screened with PCWOri+ specific primer (PCW_F ATCCCCCTGTTGACAATTAATCATC, PCW_R ACCTATAAAAATAGGCGTATCACGA) designed within the PCWOri+ sequence approximately 100 nucleotides upstream of the *Nde1* restriction site and downstream of the *Xba*I restriction site respectively. A band size of ~1800bp comprising the gene insert (1584 bp), the ompA+2 leader sequence and 100 nucleotides of PCWOri+ sequences flanking the gene insert confirmed the presence of the gene in the expression vector. Sequencing the plasmids prepared from single colonies using the ompA+2F, ompA+2-CYP9J7R, PCWOri+ forward and reverse primers validated the colony PCR results.

4.2.4.6. Co-transformation with pB13 ompA+2*CYP9J7* and pACYC-184-*An. gambiae* Cytochrome P450 reductase

To express both *CYP9J7* and cytochrome P450 reductase, 50µl of high efficiency *E. coli* JM109 cells (Promega, UK) were co-transformed with 4µl pCWOri+ (pB13) plasmid containing *Ae. aegypti CYP9J7* and 2µl pACYC-184 bearing *An. gambiae* cytochrome P450 reductase in a ratio of 2:1. Transformation was as previously described in 4.2.4.3 but in this step, the agar LB selection plates were supplemented with both ampicillin and chloramphenicol to a final concentration of 50 µg/ml and 25 µg/ml respectively. The plates were incubated at 37^{o}C overnight (14h) and individual colonies were suspended in 20µl distilled water (Sigma, UK). The colonies were screened using KAPA PCR as described in section 4.2.4.3 using the PCWOri+ (PCWF/R) primers to confirm presence of P450 insert and internal primers for cytochrome P450 reductase CPR_F CTACTCGATCCATATGACGACGGTGAACAC CPR_R TACGGATCCTACAGCACATCCTCGCCCGTGCTC. The amplification product obtained using CPR internal primers was ~600bp on 1.5% agarose gel stained with PeqGREEN.

4.2.4.7. Heterologous co-expression of pB13-ompA+2-*CYP9J7* and pACYC-184-Ag-CPR in *E. Coli* JM109

Colonies that were positive for both genes were suspended in distilled water (Sigma, UK) and 4µl of the suspension used to inoculate 8ml LB broth medium containing 8µl (50mg/ml) ampicillin and 8µl of (25mg/ml) chloramphenicol to prepare a starter culture. The cells were cultured at 37^{o}C in a shaking incubator at 200 rpm for 14-16h. The following day, 3ml of starter culture was inoculated in 200ml of pre-warmed (37^{o}C) terrific broth medium containing 300µl (50mg/ml) ampicillin and 300µl (25mg/ml) chloramphenicol, 200µl (1M) of thiamine hydrochloride and 50µl of 4000X trace elements. The culture flask was incubated at 37^{o}C with shaking speeds at 200 rpm until the optical density (OD) of the cells- scanned at 600 nm - reached 0.4, at which point the heme precursor, δ-Aminolevulinic acid (ALA) (Sigma, UK) was added to the culture medium. At this point, the incubation temperature and shaking speed was adjusted to 21^{o}C and 150rpm respectively. The optical density of the culture was monitored every 2h until it reached 0.8 when the cells were at the exponential phase. This is the growth stage when optimum expression of genes is expected hence expression of both enzymes was induced by adding Isopropylβ-D-thiogalactopyranoside (IPTG) (Sigma, UK) to a final concentration of 1mM. After 20h, the cultures were monitored for P450 expression using a spectrophotometer as described in section 4.2.4.9.

4.2.4.8. Purification of P450 and AgCPR from bacterial cultures

Twenty-eight hours post-induction, the bacterial membranes containing both *CYP9J7* and CPR were harvested as described by Pritchard et al., (2006). In brief, the culture was stopped by transferring the culture into centrifuge bottles and incubating them on ice for 1 min. Cells were pelleted by centrifugation at 2800g for 20 min while maintaining the temperature at 4°C. The pellet was resuspended in 20ml ice-cold (1X) Tris Sodium EDTA (TSE buffer). Lysozyme was added to the resuspended cells to digest the bacterial cell wall to a final concentration of 0.25mg/ml and incubated at 4°C for 1h on a shaker set to 75 rpm. The spheroplasts were pelleted at 2800g for 25min at 4°C and then re-suspended in 8ml ice-cold spheroplast resuspension buffer. Protease inhibitors; leupeptin (1µg/ml), phenylmethylsulfonyl fluoride (1mM) and

aprotinin (1µg/ml) were added to the resuspended spheroplasts before they were sonicated at 30sec bursts for a total of 2min to lyse the spheroplast membranes. The lysate was centrifuged at 30, 000g for 20 min at 4°C to separate the membranes from the cell debris. The supernatant containing the membranes was transferred to ultracentrifuge tubes, topped up with 1X Tris Sodium EDTA (TSE) buffer and centrifuged at 180, 000g for 1h at 4°C to pellet the membranes. The supernatant was discarded, the membranes resuspended by adding 1ml of ice-cold (1X) TSE buffer and ground using 6-8 strokes using a Dounce tissue grinder (Wheaton). The membranes were stored at -80°C until needed.

4.2.4.9. Cytochrome P450 and CPR quantification

The P450 concentration was quantified via spectral activity by measuring the spectra difference of reduced P450-carbon monoxide complex (Fe2+-CO) with reduced non-complexed P450 (Fe2+) (Omura and Sato, 1964). To detect the concentration of P450 in cell cultures, 1000µl of culture was transferred into 1.5 microfuge tubes and centrifuged at 4^{o}C and 16,400 rpm for 20min to pellet the cells. The cells were resuspended in 1000µl (1x) P450 spectrum buffer. The mix was split into two spectrophotometer cuvettes: reference and experimental (500µl each). A baseline reading was taken from 500 to 400nm with a dual beam Cary UV Vis spectrophotometer. The experimental cuvette was bubbled with carbon monoxide in a fume hood, at the rate of one bubble a second for 50-80s. In both reference and experimental cuvettes, a few grains of sodium dithionate were added and mixed gently by inversion and replaced into the machine. The concentration of active P450 was computed by subtracting the peak measurements at 490nm from 450nm then dividing the difference with an extinction coefficient (e_{cyt450}=0.091µM^{-1}cm^{-1}) for P450s. In purified membranes, 50µl of the membranes were added to 950µl of 1X P450 spectrum buffer and measured as described above. The measured quantity of active P450 was multiplied by the dilution factor (20) to obtain the concentration of the enzyme per ml.

The concentration of CPR was determined indirectly by determining CPR cytochrome c reduction activity per minute per milligram of protein in the membrane. Protein concentration in the membranes was measured using the CB™ Protein Bradford

Assay kit according to manufacturer's protocol. CPR Cytochrome C reduction activity was determined by adding 2µl of the membranes in 150µl of Cytochrome c (Sigma, UK) dissolved in 0.3M potassium phosphate buffer PH7.7. The reaction was initiated by adding 150µl 0.1mM NADPH or potassium phosphate buffer in test and controls respectively. The activity was monitored for 15 min by taking spectral readings at 550nm using a microplate UV-Vis reader. By plotting absorbance against time, CPR cytochrome c reduction activity nmoL/min/ml was determined by calculating the slope of the graph multiplied by the dilution factor (500) divided by the adjusted extinction coefficient of cytochrome c at 550nm (pathlength*0.021). The activity was then divided by the estimated protein concentration to determine the activity in nmol/min/mg of proteins in the membranes.

4.2.4.10. Metabolism assays

To confirm the insecticide metabolism activity, *in vitro* insecticide metabolism assays were conducted using the P450 enzymes expressed in *E. coli*. Reactions were conducted in four technical replicates. Each replicate was done in the presence and absence of cytochrome b5. In each category (±b5), a test reaction in which the complete NADPH regeneration mix was added and the control in which NADP was carried out. The reaction mixtures were as follows; **i)** 50µl of NADPH regeneration components (50mM potassium phosphate buffer PH7.4,1mM glucose-6-phosphate (G6P), 0.25mM $MgCl_2$, 0.1mM NADP and 1U/ml glucose-6-phosphate dehydrogenase (G6PDH), **ii)** 50µl NADPH regeneration components minus NADP (50mM potassium phosphate buffer PH7.4), 1mMG6P, 0.25mM $MgCl_2$, 1U/ml glucose-6-phosphate dehydrogenase (G6PDH), **iii)** 48µl enzyme buffer mixed with cytochrome b5 (50mM potassium phosphate buffer PH 7.4), 0.05µM P450, 0.4µM cytochrome b5 and insecticide and **iv)** 50µl enzyme buffer without cytochrome b5 (50mM potassium phosphate buffer PH7.4), 0.05 µM P450 and 10 µM insecticide. The reactions were started by adding the regeneration mixture to the enzyme mix containing the insecticide and the enzymes, incubated at 30°C for 5min to activate the membranes and a second incubation at 30°C in a shaking incubator set at 1200rpm for 2h for insecticide metabolism. Reactions were quenched with 100µl of acetonitrile (HPLC grade, Fisher scientific). The tubes were vortexed and inverted several times to

dissolve insecticide with acetonitrile and then centrifuged at 13,400g for 20min to pellet the membranes.

4.2.4.11. HPLC analysis

150µl of the supernatant was transferred to an HPLC vial (Thermofisher, Germany). The solvent ratios for each insecticide and wavelength (measured using an Agilent 1260 Infinity HPLC system) are presented in **Table 4.1**. 100 µl of sample was loaded into an isocratic mobile phase of acetonitrile and water with a flow-rate of 1 ml/min. Substrate peaks were separated on a 250mm C18 column (Acclaim™ 120, Dionex) at 23°C. Metabolism was calculated from the difference of insecticide concentration in the test vials (reactions with NADPH) from that of control (reactions without NADPH) which represent the % depletion of insecticides in 2h by 0.05 µM *CYP9J7*.

Table 4.1 The solvent ratio and wavelength used for each insecticide during HPLC analysis.

Run condition						
	Permethrin	Deltamethrin	Fenitrothion	Malathion	P. methyl	Bendiocarb
Solvent ratio	85% Acetonitrile 15 % water	80% Acetonitrile 20 % water	70% Acetonitrile 30% water	60% methanol 40% water	70% Acetonitrile 30% water	50% Acetonitrile 50% water
Wavelength	226	232	264	232	232	226

4.2.4.12. Homology modelling and molecular docking simulation

A 3D model of *CYP9J7* was determined by submitting the amino acid sequence to the I-TASSER server (https//zhanglab.ccmb.med.umich.edu/I-TASSER/). The model was used to predict the pattern of molecular interaction of the enzyme with insecticides. Virtual datasets of ligand insecticides cis-permethrin (CID40463), trans-permethrin (CID40326), deltamethrin (CID40585), bendiocarb (CID2314), malathion (CID4004), pirimiphos-methyl (CID34526) and fenitrothion (CID 31200) were retrieved from the Pubchem database (https//pubchem.ncbi.nlm.nih.gov/). Docking simulations were carried out using default docking parameter of CLC Drug Discovery Workbench4 (http//www.clcbio.com/products/clc-drug-discovery-workbench/).

CYP9J7 was prepared for docking by correctly positioning heme on the catalytic pockets through structural alignment with the top templates (Itqn) used earlier to model the structures. The binding site was set to 20 Å around the heme molecule.

PyMOL™ 2.2.0 was used to measure the docking distances and preparing the docking figures (Delano, 2016).

4.2.5. Insecticide cross-resistance

The gene expression profiles of Makkah and Jeddah strains selected for resistance to insecticides were investigated to investigate whether genes might contribute to insecticide cross-resistance. Selection of organophosphate and bendiocarb resistance was carried out as described in section 4.2.2. The expression profile of the top candidate genes *CYP9J7*, *CYP9J27*, *CYP9J26*, AAEL014614-RA (CYP9P450), AAEL006013-RA, AAEL006953-RA that potentially linked to deltamethrin resistance was assessed by qRT-PCR in Makkah strains selected for malathion resistance. The same qRT-PCR method was used as described in section 4.2.3.6.

4.2.5.1. Role of P450s in bendiocarb and malathion selection assessed using synergist piperonyl butoxide (PBO)

Piperonyl butoxide is a cytochrome P450 monooxygenases inhibitor. The hypothesis was that pre-exposure to PBO would increase the rate of mortality when the strains were later exposed to insecticides due to reduced P450 insecticide metabolism activity. In Chapter 2 section 2.2.3.5, I looked at the effect of PBO pre-exposure on mortality when F1 Jeddah and Makkah mosquitoes were later exposed to 0.05% deltamethrin. In this section the synergist assay was extended to test other insecticides. Standard WHO susceptibility test was conducted on 3-5 days old bendiocarb and malathion selected Jeddah and Makkah strains pre-exposed to PBO as described in Chapter 2 section 2.2.3.5. After 24h, the final mortality was recorded.

4.3. RESULTS

4.3.1. Selection of insecticide resistance

Different protocols were trialed sequentially to determine the most effective method for selecting a high level of insecticide resistance within a few generations. Single insecticide exposure has little impact on insecticidal mortality **(Table 4.2)**.

Table 4.2 Mortality rate 24h post-exposure to WHO papers impregnated with 0.05% deltamethrin in Jeddah strain selected for deltamethrin resistance using a single exposure approach in both female and male mosquitoes.

A) Single female and male selection exposure											
				Biological Replicate 1				Biological Replicate 2			
Test colonies	Exposure Time	Sex	Exposure Type	Total	Survivors	Dead	Mortality (%)	Total	Survivors	Dead	Mortality (%)
F3	4h	Female	Single	419	102	317	75.5	388	47	341	88.4
F3	2h	Male	Single	253	53	253	82.3	329	59	270	81.9
F4	4h	Female	Single	92	24	68	73.3	131	36	95	72.7
F4	2h	Male	Single	115	28	87	74.8	132	29	103	79

Using a protocol involving multiple exposures, selection efficiency improved with bioassay mortality reducing from 77.8% in the starting generation of biological replicate 2 to 16.3% in the last generation **Table 4.3**. I focused on replicate 2 on the fifth generation because it had higher numbers of survivors of both males and females after multiple exposures in the fourth generation than replicate 1. A multiple exposure approach was adopted for all subsequent selections of insecticide resistance.

Table 4.3 Mortality rate 24h post-exposure to WHO papers impregnated with 0.05% deltamethrin in Jeddah strain selected for deltamethrin resistance based on a multiple exposure approach.

Multiple female and male selection exposure											
				Biological replicate 1				Biological replicate 2			
Test colonies	Exposure Time	Sex	Exposure Type	Total	Survivors	Dead	Mortality (%)	Total	Survivors	Dead	Mortality (%)
F3	4h	Female	Single	225	27	198	87.9	241	52	189	77.8
F3	2h	Male	Single	242	51	191	78.5	257	73	184	71.1
F4	2h	Female	Double	143	51	92	66.5	245	99	146	58.7
				51	35	16	28.6	74	62	32	32.6
F4	2h	Male	Double	119	38	81	68.1	209	61	148	70.5
				38	21	17	43.7	61	39	22	36.3
F5	2h	Female	Single	Not Performed				163	137	26	16.3

4.3.2. Microarray

Thirty-eight genes (38) were overexpressed with a fold change >2 out of which 36 were significantly overexpressed in all strains (T test pairwise comparison, $P< 0.05$). In contrast, 20 were under expressed with a log fold change <2 out of which 18 genes were significantly under expressed (T test pairwise comparison, $P<0.05$). The annotation terms for the top differentially expressed genes were analyzed in David (Huang et al., 2008b, Huang et al., 2008a) to identify the terms and associated genes types that were enriched in resistant populations. Selection of the candidate genes for the enrichment analysis was based on significantly overexpressed gene (212) and under expressed genes (205). The enrichment analysis for significantly overexpressed genes generated two significant clusters with enrichment scores of 5.73 and 1.71 respectively **(Figure 4.9; Appendix 1: Table 8.7)**. Cluster 1 was enriched for monooxygenase, cytochrome P450, iron binding, metal binding, heme binding and oxidoreductase GO terms while cluster 2 was enriched for zinc ion binding, zinc fingers and nucleic acid binding **(Figure 4.9)**. Annotation terms describe Cytochrome P450 enrichment in cluster 1 and transcriptional factor in cluster 2. The enrichment analysis of the under expressed genes generated 1 significant cluster (**Figure 4.9; Appendix 1: Table 8.8)**. Annotation terms enriched in the significant cluster were

tetraspanin and peripherin **(Figure 4.9)**. The terms are linked to intermembrane proteins and neuronal intermediate filament proteins respectively.

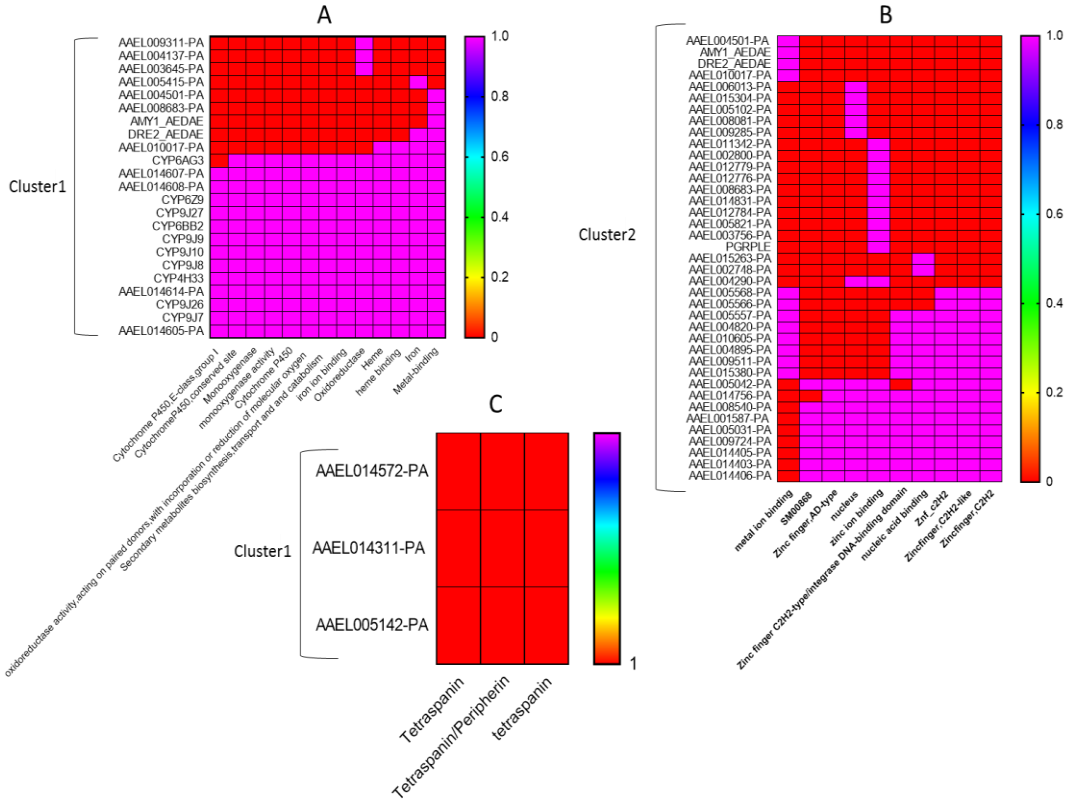


Figure 4.9 DAVID functional enrichment analysis in *Ae. aegypti* in Jeddah and Makkah strains tested in microarray. The analysis was conducted on the significantly up-regulated genes (A and B) and down regulated genes C) found in resistant relative to susceptible strains. The purple color in the box corresponds to a GO term positively associated with the gene while the red color corresponds to a GO-term not associated with the gene.

Among the cytochrome P450s genes in cluster 1, *CYP9J7*, *CYP9J10*, *CYP9J27v1*, *CYP9J27v2*, AAEL014614-RA (*CYP9450*) were also in the top 36 genes that were significantly overexpressed in all strains relative to both susceptible strains with a fold change of 2 or more **(Figure 4.10; Appendix 1: Table 8.10)**. The other P450s such as *CYP9J9*, *CYP9J26* and *CYP6Z9* were significantly over expressed but the fold change was less than 2. GSTs and CCEs had low fold change in all tested strains and most of them were down regulated in pyrethroid resistant strains relative to susceptible strains. The over representation of P450 genes in the top significantly expressed genes suggest CYPs are important in pyrethroid resistant strains. The most over-expressed gene was AAEL006953; a protein associated with chitin binding activity and metabolism **(Figure 4.10; Appendix 1: Table 8.10)**. The molecular and biological GO

terms and the identity scores for top hits in NCBI blast hints the gene codes for peritrophin-like proteins, which are found on the peritrophic membrane surrounding the insects' midgut. The other genes that were up regulated are associated with GO terms for lipid and carbohydrate metabolism, gene regulation, transport, DNA repair and other functions summarised in **Appendix 1: Table 8.10**. Most of the down regulated genes were unknown **(Figure 4.10; Appendix 1: Table 8.11)**. Most of them have GO terms for membrane proteins and protein binding **(Appendix 1: Table 8.11)**.

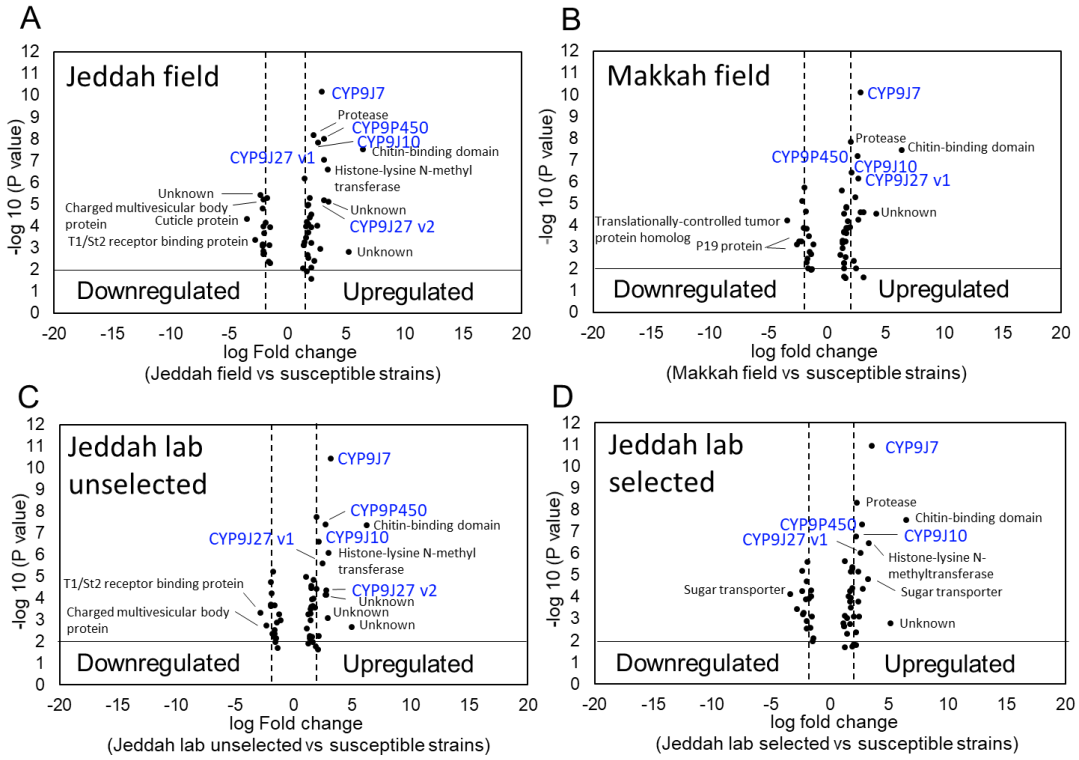


Figure 4.10 Significantly up regulated (36, *P*<0.05 and FC>2) and down regulated genes (18, *P*<0.05 and FC<2) in resistant *Ae. aegypti* strains relative to the average of New Orleans and Rockefeller. The x-axis denotes expression ratio and the y-axis is the negative log10 scale of the *P* value of the t-test of the fold change between the groups. Vertical dotted lines indicate two-fold expression differences in either direction. The horizontal line represent the significance threshold of *P* <0.001 adopted for the one sample t-test. CYP9 P450s are shown in green and Epsilon GSTs are in red. Selected genes are named. The data set is available at ArrayExpress (accession no. A-MEXP-623). Panel A) expression levels in Jeddah field strain adults versus the average of susceptible strains, panel B) adult Makkah field relative to the avarge of susceptible strains, panel C) Jeddah lab unselected versus to the avarge of susceptible strains and panel D) adult Jeddah lab-selected relative to the avarge of susceptible strains.

4.3.2.1. Validation of candidate genes with Quantitative RT-PCR

Real-time quantitative PCR was used to validate the microarray results versus the New Orleans, Rockefeller and Liverpool strains after normalisation with two housekeeping genes, RPS3 and Ae60sL8 **(Appendix 1: Table 8.4)**. The Liverpool strain was added as an additional check because of generally much lower qPCR fold-change values in Rockefeller than New Orleans.

Four CYP450s (*CYP9J7*, AAEL014614-RA (*CYP9P450*), *CYP9PJ26*, *CYP9J27*), were chosen along with AAEL006013-RA (histone lysine N methyltransferase) and AAEL006953-RA, due to the consistently high fold change in both susceptible strains (New Orleans and Rockefeller) in the microarray analysis. *CYP9J26* was selected because it showed amongst the highest fold changes against New Orleans but was lower against Rockefeller, it was included in the validation because it metabolises pyrethroids (Stevenson et al., 2012).

The quantitative real time PCR results confirmed the over-expression patterns detected by microarray, but with higher fold change values except in AAEL006953-RA. A significant over-expression in all four resistant populations was confirmed for six genes relative to New Orleans strains. Four genes AAEL014614-RA, *CYP9PJ26*, *CYP9J27*, AAEL006013-RA were significantly overexpressed in all resistant populations relative to Rockefeller while relative to the Liverpool strain, only *CYP9J26* was significantly overexpressed in all resistant populations.

In comparison with three susceptible strains, only *CYP9J26* and *CYP9J27* were significantly over-expressed in all populations **(Figure 4.11C and D)**. The expression level of both genes was also higher in Jeddah strains selected for deltamethrin resistance compared to unselected Jeddah strains **(Figure 4.11C and D)**. A significant difference in expression levels of *CYP9J7* (t-test=-3.1,df=4, *P*=0.035), *CYP9J26* (t-test=-2.9, df=4, *P*=0.044) and AAEL006013-RA (t-test=3.1, df=4, *P*=0.035) was observed in the Jeddah deltamethrin selected strain compared to unselected suggesting overexpression may have occurred in response to selection **(Figure 4.11A, C and F)**.

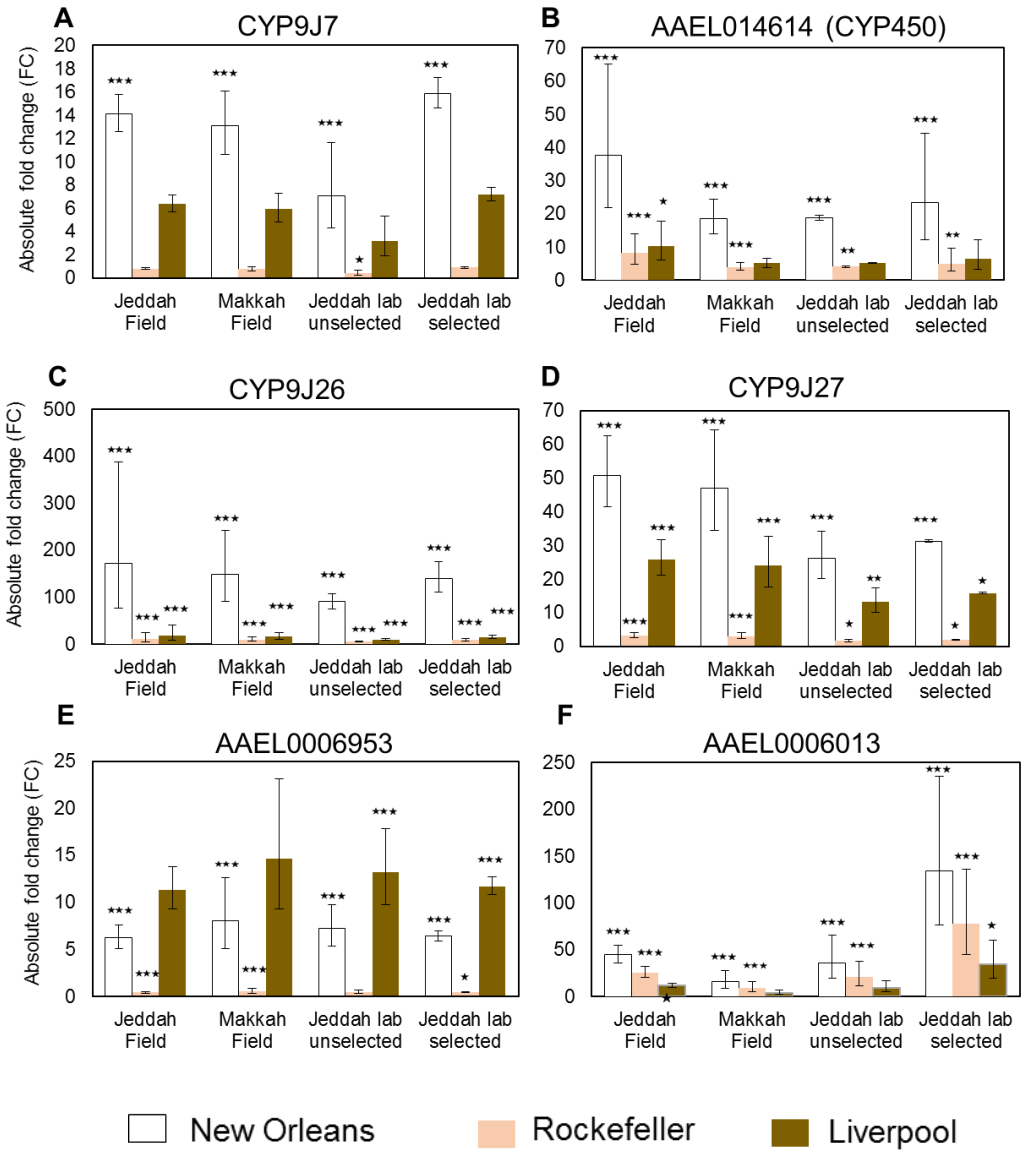


Figure 4.11 Relative fold-change of candidate genes from qRT-PCR analysis compared to susceptible strains New Orleans, Rockefeller and Liverpool. Error bars represent 95% confidence intervals. Statistical significance is indicated by *P < 0.05, **P < 0.01 and ***P < 0.001.

