MOLECULAR MECHANISMS OF MULTIPLE INSECTICIDE RESISTANCE IN *Anopheles funestus sensu stricto* AT AKAKA-REMO, SOUTHWESTERN NIGERIA

BY

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ABSTRACT

The use of insecticides against malaria vectors has been a good approach to control malaria, but its efficacy is threatened by mosquito resistance to the lethal doses. In Nigeria, *Anopheles funestus* is gradually becoming an important malaria vector, especially in rural areas. However, despite recurring cases of Insecticide Resistance (IR) in some *Anopheles* species, there is a dearth of information on the molecular mechanisms of IR in *An. funestus*. Therefore, this study was designed to investigate the molecular mechanisms of IR in *An. funestus* at Akaka-Remo, Southwestern Nigeria.

Early morning collections of adult mosquitoes were conducted from October 2014 to April 2015 in 30 rooms (25 houses) at Akaka-Remo (population density = 5,585). The mosquitoes were identified with standard keys, but only *An. funestus* (F_0) was identified to sub-species level by Polymerase Chain Reaction (PCR). The F_0 was analysed for *Plasmodium* Infection Rate (PIR) by TaqMan real-time PCR, and screened for Knockdown resistance (*Kdr*) mutations using next-generation sequencing. The gene sequences generated were analysed with bioinformatics tools. The F_0 was further subjected to forced-egg laying technique to generate the F_1. The mortality rate of 2-5 day old F_1 exposed to permethrin- (0.75%), deltamethrin- (0.05%), DDT- (4%), dieldrin- (4%) and bendiocarb (0.1%)-treated papers was determined using WHO standard procedures. The contribution of detoxifying enzymes to IR was assessed by first exposing the F_1 to three standard synergists: Piperonyl Butoxide (PB:4%), S,S,S-Tributyl Phosphorotrithioate (STP:0.25%) and Diethyl Maleate (DM:8%); and to permethrin and DDT, respectively. Microarray and real-time PCR were used to identify differentially expressed genes in permethrin and DDT-resistant populations. The frequency of resistant and susceptible alleles in DDT (L119F-GSTe2) and dieldrin (A296S-RDL) resistance markers was determined in the F_0 and F_1 genotype using TaqMan real-time PCR. Data were analysed using descriptive statistics and Student's t-test at $\alpha_{0.05}$.

A total of 375 mosquitoes were collected and identified as *An. funestus* (83.8%),

An. gambiae (6.9%), *Culex* species (5.6%), *Aedes* species (1.3%) and *Mansonia* species (2.4%). All *Anopheles funestus* were further identified as *An. funestus sensu stricto*, with a PIR of 8.0%; and no *Kdr* mutations. Permethrin, deltamethrin, DDT, dieldrin, and bendiocarb induced 68.0, 87.0, 10.0, 8.0 and 84.0 % mortalities, respectively in the F_1, indicating resistance. Exposure of the F_1 to the three synergists with permethrin induced 100% mortality each; while PB, STP and DM with DDT, respectively induced 30.0, 81.8 and 71.4% mortalities. These suggest that detoxifying enzymes contributed to IR in the mosquitoes. Overexpressed genes in the resistant populations were *GSTe2*, *GSTd3*, *GSTd1-5*, *GSTU2*, *CYP6P2*, *CYP6P9a*, *CYP6P4a* and *CYP9K1*. The frequency of resistant alleles in L119F-GSTe2 and A296S-RDL was significantly high: F_0=77.0%, F_1=80.0% and F_0=76.0%, F_1=90.0%, respectively compared to the susceptible alleles: F_0=23.0%, F_1=20.0% and F_0=24.0%, F_1=10.0%, respectively.

Overexpression of detoxifying genes and the high frequency of resistant-associated mutations were responsible for multiple insecticide resistance in the *Anopheles funestus* at Akaka-Remo.

Keywords: Akaka-Remo, *Anopheles funestus*, Insecticide resistance, *Plasmodium* infection rate, Resistant alleles

Word count: 470

DEDICATION

I dedicate this work to Almighty GOD, for he gives man the power to do great things.

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CERTIFICATION

This is to certify that this work was carried out by Mr. Seun Michael ATOYEBI in Cell Biology and Genetics unit of the Department of Zoology, University of Ibadan, Nigeria

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WHO:	World Health Organisation
DDT:	Dichloro-Diphenyl-Trichloroethane
PCR:	Polymerase Chain Reaction
DDE:	Dichlorodiphenyldichloroethylene
CDC:	Center for Disease Control and Prevention
YG:	Your Genome
UE:	Understanding Evolution
UNICEF:	United Nations Children's Fund
F_0:	Parent generation
F_1:	First filial generation
NMCPS:	National Malaria Control Programs
FMOH:	Federal Ministry of Health
USAID:	United States Agency for International Development
NIAID:	National Institute of Allergy and Infectious Disease
NPC:	National Population Commission
ICF:	International Classification of Funtioning
MS:	Malaria Site
SIT:	Sterile Insect Technique
QPCR:	Quantitative polymerase Chain Reaction
Kdr:	Knockdown resistance
KD:	Knockdown
RT-PCR:	Real-Time Polymerase Chain Reaction
IRS:	Indoor residual Spraying
LLINs:	Long Lasting Insecticide-treated Nets
DEET:	N,N-Diethyl-meta-toluamide
$C0_2$:	Carbon iv oxide
ATSDR:	Agency for Toxic Substances and Disease Registry
PB:	Piperonyl Butoxide
ACh:	Acetylcholine

AChE:	Acetylcholine enzyme
Ch:	Chlorine
RDL:	Resistance to dieldrin
HITSS:	High through-put Screening System
WHOPES:	World Health Organisation Pesticide Evaluation Scheme
ERB:	Excito-repellency box
GSTs:	Glatathione-S-Transferases
CYP:	Cytochrome P 450
GSH:	Reduced glutathione-S-Transferases
VGSC:	Voltage-gated Soddium channel
GABA:	Gamma aminobytyric acid
EIR:	Entomological inoculation rate
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
UV:	ultraviolet/universal
cDNA:	Complementary Deoxyribonucleic acid
C_T:	Threshold cycle
Tm:	melting temperature
PNA:	Peptide nucleic acids
NGS;	Next-generation sequencing
GPS:	Global positioning system
FGD:	Focus group discussion
IITA:	International Institute of Tropical Agriculture
LSTM:	Liverpool School of Tropical Medicine
EST:	Expressed Sequence Tags
NAFDAC:	National Agency for Food and Drug Administration and Control
DM:	Diethyl maleate
STP:	S,S,S-tributyl phosphorotrithioate
Bp:	Base pair
Kb:	Kilo base pair
USA	United States of America

CHAPTER ONE
INTRODUCTION

Malaria is the most prominent human infectious disease in Africa. It is widespread throughout the sub-Sahara Africa and considered to be a major public health challenge in Nigeria (CDC, 2015; WHO 2016a, 2017a). This disease has claimed the lives of millions since its emergence (Arrow *et al.*, 2004; WHO, 2017a). Remarkbly, between 2010 and 2015, malaria incidence among populations at risk (the rate of new cases) fell by 21 % globally. In the same period, malaria mortality rates among the same population group reduced by 29 % globally among all age groups and by 35 % among children under 5, which is good enough to avert an estimated 6.8 million deaths globally since 2001(WHO, 2017a).

Unfortunately, Africa regions still retain high portion of the global malaria burden with 90 % of malaria cases and 92 % of malaria deaths (WHO, 2017a). Presently in Nigeria, malaria is responsible for about 300,000 deaths annually, and has kept about 97 % of the national population at risk (CDC, 2016). Furthermore, malaria disease recently contributed to around 11 % maternal and 25 % infant mortalities in the country (WHO, 2016b).

This disease is developed when *Anopheles* mosquitoes successfully transmit the protozoan parasite of the genus *Plasmodium* (*Plasmodium falciparum, P. ovale, P. vivax* and *P. malariae*) to humans through bites. The most severe of the human protozoan parasites, *P. falciparum* is responsible for almost 90 % of all malaria cases (Owusu *et al.*, 2017; WHO, 2017a). Malaria transmission varies in different geographical location depending on suitable climatic conditions that affect the number and survival of mosquitoes, parasite predominance in the environment, density of *Plasmodium*-transmitting mosquitoes, resting (endophilic or exophilic) and feeding (anthropophilic or zoophilic) behaviours of *Anopheles* mosquitoes as well as low human immunity to infections (WHO, 2013a, 2015a).

There are over 400 different species of *Anopheles* mosquitoes, out of which 30 are malaria vectors of major importance (WHO, 2017a). In Nigeria, there are 45 species of *Anopheles* mosquitoes but only few of them: *Anopheles gambiae sensu stricto*, *An. couluzzi*, *An. arabiensis* and *An. funestus s.s* are involved in malaria transmission in humans (Awolola *et al.*, 2005a; Oyewole and Awolola, 2006; Samdi, 2012; Djouaka *et al.*, 2016).

An. gambiae complex are known malaria vectors in Nigeria. Some key members of the complex (*An. gambiae s.s.* and *An. arabiensis*) are widely spread and are involved in malaria transmission in the southwest region of the country (Awolola *et al.*, 2005a; Okwa *et al.*, 2007; Noutcha and Anumudu, 2010; Oyewole *et al.*, 2010; Okorie *et al.*, 2011). However, *An. funestus*, which is the other malaria vector in Nigeria, has been understudied, due to difficulties to locate suitable breeding habitat (Dia and Guelbeogo, 2013). There are few studies on its density and capacity to transmit *Plasmodium* parasites (Awolola *et al.*,2005b; Oyewole *et al.*, 2005; Oyewole and Awolola, 2006; Oduola *et al.*, 2012).

Awolola *et al.* (2005b) and Oduola *et al.* (2012) have reported the presence of *An. funestus* in Oyo state. Oyewole *et al.* (2005) also revealed that this mosquito species is infected with *Plasmodium* parasite and could be involved in malaria transmission at Akaka-Remo, Ogun state. Beyond Nigeria, there have been other reports that *An. funestus* could even be more involved in malaria transmission than *An. gambiae* in South Africa (Coetzee and Fontenille, 2004; Cohuet *et al.*, 2004).

Overall, malaria transmission in Nigeria has been attributed mainly to *An. gambiae s.s.* and *An. funestus s.s.* with consistent *Plasmodium* infection rates of 1.0–4.5 % (*An. funestus*) and 3.0–8.1 % (*An. gambiae*) in Ogun, Oyo and Lagos states mosquito populations (Awolola *et al.*, 2005b; Oyewole *et al.*, 2005; Oyewole and Awolola, 2006; Oduola *et al.*, 2012). Moreover, there was also another case of a high sporozoite infection rate of 25 % in an *An. funestus* population from Lagos state (Okwa *et al.*, 2007). The use of malaria vector control interventions to fight malaria disease has been quite effective, helping to reduce global malaria mortality by 26 % in the last one and half decades (UNICEF, 2013). The use of chemical insecticides, either through the use of Long Lasting Treated Nets (LLTNs) or Insecticide Residual Sprays (IRS) has been the master stroke of this strategy (CDC, 2016; WHO, 2016b).

There are four public health insecticide classes approved by WHO for mosquito control, they include Pyrethroids, Organochlorines, Carbamates and Organophosphates (Ops) (WHO, 2016c). From these insecticide classes, malaria control in Nigeria depends only on pyrethroids for LLTNs, where the bulk of IRS in markets are pyrethroid-based (WHO, 2016b). The two main types of pyrethroids used for malaria control are the type 1 (permethrin) and the type 2 (deltamethrin) (WHO, 2013b). The main difference between these two groups is the presence of cyano- group in the type 2 pyrethroids, which makes them more effective to kill exposed organsims than type 1 pyrethroids. As an alternative to pyrethroids, carbamate-based insecticides were later introduced for mosquito control in West Africa (Akogbeto *et al.*, 2010). In addition, DDT-based insecticides were also re-introduced for indoor use in Africa due to its high efficacy (WHO, 2006, 2011; Brooke *et al.*, 2013). Till date, DDT-based insecticides are preferred in southern Africa, and if applied appropriately in other regions, it could help to fast track malaria elimination in Africa.

The main challenge with the use of insecticides for malaria vector control is the resistance developed by *Anopheles* mosquitoes against commonly used insecticides. In Nigeria, there are reports that *An. gambiae s.s* have developed resistance to insecticides, notably against pyrethroids (Djouaka *et al.*, 2008), organochlorines, (Oduola *et al.*, 2010) and carbamates (Ibrahim *et al.*, 2013). However, little is known so far on the insecticide susceptibility pattern of *An. funestus s.s.* in Nigeria.

Generally, *An. funestus* prefers to breed in calm water bodies surrounded with different vegetation. Due the difficulties of getting *An. funestus* larvae in the wild, Morgan *et al.* (2010) developed a tool that is directed towards targeting adult mosquitoes resting indoors rather than focusing only on larvae collections. After collection, gravid mosquitoes will be forced to lay eggs (Cuamba *et al.*, 2010 and Morgan *et al.*, 2010), so as to generate the first filial generation, F_1 mosquitoes for WHO susceptibility tests (WHO, 2016c). This technique has assisted studies on *An. funestus* in Africa.

In recent years, *An. funestus s.s.* populations have increasingly been reported to have developed resistance to insecticides in countries like Uganda in East Africa (Mulamba *et al.*, 2014); Mozambique, Zambia, Zimbabwe and Malawi in southern Africa (Hargreaves *et al.*, 2000; Casimiro *et al.*, 2006a; Amenya *et al.*, 2008; Samb *et al.*,

2012; Vezenegho *et al.,* 2013; Choi *et al.,* 2014; Riveron *et al.,* 2015). Also, Cameroon in Central Africa (Wondji *et al.,* 2011; Menze *et al.,* 2016) and some West African countries including Benin (Djouaka *et al.,* 2011, 2016), Ghana (Okoye *et al.,* 2008) and Burkina-Faso (Dabire *et al.,* 2007).

The pattern of resistance against these insecticides varies across different regions. For example, *An. funestus* was resistant to pyrethroids and carbamate but fully susceptible to organochlorines (DDT and dieldrin) in southern Africa (Cuamba *et al.,* 2010; Wondji *et al.,* 2011). However, a more recent study in Malawi showed that this mosquito species has now began to develop resistance to organochlorines (Riveron *et al.,* 2015). In East Africa, *An. funestus* is resistant to pyrethroids and DDT but remains susceptible to carbamates in Uganda and western Kenya (Mulamba *et al.,* 2014). High resistance was recorded with dieldrin in Cameroon (Central Africa) (Wondji *et al.,* 2011). In Benin, pyrethroids and DDT resistance was first reported in 2011 (Djouaka *et al.,* 2011) at the coast region (Pahou) of the country. It was later shown to have extended to the inland region, as resistance was recorded to all insecticide classes apart from organophosphates in Kpome (Djouaka *et al.,* 2016).

Understanding the mechanisms used by *An. funestus* to survive lethal doses of insecticides is very important to manage existing resistance, and could also help to improve on future malaria control tools. The pertinent information about this species is that each mosquito population displays its peculiar mechanisms to withstand insecticides exposure (Mulamba *et al.,* 2014; Riveron *et al.,* 2015, 2017; Samb *et al.,* 2016). Nevertheless, populations within the same region could share similar resistance mechanisms, which might be a consequence of gene flow across these regions (Mulamba *et al.,* 2014).

So far, metabolic resistance mechanisms have been the main driver of insecticide resistance in *An. funestus* across Africa (Mulamba *et al.,* 2014; Riveron *et al.,* 2014a). Cytochrome P450 genes (P450s) and Glutathione-S-transferase (GSTs) are the two main gene Classes associated with resistance in this mosquito species. The P450s such as *CYP6P9a, CYP6P9b, CYP6M7, CYP9K1* and *CYP6Z1* have played various roles in pyrethroids resistance (Riveron *et al.,* 2015; Ibrahim *et al.,* 2016). It is also evident that as more P450 genes such *CYP6P4a*, *CYP6P4b*, *CYP6K1* keep evolving in resistant mosquitoes, resistance gets stronger (Ibrahim *et al.,* 2016; Samb *et al.,*2016).

Without any doubt, cytochrome P450s, which are classified under phase I detoxification genes have been heavily involved in pyrethroid resistance and also in cross-resistance with carbamates in southern African (Ibrahim *et al.*, 2016) as previously reported for *An. gambiae* (Edi *et al.*, 2014).

The GSTs, which are phase II detoxification gene family, have been more involved to drive both permethrin and DDT resistance. The GST gene that has been mostly associated with resistance is the epsilon class, especially the *GSTe2* gene. They have shown association either through overexpression in permethrin and DDT-resistant mosquitoes (Riveron *et al.*, 2014a; Samb *et al.*, 2016) or mutation on the *GSTe2* gene resulting to a *GSTe2* protein with greater DDT assess and metabolism (Riveron *et al.*, 2014a). Riveron *et al.* (2014) further highlighted that *An. funestus* mosquito carrying the mutant form of *GSTe2* produce more *GSTe2* proteins when exposed to pyrethroids or DDT, and as a result develop higher resistance. This point mutation on position 119 of the *GSTe2* gene, changing Leucine (L) to phenylalanine (F) (L119F) is rapidly developing in *An. funestus* populations (Riveron *et al.*, 2014a; Djouaka *et al.*, 2016).

Generally, a preliminary study using synergist test, where mosquitoes are first exposed to synergists before insecticides have provided key information on possible enzymes that could be expressed in response to insecticide exposure in resistant mosquitoes. The use of piperonyl butoxide (PB), S,S,S-tributyl phosphorotrithioate (STP) or Diethyl maleate (DM) to implicate oxidase, esterase and GSTs, respectively in insecticide resistance has assisted resistance mechanisms studies (Mulamba *et al.*, 2014; Djouaka *et al.*, 2016). Also, genome wide transcriptional and reverse transcriptase analyses have helped to identify set of detoxifying genes that are associated with insecticide resistance in *An. funestus* mosquito species (Ibrahim *et al.*, 2016; Menze *et al.*, 2016).

Till date, no target-site mechanism through L1014F-kdr gene mutation (main driver of insecticide resistance in *An. gambiae*) has been implicated in the resistance of *An. funestus* species (Djouaka *et al.*, 2011; Mulamba *et al.*, 2014; Menze *et al.*, 2016; Samb *et al.*, 2016). Also, there has not been any association between G119S and F455W mutations on the *Ace-1* gene (target-site of carbamates and Ops insecticides) and carbamate resistance in this mosquito species (Mulamba *et al.*, 2014). However, there was a recent discovery of a new *Ace-1* mutation (N485I) in carbamate resistant-

An. funestus populations from southern African (Ibrahim *et al.*, 2016). Also, the presence of the A296S-RDL mutation on the GABA receptor gene (target site of dieldrin insecticide) is common to dieldrin resistance in *An. funestus* (Wondji *et al.*, 2011). These are evidences that target-site resistance mechanism contributes to insecticide resistance profiles *An. funestus*.

Southwest region of Nigeria has several vegetation, suitable climatic conditions for human and animal survival (Chinago-Budnuka *et al.*, 2015). As a consequence, these conditions facilitate the breeding of insects like mosquitoes thereby increasing the intensity of malaria burden. (FMOH, 2008; WHO, 2016b). With malaria morbidity growing with higher human population at risk of malaria disease by day, there is a need to monitor the growing rate of *Plasmodium* infection in *An. funestus*.

Akaka-Remo, a rural community/Agricultural settlement in Remo-North local government area of Ogun state, Nigeria is enriched with vegetation such as bananas, vegetables, maize, shrubs, trees and crops. The vegetation borders the community along the surrounding water bodies, where several domestic and agriculture activities are constantly carried out. These environmental components make it possible for *An. funestus* to breed at Akaka-Remo (Oyewole *et al.*, 2005). Oyewole *et al.* (2005) did not only report the presence of *An. funestus* but also its potential capacity to transmit malaria parasite at Akaka-Remo. It is important to strictly follow up this study considering the high incidence of malaria cases in Ogun state (Olasehinde *et al.*, 2015). It is also vital to know the *Anopheles* mosquitoes that are responsible for malaria transmission in this community, considering the fact that over 30 % of human population visits the community health facility due to malaria annaully. High malaria incidence recorded in this region thereby calls for adequate monitoring of existing malaria vectors.

Hypothesis of the study

The hypothesis of this study is that *An. funestus*:

a. contribute to malaria transmission at Akaka-Remo

b. have developed resistance to public health insecticides commonly used to control malaria vectors

c. have developed several mechanisms to survive lethal doses of insecticides

Aim

The aim of this study is to investigate the *Plasmodium*-infection rate and molecular mechanisms of insecticide resistance in *Anopheles funestus* at Akaka-Remo, SouthWest Nigeria**.**

Objectives

The specific objectives of the study are to:

1. assess the *Plasmodium* infection rate of *An. funestus*
2. evaluate the susceptibility of *An. funestus* to public health insecticides
3. investigate the involvement of target-site mutations in insecticide resistance of *Anopheles funestus*
4. determine the role of detoxifying enzymes in insecticide resistance of *Anopheles funestus*

Significance of the study

Due to the contribution of malaria disease to human mortality, it is important to assess the efficacy of insecticides commonly used to control its vectors. In addition, mechanisms developed to withstand lethal doses of common insecticides in mosquitoes should be understood. The findings of this work will help the National Malaria Control Programmes (NMCPs) to know the present status of existing malaria control interventions in terms of effectiveness and efficiency, understand the pattern of resistance in *An. funestus* mosquitoes as well as guiding them to maintain existing and develop new tools for efficient and effective malaria control in Nigeria.

CHAPTER TWO
LITERATURE REVIEW

2.1 Malaria Disease

Malaria is an ancient and one of the most significant vector borne disease in the world. It has threatened the lives of more than one-third of the world population since its outburst in the 1880s and still thrives in the tropical Regions of Asia, Africa, Central and South America (WHO, 2016d). It has been a major cause of morbidity and mortality in Africa (WHO, 2016b). Its operation and spread in Africa is influenced by factors such as the presence of numerous *Anopheles* mosquito breeding sites leading to high mosquitoes density, mosquitoes life span, favorable climatic conditions, geographical locations, *Plasmodium* parasite predominance, human life styles and low immunity to infections in human (WHO, 2013b, 2015a).

2.2 Malaria Disease in Nigeria

Nigeria suffers the world's greatest malaria burden (approximately 30% of the total malaria burden in Africa), with approximately 51 million cases and 207,000 deaths reported annually; while 97% of the total population (approximately 173 million) is at risk of the infection (WHO, 2014). Malaria disease is responsible for 60% of patients that visits the health facilities, 30% childhood deaths, 25% of deaths in children less than one year and 11% maternal death (FMOH, 2008; WHO, 2014). This disease affects the country's economic productivity resulting in an estimated monetary loss of approximately 132 billion Naira (~700 million USD) for treatment, prevention and other direct costs (WHO, 2012).

National Malaria Control Programmes (NMCP) in Nigeria has strategically planned to reduce malaria burden by 50%. The target was to increase the coverage of LLINs by 80% and supplying 20% of houses in targeted areas with IRS, as well as treatment with two doses of intermittent preventive therapy (IPT) for 100 % of pregnant women who visit clinics for antenatal care (NPC and ICF, 2013). However, the report of malaria incidence per human polulation in Nigeria has been similar year in year out

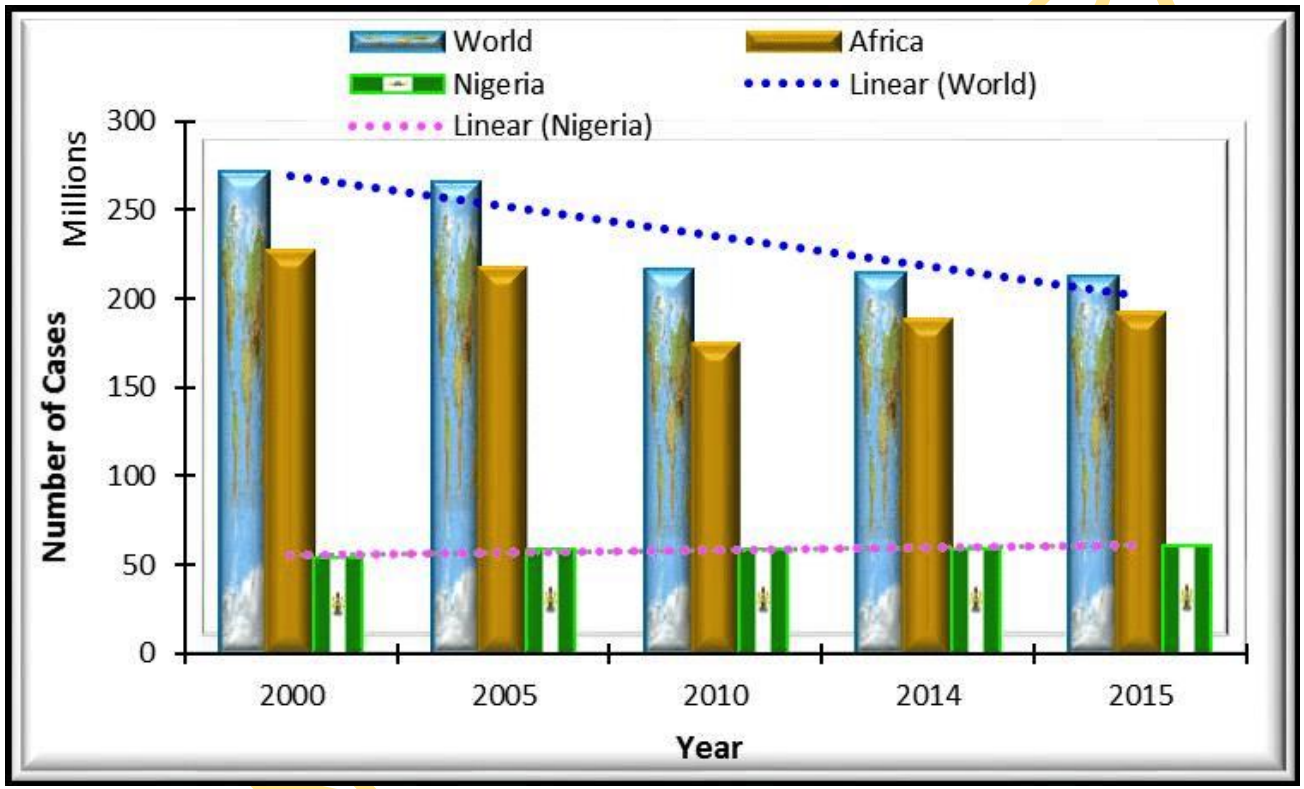

Figure 2.1: Report of malaria cases in Nigeria between 2000 and 2015.

Source: Adepoju and Akpan (2017).

since 2013 (Figure 2.1) and malaria reductions has not been heavily felt due to increase in human population (Adepoju and Akpan, 2017).

2.3 Human Malaria Parasite

Malaria is caused by a single-celled parasite from the genus *Plasmodium*. There are over a hundred species of *Plasmodium* that have the potential to give rise to malaria in animals such as birds, reptiles and mammals including humans (Dechamps *et al.*, 2010; CDC, 2016). Five species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) commonly infect humans worldwide with each possessing a distinctive appearance under microscope and produce different types of symptoms (NIAID, 2007) Their distributions differ across different geographical locations (CDC, 2016). All human malaria parasites except *P. knowlesi* are found in Africa. (CDC, 2016). *Plasmodium knowlesi* is found only in the South-East Asia region, and is well known to cause severe malaria in humans and macaque (Vythilingam and Hii, 2013; Ahmed and Cox-Singh, 2015; Millar and Cox-Singh, 2015; Brock *et al.*, 2016).

Plasmodium falciparum is the most pravelent of them all whereas the other three parasites are rarely found in tropical Regions of Africa. Most screening carried out on patients in Nigerian hospitals showed the presence of *P. falciparum*, thereby revealing the level of its prevalence in the Nigerian environment (Agboola *et al.*, 2010; Olasehinde *et al.*, 2010; Adeyemo *et al.*, 2013; Kalu *et al.*, 2012; Peletiri, 2013; Udeze *et al.*, 2013; Zama *et al.*, 2013). *Plasmodium falciparum* can multiply rapidly in the blood, thus causing severe blood loss and clogging of small blood vessels (CDC, 2016).

Unlike the *P. falciparum*, infection with *P. vivax* is considered to be very rare in Nigeria but prominent in the Central Africa (WHO, 2018). The reason for this in the Nigerian populace, just like other West Africa countries is because of the absence of Duffy blood group antigens on the erythrocytes surface (Peiper *et al.*, 1995). Recently, the perception of the Duffy-negativity protecting effect against *P. vivax* infection in West Africa has been challeneged. *Plasmodium vivax* infection was detected in the blood samples of some Beninese population in West Africa, so this perception should be reviewed to ascertain the present burden of *P. vivax* in Africa (Poirier *et al.*, 2016).

Plasmodium ovale malaria is endemic to tropical Western Africa. It is relatively unusual outside of Africa and comprises of less than 1% of isolates where found. Although, the *P. ovale* parasite is less severe and rare compared to *P. faciparum,* one peculiar feature of this species just like *P. vivax* is that it can enter a dormant stage for a long time in the liver before invading the red blood cells, causing a relapse. This stage of the parasite called hypnozoites can cause unpleasant damages in severe cases (CDC, 2016). Generally, *P. malariae* seems to pose less problems but could cause serious complications if untreated, leading to long-lasting chronic infections and sometimes can result to nephritic syndrome (CDC, 2016).

2.3.1 Life Cycle of Malaria Parasite

Humans are only exposed to malaria parasites through *Anopheles* mosquito bites and the successful transmission of these parasites to humans (secondary host) requires a series of complex developmental transformation inside the mosquito vector (primary host) (Lefevre *et al.,* 2013). Generally, the life cycle of the human malaria parasite involves two hosts: human and female *Anopheles* mosquitoes. These steps begin when mosquito ingests *Plasmodium* infectious blood meal from infected individual, which initiate the sporogonic cycle (parasite multiplication in the mosquito). *Plasmodium* male and female gametes fuse to form zygotes within the mosquito midgut (Figure 2.2). Each zygote then develops into motile ookinetes that can penetrate the walls of the mosquito to form oocysts. Oocysts now undergo several mitotic divisions that lead into hundreds of sporozoites, which are released into the haemoceol (8 to 22-day post infection depending on the *Plasmodium* species). At this stage, the parasites migrate to the salivary glands and during subsequent blood meal, saliva-containing sporozoite is inoculated into the human host.

In humans, injected sporozoites invade and infect the liver cells and mature into schizonts, which rupture before they are released to form merozoites. After replication in the liver, merozoites later invade red blood cells; at this point, clinical manifestations of malaria disease are always noticed (Figure 2.2). The *P. vivax* and *P. ovale* may later develop into hypnozoites, which are dormant stages in liver. These stages can be reactivated in weeks, months, or many years after the initial infection before invading the blood stream, causing a disease relapse. Some parasites further differentiate into the sexual erythrocytic stages (gametocytes: the male

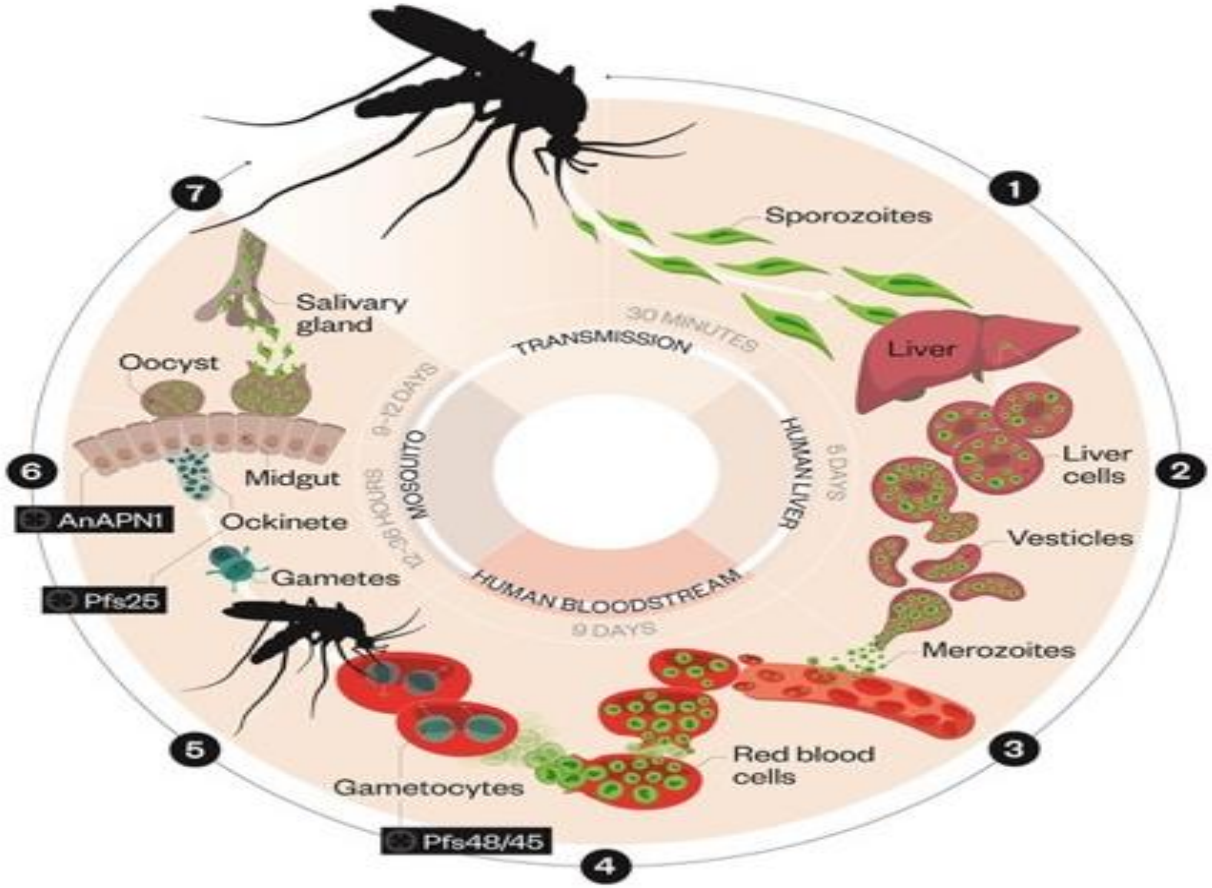

Figure 2.2 Life cycle of the malaria parasite

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microgametocytes and female macrogametocytes), which are ingested by the *Anopheles* mosquito during blood meal (CDC, 2016)

2.4 Malaria Vectors

Mosquitoes are important vectors of many diseases including malaria, dengue and yellow fever (WHO, 2017a). About 430 *Anopheles* mosquito species exist worldwide, where only around 60 to 70 are competent to transmit malaria parasites out of which only 40 are of medical importance (Stevenson and Norris, 2017).