There was a slight negative correlation among transcription patterns obtained by qRT-PCR and microarray techniques in all reported genes relative to New Orleans (Correlation=-0.32, P=0.13; **Appendix 1: Figure 8.3A**) and Rockefeller (Correlation=-0.18, P=0.39; **Appendix 1: Figure 8.3B**) **(Appendix 1: Figure 8.2)**. When extreme value obtained in the microarray but not in qPCR are removed the correlation between the two techniques relative to New Orleans was (Correlation=0.38, P=0.097; **Appendix 1: Figure 8.3C**) and Rockefeller (Correlation=0.45, P=0.04; **Appendix 1: Figure 8.3D**).

Overall therefore, there was some qualitative validation of results, but quantitatively the match between qPCR and microarray fold change values was quite poor.

4.3.3. Insecticide cross-resistance

4.3.3.1. Effect of the synergist piperonyl butoxide (PBO) on bendiocarb and malathion mortality

Exposure to bendiocarb (0.1%) for 1h in WHO tubes resulted in 26.6% mortality with Jeddah but no mortality in Makkah. No mortality was observed when both strains were exposed to PBO alone but PBO-bendiocarb exposure resulted in significantly higher mortality in Jeddah 86.6% (*P*=0.00002) and Makkah 25.3% (*P*=0.002) when compared to exposure to bendiocarb alone **(Figure 4.12A)**. The mortality rate to malathion was 90.9% and 68.8% in Jeddah and Makkah respectively. Susceptibility to malathion post-PBO exposure increased significantly in Makkah (*P*=0.002) but not significantly in Jeddah **(Figure 4.12B)**.

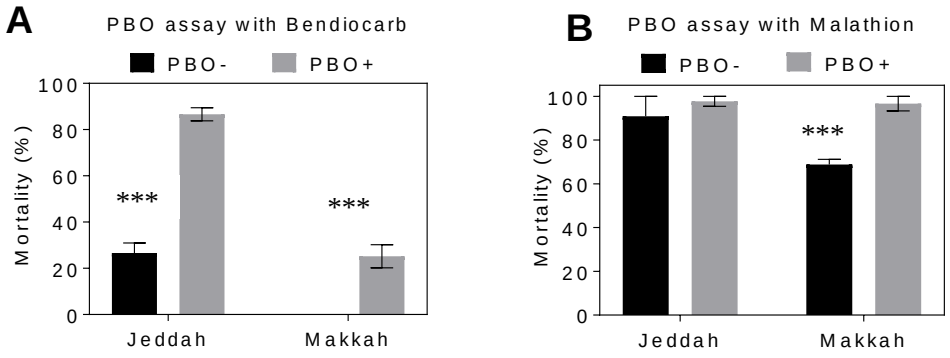


Figure 4.12 Figure shows A) bendiocarb and B) malathion 60 min bioassays with and without 60 min pre-exposure to the synergist PBO (PBO-, PBO+). Statistical significance is indicated by ***$P < 0.001$. Error bars are ±SEM.

4.3.3.2. Selection of insecticide resistance

To generate selected lines in which overexpression of specific genes could be investigated that might link with carbamate and organophosphate survival which were inhibited by PBO resulting in higher mortalities, selection with bendiocarb and malathion was attempted. Selection was not successful for bendiocarb even when the protocol optimised for deltamethrin selection was adjusted by changing the exposure time, perhaps because resistance was already very high and lacked variability and therefore was not considered further **(Appendix 1: Table 8.9)**. A reduction in mortality was achieved in the initially more resistant Makkah strain

when selected for malathion resistance. The female mortality rate reduced significantly (t test=7.3, *P*=0.00001) from 81.4% in the starting generation to 20.8% in the last generation (**Table 4.6**). For Jeddah, malathion selection was unsuccessful **(Table 4.7)** perhaps because of limited genetic variability resistance to organophosphates.

Table 4.4 Mortality rate 24h post-exposure to WHO papers impregnated with 5% Malathion in Makkah strain selected for Malathion resistance based on the multiple exposure approach on both female and males.

Selected Makkah malathion colony										
			Female				Male			
Generation	Exposure Time	Exposure Type	Total	survivors	Dead	Mortality (%)	Total	Survivors	Dead	Mortality (%)
F2	30min	Single	370	68	302	81.4	281	45	236	83.2
F3	30min	Double	385	135	250	63.9	201	51	150	74.2
	30min	Double	143	40	103	67	48	12	36	77.5
	15min	Double	Not Performed				370	107	263	67.5
	15min	Double	Not Performed				109	30	79	70.9
F4	30min	Single	130	104	26	20.8	Not Performed			

Table 4.5 Mortality ratios of multiple exposures to WHO tube impregnated with 5% malathion against *Ae. aegypti* Jeddah female and male mosquitoes (post 24h exposure).

Selected Jeddah malathion colony										
			Female				Male			
Generation	Exposure Time	Exposure Type	Total	survivors	Dead	Mortality (%)	Total	Survivors	Dead	Mortality (%)
F2	30min		266	56	210	79.1	84	14	70	81.6
	15min	Single	Not Performed				33	12	21	63.6
F3	30min	Double	40	32	8	20.1	Not Performed			
F3	30min	Double	32	0	32	100	Not Performed			
	25min	Double	167	58	109	64.6	49	13	36	73.2
	25min	Double	58	7	51	86.96	36	0	36	100
	20min	Double	Not Performed				93	44	49	52.1
	20min	Double	Not Performed				44	2	42	95.8
	15min	Double	61	37	24	43.5	59	19	40	67.1
	15min	Double	106	22	84	79.98	19	2	17	90.9
F4	30min	Single	108	30	78	71.7	Not Performed			

4.3.3.3. Differential gene expression in malathion selected strains

Gene expression profiles for the candidate genes *CYP9J7*, *CYP9J27*, *CYP9J26*, AAEL014614-RA (*CYP9P450*), AAEL006013-RA, AAEL006953-RA in the malathion selected Makkah strain were assessed by qRT-PCR. The expression level was compared to New Orleans, Rockefeller and Liverpool to calculate the fold change (FC). The results showed that expression level of all candidate genes *CYP9J7* (t-test=-4.8,*P*=0.009), *CYP9J27* (t-test=-5.5,*P*=0.005), *CYP9J26* (t-test=-3.5,*P*=0.03), AAEL0006953 (t-test=-5.5,*P*=0.005) and *CYP9P450* (t-test=-2.1,*P*=0.09) relative to the susceptible strains were lower in the malathion selected strain compared to the unselected (**Figure 4.13**) apart from AAEL006013-RA, which was slightly but not significantly higher(t-test=2.4,*P*=0.08) (**Figure 4.13**). Down regulation of the P450s in the malathion selected strains compared to unselected indicates these genes cannot contribute to cross resistance as they are highly expressed in pyrethroid resistance as observed in both strains in microarray study but downregulated in malathion resistance when assed by qPCR hence a form of antagonistic relationship or negative cross-resistance.

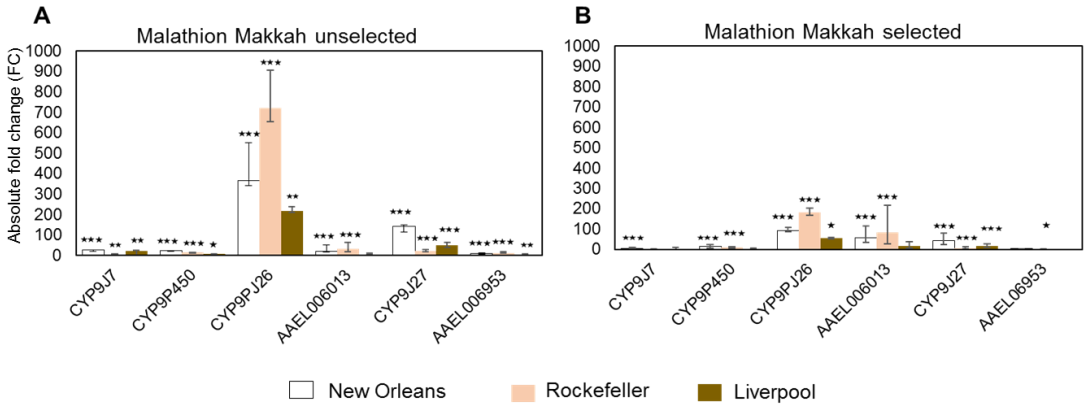


Figure 4.13 Quantitative RT-PCR analysis of candidate *Aedes* resistance genes. Relative-fold changes in malathion selected and unselected compared to the susceptible New Orleans, Rockefeller and Liverpool strains were calculated after normalisation with two endogenous reference genes. Error bars represent 95% confidence intervals (two tailed t-test, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001).

4.3.4. Gene expression analysis

4.3.4.1. Age dependent expression of metabolic genes

In chapter two, an age dependent mortality to deltamethrin was observed. Here through qPCR analysis, we evaluated if the outcome was influenced by differential

expression of some of the top overexpressed genes in the pyrethroid resistant Jeddah strain from Saudi. Comparing the expression level of each gene in mosquitoes aged 3, 5, 10 and 14 days relative to the susceptible strains showed no significant difference between the different ages **(Table 4.8; Figure 4.14).**

Table 4.6 The difference of gene expression in Jeddah strain aged 3, 5, 10 and 14 days relative to the three susceptible strains using one-way ANOVA.

Gene	F test	df	*P* value
CYP9J7	2.8	3	0.06
CYP9J27	0.58	3	0.6
CYP9J26	1.4	3	0.3
AAEL006953	0.49	3	0.05

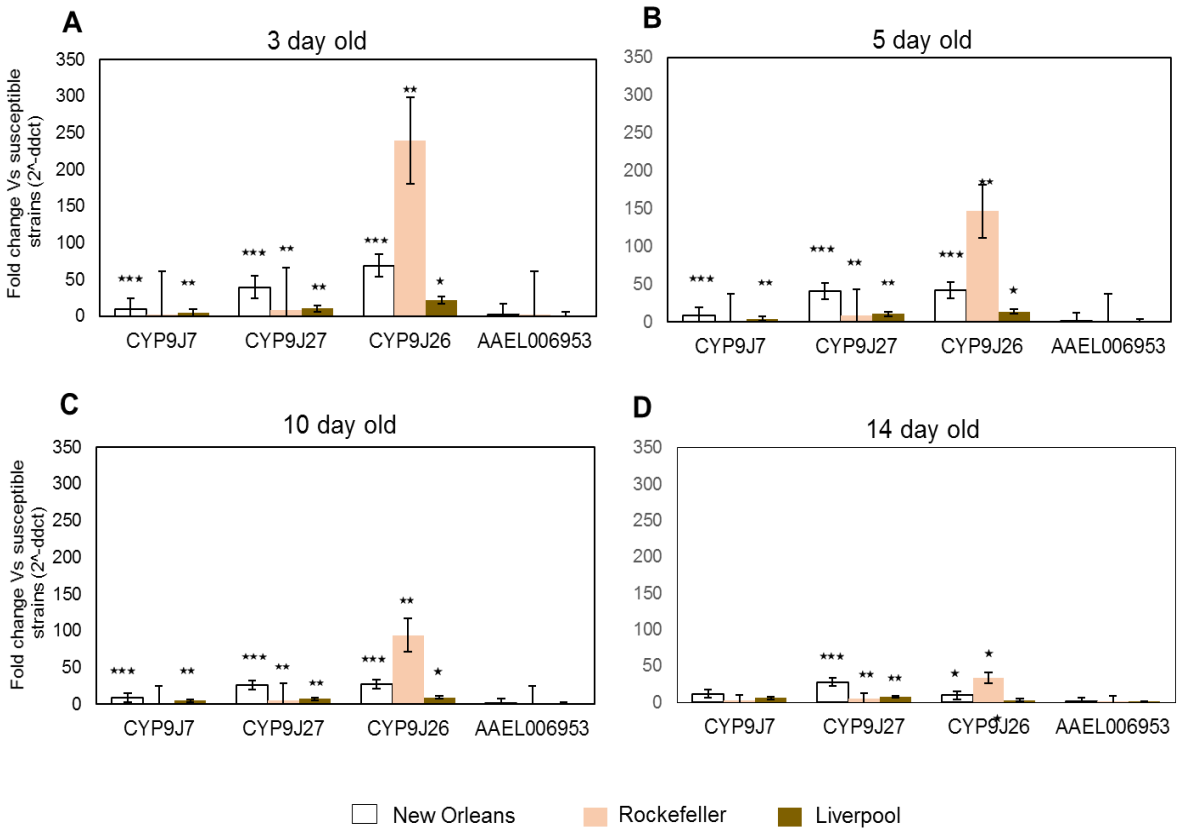


Figure 4.14 Quantitative PCR analysis of candidate genes on age-dependant mortality. Relative-fold changes compared to three susceptible strains (New Orleans, Rockefeller, Liverpool) are shown following normalisation to two endogenous reference genes. Error bars represent Standard Error (±SE). (two tailed t test, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001).

4.3.4.2. Effect of multiple or repeated exposure on gene expression

Lower mortality to deltamethrin was observed when mosquitoes were repeatedly exposed compared to unexposed mosquitoes (Chapter 2). In those repeatedly

exposed to deltamethrin, the expression level of the following tested genes *CYP9J7*, AAEL014614-RA (*CYP9P450*) and AAEL006953-RA was higher (not significant) in comparison to controls but no difference was seen in *CYP9J27* and *CYP9J26* **(Figure 4.15)**. AAEL006013 was significantly overexpressed in repeated exposure compared to control (*P*=0.03) **(Table 4.9).**

Table 4.7 The difference of gene expression between repeated exposure to deltamethrin and control relative to the three susceptible strains using a t test.

	Repeated non-exposure compared to repeated exposure			
	F test	t value	df	*P* value
CYP9J7	0.384	1.218	16	0.241
CYP9P450	0.728	0.96	16	0.351
CYP9J26	0.909	0.546	16	0.592
AAEL006013	0.869	2.378	16	0.030
CYP9J27	0.823	1.619	16	0.125
AAEL006953	0.488	0.193	16	0.848

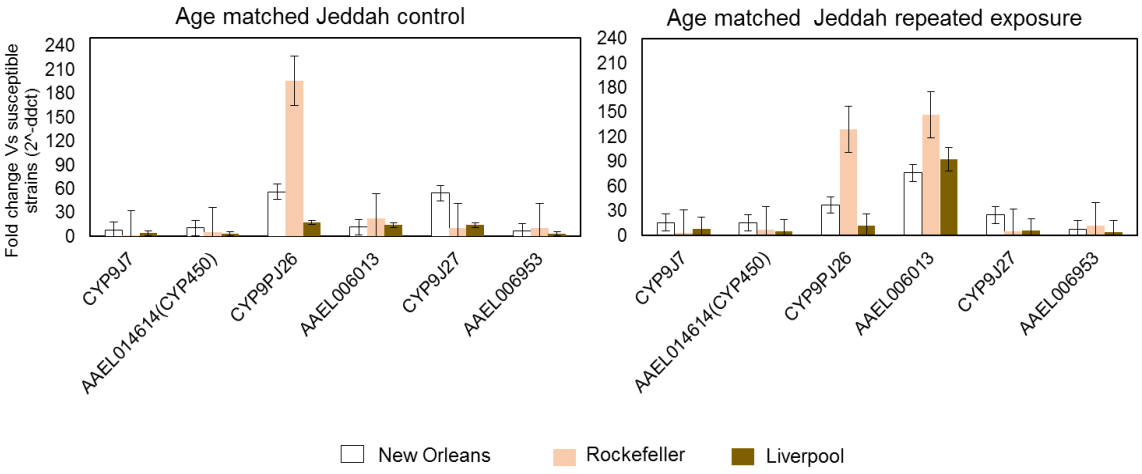


Figure 4.15 Quantitative PCR analysis of candidate genes following multiple exposure. Relative-fold changes compared to three susceptible strains (New Orleans, Rockefeller, Liverpool) are shown following normalisation to two endogenous reference genes. Error bars represent Standard Error (±SE). Same aged Jeddah control and exposed were compared.

4.3.5. *In silico* prediction of insecticide-binding parameters and conformation

Molecular docking simulations were carried out to better understand variability in the capacity of *CYP9J7* to metabolise different insecticides using a model of the P450 with pyrethroids (permethrin and deltamethrin), carbamate (bendiocarb) and

organophosphates (fenitrothion, primiphos-methyl and malathion). Docking the model with permethrin and deltamethrin predicted productive orientations above the heme such as the carboxylic group between phenoxybenzyl rings and the cyclopropane moiety, the 4' carbon of the phenoxy ring and the trans-methyl groups **(Figure 4.16A-C)** which are common routes of pyrethroid metabolism (Stevenson et al., 2011). Bendiocarb docked with C-8 of the aromatic ring oriented above the heme iron **(Figure 4.16D)** but depletion of bendiocarb was not observed in *vitro* thus suggesting that metabolism of bendiocarb may not proceed via ring hydroxylation. The organophosphates malathion, pirimiphos methyl and fenitrothion docked with the thiol group pointing away from the heme (**Figure 4.16E-G**) which suggest apart from desulfuration (activation to oxon), *CYP9J7* has alternative routes of organophosphates metabolism which may include dearylation (detoxification).

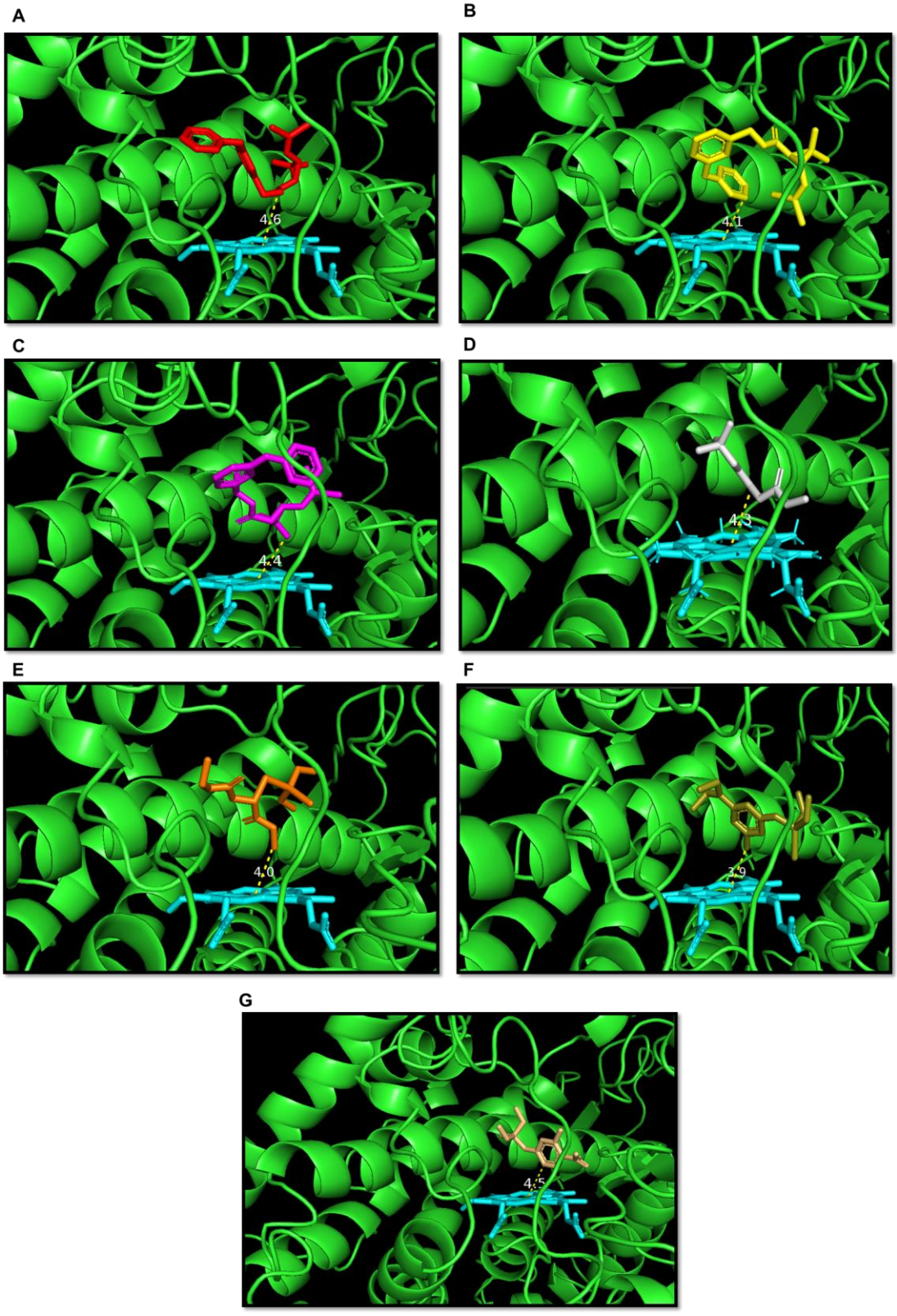


Figure 4.16 Predicted binding mode of A) cis-permethrin, B) trans-permethrin, C) deltamethrin, D) bendiocarb, E) malathion, F) pirimiphos-methyl, G) fenitrothion. *CYP9J7* helices (green) are presented in a cartoon format and heme atoms (cyan) are in stick format. Distance between insecticide and heme iron are annotated in Angstrom.

4.3.6. *In vitro* functional characterization of *CYP9J7* candidate resistance gene

4.3.6.1. Expression pattern of recombinant *CYP9J7* in *E. coli*

Recombinant *CYP9J7* protein was successfully expressed at 21°C and 150rpm and harvested 26h post-induction. Co-expression of the *CYP9J7* with NADPH cytochrome P450 reductase in *E. coli* produced a CO-spectrum **(Figure 4.17)** typical of a good-quality functional enzyme expressed predominantly as P450 with low P420 content, with a concentration of 2.5 nmoL/ml while NADPH cytochrome P450 reductase reduction activity was 165.7 nm/min/mg of cytochrome c.

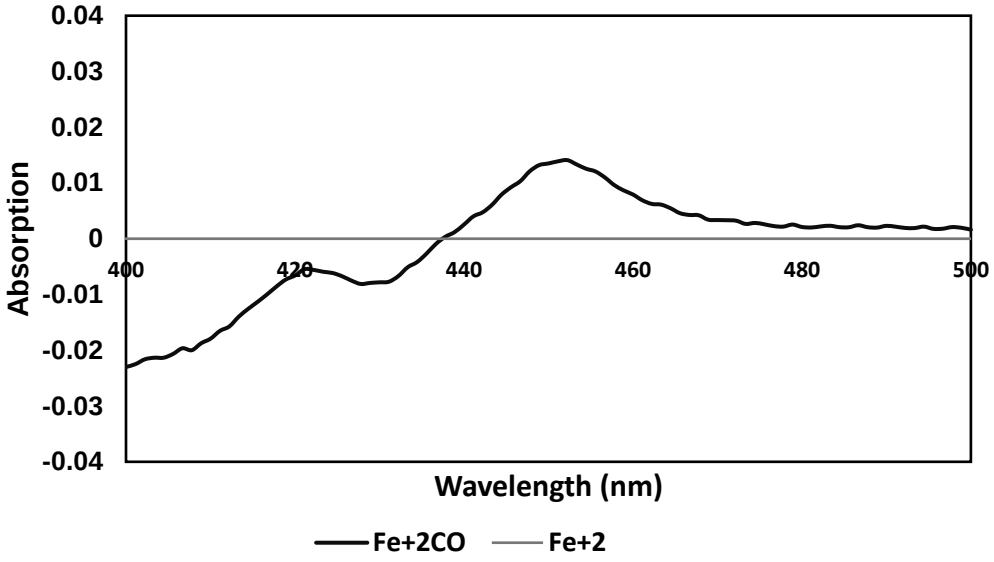


Figure 4.17 Fe2+-Co vs Fe2+ difference spectrum determined from *E. coli* membranes containing *CYP9J7*. The spectrum was determined from 50µl of purified membranes in 950 µl of 100mM Tris-sodium-EDTA, PH 7.4.

4.3.6.2. Metabolism assays with *Ae. aegypti CYP9J7*

Heterologous expression of *CYP9J7* and *in vitro* metabolism assays were carried out to validate its metabolic activity against pyrethroids (permethrin and deltamethrin), carbamate (bendiocarb) and organophosphates (primiphos-methyl, malathion, fenitrothion). The assays demonstrated that recombinant CYP9J7 cannot metabolise permethrin and bendiocarb which both showed depletion rates of 0%. Minimal activity toward deltamethrin (depletion7.9% ±8.2 (mean,±SD) was observed while more pronounced, but moderate metabolism of pirimiphos-methyl, malathion and fenitrothion, with depletion rates of 25.5% ±4.7, 38.3% ±2.1 and31.6% ±6.2 (mean, ±SD) respectively was recorded **(Figure 4.18)**. In reactions supplemented with

cytochrome b5, *CYP9J7* had a significantly higher metabolism of pirimiphos-methyl and malathion compared to reactions without it **(Figure 4.18)**.

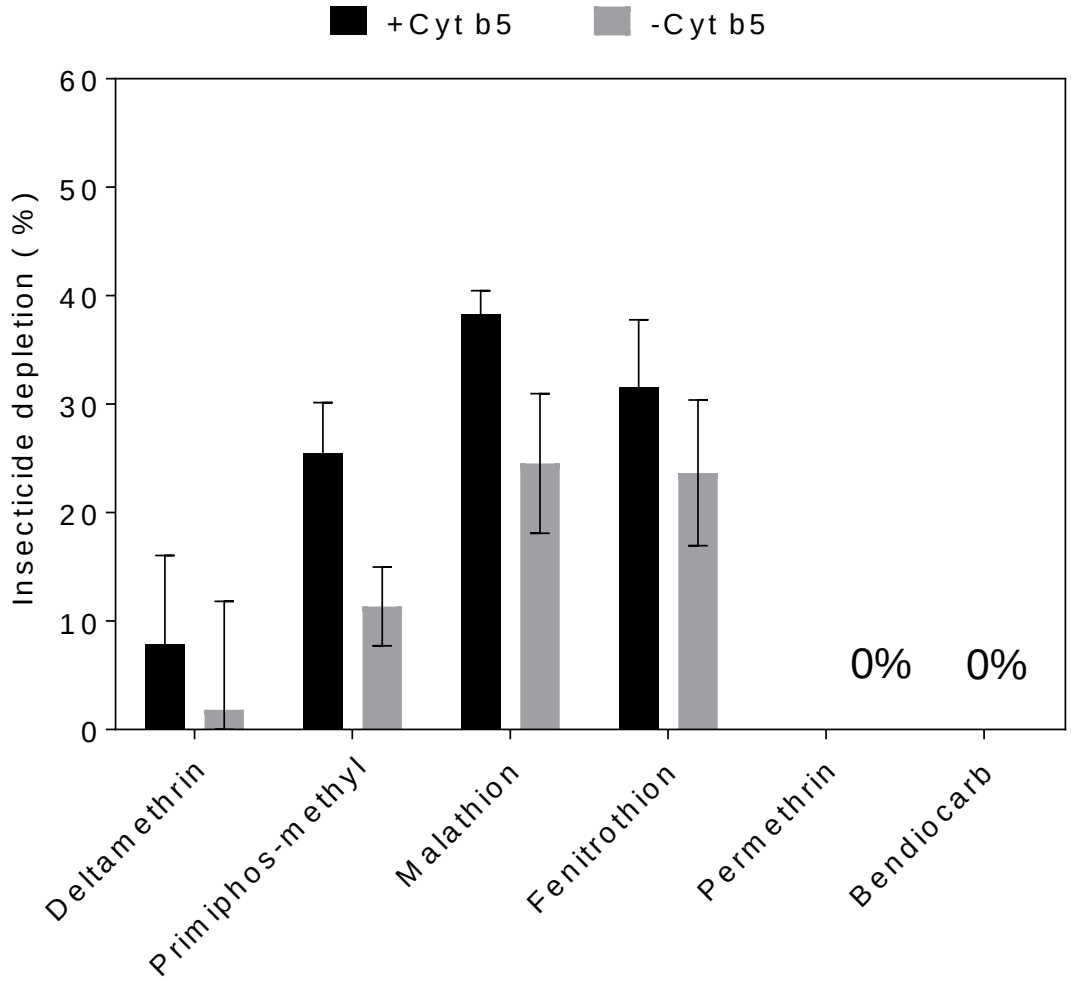


Figure 4.18 Depletion rate of pyrethroids (permethrin and deltamethrin), carbamate (bendiocarb) and organophosphate (fenitrothion, pirimiphos-methyl and malathion) by *CYP9J7 in vitro*. Cytochrome b5 enhances metabolism significantly in each case (P<0.01). Error bars represent ± SD.

4.4. DISCUSSION

Understanding the mechanisms underlying insecticide resistance and potential cross-resistance patterns is a crucial step for the design of insecticide resistance management programs. *Aedes aegypti* mosquitoes from Jeddah and Makkah exhibited resistance to multiple insecticides contributed by different resistance mechanisms as documented in Chapter 2 section 2.3.2.1 and Chapter 3 section 3.3.1. The present study aimed to delineate the metabolic genes involved in pyrethroid resistance in these strains. In the gene expression study, cytochrome P450s represented many of the top overexpressed genes in all strains and featured exclusively in the cluster with the highest enrichment score in the functional annotation analysis. These findings were further investigated by selecting the Jeddah strain for higher levels of deltamethrin resistance. Compared to the unselected Jeddah strain, the expression levels of the CYPs analysed by qRT-PCR was higher in deltamethrin selected Jeddah strains. These findings suggest a link between the differentially expressed CYPs and deltamethrin resistance in these strains.

In vitro metabolism assays with *CYP9J7*, one of the genes that was overexpressed in all strains in this study, particularly in the deltamethrin selected Jeddah strain, when compared to the pyrethroid susceptible strains and also found overexpressed in a previous study analysing gene expression in pyrethroid resistant strains from three French overseas territories (Dusfour et al., 2015) yielded unexpected results. The enzyme did not metabolise pyrethroids despite *in silico* docking analysis predicting 4' hydroxylation of phenoxybenzyl rings in permethrin which is the main route of pyrethroid metabolism (Stevenson et al., 2011). Thus, it is not clear why the gene is overexpressed in all resistant strains in this study and in the deltamethrin selected strain in qRT-PCR. One explanation could be the enzyme is important in metabolism of secondary metabolites. In pyrethroid resistant *An. gambiae*, *CYP6Z2* has also been found consistently overexpressed but it has minimal metabolic activity against the primary pyrethroids compound (Mclaughlin et al., 2008). However, this enzyme and its orthologue *CYP6Z8* in *Ae. aegypti* play a significant role in clearance of pyrethroid metabolites (Chandor-Proust et al., 2013). We did not investigate if *CYP9J7* had a similar function hence this needs to be confirmed as an explanation for its overexpression in all strains. *CYP6Z2* high affinity to primary pyrethroid compounds

has also been suggested to be important in sequestration which could be the same case with *CYP9J7,* which was predicted *in silico* to have high affinity to pyrethroids based on the low binding scores (Mclaughlin et al., 2008).

Extremely high levels of bendiocarb resistance and slight organophosphate resistance was also observed in both Saudi strains (Chapter 2, section 2.3.2.1). A significant recovery of susceptibility was observed when Jeddah (60%) and Makkah (25.2%) were pre-exposed to PBO before conducting the standard bioassay with bendiocarb. There was also recovery of susceptibility in synergistic bioassays with malathion, which was significant in Makkah strains (27%). The partial recovery of susceptibility and absence of the *Ace1* mutations (Chapter 3, section 3.3.1.2) associated with organophosphate and carbamates resistance in this populations suggest metabolic genes such as CYPs and other unknown genes, which are inhibited by the synergist PBO at least partially contribute to bendiocarb and organophosphate resistance in these populations. Esterases are known to contribute to organophosphate resistance (Edi et al., 2014) but no members of this class of metabolic genes were significantly differentially expressed in the Saudi strains. It is possible that expression level of esterases may have been altered during selection of malathion resistance in Makkah strain, but this was not assessed in this study.

In *Anopheles* species, CYPs have been associated with cross resistance to carbamates and pyrethroids. Cytochrome *CYP6Z1* which is commonly overexpressed in pyrethroid resistance populations (Edi et al., 2014, Ibrahim et al., 2016), has been validated in *vitro* as a potent metaboliser of different classes of pyrethroids and bendiocarb. In *vitro* metabolism assays indicate *CYP9J7* cannot cause cross resistance with bendiocarb as no depletion was detected, but it might possibly cause cross resistance with organophosphates if the metabolism observed involves diarylation (i.e. detoxification). However, this gene was downregulated in the malathion-selected Makkah strains, which suggests *CYP9J7* may have higher desulfuration activity that lead to activation of organophosphates to the toxic oxon form, which could make it a candidate gene for negative cross resistance between organophosphates and pyrethroids. Such a gene could be a useful marker in insecticide resistance management programmes where progressive elevation could indicate a switch point from pyrethroid to organophosphate and *vice versa*. Cross

resistance to bendiocarb may be facilitated by the other overexpressed CYPs and genes in cluster associated with transport, which have not been functionally validated. A possible candidate is *CYP9J27* which was highly overexpressed in all pyrethroid resistant strains investigated in this study and in other studies (Bariami et al., 2012, Ishak et al., 2017, Kasai et al., 2014). Through *in silico* modelling and docking analysis, the enzyme was predicted to be a potential bendiocarb metaboliser, however this should be confirmed *in vitro* or *in vivo* (Ishak et al., 2017).

Downregulation of metabolic genes associated with insecticide resistance may explain why susceptibility increases with age (Glunt et al., 2011, Rowland and Hemingway, 1987). For instance, although not significant, the expression levels of the cytochrome P450 *CYP9J26* in the Jeddah strain, which has been shown to metabolise deltamethrin *in vitro* (Stevenson et al., 2012) and is frequently overexpressed in pyrethroid resistance populations (Moyes et al., 2017), decreased from a fold change of 68.6 relative to New Orleans (3 day old), 42 in 5 day old, 27 in 10 day old and 9.6 in 14 day old mosquitoes when assessed by qRT-PCR **(Figure 4.14)**. A similar reduction in expression level of this gene relative to Rockefeller and Liverpool strains was observed **(Figure 4.14)**. Regulation of this gene and *CYP9J27* is highly likely to be age dependent rather than induced by insecticides since the genes were expressed in low levels in older mosquitoes that had been repeatedly exposed to insecticides. Other studies have reported similar finding where metabolic genes such as GSTE2, GSTE1, *CYP6P3*, *CYP6P4*, *CYP6Z3*, *CYP6M2* and COEAE1A that have consistently been found overexpressed in resistant population were found to be stage or developmentally regulated (Strode et al., 2006, Marinotti et al., 2006, Brent et al., 2013, Chen et al., 2008).

By analysing the gene expression profile of the different field and insecticides selected strains relative to susceptible strains, this study has identified metabolic resistance-related genes that may be involved in pyrethroid resistance in Jeddah and Makkah. The study did not find conclusive evidence for the specific genes causing cross resistance to bendiocarb and organophosphate. However, several of the top genes significantly overexpressed in all resistant strains remain to be validated *in vitro* and should be assessed in future studies.

4.5. CONCLUSION

Although *Vgsc* mutations were strongly associated with deltamethrin resistance in both populations in Chapter 3, section 3.3.1, metabolic resistance appears to play a role not only to pyrethroids, but also organophosphate and carbamate. I demonstrated resistance to insecticides could respond quickly to selection and involves overexpression of potential metabolic resistance genes. Through selection, I also demonstrated the strains (Makkah) can quickly develop resistance to malathion, although the mechanism was not clear, but was not a result of the candidate CYPs, which were downregulated hence the possibility of a negative cross resistance with pyrethroids. These findings should be considered in management of insecticide resistance.

CHAPTER 5
BEHAVIOUR OF PYRETHROID SUSCEPTIBLE AND RESISTANT *AEDES AEGYPTI* IN RESPONSE TO INSECTICIDE-TREATED NETTING IN A LABORATORY BIOASSAY

5.0. ABSTRACT

Background Insecticide-treated materials (ITMs) have the potential to help control arboviruses by preventing vectors entering the domestic environment. The development of pyrethroid resistance in *Ae. aegypti* threatens the efficacy of ITMs, which are primarily pyrethroid treated. The relationship between biochemical resistance and behavioural changes is poorly understood but is crucial to understand the efficacy of ITMs against resistant mosquito populations. The aim of the study reported in this chapter was to investigate whether resistant and susceptible *Ae. aegypti* strains exhibit behavioural differences during host-seeking at insecticide-treated netting.

Methods New Orleans (pyrethroid-susceptible), Jeddah and Makkah (pyrethroid-resistant) strains were exposed to nets (deltamethrin-PermaNet2.0, alpha-cypermethrin-DuraNet and untreated controls) for 10 minutes as the mosquitoes attempted to reach and feed on a human bait (a thumb) in a small-box assay.

Results Mosquitoes spent significantly less time resting and probing at treated nets compared to untreated controls, but there was no difference between resistant and susceptible strains. After contact with treated nets, all strains exhibited elevated flight activity, with most mosquitoes preferring to rest away from the treated surface.

Conclusion The results indicate that physiologically-resistant *Ae. aegypti* did not differ from susceptible when attempting to feed through an ITM.

5.1. INTRODUCTION

Vector control tools are crucial for control of *Aedes*-borne arboviruses and most successful methods rely on insecticide-based approaches such as targeting immature *Aedes* mosquitoes in water containers, or targeting adult mosquitoes by space spraying and thermal fogging. Additional tools for adult *Ae. aegypti* control are needed, which can be used for prevention rather than as an outbreak response (Dave, 1985, Esu et al., 2010). The low efficacy of fogging and space spraying is at least partially attributed to *Ae. aegypti*'s ability to exploit the human indoor environment to feed and rest (Manda et al., 2011).

Insecticide-treated materials (ITMs) such as insecticide-treated curtains (ITCs) and insecticide-treated window screens (ITSs) have potential to target indoor populations and provide protection at the personal/individual level as well as the community by preventing entry to, or killing mosquitoes attempting to, enter houses (Golding et al., 2015, Kroeger et al., 2006, Loroño-Pino et al., 2013, Manrique-Saide et al., 2015, Paz-Soldan et al., 2016, Vanlerberghe et al., 2011) . Recent studies reported that ITS usage in combination with peri-domestic larval control reduced indoor resting *Ae. aegypti* by more than 50% in Acapulco and Merida, Mexico (Che-Mendoza et al., 2015, Che-Mendoza et al., 2018). Earlier studies reported that ITCs reduced the domestic abundance of *Ae. aegypti* in Mexico, Venezuela and Thailand (Kroeger et al., 2006, Aponte et al., 2013, Vanlerberghe et al., 2013). Despite these promising results, ITM efficacy also faces challenges. For example, ITCs have limited efficacy when curtains remain opened during the day or if house entry points are not protected (Che-Mendoza et al., 2018). Another major challenge is that insecticide resistance to the pyrethroids used to treat ITMs is widespread in *Ae. aegypti* (Moyes et al., 2017).

Behaviour of mosquitoes is governed by interactions between the nervous system and the environment (Marriel et al., 2016). Pyrethroids are neurotoxins that can affect mosquito physiology and behaviour even at low concentrations (Marriel et al., 2016). Additionally, pyrethroids can produce "contact irritancy" (sometimes termed "excito-repellency"), a sub-lethal effect following brief contact with treated surfaces resulting in avoidance of treated areas (Evans, 1993, Kongmee et al., 2004). This is different to true "repellency" which is where a mosquito exhibits oriented movements away from a treated surface without any contact (Roberts et al., 2000).

Several studies have demonstrated that the primary action of pyrethroids is contact irritancy rather than repellency (Kongmee et al., 2010, Thanispong et al., 2009, Achee et al., 2009). Pyrethroids alter the gating kinetics of the *Vgsc*, and contact irritancy in mosquitoes may result from effects on the nervous system (Deletre et al., 2013), with mosquitoes becoming agitated following contact with treated materials and responding with increased flight activity (Manda et al., 2011).

Whilst biochemical mechanisms of pyrethroid resistance are reasonably well documented (Smith et al., 2016), studies focussing on changes in behavioural responses are limited. Given the key importance of pyrethroids for ITMs, the current study aimed to compare the behaviour of pyrethroid resistant Saudi Arabian strains with the standard susceptible New Orleans strain, during host-seeking at a human - baited ITM. These experiments used a new bioassay method, the "Thumb Test", a benchtop system that was originally designed to video-record activity of *Anopheles* mosquitoes at the bed net interface. The test attempts to simulate a natural situation in which blood-seeking females are prevented from reaching a human host by an insecticide-treated screen.

5.1.1. Objectives

1-Determine the efficacy of insecticide-treated netting using the WHO cone bioassay, and verify expectations of differential resistance from the earlier WHO tube bioassays in Chapter 2.

2- Characterise behavioural responses of host-seeking *Ae. aegypti* when near to, and in contact with, insecticide-treated netting.

3-Compare the behavioural repertoire, and the impacts of exposure to insecticide-treated netting, between resistant and susceptible strains of *Ae. aegypti.*

5.2. MATERIALS AND METHODS

5.2.1. Mosquito strains

All tests were carried out using three different *Ae. aegypti* strains. Resistant F2 mosquitoes from Jeddah (21°35'2.13"N,39°13'9.42"E) and Makkah F2 (21°45'2.13"N, 39°92'1.96"E) were originally collected in March 2016 **(Chapter 2, Table 2.1)**. Fully susceptible mosquitoes from New Orleans, which possess no known insecticide resistance mechanisms (Bariami et al., 2012), is a standard insecticide susceptible strain held at the Liverpool School of Tropical Medicine (LSTM). The mosquitoes were reared under insectary conditions of 27±1°C,70–75% RH and 12:12 light and dark cycle. Chinchilla pellets (Maltby's, Hull, UK) were used to feed larvae and 10% glucose solution to feed adult females. All tests used non-blood fed females, aged 3-7 days post-emergence. Mosquitoes were starved of sugar for 16-18h before testing to encourage host-seeking behaviour.

All tests were carried out in an insectary at LSTM from 10.00 and 14.00h, between 23/09/2016 to 13/11/2016.

5.2.2. Insecticide-treated netting

Mosquito behaviour was examined in the presence of three types of the net. Two long-lasting pyrethroid-treated insecticidal nets PermaNet 2.0, a polyester net coated with deltamethrin (55mg/m^2, Vestergaard, Lausanne, Switzerland), and DuraNet, a polyethylene net impregnated with alphacypermethrin (261mg/m^2.; Tamil Nadu, India), and an untreated polyester net (Abakhan Fabrics, Liverpool UK). The untreated net was tested prior the experiment to confirm it was uncontaminated with any insecticide as described in section 5.2.3. Prior to the experiment, all new untreated and treated nets were cut into appropriate-sized pieces (15x15cm^2) for the use in the thumb box apparatus and stored separately in airtight boxes at 5°C to avoid contamination and minimise insecticide degradation. Each piece of net was used on a single test day, then discarded.

5.2.3. WHO Cone bioassay

Following the standard WHO protocol (World Health Organization, 2013a), groups of five non-blood-fed female mosquitoes from each strain aged 3–5 days post emergence were introduced into a WHO plastic cone fixed onto a 15 x 15cm section of PermaNet 2.0, DuraNet, or untreated net for 3 min. After 3 min, mosquitoes were

transferred by a manual aspirator into holding cups, with 10% sugar solution soaked into cotton. Knockdown and mortality were recorded after 1h and 24h respectively (World Health Organization, 2006). A total of 10 test replicates was carried out for each strain. Cone bioassays were conducted under insectary conditions of 27°C and 70-75% relative humidity (World Health Organization, 2013a). At the end of each assay, all materials were soaked in 5% Decon 90 detergent for 2h, rinsed with tap water 3 times and once with deionised water, and dried before re-use.

5.2.4. Thumb box

The Thumb test was designed originally to investigate behaviour of *Anopheles sp.* at an insecticide-treated net surface (Hughes, 2018) and was used here without modification. The box is a cube with 100 mm sides, with a five-sided clear acrylic display case and a white acrylic base. One side of the box has a 26mm diameter circular port protruding into the box, which was covered with a 50x50mm piece of the netting being tested. A secondary fine-mesh net was fitted 5mm behind the test net to separate the volunteer's thumb from the test net, which prevents blood feeding but allows mosquitoes to detect the host and begin probing in an attempt to feed whilst in contact with the experimental net **(Figure 5.1)**. On the opposite side of the box a second circular port (26mm diameter) was used to introduce mosquitoes into the chamber **(Figure 5.1)**. Throughout the test, the volunteer kept their hand steady to avoid disturbing the mosquitoes. One volunteer (the operator) was used for all tests, and perfumes and scented toiletries were avoided on test days. In addition, eating or washing the hand with soap was avoided for at least 3h prior to testing. Separate boxes were used for each net type to prevent cross-contamination.

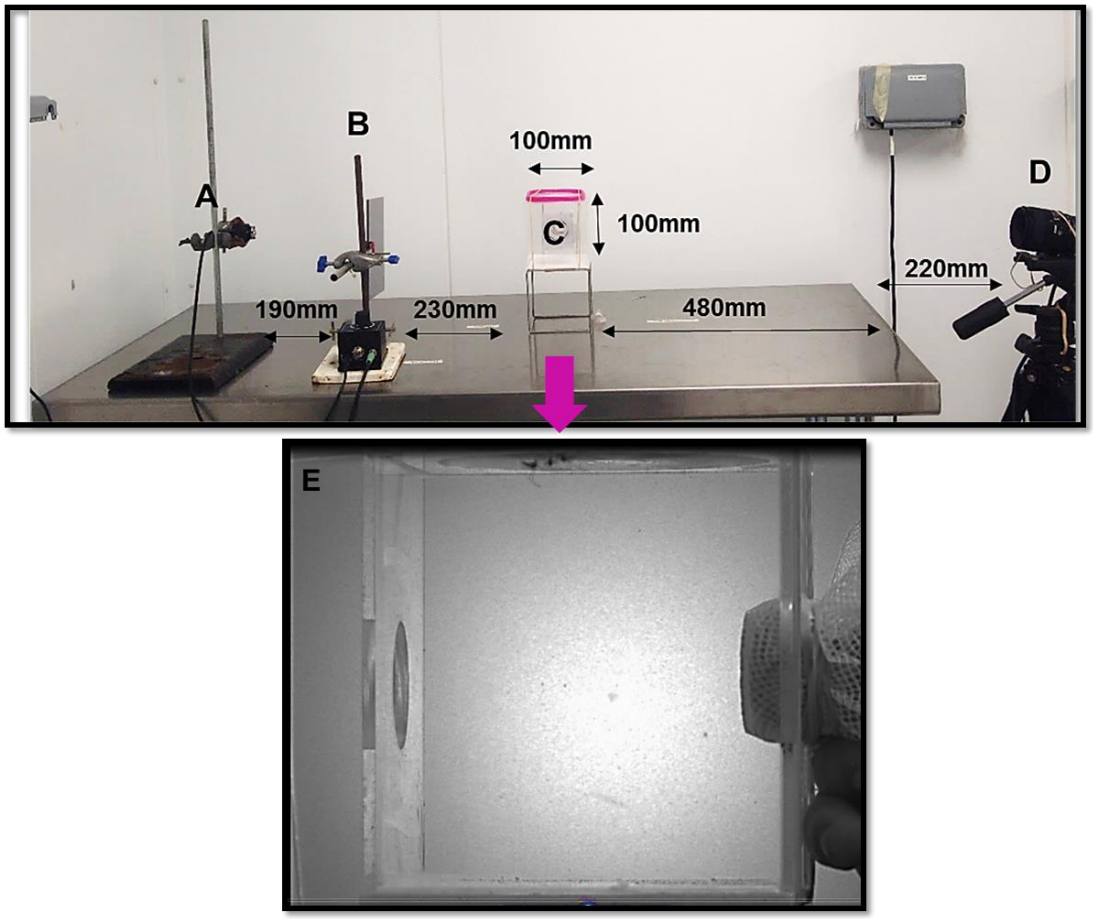


Figure 5.1 Photographs showing the thumb test apparatus in the insectary A) Light source, B) Diffuser, C) Thumb test box, D) Camera, E) The thumb box test arena showing the mosquito entry port on the left and the test netting on the right.

5.2.5. Video recording system

A Lambda Photometrix MQ013RG-E2 camera fitted with a Nikon Ai AF Micro-Nikkor 60mm/ F2.8D lens (set at f11) was used in all tests. An infrared LED (850nm; ThorLabs M850L) illuminated the test arena through a 25 x 25cm 3mm thick acrylic diffuser (Comar optics, UK) (**Figure 5.1)**. Video recording speed was 30 frames per second (s) and test videos stored as. avi files.

5.2.6. Computer hardware and software

Video recordings were analysed using NorPix StreamPix version 5. All data were stored on an external hard drive (4TB, Amazon, UK). Initial preliminary trials with individual mosquitoes were analysed using BORIS software version 6.0.6 (Friard et al., 2016). However, this was not possible for tests involving multiple mosquitoes, as there was too much activity to track each individual mosquito reliably. Consequently, the main experiments with multiple mosquitoes were analysed manually by scan

sampling at 10s intervals. The scan sampling method involves allocating each mosquito's behavior to a behavioral category in a sampled video frame throughout the 10min assay at 10s intervals.

5.2.7. Experimental set up

5.2.7.1. Optimisation tests

Prior to beginning experiments, a set of initial tests were undertaken to optimise the thumb test assay. The susceptible New Orleans and resistant Jeddah strains were used in test 1, but Jeddah alone was used in tests 2, 3 and 4. This decision was made because results of the first optimisation (test 1) showed that 0/10 Jeddah mosquitoes tested landed on the untreated net, and 2/10 landed on PermaNet 2.0. In contrast, 8/10 New Orleans strain landed on the untreated and 10/10 landed on the PermaNet2.0. Therefore, initial work aimed at finding suitable conditions that increased responsiveness of the Jeddah strain on untreated nets.

Four tests were carried out, with variations in: i) number of mosquitoes (single or groups) tested simultaneously; ii) a light source (normal brightness or dim); and iii) duration of mosquito starvation prior to testing (short or long). Prior to all tests, mosquitoes were transferred from the cage to a plastic cup by an aspirator and retained in the testing room for acclimatisation for one hour. After the acclimatisation period, Individual mosquitoes in tests 1-3 and 5 mosquitoes in test 4 and in the actual experiment were gently introduced into the thumb box by manual aspiration. Video recording began when mosquito(es) entered the box and continued for 10min. At the end of the test, the mosquitoes were transferred to a plastic cup and provided with 10% (w/v) sugar on a cotton wool pad.

For the final (optimised) experiment, mortality post-exposure was recorded 1h and 24h after the test. All boxes were soaked in Virkon solution for 24h and rinsed in water before re-use.

Optimisation tests

5.2.7.1.1. Preliminary Test 1 (individual mosquitoes; short starvation; normal light)

Mosquitoes were sugar-starved for 2-3h prior to testing, to encourage higher responsiveness. Tests were conducted under normal insectary lighting (Lumsden, 1957), which has been reported to promote *Aedes* activity (Taylor and Jones, 1969). An individual Jeddah or New Orleans mosquito was aspirated from the plastic acclimation cup and transferred into the thumb box then left for 10min. The test was repeated with 10 different mosquitoes using either untreated net or PermaNet 2.0.

5.2.7.1.2. Test 2 (individual mosquitoes; longer starvation; normal light)

Test 2 aimed to determine whether a longer starvation period would promote host seeking behaviour compared to the short starvation period used in test 1. The Jeddah strain was used in this test and subsequent optimisation tests. The mosquitoes were sugar-starved for 16-18h prior to the experiment and tested as in test 1.

5.2.7.1.3. Test 3 (individual mosquitoes; longer starvation; dimmer light)

In this test, the effect on host seeking behaviour of longer starvation and reduced light intensity was investigated based on observations that *Ae. aegypti* exhibit both diurnal and nocturnal feeding behaviour (Chadee and Martinez, 2000, Lumsden, 1957). The assay was conducted as described above but with light levels reduced to a single 7W bulb, facing away from the arena.

5.2.7.1.4. Test 4 (groups of mosquitoes; longer starvation; normal light)

Since no improvements in responsiveness were observed in the previous tests, the design was switched to test groups of 5 mosquitoes previously starved 16-18h instead of a single mosquito. This decision was based on anecdotal reports within LSTM and on a published report that feeding activity can be higher in group of mosquitoes (Charlwood et al., 2014). The optimisation results are presented in **Appendix 2**.

5.2.7.2. Experimental protocol

As observed in the optimisation tests, groups of mosquitoes were more responsive than individual mosquitoes. Therefore, the actual experiment followed the conditions in test 4, and was conducted under normal ambient lighting conditions, with a group of five mosquitoes that were previously starved for16-18h.The experiment compared the behaviour of the three *Ae. aegypti* strains to PermaNet 2.0, DuraNet and untreated nets based on analysis of the video recordings of their responses in the bioassay. The three experimental tests (untreated net, PermaNet 2.0 and DuraNet assays) were conducted on separate days.

The number of replicates per test for each strain are as presented in **Table 5.1**.

Table 5.1 Number of the susceptible New Orleans and resistant Jeddah and Makkah mosquitoes tested on three different nets (untreated net, PermaNet 2.0, DuraNet) into thumb assay.

Strain	Untreated net		PermaNet 2.0		DuraNet	
	Number of mosquitoes	Number of groups tested	Number of mosquitoes	Number of groups tested	Number of mosquitoes	Number of groups tested
New Orleans	115	23	100	20	105	21
Jeddah	100	20	125	25	105	21
Makkah	105	21	100	20	100	20

5.2.8. Categorisation of behavioural events

A set of quantifiable behavioural events were defined based on initial observations of *Ae. aegypti*, and on those defined for *Anopheles gambiae* in earlier thumb test studies (Hughes, 2018).

1- **Flight pre-net contact:** flying at any time in the assay before contact with the net.

2- **Flight post-net contact:** flying at any time during the assay after contact with the net.

3- **Resting on the walls** standing anywhere within the box other than on the experimental netting.

4- **Resting on the net:** standing on the experimental netting.

5- **Probing (always on the net):** Extending proboscis through the net.

6- **Grooming on the walls:** body cleaning behaviour while standing anywhere in the box other than on the experimental netting.

7- **Grooming on the net:** grooming while standing on the net.

8- **Knocked down:** unable to fly, stand or move in coordinated manner.

5.2.9. Statistical analysis

Count data (e.g. from cone bioassays) were analysed using Pearson χ^2 tests. The Kolmogorov-Smirnov test was applied on log transformed data and actual data to test normality. Data that were not normally distributed were analysed using non-parametric statistics. The amount of time spent engaged in different behaviours was analysed by using General Linear Model (GLM) with strain and net type as factors, and post-hoc t-tests in SPSS version 24.

Probing behaviour in the treated nets and flying pre-net contact and resting behaviour on the net in DuraNet were not normally distributed; hence, ranking was done to allow implementation of the GLM using non-parametric data. Due to the limited data obtained for walking and grooming on the walls, for statistical analysis, data were combined with resting on the walls. Likewise, time spent in walking or grooming on the net was combined with time spent resting on the net. Principal components analysis (PCA) with Varimax rotation (to aid interpretation of principal components) was run to identify groups of related variables in the correlated data (Huntingford, 1976). GraphPad Prism7 was used to produce figures for the mean time duration in each behaviour using the actual data and confidence intervals (CIs).

5.3. RESULTS

5.3.1. Cone bioassay

All mosquitoes from each strain survived 3 min exposure to an untreated net in a WHO cone assay. Exposure to PermaNet2.0 and DuraNet exposure (for 3 min) resulted in 100% mortality of the New Orleans strain **(Figure 5.2)**. Mortality rates of Jeddah and Makkah strains were 75% and 10% with PermaNet2.0 and 35% and 17% with DuraNet, respectively **(Figure 5.2)**, consistent with the greater resistance of the Makkah strain detected in tube bioassays (Chapter 2). There was a significant difference in mortality between strains for both PermaNet2.0 ($\chi 2=81.9$, df=1, $P<0.0001$) and DuraNet ($\chi 2=79$, df=1, $P<0.0001$), though overlap of confidence intervals suggests that the mortality rates of the two Saudi strains did not differ significantly for DuraNet (**Figure 5.2**).

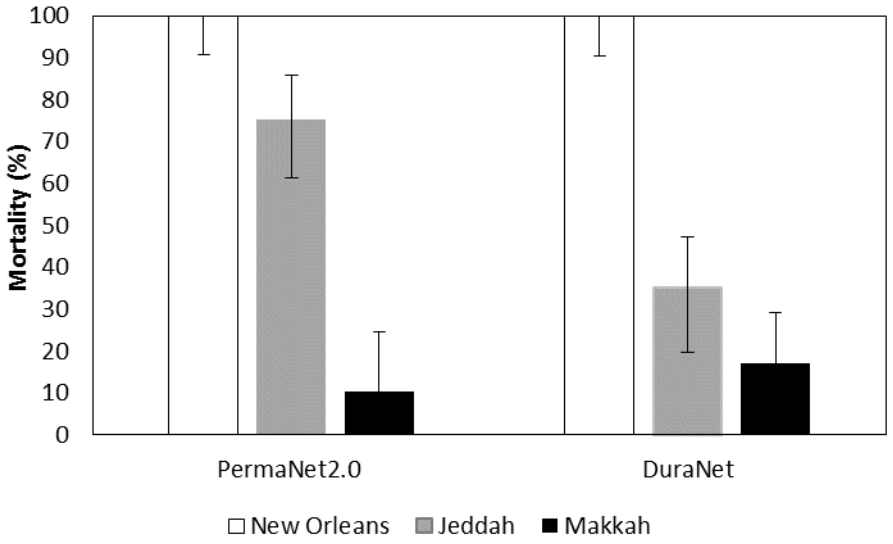


Figure 5.2 WHO cone bioassay results testing the susceptibility of the three *Ae. aegypti* strains to PermaNet 2.0 and DuraNet. Mortality (y-axis) was recorded after 24h. Error bars are 95% confidence intervals.

5.3.2. Thumb test

A total of 955 mosquitoes were tested; 320 with the untreated net, 325 with PermaNet 2.0, and 310 with DuraNet.