This implies that these mosquito species support the completion of *Plasmodium* parasite developmental stages, from the gametes fusion and oocysts formation to the invasion of salivary glands and sporozoite transmission. overall, vector competence to transmit malaria parasite is actually a combined estimation of parasite infectivity and vector susceptibility. Thus, it encompasses both the host resistance mechanisms that are used to fight infections and parasite infective mechanisms used to overcome the host defenses.

2.4.1 Major Malaria Vectors in Nigeria

Anopheline malaria vectors are available in almost every region worldwide (Kiszewski *et al.*, 2004). However, most malaria cases in the world are now found in Sub-Saharan Africa (WHO, 2017b), where three main African malaria vectors co-exist (Oluwasogo and Adeyemi, 2016). This is also the case in Nigeria, where *An. gambiae* Giles 1902, *An. arabiensis* Patton 1905 and *An. funestus* are responsible for most of the malaria cases (Okwa *et al.*, 2007; Noutcha and Anumudu, 2010; Oyewole *et al.*, 2010; Okorie *et al.*, 2011). The predominance and highly anthropophilic behaviour of these vectors make them efficient malaria vectors of medical significance (Ebenezer *et al.*, 2012; Oluwasogo and Adeyemi, 2016).

Secondary vectors such as *An. rivulorum*, *An. parensis* and *An. ziemanni* also play important roles in certain ecological niches (Afrane *et al.*, 2016). Many of the Anopheline vectors of malaria parasites belong to the cryptic species complexes. By definition, cryptic species are reproductively isolated from each other but are difficul to distinguish morphologically from one other. *Anopheles* species complexes typically include both vector and non-vector species, with two or more members of the complex/group often being found to exist in sympatry (Vijay *et al.*, 2011).

2.4.1.1 *Anopheles gambiae* Complex

Anopheles gambiae complex was first noticed by Davidson in 1964 (Davidson, 1964) and the names: *An. gambiae* Giles, 1902 and *An. arabiensis* Patton, 1905 were assigned to the two most abundant members of the complex (White, 1970). *Anopheles gambiae sensu lato* was later described to consist at least seven species namely *An. gambiae sensu stricto*, *An. arabiensis*, *An. melas*, *An. merus*, *An. bwambae*, *An. quadriannulatus* and *An. quadriannulatus* species B (Coluzzi *et al.*, 1979). Members of this complex can only be distinguished only by a species-specific molecular technique. Molecular technique such as the restriction fragment length polymorphism (RFLP) (Collins *et al.*, 1987), the multiplex Polymerase Chain Reaction (PCR) (Scott *et al.*, 1993) or simple PCR (Cohuet *et al.*, 2003) have been very successful.

An. gambiae species complex are native to Nigeria and are predominant malaria vectors in the southwest region of the country (Ebenezer *et al.*, 2014; Lamidi *et al.*, 2017). *Anopheles gambiae s.s.* and *An. arabiensis* have the most widespread distribution, and most times live in sympatry in these localities (Ebenezer *et al.*, 2012; Oluwasogo and Adeyemi, 2016). Generally, *An. gambiae s.s.* is known to favour areas with high nocturnal humidity and *An. arabiensis* thrives better mostly in arid savannas and steppes regions (White, 1970). However, the status of this species as a main malaria vector in Nigeria is due to its susceptibility to *Plasmodium* parasites invasion (Okwa *et al.*, 2007).

2.4.1.2 *Anopheles funestus* Group

Anopheles funestus is less prevalent in Nigeria compared to *An. gambiae* (Awolola *et al.*, 2005b; Oyewole *et al.*, 2005; Oduola *et al.*, 2012). Their larvae can only be found in water sources such as swamps, rice fields, edges of streams or rivers as well as in puddles and water filled hollows such as hoof prints (Minakawa *et al.*, 2008). This species thrives so well in aquatic habitats covered with vegetation with little or no disturbance on water bodies. There are nine members of the *An. funestus* group that exist in Africa; they are *An. parensis Gillies*, *An. aruni Sobti*, *An. confusus Evans and Leeson*, *An. funestus s.s.*, *An. vaneedeni Gillies and Coetzee*, *An. rivulorum Leeson*, *An. fusciveno- sus Leeson*, *An. leesoni Evans*, and *An. brucei Service* (Gillies and Coetzee, 1987). Members of this group are difficult to distinguish morphologically, however, with molecular tools such as the use of PCR, sub-species identification

becomes easy and accurate (Koekemoer *et al.*, 2002). Apart from their morphological similarities, siblings of the *An. funestus* mosquitoes always differ in their biology and vectorial competence (Oyewole *et al.*, 2005; Djouaka *et al.*, 2016).

Anopheles funestus s.s is the most widely spread member of the *An. funestus* group, with *An. lesson* and *An. rivulorum* having moderate/low distribution (Awolola *et al.*, 2005b; Oduola *et al.*, 2012). The reason *An. funestus s.s.* is so important in malaria transmission is its anthropophagic (prefers to feed on human blood) and endophilic (prefers to rest indoors after taking a blood meal) behaviours (Oluwasogo and Adeyemi, 2016). On the other hand, other members of *An. funestus* group prefer to rest outdoor (exophilic) and feed on animals (Zoophagic) (Kweka *et al.*, 2013).

An. rivulorum have earlier been shown to have preference for human blood (Awolola *et al.*, 2005b), *An. vaneedeni* prefers to feed outdoors and have been found to have the potential to carry *P. falciparum* under laboratory conditions. However, its role in malaria transmission in natural settings has not been established (De Meillon *et al.*, 1977). Kamau *et al.* (2003) reported that *An. parensis* resting indoors has a little affection for human blood and lacked the capacity to be infected with circumsporozoite protein in Kenya. Whereas, analysis of another *An. parensis* population showed that they are infected with sporozoites (Hargreaves *et al.*, 2000).

The period of the year when this mosquito species is involved in malaria transmission in Nigeria is still not very clear. However, there is a feeling that *An. funestus* compensates for *An. gambiae* during dry season, when the density of *An. gambiae* is very low. In Tanzania and Burkina Faso, the density of *An. funestus* population is always high at the end of the rainy season (Costantini *et al.*, 1999; Dabire *et al.*, 2007). In this region, *An. funestus* might be assisting the transmission of malaria and extending it to the dry season. *An. rivulorum* is an important malaria vector in Tanzania (Wilkes *et al.*, 1996) and Kenya (Kawada *et al.*, 2012). In sub Saharan Africa, *An. funestus s.s.* co-habit with other *An. funestus* sub-species and most of the time overlap with *An. rivulorum*, *An. leesoni* and *An. parensis* (Kamau *et al.*, 2003; Awolola *et al.*, 2005b; Temu *et al.*, 2007).

2.5 Factors Affecting Malaria Transmission

Malaria disease is controlled by local factors affecting its transmission, which is perculiar to different environments. Generally, mosquitoes in their habitats are constantly challenged with various biotic and abiotic pressures including resource limitation, competition, predation, climatic factors, tree canopy, flora fauna, high organic content in breeding pools and pesticides use, which affect their reproduction and survival (Lefevre *et al.*, 2013). Some vectors prefer considerable clean water for breeding, which is most time very easy to come by in rural areas (De Silva and Marshall, 2012). Other factors include distance of residential houses to the nearby forests/bushes, impact of deforestation and reforestation, effect of forest/bushes on microclimate, vector bionomics, *Plasmodium* species survival and human activities in forests (Kar *et al.*, 2014).

Often times, irrigation systems on farms unknowingly help to create more artificial mosquito breeding sites (Philbert *et al.*, 2014). Mosquito competence to transmit *Plasmodium* parasites varies with these factors and some other factors such as diet and infection history. However, the underlying mechanisms of these factors on the vectorial competence of *Anopheles* mosquitoes are complex and remain indefinable (Lefevre *et al.*, 2013).
Some common factors are as follows:

2.5.1 Climate

Climate is a determining factor in the spacial and temporal distribution of vectors and pathogens (Dhimal *et al.*, 2015). A change in climate affects the geographical range, seasons and the incidence rate of transmissible diseases (Ayanlade *et al.*, 2013). In a real sense, climate change leads to latitudinal and altitudinal temperature increases (Afrane *et al.*, 2012). Such a temperature increase alters the biology and ecology of many mosquito vectors and subsequently the dynamics of the diseases they transmit. Moreover, changes in malaria incidence rate and other related diseases with mosquito vectors have been associated with the patterns of rainfall, temperature and humidity (Kar *et al.*, 2014).

2.5.2 Temperature

The overall traits of mosquitoes including larval development, adult survival and immune response strongly depend on the surrounding temperature (Lyons *et al.*, 2013). This might be due to the ectothermic nature of insects and because mosquito whole body system is always sensitive to temperature as do all invading pathogens like the malaria-borne pathogens (Murdock *et al.*, 2012).

Studies have shown that the permissive temperature for *Plasmodium* sporogonic development is between 18 and 32 ^{o}C (Ayanlade *et al.*, 2013; Simon-Oke and Olofintoye, 2015), which suggest that malaria transmission within this temperature ranges is permitted. Temperature below 18 °C decreases parasite development significantly, while temperature beyond 32 °C compromises the survival of the mosquitoes. In rare cases, temperature around 15 °C and up to 40 °C with a humidity between 55 and 80% could be successful for the completion of the *P. falciparum* and *P. vivax* life cycle (Zhou *et al.*, 2004).

Normally, *Plasmodium* parasites should develop faster at higher temperatures (Corbyn, 2011), which should portray an intense malaria transmission. However, experiments conducted within the permissive temperature range showed that as mosquitoes became more infectious with the rise in temperature, their vectorial competence reduces (Okech *et al.*, 2004). These might be showing that malaria transmission may fall rather than rise at higher temperatures. Nevertheless, these results should be interpreted and explained with caution because mosquitoes do not experience constant temperature in nature. For example, the sporogonic cycle takes about 9-10 days at a temperature of 28 °C (Craig *et al.*, 1999; Beck-Johnson *et al.*, 2013). The gonotrophic cycle of a mosquito, which is the period between the blood feeding, digestion of the blood meal until oviposition or egg laying (Costa *et al.*, 2010) is shorter at higher temperatures because of the increase in the digestion speed (Haque *et al.*, 2010). Therefore, higher environmental temperatures always result in more frequent vector-host contact. In highlands, transmission is unstable due to fluctuations in temperatures that are normally low (Lefevre *et al.*, 2013).

The developmental rate of immature *Anopheles* mosquitoes is highly dependent on temperature. Low temperature conditions could result in severe delays in larval

development and can also result in high larvae mortality (Stanaway and Mayer, 2011). When temperature is at or below 14 °C, larval development of *An. gambiae* usually stops, resulting into larvae mortality (Koenraadt *et al.*, 2006). Also, when water temperature rises beyond normal, the larvae take a shorter time to mature (Munga *et al.*, 2007) and consequently, there is a greater capacity to produce more offspring. Overall, temperature is considered as a focal factor because it does not only affect the duration of the sporogonic cycle of the *Plasmodium* parasite in the *Anopheles* vector, it also affects the duration of aquatic stages, their survival and feeding frequency of adult female (Stanaway and Mayer, 2011).

2.5.3 Rainfall and Humidity

Rainfall is another important climate change-influenced factor that impact malaria cases in Africa (Abeku, 2007). It prepares breeding sites for mosquito to lay their eggs and ensures a suitable relative humidity of at least 80% to prolong mosquito survival (Walker *et al.*, 2013). Relative humidity below 60% has been linked with shortened life span in mosquito vectors (Rogers and Randolph, 2006). There are situations where the ratio of rainfall over precipitation/potential evapotranspiration were the main driving force for increasing *An. gambiae* and *An. arabiensis* population in the environment (Koenraadt *et al.*, 2004). Furthermore, rainfall has played a role in the rapid increase of *Anopheles* mosquito vectors towards the end of the dry season and maximally after onset of rains when humidity increases (Jawara *et al.*, 2008). During rainfall, trees in the forests add moisture to the air by transpiration and help to lower temperature, thus increasing precipitation, leading to increase in mosquito population.

Rainfall and humidity spring up the gonotrophic cycle and in the process influence malaria transmission. A decrease in the duration of the gonotrophic cycle leads to an increase in human biting frequency from an average of one in every 5 days to one every 3 days (Afrane *et al.*, 2012; Paaijmans *et al.*, 2013). An increase in the biting frequency means that malaria vectors will feed more frequently on humans and might enhance malaria transmission. Another clear observation is that a rise in rainfall could influence oocyst and sporozoite development by one and one and a half days respectively (Afrane *et al.*, 2012).

Some of the microclimatic factors such as deforestation have the potential to influence

rainfall and humidity leading to increase or decrease in the density of malaria vectors (Afrane *et al.,* 2012). For example, *An. gambiae* mosquito prefers areas with high humidity, which can be achieved by deforestation. Deforestation can lead to a decrease in indoor humidity, thereby decreasing the median survival of *An. gambiae*. However, despite the decreased survival of mosquitoes due to the effects of deforestation, mosquitoes still exhibit an enhanced reproductive fitness by 40 % over the period of their life span (Afrane *et al.,* 2006). The implication of these is that *An. gambiae* could increase its population within a short time when breeding sites are available, which potentially leads to an increase in malaria transmission when infected humans are available.

2.5.4 Vegetation

Vegetation near human habitation increases the population of malaria vectors and thus increases malaria transmission within humans living in the environment. Villages with more broadleaf forests and wetland vegetation are always known to have higher malaria rates due to the effective density of *Anopheles* mosquitoes (Haque *et al.,* 2010, 2011). Some malaria vectors like *An. funestus* prefers to breed in water bodies that are not disturbed and thrive more when there are tree canopy coverages, which serve as shelters for mosquitoes (Afrane *et al.,* 2006; Dia and Guelbeogo, 2013).

Forest floras with sugar availability have also been shown to be crucial determinants for vectorial capacity of mosquitoes. The availability of plant sugar increased egg numbers and survival potential of *An. gambiae*, which influenced their tramission potential (Gary and Foster, 2001; Manda *et al.,* 2007). In addition, leaves falling into larval habitats provide sustainable micro-climatic conditions and food for larvae, which also favor vectors and pathogens activities (Obsomer *et al.,* 2007).

2.5.5 Diet

The availability and quality of food is an important factor for insect host immunity and infection (Cory and Hoover, 2006). *Anopheles* mosquito meal influences infections either through the consumption of toxic secondary metabolites or differences in nutritional values that in turn affect host immunity (Alaux *et al.,* 2010). Although, it has been shown that nutrition can influence mosquito competence, but it is it is not very clear if diet really affects Mosquito-*Plasmodium* interactions (Koella *et al.,* 2002;

Lambrechts *et al.,* 2006). This is necessary because the relationship between nutrition, immunity and infection are complex and because studies have associated low mosquito's competence and immune response to undernourishment (Koella *et al.,* 2002; Okech *et al.,* 2004; Lambrechts *et al.,* 2006). Nutrition may indirectly influence *Plasmodium* development by mediating changes in the gut microbiota (Linenberg *et al.,* 2016).

2.5.6 Gut Microbiota Infection History and Immune Priming

The presence of bacteria in the mosquito gut could also influence the vectorial competence of the mosquito (Foster, 1995). There are specific bacteria that can play some roles in the early development of *Plasmodium*, a process which is not fully understood yet (Dong *et al.,* 2009; Meister *et al.,* 2009; Boissiere *et al.,* 2012). Enterobacterium isolated from wild mosquitoes was shown to stir up the production of reactive oxygen molecules, which makes it difficult to get rid of *P. falciparum* from the human body (Cirimotich *et al.,* 2011).

Generally, the understanding of Microbiota-Mosquito-*Plasmodium* interactions is still limited, especially when determining the contribution of natural microbial flora of vectors to their competence for malaria parasites. What is clear is that mosquitoes usually habour a wide range of organisms which may have an impact on *Plasmodium* development (Blanford *et al.,* 2005; Bargielowski and Koella, 2009)

2.5.7 Human Activities

Anthropogenic environmental changes such as deforestation, urbanization and agricultural practices may have significant effects on mosquito availability, microclimatic conditions of the aquatic habitats and human residences (Afrane *et al.,* 2012). Apart from influencing rainfall, temperatures and humidity, human intervention in environments also directly affect mosquito densities. Deforestation for the purposes of logging and self-subsistence agriculture is a serious problem in the tropical regions of Africa (Aribodor *et al.,* 2016). These changes in regional climate and in the microclimatic conditions of mosquito habitats cause abundant changes in the existing mosquito species and may make some areas permissive to the proliferation of new species. Deforestation in Cameroon caused the introduction of *An. gambiae* into a habitat that was previously dominated by *An. moucheti* (Manga *et al.,* 1995). Also in

northern Brazil, *An. marajoara*, a species that was previously of minor importance became the principal malaria vector following changes in land use (Conn *et al.*, 2002).

Changes in the land use and land cover have been linked to changes in vector ecology and malaria transmission (Stefani *et al.,* 2013). It has also been shown to affect the microclimatic condition of mosquito's larval habitats. In a study that compared the microclimatic conditions and *An. gambiae* larval development as well as survivorship in semi-natural larval habitats under three land cover types (farmland, forest, and natural swamp). There were significantly higher water temperatures in farmland habitats compared to other land cover types (Munga *et al.*, 2007). The mosquito pupation rate was also significantly greater in farmland habitats than in swamp and forest habitats with the larval-to-pupa development also significantly shorter. Tso, it may be that the land cover type may affect larval survivorship and habitat productivity through its effects on water temperature and nutrients in the aquatic habitats (Kweka *et al.*, 2016).

2.5.8 Water Bodies

Mosquitoes mature in water bodies and disperse according to their flight range. *Anopheles gambiae* and *An. funestus* populations were observed to be decreasing with increasing distance from the Yala River in Kenya (Zhou *et al.*, 2004). It has also been clearly shown that even a small change in the distance from breeding water can influence malaria transmission (Walker *et al.*, 2013). *Anopheles fluviatilis*, *An. maculatus* and *An. minimus* were shown to be prevalent near streams of water in forested areas having cooler climate and tree canopy, while *An. dirus* larvae grow well in small, clear and stagnant bodies of water in forested areas of Asia (Lindsay *et al.*, 2004; Obsomer *et al.*, 2007). *Anopheles gambiae s.s* larvae have also been discovered to grow better in water bodies under dense forest canopy rather than sparse forest coverage (Tuno *et al.*, 2005). Overall, larvae of malaria vectors develop better in water bodies under tree canopy where the water temperature is buffered and are usually around 3–3.5 ^{0}C lower than the temperature of the present sunlight (Tuno *et al.*, 2005).

2.5.9 Seasonal Variation

Understanding the duration, start and end of malaria transmission season helps to determine the transmission potential and capacity, and to plan effective control

strategies for malaria prevention (Roca-Feltrer *et al.,* 2009). Malaria cases are believed to be highest during rainy season when temperature is relatively low. This is largely due to the increased number of stagnant waters creating several mosquitoes breeding sites (Oluleye and Akinbobola, 2010). However, the intensity, timing and duration of rains at a particular malaria endemic area, which could vary from year to year could help to identify the peaks of malaria transmission and malaria cases within a study period (Roca-Feltrer *et al.,* 2009).

2.6 Vectorial Capacity and Competence of *Anopheles* Mosquitoes

In human malaria, transmission intensity is highly dependent on the vectorial capacity and competence of local mosquitoes. Most mosquitoes are dead ends for *Plasmodium* parasites, but only limited range of *Anopheles* mosquitoes are able to transmit *Plasmodium* to humans (Cohuet *et al.,* 2010). The vectorial capacity of a mosquito population largely determines the intensity of vector-borne disease transmission while the vector competence is a crucial parameter for the pathogen to be transmitted.

There are several parameters that are used to evaluate *Anopheles* mosquito capacity to transmit malaria parasites. These parameters range from mosquito abundance, mosquito aggressivity, mosquito infection rates, blood meal type of mosquitoes, parous determination and longevity to entomological inoculation rate (Animut *et al.,* 2013; Petrić *et al.,* 2014) In addition, a number of evolutionary forces such as the duration of sporogonic development, the contact between the mosquito and vertebrate host suitable for the parasite and the susceptibility/resistance of the vector to the parasite influence malaria transmission (Cohuet *et al.,* 2010). Some quantitative features, where the roles of each species in malaria transmission could differ from one another is also common. Even at the sub-species level, some populations or individual mosquitoes can have more impact on transmission (Manguin *et al.,* 2008). Moreover, evolutionary pressures on vectors, including the forces exerted by the parasites they transmit can have major consequences on malaria transmission.

Understanding the dynamics of malaria transmission in a population is critical. it gives insight into the magnitude of the problem, helps to define when and where the greatest risk occurs and facilitates the development of appropriate control strategies (Smith and McKenzie, 2004). Furthermore, it is important to determine how the level of risk

within a population may compare with other populations, this will help to identify key differences or/and similarities and highlights corresponding risk factors. Measuring transmission over longer periods can also help to define intra- and inter- annual variability as well as assess the impact of changes within a population such as the introduction of a particular intervention. These interventions could range from the use of indoor residual spraying of insecticides, distribution of ITNs, migration, and/or changes in climate to land use patterns (e.g. irrigated agriculture and urbanization) (Killeen, 2014; Killeen *et al.*, 2017).

2.6.1 Vector Longevity

The development of malaria parasite in mosquito vectors requires the passage of two epithelia (Vaughan, 2007). In the process, the sporogonic development might impose some degree of virulence and affect the fitness of the vector host. The mechanisms of *Plasmodium* virulence to mosquito vectors are outlined below:

(a) the passage of parasites through the mosquito epithelia can cause cell damage. From observation, *Plasmodium* species causes host cell damage, which is followed by apoptosis. However, the effect of infection-induced apoptosis on mosquito survival remains unclear (Hurd and Carter, 2004; Vlachou *et al.*, 2004).

(b) parasite invasion of mosquito vectors in model and natural systems induces a massive immune response (Dong *et al.*, 2006; Mendes *et al.*, 2008). Studies in insects have demonstrated that such an induced response can affect fecundity (Schwartz and Koella, 2004) or longevity (Armitage *et al.*, 2003). This suggests that developing an immune response induces a fitness cost but its effect on mosquito survival is still not clear.

(c) Another potential cost of infection is that the parasite can affect mosquito behavior, leading to mortality at extreme cases (Cator *et al.*, 2014). In the natural *An. gambiae*–*P. falciparum* system, a higher feeding associated mortality can be noticed, probably as a result of a decreased efficiency in blood meal intake and increased feeding activity. This is, in fact, the only effect of infection on mosquito survival observed in this natural system. It could be the result of compensatory behavior by the mosquito to obtain sufficient blood for its trophogonic cycle or a manipulation of the behavior of the mosquito by the parasite to increase its transmission by multiplying mosquito blood feeds (Koella *et al.*, 1998). Exploiting compensatory behavior can be considered

a particularly well-developed strategy of manipulation by the parasite (Lefevre *et al.*, 2009).

In addition, reduction of fecundity is a more common cost of infection and has only a minor effect on transmission. It is likely that evolutionary forces selected parasites affect mosquito fecundity rather than affecting mosquito survival, which does not pose so many problems on parasite transmission. Moreover, from literature, infection-induced mortality is less likely to be found in natural systems (Ferguson and Read, 2002), which could be explained by evolutionary forces acting between co-evolved parasites and hosts. As both vector and parasite share the interest of vector survival, parasites might evolve towards low virulence.

Whereas, in parallel, mosquitoes that are less affected with infection would be selectively at an advantage (UE, 2016). For this, it is important to note that virulence in *Plasmodium–Anopheles* interactions must have a genetic basis with both parasite and mosquito genetics affecting infection success (Ferguson and Read, 2002). On the other hand, the evolution of virulence could also be highly dependent on environmental factors (Ferguson and Read, 2002; Lambrechts *et al.*, 2006). For example, the fact that *P. falciparum* and *An. gambiae* share an evolutionary history, by contrast to laboratory model systems, might have led to the selection of mechanisms in both the parasite and the mosquito to reduce any negative impact on host survival and subsequently increase the chances of parasite transmission.

Tolerance might play a role in host defense against the parasite in parallel to resistance. Tolerance is expected to impact survival or fecundity as the host spends resources to repair the damages that the infection has caused (Cohuet *et al.*, 2010). But, unlike resistance, tolerance to the effects of disease-induced mortality has a positive effect on parasite development (Best *et al.*, 2008; Read *et al.*, 2008) and could be an influencial mechanism in *Plasmodium–Anopheles* interactions.

Overall, there is a crucial need to translate parasite effects on mosquito fitness, the mechanisms involved, and their genetic and environmental determinants to predict how parasite virulence evolves (Lefevre *et al.*, 2013). This is particularly relevant for the prospect of using Genetically Modified Mosquitoes (GMMs) to control malaria transmission. The potential success of the GMM strategy in the wild will be highly dependent on the balance between the cost and benefit to the mosquito, an area closely

linked to the prevalence and virulence of infection. Moreover, vector longevity is likely to become a major target to control malaria transmission, for which we need a better understanding of the interactions between vectors and pathogens in natural conditions.

2.6.2 Human–Mosquito Contact and Human Biting Rate

The density of malaria vectors that are in contact with human and vertebrate hosts for blood meals is rapidly increasing (Pollitt *et al.,* 2015). This has been heavily implicated to contribute to the high malaria cases across Africa (Ndo *et al.,* 2016). Also, enhanced contact between humans and *An. gambiae* was observed to have driven the drastic increase in vectorial capacity (Ayala and Coluzzi, 2005). However, little is known on the population dynamics of infected mosquito bites and patency (Churcher *et al.,* 2017). Perhaps, we could insinuate that the risk of getting down with malaria is driven by the contacts between infected *Anopheles* mosquitoes and humans (Churcher *et al.,* 2015).

2.6.3 Susceptibility and Refractoriness to Infection

A mosquito is considered as a competent malaria vector if the *Plasmodium* parasite can internally complete its cycle from gametocytes in the blood to sporozoites in the saliva. We can distinguish different levels of susceptibility for human malaria parasites, a qualitative level with complete refractoriness of non-*Anopheles* species and a quantitative level among *Anopheles* species. Numerous culicidae are in close contact with humans, which has brought them in a good position for more transmission of infectious agents (Churcher *et al.,* 2017). It is possible that the density and human biting rates of non-malaria vector mosquitoes can be high in areas of intense malaria transmission. For this action to be consistent, the ingestion of *P. falciparum* by non-vector species must be frequent (CDC, 2015). This suggests that crucial factors avoiding infection in non-competent mosquito species must be highly differentiated from competent ones.

In real sense, the outcome of infection depends on the mosquito response and the ability of the parasite to evade it. The use Parasite–Vector system models have partially (Mendis *et al.,* 2000) deciphered the *An. gambiae* immune system (Blandin *et al.,* 2009; Povelones *et al.,* 2009). There are genes that play certain roles in the

mosquito response to *P. falciparum* (Dong *et al.*, 2006; Mendes *et al.*, 2008). A genetic island of resistance was mapped, demonstrating that genetic variants play a role in susceptibility and resistance of mosquito vectors and a candidate gene encoding a leucine-rich protein (APL1) showed allele-dependant resistance (Riehle *et al.*, 2006; Mitri *et al.*, 2009). The comparisons of different Vector–Parasite combinations revealed that parts of the mosquito response mechanism are general and effective against several infectious agents (Dong *et al.*, 2006; Mendes *et al.*, 2008), whereas others are more specific (Cohuet *et al.*, 2006; Dong *et al.*, 2006).

For instance, melanization appeared to be a baseline response to *Plasmodium* infection or other foreign bodies such as sephadex beads, *An. gambiae* (Lambrechts *et al.*, 2007) just like other mosquito species (Habtewold *et al.*, 2008). However, melanization does not appear as the major infection control mechanism, although, it is occasionally observed in experimentally infected mosquitoes (Schwartz and Koella, 2002; Riehle *et al.*, 2006). Also, silencing of key melanization genes did not affect the level of infection by *P. falciparum* (Cohuet *et al.*, 2006). Thus, the mechanism of melanization appears to be a highly efficient mosquito response against non-self except against the co-adapted human malaria parasite. This suggested that *Plasmodium* either suppresses the immune response or develops evasion mechanisms (Lambrechts *et al.*, 2007). The fact that key immune related genes in mosquito are upregulated during *P. falciparum* infection of mosquitoes favors the hypothesis of evasion (Cohuet *et al.*, 2006; Dong *et al.*, 2006; Mendes *et al.*, 2008).

The evolutionary arms race between parasites and hosts predict constant evolution of mosquito refractoriness and parasite evasion mechanisms. The high diversity and plasticity of *Plasmodium* in vertebrate hosts led to the assumption that parasite evolves faster and adapts more rapidly than the host. In this case, the parsite is expected to be transmitted more successfully through a local co-evolved vector population rather than a non-local one. The colonization of *P. falciparum* by *An. gambiae* was clearly shown as they were tested and showed more vector competence (showed high numbers of parasite development) compared to non-African *Anopheles* species (De Zulueta *et al.*, 1975).

Thus, the high vector competence of *An. gambiae* could partially be due to the fact that African *P. falciparum* is well adapted to this vector, and the frequency and

intensity of contacts between the parasite and the mosquito have determined the level of co-adaptation. This could also explain the efficient transmission of *P. falciparum* by its local vectors, but predicts that an introduction of *P. falciparum* in areas lacking this parasite where vector populations are not co-adapted would not rapidly lead to a high level of transmission. However, it cannot be neglected that new contacts between *P. falciparum* and *Anopheles* mosquitoes are sometimes efficient; studies on non-natural host–parasite combinations sometimes reveal high vector susceptibility (Collins and Robert, 1991; Nace *et al.*, 2004).

The outcome of infection is also parasite genotype–vector genotype dependent; no parasite has optimal transmission in all hosts and no host resists all parasites. For instance, *An. gambiae* iso-female lines can be highly susceptible to some parasite genotypes and at the same time highly resistant to others (Lambrechts *et al.*, 2005). *Plasmodium* infectivity and mosquito susceptibility are also environment-dependent (Tripet *et al.*, 2008). Such genotype–genotype environment interactions occur when one parasite genotype is more successful with one host genotype and less with another but these interactions change when the host and parasite are exposed to different environments (Wolinska and King, 2009).

Parasite genetics are surely important factors in mosquito invation. So, the efficiency of potential mosquito resistance genes in natural situations might differ depending on environment and parasite genetics (Boëte, 2009). A full consideration of the effect of the environment and parasite diversity on the evolution of vector competence is crucial to fully understand *Anopheles–Plasmodium* interactions.

2.7 Malaria Vector Control

For a much more preventive approach against malaria disease, the WHO recommended an integrated strategy that combines clinical control of the malaria parasite with the use of chemical insecticides to control malaria vectors (WHO, 2016b). Since *Anopheles* mosquitoes are the vectors that spread malaria disease, engaging on strategies that will help to decrease their population in the environment will lead to reduction in malaria morbidity and mortality. There are three methods that are used to control malaria vectors, which are physical, biological and chemical methods. However, chemical methods through the use of LLITNs, IRS, repellents and attractants have been the main driving force to combat malaria disease in Nigeria

(WHO, 2017b). Also, the careful design of human settlements, especially in urban and sub-urban areas has helped to reduce malaria mortality (Kabaria *et al.*, 2017).

2.7.1 Physical Control of Mosquito Vectors

Physical methods of controlling vectors comprise of techniques that prompt changes in vector behaviours, limit vector access to humans or cause direct damage that leads to vector death. The different approaches to this method are either through passive, active, thermal, electromagnetic, flooding, or mechanical actions (Vincent *et al.*, 2009). Overall, these tools are either used directly to remove or kill pests/vectors or physically keep insect vectors from reaching their hosts by means of a barrier or trap (Ojo and Omoloye, 2016). Some methods, however alter the physical environment to make it unfavorable for pests. Although, physical control methods are considered to have relatively little impact on natural vectors and other non-target organisms, they can be rapid and effective, and are well suited for different landscapes (WHO, 2017a). This method was used extensively in the past before the advent of the first set of chemical insecticides and provided an effective way to fight malaria (MS, 2015a).

A key target of this method is to sterilize male mosquitoes; while exposing the male pupae or adult mosquitoes to high energy ionising radiation (Helinski *et al.*, 2009). This will cause damage to germ cells of the male mosquito, making them sterile. After this process is successful and a female mosquito mates with the infertile male, she becomes infertile for the rest of her lifespan. Sustainable act of releasing large numbers of sterile males over a period of time that covers several generations of the target population could lead to a gradual decrease in the productive capacity of the mosquitoes. There will only be few fertile mosquitoes, which means low/no fecund mating events and the population could eventually be eliminated.

2.7.2 Biological Control of Mosquito Vectors

Biological agents such as parasites, pathogens and predators can be used to target various life stages of the mosquito (Kamareddine, 2012). Bacteria, fungi, viruses and fish have been used at different levels to decrease mosquito larvae populations. These agents are actually inexpensive to access as well as safe to humans and non-target organisms, they therefore provide a potential environmentally friendly option. Some

fishes such as *Gambusia affinis* and *Gambusia Holbrook* have been used to feed mosquito larvae for vector control purpose (Lawrence *et al.*, 2016).