5 3.2.1. Mortality following the thumb test

None of the mosquitoes died at any time during or after trials with exposure to untreated net. At 1hour post-exposure in trials with treated nets, none of the Saudi mosquitoes died, but the mortality rate of New Orleans was 54% when exposed to PermaNet 2.0 and 39% for DuraNet **(Figure 5.3A)**. The difference in 1h mortality among strains was significant for trials with each treated net (PermaNet 2.0 χ^2=145.7, df=1, *P*<0.0001; DuraNet χ^2=98.3, df=1, *P*<0.0001). The mortality 24h post-exposure to PermaNet2.0 was 100%, for New Orleans, 4.8% for Jeddah and 2% for Makkah **(Figure 5.3B)**, with very similar mortality recorded after DuraNet exposure (96% for New Orleans, 3% for Jeddah and 2.6% for Makkah) **(Figure 5.3B)**. These differences in mortality among strains at 24h post-exposure were significant (PermaNet 2.0 χ^2=290.4, df=1, *P* <0.0001; DuraNet χ^2=270.8, df=1, *P*<0.0001), but as is evident from the overlapping confidence intervals in **Figure 5.3B,** there was no significant difference in mortality rates between the Saudi strains.

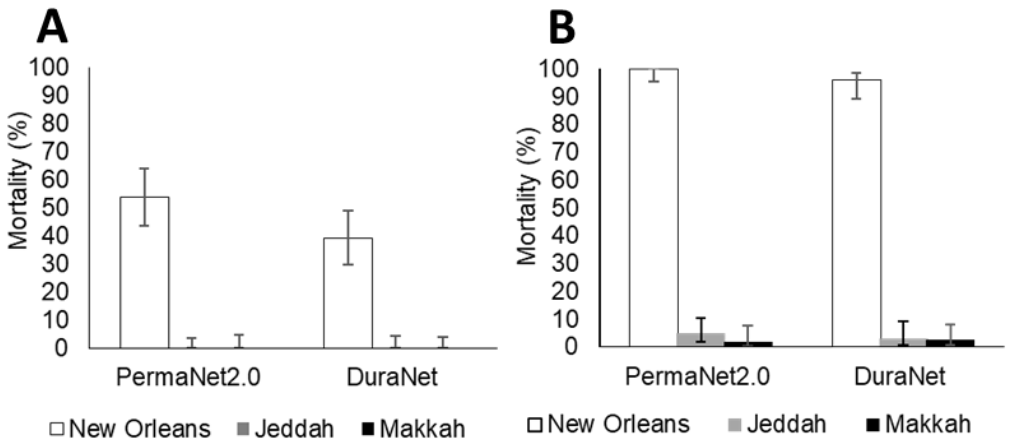


Figure 5.3 Mortalities following 1h exposure (A) and 24h exposure (B) to PermaNet 2.0 and DuraNet of New Orleans, Jeddah and Makkah strains after the thumb test.

5.3.3. Behavioural profiles during the thumb test assays

5.3.3.1. Behavioural time budgets for all activities

There was a 100% response (approaching and resting on the barrier netting) in the groups of five mosquitoes (though not necessarily all individuals) in all strains to both untreated and treated netting **(Figure 5.4)**. Behaviour profiles showed a clear influence of the use of a treated or untreated net. Most notably far more time was spent in probing and the other 'on the net' behaviours, and less in 'on the wall' behaviours in the assays with untreated net than either type of treated net (upper row of panels compared to others in **Figure 5.4**. Owing to their limited occurrence some categories from **Figure 5.4** were pooled for statistical analysis (i.e. resting includes resting, grooming and walking). In contrast the category flying was subdivided to include flying before net contact and after net contact, based on the hypothesis that net contact may affect subsequent flight.

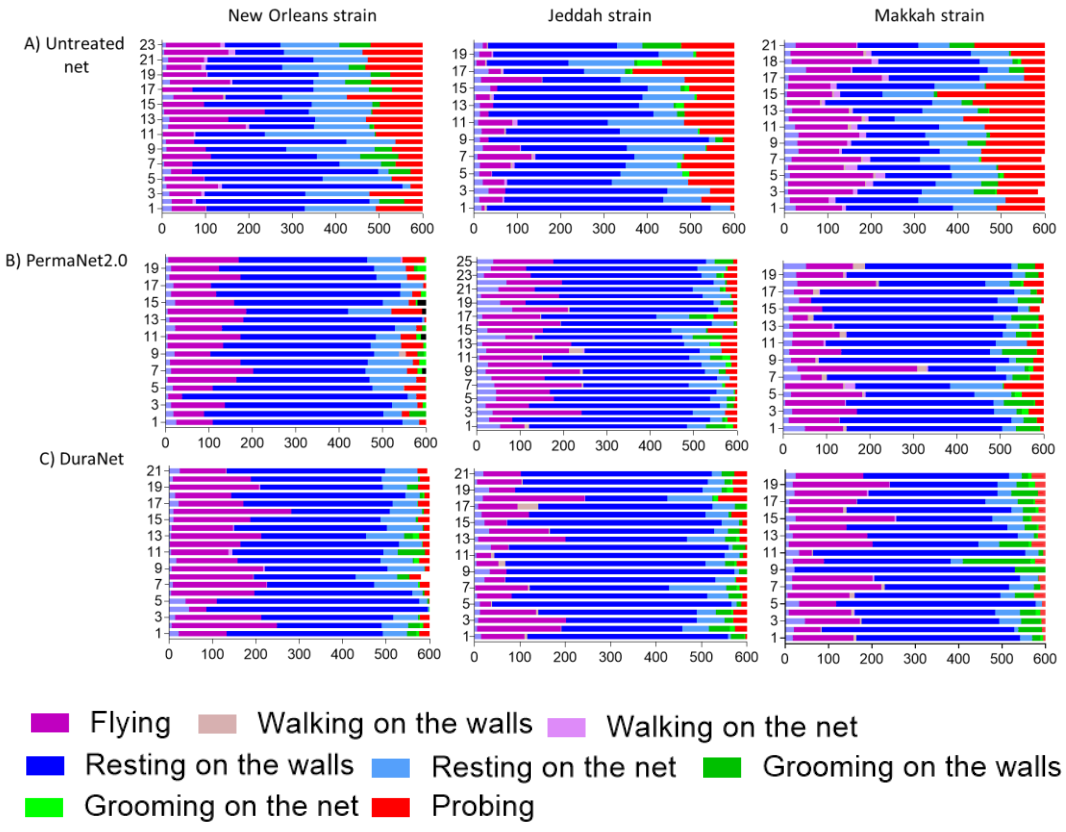


Figure 5.4 Total time spent (mean time in seconds) on each behavioural event by New Orleans, Jeddah and Makkah in the test nets; A) Untreated net, B) PermaNet 2.0, C) DuraNet. Each individual is illustrated in a separate bar. Note that behaviours are ordered according to categories and are not displayed sequentially in order of occurrence during the trial. The x-axis represents the time (s) spent in each behavioural category. The y-axis represents mosquito identification (ID).

5.3.3.2. Statistical analysis of behavioural categories

Results from GLM analyses of behaviours (or pooled behaviour categories as noted above) in relation to mosquito strain and net (with separate analysis for each treated net brand) are shown in **Table 5.2**. Differences between strains are not unexpected but of principal interest in the analyses are the effect of net (treated/untreated) and whether there are strain*net interactions which would indicate a differential response to net type among strains **(Table 5.2)**.

The key behaviours in the thumb test are probing and resting on net (which includes all non-probing 'on the net' behaviours. As suggested in **Figure 5.5**, both are significantly impacted by the presence of insecticide on the net (greatly reduced; means are shown in **Figures 5.4** and **5.5** for each net type). However, differences among strains are small and only for resting on the net (DuraNet vs untreated) was a significant result obtained. Crucially, there was no significant interaction term for either resting on the net or probing indicating that the change in behaviour induced by treated nets was the same across strains, irrespective of their resistance status **(Table 5.2)**. Flying post net contact and resting on the walls were also strongly affected by net, with a general reduction increase in time spent on each in the presence of insecticide **(Table 5.2; Figures 5.4, 5.5)**. For these behaviours there was more variation among strains and also significant interaction terms (**Table 5.2**), but not in a manner consistent with a differential strain response associated with resistance status (for example a greater increase in flying or resting in New Orleans than the Saudi strains with treated nets vs untreated) (**Figures 5.4, 5.5**).

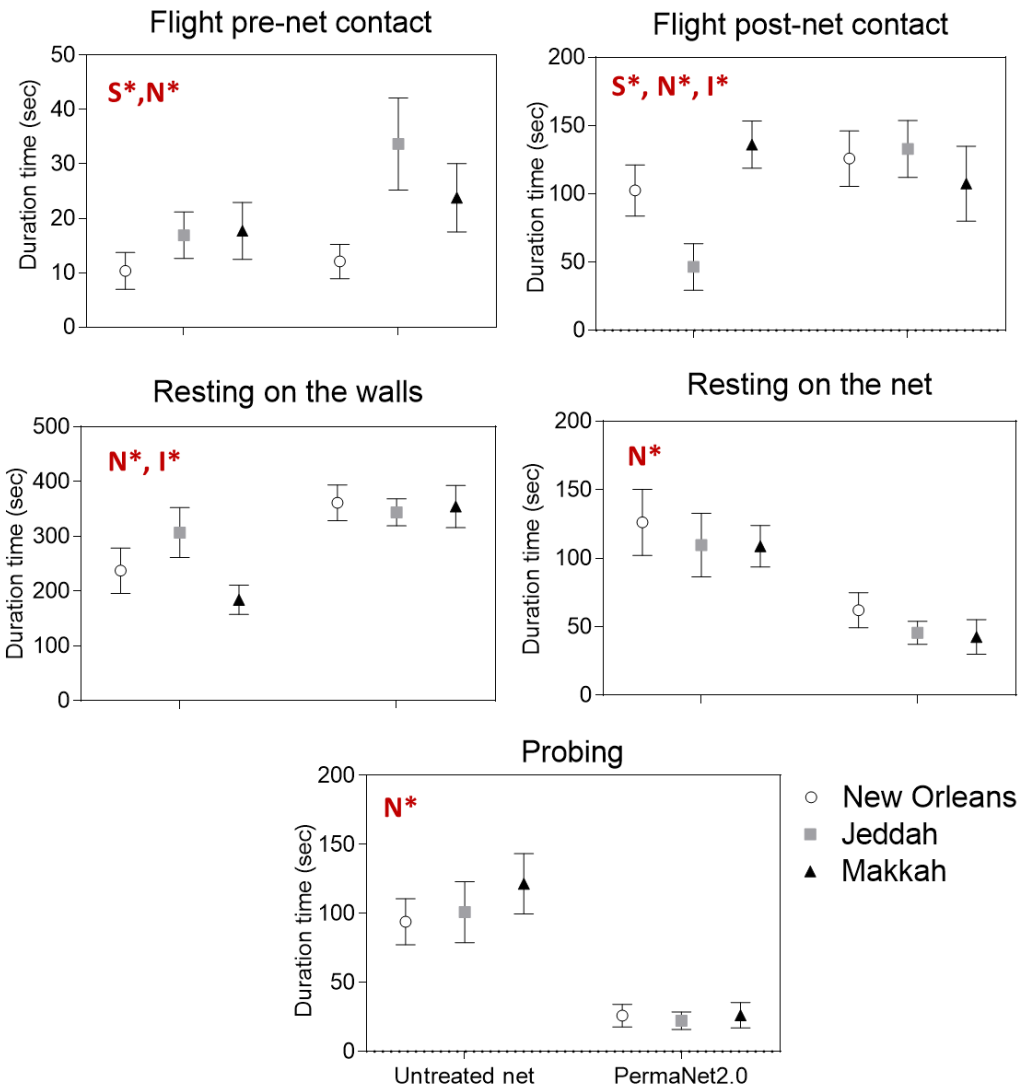
Flying prior to net contact was partitioned as a behaviour since more flight might be indicative of repellency. Across both net brands there was a significant strain effect with New Orleans spending the least amount of time in this behaviour (consistently only around 10s), but more time was spent flying before net contact only with the PermaNet barrier (and not the DuraNet) (**Figures 5.4 and 5.5**). In neither case was there a significant interaction term, with the resistant strains consistently flying more before net contact than New Orleans (**Table 5.2**). Full details of pairwise tests between strains for all behaviour types and associated parameter values are given in Appendices **Tables 8.12 - 8.14.**

Table 5.2 ANOVA table for behavioural events between three strains (New Orleans, Jeddah, Makkah) on untreated and treated nets (PermaNet 2.0, DuraNet) throughout the 600s test period of thumb test assay, showing significant effects among nets, strains and interaction.

Behaviour	Net type	Degree of freedom (df)	Strain		Net		Strain*net	
			F	*P*	*F*	*P*	*F*	*P*
Flight pre-net contact	PermaNet 2.0	123	14.1	0.000003	9.6	0.002	1.5	0.23
	DuraNet	120	4.8	0.01	0.13	0.73	0.41	0.67
Flight post-net contact	PermaNet 2.0	123	5.8	0.004	11.2	0.001	16.9	$3.3x10^{-7}$
	DuraNet	120	22.2	$6.1x10^{-9}$	18.3	0.00004	4.5	0.01
Resting on the walls	PermaNet 2.0	123	2.6	0.08	59	$4.1x10^{-12}$	8.5	0.0004
	DuraNet	120	9.3	0.0002	60.2	$3.2x10^{-12}$	4.4	0.02
Resting on the net	PermaNet 2.0	123	1.6	0.2	91.4	$1.6x10^{-16}$	0.18	0.84
	DuraNet	120	3.5	0.03	95.3	$6.3x10^{-17}$	0.11	0.90
Probing	PermaNet 2.0	123	2.1	0.13	182.8	$4.4x10^{-26}$	1.7	0.18
	DuraNet	120	1.8	0.17	224.1	$3.2x10^{-29}$	2.2	0.12

*The hypothesis df are 2 for strain and strain*net and 1 for the net.

A) PermaNet 2.0



B) DuraNet

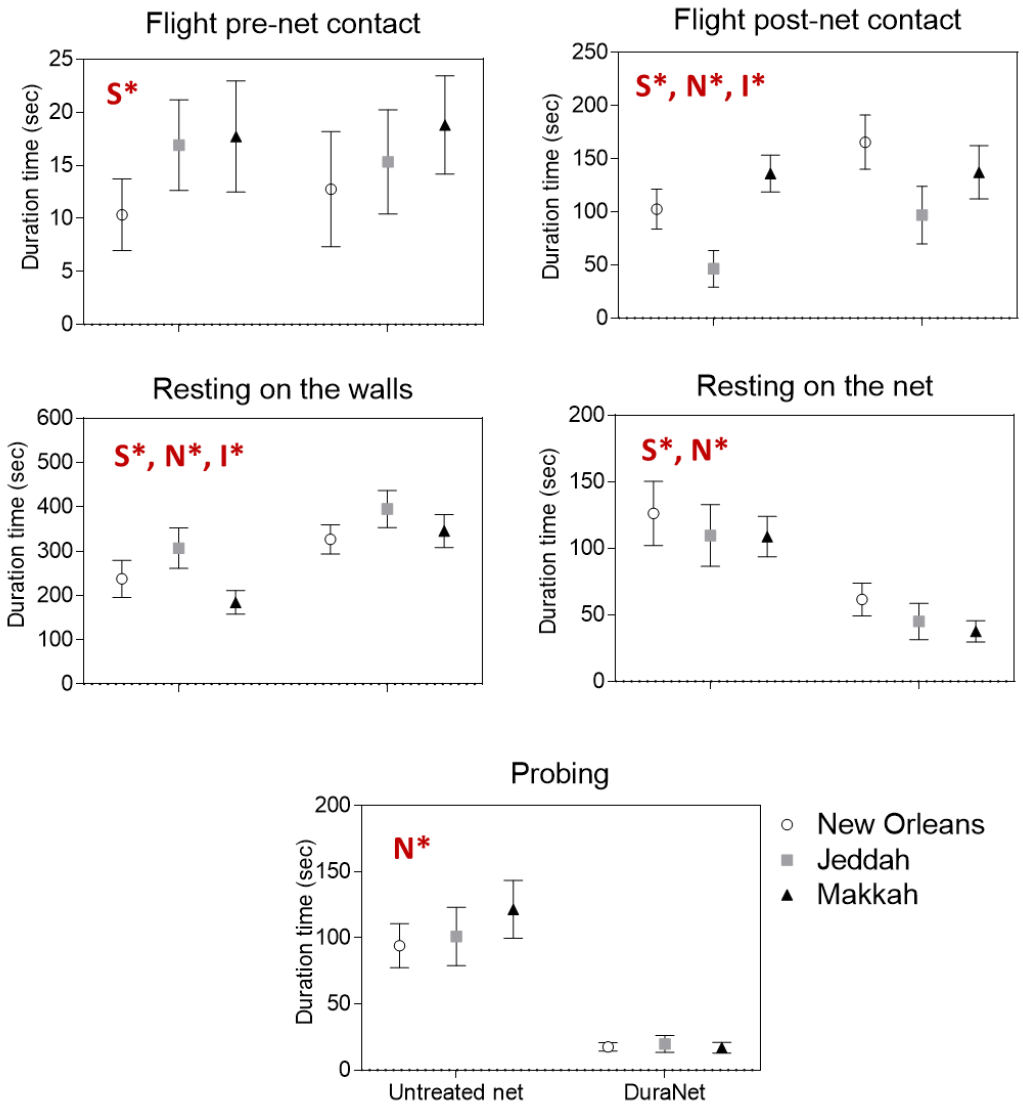


Figure 5.5 The mean time spent by each strain in each behaviour (out of 600s total) with confidence intervals (CI) on the A) PermaNet 2.0 vs untreated and B) DuraNet vs untreated in the thumb test. Red letters S*, N*, I* indicate a significant effect of Strain (S), Net (N) or a strain*net interaction (I), respectively (see Table 5.2).

5.3.3.3. Unified analysis of behaviour patterns using principal components analysis

Owing to the correlated nature of the behaviours shown within the fixed duration assay, a holistic analysis was performed using principal components analysis. Two significant principal components (eigen value>1) were detected in both the PermaNet2.0 and DuraNet analyses, which explained 77.3% and 78.7% of the variation in each case **(Table 5.3).** Principal component 1 (PC1) showed a strong

positive correlation with resting on the net and probing, but a negative correlation with resting on the walls, and thus was readily interpretable in terms of the major categories of on or off the net behaviours **(Table 5.4)**. Clear and substantial differences between treated vs untreated nets are evident (**Figure 5.6)** but the small strain effect in the PermaNet analysis was not consistent with a resistant vs susceptible interpretation and there were no significant interaction terms **(Table 5.5)**. This confirms and clarifies results from the previous univariate analysis in suggesting that insecticide on a bednet exerted major effects on behaviour but not in a way that was impacted by resistance in the strains. On the other hand, PC2 is difficult to interpret biologically and clearly for PermaNet 2.0 and DuraNet analysis, the correlation is completely different in same behaviours **(Table 5.4)**.

Table 5.3 Significant principal components (PC) obtained in separate analysis for PermaNet 2.0 and DuraNet assays.

		Rotation Sums of Squared Loadings		
Principal Component	Net type	Total	% of Variance	Cumulative%
PC1	PermaNet2.0	2.6	52.72	52.72
	DuraNet	2.5	49.14`	49.14
PC2	PermaNet2.0	1.23	24.6	77.3
	DuraNet	1.5	29.6	78.7

Table 5.4 Factor loadings (correlations) of behaviours with principal components for PermaNet 2.0 and DuraNet assays. Strong and consistent correlations are highlighted in bold.

Behaviour	Net type	PC1	PC2
Flight pre-net contact	PermaNet2.0	-0.51	0.07
	DuraNet	-0.130	**0.68**
Flight post-net contact	PermaNet2.0	0.27	**0.99**
	DuraNet	-0.007	**-0.889**
Resting on the walls	PermaNet2.0	**-0.86**	-0.46
	DuraNet	**-0.87**	0.42
Resting on the net	PermaNet2.0	**0.92**	-0.04
	DuraNet	**0.91**	-0.16
Probing	PermaNet2.0	**0.89**	-0.14
	DuraNet	**0.92**	0.18

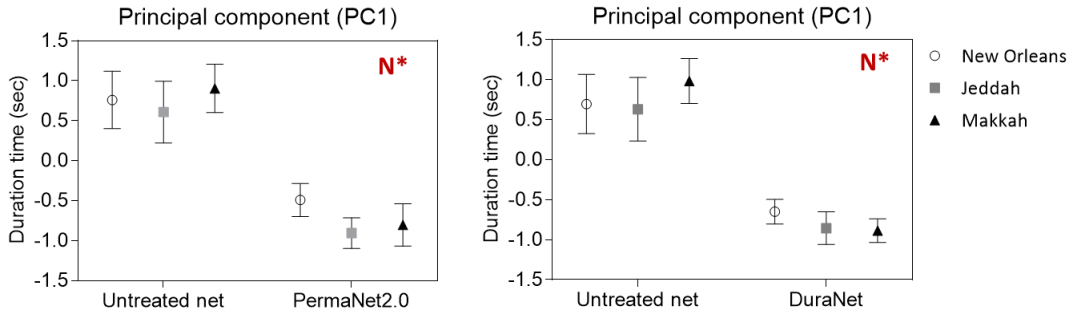


Figure 5.6 Principal component 1 scores (mean and confidence intervals) for each strain in assays with each net type.

Table 5.5 ANOVA table for principal components between three strains (New Orleans, Jeddah, Makkah) on untreated and treated nets (PermaNet 2.0, DuraNet) throughout the 10min test period of thumb test assay. Significant effects are highlighted in bold.

		Strain		Nets		Strain*net	
Principal component (PC)	Net type	*P* value	F test	*P* value	F test	*P* value	F test
PC1	PermaNet 2.0	0.12	2.2	**$7.7x10^{E-25}$**	168.9	0.6	1.3
	DuraNet	0.45	0.8	**$1.4x10^{E-27}$**	203.0	0.14	2.03

5.4. DISCUSSION

The reduced mortality in Makkah and Jeddah strains compared to the New Orleans strain in the WHO cone bioassay indicates that the Makkah is more resistant to pyrethroid net products than Jeddah, but that both display appreciable resistance. These results are consistent with the results of the WHO tube bioassay, as reported in Chapter 2 section 2.3.2. An important question is how this pyrethroid resistance may impact on control interventions, which will depend not only on capacity to survive insecticide but also modifications to behaviour. This study aimed to investigate one aspect of this by using the thumb test assay to quantify whether these resistant *Ae. aegypti* strains exhibited differences in behaviour in the presence of an insecticide treated net compared to a susceptible strain.

It was noted during optimisation tests that individual mosquitoes of the strains newly colonised from Saudi Arabia were not responsive in the thumb test in contrast to the susceptible New Orleans strains, which has been in colony for many years. An explanation is that the lab colonised strains are likely to be well adapted to small cages, and artificial feeding procedures, thus more likely to readily respond to host cues in a small test arena (Ross et al., 2017). However, when the strains from Jeddah, Saudi Arabia were tested in groups of five on untreated net **(Appendix 2: Figure 9.4)**, at least one individual in each group responded by approaching the thumb and landing on the untreated net barrier.

A group of mosquitoes responding better to host cues than an individual is a phenomenon observed in nature that has been termed the invitation effect (Charlwood et al., 2014). Normally mosquitoes orient to the host by kairomone (chemicals) cues emitted by the host along with other factors like body heat. The invitation effect is suggested to be associated with chemicals released by feeding mosquitoes which attracts others. The invitation effect phenomenon was described in 1977 by Alekseev and his colleagues when they observed the presence of an initial group of blood feeding individuals attracted more host seeking mosquitoes to that host by odour of the blood or the mosquitoes release odour from their body during feeding. This has also been reported in sand flies, which respond to volatiles released by feeding females from their mouthparts or maxillary palps (Schlein et al., 1984). Kairomones emitted by the host such as carbon dioxide or other chemicals from

irritation reactions have been suggested to increase during the mosquito feeding process (Ahmadi and Mcclelland, 1985). An invitation effect has also been observed in other groups of insects such as blackflies (Mccall and Lemth, 1997). In blackflies, a higher blood feeding rate was observed in chambers with more than 20 flies compared to those with four flies, hence evidence of the invitation effect (Ahmadi and Mcclelland, 1985). However, in our study, there is no blood feeding because of the barrier that prevents mosquitoes from reaching the thumb, thus the mechanism proposed for an invitation effect is not valid. An alternative explanation could be that mosquitoes touch or bump into each other which stimulates flight. Flight creates a turbulence which may distribute host odour in the box stimulating the other mosquitoes to fly towards the host. When mosquitoes move close to the host, they detect higher temperature near the barrier net making them land on it.

Withholding of sugar for a prolonged period prior to the test also increased responsiveness to some degree and was employed in the final protocol. This is because sugar meals inhibit host-seeking and blood-feeding behaviour (Straif and Beier, 1996). *Aedes aegypti* that previously fed on sugar, can remain quiescent and unresponsive to human odour and movements for 2-5h (Jones and Madhukar, 1976). The authors suggested the inhibition arose from distension of the abdomen after sugar feeding which in turn stimulates stretch receptors in the abdomen and hence could produce a primary inhibition in avidity for blood. However, sugar feeding inhibition can be inconsistent, with no difference observed in the total number of *An. gambiae* that blood fed regardless of their sugar feeding status (Straif and Beier, 1996).

In this study, no difference in host seeking response was observed between normal and dim lighting. *Ae. aegypti* preferentially feed bi-diurnally but on rare occasions, nocturnal feeding has been observed. For example, one study observed that 90% of female *Ae. aegypti* landed on human-baits during the day compared to 10% of the total catch at night (Chadee and Martinez, 2000). Moreover, Corbet and his colleagues reported that 99.8% (N=1190) of Tanzanian female *Ae. aegypti* were collected between 6 to 7am (post-sunrise) and 5 to 6pm (pre-sunset) through the human landing catch method (Corbet and Smith, 1974). In Kenya, Ae. *aegypti* females were also mainly collected inmornings and evenings by human landing catch.

However, human landing catches were significantly higher in the afternoon than morning (Ndenga et al., 2017). It has been reported flight activity of *Ae. aegypti* decreases in the darkness which deactivates the host-seeking activity (Kawada et al 2005, Taylor & Jones 1969) Whereas, increased nocturnal feeding activity in a closely related species, *Ae. albopictus* has been observed in Japan (Higa et al., 2000). The widely observed diurnal activity of *Ae. aegypti* informed my decision of using normal lighting in the actual experiment.

The most notable behavioural changes detected in the thumb test were reduced probing and resting on the insecticide treated nets compared with untreated nets. In contrast (and shown to be negatively associated in the PCA analysis), all strains spent more time resting on the side of the walls away from the treated net compared to untreated nets. These observations suggest pyrethroids induce avoidance response either through contact irritancy which reduces the willingness or capacity to feed owing to sublethal intoxication. Asian strains of *Ae. aegypti* exhibited escape responses after contact with 0.02g/m2 deltamethrin-treated papers before acquiring a lethal dose of insecticide (Kongmee et al., 2004). Evidence for behavioural avoidance has also been observed in *An. gambiae.* Huts treated with DDT had 50% less *An. gambiae* entry compared to control huts without insecticide (Chareonviriyaphap, 2012). The class of insecticides to which mosquitoes are resistant has also been associated with differences in behavioural responses. A permethrin-resistant *Ae. aegypti* strain from Thailand showed a significantly lower irritancy response than a deltamethrin-resistant strain in a 3 min test period in an excito-repellency test chamber with permethrin. The difference in behaviour response was thus driven by the resistance mechanism each strain possessed (Paeporn et al., 2007).

In my experiment there was no evidence of differences in probing or resting on the net between resistant and susceptible strains in their response to treated nets vs. untreated nets. The similarity in response across strains suggests that the effect of insecticides (presumably the contact irritancy) was equally effective despite differences in insecticide resistance status.

The thumb test results for flying pre-net-contact did not indicate a clear repellency effect for either the deltamethrin or alphacypermethrin treated nets. This may be due to most pyrethroids acting mainly as contact irritants unlike DDT which has more of a spatial repellent effect (Achee et al., 2009). For instance, a High Throughput Screening System (HITSS) estimating movement of *Ae. aegypti* away from an insecticide-treated net indicated that DDT was repellent to mosquitoes at doses as low as 9 mg/m^2, however, no repellency was detected for deltamethrin at doses up to 1.2 g/m^2 (Thanispong et al., 2009, Kongmee et al., 2010, Grieco et al., 2007, Achee et al., 2009). Another study measured landing of *Ae. aegypti* and other mosquitoes on insecticide-treated paper and also failed to detect evidence for spatial repellency by deltamethrin albeit at lower doses (20 mg/m^2) (Cooperband and Allan, 2009). The previous studies showed that the primary action of pyrethroid is contact irritancy followed by toxicity, whereas the primary action of DDT is spatial repellence with contact irritancy as the secondary action. Therefore, both insecticides can alter the behaviour of vectors either through contact irritation in the case of pyrethroids or spatial repellency for DDT which in turn translate to reduced human-vector contact (Achee et al., 2009). However, it should be noted that the measure of repellency used in our assays – flying pre-net contact – was imperfect because if there was a stronger motivation to feed in one strain, e.g. New Orleans, which showed the lowest time in this behaviour, it might confound weak repellency effects.

The outcome of the current study is consistent with another study which investigated host seeking responsiveness of *An. gambiae* in a thumb box supplemented with PermaNet 2.0. Parker reported a significant effect on host-seeking behaviour such as reduced probing and resting on the net in thumb assays with treated nets compared to untreated nets (Parker, 2015). Obtaining similar results in distinct mosquito taxa suggests that pyrethroid treated materials can still be effective in providing an extra layer of protection in addition to a physical barrier through their contact irritancy effects. This additional layer of protection is important in reducing human vector contact hence increased personal protection. However, the dramatically reduced mortality in resistant mosquitoes after contact with the net means they are still able to feed on unprotected persons thus sustaining transmission in areas of low

insecticide treated material coverage and thus community protection is greatly reduced.

5.5. CONCLUSION

The thumb test assay was carried out to evaluate behavioural effects of insecticide on resistant and sensitive *Ae. aegypti*. The assay showed strong behavioural shifts resulting from exposure to insecticide-treated materials (PermaNet 2.0/DuraNet), consistent with contact irritancy of the pyrethroids. Despite significant differences in mortality after the assay between the strains tested, there was no evidence that resistant strains differed from susceptible strains in their behavioural responses to pyrethroids.

CHAPTER 6
WIND TUNNEL STUDIES ON THE EFFECT OF INSECTICIDE TREATED MATERIALS ON *AEDES AEGYPTI* HOST LOCATION BEHAVIOUR

6.0. ABSTRACT

Background Insecticide treated materials (ITMs), such as bed nets, curtains and doorway and window screens can reduce domestic infestations of *Ae. aegypti* and potentially impact dengue transmission. All ITMs depend on pyrethroids, but as shown elsewhere in this thesis, strong pyrethroid resistance exists in *Ae. aegypti* populations from Makkah and Jeddah. This chapter reports on investigations into the behaviour of host-seeking adult females of these resistant populations as they respond to a host behind an ITM barrier containing holes.

Methods Field-collected insecticide resistant mosquitoes from Jeddah and Makkah, and a fully susceptible New Orleans strain were released individually into a wind tunnel to fly upwind towards holed nets (untreated/PermaNet 2.0). The behavioural events (Flying, Resting, Bouncing, Visiting) were digitally recorded for 20 min and analysed.

Results ln a wind tunnel bioassay, 100% of Makkah females, 87.5% of Jeddah and 60% of New Orleans (control susceptible strain) mosquitoes successfully passed through the holes in an untreated net within the 20 min trial period. There was a significant reduction in the number of mosquitoes that passed through the treated net compared to untreated net (P<0.0005). This reduction was significantly greater for the New Orleans susceptible strain (85%) compared to the resistant strains, Jeddah (59%) and Makkah (42%) (P<0.01). All New Orleans were knocked down by the end of the assay, Jeddah and Makkah were not knocked down but 90% and 45% mortality, respectively was recorded in the 24h post assay assessment. Analysis of specific behavioural events showed an increase in resting on the wind tunnel walls post contact with insecticide treated net and a reduction in bouncing and visiting the net, suggesting an impact of contact irritancy or a sub-lethal effect from deltamethrin.

Conclusion These data indicate that a PermaNet 2.0 net might fail to protect against the resistant Makkah and Jeddah mosquitoes. However, further behavioural studies

are needed to understand mosquito behaviour to ITNs alongside other vector control interventions. Overall, this indicates that physiological resistance enabled resistant mosquitoes to pass throught the holed treated nets better by surviving long enough to do so, rather than by changing behaviours with patterns either similar between strains or not varying in a manner consistent with resistance level.

6.1. INTRODUCTION

Evidence from several studies shows that integration of insecticide treated materials such as curtains, window screens and bed nets in control of *Ae. aegypti* can substantially reduce infestation (Kroeger et al., 2006, Lenhart et al., 2008, Vanlerberghe et al., 2011). For example in Cambodia, the use of insecticide treated water jar covers resulted in significantly fewer pupae per house in intervention sites compared to control sites (6.6 and 31.9, respectively, $P<0.01$) (Seng et al., 2008). The term insecticide-treated material refers to any material or fabric that has been impregnated with insecticide to repel, kill or provide a barrier against disease vectors, *e.g.* woven cloth, netting, plastic sheets, etc. There are potentially three modes of action by which ITMs may act on insects contact irritancy, non-contact repellency and acute toxic effect (Thanispong et al., 2009). Contact irritancy refers to a rapid take off or movement away from insecticide treated surface soon after physical contact before absorbing a lethal dose of insecticide. In contrast, non-contact (spatial) repellency refers to changes in behaviour when an insect interacts with an airborne chemical volatile which causes it to move away from the source of volatiles. Both the rapid take-off induced by contact irritancy, and switch in direction or interference in host detection with non-contact repellents are important potential impacts of insecticide that can reduce vector-host contact and increase protection beyond the physical barrier provided by the material (Roberts et al., 2000, Evans, 1993, Grieco et al., 2007, Kongmee et al., 2004). However, these two modes of action provide only personal protection to the ITM users and have no benefit for the unprotected community members, who may be bitten by the diverted mosquitoes. If the mosquitoes succumb after exposure to initially sub-lethal effects, then community protection achieved through personal protection may be lost. On the other hand, insecticide action through acute toxic effect, *i.e.* the series of events that follow uptake of insecticide by an insect, leads to knockdown or death and provides personal protection as well as protection to those individuals in the community that have no protection from ITMs (Achee et al., 2009).

The use of ITMs showed some promise in reducing the population of adult *Ae. aegypti* in Mexico, Venezuela, Thailand and Cambodia, when used as window and door curtains, and in Haiti as insecticide treated nets (ITNs)(Kroeger et al., 2006,

Lenhart et al., 2008, Seng et al., 2008, Vanlerberghe et al., 2010) For example, in Trujillo (Venezuela) and Veracruz (Mexico) insecticide-treated window curtains and domestic water container covers reduced the density of adult female *Aedes* mosquitoes with evidence of a possible reduction in dengue transmission (Kroeger et al., 2006). A pilot study distributing permethrin-impregnated bed nets to houses in Haiti found that after five months the Breteau, house, and container indices and also pupae per person were lower in treated than untreated homes (Lenhart et al., 2008). In Venezuela, houses with deltamethrin-treated window curtains had reduced numbers of indoor resting or host seeking *Aedes* possibly due to toxic effect of deltamethrin or contact irritancy deterring incoming mosquitoes (Vanlerberghe et al., 2011).

The widespread occurrence of physiological pyrethroid resistance in *Ae. aegypti* globally (Moyes et al., 2017), is a concern for the effectiveness of ITMs but predicting its impact on vector control strategies using ITMs is difficult. For instance, in laboratory settings, *An. gambiae* homozygous for the *kdr* 1014F mutation, which it can be assumed have more target-site insensitivity than heterozygotes and homozygous wildtype, were less effective at passing through holes in nets compared to heterozygotes (which were most successful) but better than the homozygous wildtype (Diop et al., 2015, Porciani et al., 2017). This example illustrates the difficulty in making simple predictions of impact of resistance on control based on insecticide resistance status. However, whilst resistant mosquitoes may survive exposure to insecticide treated materials the insecticides may have a subsequent delayed or sub-lethal impact on their ability to sustain transmission (Viana et al., 2016). Strong and potentially rising pyrethroid resistance in *Ae. aegypti* in Saudi Arabia (Chapter 2, section 2.3.2) raises concerns over how effective ITMs will be now and in future when integrated with current control interventions. In this study, I investigated how *Ae. aegypti* insecticide resistance status impacts passage through insecticide treated net barrier containing holes during host seeking activity.

6.1.1. Objectives

1-To evaluate the impact of a long-lasting insecticidal net (PermaNet2.0) on susceptible and resistant *Ae. aegypti* mosquitoes using the conventional WHO cone bioassay.

2-To quantify the ability of pyrethroid-resistant host-seeking *Ae. aegypti* to locate a hole in a human-baited insecticide-treated net barrier, cross the barrier and survive exposure at higher rates than pyrethroid-susceptible strains.

3-To characterise and compare behavioural events during and after interaction with the ITM surface by pyrethroid-susceptible and resistant mosquitoes.

6.2. MATERIALS AND METHODS

6.2.1. Mosquito strains

All tests were carried out using three *Ae. aegypti* strains: pyrethroid resistant Jeddah and Makkah F4 strains **(Chapter 2: Table 2.1)** and the susceptible New Orleans strain. Details on their origins and methods for colony maintenance are as described in Chapter 5, section 5.2.1.

6.2.2. Insecticide-treated Materials (ITMs)

The behaviour of each mosquito strain was investigated in response to PermaNet 2.0 polyester nets coated with 55mg/m^2 deltamethrin (Vestergaard, Lausanne, Switzerland), and untreated polyester net (Abakhan Fabrics, Liverpool, UK). All nets were new and unwashed. After cutting to size they were stored in separate boxes at 5°C to eliminate contamination risk and to prevent insecticide degradation. Each piece of netting was used 3 times and then discarded.

6.2.3. Wind tunnel assay

Wind tunnel experiments were carried out in a dedicated insectary from September to November 2017. All experiments were performed between 10.00 and 14.00h. The aim of the wind tunnel assay was to investigate the impact of insecticide resistance status on host seeking behaviour of *Ae. aegypti* when presented with a treated and untreated net barrier containing holes as they fly towards the host. A simple benchtop wind tunnel originally designed and constructed for studies on *Anopheles* was adapted for use in this study. The interior measured 30cm x 30cm in cross-section and 85 cm in length, and was divided into three chambers: chamber 1, downstream rear funnel section housing the fan; chamber 2, the central 65 cm flight tunnel with transparent Perspex walls, measuring 65 cm long with the test netting at the upstream end; chamber 3, a 20 cm long upstream section closest to the human bait with the test netting at its downstream end **(Figures 6.1 & 6.2)**. Mosquitoes were released by pulling out a cotton ball from a falcon tube oriented horizontally in the centre of the airflow, 40 cm downstream of the test netting **(Figure 6.2)**. Mosquitoes were lured towards the test netting by the attractants emitted by a human volunteer seated at the upstream end, immediately outside the wind tunnel **(Figure 6.2)**. A barrier of untreated or insecticide-treated netting was fitted across the entire cross-sectional area of the wind tunnel, 20 cm from the upstream end. Five holes (2 cm

diameter) were cut in this net barrier one at the centre of the net barrier, and the other four equidistant and located 3cm from the border (two at the top and two at the bottom). The same human volunteer (the author) acted as volunteer for the attractant source, to avoid any potential variation between different volunteers **(Figure 6.2)**.

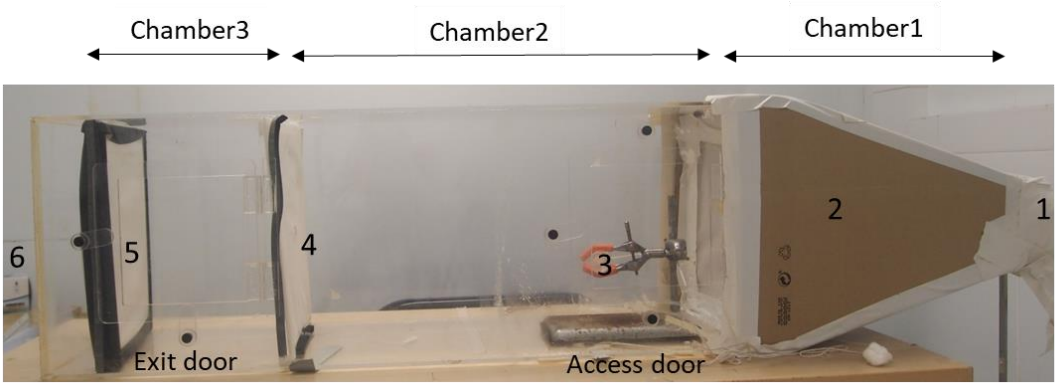


Figure 6.1 Components of the wind tunnel apparatus described. 1) Fan; 2) Rear cardboard funnel housing the fan; 3) Falcon tube; 4) Untreated or treated net barrier; 5) Host barrier; 6) Human volunteer.

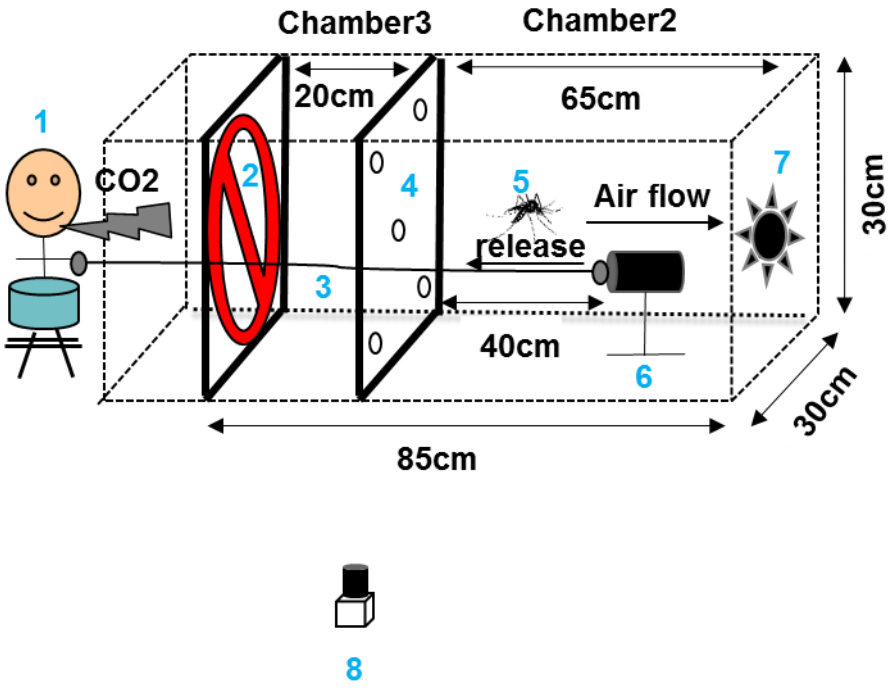


Figure 6.2 Diagram showing the wind tunnel bioassay chambers and associated components. 1) Human volunteer, 2) Host barrier, 3) A release cord, 4) Untreated and treated net materials, 5) *Ae. aegypti* mosquito, 6) Tube stand, 7) Fan, 8) Camera.

Test mosquitoes were placed into plastic cups to acclimatise for 1h in the testing room prior to use. A single mosquito was aspirated from the plastic cup and transferred into the cotton-plugged holding tube in the tunnel **(Figures 6.1 and 6.2)**, and the fan was switched on. Air flow speed during tests was maintained at 0.2ms^{-1}, measured using an anemometer. After 2 min, the cotton plug was removed by pulling on a cord to allow the mosquito to enter the wind tunnel. The mosquito was allowed to fly without interference for 20 min. Activity was recorded as described below. At the end of the assay, mosquitoes were removed using a manual aspirator and transferred to a plastic cup with 10% sugar solution.

At the end of each test session, the interior walls of the wind tunnel were wiped with 5% Decon 90 and rinsed with water to minimise risk of any insecticide contamination. Post-testing, mosquitoes exposed to treated nets were maintained on sugar solution under normal insectary conditions and monitored daily until death. Those exposed to untreated nets were followed for up to 12 days. The assay was repeated for twenty-five replicates for each strain and net treatment.

6.2.4. Video recording system

The Camera system used a Lambda Photometrix MQ013RG-E2, with a Nikon Ai AF Micro-Nikkor 20mm F2.8D lens positioned to capture all mosquito movement within the wind tunnel (**Figure 6.2**). The tests were carried out in normal room lighting. Mosquito behaviour was recorded at 50 frames/second using StreamPix software version 5 (www.norpix.com) and data saved as.*seq* files. All videos records were stored in duplicate on different external hard drives (Seagate Backup Plus 4TB Portable External Hard Drive, Amazon, UK) the extra copy serving as back up.

6.2.5. Behaviour categorisation

Mosquito behaviours in the wind tunnel assay were classified into the following defined activities

1- **Flight pre-net contact** mosquito in flight inside chamber 2 from release until first contact with the test net barrier upstream.
2- **Flight post-net contact** mosquito in flight inside in chamber 2 or 3 after first contacting the net until the end of the experiment.
3- **Resting pre-net contact:** mosquito rested in chamber 2 from time of release until contacting the test net barrier.

4- **Resting on the net:** mosquito rested on the test netting (untreated net or PermaNet 2.0).
5- **Resting on the walls:** mosquito rested anywhere (except net) in chamber 2 or 3 after first contact with the test net until the end of the experiment.
6- **Resting on the host barrier:** mosquito rested on the netting barrier between chamber 3 and the host.
7- **Visiting:** mosquito makes single contact on the test netting but contacts the net only once during flight.
8- **Bouncing:** mosquitoes perform multiple rapid contacts on the test netting.
9- **Knockdown:** mosquito knocked down and appears paralysed or dead.

In addition to these behaviour categories, the following data were collected

- Numbers of mosquitoes arriving at the treated net, passing through a hole and reaching the host 'barrier'.
- Number of net contacts (visiting, bouncing) per mosquito.
- Time taken to (i) reach the net, (ii) pass hole, (iii) reach the final barrier.

6.2.6. Statistical analysis

All recorded behavioural events were analysed using Boris software (Friard et al., 2016). Pearson chi-square (χ^2) tests were used to compare numbers of mosquitoes passing through the holes in different treatments as shown in **Table 6.1**. A Kolmogorov-Smirnov (K-S) test was used to test the distribution of the data; most proved to be non-normally distributed, and so a natural logarithm transformation (LN) was applied, and data re-tested using a K-S. The proportion of time spent on each behaviour was obtained by dividing the time spent on each behaviour by the total time of the assay (1200s, or until knockdown occurred). The proportion of periods spent in flight, resting (the net barrier/ walls/ host barrier), visiting and bouncing were analysed using General Linear Models (GLM) for tests of individual behaviours using SPSS Version 24. To identify groups of related variables, principal components analysis (PCA) with Varimax rotation was performed on the behavioural categories (Huntingford, 1976). TraMineR (R package version 3.3.1) was used to produce chronograms depicting the sequence and duration of behaviours for

individual mosquitoes. GraphPad Prism7 was used to produce figures for the average proportion of time spent in each behaviour and confidence intervals (CI).

6.4. RESULTS

6.4.1. Wind tunnel assays

In total, 139 mosquitoes were tested in the wind tunnel, of which 82 passed through the hole in the netting. One mosquito belonging to Makkah strain was excluded from analysis because it passed under the untreated net instead of the required holes. The proportions of Makkah, Jeddah and New Orleans that passed through the holed untreated net barrier was 100%, 87.5% and 60% respectively, falling to 58%, 36% and 9% with the treated net **(Table 6.1)**. There was a significant difference among strains in the number of mosquitoes that penetrated both untreated nets (χ^2=13.34, df=1, *P*=0.0003) and PermaNet2.0 (χ^2=12.2, df=1, *P*=0.0005). For each of New Orleans, Jeddah and Makkah (separately) there was a significant reduction in the number of mosquitoes passing the treated net compared to the untreated (Fisher exact test, *P*<0.001 in each case). Importantly, there was a significant strain*net type interaction characterised by significant reduction in the proportion of mosquitoes within or between strains that crossed the insecticide treated barrier compared to the untreated barrier (homogeneity χ^2=8.05, df=2, *P*=0.018). The relative ratio of passing through the treated/untreated net was lowest for New Orleans (0.15) followed by Jeddah (0.41) then Makkah (0.58).

Table 6.1 Rates of passage through treated or untreated net barrier with holes by Jeddah, Makkah and New Orleans *Ae. aegypti* strains.

Untreated Net	New Orleans	Jeddah	Makkah
tested	25	24	22
Crossed barrier	15 (60%)	21 (87.5%)	22 (100%)
PermaNet2.0	New Orleans	Jeddah	Makkah
tested	22	22	24
Crossed barrier	2 (9%)	8 (36.4%)	14 (58.3%)

6.4.2. Mortality following the Wind test

No mosquitoes were knocked down in the untreated net trials **(Figure 6.3A-C; Appendix 1: Figure 8.4)**. However, with PermaNet2.0, 100% of New Orleans and 36% of Jeddah mosquitoes were knocked down during the experiment, though none of the Makkah mosquitoes were knocked down **(Figure 6.3D-F; Appendix 1: Figure 8.4)**. No mortality was observed in any strain 24h after the assays with an untreated barrier net and all remained alive until the end point which was 12 days post-assay, With the PermaNet2.0 barrier net, mortality in New Orleans, Jeddah and Makkah was 100%, 90% and 45% respectively (χ^2=22.77, df=1, *P*<0.001).

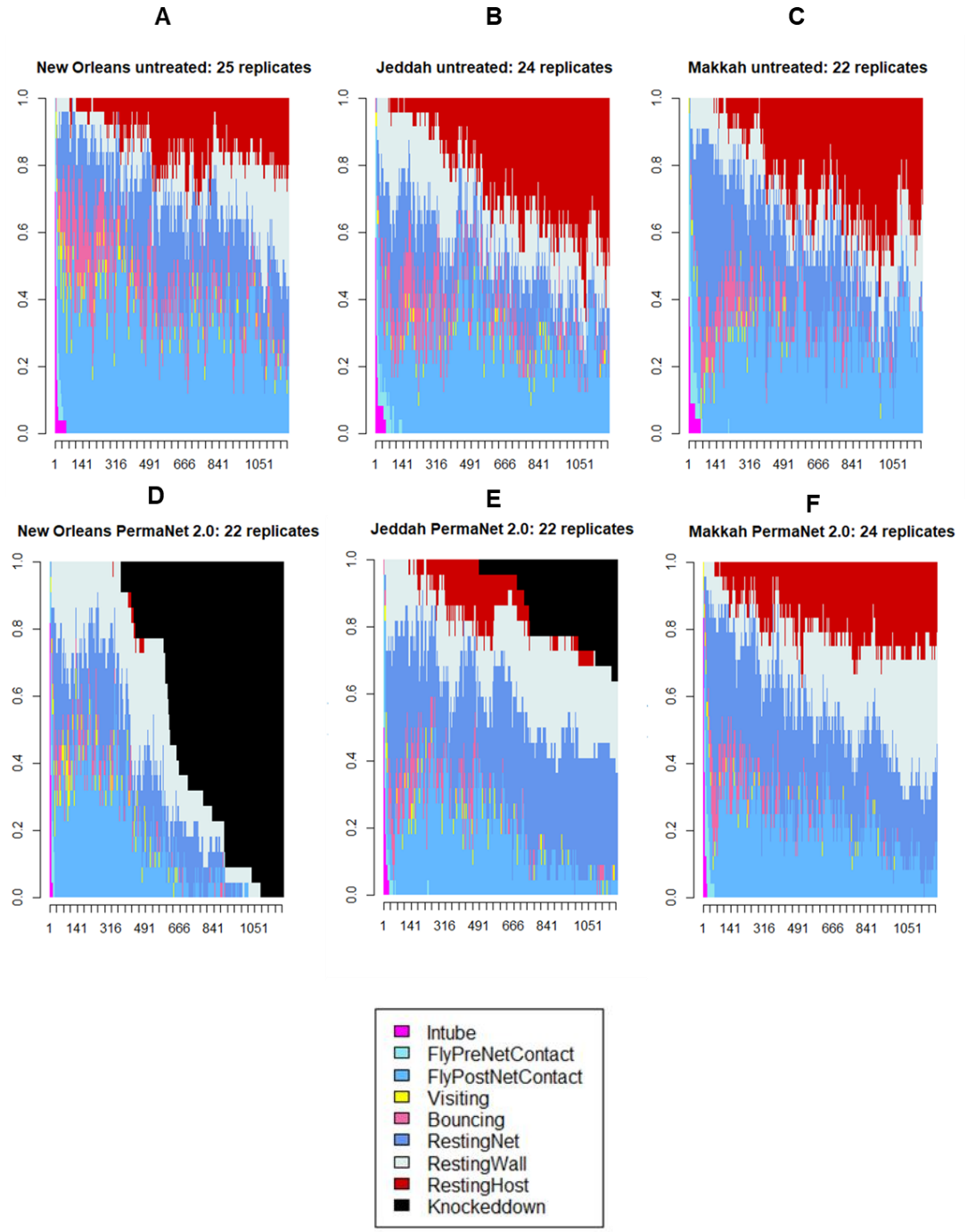


Figure 6.3 Chronogram showing frequency of different behavioural events recorded during host seeking flights by *Ae. aegypti* adult females in a wind tunnel assay. New Orleans (A), Jeddah (B), Makkah (C) with untreated net; New Orleans (D) Jeddah (E), Makkah (F) with PermaNet 2.0. The y-axis represents the proportionate frequency of total tested mosquitoes and x-axis represents the total duration (s) of each behavioural event.

6.4.3. Pre-net exposure events

If repellency were a factor affecting host location and success of passage through the barrier, an alteration in behaviour before first contact with the net would be predicted, with a greater time in the PermaNet trials. The activities observed before net contact were resting on the walls of chamber 2 and flying. The proportion of total time (duration) spent on flying and resting by all strains before net contact was less than 1.5% of total time of assay **(Appendix 1: Table 8.16; Figure 6.3)**. There were no significant differences among strains in either behaviour type, nor between nets once multiple testing correction was applied **(Table 6.2)**

6.4.4. Net barrier contact events

At the net barrier, the activities observed were visiting (which involves only a very brief net contact), bouncing (involving multiple net contacts), and resting on the barrier (which involves more prolonged contact). On average, the three activities made up more than 20% of the total time of the assay **(Figure 6.4)**. The number and total duration of visiting events differed significantly among the strains but was unaffected by net-type and generally showed a rank order pattern of New Orleans, Jeddah and Makkah **(Table 6.2; Figure 6.5 C, I; Appendix 1:Table 8.16)**. The number of bouncing events was highest for New Orleans, followed by Jeddah and Makkah, and decreased in the same way for all strains when faced with the PermaNet2.0 barrier (relative to the untreated net). The duration of bouncing showed a similar pattern but was less consistently variable among strains **(Table 6.2; Figure 6.5 D, J; Appendix 1: Table 8.16**). There was slight variation (not significant after correction for multiple testing) in the number of resting events, with a similar pattern across strains, but surprisingly all strains spent slightly more time resting on the treated net compared to untreated nets **(Table 6.2; Figure 6.5 E, K; Appendix 1: Table 8.16**). Although this difference was not significant after correction, the result remains surprising given the relatively prolonged time spent in resting on the net and amount of contact with insecticide that would ensue in the PermaNet2.0 treatment (**Figure 6.3, 6.5)**. Importantly there were no significant strain*net interaction effects for these behaviours demonstrating that the response of the strains to treated (vs. untreated) nets was similar (**Table 6.2**).

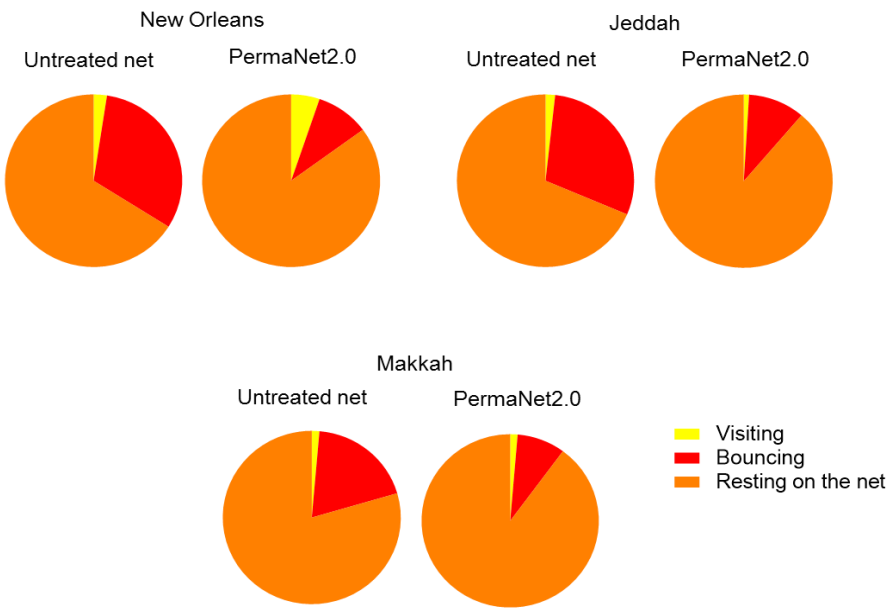
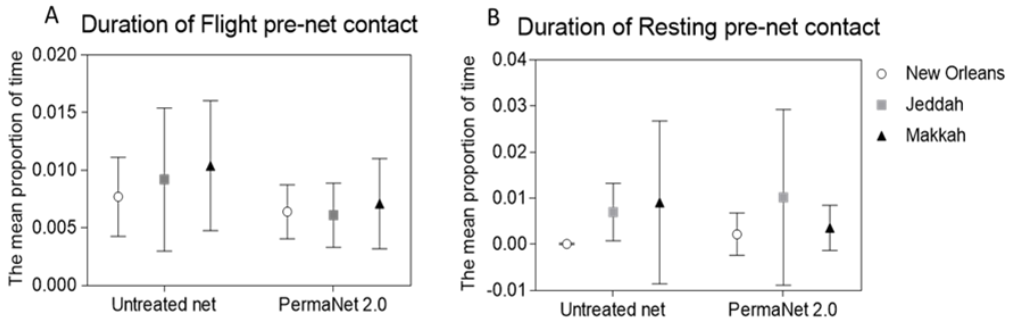
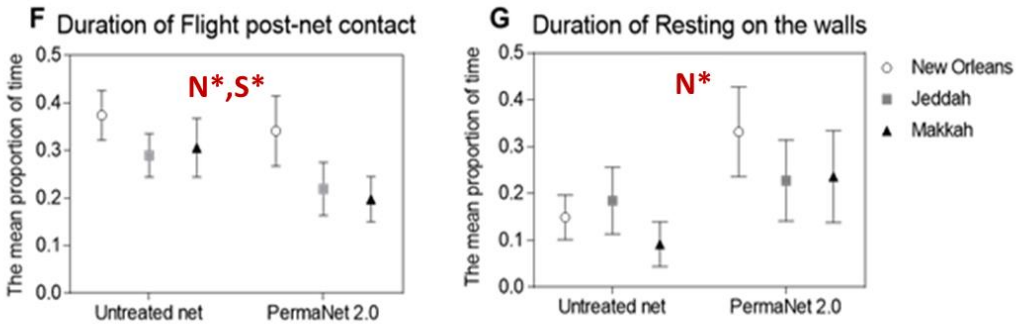


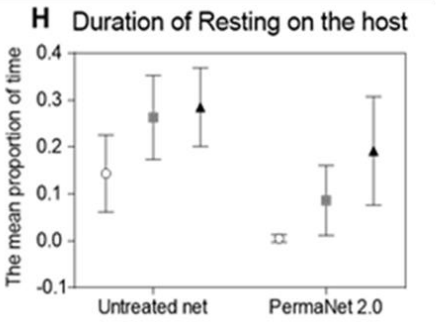
Figure 6.4 The proportion of time spent on bouncing, visiting and resting on the net in wind tunnel assays with insecticide treated nets and untreated nets of New Oreleans (insecticide-susceptible), Jeddah and Makkah mosquito strains.