There are reports that pathogenic bacteria, such as *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* are highly effective in controlling mosquito larvae at very low doses (Mittal, 2003). Also, some fungi have been used to control mosquito larvae and adults. Such entomopathogenic fungi, for example, the aquatic *Coelomomyces* species and soil fungi *Beauveria bassiana* can kill *An. gambiae* through tarsal contact (Scholte *et al.*, 2004). Unlike bacteria, microsporidia and viruses, fungi can infect and kill without being ingested. The spores germinate on the insect cuticle and immediately penetrate into the mosquito hemolymph and can grow there within one to two weeks. This has been shown to reduce malaria transmission up to 90 % as soon as the mosquitoes become infected with the fungus after ingesting *Plasmodium*. Transgenic fungi that can produce antimalarial peptides in the mid-gut of the mosquito have also been reported (Fang *et al.*, 2011).

2.7.3 Chemical Control of Mosquito Vectors

The use of chemical insecticides to eliminate mosquito vectors is a very common and reliable control tools. Larviciding (to control mosquito larve), the use of IRS and LLITNs to control adult mosquitoes are common tools currently in use to eliminate malaria vectors (WHO, 2017a). Ultra-low spraying is another tool that is used to target exophilic mosquitoes (Boubidi *et al.*, 2016; Pryce *et al.*, 2017).

The mosquito life cycle has four stages, of which three are spent in a stagnant aquatic environment, so destroying larvae while the mosquito is still in the aquatic environment could be an effective vector control method. The earliest chemical larvicide, cupric acetoarsenite was successfully used to eradicate *An. gambiae* in Brazil before World War II (Killeen *et al.*, 2002). Temephos, an organophosphate larvicide was considered much safer to kill mosquito predators. In recent years, insect growth regulators have been implemented for use as larvicides (Sadanandane *et al.*, 2012). Insect growth regulators work by binding onto larvae hormone receptors, preventing the insects from reaching the next stage of development. A significant advantage of an insect growth regulator is that it is target-specific and has shown a

good margin of safety on non-target organisms. However, these agents could be toxic to immature stages of other aquatic insects.

Before the advent of DDT and pyrethroids in the 1950s and 1970s respectively, researchers focused on diverting the female mosquitoes from human beings. This was achieved by repelling them, but with the initial perceived success of DDT and pyrethroid insecticides, research in this field became subdued. The controversy of the use of DDT actually opened the door to the emergence to pyrethroids as alternative insecticides for mosquitoes control (Nardini *et al.*, 2013). The mechanisms of repellents function are not yet clearly understood. They include chemical products with an offensive smell or taste to mosquitoes. Plant-derived compounds with repellence properties are most likely chemicals that are produced in defense against insects that pose a threat to the plant itself. These chemicals can be grouped into different categories based on the functional groups. They include nitrogen-containing compounds, terpenoids, phenolics, proteinase inhibitors and growth regulators (Maia and Moore, 2011).

Plants with better repellent properties fall into distinct families with the *Poaceae* family (citronella-based) being the pre-eminent one. Species of *Lamiaceae*, *Fabaceae* and *Asteraceae* also showed promising results. Prior to the extensive use of synthetic repellents, aromatic/essential oils were commonly used (Maia and Moore, 2011). Back then, Soldiers were given creams containing citronella, camphor and paraffin, which were products of essential oils. Numerous essential oil-producing plants from the *Lamiaceae*, *Poaceae*, *Rutaceae* and *Myrtaceae* families have very well known repellent activity. Synthetic products that have been used as repellents include Indalone, dimethylpthalate 2-ethyl-1, 3-hexane diol (Rutgers 612) and *N*,*N*-diethyl-*m*-toluamide (DEET), although, DEET is considered the most effective and widely used repellent (Fürstenberg-Hägg *et al.*, 2013).

Generally, attractants present an option for controlling mosquito vector populations via mosquito traps. For instance, *An. gambiae* is always attracted to ammonia, lactic acid and other carboxylic acids naturally present in the body odour and sweat produced by warm-blooded animals (Okumu *et al.*, 2010). Some of these compounds were used as synergist, blends together to attract more mosquitoes than individual compounds (Smallegange *et al.*, 2005). However, relative to human sweat, these

blends are still less attractive. This implies that there are additional compounds, yet to be discovered, with synergistic activity for attracting mosquitoes. Carbon dioxide is another common compound that plays an important role in mosquito host-seeking behaviour. In addition, a blend of 2-butanone was recently showed to be a very effective synthetic odour-baited traps for *An. gambiae* and *An. funestus* (Mburu *et al.*, 2017).

The WHO has always promoted the use of LLINs and IRS as a primary operational vector control intervention to prevent malaria transmission. However, African countries favour LLITN programmes as they are inexpensive and unlike IRS programmes, are easy to implement (WHO, 2017b). The challenge with LLITNs is that protection is only offered during sleeping time. Also, washing of treated nets could gradually diminish the insecticidal activity of the nets (Mutuku *et al.,* 2013). From the 12 insecticides approved by WHO for IRS, 6 are classified under pyrethroids (permethrin, alphacypermethrin, betacyfluthrin, bifenthrin, deltamethrin, etofenprox and lamdacyhalothrin), 3 are organophosphates (malathion, fenitrothion and pirimiphos-methyl), 2 as carbamates (propoxur and bendiocarb) and the DDT (organochlorine).

2.8 Common Insecticides in use for Malaria Vector Control

Insecticides use is integral showpiece in mosquito control programmes (WHO, 2016c). It all started in the ancient china and in the Middle Ages in Persia, where botanical preparation set-ups were first introduced (Davies *et al.*, 2007). However, in Europe, dried, ground dalmatian pyrethrum as well as other plants possessing extermination properties were used against cockroaches, bedbugs, flies, and mosquitoes (Isman, 2006; Davies *et al.,* 2007). For example, elderberry flowers were showed to be effective against cockroaches, aqueous extract of tobacco against aphids, and an aqueous extract of wormwood against weevils (Aslam *et al.,* 2015; Oberemok *et al.,* 2015). Inorganic sulfur (via fumigation) was another natural chemical used for insect control (Oberemok *et al.,* 2015). After all the use of these ancient natural compounds, there was an evolution of new sets and combinations of synthetic chemicals, which became so effective to control insects including malaria vectors.

2.8.1 Organochlorine Insecticides

The wider use of plant protection chemicals started in the middle of the 19th century. In the battle against the Colorado potato beetle, Paris green (mixed copper

acetoarsenite) was successfully applied in 1871 (Alyokhin, 2009). Paris green was widely used in many countries around the world until the middle of the 20th century, in particular for the control of the mosquitoes of the genus *Anopheles* (MS, 2015b). In 1874, an Austrian student and chemist Othmar Tseidler synthesised DDT (MS, 2015b). Its insecticidal properties were later found in 1939 by Swiss chemist Paul Müller working at that time in J. R. Geigy Ltd. (Davies *et al.*, 2007). In 1948, Paul Müller won the Nobel Prize in Medicine for the discovery of the high efficiency of DDT as a contact poison.

As it turned out later, DDT was effective in action and dangerous not only against pest insects but also for many other groups of organisms, such as mammals, birds, and reptiles (Sanchez-Bayo and Hyne, 2011). DDT was the most preferred pesticide for indoor residual spraying as at then because it has an efficacy of 12 months or more (Dos Santos *et al.*, 2007). The longer-lasting efficacy of DDT provides a low-cost option as one spray cycle per annum was sufficient, while two or more spray cycles may be required for the alternative insecticides. In South Africa, DDT was temporarily replaced with deltamethrin between 1996 and 1999 but failed. The failure of the pyrethroid was attributed to the reemergence of *An. funestus* mosquito strains. However, DDT was the reintroduced in 2000 when malaria transmission reached epidemic proportions. The stabilisation of DDT alternatives for indoor residual spraying includes formulations based on micro-encapsulated insecticides that have been tested with great success. This revealed that shielding the insecticides from the outside environment stabilises them against premature degradation. However, the higher costs of other classes of insecticide were still one of the draw backs associated with their widespread implementation as replacements for DDT.

Globally, the era of DDT in plant protection lasted until the second half of the 20th century when the use of organophosphates (dichlorvos, cyanophos, fonofos) and carbamates (carbaryl, carbofuran, aldicarb) were released as alternatives (Oberemok *et al.*, 2015). Despite harming the environment with carbamates and organophosphates, these insecticides are still among the widely used classes of insecticide preparations (19 % of insecticides in the world market) and also play a major role in the control of pest insects (Casida and Durkin, 2013). Due to the high efficacy of DDT, replacing this synthetic insecticide, although golden and offers advantages and novel

possibilities, became very difficult. This is partly because other insecticides have different modes of action and they degrade rapidly in the environment.

2.8.2 Pyrethroids Insecticides

Pyrethrins are combinations of six active insecticidal properties compounds found inside pyrethrum extracts (Gunasekara, 2005). Pyrethrins are only slightly soluble in water, but can dissolve in organic solvents like alcohol, chlorinated hydrocarbons, and kerosene (ATSDR, 2003a). It breaks down quickly in the environment, especially when exposed to natural sunlight. Pyrethrum is a naturally occurring mixture of chemicals found in certain chrysanthemum flowers (Breedlove and Arguin, 2016). It was first recognized to possess insecticidal properties around 1800 in Asia and was used to kill ticks and various insects such as fleas and mosquitos.

Pyrethroids are manufactured chemicals that are very similar in structure to the pyrethrins, but are often more toxic to insects as well as to mammals, and last longer in the environment than pyrethrins (Palmquist *et al.,* 2012). More than 1,000 synthetic pyrethroids have been developed but less than a dozen of them are currently in use. Pyrethrins and pyrethroids are often combined to synergists to enhance their insecticidal activity (NPIC, 2016). Synergists prevent some certain detoxifying enzymes from breaking down the pyrethrins and pyrethroids, thus increasing their toxicity. Most commercial pyrethroids are not one single molecule but several molecules. They have the same chemical formula that have their atoms joined together in the same sequence, but have a different arrangement of the atoms in space (stereoisomers).

If the stereoisomers are not mirror images of one another, they are called diastereomers, which have different physical properties like boiling point, melting point and solubility. If they are non-super imposable mirror images of each other, they are referred to as enantiomers and properties like boiling point, melting point and solubility are identical. However, both diastereomers and enantiomers can have different insecticidal properties and different toxicities (Liu and Gan, 2004).

Pyrethrins and pyrethroids are primarily released into air because of their use as insecticides. They were sometimes sprayed on crops from planes and helicopters or sprayed to the ground by trucks, tractors, or hand-held applicators (ATSDR, 2003b).

They can be use to control flying insects such as mosquitos and flies, on livestock and pets. In air, six of the pyrethrins and many of the pyrethroids are broken down or degraded rapidly by sunlight or other compounds found in the atmosphere (ATSDR, 2003b). They often last only 1 or 2 days before being degraded. Since many of these compounds are extremely toxic to fish, they are usually not sprayed directly onto water but they can enter lakes, ponds, rivers, and streams from rainfall or runoff from agricultural fields.

These compounds bind strongly to dirt and usually are not very mobile in soil. They are not easily taken up by the roots of plants and vegetation because they are strongly bound to the soil. However, they are often sprayed directly onto crops and plants so they may be found to protect leaves, fruits, and vegetables against pest attacks. Because these compounds adsorb so strongly to the soil, they usually do not leach into groundwater, do not contaminate drinking water supplies, and are volatilize from soil surfaces slowly (Agrawal *et al.,* 2010). These compounds could eventually be degraded by the microorganisms in soil and water. They can also be degraded by direct sunlight at the surfaces of water, soil, or plants. Although, some developed pyrethroids was reported to persist in the environment for a longer time before they are degraded (Dos Santos *et al.,* 2007).

2.8.3 Organophosphates and Carbamates Insecticides

Carbamate insecticides are derivatives of carbamic acid, HOC(O)NH2. Carbamates vary in their spectrum of activity, mammalian toxicity and persistence in the environment. They are relatively unstable compounds that break down in the environment within weeks or months (Oberemok *et al.,* 2015). The organophosphate and carbamate insecticides are represented by a wide variety of chemical structures having different chemical and physical properties. The toxicity of these materials to insects and mammals is determined by a number of factors that may influence the insecticides as they are absorbed, translocated to the target site and as they inactivate the target, leading to poisoning.

Organophosphates poison insects and other animals including birds, amphibians and mammals primarily by phosphorylation of the acetylcholinesterase enzymes (AChE) at nerve endings. This results in a loss of available AChE, so the effector organ becomes overstimulated by excess acetylcholine (Ach, the impulse-transmitting

substance) in the nerve ending. The AChE enzyme is critical to normal control of nerve impulse transmission from nerve fibers to smooth and skeletal muscle cells, glandular cells and autonomic ganglia as well as within the central nervous system mass must be inactivated by phosphorylation before symptoms and signs of poisoning becomes manifest.

At sufficient dosage, loss of enzyme functions allows accumulation of Ach peripherally at cholinergic neuroeffector junction (muscarinic effects), skeletal nerve-muscle junctions and autonomic ganglia (nicotinic effects). At cholinergic nerve junction with smooth muscle and gland cells, high Ach concentration causes muscle contraction and secretion. At skeletal muscle junction, excess Ach may be excitatory (causes muscle twitching), but may also weaken or paralyze the cell by depolarizing the end-plate. In the central nervous system, high Ach concentration causes sensory and behavioral disturbances, incoordination, depressed motor function and respiratory depression. Increased pulmonary secretions coupled with respiratory failure are the usual causes of death from organophosphate poisoning. Recovering depends ultimately on generation of new enzyme in all critical tissues.

The carbamate insecticides act similarly to the organophosphates, in that they inhibit acetylcholinesterase (AChE) at nerve synapses and neuromuscular junctions and their effects are more reversible and less severe.

2.9 Malaria Control Tools: IRS and LLINs

Since the beginning of the 21st century, the number of annual deaths attributed to global malaria cases has reduced due to significant investment on improved case treatment and insecticide-based vector control strategies (WHO, 2015b). It is perceived that only through this multifaceted approach will malaria control and elimination succeed (Coleman *et al.*, 2017). Effective vector control is a key component of this strategy with insecticides playing a central role in most malaria control programmes.

In recent years, malaria vector control interventions have been scaled up in many countries including Nigeria, resulting in considerable reductions in disease mortality (WHO, 2016d). However, in Nigeria, there has been considerable reduction in malaria mortality (WHO, 2017a) but no significant reduction in the morbidity since the turn of

the 21st century (Adepoju and Akpan, 2017). Deficiencies of these strategies include the lack of guidelines for pesticide registration, gaps in pesticide procurement practices, and lack of training on vector control decision makers and applications (Matthews *et al.*, 2011; Van Den Berg *et al.*, 2011). These shortcomings have hindered the optimal selection and use of insecticides, and application methods for vector control undermining the effectiveness and safety of operations.

Also, the extensive use of LLITNs and IRS has raised concern over the development of insecticide resistance and adverse effects to the environment and human health (Ranson *et al.*, 2008). Genes identified to confer insecticide resistance in insects have been spreading in vector populations, particularly in vectors of pathogens causing malaria and dengue (Ranson *et al.*, 2011). Monitoring data on malaria vectors in Africa confirmed that levels of resistance are increasing and more cases of resistance are being recorded in new locations (Samb *et al.*, 2016).

There were clamour for alternatives to the use of the organochlorine, DDT for vector control, given its toxicity, environmental persistence, bioaccumulation, vectors building resistance and potential for transboundary movements, then pyrethroids were fully introduced. Pyrethroids are currently the only class of insecticides approved for treating netting fabric because of their rapid effects on mosquitoes at low dosages combined with their relatively low health risk (Zaim *et al.*, 2000; WHO, 2016b). Now, there are lots of emphases on the search for alternatives to pyrethroids because of the level of resistance that malaria vectors have built against them (Nkya *et al.*, 2014). There is always going to be the need to locally appropriate cost-effective alternatives for a sustainable transition from pyrethroids, especially for IRS (WHO, 2013c). This will guide against the pressure on pyrethroids and a mode of switching to other insecticides in case the challenges of using this insecticide class persist.

2.10 Insecticides Mode of Action

Most insecticides target the nervous system of the insect (Davies *et al.*, 2007). However, organophosphate (e.g. chlorpyrifos, diazinon, fenitrothion, fenthion, malathion and temephos) and carbamate (e.g. propoxur, bendiocarb, carbaryl) insecticides serve as cholinesterase inhibitors (Das, 2013). Similarly, cyclodienes (e.g.

dieldrin) insecticides attack insects by affecting the chloride channel; they work by inhibiting the gamma aminobutyric acid (GABA) receptor of the insect (Tong, 2010).

The insects' voltage-gated sodium channels are the targets for DDT and pyrethroids, which are responsible for the rising phase of action potentials in the membranes of neurons and most electrically excitable cells. Pyrethroids (e.g. permethrin, deltamethrin, cypermethrin) and DDT act on the sodium channel by preventing the channels from closing. The alteration on the gating properties keep the sodium channel open for unusual long time, thereby causing a prolonged flow of sodium current (nerve impulse transmission). This initiates repetitive discharges and prevents the repolarization phase of action potentials leading to tremors and eventually death (Davies *et al.,* 2007)

The common feature found in sodium channels is that relatively small changes, such as point mutations or substitutions, short sequence insertions or deletions or alternative splicing in the structure of these channels significantly affect their behavior and are sufficient to change neuronal firing resulting in different phenotypes. Modifications of the insect's sodium channel structure can make the channel insensitive to DDT and pyrethroids binding, via a reduction in or an elimination of the binding affinity of the insecticides to proteins and hence result in the development of insecticide resistance.

Organophosphorus and carbamate insecticides are toxic to insects and mammals by virtue of their ability to inactivate the enzyme acetylcholinesterase, which is a class of enzymes that catalyzes the hydrolysis of the neurotransmitting agent acetylcholine (ACh) (Fukuto, 1990; Costa *et al.,* 2008). The inhibition of AChE and related esterases often referred to as serine hydrolases, has been demonstrated to be the result of a chemical reaction between the enzyme and the organophosphate or carbamate ester. The phosphorylated or carbamylated enzyme will no longer be capable of affecting the hydrolysis of ACh; this will result in a buildup of the neurotransmitter at a nerve synapse or neuromuscular junction (Fukuto, 1990; Talley, 2001).

AChE is present and has been isolated from a wide range of animals including mammals, birds, fish, reptiles, and insects. It is responsible for the rapid hydrolytic degradation of the neurotransmitter ACh into the inactive products choline and acetic acid (Fukuto, 1990). Physiologically important neurotransmitting agents are involved

in the transmission of nerve impulses to effector cells at cholinergic, synaptic, and neuromuscular junctions. The presence of AChE has been demonstrated in a variety of animal tissues and enzymes from a number of different sources including fish electric organs, mammalian erythrocyte, insect and mammalian brain, and other tissues (Xu *et al.,* 2017). AChE is virtually aubiquitous enzyme but in mammals, it is localized in certain areas of the central nervous system and in organs and glands that are controlled by the parasympathetic division of the autonomic nervous system.

When a nerve impulse moves down a parasympathetic neuron and reaches a nerve ending, the ACh stored in vesicles in the ending is released into the synaptic or neuromuscular junction. Within 2 to 3 msec, the released ACh interacts with the ACh receptor site on the postsynaptic membrane, causing stimulation of the nerve fiber or muscle. This has made AChE to serve as a regulating agent of nervous transmission by reducing the concentration of Ach in the junction through which AChE catalyze the hydrolysis of ACh into choline (Ch) and acetic acid (A). These products do not stimulate the postsynaptic membrane. When AChE is inactivated by an organophosphorus or carbamate ester, the enzyme is no longer able to hydrolyze Ach. This makes the concentration of ACh in the junction to remain high and continuous stimulation of the muscle or nerve fiber occurs, eventually resulting into exhaustion and tetany (Oberemok *et al*., 2015).

2.11 Insecticide Resistance

Insecticide resistance as defined by the WHO is the ability of an organism to tolerate doses of a toxicant, which would prove lethal to a majority of individuals in a normal population of the same species (WHO, 2016d). The development of resistance is always influenced by many factors such as (a) genetic factors, including the level of resistance conferred by the resistance allele(s), mutation and relative dominance of the characters (b) biological factors, including the fitness of the heterozygous and homozygous resistant phenotypes and initial population size (c) reproductive factors, including the rate of increase and fluctuations in population size and (d) operational factors, including previous selection with other insecticides, mode of insecticide application, proportion of population exposed to selective doses, dosage of insecticide taken up by exposed insects and the life stage of the mosquito selected (Siegwart *et al.,* 2015).

The mechanism of insecticide resistance adopted by an organism depends on the prevailing pressure and the mode of action of the insecticide in use. In general, response to insecticides can be categorized into two major types: physiological resistance and behavioral avoidance (Chareonviriyaphap *et al.,* 2013; Nasen *et al.,* 2016) Physiological resistance is the ability of an insect population to survive the exposure of insecticide concentration that normally should result in killing the insect completely. This could be achieved mainly through increased metabolic detoxification and/or decreased target site sensitivity (Dang *et al.,* 2017; Kaplanoglu *et al.,* 2017).

In contrast, behavioral avoidance also known as deterrence is the ability of an insect to move away from an insecticide-treated area, often without lethal consequence. This type of response can be further divided into direct contact excitation (sometimes referred to as irritancy) and non-contact spatial repellency (Chareonviriyaphap *et al.,* 2013). The term contact irritancy involves an insect leaving an insecticide treated area only after making physical or tarsal contact with the chemical, whereas spatial repellency is when insects move away from the insecticide-treated area without making direct contact.

Since the 1940s, resistance has appeared in most major insect vectors from every genus. More than 100 mosquito species including 56 species of anopheline and 39 species of culicine mosquitoes are known to have developed resistance to one or more insecticides. Pyrethroids resistance have been detected in various *Anopheles* species including *An. gambiae* (Corbel *et al.,* 2007; N'Guessan *et al.,* 2007; Okorie *et al.,* 2015), *An. albimanus* (Brogdon and Barber, 1990), *An. arabiensis* (Casimiro *et al.,* 2006b) and *An. funestus* (Djouaka *et al.,* 2016). Organophosphate resistance has been recorded in *Culex* and *Anopheles* vectors species including *An. arabiensis* (Hemingway, 1983), *An. culicifacies* (Herath *et al.,* 1987), *An. stephensi* (Hemingway, 1982) and *An. sacharovi* (Hemingway *et al.,* 1985). Carbamate resistance has also been detected in *An. gambiae* (N'Guessan *et al.,* 2003), *An. sacharovi* and *An. albimanus* (Hemingway *et al.,* 1992).

Reports of resistance in *An. funestus* mosquitoes have also emerged, cases of dieldrin resistance in Cameroon and Burkina Faso (Wondji *et al.,* 2011), DDT resistance in Benin and Uganda (Mulamba *et al.,* 2014; Djouaka *et al.,* 2016). Other reports of resistance in *An. funestus* include resistance to DDT and bendiocarb in Obuasi, Ghana

(Coetzee *et al.*, 2005), deltamethrin in KwaZulu-Natal, South Africa and southern Mozambique (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001) as well as to pyrethroid and carbamates in Mozambique (Casimiro *et al.*, 2006a; Cuamba *et al.*, 2010).

2.11.1 Behavioral Responses to Insecticides

For observing the behavioral responses of mosquitoes to insecticides, WHO developed the first test box, the excito-repellency test box to evaluate the excitation (irritability) of exposed mosquitoes following physical contact with insecticides (WHO, 1970). The test system was further modified by investigators interested in behavioral avoidance responses to DDT and some of the early synthetic pyrethroids (Ree and Loong, 1989; Roberts *et al.*, 1997). Thereafter, a light-proof test chamber was designed to study the irritant response of *An. gambiae* to several chemical compounds (Evans, 1993).

One limitation associated with all of these earlier test systems was the procedural difficulty for introducing and removing test specimens with the assay designs. Other concern was the inability to evaluate various physiological states found in wild-caught mosquitoes and selecting the ideal range of concentrations for chemical evaluation (Chareonviriyaphap *et al.*, 2013). Moreover, there was no universal analysis or set of statistical methods for interpretation of data to discriminate between contact excitation and non-contact repellency responses (Roberts *et al.*, 1997).

All these led to the development of an improved excito-repellency test device that was able to better differentiate between excitation and spatial repellency, which was first tested against field populations of *An. albimanus* in Central America (Roberts *et al.*, 1997). Unfortunately, this prototype was cumbersome to handle and required considerable time for attaching the insecticide-treated test papers inside the chamber. Eventually, a more field deployable test system that was collapsible and easily transportable was designed (Chareonviriyaphap *et al.*, 2002). This system is now extensively used to investigate the behavioral responses of various mosquito species (Kongmee *et al.*, 2004). In addition, a more compact, modular assay system known as the High Through-put Screening System (HITSS) was developed for mass screening of chemicals and adult mosquito responses including contact irritancy, spatial repellency and toxicity depending on the specific design set-up.

This modular system, now approved by WHO Pesticide Evaluation Scheme

(WHOPES) for testing efficacy of new active ingredients intended for spatial repellent is of a reduced size compared to the previous excito-repellency box devices and greatly minimizes the treated surface area required thereby reducing the amount of chemical required for handling and testing (WHO, 2012). Unlike physiological resistance, accurately measuring of behavioral responses remains elusive and sometimes this response is best suited as behavioral avoidance (Chareonviriyaphap *et al.,* 2013). This is because it is an innate, involuntary response to external stimuli rather than a permanent, genetically-based shift in behavior as the development of apparent fixed behavioral changes.

2.11.2 Cuticle Resistance Mechanism

Cuticle thickening has been shown to contribute to insecticide resistance in mosquitoes (Wood *et al.,* 2010; Yahouédo *et al.,* 2017). There are also mechanisms that have been used to augment cuticle thickening, leading to a very high reduced penetration of insecticides (Bass and Jones, 2016; Lilly *et al.,* 2016). Ordinarily, reduced penetration does not by itself, impart a high degree of resistance, but it could contribute to high level of insecticide resistance, leading to a wider variety of cross-resistance insecticides (Yu *et al.,* 2008), increasing the efficiency of metabolic detoxification (Ahmad *et al.,* 2006; Mamidala *et al.,* 2012) or delaying the onset of knockdown (Wood *et al.,* 2010). However, the expression of one or more resistance genes does not necessarily predicate a corresponding change in expression of cuticle proteins (Lilly *et al.,* 2016).

Although, it appears that, conversely, reduced penetration is typically found so significant only when other mechanisms are present and active (Wood *et al.,* 2010). Thicker cuticles lead to slower rates of insecticide absorption, which is likely to enhance the efficiency of metabolic detoxification. Whereas, slower insecticide penetration across the cuticle (though not necessarily the result of cuticle thickening) has been associated with insecticide resistance in the cotton bollworm *Helicoverpa armigera* (Ahmad *et al.,* 2006).

Measurable cuticle thickening has been associated with pyrethroid resistance in the Chagas disease vector *Triatoma infestans* (Pedrini *et al.,* 2009) and was inferred from microarray gene transcription analysis in *An. stephensi* (Vontas *et al.,* 2007).

Measurements of rates of Lowered insecticide penetration have also been linked to thickened cuticles as well as other structural components of the cuticles such as relative amounts of surface hydrocarbons. This decreased rates of penetration across the cuticle slows the insecticide inoculation in internal organs and sufficiently allowed for effective metabolically-mediated detoxification (Pedrini *et al.,* 2009). A link between cuticle thickness and response to insecticide exposure is thus based on the assortment of phenotypes, where the insecticide tolerant in resistant groups were associated with thicker cuticles (Hunt *et al.,* 2005).

2.11.3 Metabolic Resistance Mechanism

Increased metabolic detoxification is one of the common mechanisms of insecticide resistance (Menze *et al.,* 2016; Samb *et al.,* 2016). There are three enzyme classes that are commonly involved in insecticide detoxification: the monooxygenases (cytochrome P450s), esterases and glutathione *S*-transferases (GSTs) (David *et al.,* 2013).

2.11.3.1 Monooxygenases

The monooxygenases are a complex of detoxifying enzymes found in most aerobic organisms including insects and are critical in the regulation of endogenous compounds such as drugs, insecticides and plant toxins (Hemingway and Ranson, 2000; Werck-Reichhart and Feyereisen, 2000). Cytochrome P450 monooxygenase mediated detoxification is very important because they metabolize virtually all insecticides (Hemingway and Ranson, 2000). The spectrum of insecticides that monooxygenases have conferred resistance for include pyrethroids, organophosphates and to a low degree in carbamates (Riveron *et al.,* 2013, 2014b). Monooxygenases-mediated resistance has been reported for pyrethroid resistance in *An. funestus* populations (Wondji *et al.,* 2011; Mulamba *et al.,* 2014; Djouaka *et al.,* 2016; Riveron *et al.,* 2016; Samb *et al.,* 2016), *C. quinquefasciatus* (Chandre *et al.,* 1999), *An. gambiae* and *An. arabiensis* (Okorie *et al.,* 2015).

Cytochrome P450 enzymes bind molecular oxygen and receive electrons from nicotinamide-adenine dinucleotide phosphate (NADPH) to introduce an oxygen atom into the substrate and to form water with the oxygen atom:

Substrate (S) + (NADPH + H^+) + O_2 = P(O) + $NADP^+$ + H_2O

Note: S = substrate; P = product

The electrons necessary for this reaction are transferred from NADPH on the substrate by an NADPH cytochrome P450 reductase. Although, the stability of the initial product [S(O)] can vary leading to a final reactions as diverse as hydroxylation, epoxidation, O-, N-, and S- dealkylations, N- and S- oxidations and to such various chemical reactions, and products that these enzymes have been called diversozymes.

The key protein of this enzymatic system is in each case a cytochrome P450 that is responsible for the specificity of the reaction. This protein has an absorption at 450 nm when reduced and saturated with CO, hence its name. If P450 monooxygenase activities are exerted on such a significant diversity of substrates (steroids, juvenile hormone, hydrocarbons, pesticides or insecticides), it is because there is a high number of cytochromes P450 in that organism. To cope with such a diversity, a nomenclature based on sequences homologies of P450 was adopted and now universally accepted.

Cytochrome P450 (CYP genes) constitute one of the largest gene family with representatives in virtually all living organisms, from bacteria to protists, plants, fungi, and animals (Werck-Reichhart and Feyereisen, 2000). The human genome carries about 57 CYP genes and about 170 CYP in a mosquito (Feyereisen, 2012). The designates gene members of the P450 super-family carries a CYP prefix, followed by a numeral for the family, a letter for the subfamily, and a numeral for the individual gene. All members of a family share more than 40 % identity and members of a subfamily share more than 55 % identity at the amino-acid sequence level (Sello *et al.*, 2015). Genes are always described in italics, whereas the gene product, mRNA and enzyme are in capital letters. Six CYP families have been assigned as Insect P450s; five are insect-specific (CYP 6, 9, 12, 18 and 28), and one, the CYP 4 is shared with sequences from other organisms. Each P450 protein is the product of a distinct CYP gene and P450 diversity is the result of successive gene (or genome) duplications followed by sequence divergence.

P450s also show activities as oxidases, reductases, desaturases, isomerases (Mansuy,

1998; Guengerich, 2001). Because of their complex catalytic mechanism, P450 enzymes often generate superoxide or hydrogen peroxide from unsuccessful or uncoupled reactions leading to oxidative stress in cells, which are much more toxic than the initial insecticide (Fabbri, 2014). There are soluble forms of P450 in bacteria, and membrane-bound forms in microsomes and mitochondria of eukaryotes. Most animal P450s are dependent on redox partners for their supply of reducing equivalents (NADPH cyt P450 reductase and cyt b5 in microsomes, a ferredoxin and a ferredoxin reductase in mitochondria). Some bacterial and fungal P450 enzymes are fusion proteins with a variety of redox partners, whereas some P450 enzymes act directly on their substrates without the need for dioxygen or reducing equivalents (Hannemann *et al.,* 2007; Munro *et al.,* 2007).

Foreign compounds, as well as endogenous metabolites can induce the transcription of P450 genes through complex interactions with members of the nuclear receptor family and with PHLH-PAS proteins, such as the PXR (pregnane X receptor) and the Ah (aryl hydrocarbon) receptors of vertebrates, respectively. The regulation of P450 gene expression by chemicals allows animals to respond to changing environments directly by mounting a detoxification defense and indirectly by adapting their basal metabolism, hormone balance, and hence, rate of development and reproduction (Kirchmair *et al.,* 2012).

2.11.3.2 Esterases

Esterases have been able to detoxify a broad range of insecticide through sequestration of the insecticide rather than metabolizing the insecticide (Hemingway and Ranson, 2000; Montella *et al.,* 2012). They have also provided a narrow range of insecticide resistance through metabolism of a few insecticides with an ester bond. In mosquitoes, esterase-based resistance mechanisms occur when the esterase is modified so that they can metabolize insecticides more efficiently or the esterase level is elevated, primarily through gene amplification (Montella *et al.,* 2012).

Esterases are known to detoxify organophosphates and carbamates but are almost considered to be important but less in pyrethroids resistance (Pasteur and Raymond, 1995; Hemingway and Ranson, 2000; Russell *et al.,* 2011). Esterases-mediated detoxification has been described in *An. albimanus* from southern Mexico (Penilla *et*

al., 1998), pyrethroid resistant populations of *An. gambiae* and *An. arabiensis* from Mozambique (Casimiro *et al.*, 2006) and Tanzania (Matowo *et al.*, 2010) and a temephos resistant strain of *C. quinquefasciatus* from Colombia (De Silva and Hemingway, 2002).

Resistance to temephos and chlorpyrifos has been associated with esterases in *Ae. Aegypti* (Mazzarri and Georghiou, 1995). This enzyme family has also been associated with malathion resistance in *C. quinquefasciatus* from Cuba (Coto *et al.*, 2000) and in *C. quinquefasciatus* and *C. tritaeniorhynchus* from Sri Lanka (Karunaratne and Hemingway, 2001). Esterases have been found to confer permethrin resistance in *An. gambiae* from Kenya (Vulule *et al.*, 1999) and Benin (Aïzoun *et al.*, 2013).