Pre-net exposure events



Post-net exposure events





Net barrier contact events

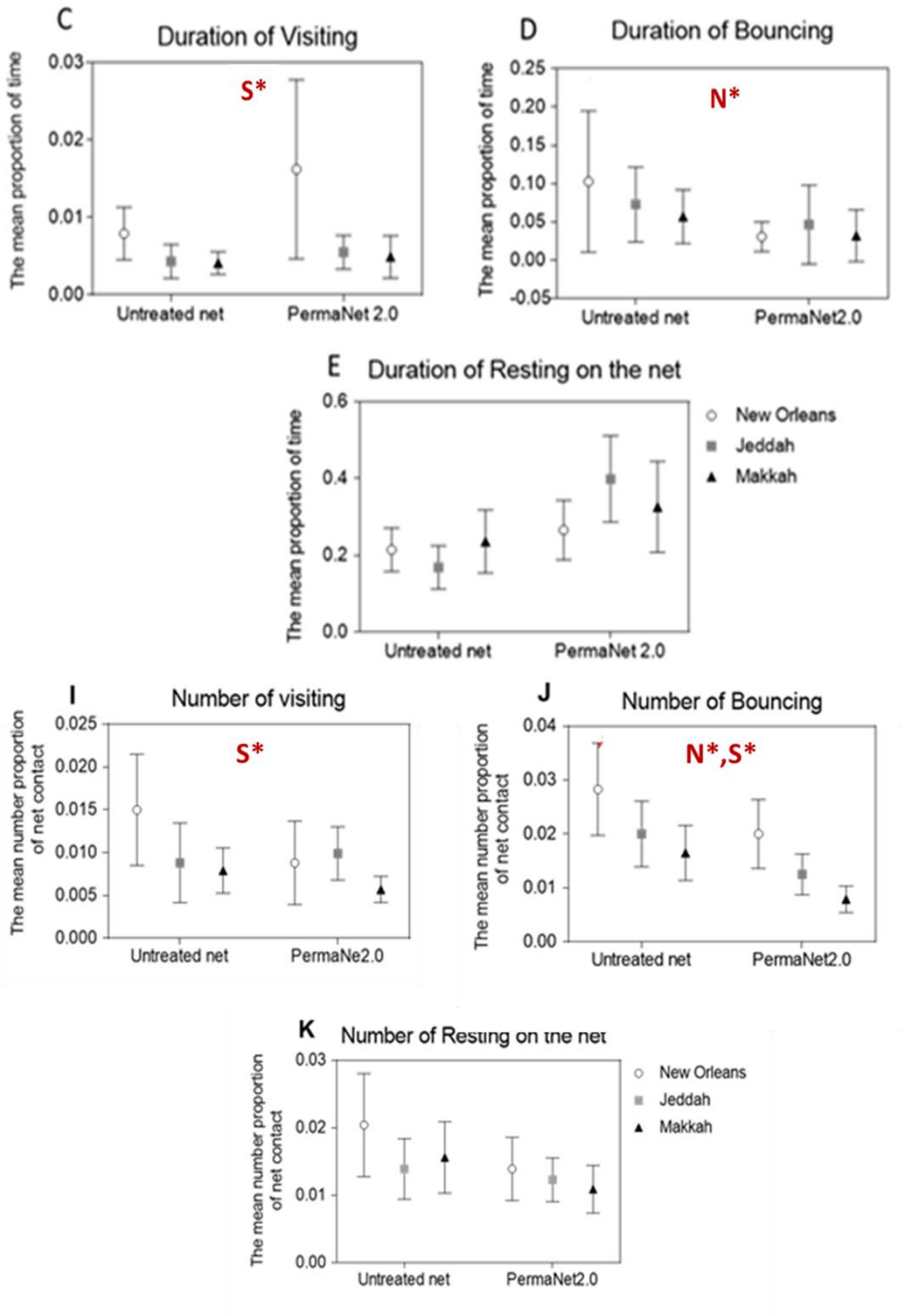


Figure 6.5 The responses of three *Ae. aegypti* strains interacting with untreated or PermaNet 2.0 barriers in a wind tunnel assay. Figures A-B) mean flight duration and resting pre-net exposure, Figures C- E) mean duration for visiting, bouncing and resting on the net and Figures F to K show mean duration and number of net contacts made by *Ae. aegypti* mosquitoes post net exposure on untreated and PermaNet 2.0. The y-axis presents the mean duration or number of the net contacts for each behaviour and the x-axis presents net type (untreated, PermaNet 2.0). A symbol (N*) indicates the mean duration is significantly difference among strains in nets**,** (S*) is significantly difference among strains and (I*) the interaction is significant. Error bar represents 95% confidence intervals.

Table 6.2 General linear model (GLM) analysis testing differences in each behaviour between strains (New Orleans, Jeddah, Makkah) and nets (untreated net and PermaNet 2.0).

	Among strains		Between nets		Strain*net	
Pre-net exposure events						
Behaviour	*P* value	F test	*P* value	F test	*P* value	F test
Flight pre-net contact	0.68	0.39	0.46	0.56	0.79	0.24
Resting pre-net contact	0.16	1.9	0.04	4.15	0.44	0.82
Net barrier contact events						
Behaviour	*P* value	F test	*P* value	F test	*P* value	F test
Visiting	**<0.0001**	12.1	0.3	1.1	0.25	1.4
Bouncing	0.3	1.2	**<0.0001**	19.3	0.93	0.07
Resting on the net	0.95	0.06	0.014	6.2	0.07	2.8
Post-net exposure events						
Behaviour	*P* value	F test	*P* value	F test	*P* value	F test
Flight post- net contact	**0.0005**	8.1	**0.001**	12.7	0.47	0.76
Resting on the walls	0.005	5.6	**0.001**	12.7	0.21	1.6
Resting on the host	0.35	1.1	0.29	1.7	0.798	0.23
Number of Visiting events	**<0.0001**	21.1	0.18	1.8	0.02	4.4
Number of Resting on the net events	0.03	3.7	0.62	0.24	0.13	2.1
Number of Bouncing events	**0.002**	6.6	**0.002**	9.5	0.28	1.3

The significance threshold is corrected for multiple testing, treating each effect–strain, net or interaction as a separate hypothesis by the Bonferroni method as critical *P*=0.05/11. Highlighted values are significant.

6.4.5. Post-net exposure events

The events after contact with the net included flying, resting on the walls and on the host barrier after passing through the net. Flying duration in all strains was significantly higher in assays with untreated nets than treated nets **(Table 6.2; Figure 6.5)**. The New Orleans strain spent more time in post-net-contact flying compared to the other strains in both nets **(Appendix 1: Table 8.16)**. All strains spent a longer duration resting on walls after contact with treated nets compared to untreated nets but upon correction the difference among strains was not significant **(Figure 6.5G; Appendix 1: Table 8.16)**. There was no significant difference in resting duration on the host barrier among the strains and between the treated and untreated net assays (**Table 6.2)** between nets *P*=0.29, between strains *P*=0.35).

In summary, the duration spent on behaviours such as bouncing, which are aimed at finding access to the host (holes on nets), resting on the net barrier and re-orienting to host stimuli (post exposure flying) in all strains was much lower when exposed to treated net compared to untreated nets **(Figures 6.3 and 6.5)**. Moreover, a

significantly longer duration was spent resting on walls after contact with treated nets than with untreated nets.

6.4.6. Analysis of overall behavioural time budgets

6.4.6.1. Principal components analysis

Four significant principal components (eigen values >1) were detected following varimax rotation, which aided interpretation with respect to the input variables. The four components respectively explained 25.6%, 22.1%, 16.8% and 12.8% of the total variance. The key principal components are 1 or 2, which explained >20% of variation in behaviour outcome each. The PC1 presented a strong positive correlation between the most net-tactic host-seeking behaviours bouncing (number and duration), and resting visits to the net but interestingly no correlation with duration of resting on the net **(Table 6.3, Table 6.4, Figure 6.6)**. PC2 showed a strong correlation of less net-associated host seeking behaviours (flying post net contact and visiting).

Table 6.3 Factor loadings of principal components analysis by varimax rotation method. The table reveals relationships between behaviour variables. A symbol a,b,c indicates that behaviour is strongly associated with each other.

Behaviour	Component	
	1	2
Flight Pre-Net contact	0.06	-0.1
Flight Post-Net contact	0.1	0.7[b]
Resting on the walls	-0.13	-0.1
Resting on the Net	0.07	-0.1
Visiting	-0.01	0.8[b]
Bouncing	0.9[a]	-0.1
Number of Visiting events	0.2	0.9[b]
Number of Resting events on the net	0.8[a]	0.2
Number of Bouncing events	0.9[a]	0.2

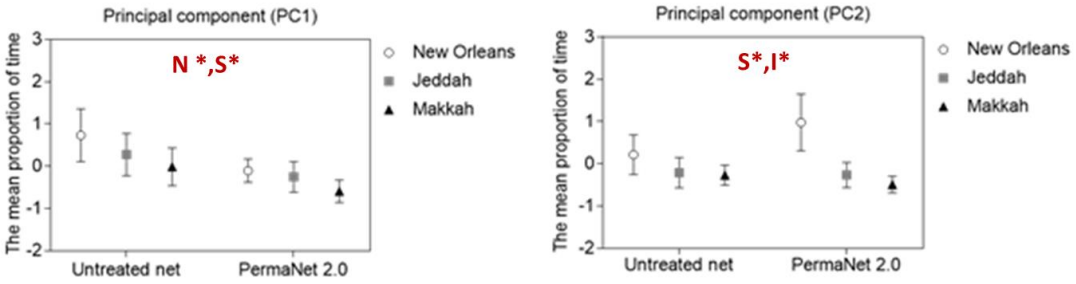


Figure 6.6 Major principal components with untreated and PermaNet 2.0. A) PC1, B) PC2.The x-axis represents a net type (untreated, PermaNet 2.0). The y-axis represents the principal component score. (S*) is significantly difference among strains, (N*) is significantly difference among nets and (I*) the interaction is significant.

For both principal components there was a significant effect of strain, with strains following the order New Orleans > Jeddah > Makkah, irrespective of net type, i.e. for both more- and less- net-tactic host seeking behaviours the proportion of time spent in each followed the reverse order to the resistance profile across strains. For PC1 but not PC2 there was also a highly significant net effect, representing a reduction in time spent in more net-tactic behaviours, which was the same across strains, evident from the lack of a significant interaction term **(Table 6.4)**.

Table 6.4 General linear model (GLM) analysis of differences in each principal component between strains, and nets. Significant effects are highlighted in bold.

Principal component	Among strain		Among nets		Strain*net	
	P value	F test	*P* value	F test	*P* value	F test
PC1	**0.01**	4.5	**0.0002**	14.8	0.72	0.33
PC2	**0.000002**	14.6	0.32	1.0	**0.03**	3.7

Critical *P*=0.025

6.5. DISCUSSION

The outcome of WHO cone assays showed a reduced efficacy of holed PermaNet2.0 against *Ae. aegypti* strains from Jeddah and especially Makkah in comparison with New Orleans. This is in line with the results of the high levels of deltamethrin resistance in *Ae. aegypti* detected in WHO tube assays (Chapter 2 section 2.3.2). The highest proportion of mosquitoes that crossed PermaNet 2.0 belonged to the Makkah strain, followed by Jeddah with New Orleans having the lowest proportion of mosquitoes that crossed the treated barrier (**Table 6.1**). The Saudi *Ae. aegypti* strains, both possess high frequencies of the paired 1016G and 989P *Vgsc* mutations, which are strongly associated with survival upon long exposure to deltamethrin (Chapter 3, section 3.3.1). The L1014F mutation, situated nearby has been reported to influence host seeking responses with heterozygotes generally most responsive to host cues followed by homozygous wildtype while homozygous mutants were the least responsive (Diop et al., 2015, Porciani et al., 2017). These findings may show some correspondence with results for the strains studied here Makkah has a significantly higher proportion of homozygous mutants (989P and 1016G) than Jeddah and based on the host seeking behaviours resting on the net, bouncing and visiting (summarised in principal components 1 and 2) does appear less active than Jeddah (Chapter 3, section 3.3.1). New Orleans was the most active in host seeking, which does not correspond to results from the above studies, but it is also a long-adapted lab strain, which may influence responsiveness, as discussed in Chapter 5.

However, the success in the host response (crossing the barrier) results in the current study differs from what has been reported previously in other studies (Diop et al., 2015, Porciani et al., 2017) and it is also important to consider the toxic effect of insecticide on the strains. Mosquitoes that can withstand a longer duration of insecticide exposure during net-contact behaviours such as bouncing as they seek an entry are more likely to be successful in crossing the barrier.

In the WHO cone and also the wind tunnel assays, none of the Makkah strain were knocked down; Jeddah showed intermediate knockdown while all New Orleans strains were knocked down. Although the limited number of strains precludes a firm conclusion, the knockdown rate among the strains mirrors the proportion of individuals that passed through the treated barrier. Strains with a higher proportion

of homozygous *kdr* mutants (equating to higher resistance given strong dependence on *kdr* for their pyrethroid resistance Chapter 3) will be more successful in crossing the barrier due to more time allowed for searching before knockdown. Nonetheless the strong phenotypic resistance in the cones assays is in line with the bioassay results obtained earlier.

An alternative explanation is behavioural differences among the strains when they encounter any physical barrier. The field collected strains may have an intrinsic advantage over the lab colonised strain in locating the host even in the presence of a physical barrier, i.e. they are more efficient in host seeking. Similar results were observed in crossing untreated nets where Makkah had higher proportions of individuals that crossed the barrier followed by Jeddah and the least being New Orleans, despite apparently greater effort in host seeking behaviours in the former (PC1 and PC2), evident as more time in active host seeking behaviours. This effect may have been absent from other studies because they used strains from a similar genetic background (Diop et al., 2015, Porciani et al., 2017). In addition, the other studies utilised *An. gambiae* in which effects of *kdr* genotypes on host seeking behaviour may differ from those in *Ae. aegypti* (Reidenbach et al., 2009).

As observed in the thumb test study (Chapter 5), contact irritancy from pyrethroid treated nets appears to impact the behaviours recorded in the wind tunnel assay. The reduced duration of bouncing and other high net contact behaviours (PC1) in insecticide treated net treatments may result from contact irritancy. This may explain why the number of visits in all strains (mosquito touching the net once and flying away immediately) although not significant, were higher in treated nets compared to untreated nets. An increased number of visits to treated nets may result from multiple unsuccessful attempts to find a way to the host, if visits are not as efficient as bouncing in finding holes.

Overall, bouncing on the treated net was significantly lower compared to untreated nets. This can be compared to the average time spent probing in thumb test assays with treated nets which was significantly lower compared to the thumb test assay with untreated nets. The reduction in bouncing in wind tunnel and probing in thumb test in treated nets compared to untreated nets further supports contact irritancy has a significant effect on host seeking behaviour of both resistant and susceptible

strains. Similar findings have been reported in *An. gambiae* when comparing exposure between treated and untreated nets (Parker et al., 2015). Surprisingly the total duration of resting events on the net was significantly higher in the presence of treated than untreated nets, though the number of resting events was not. Though speculative, this might result, in some cases at least from a toxic, semi-knockdown effect of the insecticide leading to an increased resting behaviour.

The effect of spatial repellency was more pronounced in the thumb test where a difference in pre-net contact flight duration in the treated assay was observed unlike the wind tunnel assay. It has been suggested that pyrethroids are semi volatiles and hence in small compartments, the volatile molecules may be concentrated enough to influence host seeking behaviour (Parker, 2015, Cohnstaedt and Allan, 2011). In support of this hypothesis, the concentration of DDT from treated material in the air within a small behaviour chamber (30.5cm × 30.5cm × 30.5cm) assay was found to be higher than the concentration in the air of a room-scale semi-field experimental huts (4.0m width, 5.0m length, 2.5m height) (Martin et al., 2013). The effect of treated nets (PermaNet 2.0) on pre-net flight duration in the thumb assay was more pronounced in the resistant strains suggesting they may be more tuned to detecting insecticides compared to susceptible strains but it was not detected in wind tunnel thus indicating it may be less important in larger spaces hence of little significance to vector control.

Overall, the wind tunnel and thumb test findings indicate insecticides have a significant effect on host feeding behaviour of *Ae. aegypti* irrespective of insecticide resistance status. The effect appears largely due to contact irritancy, which reduces the time spent in net-tactic search behaviours such as bouncing and probing on treated nets. However, increased tolerance to insecticides increased the chances of resistant mosquitoes accessing a blood meal when the ITMs are damaged. The ability to cross damaged ITMs and survive post treated net exposure indicate insecticide resistance could have an impact on both personal and community protection provided by ITMs.

6.6. CONCLUSION

Makkah and the Jeddah mosquitoes penetrated through holes in PermaNet 2.0 more successfully than the New Orleans strain. They also had lower knockdown during assays and higher survival rates after wind tunnel assays. These observations suggest that in an area with a higher frequency of resistant mosquitoes, the overall protection to the community and at a personal level provided by insecticide treated materials may be reduced. This study shows how behavioural studies can give insight into the way in which physiological resistance may impact the efficacy of control interventions, whilst also highlighting the need for alternative insecticides for ITMs.

CHAPTER 7

GENERAL DISCUSSSION

Dengue fever in Saudi Arabia was first reported in Jeddah city in 1994. This was followed by a larger outbreak in 1995 which affected other cities in the western and southern regions of the country (Alshammari et al., 2018). Since these initial outbreaks cases of dengue fever have been reported annually in the country (Aziz et al., 2014). The most affected regions remain in the western and southern region which include Makkah and Madinah which are popular tourist destinations for Muslims who visit annually for Hajj or Umrah (Al-Tawfiq and Memish, 2018). The city of Jeddah which has the highest annual cases of dengue, hosts ports and an international airport which receives tourist and workers in transit to other cities in western and southern regions (Fakeeh and Zaki, 2003). The economic importance of this region has pushed the government to invest heavily in control of dengue fever (Aziz et al., 2014) primarily via heavy use of insecticides to reduce *Ae. aegypti* densities (Saggu et al., 2016). Although accumulating evidence, albeit mainly from studies of malaria, suggests that insecticide resistance can reduce the effectiveness of insecticide-based vector control (discussed in Chapter 1 section 1.12), prior to this study, very little information was available on the insecticide resistance phenotype of *Ae. aegypti* in Saudi Arabia. No previous information was available on the mechanisms of insecticide resistance or the impact of insecticide resistance on host seeking behaviour, which remain quite rare generally.

In a series of studies, I characterised the resistance profile of Makkah and Jeddah strains to different adulticides and larvicides, the mechanisms contributing to insecticide resistance in both strains established from each population and evaluated the impact of resistance on host seeking behaviour of both populations in relation to a susceptible strain.

The key findings were as follows;

- Both populations had a high prevalence of deltamethrin, permethrin and bendiocarb resistance, showed some evidence of resistance to fenitrothion and malathion, but were susceptible to temephos and *Bti.*

- Age and exposure duration had a significant effect on survival of both strains whereby susceptibility to insecticides increased with age and duration of exposure but decreased after repeated exposures.
- The S989P, V1016G and F1534C *kdr* mutations were detected for the first time in the Middle Eastern region. S989P and V1016G markers were also detected for the first time outside of Asia and were in perfect linkage disequilibrium (LD) and strongly predicted deltamethrin resistance, but in negative LD with F1534C which likely as a consequence was negatively associated with resistance.
- The top overexpressed genes in microarray analysis were significantly enriched for cytochrome P450s. These included genes previously implicated in pyrethroid resistance in multiple transcriptomic studies and the relatively novel candidate, *CYP9J7*, which was consistently the strongest candidate in the current experiments. No GSTs or CCEs were identified as significantly overexpressed using the strict replication criteria applied.
- *CYP9J7* displayed no metabolic activity against pyrethroids but metabolised the organophosphates, pirimiphos methyl, malathion and fenitrothion. This appears to be the first *Aedes* P450 demonstrated to metabolise OPs and downregulation in a selection experiment suggests that it may be involved in desulfuration (activation) of the insecticides.
- Neither the thumb test or wind tunnel assay found evidence of altered host seeking behaviour in Jeddah and Makkah females compared to a susceptible strain, with the behaviour of all strains strongly affected by the presence of a treated barrier, suggesting that contact irritancy remains despite resistance. However, more resistant mosquitoes passed more successfully through a holed treated net, as a result of their greater capacity to resist pyrethroid toxicity and knockdown and carry on exploration until barrier penetration.

Resistance to pyrethroids in the Jeddah and Makkah strains was detected in WHO tube and cone assays, and also in the wind tunnel and thumb test (in which resistance was evident from knock down and mortality rate compared to the susceptible strain). However, the mortality rate varied among the assays **(Table 7.1)**. Variation in mortality rate could be attributed to the difference in exposure duration **(Table 7.1)**

and insecticide concentrations on the treated net (WHO cone, thumb and wind tunnel) or paper (WHO tube assay).

The difference in mortality observed in WHO cone, wind tunnel and thumb assays which were based on PermaNet 2.0 which had the same deltamethrin concentration **(Table 7.1)** can be attributed to duration of exposure. The duration of exposure to the net in the thumb test (total time spent on probing, resting, walking and grooming on net in New Orleans was 1.6 min, Jeddah 1.2 min and Makkah 1.3 min) was lower than in the wind tunnel assay (total time spent on visiting, bouncing and resting on the net in New Orleans was 7 min, Jeddah 9 min and Makkah 7 min). This longer exposure to insecticide in the wind tunnel assay is likely to be a major factor contributing to the higher knockdown and mortality recorded during and after this assay, respectively compared to the thumb assay, in which there was no within-assay knockdown. The time spent in contact with treated material in cone bioassay wasn't recorded in the current study hence the method cannot be compared with thumb and wind tunnel assays, and this is a major current limitation of the assay which can be overcome using a video-recorded modification to the method (Hughes, 2018).

Table 7.1 Summary of mortality rates observed in mosquitoes (Jeddah, Makkah and New Orleans strains) exposed to all assays conducted in this thesis.

Strain	Mortality				
	WHO tube bioassay (60 min)	WHO cone bioassay (3 min)	Thumb test assay (10 min)	Wind tunnel assay (20 min)	PBO assay (60 min)
New Orleans	100	100	54	100	-
Jeddah	21.4	75.3	0	90	46.6
Makkah	4.4	10.3	0	45	20.7

In all assays involving pyrethroids, the Makkah strain consistently had lower mortality rates than the Jeddah strain. The Makkah strain had higher frequencies (67%) of the dual knockdown resistant mutations (989P and 1016G) compared to 46% in Jeddah strains. The dual mutations were strongly linked to pyrethroid resistance in the current study and also in other studies (Sayono et al., 2016, Li et al., 2015, Fernando et al., 2018, Srisawat et al., 2010). Pre-exposure to the synergist PBO did not

significantly increase susceptibility in the Makkah strain, despite the higher resistance, but significantly increased mortality in Jeddah. Thus, the effect of the dual mutations on survival seems to be larger than that attributable to metabolic resistance. The dual mutations can serve as markers for prediction of pyrethroid resistance in *Ae. aegypti* population in Saudi Arabia.

While the 1534C mutation has been reported in the Americas, Africa and Asia, our study contributes to knowledge of the boundaries for each of the V1016 mutations, with the most westerly report of the 1016G mutation to date. V1016I has recently been detected in Ghana (Kawada et al., 2016) and Burkina Faso (Sombié et al., 2019) alongside the F1534C substitution, suggesting a possible contact zone between the 1016G and 1016I mutations located between Saudi Arabia and West Africa. The impact of the interaction of the 1534C+1016I and 989P+1016G+1534F haplotypes on *Ae. aegypti* resistance phenotype if unknown and where interaction may occur is an area that needs to be explored. Other important *kdr* mutations which were not detected in the present study but should be monitored in future are I1011 M and V410L (Haddi et al., 2017, Du et al., 2013). Both have been linked to pyrethroid resistance in Latin America (Moyes et al., 2017) and the V410L mutation has been shown to directly confer resistance to permethrin and deltamethrin alone and can provide multiplicative resistance in combination with F1534C in transformed *Xenopus* oocytes (Haddi et al., 2017). Surveillance of *kdr* mutations will be important, as their different combinations can lead to qualitatively and quantitatively different impacts on pyrethroid resistant phenotype.

Jeddah and Makkah exhibited a low prevalence of resistance to the organophosphates, fenitrothion and temephos. The G119S mutation in *Ace1* gene which is strongly linked to organophosphate resistance in other mosquito species (Bkhache et al., 2018, Essandoh et al., 2013) was not detected in both populations. In the microarray analysis, esterases, a class of enzymes linked to temephos and other organophosphates resistance (Grisales et al., 2013, Marcombe et al., 2012, Poupardin et al., 2014, Grigoraki et al., 2016) were not significantly differentially expressed in the Jeddah and Makkah populations. The absence of the target site mutation and low expression of esterases may explain the relatively low prevalence of organophosphate resistance. However, a different class of enzymes may be

associated with the slight resistance to organophosphates especially in Makkah where a statistically significant (*P*=0.002) recovery of susceptibility was observed when pre-exposed to PBO before conducting the standard bioassay with malathion. The recovery of susceptibility with PBO, but lack of significant overexpression of esterases suggests that cytochrome P450s might play some role in the limited organophosphate resistance phenotype observed. Selection for malathion resistance in the Makkah strain greatly reduced mortality over a few generations, but surprisingly was associated with reduced expression of all the candidate CYPs assessed by qPCR (*CYP9J7*, *CYP9J27*, *CYP9J26*, AAEL014614-RA (CYP9P450)) suggesting involvement of other genes or a different mechanism of action of the PBO such as enhanced penetration resistance (Bingham et al., 2011). It was only possible to functionally-evaluate one of these candidate genes in the present study, but for *CYP9J7*, I detected significant metabolism of all three organophosphates tested, but not pyrethroids. Coupled with downregulation of the gene when under malathion selection, this finding is consistent with a role in activation of the organophosphates to their toxic oxon form, which requires desulfuration mainly catalysed by CYPs (Hollingworth, 1971).

Pyrethroid resistance was strongly linked to overexpression of CYPs in both strains. *CYP9J10*, *CYP6BB2* and *CYP9J26* were among the overexpressed CYPs, and have been validated as pyrethroid metabolisers (Kasai et al., 2014, Stevenson et al., 2012). Selection of metabolic resistance to pyrethroids may thus negatively correlate with selection of malathion resistance. Negative cross resistance to pyrethroids and organophosphate may explain why low prevalence of organophosphate resistance was observed in both population even though temephos is used for larviciding in both cities (Aziz et al., 2011). Yet negative cross resistance between the organophosphates temephos and malathion has also been reported whereby selection of malathion resistance in a lab strain that was previously highly resistant to deltamethrin and temephos led to increased susceptibility to both of these insecticides (Viana-Medeiros et al., 2018). Moreover, resistance to both pyrethroids and organophosphates in a single strain has been observed in the wild (Goindin et al., 2017, Lima et al., 2011).

A common observation in these multi-resistant strains was overexpression of esterases such as CCEae3a, glutathione transferases such as GSTe2 and high frequencies of the target site mutations V1016I, F1534C and I1011M. Though it is unclear whether individual mosquitoes were resistant to both pyrethroids and organophosphates in these strains, such findings in combination with those from my study suggest that whilst pyrethroid-malathion negative cross resistance can occur in an *Ae. aegypti* population it may depend on the specific mechanisms present.

In common with most previous studies in *Anopheles* and *Aedes*, susceptibility to pyrethroids in the Jeddah strain increased with age (Chapter 2 section 2.3.2). Most arboviruses require an extrinsic incubation period in the vector that ranges between 7 to 14 days (Kuno and Chang, 2005); over this period susceptibility to pyrethroids increased significantly in Jeddah (38.6% increase mortality in 14 day old mosquitoes compared to 5 days old). Therefore, if pyrethroids are used to control *Aedes*, and they are applied correctly (with respect to dose, timing, etc), increased susceptibility with age could help to reduce arbovirus transmission. But vertical transmission of dengue virus occurs in nature hence age dependent mortality may not have an impact on dengue transmission unless if the mosquitoes die before reproducing hence interrupting vertical transmission of the virus to their progeny (Ferreira-De-Lima and Lima-Camara, 2018). We observed a reduction in mortality in 14 day old females that had been repeatedly exposed to deltamethrin every 24h compared to a three day old cohort when first exposed. This result contrasts with those in the age-dependent mortality study. Repeated exposure every 24h may have led to either selection of the most genetically resistant individuals and/or induction of detoxification genes. Poupardin and colleagues, demonstrated repeated exposure of *Ae. aegypti* to xenobiotics and insecticides leads to induction of CYPs and GSTs (Poupardin et al., 2008). Irrespective of the mechanism, given the importance of adult longevity for disease transmission (Ernst et al., 2016), the finding that repeated exposure can break the normal age-dependent susceptibility relationship in the population is a concern for pyrethroid-based control, and it will be important to determine whether the same trend holds for other insecticides.