2.11.3.3 Glutathione-S-Transferases

GSTs confer resistance by conjugating reduced glutathione (GSH) to a large range of xenobiotics aiding in their detoxification and excretion (Hemingway and Ranson, 2000; Ramsay and Dilda, 2014). For example, most GSTs detoxify and transform DDT to the non-toxic metabolite DDE (Pasteur and Raymond, 1995).

Elevated GSTs have been found in many resistant mosquito species (Riveron *et al.*, 2017). Examples include DDT resistant populations of *An. arabiensis* (Hargreaves *et al.*, 2003), *An. gambiae* (Prapanthadara *et al.*, 1996), *An. atroparvus* and *An. sacharovi* (Hemingway *et al.*, 2004), *An. albimanus* (Penilla *et al.*, 1998) and *An. funestus* (Riveron *et al.*, 2014a). There are also cases of cross-resistance between insecticides, a case of permethrin and carbamate (Ibrahim *et al.*, 2016). GST-based DDT resistance often acts as a secondary resistance mechanism for fenitrothion with a monooxygenase or esterase-based resistance mechanism taking the primary actions (Hemingway and Ranson, 2002). In addition, GST has been engine for permethrin detoxification in resistant *An. funestus* (Riveron *et al.*, 2013).

2.11.4 Target-site Resistance Mechanisms

Modification of gamma aminobutyric acid (GABA)-gated chloride channel and the voltage-gated sodium channel (VGSC) has influenced insecticide resistance in mosquitoes. The VGSC-associated resistance is common in *An. gambiae* population (Mitchell *et al.*, 2014; Ochomo *et al.*, 2015). However, it is still not found in *An.*

funestus mosquitoes (Menze *et al.*, 2016). On the othe side, GABA channel resistance has been prominent to dieldrin resistance (Wondji *et al.*, 2011). Point mutation on *GSTe2* gene, leading to phenylalanine replacing leucine has also been associated with DDT resistance in *An. funestus* (Mulamba *et al.*, 2014). *Kdr* mutation might be completely absent in *An. funestus* populations but some novel point mutations that may contribute to resistance in the future are gradually coming up in *An. funestus* (Menze *et al.*, 2016).

2.11.4.1 Altered Acetylcholinesterase Enzyme

The mode of action of organophosphates and carbamate insecticides involves alterations in acetylcholinesterase (AChE) (Brogdon *et al.*, 1999; Hemingway and Ranson, 2000). This mechanism is due to a change in the AChE, reducing its affinity to bind insecticides. Altered AChE has been found to confer resistance of insects to different insecticides such as to propoxur and organophosphate insecticides (temephos, chlorpyrifos and malathion) in *C. pipiens* from Portugal (Bourguet *et al.*, 1996).

The presence of altered AChE has also been found in an organophosphate (ethyl parathion) and carbamate (propoxur) resistant strain of *An. albimanus* (Hemingway and Georghiou, 1983); carbamate resistant populations of *An. albimanus* from southern Mexico (Penilla *et al.*, 1998) and field populations of *An. nigerrimus*, *C. tritaenorhynchus* and *C. gelidus* (Hemingway *et al.*, 1986). The altered AChE gene is also present but at low frequency in pyrethroid resistant populations of *An. gambiae*, *An. funestus* and *An. arabiensis* from Mozambique (Casimiro *et al.*, 2006a; 2006b).

2.11.4.2 Gamma Aminobutyric Acid (GABA) Receptors

The gamma aminobutyric acid (GABA) receptor is a chloride-ion channel in the insect's central nervous system and neuromuscular junctions (Hemingway and Ranson, 2000). Mutations on the GABA receptor are implicated as a site of action for ivermectins and cyclodienes (Hemingway and Ranson, 2000).

A single mutation replacing alanine with a serine on position 302 in the M2 transmembrane domain of the GABA gene was associated with dieldrin resistance of *Drosophila melanogaster* (Ffrench-Constant and Rocheleau, 1993). This mutation was also found in dieldrin resistant strains of the yellow fever mosquito *Aedes aegypti*, the

house fly *Musca domestica*, the red flour beetle *Tribolium castaneum*, the coffee pod borer *Hypothenemus hampei* and the American cockroach *Periplaneta americana* (Ffrench-Constant and Rocheleau, 1993). In addition, another mutation changing alanine to glycine or serine on position 296 has been associated with dieldrin resistance in *An. gambiae* species (Kaiser, 2015).

2.11.4.3 Sodium ion Channels [Knockdown resistance (*kdr*)]

Voltage-gated sodium channels are the target site of pyrethroids and DDT insecticides. It functions by altering the sodium channels in nerve membranes (Soderlund and Bloomquist, 1989; Soderlund and Knipple, 2003; Ranson *et al.*, 2011). Reduced sensitivity of the sodium channel to DDT and pyrethroids is expressed as knockdown resistance (*kdr*) (Soderlund and Knipple, 2003). Knockdown resistance was first described in the house fly, *Musca domestica* with the single nucleotide substitution changing leucine (TTA) to phenylalanine (TTT), hence the *kdr* mutation; this occurs on domain II, segment 6 of the sodium channel gene and also considered the most common *kdr* gene mutation (Soderlund and Knipple, 2003; Davies *et al.*, 2007; Mitchell *et al.*, 2014; Ochomo *et al.*, 2015).

Two alternative substitutions at this position also confer resistance to DDT and pyrethroids. A leucine to histidine substitution was associated with pyrethroid resistance in the tobacco budworm *Heliothis virescens* (Park and Taylar, 1997), and a leucine to serine substitution confered DDT resistance and low levels of permethrin resistance in *C. pipiens* strain from China (Martinez-Torres *et al.*, 1999a, 1999b), East African *An. gambiae* (Ranson *et al.*, 2000) and *An. sacharovi* (Luleyap *et al.*, 2002). Four different mutations of leucine to tryptophan, isoleucine to methionine, glycine to valine, and valine to glycine were also identified on domain II of the sodium channel gene in pyrethroid resistant field population of *Ae. aegypti*, where the common *kdr* mutation was absent (Brengues *et al.*, 2003).

Reports of *kdr* mutation in pyrethroid resistant populations of *An. gambiae* exist in several African countries. These include Burkina Faso (Martinez-Torres *et al.*, 1998; Diabate *et al.*, 2004), Cameroon (Etang *et al.*, 2006), Cote d'lvoire (Chandre *et al.*, 1999) and Equatorial Guinea (Reimer *et al.*, 2005; Sharp *et al.*, 2007). Others include Benin (Weill *et al.*, 2000), Gabon (Pinto *et al.*, 2006), Ghana (Yawson *et al.*, 2004),

Kenya (Ranson *et al.*, 2000), Mali (Fanello *et al.*, 2003), Senegal (Weill *et al.*, 2000), Uganda (Verhaeghen *et al.*, 2006) and Nigeria (Awolola *et al.*, 2007). The *kdr* mutation was also found in *An. stephensi* (Enayati *et al.*, 2003), *An. arabiensis* (Verhaeghen *et al.*, 2006; Matambo *et al.*, 2007), *An. sacharovi* (Luleyap *et al.*, 2002), *C. pipiens* (Martinez-Torres *et al.*, 1999a; McAbee *et al.*, 2004) and *C. quinquefasciatus* (Xu *et al.*, 2005).

Another mutation changing methionine to threonine known as the super-*kdr* mutation occurs between the segments 4 and 5 of domain II and results in a much higher resistance than *kdr* in house flies (Miyazaki *et al.*, 1996; Williamson *et al.*, 1996); horn fly (Guerrero *et al.*, 1997) and tobacco whitefly (Morin *et al.*, 2002). These super-*kdr* mutations are usually found in combination with the *kdr* mutation (Soderlund and Knipple, 2003). More than twenty different sodium channel mutations have been identified in different resistant arthropods and all these mutations so far have been identified in resistant mosquitoes at the domain II of the sodium channel gene (Soderlund and Knipple, 2003; Hemingway *et al.*, 2004; Liu *et al.*, 2006; Mitchell *et al.*, 2014; Ochomo *et al.*, 2015).

2.12 Review of Methods

2.12.1 Mosquitoes Collections Techniques

Mosquito collections are carried out mainly for research purposes. Entomologists have designed different methods of collections depending on research objectives and targets. There are steps for collections and collations of data.

2.12.1.1Entomological Surveillance

Entomological survey is an essential component of malaria vector control programmes operational activities and research. There are four main types of surveys used in vector studies namely: (i) preliminary surveys (ii) routine or trend observations (iii) spot checks (iv) focal investigations (WHO, 2013d; 2014).

i. Preliminary surveys

Preliminary surveys are original and basic; they are considered as short-term surveys that are used to gather baseline data for planning vector control measures. They

provide information on the identity of vector species, their resting and feeding habits, densities, longevity, different types of water bodies for breeding and their sensitivity to available insecticides.

ii. Regular or trend observations

These are long-term observations carried out regularly; for example, monthly, quarterly or yearly. This is ususally done for the purpose of monitoring and evaluating the impact of control measures. They provide information on changes in vector density, infection rates, behaviour and susceptibility of vectors to insecticides.

iii. Spot checks

Spot checks are carried out in localities that are chosen at random. As the fixed stations often used to monitor mosquito populations may not be representative of all areas, spot checks may be conducted randomly in selected areas to supplement routine observations or obtain a clearer indication of the effects of control measures.

iv. Focal investigations

Focal investigations are undertaken in areas of new or persistent malaria transmission to investigate on why there is transmission or why the disease is not responding to the measures being applied and to identify the best approache(s) for control.

The aim of entomological surveillance is to gather baseline data for planning anti-vector measures including distinguishing *Anopheles* from other insects, identification of malaria vector species, vector population density, rate of infection (sporozoite rate, oocyst rate), longevity of vector (parous, nulliparous), feeding habit (zoophilic, anthropophilic), behaviour (exophilic, endophilic, exophagic, endophagic), seasonal activities, larval habitat, type of breeding water and determining susceptibility to insecticides.

2.12.2 Adult Mosquito Collection Methods

There are several methods that have been designed to collect *Anopheles* mosquito on the field. Collection technique could vary depending on the type of study and kind of mosquitoes that are required for the study.

2.12.2.1 Hand Collection of Indoor resting Mosquitoes

Many of the anopheline species, which are malaria vectors rest indoors (Animut *et al.*, 2013; Lobo *et al.*, 2015). Hand collection, using WHO aspirators and some other modified forms were efficient for collecting mosquitoes, and could help to generate information on usual resting places, resting density and changes in density (Harbison *et al.*, 2006; Maia and Moore, 2011). It also provides live specimens for susceptibility and bioassay testing and for observations on mortality among mosquitoes from houses where insecticide is present on bed nets or on the walls.

Equipment

Sucking tube (also referred to as WHO aspirator (Figure 2.3), ash light, paper cups with covering net, cotton wool, rubber bands, mosquito cages, a card box container or insulated picnic box, chloroform and towels are needed for this collection method (WHO, 2013d).

How to use a sucking tube:

a. With the mouthpiece in the mouth, hold the sucking tube with its opening 1–2cm away from the mosquito.

b. Move the end of the sucking tube closer to the mosquito and at the same time, suck gently but quickly so as to draw the mosquito into the tube.

c. Place a finger over the tube to prevent the mosquito from escaping.

d. Place the end of the tube with the finger still in position, near the hole in the mesh covering the paper cup. Remove the finger and quickly put the tube into the hole.

e. Blow gently into the mouthpiece so as to transfer the mosquito to the paper cup; at the same time, tap the tube with the index finger to disturb resting mosquitoes (WHO, 2013d).

It is important to note that a maximum of five mosquitoes should be sucked at once into one tube before transferring them to the paper cup to avoid congestion.

There is a modified form of this mosquito collection tool, which is considered to be less laborious and has the capability to collect much more mosquitoes at a time than the mouth aspirator. This technological modified tool, known as an electric aspirator is very effective and efficient for live mosquito collections (WHO, 2013d).

Some tips should be known prior to collecting mosquito with WHO aspiration/hand collection method:

a. Mosquitoes should be collected early in the morning after the house occupants are up and dressed. In villages, at least 10 houses should be searched in order to provide a representative sample. The consent of the households should be obtained in advance of the search and occupants should not open the windows in the morning prior to mosquito collections. However, there are possibilities of carrying out a night collection studies to determine the aggressiveness of disease vectors and other mosquito density parameters such as the entomological inoculation rate (EIR). This is also a good entomological target that can give information on the peak of mosquito bites.
b. Mosquitoes caught alive in houses may be kept for 24 hours. This will allow a check on the 24-hour mortality rate among mosquitoes from houses with insecticide-treated nets or collected from sprayed houses.
c. The whole house should be examined or if the house is too large, spend up to 15 minutes searching room by room. Pay special attention to rooms that people slept the previous night. With the aid of the flash light, look for mosquitoes on walls, on the ceiling, behind and under furniture, inside large pots and jars, and under beds. Conduct a systematic search of the house starting at the main door and searching to the left moving clockwise around the inside of the house.
d. Use separate cups for each house. Cups must be clearly labelled in pencil with at least the following information: locality, date and time of collection, minutes spent on collecting, house number or household head's name, type of structure (house, animal shelter, store, etc.), whether sprayed and if so, when, number of people and/or animals in the room during the previous night and the collector's name.

Keeping mosquitoes alive on the field

If mosquitoes are to be kept for some time on the field and during transport, the

following precautions should be taken to ensure they are intact:

a. Soak pieces of cotton wool in 8-10 % sugar solution, squeeze out excess sugar solution and place the cotton wool over the tops of the cups.

b. Place cups holding mosquitoes upright in a cardboard box or preferably in an insulated cold box so the mosquitoes do not become heated.

c. Cover the cups with a damp towel and keep the towel damp until the mosquitoes reach the insectary.

d. Make sure that mosquitoes are kept in places that are free from insecticide contamination and away from ants.

e. Before transport, pack newspaper or other material between the cups to minimize movement and drive slowly and carefully.

2.12.2.2 Pyrethrin spray collection of indoor resting mosquitoes

Pyrethrin spray collection involves using a pyrethrin space spray to knock down mosquitoes resting inside a house and collect them on white sheets spread on the floor and other at surfaces in the house prior to insecticide spray (WHO, 2013d, 2014). It is unlikely that all the mosquitoes resting in a house would be obtained using the hand collection method. However, using the spray sheet collection method, it could be possible to collect almost all the mosquitoes from a well-closed room sprayed with a fine mist of pyrethrin solution.

This method of collection allows quantitative studies to be undertaken including measurement of

1. indoor resting density (the number of mosquitoes resting indoors during the day)
2. human-biting density
3. seasonal changes in indoor resting density
4. number of mosquitoes remaining in a given room following a hand collection

This kind of collection technique cannot be used for sampling when bioassay studies are part of the targets of the study.

Equipment/materials needed for this collection method

White cotton sheets (sizes: 2 x 1 m, 2 x 2 m and 2 x 3 m); hand sprayers, aerosol insecticides, pyrethrin solution, kerosene, small Petri dishes, paper cups, hand lens, forceps, a container (or preferably a cold box) for transporting mosquitoes, cotton wool, filter paper and a torch will always be needed for this collection method. The hand sprayers should be of the double-action type with an air valve (Figure 2.4). The pyrethrin solution should be prepared at a concentration of 0.2 – 0.3 % in kerosene (WHO, 2013d).

Preparation of rooms for Pyrethrin spray collection

The technique is usually carried out by a team of three or four people so that required rooms (8-10) in each locality could be covered. Ensuring that all resting mosquitoes are disturbed as little as possible, prepare a room for spraying as follows:

1. Remove all people and animals.
2. Remove or cover all food, water and equipments
3. Remove all small items of furniture
4. Cover all openings and eaves with cloth or mosquito netting
5. Spread the white sheets so that they completely cover the floor and all flat surfaces of the remaining furniture; sheets should also be spread under tables, beds and other places where mosquitoes may hide.
6. Close all windows and doors before spraying to avoid target mosquitoes escaping from the house.

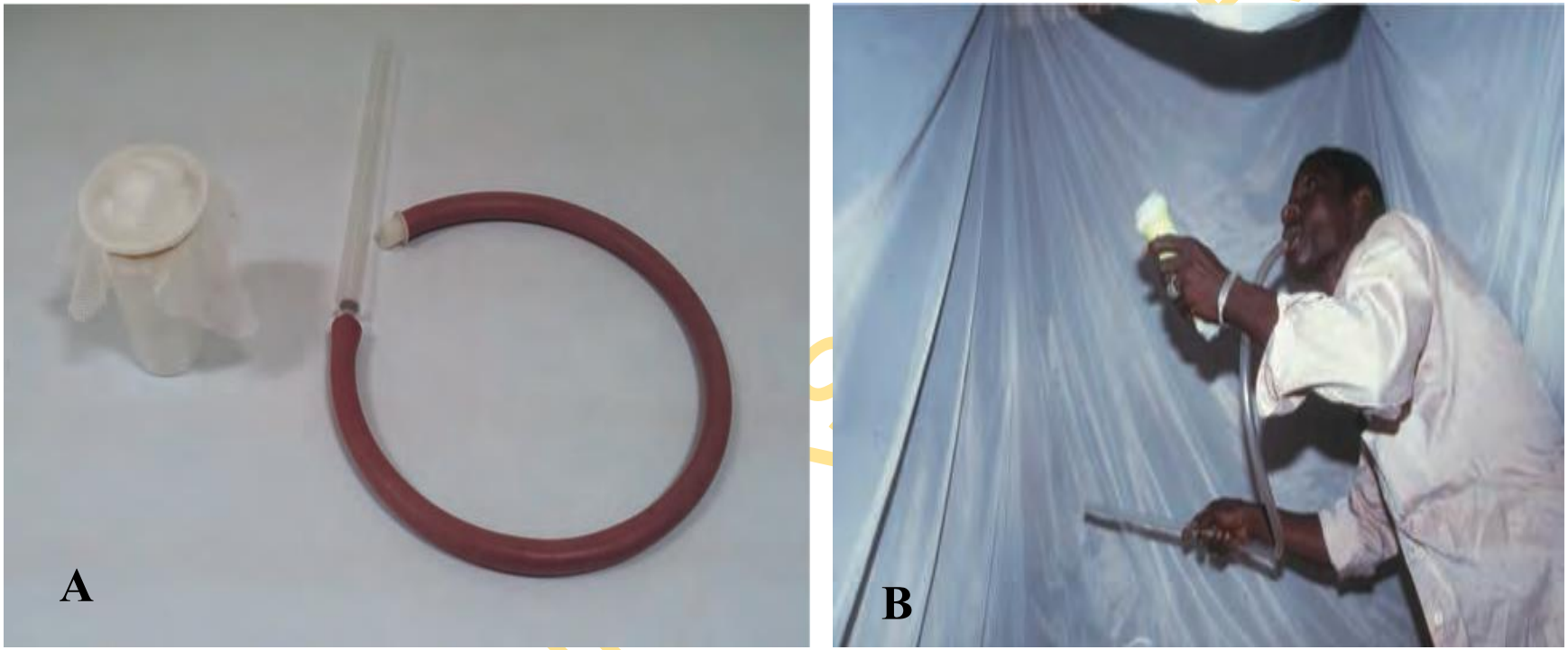


Figure 2.3: (a) Sucking tube and paper cup for hand collection of adult mosquitoes (b) Indoor mosquito collection using WHO aspirator
Source: WHO (2013).

Space spraying and collection of mosquitoes.

One of the team members should walk around the outside of the room and spray in open spaces or holes in the walls and eaves. Another member of the team should then enter the room, close the door and moving in a clockwise direction, apply spray towards the ceiling until the room is filled with a fine mist. The operator should leave the room quickly and make sure that the door remains closed for at least 10 minutes (WHO, 2013d). Starting from the doorway, pick up the sheets one at a time by their corners and carry the sheets outside (Figure 2.5a). Collect the knocked down mosquitoes outside in daylight using forceps. Place collected mosquitoes in a labelled Petri dish with a layer of damp cotton wool and filter paper on top of the cotton wool. Use separate Petri dishes for each house, and label the dishes with all the essential information (Figure 2.5b).

Other commonly used methods for indoor and outdoor collections of mosquitoes are described in detailed (WHO, 2013d).

2.13 The WHO Susceptibility Test for Adult Mosquitoes

Apart from the WHO susceptibility test, there are other standard bioassay methods such as the CDC Bottle bioassay method that are used to test the susceptibility of mosquitoes to insecticides (CDC, 2015). The WHO susceptibility bioassay is a direct response-to-exposure test; it measures mosquito mortality to a known standard dose of a given Insecticide (that is the diagnostic or discriminating concentration). Discriminating concentrations that are usually assessed have been established under standardized laboratory conditions for all insecticides (WHO, 1998).

2.13.1 Measuring Insecticides' Susceptibility in Adult Mosquitoes

WHO bioassay test procedure are stated below:

1. Sheets of clean white paper (12 x 15cm) rolled into a cylindrical shape are inserted into holding tubes and fastened into position with a steel spring-wire clip.

2. To test the susceptibility to insecticide, at least 120–150 active female mosquitoes are aspirated (in batches) from a mosquito cage into six holding tubes to give six replicate samples of 20–25 mosquitoes per tube including the control.

Figure 2.4: (a) A typical hand sprayer for mosquito collection (b) Hand spray in use.

Source: WHO (2013).

Figure 2.5: (a) Pyrethrin spray collection used for sampling indoor mosquitoes (b) Mosquito retrieved from spraying stored in petri dishes

Source: WHO (2013).

3. Once the mosquitoes have been transferred into each tube, the slide unit is closed and the holding tubes are set in an upright position for at least one hour. After one hour, the damaged insects are removed.

4. Exposure tubes are prepared in much the same way. The red-dotted tubes are always used for the insecticide-impregnated papers while the yellow/green-dotted tubes are used for control papers.

5. Empty tubes are attached to the vacant position on the slides and with the slide unit open, the mosquitoes are blown gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, the slide unit is closed and the holding tubes can be detached and set to one side. Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost for a period of 1 hour.

6. After 1hour exposure period, the mosquitoes are transferred back to the holding tubes. The exposure tubes are detached from the slide units and a pad of a cotton wool soaked in sugar water is placed on the mesh-screen end of the holding tubes.

7. Mosquitoes are maintained in the holding tubes for 24 hours (the recovery period). During this time, it is important to keep the holding tubes in a shady, sheltered place free from extreme temperatures (a standard insectary is ideal). Temperature and humidity should be monitored during the recovery period.

8. After 24-hour post-exposure, the number of dead mosquitoes in exposed and control set-ups are counted and recorded. An adult mosquito is considered to be alive if it is able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they have lost legs or wings, are considered moribund and are counted as dead.

9. On completion of the susceptibility test, mosquitoes may be transferred into individual, clearly labelled eppendorf tubes (separating dead and live mosquitoes into separate tubes) for storage at -20 ^{0}C and further analysis (WHO, 2013d).

2.13.2 Mosquito Specimens to use for Insecticide Susceptibility Test

The age, physiological status and gender of mosquitoes are factors that can influence the outcomes of susceptibility tests (WHO, 2013d, 2016c). The use of male mosquitoes is not always recommended for monitoring resistance because they are usually smaller and more fragile than female, therefore tend to have higher mortalities than normal. However, they can be used to determine the extent to which a mosquito species have developed resistance to an insecticide (Djouaka *et al.*, 2016).

In addition, susceptibility test focus on female mosquitoes because they are the mosquito sex that transmit diseases. Studies using adult female mosquitoes have repeatedly shown that both age and physiological status (i.e. unfed or blood-fed, semi-gravid or gravid) have a marked effect on insecticides susceptibility. It was observed that older mosquitoes are sometimes more susceptible to insecticides, especially when resistance is conferred by the presence of a detoxifying enzyme, the activity of which tends to decline with age. Consequently, it is recommended that susceptibility tests should be performed on non-blood fed females and age not more than 2 to 5-day post emergence (WHO, 2016c).

In the interest of obtaining age-standardized results, it is recommended that susceptibility tests be performed using either adult females derived from larval collections (the preferred option) or if larval collections are not possible, the F_1 progeny of wild-caught female mosquitoes (Cuamba *et al.*, 2010; Morgan *et al.*, 2010). If using larval collections, samples from the same place and the same type of breeding site may be pooled before testing in order to provide a sufficient number of test subjects (WHO, 2013d). However, larval collections should ideally be made from a number of different breeding sites in order to avoid sampling individuals from single egg batches, which might otherwise result in a high proportion of siblings in the test population.

Since the genotypic variability of the progeny of one adult female is likewise limited, wild caught females should also be collected from a number of different sections so as to ensure a broadly representative sample of the local population. In practice, this means that at least 30 batches of eggs, more if there is a mixture of species, should be harvested from the wild-caught females and incubated. When relying on larval

collections to provide young adult females for resistance monitoring, it is important to record the type of breeding site (e.g. rice field, rain water collection, irrigation channel, well) from which the larval collection was made.

The main advantage of using wild-caught females directly is convenience. Whereas, the disadvantage is that their age is unknown, which may lead to greater variation in susceptibility test results (and most likely an underestimation of resistance) depending on the species distribution and the insecticide being tested.

2.13.3 Required Sample Size and Standard Conditions for Bioassays

The required mosquitoes to conduct a single set of WHO bioassay tests for a single insecticide is between 120 to150 adult female mosquitoes; 100 will be exposed to the insecticide that is being tested (in 4 replicates each of around 25 mosquitoes or 5 replicates of around 20 mosquitoes). The remaining 20 or 50 will serve as controls (that is 1 or 2 replicates each of around 20 or 25 mosquitoes). The control mosquitoes are exposed to papers impregnated with the appropriate carrier oil only or a plane paper free from contaminations and insecticide treatment.

In all other respects, the control mosquitoes are treated in the same way as the exposed mosquitoes; they are tested simultaneously and under the same conditions. The purpose of the inclusion of the controls is to provide an estimate of natural mortality during the test and to account for all variables that may induce mortality other than the insecticide being tested (WHO, 2013d). When relying on pooled samples, mosquitoes should be provided with access to a 10 % sugar meal until the bioassay is carried out. Ambient temperature can influence the toxicity of insecticides; similarly, relative humidity has been shown to affect the survival of mosquitoes during the holding period. For standard experiment, it is ideal for tests and holding tube set-ups be placed at a temperature of 25 ± 2 °C and 80 ± 10 % relative humidity (WHO, 2013d, 2014). During the 1hour exposure period and the subsequent 24 hour holding period, both the temperature and relative humidity should be monitored.

The efficacy of impregnated papers declines with the number of uses and the number of mosquitoes tested (WHO, 2013d). This is especially true of the pyrethroid-impregnated papers. The current recommendation is that no insecticide-impregnated

Table 2.1: Discriminating concentrations of insecticides for adult Anopheline mosquito susceptibility tests (WHO 2017).

Insecticide class	Insecticide	Discriminating concentration (1-hour exposure period)
Organichlorines	DDT	4%
	Dieldrin	0.40%
		4%
Pyrethroids	Permethrin	0.75%
	Deltamethrin	0.05%
	Lambda-cyhalothrin	0.05%
	Cyfluthrin	0.15%
	Etofenapyr	1%
Carbamates	Propoxur	0.10%
	Bendiocarb	0.10%
	Carbosulfan	0.40%
Organophosphate	Malathion	5%
	Fenitrothion	1%
	Pirimiphos methyl	0.25%
Pyrroles	Chlorfenapyr	5%
Phenyl pyrazoles	Fipronil	2%

paper should be used more than 4 times, the equivalent of exposing around 100 mosquitoes of 25 mosquitoes per replicate (WHO, 2016c).

2.13.4 Calculation of Mortality and Knockdown Rates

Standard form for recording and reporting results of bioassays, both mortality and knockdown rate is available (WHO, 2013d). However, individuals can develop a suitable form to take recordings as long as it comprises all the required information needed for comprehensive reports. The assessment of mortality, that is the number of dead mosquitoes in both the exposure and the control tubes is made 24hour post-insecticide exposure (WHO, 2013d). Observed mortality after bioassays is calculated as below:

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

A similar calculation should be made in order to obtain a value for the control mortality. If the control mortality is above 20 %, the tests must be discarded (WHO, 2013d). When control mortality is greater than 5 % but less than 20 %, then the observed mortality has to be corrected using Abbots formula, as follows:

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

If the control mortality is below 5 %, it can be ignored and no correction is necessary. When reporting mortality counts, the sample size should always be given and preferably an estimate of the 95 % confidence interval.

Pyrethroids and DDT are fast-acting insecticides, which have a knockdown effect. When *kdr* is involved, the rate of knock down (KD) is a clear sensitive indicator for early detection of resistance. Observation of the number of KD mosquitoes is made during the hour-long exposure period (WHO, 2013d). It is recommended that observations are made at regular intervals, usually after 10, 20, 30, 40, 50 and 60 minutes into the exposure period, with the last observation just before transfer to the observation tube. However, in some cases, it is advisable to assess KD effect every 5

minutes, this gives more information about the mosquito's response to insecticides especially if resistance is high in the mosquito population.

From the observed KD counts, it is possible to calculate knockdown rates for 50% as well as 95% of mosquitoes (KD 50 and KD 95, respectively), either graphically using log-probit paper or by computer using a log time-probit statistical model. Although, the calculation of KD 50 and KD 95 values is a relatively simple procedure, these measures are not widely used for routine monitoring of susceptibility for operational purposes.

This interpretation of the result of bioassay according to WHO (2013) is as follows:

- Mortality in the range 98 - 100 % indicates susceptibility.
- If the observed mortality is between 90 - 97 %, it suggests suspected resistance.
- If mortality is less than 90 %, it indicates resistance.

2.14 Review of Molecular Techniques used for Insecticide Resistance Investigations

Several molecular techniques are used to investigate the mechanisms of insecticide resistance in mosquito vectors. They are not only useful for the molecular identification of mosquito species but also reveal sensitive information needed to understand why mosquitoes have developed resistance to insecticides (Riveron *et al.*, 2017).

Insects are very important economically and ecologically to human due to their various roles; as pests, decomposer in nutrition cycle, vector for the transmission of diseases and a source of medically and economically important products. To identify insects such as mosquitoes and for other research purposes, different molecular techniques are used. For each molecular technique, high quality DNA/RNA/protein is required, which could be extracted from mosquito samples using different techniques. The selection of DNA extraction technique is dependent on specimen under study, time required for extraction, economical considerations of technique due to reagents and equipment used for extraction and most importantly extracted DNA quality and quantity (Asghar *et al.*, 2015).

There are some techniques through which DNA was extracted in 20 min from coleopteran, Diptera and Hemiptera without any structural damage or discoloration (Castalanelli *et al.*, 2010). It was also found that DNA of different small insects with short storage time in 95 % ethanol can be separated more successfully by technique which is simpler, reliable, economical, needs less equipment and reagents. Techniques used for extracting DNA from insects have been well described (Asghar *et al.*, 2015; Psifidi *et al.*, 2015).

Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes (Lee *et al.*, 2012). Agarose is isolated from the seaweed genera Gelidium and Gracilaria, and consists of repeated agarobiose (L- and D-galactose) subunits 2. During gelation, agarose polymers associate non-covalently and form a network of bundles whose pore sizes determine a gel's molecular sieving properties. The use of agarose gel electrophoresis revolutionized the separation of DNA.

Prior to the adoption of agarose gels, DNA was primarily separated using sucrose density gradient centrifugation, which only provided an approximation of size. To separate DNA using agarose gel electrophoresis, the DNA is loaded into pre-cast wells in the gel and a current is applied (Lee *et al.*, 2012). The phosphate backbone of the DNA (and RNA) molecule is negatively charged, therefore when placed in an electric field, DNA fragments will migrate to the positively charged anode. Because DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight (EDVOTEK, 2014).

The leading model for DNA movement through an agarose gel is biased reptation, whereby the leading edge moves forward and pulls the rest of the molecule along. The rate of migration of a DNA molecule through a gel is determined by the size of DNA molecule, agarose concentration, DNA conformation, voltage applied, presence of ethidium bromide/medori green, type of agarose and electrophoresis buffer (Lee *et al.*, 2012). After separation, the DNA molecules can be visualized under ultraviolet light after staining with an appropriate dye.

2.14.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique in molecular biology that helps to

amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a single DNA sequence (Ehtisham *et al.*, 2016).

The classical PCR was developed in 1984 by the American biochemist, Kary Mullis, which was honoured with the Nobel Prize and the Japan Prize in 1993 (Bartlett and Stirling, 2003). However, the basic principle of replicating a piece of DNA using two specific primers (forward and the reverse primers) was later described by Gobind Khorana in 1971, but progress was limited due to primer synthesis and polymerase purification issues (Kleppe *et al.*, 1971). It was later fully developed and became a common and often indispensable technique in medical and biological research investigations (Mohini and Deshpande, 2010; Ehtisham *et al.*, 2016). It has rapidly become one of the most widely used techniques in molecular biology because it is quick, moderately expensive and simple. The reaction is highly specific and extremely sensitive enabling one copy of a sequence in a single cell to be detected.

PCR can be classified as follows:

a. Traditional/Classical PCR

Traditionally, PCR is performed in a tube and when the reaction is complete, the products of the reaction (the amplified DNA fragments) are analyzed and visualized through gel electrophoresis technique.

b. Real-time/Qualitative PCR

In molecular biology, real-time polymerase chain reaction also called quantitative real time polymerase chain reaction is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a target DNA molecule (Lee *et al.*, 2012). Real-Time PCR permits the analysis of the products when the reaction is actually in progress. This is achieved by using fluorescent dyes that react with the amplified product and can be measured with the instrument. This also facilitates the quantitation of the DNA. The PCR could therefore be used to determine the presence of a DNA sequence and the number of its copies in the sample. Real-Time PCR is considered to be a more rapid assay, since it is not necessary to perform electrophoresis or other procedure after the DNA amplification reaction.

2.14.1.1 Basic Concept of PCR

As the name implies, it is a chain reaction: one DNA molecule is used to produce two copies, then four, then eight and so on. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. Polymerases require a supply of DNA building blocks, the nucleotides consisting of the four bases: adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of artificial synthesized DNA, known as the primer to initiate replication, to which they attach the building blocks. Also, a longer DNA molecule to be replicated serves as a template for constructing the new strand. When these ingredients are provided, the enzymes will construct exact copies of the template, and this selectively indicates the amplification of a particular segment of DNA.

There are three major steps involved in the PCR technique, these include denaturation, annealing, and extension (YG, 2016). To amplify a segment of DNA using a simple/classical PCR, the sample is first heated so the DNA denatures or separates into two pieces of single-stranded DNA. DNA is denatured at high temperatures (usually between 90 - 97 ^{0}C). Then, a synthesized primer that is specific to the target segment anneals/binds with the template strand to initiate replication and prime the extension at a temperature of 50 - 60 °C.

In step three, there is extension of a new strand, which is initiated at the end of the annealed primers to create a complementary copy strand of DNA (at a temperature of around 72 °C). This third step is achieved by the use of a polymerase enzyme (Taq polymerase). This process results in the duplication of the original DNA with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies and so on.

2.14.2 Real-Time Polymerase Chain Reaction

Real-time PCR was developed in 1992, as a refinement of the original PCR created by Kary Mullis and represents a significant biotechnological breakthrough for the diagnosis of infectious and parasitic diseases (Garibyan and Avashia, 2013). This system is based on the use of dyes or fluorescent probes that permit the monitoring of the amplified product (Paiva-Cavalcanti *et al.,* 2010). A dye that is widely used for

this technique is the SYBR Green I, which binds non-specifically the duplexes of DNA generated during the amplification. Another fluorescence probe that is specifically targeted to a region of internal sequence that needs to be amplified is the TaqMan probe (Nagy *et al.*, 2017). During amplification, the TaqMan probe is degraded to release a reporter that emits light and a quencher that inhibits light.

The light emission analysis is made of a light signal detector that creates a graphic with the absorption obtained after each round of PCR, the generated signal reflects the amount of product formed. The cycle in which the limit of negativity or threshold exceeds is directly related to the amount of amplified DNA and is called cycle threshold (C_T) representing the point at which the emission of fluorescence in the sample test surpasses the background (Nagy *et al.*, 2017).

The qPCR results are recorded through interconnected computer graphics generated in the thermal cycler. Basically, there are four kinds of analysis that are carried out: amplification curve, dissociation curve, spectrum and component analysis (Promega, 2017). Through the amplification curve, the C_T of each sample may be checked, which is of crucial importance for the calculation of specificity (σ) and determination of the positive samples. The C_T values ≤ 29 indicates high amount of target nucleic acid; C_T values between 30 - 37 indicates moderate amount of target nucleic acid, while C_T values between 38 - 40 indicates low amount of target sequence.

The dissociation curve gives information on the emission of fluorescence by melting temperature (Tm). Non-specific amplification of primer dimers can be verified by this analysis (Promega, 2017). Spectrum assessment shows the capture of fluorescence by certain filters. SYBR Green I, for example is captured by filters A and B, while ROX (background ABI PRISM 7000® and 7500® Applied Biosystems, USA) is read by filters C and D (Applied Biosystem, 2010). If a positive reaction occurs, the florescent absorption by A and B filters should be greater than that of C and D, which remains constant. If SYBR Green does not exceed ROX, the reaction is always considered to be negative. The component shows the behavior of the fluorescence emission of each dye used during the reaction. Consequently, the background should remain constant and the dye used for amplification should outrange it according to how many PCR cycles are being carried out.

Positive standards and negative controls should be included in all reactions. Optimal behavior of standard samples during the chemical reactions ensures the results of the other samples. The qPCR allows the completion of four types of tests: absolute quantification, relative quantification, high melting resolution analysis and allelic discrimination analysis, which present different and varied applications (Nolan *et al.*, 2013). As a diagnostic tool, the absolute quantification can be used to detect infection and quantify its etiologic agent.

As defined, the standard curve is related to concentrations of DNA standards. Through these data, the software quantifies the target DNA in the test sample. The standard curve also provides a slope and composed of the points on the curve. This finding is important to calculate the efficiency of amplification (μ). High efficiency is associated with a slope of approximately 3.32 for each dilution of 10 of the target. A slope of -3.3 is connected with 100 % efficiency, which indicates that the number of amplified molecules doubles with each PCR cycle.

2.14.2.1 Chemistries of real-time PCR

Today, using fluorescence dyes to target nucleic acids in an experiment has improved the quality and accuracy of products in molecular biology. Both sequence specific probes and non-specific labels are available as reporters. Classical intercalators interfere with the polymerase reaction and asymmetric cyanine dyes such as SYBR Green I and BEBO have become more popular. Asymmetric cyanines have two aromatic systems containing nitrogen, one of which is positively charged connected by amethine bridge. These dyes have virtually no fluorescence when they are free in solution due to vibrations engaging both aromatic systems, which convert electronic excitation energy into heat that dissipates to the surrounding solvent.

However, the dyes become brightly fluorescence when they bind to DNA, presumably to the minor groove (Nygren *et al.*, 1998). In PCR, the fluorescence of these dyes increases with the amount of double stranded products amplified, though not strictly in proportion because the dye fluorescence depends on the dye base binding ratio, which decreases during the course of the reaction. The dye fluorescence depends also to some degree on the DNA sequence. But a certain amount of a particular double-stranded DNA target in the absence of significant amounts of other double-stranded

DNAs gives rise to the same fluorescence every time. Hence, the dyes are excellent for quantitative real-time PCR when samples are compared at the same level of fluorescence in absence of interfering DNA.

Although minor groove binding dyes show preference for runs of AT base-pairs (Jansen *et al.,* 1993), asymmetric cyanines are considered sequence non-specific reporters in real-time PCR. They give rise to fluorescence signal in the presence of a double stranded DNA including undesired primer–dimer products. Primer–dimer formation occurs in qPCR when the dye interferes with the formation of specific products because of competition of the reactions for reagents and may lead to erroneous readouts. This reaction can be controlled by the melting curve analysis after completing the PCR. The temperature is then gradually increased and the fluorescence is measured as function of temperature.

The fluorescence decreases gradually with increasing temperature because of increased thermal motion, which allows for more internal rotation in the bound dye (Nygren *et al.,* 1998). However, when the desired temperature is reached at which the double stranded DNA strand separates, the dye comes off and the fluorescence drops abruptly (Ririe *et al.,* 1997). This temperature, which is referred to as the melting temperature, Tm, is determined as the maximum of the negative first derivative of the melting curve. Since primer–dimer products typically are shorter than the targeted product, they melt at a lower temperature and their presence is easily recognized by melting curve analysis (Kubista *et al.,* 2006).

Labeled primers and probes are based on nucleic acids or some of their synthetic analogues such as the Peptide Nucleic Acids (PNA) and the Locked Nucleic Acids (LNA) (Costa *et al.,* 2004). The dye labels are of two kinds: (i) fluorophores with intrinsically strong fluorescence, such as fluorescein and rhodamine derivatives (Sjöback *et al.,* 1995), which through structural design are brought into contact with a quencher molecule and (ii) fluorophores that change their fluorescence properties upon binding nucleic acids. Examples of probes with two dyes are the hydrolysis probes, popularly called Taqman probes (Holland *et al.,* 1991), which can be based either on regular oligonucleotides or on LNA (Braasch and Corey, 2001), Molecular Beacons (Tyagi and Kramer, 1996; Tyagi *et al.,* 1998), Hybridization probes and the Lion probes (Caplin *et al.,* 1999). The dyes form a donor–acceptor pair, where the donor

dye is excited and transfers its energy to the acceptor molecule if it is in close proximity. Energy transfer and quenching are distance dependent and structural rearrangement of the probe or in the case of hydrolysis probes, degradation, change the distance between the donor and acceptor and hence, the fluorescence of the system (Kubista *et al.,* 2006). Probes based on a single dye, whose fluorescence changes upon binding target DNA include the LightUp probes (Svanvik *et al.,* 2000), AllGlo, simple and Displacement probes (Li *et al.,* 2002).

SYBR green I binds to the minor groove of double stranded DNA (dsDNA), emitting 1,000 folds' greater fluorescence than when it is free in solution (Wittwer *et al.,* 1997). Therefore, the greater the amount of dsDNA present in the reaction tube, the greater the amount of DNA binding and fluorescent signal from SYBR green I. Thus, amplification of DNA in the reaction tube is measured (Valasek and Repa, 2005).

The minor groove binding asymmetric cyanine dye, BEBO is tested as sequence non-specific label in real-time PCR (Bengtsson *et al.,* 2003). The intensity of the fluorescence of BEBO increases upon binding to double-stranded DNA, allowing emission to be measured at the end of the elongation phase in the PCR cycle. BEBO concentrations between 0.1 and 0.4 mM generated sufficient fluorescence signal without inhibiting the PCR. This dye has absorbance and emission wavelengths that can be detected on the FAM channel on most common real-time PCR platforms, and shows a strong fluorescence increase when bound to dsDNA.

Hydrolysis probes also called 5'-nuclease probes, because it possesses the 5'-exonuclease activity of DNA polymerase offer an alternative approach to the problem of specificity. These are likely the most widely used flourogenic probe format (Mackay, 2004) and are exemplified as TaqMan probes. In terms of structure, hydrolysis probes are sequence-specific dually fluorophore-labeled DNA oligonucleotides (Valasek and Repa, 2005). When the quencher and reporter are in close proximity, that is, they are both attached to the same short oligonucleotide, the quencher absorbs the signal from the reporter (Valasek and Repa, 2005). This is an example of fluorescence resonance energy transfer (also called Forster transfer) in which energy is transferred from a donor (the reporter) to an acceptor (the quencher) fluorophore.

During amplification, the oligonucleotide is broken apart by the action of DNA polymerase (5' nuclease activity) and the reporter and quencher separate, allowing the reporters' energy and fluorescent signal to be liberated. Thus, hydrolysis of the oligonucleotide results in an increase of reporter signal and corresponds with the specific amplification of DNA (Valasek and Repa, 2005). Examples of common quencher fluorophores include TAMRA, DABCYL, and BHQ, whereas reporters are more numerous, some of which are FAM, VIC, NED. Hydrolysis probes afford similar precision as SYBR green I, but they give greater insurance regarding the specificity because only sequence-specific amplification is measured (Wilhelm and Pingoud, 2003). In addition, hydrolysis probes allow for simple identification of point mutations within the amplicon using melting curve analysis (Valasek and Repa, 2005).

Molecular beacons are similar to TaqMan probes but are not designed to be cleaved by the 5' nuclease activity of Taq polymerase. These probes have a fluorescent dye on the 5' end and a quencher dye on the 3' end of the oligonucleotide probe. A region at each end of the molecular beacon probe is designed to be complementary to itself, so at low temperatures, the ends anneal, creating a hairpin structure. This integral annealing property positions the two dyes in close proximity, quenching the fluorescence from the reporter dye (Espy *et al.,* 2006). The central region of the probe is designed to be complementary to a region of the PCR amplification product.

At high temperatures, both the PCR amplification product and probe are single stranded. As the temperature of the PCR is lowered, the central region of the molecular beacon probe binds to the PCR product and forces the separation of the fluorescent reporter dye from the quenching dye. The effects of the quencher dye are obviated and a light signal from the reporter dye can be detected. If no PCR amplification product is available for binding, the probe reanneals to itself, forcing the reporter dye and quencher dye together, preventing fluorescent signal (Espy *et al.,* 2006). Typically, a single molecular beacon is used for detection of a PCR amplification product and multiple beacon probes with different reporter dyes are used for single nucleotide polymorphism detection. By selection of appropriate PCR temperatures and/or extension of the probe length, molecular beacons will bind to the target PCR product when an unknown nucleotide polymorphism is present but at a slight cost of reduced specificity. There is no specific temperature for thermocycling

requirement of molecular beacons, so temperature optimization of the PCR is simplified (Espy *et al.*, 2006).

2.14.2.2 Normalization with Reference Genes

Reference genes represent the most common method for normalizing quantitative real time PCR (qRT-PCR) data. Reference genes are often referred to as housekeeping genes with the assumption that they are expressed at a constant level in various tissues at all developmental stages and are unaffected by the experimental treatment (Hendriks-Balk *et al.*, 2007; Balogh *et al.*, 2008). The use of this endogenous control theory allows for controlling all stages of the experimental protocol; its expression reflects not only the quantity and quality of RNA used, but the efficiencies of the RT and PCR. The commonly used reference genes include actin, ribosomal protein 7 (RSP 7) and 18S rRNA. The other reference genes are PGK1, B2M, GAPD, HMBS, HPRT1, RPL13A, SDHA, TBP, UBC and YWHAZ (Vandesompele *et al.*, 2002).

The initial concentration of a target is usually derived from the C_T values, which are the number of amplification cycles that their corresponding curves cross the threshold line. This line is placed at the exponential phase, so as to be clearly distinguishable from background noise. For each sample, the C_T obtained for target and reference genes must be converted to normalized expression ratio. For this, various options are available; they are either integrated in the software provided with the various qPCR instruments or as described in the literature (Pfaffl, 2001).

The relative standard curve method requires a construction of range made from a series of dilutions of a reference sample for the target gene and reference gene. The range to generate the standard curves is obtained by expressing the C_T as a function of log of the initial concentration of cDNA. Concentration values for each point of the range can be set arbitrarily in accordance with the dilution factors. Therefore, the relative amount of a target is determined by the C_T interpolation with the standard curve. The standard expression of a gene of interest is determined by the following formula:

$$R = \frac{\text{Relative amount of the gene of interest}}{}$$

Relative amount of the reference gene

The normalized ratio of each sample is divided by the normalized ratio of the calibrator. Thus, the calibrator becomes the reference 1x, and all other samples are expressed as a ratio relative to the calibrator. The method of ΔΔCT uses a mathematical formula to calculate the ratio of expression of a target gene between two samples, normalized with reference gene. First, the differences, ΔCT between the values of C_T target gene and reference gene were determined for the test sample and control.

ΔCT (sample) = C_T (target sample) - C_T (reference sample)

ΔCt (control) = C_T (target control) - C_T (reference control)

Next, the $\Delta\Delta C_T$ between control and the sample is calculated: $\Delta\Delta C_T = \Delta C_T$ (control) - ΔC_T (sample)

Finally, the normalized ratio of expression of a target gene is determined by the formula: $2 - \Delta\Delta C_T$.

Unlike the relative standard curve method, where the amplification efficiency (E) of target and reference genes is directly taken into account when building ranges, the method of $\Delta\Delta C_T$ is assumed that the efficiencies of the two genes are equal to 100% (E = 2, with each cycle of the exponential phase, the concentration of PCR products is doubled). However, a difference in PCR efficiency of 3% (ΔE = 0.03) between the two genes results in an error of 47 % for the ratio of expression if E target < E ref and 209 % if E target > E ref after 25 cycles. In addition, the error increases exponentially with larger variations of efficiency and a greater number of cycles.

However, there are other models that take the efficiency of PCR target gene and reference gene into account. The most common is the model of Pfaffl (Pfaffl, 2001), where the relative expression ratio (R) of a target gene between a sample and control is determined by the formula below:

$$R = \frac{(E_{target})^{\Delta CTtarget\ (control - samples)}}{(E_{reference})^{\Delta CTreference\ (control - samples)}}$$

In Pfaffl model, the efficiency of PCR for a given gene is calculated from the construction of a calibration curve using the following formula: E = 10 [-1/ gradient]. This method gives a good estimate of effectiveness. However, this approach assumes that the amplification efficiencies between the diluted samples are identical, creating a linear relationship between C_T and amount of cDNA in the beginning. This modified form analysis was developed and was able to incorporate more accurate estimates and provided improved individual efficiency corrected calculation (Rao *et al.*, 2013).

The different models of normalization with reference genes therefore have all the advantages and disadvantages. At present, there is no time-honored method for the treatment of the results of RT-qPCR. Normalization to a reference gene is a simple method and frequently used because it can control many variables (Schmittgen and Livak, 2008; Rao *et al.*, 2013). Another advantage of reference genes as compared to total or ribosomal RNA is that the reference gene is subject to the same conditions as the mRNA of interest (Hendriks-Balk *et al.*, 2007).

What has become apparent over the years is that there is no single reference gene for all experimental systems. Quantified errors related to the use of a single reference gene as more than three-fold in 25% and more than six-fold in 10% of samples. It is important that reference genes must be carefully validated for each experimental situation and those new experimental conditions or different tissue samples require re-validation of the chosen reference genes (Balogh *et al.*, 2008). If inappropriate reference genes are used for normalization, the experimental results obtained can differ greatly from those using a validated reference gene. Validation of a reference gene requires removal of any non-specific variation in expression. Because of the inherent variation in the expression of reference genes, the use of multiple reference genes rather than one reference gene is recommended to ensure reliable normalization of real-time PCR (Hendriks-Balk *et al.*, 2007).

2.14.3 Microarray Experiment for Genome-wide Association/Expression Studies

Microarrays consist of a collection of nucleic acid sequences immobilized onto a solid support so that each unique sequence forms a tiny feature called a spot or target, which are deposited onto microarray slides (Nsofor, 2014). The size of these spots varies from one system to another but it is usually less than 200µl in diameter. A glass slide

acts as the solid support onto which up to tens of thousands of spots can be arrayed in a total area of a few square centimeters.

Preparation of DNA targets

DNA targets that will be used for microarray spotting must first be amplified and purified. Universal primers that are complementary to target sequences are used to amplify only part of the DNA target or clone (Nsofor, 2014). Amplifying only the target region reduces/removes the amount of sequences that might compromise hybridization specificity. The amplified DNA needs to be purified to remove enzymes, nucleotides and buffer components, all of which can interfere with the microarray analysis if present in target solution. Column purification methods like the Gel Band Purification Kit, GF PCR DNA or other available purification kits can be used. Whatever method that is used for amplification and purification, it is most important to verify that the amplified fragments are of the right size, do not contain other contaminating sequences and that they are present in the right quantity and quality.

Design of oligonucleotides

The design of oligonucleotide targets should take into account factors that influence the specificity and strength of hybridization with labelled probes in microarray experiments (Liu *et al.*, 2010). The specificity can be estimated by comparing the oligonucleotide sequence with known gene sequences printed on microarray probes. An optimal length for DNA targets should be between 300-800 nucleotides (Liu *et al.*, 2010; Nsofor, 2014). Fragments of this length can be efficiently attached to the microarray slide surface, where they form specific and stable hybrids.

Probe labeling

The sample that is to be analyzed, whether it is mRNA for a gene expression study or DNA derived from genomic analysis, is converted to a labelled population of nucleic acids using probes. Probes consist of several thousands of different labelled nucleic acid fragments (Liu *et al.*, 2010). Fluorescent dyes, especially the cyanine dyes Cy3 and Cy5, have been adopted as the predominant label in microarray analysis. Fluorescence has the advantage of permitting the detection of two or more different signals in one experiment. This has allowed investigators to perform comparative

analysis of two or more samples in one microarray. It has also increased the accuracy and throughput of microarray analysis over filter-based macroarrays in which only one radioactively labelled sample can be conveniently analyzed at a time.

Microarray hybridisation

In a microarray hybridisation, the labeled fragments in the probe are expected to form duplexes with their immobilized complementary targets (Kumar, 2009). This requires that the nucleic acids are single-stranded and accessible to each other. The number of duplexes formed reflects the relative number of each specific fragment in the probe as long as the amount of immobilized target nucleic acid is in excess and not limiting the kinetics of hybridisation. Two or more samples labelled with different fluorescent dyes can be hybridised simultaneously at each target spot. By measuring the different fluorescent signals associated with each spot, the relative abundance of specific sequences in each of the samples can be determined.

Microarray scanning and data analysis

Microarray scanners typically contain two different lasers that emit light at wavelengths, which are suitable for exciting the fluorescent dyes used as labels. A confocal microscope attached to a detector system records the emitted light from each of the microarray spots, allowing high-resolution detection of the hybridisation signals (Riveron *et al.*, 2014b). Despite their small size, microarray generates large quantities of data even from a single experiment. As a typical experiment will involve the use of several analyzed samples on replicate arrays, the use of computerized data processing is necessary in order to handle the amount of data generated and to gain maximum information from the experiment. This can be achieved by specialized software that extracts primary data from scanned microarray slide images, normalizes this data to remove the influence of experimental variation and finally manipulates the data so that biologically meaningful conclusions can be made.

Gene expression analysis with microarrays

A typical microarray gene expression analysis experiment compares the relative expression levels of specific transcripts in two unique different samples (Riveron *et al.*, 2014b). One of these samples is always a control and the other is derived from

cells whose response or status is being investigated (experimental sample). Each sample is labelled with a different fluorescent dye and equal amounts of the labelled samples are combined and hybridised on the microarray slide. The fluorescent signals corresponding to the two dyes are measured independently from each spot after hybridisation. After normalization, the intensity of the two hybridisation signals will be compared.

Microarray analysis does not give information about absolute gene expression levels in the samples. This is because the intensity of the fluorescent signals is not only proportional to the number of hybridised fragments but also to the length of these fragments and the number of fluorescent labels on each fragment, that is labelling density. As thes are determined by the unique nucleotide sequence of each gene and transcript, they will vary from gene to gene. If two samples have been labelled under similar conditions, the length and labelling density of specific transcripts will be similar in the two samples, making it possible to compare the relative abundance of the transcripts in the two samples. A strong hybridisation signal from microarray analysis does not necessarily correspond to a highly expressed gene; it could be derived, for example, from a gene that is expressed at a relatively low level but yields long, highly labeled probe fragments. Gene expression analysis using microarray techniques have been applied to numerous mammalian tissues, plants, yeast, bacteria and insects.

2.15 Sequencing Technique

Since the fundamental discovery of the structure of DNA (Watson and Crick, 1953) and the pioneering development of methods to detect the sequence of DNA bases by foundational approaches such as Maxam and Gilbert's technique (Maxam and Gilbert, 1977) and Sanger sequencing (Sanger and Coulson, 1975), the field of DNA sequencing has rapidly evolved in capacity, capability and applications. As with many technologies, advances across multiple fields were brought together to achieve routine sequencing at the genome scale. The development of the PCR (Saiki *et al.*, 1988), and the development of fluorescent automated DNA sequencing have enabled studies on the genomes of living organisms to be interesting with more understanding (Pareek *et al.*, 2011).

A lot of next-generation sequencing companies and technologies have been created ever since, and the corresponding field of bioinformatics has exploded as a major scientific and training discipline. Science is growing very fast and next generation sequencing technologies are currently the hottest topic in the field of human and animals' genomics researches (Pareek *et al.,* 2011). Early efforts at sequencing genes were painstaking, time consuming, and labor intensive. This situation began to change from the mid-1970s, when Frederick Sanger developed the then faster and more efficient techniques to sequence DNA. Indeed, this work was ground breaking that it led to his receipt of the Nobel Prize in Chemistry.

Meanwhile, a more robust sequencing technique known as the Next Generation Sequencing (NGS) is a type of DNA sequencing technology that uses parallel sequencing of multiple small fragments of DNA to determine sequence (Rizzo and Buck, 2012). In contrast to Sanger sequencing, the speed of sequencing and amounts of DNA sequence data generated with NGS, which is considered a high-throughput technology, are exponentially greater and are produced at significantly reduced costs (Voelkerding *et al.,* 2009).

Third-generation sequencing uses parallel sequencing similar to NGS, but unlike NGS, third-generation sequencing uses single DNA molecules rather than amplified DNA as a template (Attia and Saeed, 2016). However, the starting material that provides a template NGS analysis is double-stranded nuclear DNA, this can be obtained from a variety of cell types. One good advantage of third generation sequencing is that it potentially eliminates errors in DNA sequence introduced in the laboratory during the DNA amplification process (Schadt *et al.*, 2010).

NGS can be used to sequence every nucleotide in an individuals' DNA, or limited to smaller portions of the genome such as the exome or a pre-selected subset of genes (Mardis, 2008; 2010). Many second generation HT- NGS platforms are commercially available and the race for more additional platforms will soon emerge (Pareek *et al.,* 2011). NGS has helped to study the insect genome, especially mosquito genome, to detect potential mutations that may have influenced insecticide resistance**.** This approach helps to assess the target site of insecticides in resistant mosquitoes compared to control mosquitoes as a possible mechanism of mosquito resistance to insecticides.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site and Mosquito Collection

3.1.1 Study Site Selection

Before mosquito samples collection for this study commenced, several survey were conducted at different rural communities in Oyo and Ogun states to identify a suitable site for *An. funestus* breeding. During the survey, mosquitoes were collected between the hours of 06.00am to 12noon. Eventually, Akaka-Remo was selected due to the availability of *An. funestus.*

3.1.2 Study Site Description

The coordinates (longitude and latitude) of Akaka-Remo was determined with the use of a global positioning system device (Garmin, USA). Akaka-Remo (6°57′N, 3°43′E) is a rural community in Remo-North local government area of Ogun state in the southwest region of Nigeria (Figure 3.1). The village is about 71.4 km from Lagos and 50 km from Ibadan. This settlement is surrounded with a permanent medium-size slow moving stream called Erititi stream (Figure 3.2) that leads to the popular Ona river. Erititi stream, which is the main source of water at Akaka-Remo is surrounded with different vegetation such as bananas, vegetables, maize, shrubs, trees and crops bordering the water bodies at almost all locations in and outside the village (Figure 3.2); this makes the water body a suitable breeding site for *An. funestus*.

The inhabited area at Akaka-Remo is about 0.25 square kilometer. The main human tribes inhabiting this village are the Yorubas and a small community of Eguns. The main commercial activity in the community is agriculture, which has attracted the use of pesticides/insecticides. Pesticides are usually used to control pests that attack farm crops and produces. Houses inhabited in the village are mainly made of mud and a few made of cement blocks (Figure 3.3), which are constructed at an average of 5m away

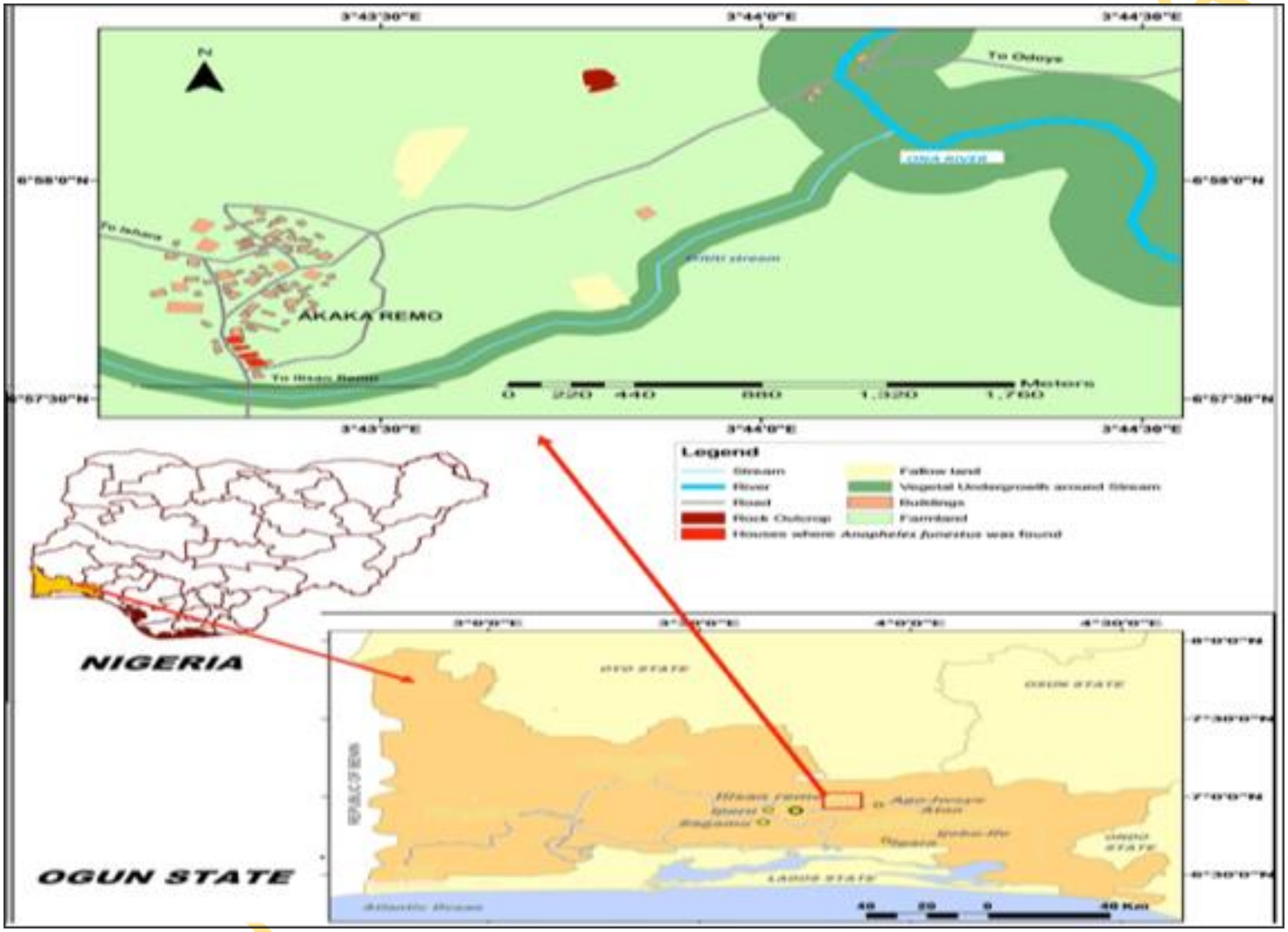


Figure 3.1: Akaka-Remo, Ogun state showing the proximity of stream and vegetation to residential houses

from one other. Most houses have either detached/destroyed or no ceilings. The selection criteria described in this section were mainly entomological as the main target for this study was to characterize only the population of malaria vectors in Akaka-Remo.

3.1.3 Mosquito collection

Only adult female *Anopheles* mosquitoes resting indoor were collected for different analysis. All houses in the village were first surveyed before using systematic random sampling method for selecting houses where mosquitoes were collected. Overall, thirty (30) rooms (twenty (20) houses) were selected, and the 30 rooms were chosen in a way to cover the various micro-ecologies at Akaka Remo. Mosquitoes samples were collected with the use of electric aspirators (Figure 3.4) and torches between the hours of 06.00 and 10.00 a.m. Samples were collected from October, 2014 (end of rainy season) to April, 2015 (beginning of rainy season) except in January, 2015 due to intense harmattan (a short period of a very dry and dusty wind observed between the end of November to early March in West Africa). Collection period was in the main climatic seasons in southwest of Nigeria: rainy season, transition from rainy to dry season, dry season and transition from dry to rainy season.

3.1.4 Ethical Statement

There was no ethical permit required for this study. Samples collected were only mosquitoes resting indoors in the early hours of the day (6-10 am). There was no form of contact with human and animals residing in houses throughout the period of the study. However, before mosquito survey and collection began, there was a focus group discussion (FGD) with the community and household heads. During this exercise, the study's aim, objectives and details of mosquito collections were thoroughly explained, questions were entertained and addressed. After being satisfied with the discussion, a go-ahead for mosquito sample collections, which also serves as a verbal consent to carry out the study in the community was given by both the community and household heads. Thereafter, the community unanimously delegated a member of the community development committee, who is also a health officer in the community health center as a facilitator. This individual was fully involved and assisted in accessing the target houses where samples were collected throughout the study period.

Figure 3.2: View of some vegetation surrounding Erititi stream at Akaka-Remo. (**a)** the only exposed point of the stream, where washing and bathing activities are carried out. **(b)** an example of how Erititi stream is covered with vegetation.

Figure 3.3: Common houses and ceilings at Akaka-Remo. **a**) Mud and un-cemented houses with no ceiling. **b**) mud and cement houses are always in sympatry at Akaka-Remo. **c**) another common ceiling at Akaka-Remo (mostly detached). **d**) few houses have ceiling and cemented walls at Akaka-Remo

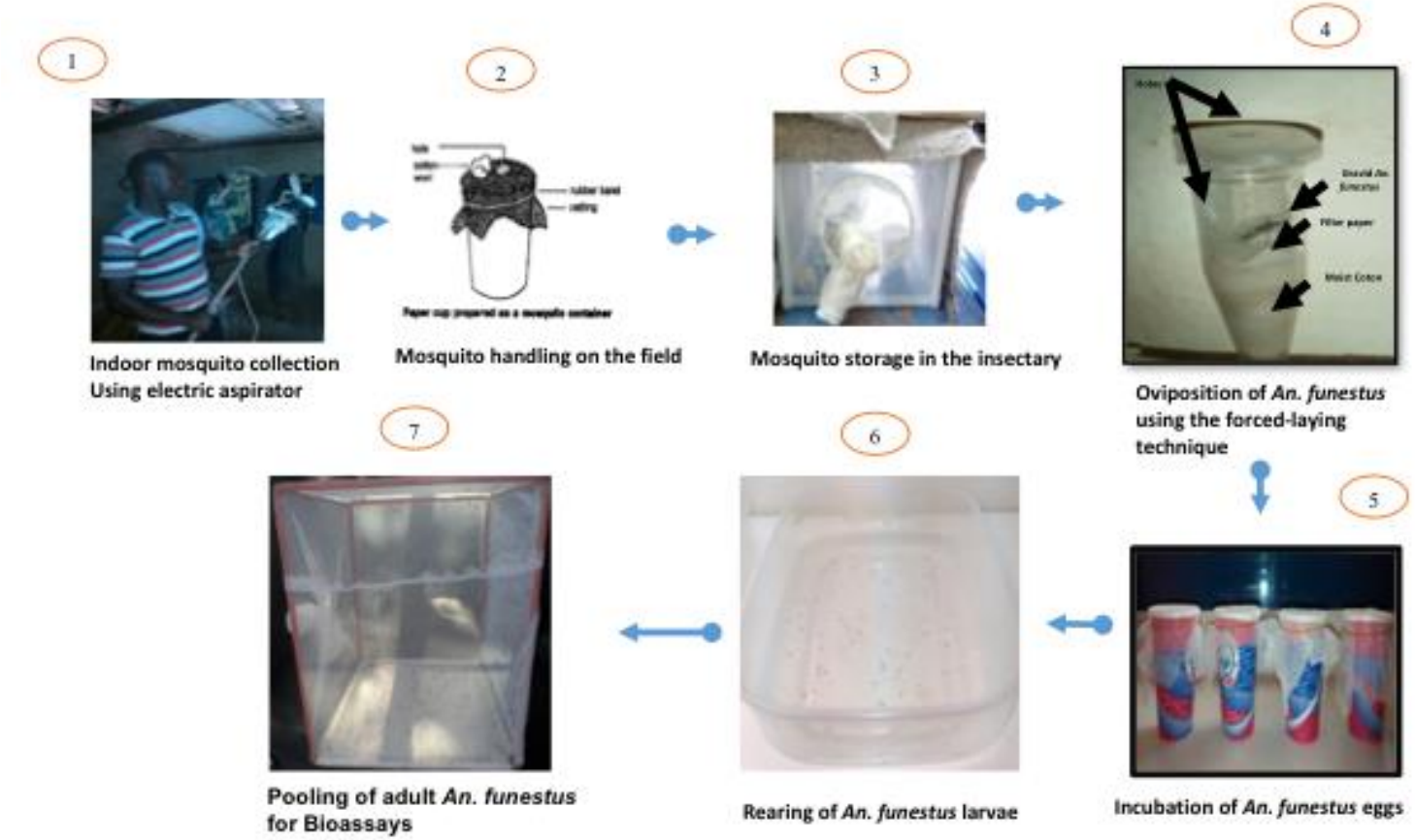


Figure 3.4: Different steps for handling *Anopheles funestus*: from indoor collection of adults through rearing to obtain first filial generations used for WHO susceptibility tests.