In the thumb and wind tunnel studies, there was a reduction in the proportion of contact with the net and feeding attempts, and of successful passage through nets, respectively, with impregnated compared to untreated nets. The reduction in time spent on these host seeking behaviours was observed in both resistant and susceptible strains, indicating that each was affected by contact irritancy and displayed similar avoidance behaviour. The main contrast between the resistant and susceptible strains was the knockdown and mortality rate observed in both assays. Lower knockdown rate enabled more Jeddah, and especially the more resistant Makkah to cross the treated net barrier compared to New Orleans. Although contact irritancy in the current and other studies (Parker, 2015, Hughes, 2018) significantly reduces host seeking-related behaviour rate successfully in resistant and susceptible strains, increased pyrethroid tolerance still can confer an advantage by potentially allowing survival for long enough to maintain the search for a blood meal even in the presence of treated materials, coupled with reduced mortality thereafter.

7.1. CONCLUSION

This research established the resistance profile of the two-primary dengue-endemic areas of Saudi Arabia, Makkah and for the first time Jeddah. The worrying outcome of this study is the evidence of high prevalence of insecticide resistance in both cities. As discussed in Chapter 1 section 1.10.5, some studies have demonstrated high level of insecticide resistance can lead to entomological control failure. It is high time that the control programs in both cities should consider including insecticide resistance monitoring as part of the vector control feedback tool to, 1) Assess the effectiveness of the ongoing control program and 2) to monitor resistance while collecting evidence to guide them making decisions on which vector control tools to implement. Although the population was still susceptible to temephos and *Bti*, the control program should explore other non-insecticide-based control tools such as biological control and larval source reduction to slow down selection of higher levels of insecticide resistance. The program should also consider or continue engaging the community through public awareness forums to participate in control activities as this can accelerate the achievement of zero dengue transmissions in Saudi Arabia.

7.2. Limitations and suggestion for future studies

The aim of this study was to characterise the behavioural and physiological mechanisms of insecticide resistance in *Ae. aegypti* population in two dengue foci cities in Saudi Arabia. Target site insensitivity and, to some extent, overexpression of metabolic genes especially in the Jeddah strain were associated with pyrethroid resistance. Contrasting results from assays with and without PBO also strongly implicated metabolic genes in resistance to carbamates and the limited resistance to organophosphates. Although not widely used at present for *Aedes* control, bendiocarb may become increasingly important as a pyrethroid-alternative for IRS (Vazquez-Prokopec et al., 2017). Future studies should investigate the specific genes contributing to the extreme bendiocarb resistance observed in this study, and also if they are similar genes that can cause cross-resistance to pyrethroids. The role of *CYP9J7* in pyrethroid resistance was not established in this study. This enzyme was overexpressed in both populations and in at least one other study of pyrethroid resistant *Ae. aegypti* from French Oceanic islands (Dusfour et al., 2015) thus suggesting a role in pyrethroid resistance. Its role in sequestration and secondary metabolism of pyrethroid metabolites should be investigated. We did not find evidence of behaviour changes (as distinct from greater resistant-linked success in the wind-tunnel assay) in either strain that could have an impact on effectiveness of insecticide treated materials. However, the study did not evaluate behaviours such as changes in feeding time, location and mating success, which can have an impact on the effectiveness of control tools. An important observation of this study was the differences in resistance mechanism between the two strains. Future studies should investigate the factors driving the evolution of different resistance mechanism in strains separated by a relatively short distance (approximately 100km). This is especially important in generating data to guide control programs when they are evaluating which insecticide-based control tools to consider. Finally, it is crucial that monitoring programmes are implemented to evaluate the efficacy of current and future control programmes in Saudi Arabia. The knowledge on factors affecting and underpinning resistance from my study, should provide valuable resources to support such work.

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8.0. APPENDIX 1

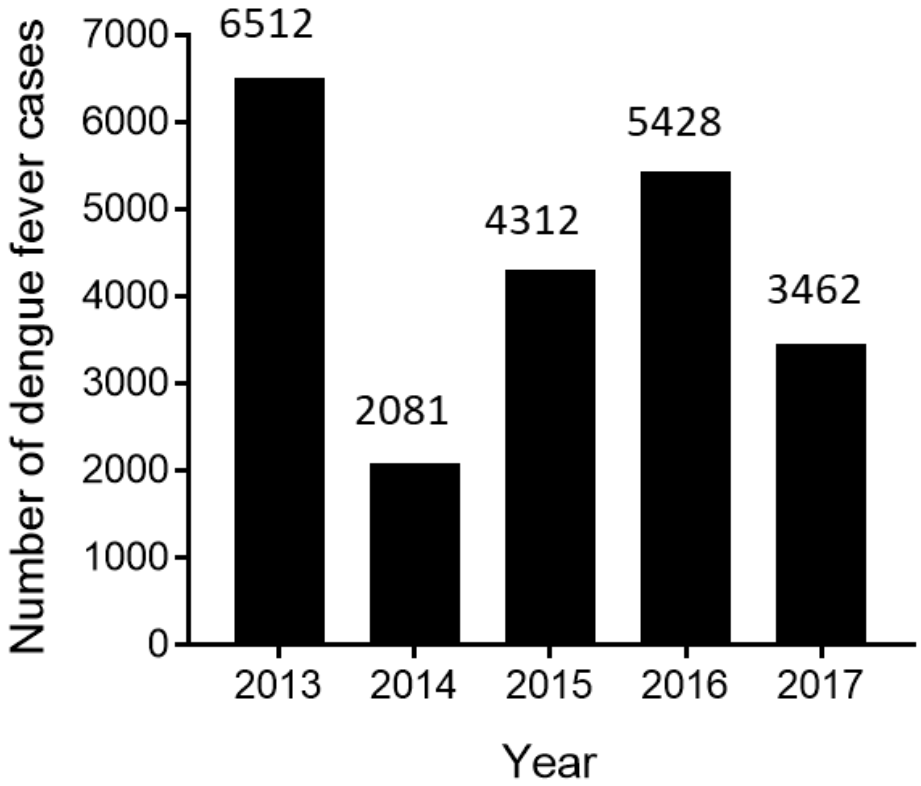

Figure 8.1 Annual number of dengue fever cases in Saudi Arabia from 1994-2017 (Ministry of Health, 2018, Fakeeh and Zaki, 2003).

Table 8.1 Temephos stock dilution with distilled water up to 200 ml to obtain the appropriate final concentrations.

Final concentration (mg/L)	Volume of stock solution (mL)	Volume of distilled water (mL)
0.08	1	199
0.07	0.875	199.125
0.06	0.75	199.25
0.04	0.5	199.5
0.03	0.375	199.625
0.02	0.25	199.75
0.01	0.125	199.875
0.005	0.0625	199.9375
0.0025	0.03125	199.96875

Table 8.2 *Bacillus thuringiensis israelensis (Bti)* stock dilution with distilled water from the stock solution at 1.2%.

C1 (stock) ppm	Volume of stock solution (mL)	Desired concentration (100mL)-ppm	Volume 2 (mL)
120	0.005	0.0059997	100.005
120	0.003	0.003599892	100.003
120	0.002	0.002399952	100.002
120	0.001	0.001199988	100.001
120	0.00075	0.000899993	100.00075
120	0.0005	0.000599997	100.0005
120	0.0002	0.00024	100.0002
120	0.0001	0.00012	100.0001

Vectobac stock (1.2%) was diluted by adding 1ml of the stock (1.2%) to 99 ml distilled water to obtain 0.012% (120pmm) which was used in the experiment.

Table 8.3 Generalised Linear Model for the effects of strain, repeated of deltamethrin exposure on mortality of *Ae. aegypti* females from Cayman and Jeddah.

	Parameter Estimates in Cayman and Jeddah strains							
Strain	Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		
				Lower	Upper	Wald Chi-Square	df	P value.
Cayman	(Intercept)	-1.19	0.24	-1.67	-0.72	23.94	1	9.89E-07
	Day	-0.22	0.063	-0.34	-0.1	12.48	1	0.0004
Jeddah	(Intercept)	-0.32	0.17	-0.67	0.02	3.4	1	0.07
	Day	-0.34	0.05	-0.43	-0.24	43.6	1	4.09E-11

Table 8.4 List of primer sequences for qRT-PCR.

Primer name	Primer sequence (5'–3')	Reference
CYP9J10 F	ATCGGTGTTGGTGAAAGTTCTGT	(Bariami et al., 2012)
CYP9J10 R	CATGTCGTTGCGCATTATCCC	(Bariami et al., 2012)
CYP9J28 F	CCACTGACGTACGATGCGA	(Bariami et al., 2012)
CYP9J28 R	GCCGATCAGTGGACGGAGC	(Bariami et al., 2012)
CYP9J32 F	CGGTCCGCTTATGACGAAGAG	Grigolaki et al. unpublished
CYP9J32 R	TTTGTTCGCTCCGAAGAGTGG	Grigolaki et al. unpublished
CYP9M6 F	TCGGTGCACAATCCAAACAAC	(Kasai et al., 2014)
CYP9M6 R	GTCGGGTACGACCAACGAAA	(Kasai et al., 2014)
ABCB4 F	GAATGGCCGCATCTGCCAG	(Bariami et al., 2012)

ABCB4 R	CGTTTCCTTGGGACCGAGCT	(Bariami et al., 2012)
RPS3 F	AGCGTGCCAAGTCGATGAA	(Kasai et al., 2014)
RPS3 R	GTGGCCGTGTCGACGTACT	(Kasai et al., 2014)
Ae60sL8 F	CTGAAGGGAACCGTCAAGCAA	(Grisales et al., 2013)
Ae60sL8 R	TCGGCGGCAATGAACAACT	(Grisales et al., 2013)

Table 8.5 Genotypes of females surviving long (4 or 6h) exposure or killed by short (1h exposure).

Strain	Individual	989 (mutant=C)	1016 (mutant= G)	1534 (mutant =G)	Genotype	Alive or Dead	Triple mutant present?
Jeddah	J01g1d1	C C	G G	T G	7	dead	Yes
Jeddah	J02g1d1	T T	T T	G G	2	dead	
Jeddah	J03g1d1	T T	T T	G G	2	dead	
Jeddah	J04g1d1	T T	T T	G G	2	dead	
Jeddah	J05g1d1	T T	T T	G G	2	dead	
Jeddah	J06g1d1	C C	G G	T G	7	dead	Yes
Jeddah	J07g1d1	C C	G G	T T	1	dead	
Jeddah	J08g1d1	C C	G G	T T	1	dead	
Jeddah	J09g1d1	T T	T T	G G	2	dead	
Jeddah	J10g1d1	T T	T T	G G	2	dead	
Jeddah	J11g1d1	T T	T T	G G	2	dead	
Jeddah	J12g1d1	T T	T T	T G	3	dead	
Jeddah	J13g1d1	T C	T G	G G	6	dead	Yes
Jeddah	J14g1d1	T T	T T	T G	3	dead	
Jeddah	J15g1d1	T C	T G	G G	6	dead	Yes
Jeddah	J16g1d1	T T	T T	T G	3	dead	
Jeddah	J17g1d1	T T	T T	G G	2	dead	
Jeddah	J18g1d1	T T	T T	G G	2	dead	
Jeddah	J19g1d1	T T	T T	G G	2	dead	
Jeddah	J20g1d1	T T	T T	G G	2	dead	
Jeddah	J21g1d1	T C	T G	T G	5	dead	
Jeddah	J22g1d1	T C	T G	G G	6	dead	Yes
Jeddah	J23g1d1	T T	T T	T G	3	dead	
Jeddah	J24g1d1	T C	T G	T G	5	dead	
Jeddah	J25g1d1	T T	T T	G G	2	dead	
Jeddah	J26g1d1	C C	G G	T T	1	dead	
Jeddah	J27g1d1	T T	T T	G G	2	dead	
Jeddah	J28g1d1	T C	T G	T G	5	dead	
Jeddah	J29g1d1	T C	T G	T T	4	dead	
Jeddah	J30g1d1	T T	T T	G G	2	dead	
Jeddah	J31g1d1	T T	T T	G G	2	dead	
Jeddah	J32g1d1	T T	T T	G G	2	dead	

Makkah	M01g1d1	T C	T G	T G	5	dead	
Makkah	M02g1d1	T T	T T	G G	2	dead	
Makkah	M03g1d1	T T	T T	G G	2	dead	
Makkah	M04g1d1	T C	T G	T G	5	dead	
Makkah	M05g1d1	T C	T G	T G	5	dead	
Makkah	M06g1d1	T C	T G	T T	4	dead	
Makkah	M07g1d1	T C	T G	G G	6	dead	Yes
Makkah	M08g1d1	T T	T T	G G	2	dead	
Makkah	M09g1d1	T T	T T	G G	2	dead	
Makkah	M10g1d1	T T	T T	G G	2	dead	
Makkah	M11g1d1	T T	T T	G G	2	dead	
Makkah	M12g1d1	T T	T T	G G	2	dead	
Makkah	M13g1d1	T T	T T	G G	2	dead	
Makkah	M14g1d1	T T	T T	G G	2	dead	
Makkah	M01g1A6	C C	G G	T T	1	alive	
Makkah	M02g1A6	C C	G G	T T	1	alive	
Makkah	M03g1A6	T C	T G	T G	5	alive	
Makkah	M04g1A6	C C	G G	T T	1	alive	
Makkah	M05g1A6	C C	G G	T T	1	alive	
Makkah	M06g1A6	C C	G G	T T	1	alive	
Makkah	M07g1A6	T C	T G	T G	5	alive	
Makkah	M08g1A6	C C	G G	T T	1	alive	
Makkah	M09g1A6	C C	G G	T T	1	alive	
Makkah	M14g1A4	T C	T G	T G	5	alive	
Makkah	M15g1A4	T C	T G	T G	5	alive	
Makkah	M16g1A4	C C	G G	T T	1	alive	
Makkah	M17g1A4	T C	T G	T G	5	alive	
Makkah	M18g1A4	C C	G G	T T	1	alive	
Makkah	M19g1A4	C C	G G	T T	1	alive	
Makkah	M20g1A4	C C	G G	T T	1	alive	
Makkah	M21g1A4	C C	G G	T T	1	alive	
Jeddah	J01g1A6	T C	T G	T G	5	alive	
Jeddah	J02g1A6	T C	T G	T G	5	alive	
Jeddah	J03g1A6	T C	T G	T G	5	alive	
Jeddah	J04g1A6	T T	T T	G G	2	alive	
Jeddah	J05g1A4	C C	G G	T T	1	alive	
Jeddah	J06g1A4	T C	T G	T G	5	alive	
Jeddah	J08g1A4	T T	T T	G G	2	alive	
Jeddah	J09g1A4	T C	T G	T G	5	alive	
Jeddah	J10g1A4	T C	T G	T T	4	alive	
Jeddah	J11g1A4	C C	G G	T T	1	alive	
Jeddah	J12g1A4	C C	G G	T T	1	alive	
Jeddah	J13g1A4	C C	G G	T T	1	alive	
Jeddah	J14g1A4	T C	T G	T G	5	alive	

Jeddah	J15g1A4	T C	T G	T G	5	alive	
Jeddah	J16g1A4	C C	G G	T T	1	alive	
Jeddah	J17g1A4	T C	T G	T G	5	alive	
Jeddah	J18g1A4	T C	T G	T G	5	alive	
Jeddah	J19g1A4	C C	G G	T T	1	alive	
Jeddah	J20g1A4	C C	G G	T T	1	alive	

Table 8.6 List of primer sequences for qRT-PCR.

Primer		Sequence	Product size
CYP9J7	CYP9J7_1F	CGGGGTTGATCAGGGATACG	145
	CYP9J7_2R	GTGGTTTCCGAGACCTCCTG	
CYP9J26	CYP9J26_2F	GTTCGGGAAGGGTGGAAGTC	76
	CYP9J26_1R	AAAGCACACGACGCAATCAC	
CYP9450	AAEL014614_1F	CCGAGAAGACACAGATCCCG	161
	AAEL014614_1R	TCACACATGCTCAACGCTTC	
AAEL006013-RA	AAEL006013_1F	CGAATCGCCGGTCATTGTTC	164
	AAEL006013_RA	TGGGTTTTTCGTCGGAGAGG	
AAEL006953-RA	AAEL006953_1F	GAGCCGGAGGTTAGAGTTCG	161
	AAEL006953_1R	TTCCCATTGGAGCCTTGTCC	

Table 8.7 Clusters of GO term based on 212 significantly overexpressed genes using David analysis.

Annotation Cluster 1	Enrichment Score 5.73	Count	*P* Value	Benjamini
UP_KEYWORDS	Iron	17	7.20E-08	5.30E-06
UP_KEYWORDS	Heme	15	1.00E-07	3.70E-06
INTERPRO	Cytochrome P450, conserved site	14	2.60E-07	7.80E-05
UP_KEYWORDS	Monooxygenase	14	3.90E-07	9.60E-06
GOTERM_MF_DIRECT	monooxygenase activity	14	4.60E-07	3.30E-05
INTERPRO	Cytochrome P450, E-class, group I	13	5.20E-07	8.00E-05
INTERPRO	Cytochrome P450	14	6.40E-07	6.50E-05
GOTERM_MF_DIRECT	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	14	1.40E-06	5.00E-05
GOTERM_MF_DIRECT	heme binding	15	2.60E-06	6.30E-05
UP_KEYWORDS	Oxidoreductase	17	7.30E-06	1.30E-04
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism	14	9.30E-06	7.50E-05
GOTERM_MF_DIRECT	iron ion binding	14	1.20E-05	2.20E-04
UP_KEYWORDS	Metal-binding	19	8.70E-03	1.20E-01
Annotation Cluster 2	Enrichment Score 1.51	Count	*P* Value	Benjamini
SMART	ZnF_C2H2	17	1.60E-03	1.00E-01

INTERPRO	Zinc finger, C2H2-like	17	5.80E-03	3.60E-01
INTERPRO	Zinc finger, C2H2	17	9.50E-03	4.40E-01
INTERPRO	Zinc finger C2H2-type/integrase DNA-binding domain	14	1.60E-02	5.10E-01
GOTERM_MF_DIRECT	zinc ion binding	20	2.70E-02	2.80E-01
INTERPRO	Zinc finger, AD-type	9	3.30E-02	7.20E-01
SMART	SM00868	8	3.80E-02	5.80E-01
GOTERM_MF_DIRECT	nucleic acid binding	17	1.30E-01	7.70E-01
GOTERM_CC_DIRECT	nucleus	15	3.50E-01	9.90E-01
GOTERM_MF_DIRECT	metal ion binding	12	3.50E-01	9.60E-01
Annotation Cluster 3	Enrichment Score 1.05	Count	*P* Value	Benjamini
INTERPRO	Nuclear transport factor 2, Eukaryote	3	1.20E-02	4.50E-01
KEGG_PATHWAY	RNA transport	6	8.70E-02	9.00E-01
KEGG_PATHWAY	mRNA surveillance pathway	4	1.50E-01	9.30E-01
KEGG_PATHWAY	Ribosome biogenesis in eukaryotes	3	4.40E-01	9.70E-01

Table 8.8 Clusters of GO term based on significantly under expressed 205 gene using David analysis.

Annotation Cluster 1	Enrichment Score 1.53	Count	*P* Value	Benjamini
PIR_SUPERFAMILY	tetraspanin	3	2.10E-02	1.90E-01
INTERPRO	Tetraspanin	3	3.00E-02	1.00E+00
INTERPRO	Tetraspanin/Peripherin	3	3.90E-02	1.00E+00
Annotation Cluster 2	Enrichment Score 1.23	Count	*P* Value	Benjamini
UP_KEYWORDS	Membrane	49	3.00E-02	8.50E-01
UP_KEYWORDS	Transmembrane helix	47	4.70E-02	6.30E-01
UP_KEYWORDS	Transmembrane	47	4.80E-02	5.30E-01
GOTERM_CC_DIRECT	integral component of membrane	47	1.80E-01	7.10E-01

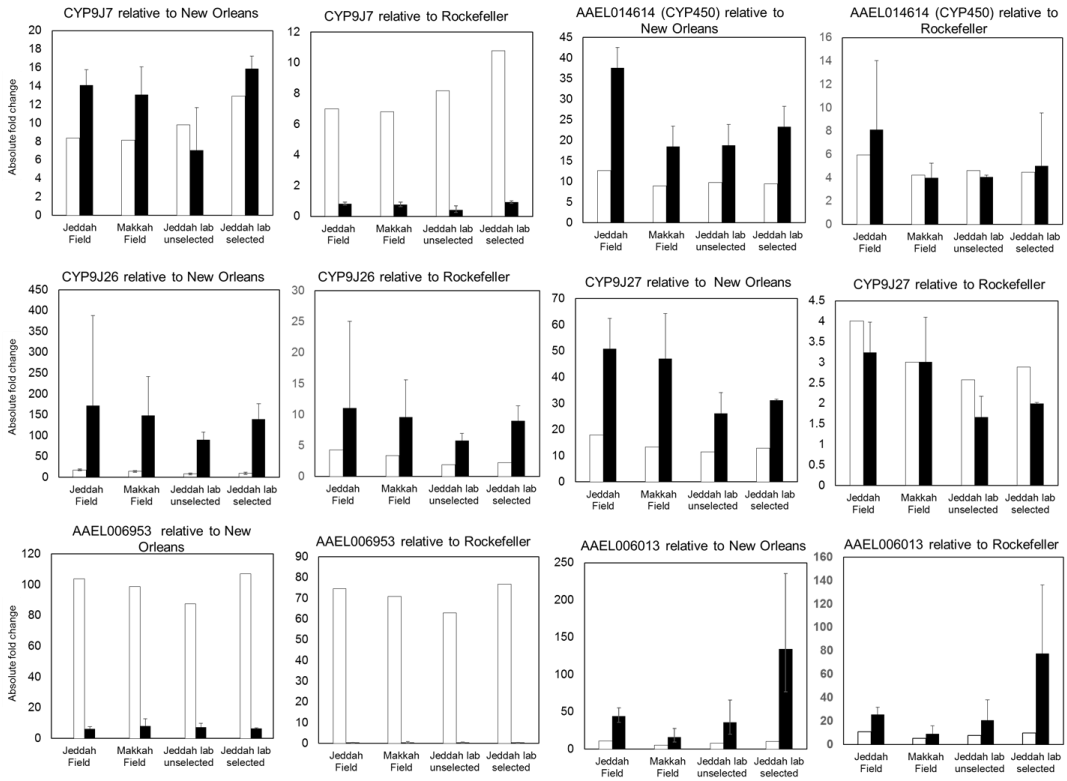

Figure 8.2 Relative fold-change of candidate genes from qRT-PCR analysis and microarray compared to the susceptible strains New Orleans and Rockefeller. Error bars represent 95% confidence intervals.

Table 8.9 Mortality ratios of multiple exposures from WHO tube impregnated 0.1% bendiocarb against *Ae. aegypti* A) Jeddah and B) Makkah female and male mosquitoes post 24h exposure.

A) Selected Jeddah bendiocarb colony										
			Female				Male			
Generation	Exposure Time	Exposure Type	Total	survivors	Dead	Mortality (%)	Total	Survivors	Dead	Mortality (%)
F2	8h	Single	135	24	111	81.5	Not Performed			
	6h	Single	68	6	62	91.1	Not Performed			
	4h	Single	157	22	135	86	Not Performed			
	2h	Single	Not Performed				124	27	97	77
F3	8h	Multiple	49	1	48	98.1	45	1	44	97.5
	8h	Multiple	1	1	0	0	1	1	0	0
	4h	Multiple	63	7	56	88.8	43	5	38	89.0
	4h	Multiple	6	4	2	33.3	6	2	4	55.6
	2h	Multiple	38	8	30	79.2	48	15	33	68.0
	2h	Multiple	8	3	5	63.3	15	10	5	36.1
F4	4h	Single	105	17	88	84.2	Not Performed			

B) Selected Makkah bendiocarb colony										
			Female				Male			
Generation	Exposure Time	Exposure Type	Total	Survivors	Dead	Mortality (%)	Total	Survivors	Dead	Mortality (%)
F2	8h	Single	199	47	152	75.9	221	49	172	78.2
	8h	Multiple	250	30	220	88.4	82	7	75	89.8
	8h	Multiple	28	17	11	29.9	7	3	4	54.2
F3	6h	Multiple	Not Performed				63	7	56	88.9
							7	7	0	0
F4	8h	Single	223	43	180	79.9	Not Performed			

Table 8.10 Significantly upregulated genes in resistant strains (P<0.05 and FC>2 in all comparisons with New Orleans and Rockefeller involving all strains). The genes ranked based on the highest fold change in Jeddah lab selected.

Protein Name	Vectorbase ID	Log Fold change				Function		
		Jeddah Field	Jeddah lab unselected	Jeddah lab selected	Makkah field	Molecular function	Biological process	Cellular component
Chitin-binding domain type 2	AAEL006953-RA	6.46	6.21	6.5	6.39	Chitin binding	Chitin metabolic process	Extracellular region
Unknown	AAEL008176-RA	5.23	4.94	5.13	3.12			
CYP9J7	AAEL014606-RA	2.94	3.16	3.56	2.89	Monooxygenase activity	Oxidation-reduction process	Integral component of membrane
Histone-lysine N-methyltransferase	AAEL006013-RA	3.44	2.98	3.32	2.4	Protein binding, histone-lysine N-methyltransferase activity - Methyltransferase activity	- Regulation of transcription - DNA templated - methylation - Histone lysine methylation	- Nucleus - Chromosome
Unknown	AAEL011980-RA	3.47	2.81	3.24	3.09	Unknown	Unknown	Unknown
CYP9J27 v2	AAEL014607-RA	3.11	2.7	2.77	2.87	Monooxygenase activity	Oxidation-reduction process	Integral component of membrane
Cytochrome P450	AAEL014614-RA	3.12	2.75	2.7	2.62	Monooxygenase activity	Oxidation-reduction process	Unknown
CYP9J27 v1	AAEL014616-RA	3.08	2.44	2.61	2.66	Monooxygenase activity	Oxidation-reduction process	Integral component of membrane
Protein YIPF	AAEL001596-RA	1.63	1.65	2.44	1.45	Unknown	Vesicle-mediated transport	Integral component of membrane
Sugar transporter	AAEL007050-RA	2.03	1.96	2.43	1.67	Transmembrane transporter activity	Transmembrane transport	Integral component of membrane
Unknown	AAEL007986-RA	2.53	2.76	2.4	2.68	Unknown	Unknown	Unknown
Protease m1 zinc metalloprotease	AAEL012779-RA	2.21	1.97	2.28	2.02	Metallopeptidase activity	proteolysis	Integral component of membrane
Nuclear movement protein nudc	AAEL001682-RA	2.01	2.14	2.24	1.61	Unknown	Unknown	Unknown

CYP9J10	AAEL006798-RA	2.6	2.17	2.22	2.12	Monooxygenase activity	Oxidation-reduction process	Integral component of membrane
Cytochrome c oxidase	AAEL003234-RA	2.03	2.09	2.22	2.47	Unknown	Unknown	Unknown
Unknown	AAEL001274-RA	2.79	2.9	2.01	4.19	Unknown	Unknown	Unknown
Neutral alpha-glucosidase ab precursor	AAEL010599-RA	1.72	1.26	2.01	1.48	catalytic activity, hydrolase activity, hydrolysing O-glycosyl compounds, carbohydrate binding	carbohydrate metabolic process	Unknown
Unknown	AAEL000861-RA	1.73	1.67	1.92	1.67	Unknown	Unknown	Unknown
Unknown	AAEL007877-RA	1.88	1.5	1.89	1.65	Unknown	Unknown	Unknown
Unknown	AAEL000560-RA	1.89	1.67	1.89	1.31	Unknown	Unknown	Unknown
ABC transporter	AAEL006717-RA	2.29	1.9	1.87	2.26	- ATPase activity - Nucleotide binding	Transmembrane transport	Integral component of membrane
S-adenosylmethionine synthase	AAEL004501-RA	1.37	1.63	1.8	1.31	- ATP binding - Metal ion binding - Methionine adenosyl transferase activity - Nucleotide binding	- One-carbon metabolic process. -S-adenosylmethionine biosynthetic process	Unknown
Unknown	AAEL005466-RA	1.67	1.5	1.77	1.48	Unknown	Unknown	Integral component of membrane
Unknown	AAEL009828-RA	2.03	1.8	1.76	1.99	Unknown	Vesicle-mediated transport	Mon1-Ccz1 complex
Unknown	AAEL005543-RA	1.56	1.63	1.7	1.75	Transferase activity	Unknown	Integral component of membrane
Trypsin	AAEL006425-RA	1.66	1.55	1.69	1.45	-Serine-type endopeptidase activity - Hydrolase activity -Serine type peptidase activity	Proteolysis	Unknown
Mitochondrial ribosome recycling factor	AAEL009225-RA	2	1.4	1.69	1.58	Unknown	Translation	Unknown

Alpha methylacyl-coa racemase	AAEL013260-RA	1.56	1.24	1.58	1.43	Catalytic activity	Unknown	Unknown
Unknown	AAEL000055-RA	1.32	1.36	1.46	1.42	Unknown	Unknown	Unknown
Unknown	AAEL014406-RA	1.79	1.42	1.45	1.57	- Zinc ion binding - Nucleic acid binding	Unknown	Nucleus
Unknown	AAEL013426-RA	1.72	1.49	1.28	1.34	Unknown	Unknown	Unknown
Methyltransferase-like 26	AAEL004990-RA	1.46	1.04	1.26	1.24	Unknown	Unknown	Unknown
Protease m1 zinc metalloprotease	AAEL012776-RA	1.76	1.56	1.24	1.43	- Aminopeptidase activity - Peptidase activity, - Metal ion binding -Metallopeptidase activity	Proteolysis	Unknown
Unknown	AAEL009445-RA	1.43	1.15	1.17	1.62	Unknown	Unknown	Unknown
Unknown	AAEL000587-RA	1.37	1.44	1.17	1.16	Unknown	Unknown	Unknown
Unknown	AAEL012866-RA	1.74	1.46	1.15	1.79	Unknown	Unknown	Unknown

Table 8.11 Top 18 significantly under expressed genes in resistant strains (P<0.05 and FC< 2 in all comparisons relative to the average of New Orleans and Rockefeller. The genes ranked based on the highest fold change in Jeddah lab selected.