3.2 Morphological and Molecular Identification of Mosquitoes

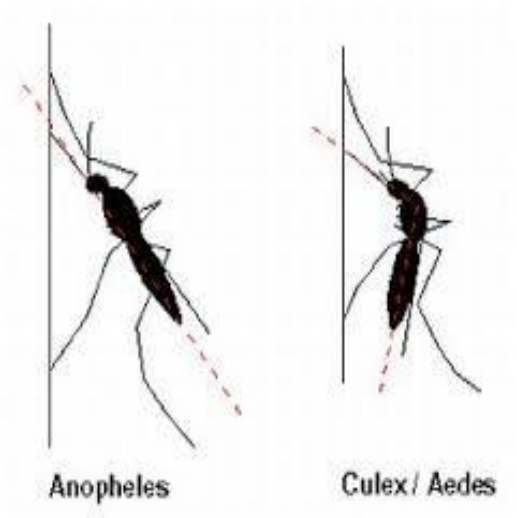
All available insects in the rooms were collected during aspiration, after which *Anopheles* mosquitoes were separated from other mosquitoes/insects collected using morphological identification keys (Gillies and Meillon, 1968). Resting position of the mosquito was used to differentiate *Anopheles* mosquito from other mosquitoes. Similar sizes of the three pale bands on the palp and fore/hind legs being entirely black in *An. funestus* were used to differentiate them from *An. gambiae* (Figure 3.5).

All *An. funestus* collected (both oviposited and non-oviposited) during the field study were further identified to sub-species level using molecular technique. PCR analysis was done to confirm the morphological identification of *An. funestus* mosquitoes and to further identify the sub-species of the *An. funestus* group. Three steps were involved in this technique: genomic DNA extraction, polymerae chain reaction and agarose gel electrophoresis.

3.2.1 DNA Extraction

Genomic DNA was extracted using the Livak protocol (Livak, 1984); (Appendix 1a) by adding 100 ml of lysing buffer containing NaCl, sucrose, HCl, EDTA and SDS (Appendix 1b-1c) to one mosquito sample per 1.5 µl eppendorf tube. Sterile laboratory pestles were used to grind and homogenise each mosquito in the test tube (Figure 3.6[1]). Homogenates were incubated at 65 °C in water bath for 30 mins, after which 14 µl 8 M potassium acetate was added to each sample for proper condensation before another 30 mins of incubation on ice. Samples were now centrifuged at 13,000 rpm (4 °C) for 20 mins to collect supernatants, which were transferred into a new 1.5 µl eppendorf tubes. A total of 200 µl of 100 % ethanol was later added for precipitation; solution in each test tube was gently mixed by vortexing and then re-centrifuged at 13,000 rpm (4 °C) for 15 mins. Supernatants were removed and discarded, the pellet was carefully rinsed with 100 µl ice cold 70 % ethanol before drying on the bench for 1 hr. After air drying, DNA extracts were re-suspended in 100 µl sterile distilled water (ddH_20), quantity of the extracts was measured using the nanodrop and samples were stored at -20 °C for PCR analysis.

1

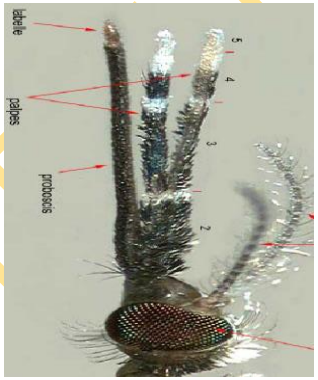


Resting position was used to differentiate *Anopheles* from other mosquitoes

Differentiation between *An. funestus* and *An. gambiae*

2

Anopheles gambiae



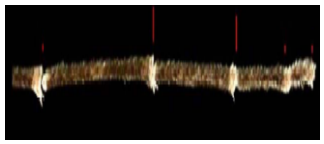
palp with 3 bands but the apical band is more conspicous

Anopheles funestus

palp with 3 bands of equal size

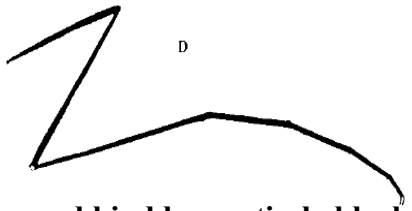
3

Anopheles gambiae



Fore legs with bands

Anopheles funestus



Fore and hind legs entirely black

Figure 3.5: Some standard keys used for morphological identification of mosquito species

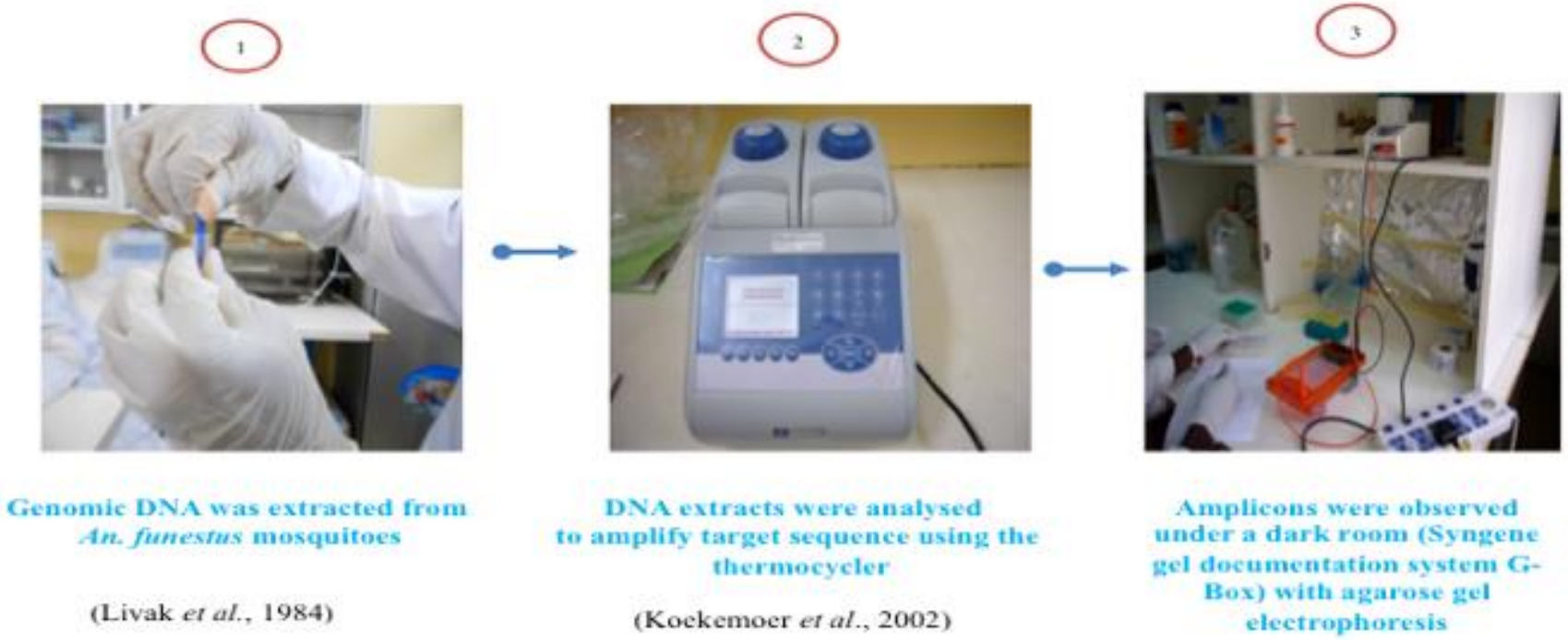


Figure 3.6: Main steps involved in the molecular identification of *Anopheles funestus* species.

3.2.2 PCR Analysis for *An. funestus* Speciation

Genomic DNA extracted from each *An. funestus* mosquito was used as template for the PCR analysis (Koekemoer *et al.*, 2002). The thermocycler (GenePro model B-480) was used to amplify target DNA fragments. Master mix prepared for each reaction contained 1.5 µl of PCR buffer A, 0.12 µl of 25 mM dNTPs, 0.12 µl of Kapa Taq, 0.9 µl of 25 mM $Mgcl_2$, 0.51 µl of each primer [Universal: forward (10 mM UV F) and *An. funestus* sub-species specific primers: *An. funestus s.s.* (10 mM Fun R); *An. vaneedeni* (10 mM Van R); *An. rivulorum* (10 mM RIV R); *An. parensis* (10 mM PAR R); *An. rivulorum*-like (10 mM RIVLIKE R); *An. leesoni* (10 mM LEES R)], 7.79 µl of ddH_20. The 14 µl master mix was added to 1.0 µl of each DNA template, making a total reaction volume of 15 µl in the PCR tubes loaded into the thermocycler (Figure 3.6^2, Appendix 2). Amplification reaction used for analysis on the machine was programmed at 94 ^{0}C for 2 mins for the initial denaturation; 35 cycles of 94 ^{0}C for 30 secs (final denaturation), 45 ^{0}C for 30 secs (primers annealing) and 72 ^{0}C for 40 secs (initial polymerase extension); and a final polymerase extension of 72 ^{0}C for 5 mins (Appendix 3).

3.2.3 Agarose Gel Electrophoresis for Viewing DNA Fragments

A 1.5 % agarose gel was used for the migration of PCR products. A 1.5 g agarose powder was dissolved in 100 ml of Tris-acetate-EDTA (TAE) buffer, heated to boiling temperature for proper dissolution. Gel solution was now left to cool at room temperature before adding 3 µl of medori green and gently swirled (Appendix 4). Medori green is less toxic compared to ethidium bromide and being a radioactive compound, it binds well to nucleotides and make them to fluoresce in the presence of ultraviolet light. After cooling, gel was carefully cast into the electrophoretic tank and was allowed to solidify at room temperature, wells were created on it with the use of electrophoretic combs. After 30 mins, 4-6 µl of PCR products were mixed with 2 µl loading dye into the different wells. Electrophoretic tank was then connected to electricity and set at 100 Voltage and allow running for a range of 30-45 mins. Molecular weight markers (ladders) ranging from 100 to 1000 base pairs (bp) were loaded at all wells at the extreme of the electrophoretic tank to interpret/determine the sizes of all sample bands (Table 3.1) and figure out the target *An. funestus* species DNA.

Table 3.1: Primers used for the molecular identification of members of *Anopheles funestus* group.

***Anopheles funestus* sub-species**	**Primer sequence**	**Molecular weight (base pair)**
***An. funestus* genus (UV)**	TGT GAA CTG CAG GAC ACA T	/
***An. funestus s.s.* (FUN)**	GCA TCG ATG GGT TAA TCA TG	505
***An. vaneeden* (VAN)**	TGT CGA CTT GGT AGC CGA AC	587
***An. rivulorum* (RIV)**	CAA GCC GTT CGA CCC TGA TT	411
***An. parensis* (PAR)**	TGC GGT CCC AAG CTA GGT TC	252
An. rivulorum like	CCG CCT CCC GTG GAG TGG GGG	313
***An. leesoni* (LEES)**	TAC ACG GGC GCC ATG TAG TT	46

UV universal primer: to amplify *An. funestus* genus fragment sequence; FUN: *An. funestus s.s.*; VAN: *An. vaneedeni*; RIV: *An. rivulorum*; PAR: *An. parensis*; LEES: *An. leesoni.*

3.3 Mosquito Rearing

Blood-fed mosquitoes retrieved from each collection were kept in experimental cups after morphological identification on the field. After each mosquito sampling, samples were immediately transported to the insectary of the International Institute of Tropical Agriculture (IITA), Benin. They were kept at 25–28 °C temperature and a relative humidity of 80 % until they became fully gravid, before subjecting them to the forced-egg laying technique (Figure 3.4) (Morgan *et al.*, 2010). The forced-egg laying technique is the method adopted to generate F_1 population from the parent (F_0) *An. funestus* collected from the wild. Eggs released on filter papers and cotton during oviposition were transferred into small cups until hatching, after which the emerging larvae were transferred into big rearing bowls (Figure 3.4).

By WHO standard, F_1 mosquito samples are best suitable for insecticides susceptibility studies (WHO, 2013d). Larvae retrieved after incubating eggs were reared in mineral water (FIFA water) and were daily fed with Tetramin baby fish. Rearing water was changed every 48 hours to reduce larvae mortality. Resulting adults were pooled into cages for WHO susceptibility tests (WHO, 2013e). Mosquito rearing, processing and bioassays were conducted at the insectaries of IITA-Benin and the vector Biology department of the Liverpool School of Tropical Medicine (LSTM), United Kingdom (UK). Some of the eggs collected from successful forced-egg laying activities at the insectary of IITA-Benin were sent via courier to LSTM for rearing into F_1 and subsequent experiments.

3.4 Mosquito Distribution

The number of mosquitoes collected during this study was enumerated. *Anopheles* mosquito density was sorted out and compared to other mosquito species that were collected. In addition, the density of *An. funestus* was compared with *An. gambiae*. Also, the density of *An. funestus* per room was determined by dividing the number of *An. funestus* collected during a period by the number of rooms surveyed in that period.

3.5 Estimation of *Plasmodium* Infection in Wild (F_0) *Anopheles funestus*

The Nested PCR and TaqMan assay were used to assess the *Plasmodium* infection rate of *An. funestus*. The TaqMan assay, a more recent method was used to

confirm/validate the result of the Nested PCR assay in case of likely under estimation of infection by Nested PCR.

3.5.1 Estimation of *Plasmodium* Infection Rate using the Nested PCR

Nested PCR (Snounou *et al.*, 1993) is a method that was designed to detect the four human malaria parasites (*P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae*) based on the sequence of their small subunit ribosomal RNA (ssRNA) genes. It involves three steps: DNA extraction, PCR analysis and agarose gel electrophoresis.

Genomic DNA extraction was done using the Livak protocol (Livak, 1984) as previously described (Appendix 1). This PCR method use a two-stepped analysis, each step was done according to the previously described analysis (Appendix 2). The first analysis was used to amplify the sequence of the *Plasmodium* genus (using a genus specific primer: Table 3.2a), while an aliquot of the product obtained was used for the second amplification of each specific parasite species using species specific primers (Table 3.2b). The second amplification reaction was only run for samples that had positive bands on agarose gel from the first amplification analysis.

For the first PCR reaction, 1 µl of the purified DNA extract was used as a template to amplify the fragment that spanned through rPLU5 and rPLU6 of the whole *An. funestus* mosquito genome. Similarly, 1 µl aliquot each from the PCR products of the first amplification was used for the second amplification, which involved four separate reactions. Each reaction test tube contained all the four species specific primer pairs. The PCR assays were performed using a heating block (PTC-100, MJ Research Inc., USA). The cycling parameters used for the first amplification reaction are 95 °C for 5 min for initial denaturation, 24 cycles of a final denaturation at 58 °C for 2 min, annealing at 72 °C for 2 min, initial extension at 94 °C for 1 min and a final extension at 72 °C for 5 min. On terminating the amplification cycle, the temperature was reduced to -20 °C to keep the PCR products in proper conditions before the second amplification. The second amplification was subjected into the same cycling parameters except for a 30 cycles (step 2-4) reaction instead of 24 cycles in the first reaction.

Agarose gel electrophoresis was run for all PCR products to estimate the target parasite sequence in each mosquito as previously described in Appendix 4. A fragment

Table 3.2a: Primers used for the *Plasmodium* genus identification (First amplification)

Genus specific primers	**Sequences**
Forward, F	5' - TTAAAATTGTTGCAGTTAAAACG -3'
Reverse, R	5'- CCTGTTGTTGCCTTAAACTC -3'

Table 3.2b: Primers used for the *Plasmodium* species identification (Second amplification)

***Plasmodium* species**	**primers**	**Primer sequence**
P. falciparum (FAL)	Forward, F	5' - TTAAACTGGTTTGGGAAAACCAAATATATT -3'
	Reverse, R	5' - ACACAATGAACTCAATCATGACTACCCGTC -3'
P. ovale (OVA)	Forward, F	5' - ATCTCTTTTGCTATTTTTTAGTATTGGAGA -3'
	Reverse, R	5' - GGAAAAGGACACATTAATTGTATCCTAGTG -3'
P. vivax (VIV)	Forward, F	5' - CGCTTCTAGCTTAATCCACATAACTGATAC -3'
	Reverse, R	5' - ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA -3'
P. malariae (MAL)	Forward, F	5' - ATAACATAGTTGTACGTTAAGAATAACCGC -3'
	Reverse, R	5' - AAAATTCCCATGCATAAAAAATTATACAAA -3'

of approximately 1.2 kb will correspond to the product of the first amplification reaction,while for the second reaction, the sizes of bands corresponding to the *Plasmodium* species are as follows: 205 bp for *P. falciparum*, 120 bp for *P. vivax*, 144 bp for *P. malariae* and approx. 800 bp for *P. ovale*.

3.5.2 Estimation of *Plasmodium* Infection Rate using the TaqMan Technique

Adult female *An. funestus* was also analysed for *Plasmodium* infection using the TaqMan assay as described by Bass *et al.* (2008). Genomic DNA extraction was first performed on whole mosquitoes using the Livak protocol (Livak, 1984) (Appendix 1). DNA extracts obtained was later used as template for TaqMan assay. TaqMan sensimix, (Applied Biosystems, California, USA), primers designed for *Plasmodium* genus target, minor groove binding (MGB) probes (Applied Biosystems, California, USA) for *Plasmodium* species targets, sterile water (St. Louis Missouri, USA) and template DNA were used for this assay.

Primer sequences that flank the probe binding site on the target *Plasmodium* genes are as follows: Forward, PlasF = 5'- GCTTAGTTACGATTAATAGGAGTAGCTTG -3' and reverse, PlasR = 5'- GAAAATCTAAGAATTTCACCTCTGACA -3'. The primers were designed to amplify only *Plasmodium* parasite sequence from mosquito genomic DNA extract while the probes were used to identify the specific *Plasmodium* species by producing fluorescence against specific *Plasmodium* species parasite. There were two MGB probes designed to bind and identify the specific *Plasmodium* parasite in each sample; the first probe, Falcip+ (5'- TCTGAATACGAATGTC -3') labeled with 6-FAM was designed to detect *P. falciparum* (*P. fal.)* while the second probe, *OVM+* (5'- CTGAATACAAATGCC -3') was labeled with VIC for the detection the combination of *P. malariae*, *P. ovale* and *P. vivax* (*P. OVM*).

Briefly, a total of 10 µl TaqMan PCR reaction containing 1 µl of genomic DNA, 5 µl of SensiMix (Quantace), 0.8 µl (800 nM) of each primer and 0.3 µl (300 nM) of probe PlasF and 0.2 µl 200 nM of probe OVM+ with 1.9 µl sterile ddH_20 were utilized. Reactions were run on a Rotor-Gene 6000TM (Corbett Research) using the temperature cycling conditions at 95 °C for 10 minutes, followed by 40 cycle reaction at 92 °C for 15 seconds and finally at 60 °C for 1 minute. The increase in VIC and FAM fluorescence was monitored in real time by acquiring each cycle on the yellow

(530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 emission) of the Rotor-Gene respectively. A negative control (water) and positive controls (known FAM and OVM) were used for discrimination between the infected and uninfected mosquitoes (Appendix 5).

3.6 Insecticide Susceptibility Tests

WHO susceptibility test (WHO, 1998) was conducted for all F_1 mosquitoes generated from forced-egg laying technique. According to WHO standard, 2–5 day old F_1 adult female and male mosquitoes pooled from different F_0 mosquitoes were used. A total of 20–25 mosquitoes per test tube (Figure 3.7) with at least 4 biological replicates were exposed to insecticide-impregnated (test groups) and insecticide free papers (control group) for 1 hr. After exposure, mosquitoes were immediately transferred into clean holding tubes free from insecticides, where they were fed with 10 % sugar solution. Mosquito samples were then monitored until mortalities were determined at 24 hrs post insecticide exposure (WHO, 2013e).

Six insecticides belonging to the four classes of insecticides used for malaria vector control were tested: the pyrethroids permethrin (0.75 %) and deltamethrin (0.05 %), the organochlorines DDT (4 %) and dieldrin (4 %), the carbamate bendiocarb (0.1 %) and the organophosphate malathion (5 %). After mortalities were determined at 24 hrs post exposure, susceptible (dead) mosquitoes were preserved singly inside eppendorf tubes on silica gel and kept at 4 ^{0}C while resistant (alive) mosquitoes were preserved on RNA later (ThermoFisher Scientific) in pools of 10 and kept at -80 ^{0}C for subsequent experiments. However, resistant mosquito samples generated from bioassays at LSTM were stored directly at -80 ^{0}C without the use of RNA later.

3.7 Investigating Potential Resistance Mechanisms of *An. funestus* to Insecticides

Target-site and metabolic resistance were explored as possible mechanisms used by *An. funestus* population to withstand the lethal doses of insecticides. The involvement of the most common mechanism in *An. gambiae* populations, Knockdown resistance mechanism was first analysed to detect the presence of a point mutation on position 1014 of the voltage-gated sodium channel (VGSC), the target site of pyrethroids and DDT insecticides. Mutation on VGSC region changes leucine to phenylalanine

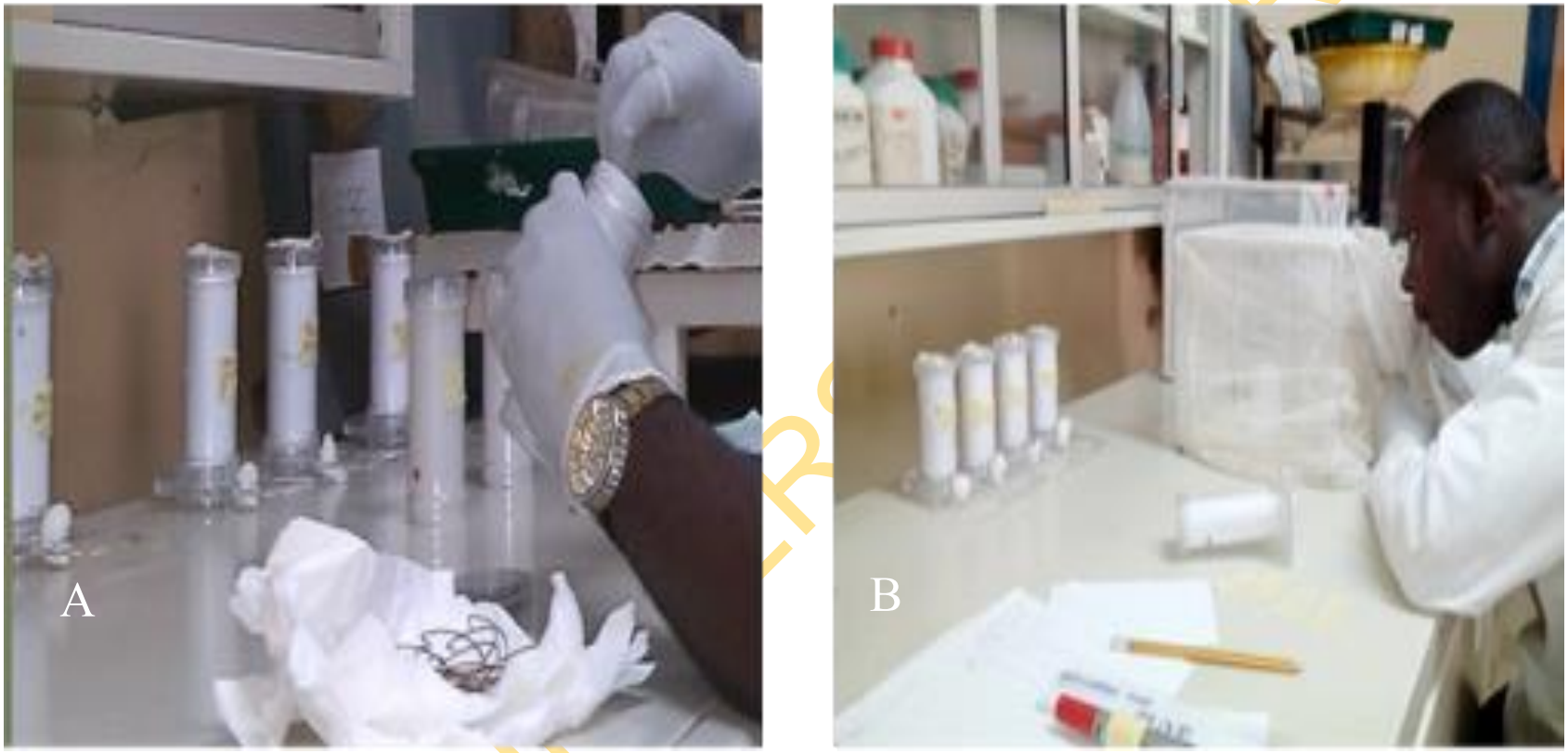


Figure 3.7: (a) Preparation of WHO susceptibility test tubes for Bioassays **(b)** Transfering pooled *Anopheles funestus* mosquitoes into test tubes.

(L1014F) or serine (L1014S). For Metabolic resistance, a preliminary study where the implication of detoxification enzymes in insecticides resistance using the synergist assay was assessed (Brooke *et al.,* 2001). Furthermore, a genome wide transcriptional analysis coupled with a reverse transcriptase polymerase chain reaction (RT-PCR) were used to quantify and implicate overexpressed detoxification transcripts in resistant mosquitoes as a possible mechanism for insecticide resistance.

3.7.1 Knockdown resistance Mutation (Kdr) Analysis in Wild F_0 *An. funestus*

Anopheles funestus (F_0) collected from Akaka-Remo, kept on silica gel and stored at -20 °C were used for this analysis. Genomic DNA was first extracted from all the mosquitoes used as previously described (Livak, 1984). A fragment, referred to as the Ex 20: domain II, segment 6 that spans the exon 20 of the VGSC, containing the 1014 codon associated with *kdr* in *An. gambiae* (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000) was amplified from the extracted DNA of ten wild female *An. funestus* mosquitoes. Amplification was performed using the KdrFunR2 primer (5'- CCG AAA TTT GAC AAA AGC AAA -3') (Cuamba *et al.,* 2010; Mulamba *et al.,* 2014). PCR products that were successfully amplified were purified using the Qiaquick purification kit (QIAgen, Hilden, Germany; Appendix 6) and were subsequently sent to University of Cambridge, London for sequencing.

3.7.2 Glutathione-S-transferase Mutation Analysis in Wild F_0 *Anopheles funestus*

Six female *An. funestus* (F_0), kept on silica gel and stored at -20 °C were used for this analysis. Genomic DNA was first extracted from all mosquitoes as previously described (Livak, 1984). A full-length of *GSTe2* (exons and introns) was amplified from female *An. funestus* mosquitoes using Phusion High-Fidelity DNA Polymerase (Fermentas, Burlington, Ontario, Canada) and the following thermal conditions: 1 cycle at 95 °C for 5 mins; 35 cycles at 94 °C for 20 secs, 57 °C for 30 secs and 72 °C for 60 secs; and 1 cycle at 72 °C for 5 mins. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and were subsequently sent to University of Cambridge, London for sequencing.

3.7.3 Genotype Analysis of L119F-GSTe2 and A296S-RDL Resistant Markers.

Insecticide resistance markers, L119F-GSTe2 and A296S-RDL were genotyped in female *An. funestus*. A custom TaqMan assay was used to genotype L119F-GSTe2, as a potential DDT resistance marker and A296S-RDL, as a potential dieldrin resistance marker in *An. funestus*. This assay is similar to what was previously described in section 3.5.2 (Bass *et al.,* 2008) to determine the *Plasmodium* infection rate of *An. funestus*. For genotyping analysis, universal primers and allele specific (for either resistant, R or susceptible, S allele) probes were used to separately genotype for potential mutations on the *GSTe2* and *Rdl* genes. Both F_0 and F_1 *An. funestus* were used for the analysis.

Sensimix, (Applied Biosystems, California, USA), minor groove binding (MGB) probes mix (Applied Biosystems, California, USA), sterile water (St. Louis Missouri, USA) and template DNA were used. The two fluorophore-labelled specific TaqMan probes were used: FAM was used to detect the homozygous resistant genotype (RR), HEX to detect the homozygous susceptible genotype (SS), with both FAM and HEX used to detect the heterozygous genotype, RS. A negative control (water) and positive controls [known FAM to detect homozygous resistant; known HEX to detect homozygous susceptible and both FAM and HEX to detect heterozygous population] were also used in a 10 µl volume reaction.

The reaction volume constituted of 5 µl 1x sensimix (Bioline, London, UK), 0.0625 µl probe mix (160x), 3.9375 µl ddH_20 and 1 µl of the template DNA. The reaction cycle conditions set in the Agilent MX3005P machine were 10 mins at 95 °C for DNA denaturation followed by 40 cycles at 92 °C for 15 secs (probes annealing and fluorescence) and 60 °C for 1 mins (reaction extension). The increase in VIC/HEX and FAM fluorescence was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channels (470 nm excitation and 510 emission) of the Rotor-Gene respectively. The endpoint fluorescence of the analysis was evaluated using the Agilent MXPro software; the relationship between the frequency of resistant alleles and insecticides (DDT and dieldrin) resistance phenotypes was evaluated.

3.7.4 Preliminary Assessment of Metabolic Resistance in *Anopheles funestus* using Synergist Tests

Three synergists: piperonyl butoxide (PB), S,S,S-tributyl phosphorotrithioate (STP) and Diethyl maleate (DM) were assessed. The F_1 *An. funestus* mosquitoes were pre-exposed to PB, STP and DM synergists before exposing the mosquitoes to insecticdes, to implicate the roles of oxidase, esterase and GSTs, respectively in insecticide resistance. This test was conducted due to the level of resistance observed in *An. funestus* to DDT, dieldrin and permethrin insecticides. Two different groups of 2–5 day old F_1 adult *An. funestus* were used including control groups (WHO, 2013e).

Group 1: This consist of *An. funestus* mosquito (20 per test tube) that were independently pre-exposed to different test tubes containing 4 % PB, 0.25 % STP and 8 % DM treated papers for 1 hr and were immediately transferred into another test tubes containing 0.75 % permethrin, 4 % DDT and 4 % dieldrin treated papers for 1 hr. This group is referred to the synergised group.

Group 2: The second group consists of mosquito samples that were only exposed to the three insecticides (Permethrin, DDT and dieldrin) used for the synergised group. This group is referred to as unsynergised group.

Two controls were used, control 1 consisted of *An. funestus* mosquitoes exposed to papers neither treated with insecticides nor synergists, while control 2 consisted of *An. funestus* mosquitoes exposed to papers treated with only synergist compounds. Mortalities observed with synergised group were then compared with the unsynergised group 24 hrs post-exposure considering the controls.

3.7.5 Microarray Experiment for the Screening of Candidate Metabolic Transcripts in Insecticide Resistance of *Anopheles funestus*

This analysis only focused of the screening of candidate metabolic genes overexpressed in permethrin and DDT resistant *An. funestus*. This genome-wide transcriptional analysis was carried out to identify set of genes that were differentially overexpressed in insecticide resistance mosquitoes as a possible mechanism for the multiple phenotypic resistance observed in *An. funestus* mosquitoes at Akaka-Remo. The flow chart of the steps involved in this analysis is presented in figure 3.8.

3.7.5.1 Composition of the Microarray Chips

The Agilent *An. funestus* chip, 8 x 60k (60mer) designed using the eArray programme (Agilent: A-MEXP-2374) was used for the microarray hybridization analysis. This chip contains the 4 x 44 array (A-MEXP-2374) (Riveron *et al.*, 2013) plus an additional 15,527 Expressed Sequence Tags (ESTs) generated from a transcriptome sequence analysis of *An. funestus* (Crawford *et al.*, 2010). Overall, each array is incorporated with 60 mer probes designed from 8,540 ESTs (2 probes for each EST) generated from *An. funestus* transcriptome 454 sequencing (Gregory *et al.*, 2011), a set of 2,850 *An. funestus* cDNAs from GenBank (2 probes for each EST), a set of P450 genes (3 probes for each gene) from the *rp1* and *rp2* QTL BAC sequence (Wondji *et al.*, 2009; Irving *et al.*, 2012) and the 13,000 transcripts of the complete *An. gambiae* genome. In addition, all of the *An. gambiae* detoxification genes present on the *An. gambiae* detox chip (David *et al.*, 2005) were added with 3 probes for each gene to explore all possible conserved gene sequences between *An. gambiae* and *An. funestus*.

3.7.5.2 Target Preparation: RNA Extraction, cDNA Synthesis and cRNA labeling

Three mosquito populations used for this experiment were defined as: resistant population, alive mosquitoes after exposure to 0.75 % permethrin (Permethrin resistant, R_{perm}) and 4 % DDT (DDT resistant, R_{DDT}); unexposed to insecticides (Control, C); fully susceptible laboratory strain FANG (Susceptible, S).

Total RNA was extracted from 3 batches of a pool of 10 *An. funestus* mosquitoes for each population using Picopure RNA Isolation Kit (Arcturus). Extraction was initiated by grinding samples in 100 µl extraction buffer in eppendorf tubes, which were immediately incubated at 42 ^{0}C for 30 mins (Appendix 7). Samples were now centrifuged at 14,000 (fresh samples stored directly without RNA later) or 16,000 rcf (samples stored on RNA later) for 2 mins, to remove supernatants into fresh 1.5 µl tubes without disturbing the pellet. A 100 µl volume of 70 % ethanol was added to each sample and mix by pipetting, before transferring samples into already prepared conditioning columns. Columns were centrifuged at 100 rcf for 2 mins to bind DNA on to columns, after which were immediately centrifuged at 16,000 rcf for 30 secs (the flow through was discarded). Columns were later subjected to several washing using wash buffers provided in the kit. Conditioning column was placed on a new 0.5 ml

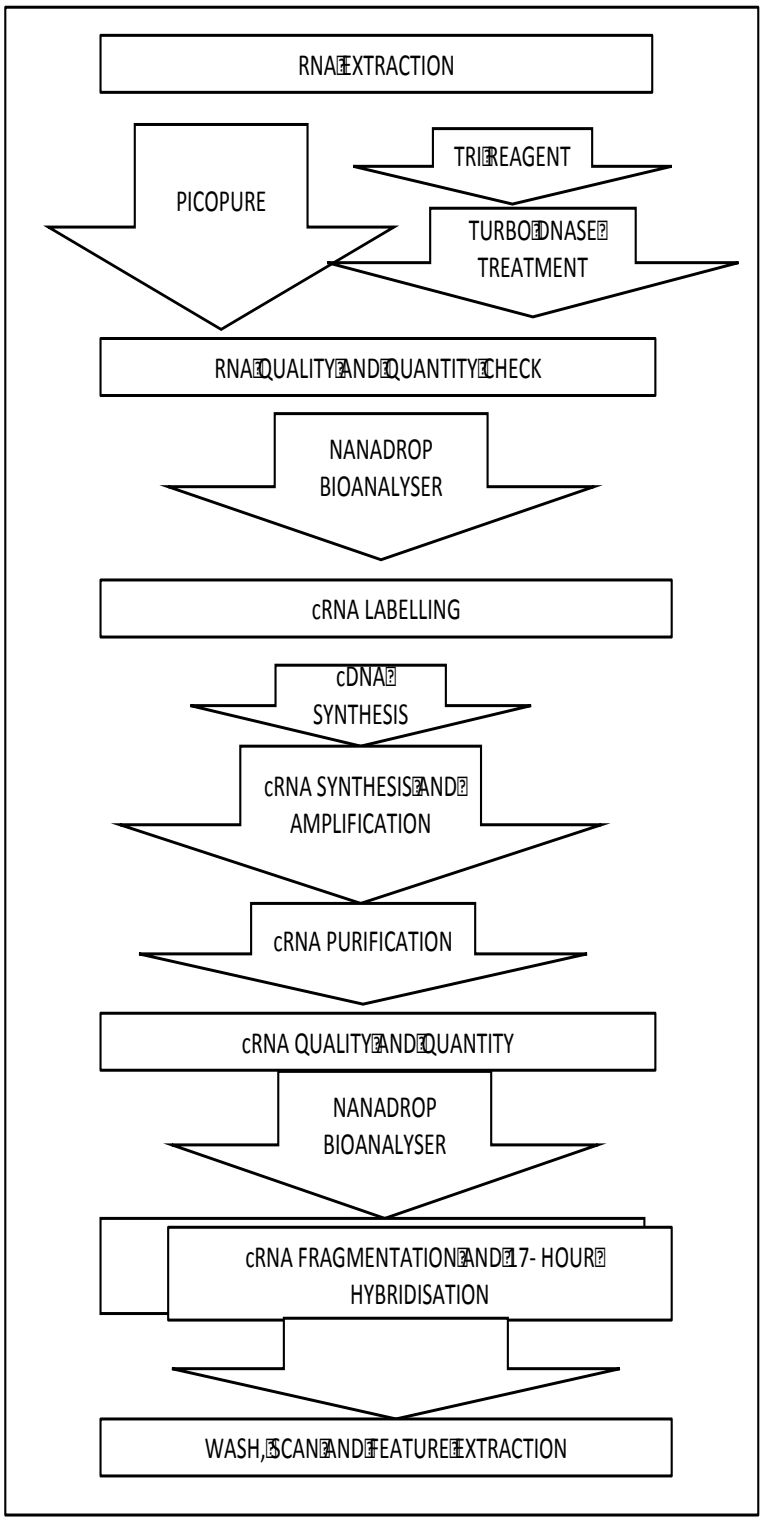


Figure 3.8: Flow chart of the steps used for microarray experiment

(provided in the kit), 30 µl volume of the elution buffer was introduced on to each membrane and left for 1 min at room temperature. Column placed on 0.5 ml tubes were centrifuged at 1,000 rcf for 1 min to distribute the elution buffer onto the column. Sample tubes were immediately centrifuged at 16,000 rcf for 1 min and eluted RNA were kept on ice for subsequent experiments or stored at -80 ^{0}C.

3.7.5.3 Total RNA Quantity and Quality Check

The quantity and quality of extracted RNA samples were measured using nanodrop, ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively.

Dye concentrate and gel aliquot were equilibrated in the dark at room temperature for 30 mins to measure the quality of total RNA. Dye mix was later vortex for 10 secs and spin briefly before adding10 µl of the dye mix to 65 µl gel aliquot. Total mix was thoroughly mixed by vortexing and centrifuged at 14,000 rpm for 10 mins. Tube was protected from light by covering with foil and stored at room temperature until use. Total RNA and ladder aliquot samples kept on ice to be measured were diluted to 50 ng and denatured in the thermocycler at 70 ^{0}C for 2 mins before samples were run on the bioanalyser (Appendix 8).

For total RNA quantity checked on nanadrop, 1 µl of each sample was introduced into each well and samples were measured as described in Appendix 11. Only samples that were still of good quantity and quality were used for microarray analysis.

3.7.5.4 Complementary RNA Labeling

All triplicate samples of different population were labeled with two different fluorescence dyes. The cyanine 3-CTP (Cy3), which is red/pink colour but fluoresced green and the cyanine 5-CTP (Cy5), which is a blue colour but fluoresced red was used for cRNA labeling. Complementary RNA (cRNA) from each extracted RNA pool was amplified by labeling resistant samples (R) with cy5 dye and susceptible strain FANG samples with cy3 using Agilent Quick Amp Labeling Kit (two-color) following the manufacturer's protocol. All the materials/steps used for RNA preparation, labeling chemical reaction and the method used to purify amplified cRNA are detailed in Appendixes 9-10.

The quality (Bioanalyser) and quantity (nanodrop) of labeled cRNA samples were further assessed as stated in 3.7.5.3.

3.7.5.5 Fragmentation and Hybridisation of Target Transcripts

Concentration of each cRNA measured with nanadrop was made up to the required 300 ng needed for fragmentation and hybridisation (Appendix 12). Samples fragmentation was done for 30 mins before the 2x GE hybridisation buffer (provided in the kit) was added. Samples were mixed by vortexing and spin at 13,000 rpm (room temperature) for 1 min. Sample mixtures were carefully dispensed into center of the gasket chambers, after which gaskets were carefully placed on microarray slides. The hybridisation assembly was firmly tightened and placed into the hybridization oven to hybridise at 65 ^{0}C for 17 hrs.

Overall, five hybridisations were done for permethrin comparisons, which comprised of three biological replicates (R_{perm}-S, R_{perm}-C and C-S) and two dye swaps. Whereas, for DDT comparisons, four hybridisations were done, this comprised of two biological replicates (RDDT-S and C-S) and two dye swaps.

3.7.5.6 Microarray Washing and Scanning

After hybridisation, each array was carefully detached from the gasket sandwich inside the wash buffer 1. Several other washing, fixing and drying of the array were done in a fume hood (Appendix 13). Array scanning and extraction of gene transcripts features was done using the Agilent scan control software.

3.7.6 Analysis of Insecticide Resistant-Associated Genes using the Reverse Transcriptase PCR (RT-PCR)

Further investigation using the RT-PCR was carried out to validate the expression patterns of some overexpressed genes identified with the microarray analysis. Some other resistant-associated genes that were not overexpressed with the microarray analysis were also assessed.

In RT-PCR analysis, expression levels of target genes in the resistant populations were compared with the control populations (Wondji *et al.*, 2009; Gregory *et al.*, 2011; Riveron *et al.*, 2014a; Samb *et al.*, 2016). Detoxification genes analysed are as

follows: Glutathione-S-transferase family (*GST2e, GSTd3, GSTd1-5*); Cytochrome P450 genes (*CYP6P9a, CYP6P9b, CYP6P4a, CYP6P4b, CYP6M7, CYP6AA1, CYP4C27, CYP9K1*); Aldehyde oxidase (*Ald oxi*) and trypsin.

This experiment was conducted using three batches of a pool of 10 resistant (permethrin: R_{perm} and DDT: R_{DDT}), 10 unexposed control (C) and 10 susceptible strain FANG (S) mosquitoes (Riveron *et al.*, 2013). There are three steps involved in this analysis: RNA extraction, complementary DNA synthesis and reverse transcriptase PCR amplification.

3.7.6.1 RNA Extraction

Total RNA was extracted from each pool of mosquitoes using the Picopure RNA Isolation Kit (Arcturus) as described in appendix 9.

Total RNA was extracted from 3 batches of a pool of 10 *An. funestus* mosquitoes for each population using Picopure RNA Isolation Kit (Arcturus). Extraction was initiated by grinding samples in 100 µl extraction buffer in eppendorf tubes, which were immediately incubated at 42 ^{0}C for 30 mins (Appendix 8). Samples were now centrifuged at 14,000 (fresh samples stored directly without RNA later) or 16,000 rcf (samples stored on RNA later) for 2 mins to remove supernatants into fresh 1.5 µl tubes without disturbing the pellet. A 100 µl volume of 70 % ethanol was added to each sample and mix by pipetting, before transferring samples into already prepared conditioning columns. Columns were centrifuged at 100 rcf for 2 mins to bind DNA on to columns and were immediately centrifuged at 16,000 rcf for 30 secs (the flow through was discarded). Columns were later subjected to several washing using wash buffers provided in the kit. Conditioning column was placed on a new 0.5 ml (provided in the kit), 30 µl volume of the elution buffer was introduced on to each membrane and left for 1 min at room temperature. Column placed on 0.5 ml tubes were centrifuged at 1,000 rcf for 1 min to distribute the elution buffer onto the column. Sample tubes were immediately centrifuged at 16,000 rcf for 1 min and eluted RNA were kept on ice for DNA synthesis or stored at -80 ^{0}C.

3.7.6.2 Complementary DNA synthesis (cDNA synthesis)

Complementary DNA samples needed for RT-PCR analysis were synthesised from each extracted RNA. The RNA concentration of each sample measured with nanadrop (appendix 12a), was diluted to make up the 8 µl volume required for cDNA synthesis (Appendix 14). A total volume of 13 µl containing 1 µl of oligodT20 (50 mM), 8 µl diluted RNA, 1 µl 10 mM dNTP mix and 3 µl sterile water was the first reaction mix step. Afterwards, 7.5 µl reaction mix containing 4 µl 5x first strand buffer, 1 µl DDT (0.1M), 1 µl RNase Out and 1.5 µl superscript III RT were added to each sample making a final volume of 20.5µl, before incubating at 65 ^{0}C for 5 mins. A cycling condition at 25 ^{0}C for 5 mins, 50 ^{0}C for 60 mins and 70 ^{0}C for 15 mins were used for synthesis in the thermocycler. A 1 µ volume of RNase H (E-Coli) was added to the resulting PCR products and incubated again in the thermocycler at 37 ^{0}C for 20 mins to obtain the final cDNA product.

3.7.6.3 Reverse transcriptase PCR amplification

The final cDNA products were diluted to a ratio of 1:50 before use. RT-PCR amplification was performed using the MX 3005P (Agilent) system.

First, a five serial dilution of cDNA samples was done and analysed to generate PCR efficiency and quantitative differences between samples, where a standard curve was generated for each gene (Appendix 15). PCR mix for a single reaction used for this experiment is as follows: 10 µl SyBr Green (ThermoFisher); 0.6 µl of both the forward and reverse 10 mM primers; 7.8 µl dH_20; 1 µl cDNA template making of total of 20 µl reaction mix. Also, thermal condition parameters used are as follows: 1 cycle at 90 ^{0}C for 3 mins for initial denaturation, 40 cycles at 95 ^{0}C for 10 secs and 60 ^{0}C for 10 secs for the amplification (final denaturation, annealing, extension and fluorescence read), and 1 cycle at 95 ^{0}C for 1 min, 55 ^{0}C for 30 secs and 95 ^{0}C for 30 secs for dissociation/melting. The primers used for all target genes are attached (Table 3.3)**.** Expression levels of two housekeeping genes: ribosomal protein S7 (RSP7; AGAP010592) and actin 5C (AGAP000651) were used as controls to normalize the expressions of target genes.

Table 3.3: List of primers used for the reverse transcriptase PCR (RT-PCR)

Gene Primers	Forward	Reverse
CYP6M7	CCA GAT ACT GAA AGA GAG CCT TCG	CAAGCACTGTCTTCGTACCG
CYP6P9a	CAGCGCGTACACCAGATTGTGTAA	TCACAATTTTTCCACCTTCAAGTAATTACCCGC
CYP6P9b	CAGCGCGTACACCAGATTGTGTAA	TTACACCTTTTCTACCTTCAAGTAATTACCCGC
CYP6P4a	AACTCGTATTCGACCCCAAA	CGTTTCCATGGAATTACATTTTCTG
CYP6P4b	AACTCGTATTCGACCCCAAA	ACAATCATTATACCACACATCTGAC
CYP6AA4	CATCTGGCTGAATGGCACTA	TCAACAATGCCATCAAATCG
CYP64C27	-	-
CYP9K1	AGGGCTTCTGGATACGGTTC	CGTACGGTTCGGTTTTGATT
Trypsin	GGCCACAACCTCAAAGTCTC	CGACAGAAATCAGTCGTTAGTACG
GSTe2	GTTTGAAGCAGTTGCCATACTACGAGG	TCAAGCTTTAGCATTTTCCTCCTTTTTGGC
GSTd3	CACGGCCAGTCCTCTTTTAG	AAGCTTCTTCGCCACCAGTA
GSTd1-5	TGGAGAAATACGGCAAGGAC	CTTGGCGAAGATTTGTGGAT
Aldehyde oxidase	GCTCTGAACATTGCACCTCA	TGGTGTCGAACGATTGTGTT
RSP7	GTGTTCGGTTCCAAGGTGAT	TCCGAGTTCATTTCCAGCTC
Actin	TTAAACCCAAAAGCCAATCG	ACCGGATGCATACAGTGACA

3.8 Data analysis

Insecticide susceptibility tests and Genotype analysis

Microsoft excel was used to compute all percentage mortalities and standard errors including all charts. Resistance status of *An. funestus* mosquitoes were classified as recommended (WHO, 2016c). This standard classification is as follows:

a. Susceptible mosquito population = Mortality > 98 %.

b. Suspected resistance in mosquito population = Mortality ranging from 90 to 98 %

c. c. Resistant mosquito population = Mortality < 90 %.

Chi-square using R software was used to test for significant difference in percentage mortalities between female and male mosquito populations used for WHO susceptibility test and the distribution of the genotype frequencies (F_1) between the resistant and susceptible mosquito samples. Had2know online statistical software was used to test for significant difference between observed genotypic frequencies (F_0) and to confirm if observed genotypic frequencies are according to Hardy–Weinberg equilibrium. VassarStats online statistical software was used to generate odd and risk ratios and confidence levels of the all frequency data.

Sequence analysis

Knockdown resistance mutation

BioEdit was used to manually trace all sequences to detect polymorphic positions and for ClustalW alignment (Thompson *et al.*, 1994) while haplotype construction and polymorphic analysis to define genetic parameters such as the nucleotide diversity π, haplotype diversity and the D and D* selection estimates was done using DnaSP v5.10 (Librado and Rozas, 2009).

Sequences generated from *An. funestus* mosquitoes at Akaka-Remo were compared to sequence sets previously obtained from *An. funestus* mosquitoes at Kpome (Tchigossou *et al.*, 2018) and Pahou (Djouaka *et al.*, 2011) in southern Benin as well as at Gounougou (Menze *et al.*, 2016) in northern Cameroon. The level of Kst of pairwise genetic differentiation between these three mosquito populations was

determined with Dnasp (version 5.10.01).

Neighbour-joining tree; a genetic analysis to determine and compare the genetic differentiation of *An. funestus* among Nigeria (Akaka-Remo), Republic of Benin (Kpome and Pahou) and Cameroon (Gounougou) mosquito populations based on genetic distance was generated using Mega 6.06. Also, Maximum Likelihood Phylogenetic (MLP) tree was constructed from the VGSC haplotypes in different mosquito sample populations with Mega 6.06 using the best constructing model (Tamura *et al.*, 2011).

GSTe2 mutation

Detection of L119F-GSTe2 polymorphic position and sequence alignments was done through a manual sequence trace analysis using BioEdit. Data were exported to DnaSp (version 5.10.01) to determine genetic differentiation of the *GSTe2* gene among different *An. funestus* mosquitoes.

Microarray analysis

Data obtained from all comparisons in the microarray experiment were analyzed using the Gene-spring GX 13.0 software: differentially expressed genes were selected at a statistical significance level of $P \leq 0.05$ with Benjamini-Hochberg correction for multiple testing of a cut-off of 2 fold-change (FC), except for DDT-S and Perm-C comparisons that differentially expressed genes were only identified with no correction multiple testing but still with T-test against zero and a cut-off at $p \leq 0.05$.

RT-PCR analysis

Calculation of the relative expression level and fold change of each target gene in resistant and control relative to the susceptible population was done according to the 2-ΔΔCT method incorporating the corresponding PCR efficiency of each gene using the Microsoft excel (Schmittgen and Livak, 2008).

CHAPTER FOUR

RESULTS

4.1 Distribution of Mosquito Species at Akaka-Remo

Overall, 376 adult mosquitoes were collecte3d indoor between October, 2014 and April, 2015 at Akaka-Remo. *Anopheles* mosquitoes were 90.69 % (n = 341): 83.8 % (n = 315) were *An. funestus* while 6.9 % (n = 26) were *An. gambiae*. Other mosquito species identified were *Culex* species (n = 21, 5.6 %), *Aedes* species (n = 5, 1.3 %) and *Mansonia* species (n = 9, 2.4 %).

Based on climatic and entomological classification, October 2014 was in the rainy season, November and December 2014 was in the transition from rainy to dry, February and March 2015 was in the dry season while April was in the transition from dry to rainy season. The highest density of *An. funestus* was recorded in April, 2015 (n = 141 out of 155 mosquitoes), a high density was observed in February and March, 2015 [n = 120 out of 136 mosquitoes] (Figure 4.1). The density of *An. funestus* in November/ December, 2014 was n = 53 out of 82 mosquitoes, with the lowest density recorded in October, 2014 (n = 1 out of 3 mosquitoes). Overall, the density of *An. funestus* was high in all the collection periods compared to *An. gambiae* except in the month of October (Figure 4.2).

The density of *An. funestus* was also the highest among all mosquito species collected except in October. The density of mosquito species in October, 2014 was very low; *An. gambiae* (n = 2) and *An. funestus* (n = 1) were the only mosquito species collected during this period (Figure 4.3). There was a moderate density of all mosquitos collected in November/December, 2014; the density of *An. funestus* n = 53, with *An. gambiae* (n = 12) having almost similar density as *Culex* species (n = 10), followed by *Mansonia* species (n = 4) and *Aedes* species (n = 3) with the lowest density (Figures 4.4). In February/March, 2015, the density of *An. funestus* was the highest (n = 120), followed by *An. gambiae* (n = 8), *Mansonia* species (n = 4), *Culex* species (n = 3) and

Aedes species (n = 1), respectively (Figure 4.5). For collections in April, 2015, the trend of the mosquito species density was from *An. funestus* (n = 141) > *Culex* species (n = 8) > *An. gambiae* (n = 4) and a joint density for *Aedes* species (n = 1) and *Mansonia* species (n = 1) (Figure 4.6).

The density of *An. funestus* per room estimated in 30 rooms in April, 2015 (4.7 m/r) was also the highest, followed by February/March, 2015 (4 m/r), November/March, 2014 (1.8 m/r) and the lowest in October, 2014 (0.033 m/r) (Figure 4.7).

4.2 *Plasmodium* Infection rate and Molecular Identification of *An. funestus* at Akaka-Remo

The nested PCR analysis showed that 4 (4 %) out of 100 F_0 *An. funestus* were positive for *P. falciparum*. However, TaqMan assay performed on the same set of sample revealed that 8 (8 %) F_0 *An. funestus s.s.* were positive for *Plasmodium* parasite (Table 4.1). A total of 7 (7 %) *An. funestus* mosquitoes were infected with *P. falciparum*, while a mixed infection of *P. ovale, P. vivax* and *P. malariae* was found in 1 mosquito (1 %).

PCR analysis conducted on all the female *An. funestus* (n = 315) mosquitoes collected at Akaka-Remo that had been morphologically identified as *An. funestus* sensu lato (*s.l.*) revealed that they all belong to *An. funestus s.s.* (Figure 4.8).

4.3 WHO Insecticide Susceptibility Test on *Anopheles funestus* at Akaka-Remo

A total of 96 F_0 *An. funestus* laid eggs out of the 196 mosquitoes that were subjected to forced egg-laying technique. Eggs recovered from the oviposition eventually resulted into 1269 F_1 *An. funestus* adults, yielding 679 females and 590 male mosquitoes. All the mosquitoes were exposed to six different insecticides. This test revealed that *An. funestus* species from Akaka-Remo have developed resistance to all the families of insecticides except the organophosphate (Figure 4.9). The highest level of resistance was recorded against organochlorines: dieldrin exposure resulted into mortalities of 8 % ± 3.24 (females) and 22 % ± 1.73 (males). Similarly, DDT exposure produced mortalities of 10 % ± 2.66 in female and 17 % ± 2.45 in male population. Resistance was also observed against both type I and II pyrethroids (without and with cyano

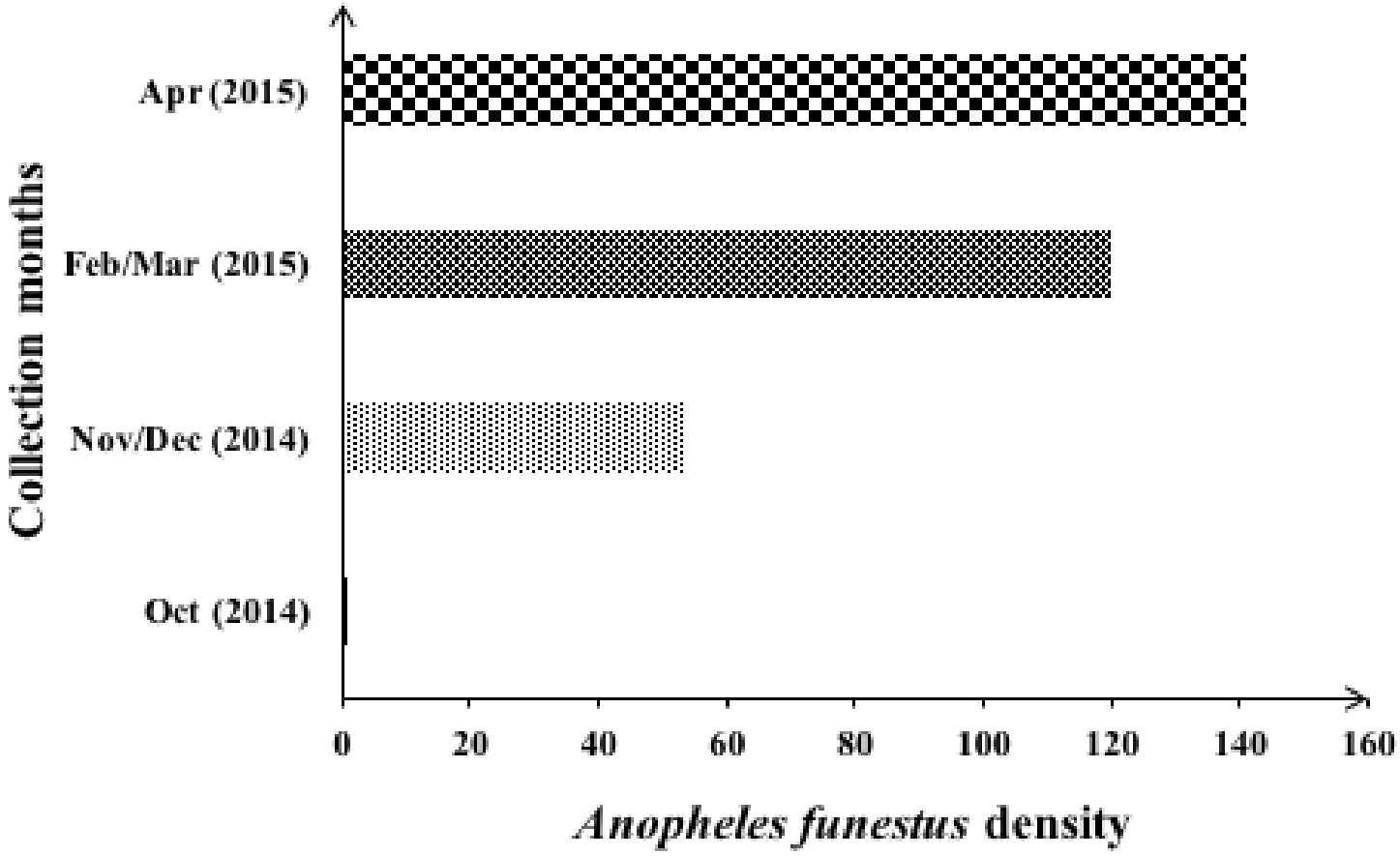


Figure 4.1: Distribution of *Anopheles funestus* in different collection months at Akaka-Remo.

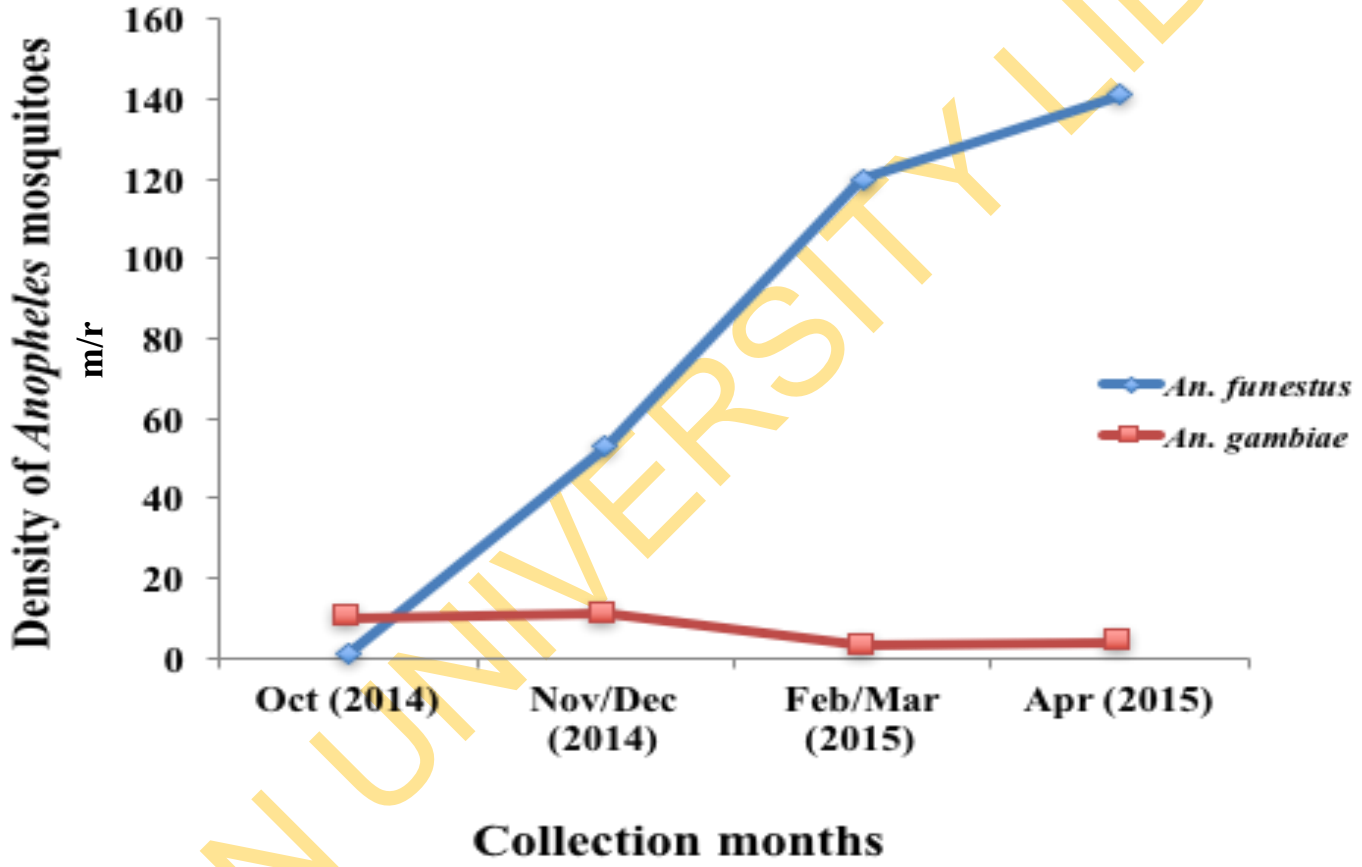


Figure 4.2: Density of *Anopheles funestus* and *Anopheles gambiae* mosquitoes at Akaka-Remo.

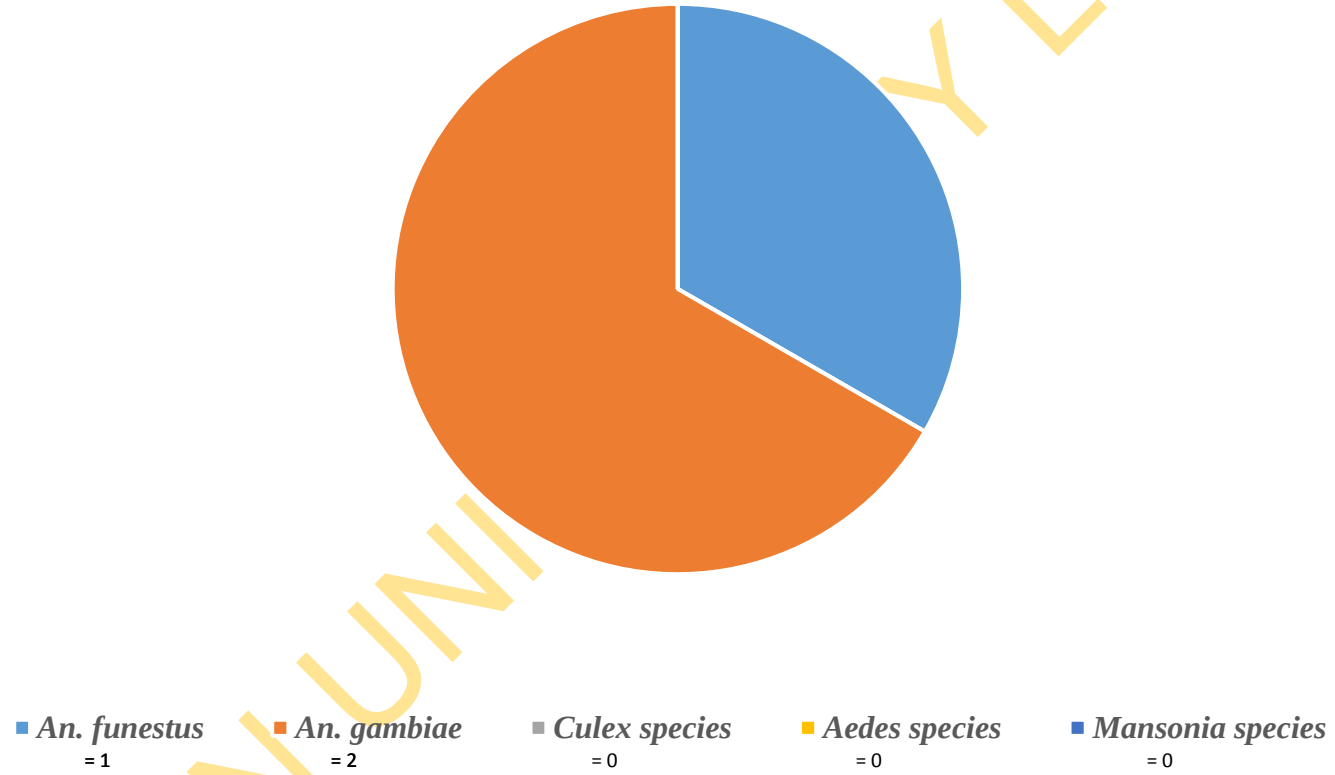


Figure 4.3: Density of mosquitoes in October, 2014 at Akaka-Remo.