Protein Name	Vectorbase ID	Log Fold change				Function		
		Jeddah Field	Jeddah lab unselected	Jeddah lab selected	Makkah fiel	Molecular function	Biological process	Cellular component
Sugar transporter	AAEL007139-RA	-1.61	-1.47	-1.44	-1.35	Transporter activity	Transmembrane transport	Integral component of membrane
Unknown	AAEL012645-RA	-2.02	-1.35	-1.5	-1.74	Chitin binding	Chitin metabolic process	Extracellular region
Unknown	AAEL006001-RA	-1.56	-1.44	-1.54	-1.34	Unknown	Unknown	Unknown
T1/St2 receptor binding protein	AAEL014029-RA	-1.53	-1.13	-1.57	-1.18	Unknown	Unknown	Integral component of membrane
Charged multivesicular body protein 2a	AAEL001336-RA	-2.11	-1.21	-1.63	-1.8	Unknown	Vacuolar transport	Unknown
Cuticle protein	AAEL009800-RA	-1.48	-1.6	-1.64	-1.29	Structural constituent of cuticle	Unknown	Unknown
P19 protein	AAEL013941-RB	-1.89	-1.65	-1.78	-1.74	Protein binding	Unknown	Unknown
Unknown	AAEL008025-RA	-1.77	-1.74	-1.89	-1.95	Unknown	Unknown	Unknown
Alanine aminotransferase	AAEL009875-RB	-2.12	-1.62	-1.96	-1.67	-Catalytic activity - Pyridoxal phosphate binding	Biosynthetic process	Unknown
Unknown	AAEL008007-RA	-2.31	-1.97	-1.97	-2.15	Unknown	Unknown	Unknown
Alanine aminotransferase	AAEL009875-RA	-2.09	-1.57	-1.97	-1.61	-Catalytic activity Pyridoxal -Phosphate binding	Biosynthetic process	Unknown
P19 protein	AAEL013941-RA	-2.09	-1.93	-2.04	-2.03	Protein binding	Unknown	Unknown
Anterior fat body protein	AAEL001022-RA	-2.12	-1.67	-2.23	-2.22	Unknown	Unknown	Unknown
Anterior fat body protein	AAEL001022-RB	-2.11	-1.83	-2.33	-2.38	Unknown	Unknown	Unknown
Unknown	AAEL006792-RA	-2.15	-1.87	-2.37	-1.56	Unknown	Unknown	Unknown

Vesicle transport protein	AAEL006010-RA	-1.99	-1.97	-2.39	-1.47	Unknown	-Protein transport -Vesicle-mediated transport	Integral component of membrane
Merozoite surface protein	AAEL001279-RB	-2.76	-2.35	-2.81	-2.59	- Nucleic acid binding - Metal ion binding	Unknown	Unknown

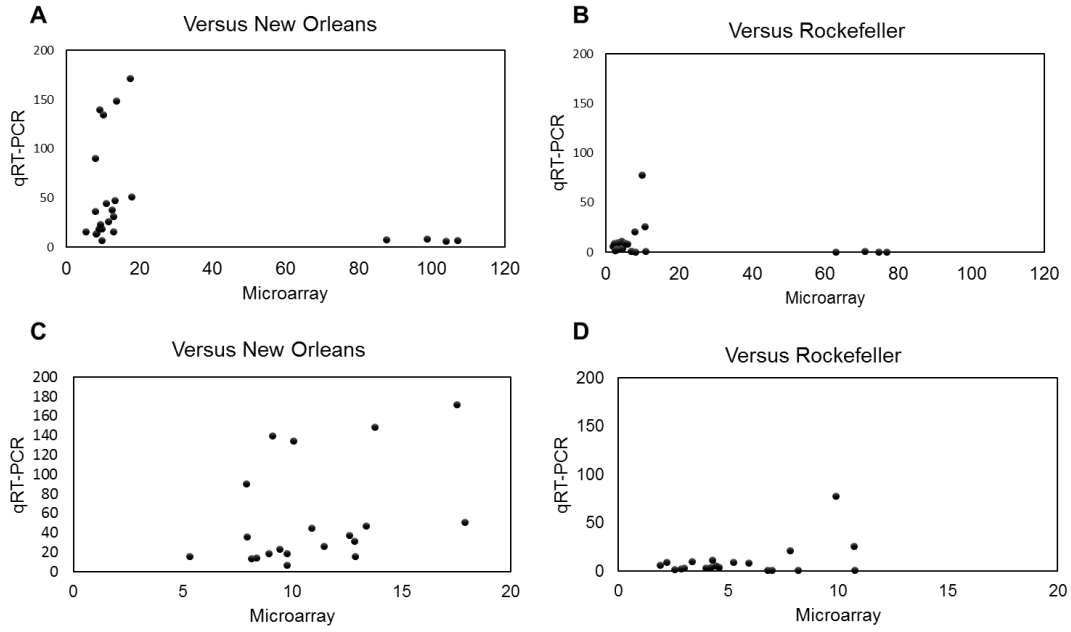

Figure 8.3 Correlation between microarray and qRT–PCR data of candidate genes selected from the list of upregulated probes. The Candidate genes were *CYP9J7*, AAEL014614 (*CYP9450*), C*YP9J26*, *CYP9J27*, AAEL006013, AAEL006953 versus A) New Orleans, B) Rockefeller. Using the same candidate genes without AAEL006953 versus C) New Orleans, D) Rockefeller to assess correlation between microarray and qRT–PCR data.

Table 8.12 The mean time spent by numbers of *Ae. aegypti* mosquitoes in New Orleans, Jeddah and Makkah in each behaviour for 600 seconds (s) and confidence interval (CI) on the untreated, PermaNet 2.0 and DuraNet in the thumb test. The n.r. indicates that behaviour is not seen during the test.

		New Orleans		Jeddah		Makkah	
Behaviour	Type of Net	Mean (s)	CI (95%)	Mean (s)	CI (95%)	Mean (s)	CI (95%)
Flight pre-net contact	Untreated	10.3	3.4	16.9	4.3	17.7	5.2
	PermaNet 2.0	12.1	3.1	33.7	8.5	23.8	6.3
	DuraNet	12.8	5.4	15.3	4.9	18.8	4.6
Flight post-net contact	Untreated	102.5	18.8	46.5	17.2	136.1	17.3
	PermaNet 2.0	125.8	20.2	132.9	20.9	107.5	27.4
	DuraNet	165.6	25.6	96.9	27.2	137.2	25.9
Resting on the walls	Untreated	237.6	41.6	306.9	45.6	184.4	26.8
	PermaNet 2.0	361.4	32.6	344	24.5	354.7	38.4
	DuraNet	326.8	33.9	395.1	41.7	345.6	37.3
Resting on the net	Untreated	126.2	24.0	109.6	23.1	108.8	15.2
	PermaNet 2.0	62.1	12.9	45.6	8.3	42.6	12.6
	DuraNet	61.6	12.3	45.0	13.6	37.6	8.2
Probing	Untreated	94.1	16.6	101	22.2	121.5	21.8
	PermaNet 2.0	26.1	8.1	22.4	6.4	26.4	9.2
	DuraNet	17.6	3.2	19.8	6.8	17	4.2
Grooming on the walls	Untreated	24.8	14.9	7.9	27	14.9	10.7

	PermaNet 2.0	3.6	14.2	13.6	9.7	31.7	10.9
	DuraNet	10.3	9.2	19.4	5.1	36.7	16.9
Grooming on the net	Untreated	0.1	0	7	9.9	1.5	3.4
	PermaNet 2.0	5.1	3.4	4.9	3.9	3.6	3.2
	DuraNet	3.05	2.6	3.5	2.5	3.8	2.3
Walking on the walls	Untreated	0.08	n.r	1.6	1	2.6	1.4
	PermaNet 2.0	0.9	88.9	2.7	7.3	6.9	4.7
	DuraNet	0.2	0	4.5	8.0	2.5	2.6
Walking on the net	Untreated	4.3	2.3	2.6	1.6	12.5	2.8
	PermaNet 2.0	0.3	n.r	0.16	n.r	2.8	4.8
	DuraNet	1	4.1	0.2	0	0.8	1.4

Table 8.13 Mean differences among New Orleans, Jeddah and Makkah mosquitoes in each behaviour with PermaNet 2.0 in the thumb test.

Dependent Variable	(I) strain	(J) strain	Mean difference (I-J)	*P* value
Flight pre-net contact	Jeddah	Makkah	5.5	0.11
		New Orleans	15.1	$5.05x10^{E-7}$
	Makkah	Jeddah	-5.5	0.1
		New Orleans	9.5	0.02
	New Orleans	Jeddah	-15.1	$5.05x10^{E-7}$
		Makkah	-9.5	0.002
Flight post-net contact	Jeddah	Makkah	-27.66	0.02
		New Orleans	-18.9	0.1
	Makkah	Jeddah	27.66	0.02
		New Orleans	8.8	0.7
	New Orleans	Jeddah	18.88	0.1
		Makkah	-8.78	0.7
Resting on the walls	Jeddah	Makkah	45.5*	0.03
		New Orleans	30.2	0.21
	Makkah	Jeddah	-45.5	0.03
		New Orleans	-15.3	0.7
	New Orleans	Jeddah	-30.25	0.21
		Makkah	15.29	0.7
Resting on the net	Jeddah	Makkah	-5.62	0.8
		New Orleans	-20.08	0.1
	Makkah	Jeddah	5.62	0.8
		New Orleans	-14.45	0.2
	New Orleans	Jeddah	20.08	0.1
		Makkah	14.45	0.2
Probing **	Jeddah	Makkah	-17.71	0.1
		New Orleans	-12.7	0.2

	Makkah	Jeddah	17.7	0.1
		New Orleans	5.01	0.8
	New Orleans	Jeddah	12.7	0.2
		Makkah	-5.01	0.8

*The mean difference is significant at the 0.05 level.**The ranked data used for Probing.

Table 8.14 Table Shows the mean differences among New Orleans, Jeddah and Makkah strains in each behaviour with DuraNet in the thumb test.

Dependent Variable	(I) strain	(J) strain	Mean difference (I-J)	*P* value
Flight pre-net contact **	Jeddah	Makkah	-11.2	0.6
		New Orleans	25.9	0.1
	Makkah	Jeddah	11.2	0.6
		New Orleans	37.1	0.003
	New Orleans	Jeddah	-25.9	0.1
		Makkah	-37.1	0.003
Flight post-net contact	Jeddah	Makkah	-64.3	$1.6x\ 10^{E-7}$
		New Orleans	-60.3	$5.5x10^{E-7}$
	Makkah	Jeddah	64.3	$1.6x10^{E-7}$
		New Orleans	4.01	0.9
	New Orleans	Jeddah	60.3	$5.5x10^{E-7}$
		Makkah	-4	0.9
Resting on the walls	Jeddah	Makkah	77.9	0.0003
		New Orleans	70.8	0.001
	Makkah	Jeddah	-77.9	0.0003
		New Orleans	-7.1	0.9
	New Orleans	Jeddah	-70.8	0.001
		Makkah	7.1	0.9
Resting on the net **	Jeddah	Makkah	0.05	1
		New Orleans	-21.7	0.1
	Makkah	Jeddah	-0.05	1
		New Orleans	-21.8	0.1
	New Orleans	Jeddah	21.7	0.1
		Makkah	21.8	0.1
Probing**	Jeddah	Makkah	-6.8	0.6
		New Orleans	-2.4	0.9
	Makkah	Jeddah	6.8	0.6
		New Orleans	4.4	0.8
	New Orleans	Jeddah	2.4	0.9
		Makkah	-4.4	0.8

*The mean difference is significant at the 0.05 level.**The ranked data used for Flight pre-net contact, Resting on the net and Probing.

Table 8.15 Mean differences among New Orleans, Jeddah and Makkah strains in the first principal component with PermaNet 2.0 and DuraNet in the thumb test.

Dependent Variable	(I) strain	(J) strain	Mean difference (I-J)	*P* value
			(PermaNet/DuraNet)	(PermaNet/DuraNet)
PC1	Jeddah	Makkah	0.4/-0.09	**0.01**/0.8
		New Orleans	0.5/-0.1	**0.002**/0.6
	Makkah	Jeddah	0.4/0.1	**0.01**/0.8
		New Orleans	-0.06/-0.04	0.9/0.9
	New Orleans	Jeddah	0.5/0.14	**0.002**/0.6
		Makkah	0.06/0.04	0.9/0.9

A significant *P* value is shown in bold type.

Table 8.16 The proportion of total time (duration) spent by numbers of *Ae. aegypti* mosquitoes in New Orleans, Jeddah and Makkah strains for each behaviour for 1200s and standard deviations (SD) on the untreated and PermaNet 2.0 in the wind tunnel assay.

	Untreated net						PermaNet2.0					
	New Orleans		Jeddah		Makkah		New Orleans		Jeddah		Makkah	
	Proportion	SD	Proportion	SD	Proportion	SD	Proportion	SD	Proportion	SD	Proportion	SD
Flight pre-net contact	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Flight post- net contact	0.37	0.13	0.29	0.11	0.31	0.14	0.34	0.17	0.22	0.13	0.20	0.11
Resting pre-net contact	0.00	0.00	0.01	0.01	0.01	0.04	0.00	0.01	0.01	0.04	0.00	0.01
Resting on the walls	0.15	0.11	0.18	0.17	0.09	0.10	0.33	0.22	0.23	0.19	0.24	0.23
Resting on the net	0.21	0.14	0.17	0.13	0.24	0.18	0.27	0.17	0.40	0.25	0.33	0.28
Resting on the host	0.14	0.20	0.26	0.21	0.29	0.18	0.01	0.02	0.09	0.17	0.19	0.27
Bouncing duration	0.10	0.09	0.07	0.05	0.06	0.04	0.03	0.02	0.05	0.05	0.03	0.03
Visiting duration	0.01	0.01	0.00	0.01	0.00	0.00	0.02	0.03	0.01	0.00	0.00	0.01
Number of Visiting events	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00
Number of Resting events	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Number of Bouncing events	0.03	0.02	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.01

Figure 8.4 20min duration from pre-releasing (in tube) and the sequential behavioural events (Flying pre-net contact, flying post-net contact, visiting, bouncing, resting pre-net contact, resting on the net, resting on the walls, resting on the host, knocked down) of pyrethroid susceptible New Orleans, resistant Makkah and Jeddah strains. The x-axis represents individual mosquito identification (ID) and y axis represents time spent (sec)for each behavioural category.

9.0. APPENDIX 2

9.1. Initial thumb test optimisation tests (1-4)

9.1.1. Preliminary Test 1 (individual mosquitoes; short starvation; normal light)

The Jeddah strain did not rest or probe at all on the untreated net but a slight response on PermaNet2.0 was recorded where 2/10 tested mosquitoes rested on the net with a mean resting and probing time of 6.0s±14.2 and 3.9s±8.5 (mean,±SD) respectively **(Figure 9.1)**. New Orleans were more responsive with 8/10 and 10/10 of the tested mosquitoes resting on the untreated and PermaNet2.0 respectively.

The average resting and probing time for New Orleans on untreated net was 36.4s ±40.3 and 174.1s±150.6, and PermaNet2.0 was 46.1s±42.1and probing 23.3s±19.6 (mean,±SD), respectively. Based on the results obtained, the decision was made to exclude the New Orleans from the rest of the optimisation tests and use Jeddah with the untreated control net for optimisation.

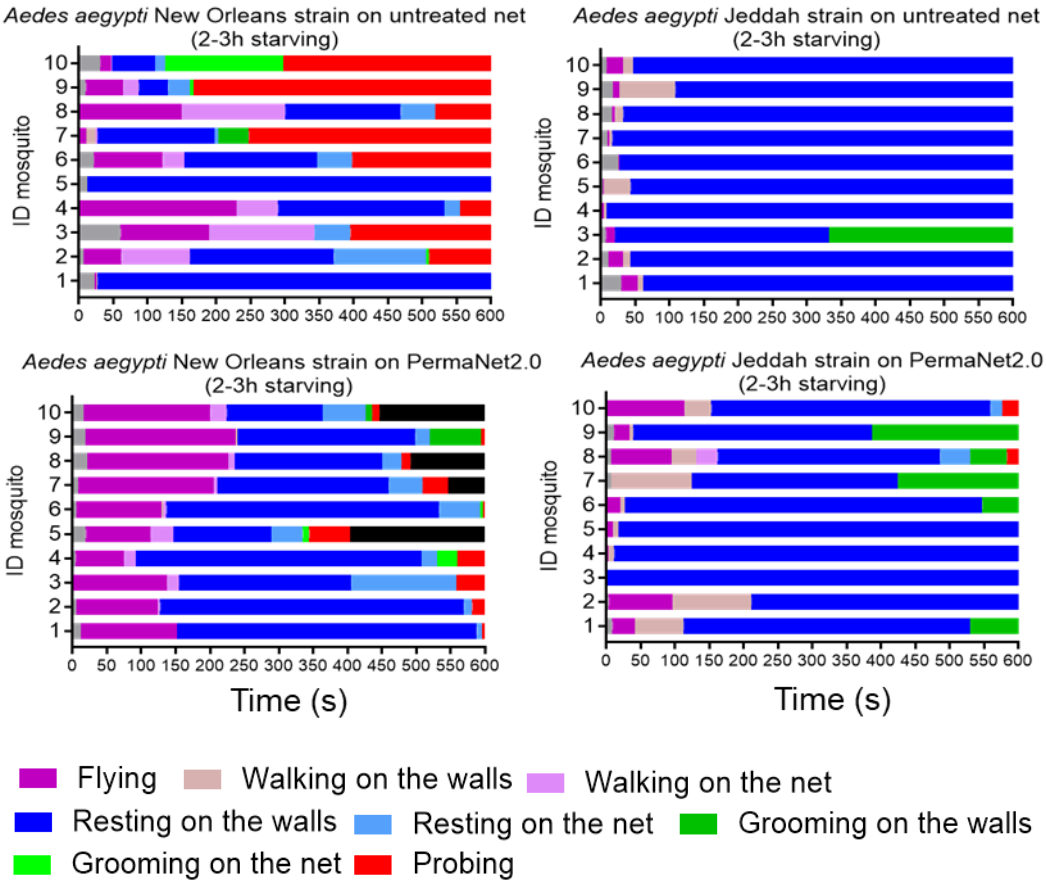

Figure 9.1 Preliminary test 1 showing the duration time (s) spent in each behaviour by of individual *Ae. aegypti* mosquitoes from Jeddah and New Orleans strains following sugar-starving for 2-3h prior to testing under daylight. The x-axis represents the time (s) spent in each behavioural event, y-axis represents number of mosquitos tested.

9.1.2. Preliminary Test 2 (individual mosquitoes; longer starvation; normal light)

As New Orleans mosquitoes had responded to untreated nets (in terms of the number of individuals responding), attention focused on the less responsive Saudi strain. Half of the mosquitoes tested (N=10) responded to the thumb bait by touching the net, although only for a short period of time, where the average resting time was 12.7s±21.8 and probing was 4.3s±8.2 (mean±SD) **(Figure 9.2)**. Despite extending the period of starvation, the difference in the average time spent at rest or probing on the untreated net was not statistically significant whether mosquitoes were starved for a short period (preliminary test 1) or long period (resting t-test, t-value=-2.0, *P*=0.06); (probing t-test, t-value=-1.8, *P*=0.08). Due to lack in responsiveness for the Jeddah strain, light intensity was adjusted in the next optimisation.

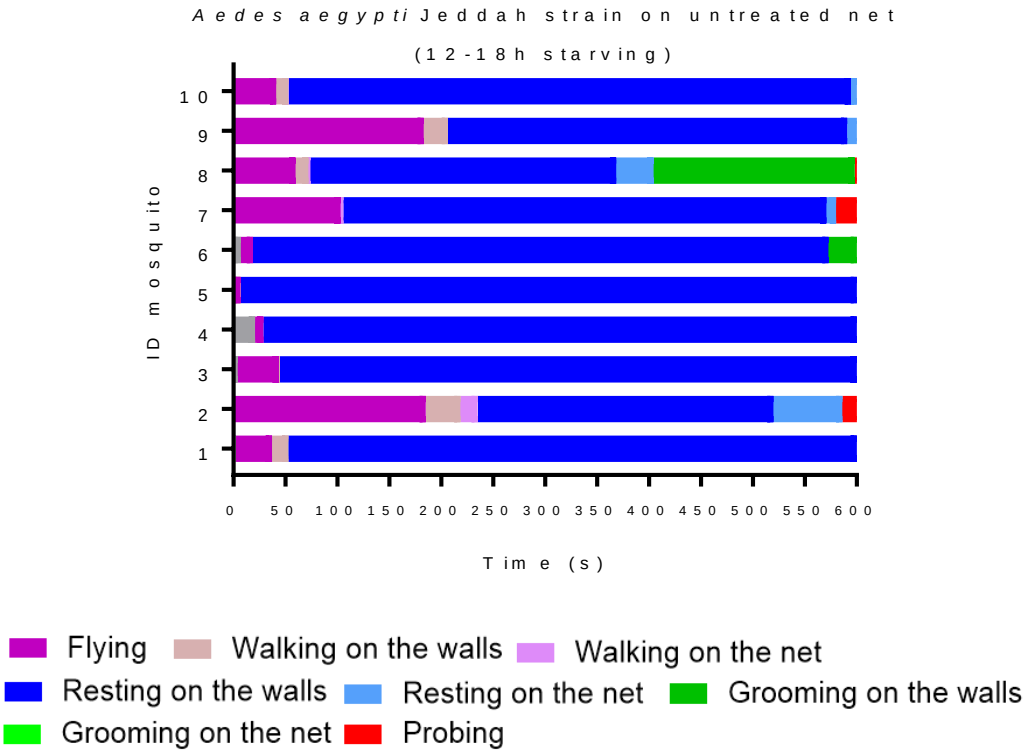

Figure 9.2 Preliminary test 2 showing the duration time (s) for each behaviour spent by individual *Ae. aegypti* mosquitoes from Jeddah strain that were sugar-starved for a prolonged time prior to the experiment and in the presence of the normal light. The x-axis represents the time (s) spent in each behavioural event, y-axis represents number of mosquitos tested

9.1.3. Preliminary Test 3 (individual mosquitoes; longer starvation; dimmer light)

In dim light conditions, half of the tested mosquitoes (N=10) reached the untreated net **(Figure 9.3)**. The average resting and probing time (mean,±SD) was 24.1±34.2 and 23.8±39.7 respectively. No significant change on host response was observed under normal light or dimmed light when the mosquitoes were starved for long (resting t-test, t-value=-0.75, *P*=0.5, Probing t-test,t-value=-1.5,*P*=0.15) or short starvation (Resting t-test, t-value=-1.8, *P*=0.09, Probing t-test, t-value=-1.9, *P*=0.07).

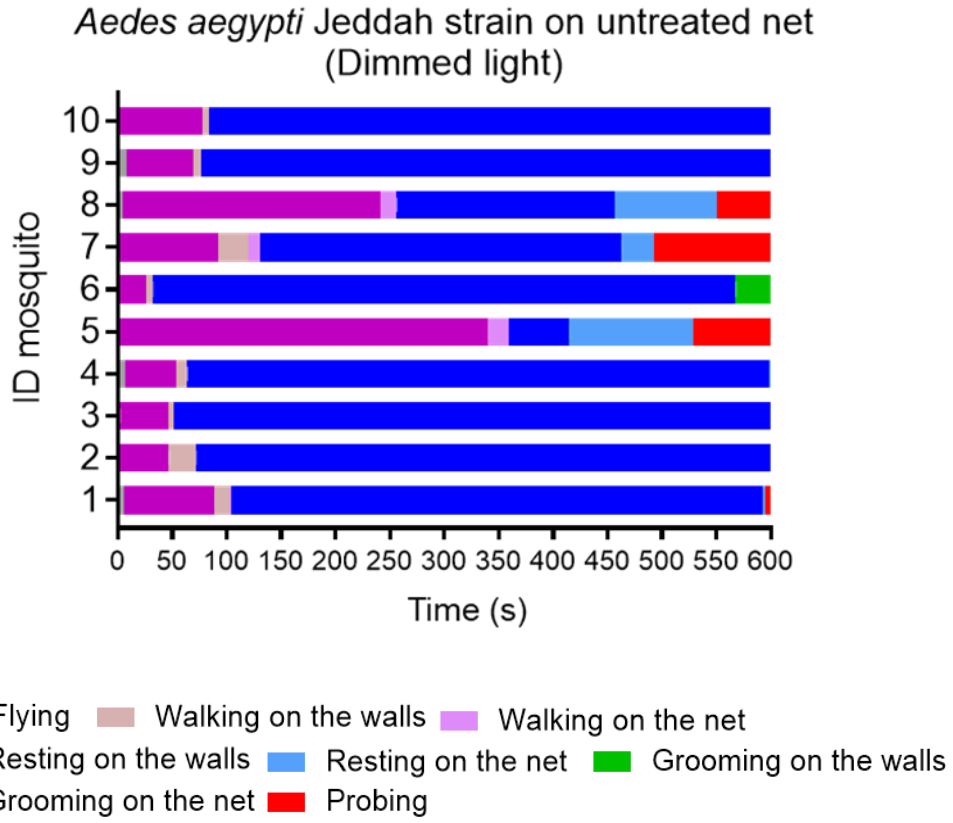

Figure 9.3 Preliminary test 3 showing the duration time (s) for each behaviour spent by of individual *Ae. aegypti* mosquitoes from Jeddah strain that were sugar-starved for a prolonged time prior to the experiment and with dimmed light. The x-axis represents the time (s) spent in each behavioural event, y-axis represents number of mosquitoes tested.

9.1.4. Preliminary Test 4 (groups of mosquitoes; longer starvation; normal light)

All mosquitoes landed on the untreated net (NO of group=10) **(Figure 9.4**). The mean resting times per five mosquitoes in the test (mean,±SD) were 115.4s±57.2 and probing were 76s±37.7. There was a significant difference in responsiveness between individual mosquitoes in test 1 and groups of mosquitoes (both resting and probing t-value=-6.38, *P*<0.00001) or individual mosquitoes in test 2 and group (resting t-test,t-value=-5.3, *P*<0.00005); (probing t-value=-5.9, *P*<0.00002).

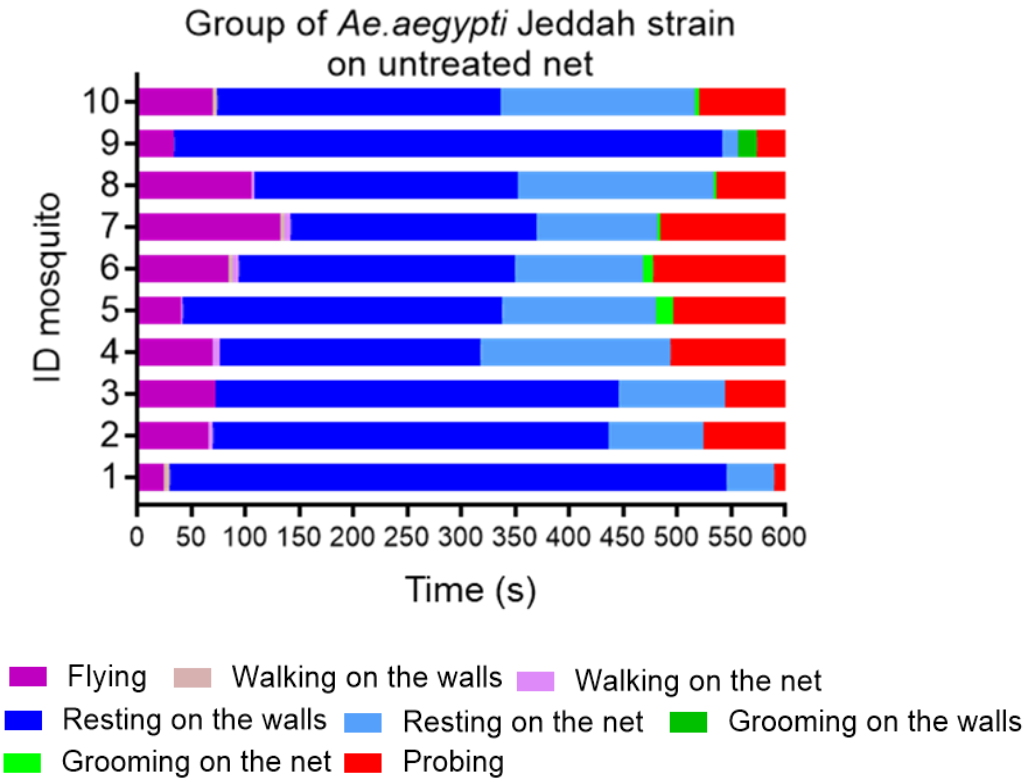

Figure 9.4 Preliminary test 4 showing the duration time (s) spent by group of *Ae. aegypti* mosquitoes from Jeddah strain that were sugar-starved for a prolonged time prior to the experiment and with normal light into the thumb test. The x-axis represents the time (s) spent in each behavioural. The y-axis represents mosquito identification (ID).