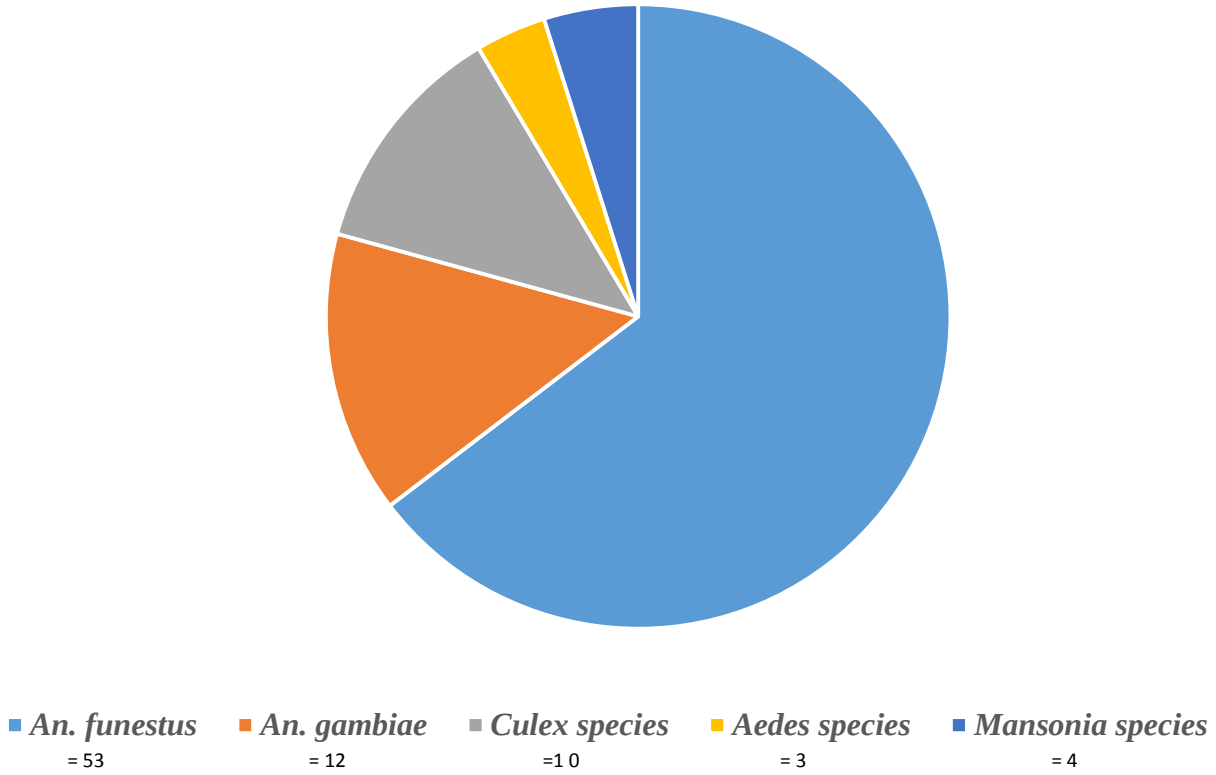


Figure 4.4: Density of mosquitoes in November/December, 2014 at Akaka-Remo.

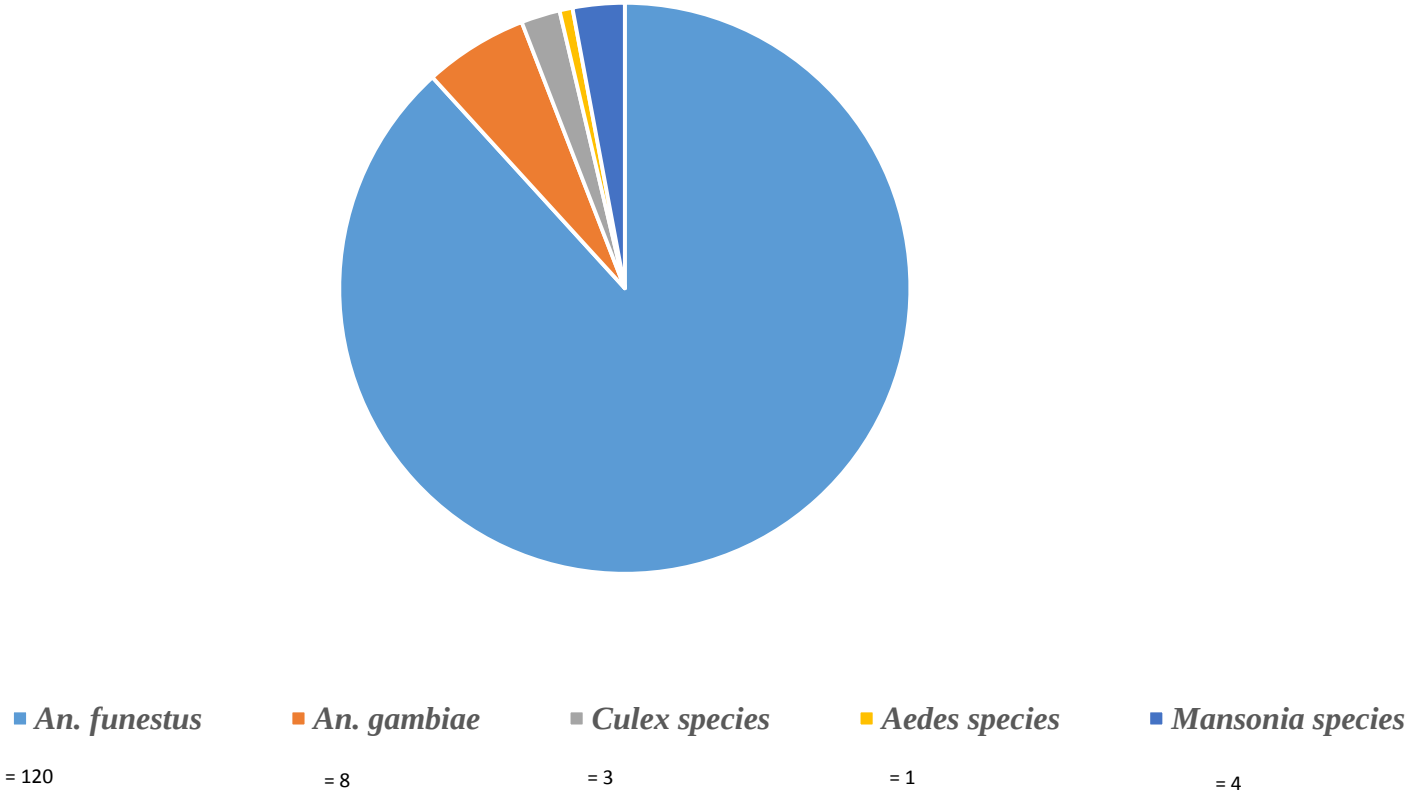


Figure 4.5: Density of mosquitoes in February/March, 2015 at Akaka-Remo.

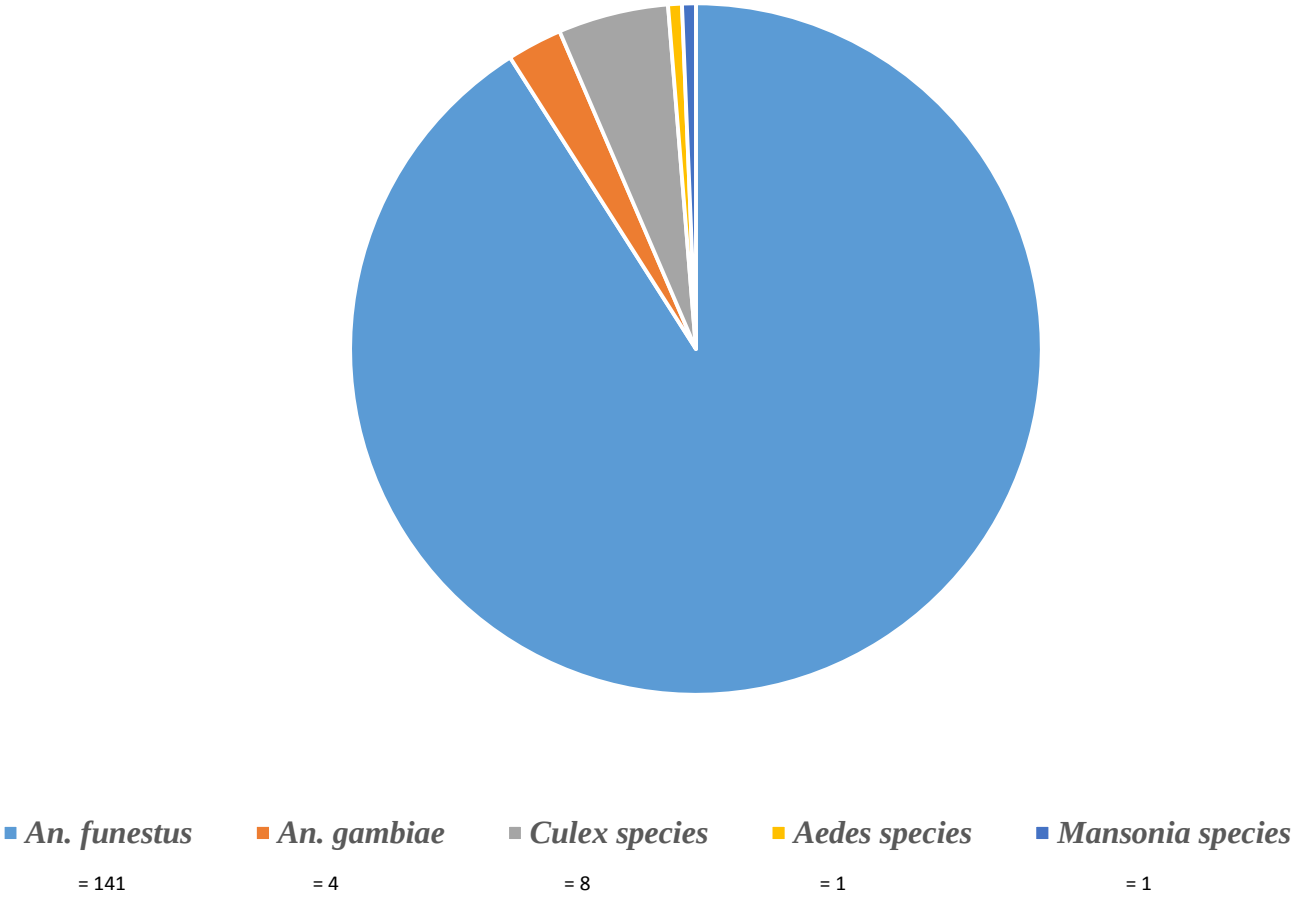


Figure 4.6: Density of mosquitoes in April, 2015 at Akaka-Remo.

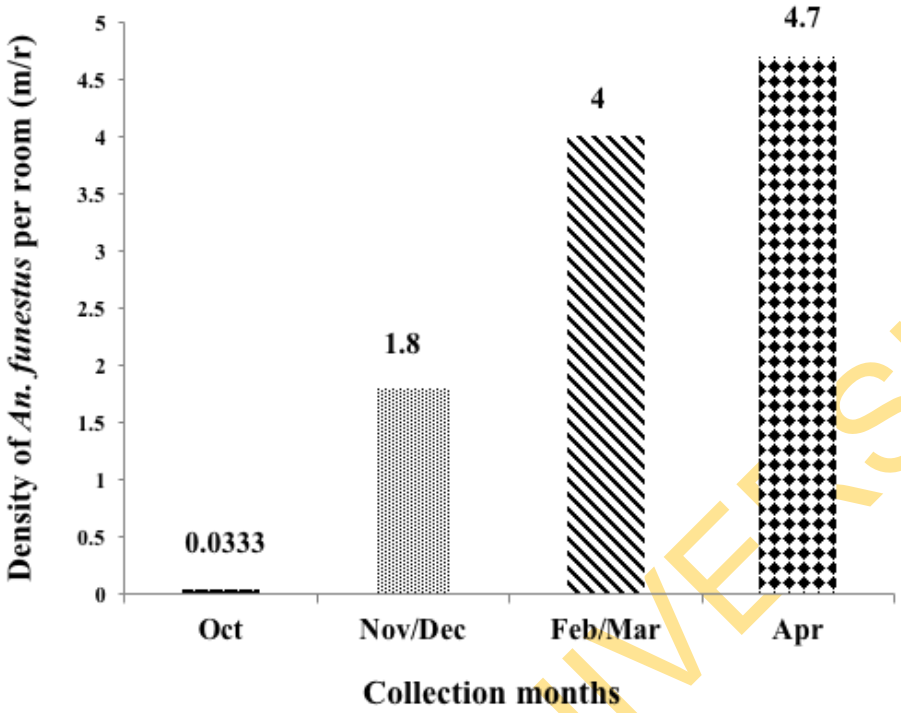


Figure 4.7: The distribution of *Anopheles funestus* per room at Akaka-Remo.

Table 4.1: *Plasmodium* Infection rate of *Anopheles funestus* at Akaka-Remo

Mosquito species Identification	**Number of *An. funestus* tested**	**Positive for *P. fal* (%)**	**Positive for *P. OVM* (%)**	**Positive for *P. fal* and *P. OVM* (%)**	**Total *An. funestus* infected (%)**
An. funestus s.s.	100	7 (7)	1(1)	-	8 (8)

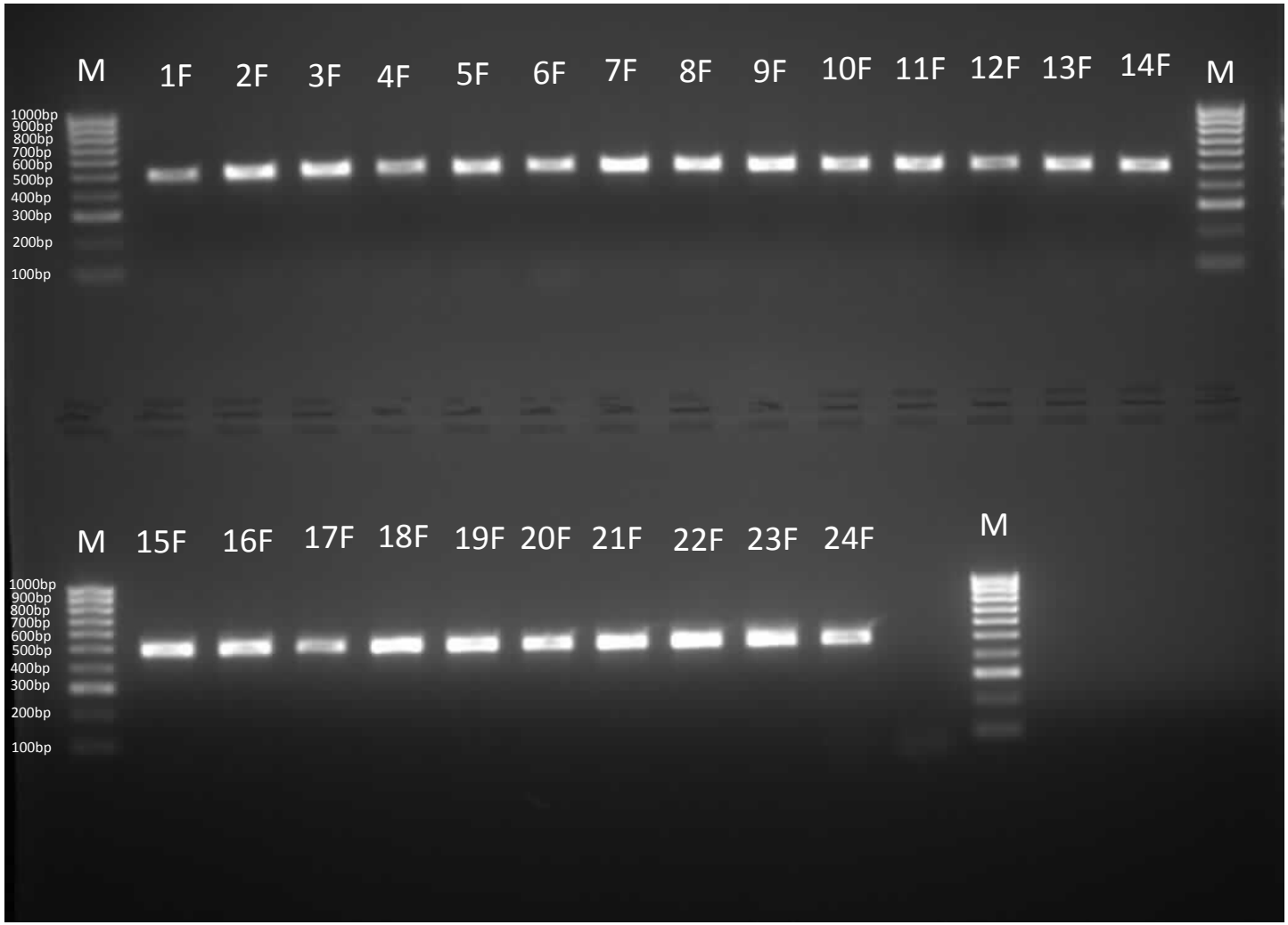


Figure 4.8: PCR species identification analysis showed that all *Anopheles funestus sensu lacto* collected at Akaka-Remo belong to *An. funestus sensu stricto* [M=DNA ladder; 1F – 24F = *An. funestus* samples].

group), with a mortality of 68 % ± 5.64 in females (85 % ± 3.15 for males) for permethrin (type I) and a mortality of 87 % ± 10.96 in females (94 % ± 3.98 for males) for deltamethrin (type II). In addition, bendiocarb (carbamate) resistance was also observed with mortalities of 84 % ± 5.67 in females and 90 % ± 2.36 in males.

In contrast, a full susceptibility of 100 % mortality was recorded in both female and male populations exposed to organophosphate malathion. Overall, there was no significant difference ($\chi 2 = 3.0922$, df = 5, P = 0.6858) in the percentage mortalities between the exposed female and male mosquitoes. Further test showed that this mosquito species survived permethrin exposure up to 90 mins; 30 mins more than the WHO standard exposure period of 60 mins. (Figure 4.10).

4.4 Analysis of the VGSC as a Potential Driver for Insecticide Resistance of *An. funestus*

The portion of the VGSC gene (924 bp) spanning intron 19 and the entire exon 20 (207 bp) located at segment 6 on domain II was successfully amplified and sequenced in eleven *An. funestus* mosquitoes from Akaka-Remo.

Analysis of the sequence set did not detect the L1014F (-TTA- to -TTT-) or the L1014S (-TTA- to -TCA-) mutations that are common to *An. gambiae* in West and East Africa, respectively (Figure 4.11). Further analysis was done with the 907 Base Pair (BP) sequence obtained from five individual *An. funestus* mosquitoes using the DNASP software. This assessment detected 20 polymorphic sites (887 monomorphic sites) and 8 haplotypes (Figure 4.12; Table 4.2 Appendix 17 and 18). Analysis of Akaka-Remo mosquito samples using the neighbour-joining (NJ) tree, with respect to geographical distance revealed a similar genetic constitution with Cameroon mosquito population but a higher differentiation than Benin mosquitoes (Figure 4.13; Table 4.2).

Also, there was no correlation in the VGSC polymorphisms of the different mosquito populations. This was shown by the lack of clustering of mosquito samples from the same locality with the MLP tree (Figure 4.14). The absence of correlation was further supported by the fact that polymorphism did not result into any amino acid change as well as the estimates of Tajima D and Fu and Li D* statistics, which were not statistically significant (Table 4.2).

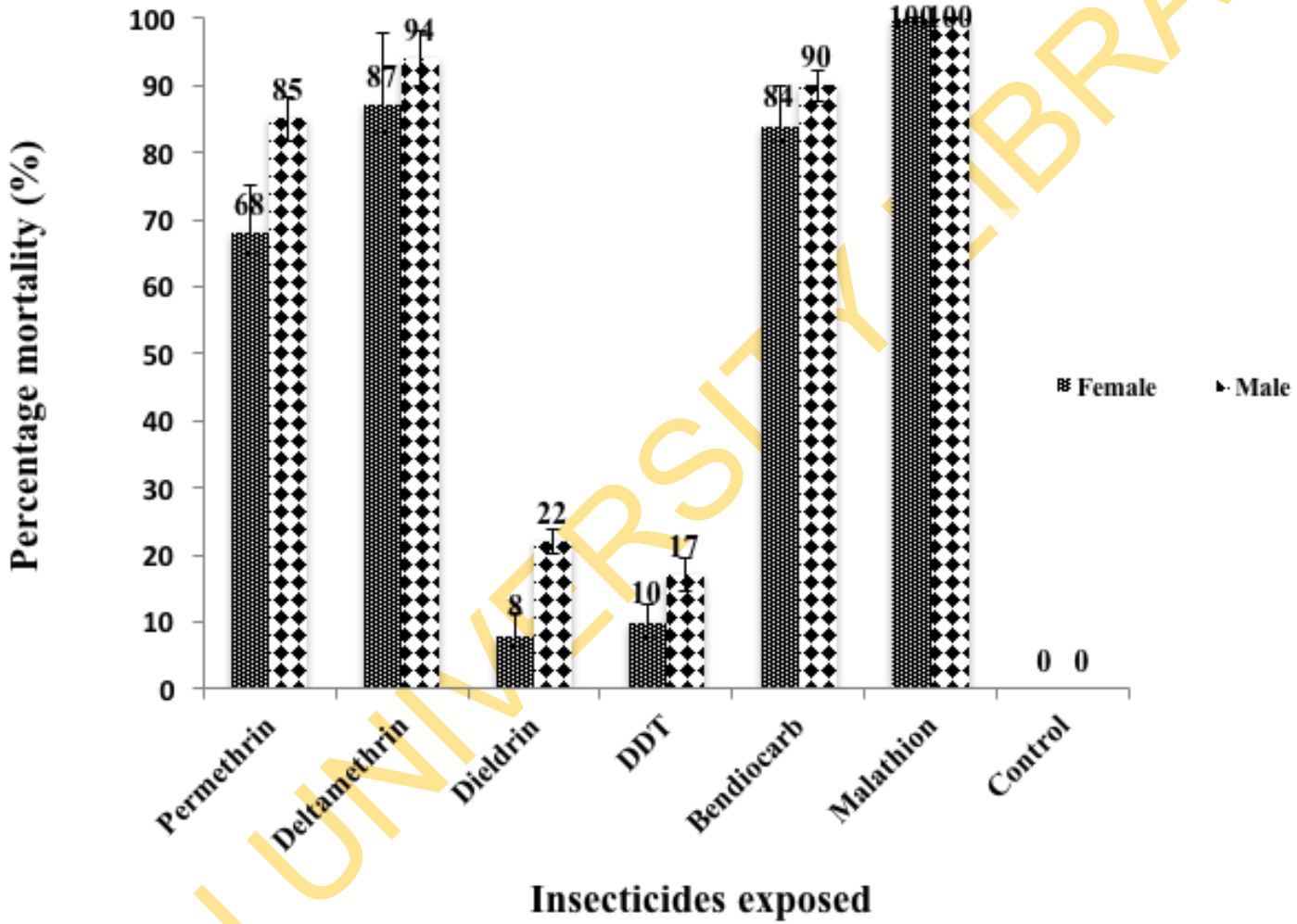


Figure 4.9: Insecticide resistance profile of *Anopheles funestus s.s.* at Akaka-Remo. No significant difference ($\chi 2 = 3.0922$, df = 5, P = 0.6858) was observed in percentage mortalities between female and male mosquitoes to all insecticides.

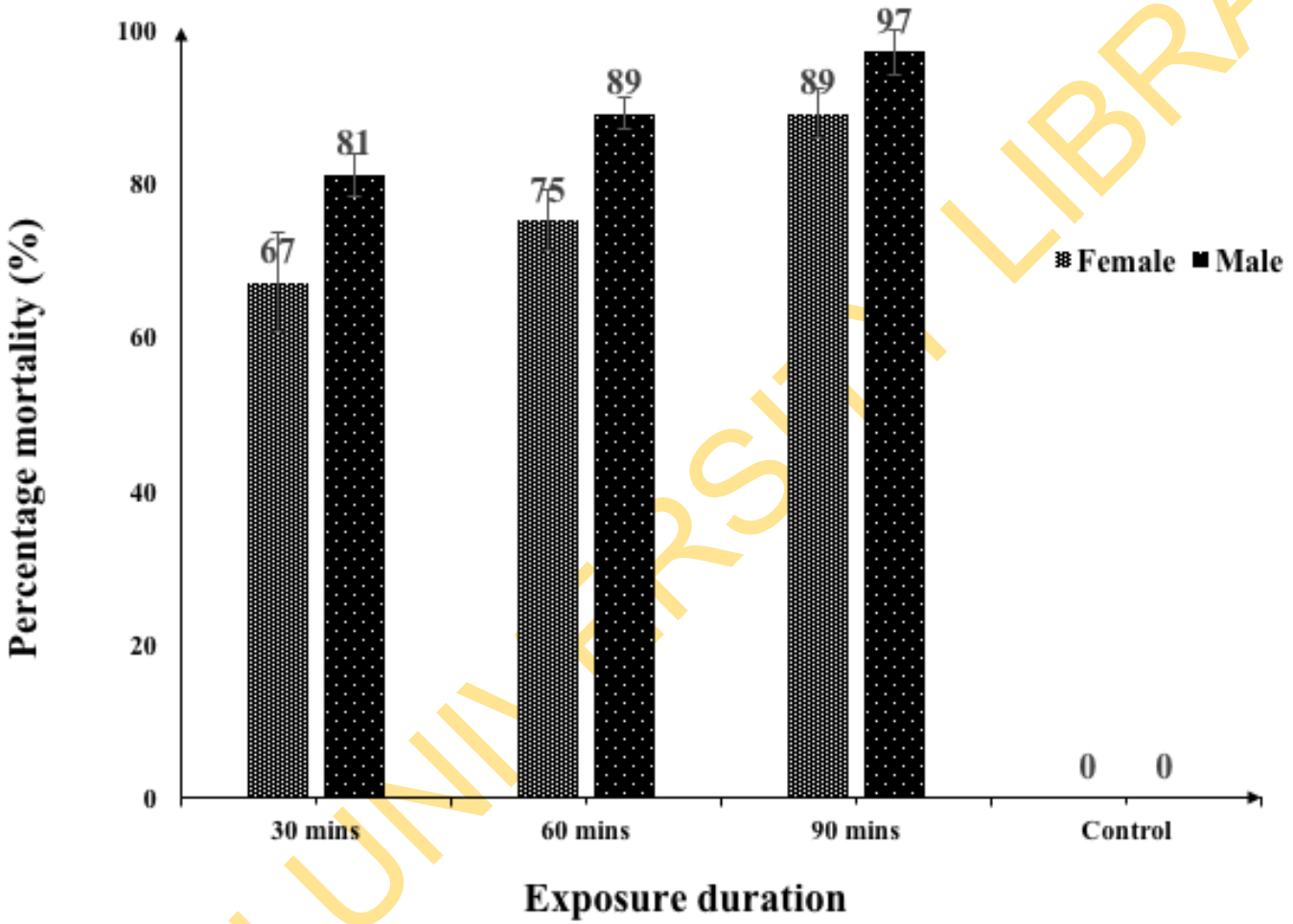


Figure 4.10: Insecticide resistance status of *Anopheles funestus s.s.* to pyrethroids (permethrin) at different exposure periods in Akaka-Remo**.**

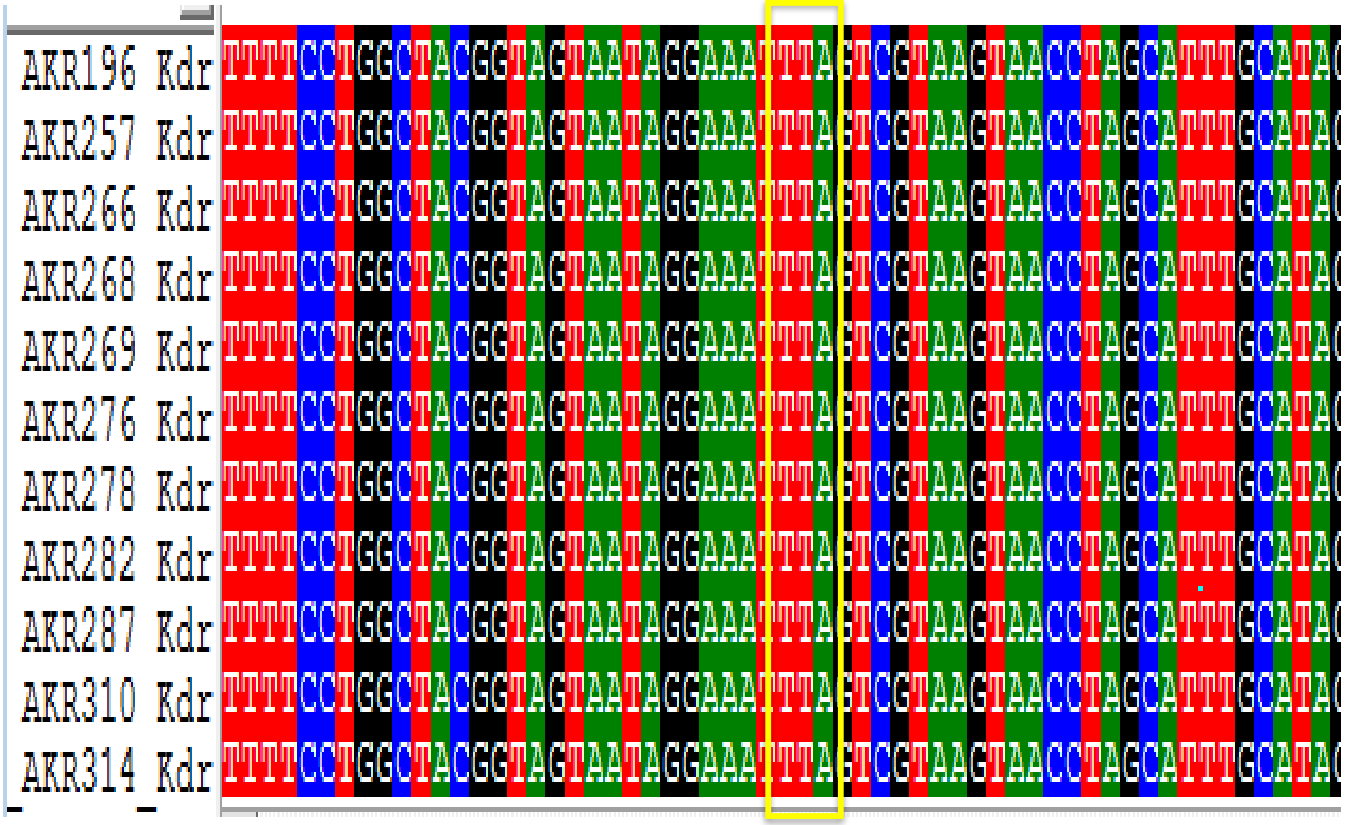


Figure 4.11: The *Kdr*, L1014F and L1014S mutations were absent on the Voltage-Gated sodium channel region of *Anopheles funestus*.

4.5 Investigation of L119F mutation on *GSTe2* as a Possible Insecticide Resistance Mechanism in *Anopheles funestus*

The L119F mutation was detected in all the F_0 *An. funestus*. No mosquito was still carrying the wild type form for *GSTe2* genes in the six mosquitoes analysed (Figure 4.15). Analysis of the 119 portion only detected the mutant -TTT-, coding for phenylalanine (119F) instead of the wild type -CTT- that codes for Leucine (L119) on the *GSTe2* gene, except for one mosquito carrying the ambiguity code, Y, that codes for either cytosine (C) of thymine (T). Further analysis using the total number of sequence generated (number of site = 739) detected 8 polymorphic sites and 731 monomorphic sites. (Table 4.3). Also, a moderate haplotype diversity, hd = 0.567 and high nucleotide diversity, π = 0.00246 was detected. However, this mutation was also not significant on the overall genetic determinants of *An. funestus* mosquito species.

4.6 Genotype and Allele Distribution of L119F-GSTe2 Mutation in the *Anopheles funestus s.s.* Population at Akaka-Remo

The L119F-GSTe2 mutation was detected in 83 (94 %) out of the 88 F_0 mosquitoes that were genotyped (Figure 4.16). Over half (n = 52; 59.01 %) of the total *An. funestus* analysed carried the homozygous resistant RR genotype, 31 (35.23 %) *An. funestus* were heterozygous RS for the *GSTe2* gene while 5 (5.68 %) carried the homozygous susceptible, SS genotype. The analysis gave a resistant allelic frequency, R of 77 % and a susceptible allelic frequency, S of 23 %.

Similarly, when the F_1 generations (25 resistant and 25 susceptible *An. funestus* after exposure to DDT) were screened for L119F-GSTe2 mutation, a genotypic frequencies of 64 % RR (n = 16), 32 % RS (n = 8), 4 % SS (n = 1) and 48 % RR (n = 12), 48 % RS (n = 12) and 4 % SS (n = 12) were produced in the resistant and susceptible populations, respectively. The L119F mutation observed in the F_1 generation of *An. funestus* gave a resistant allelic frequency of 80 % in the resistant and 72 % in the susceptible populations (Figure 4.17).

The observed genotypic frequency was shown to be at Hardy–Weinberg equilibrium (P = 0.8935). Further statistical analysis showed that there was no significant difference (χ = 1.37, *df* = 2, P = 0.5037) in the frequency of L119F-GSTe2 mutation between the susceptible and resistant samples and consequently the correlation

between these two mosquito population was not significant (OR = 1.56; P = 0.1859).

4.7 Genotype and Allele Distribution of A296S-RDL Mutation in the *Anopheles funestus s.s.* Population at Akaka-Remo

The presence of the A296S-RDL mutation was high (n = 90) in the F_0 *An. funestus* population analysed (n = 92). Homozygote, RR genotype was recorded in over half of the total *An. funestus* (n = 50; 54.35 %). Likewise, 40 (43.35 %) *An. funestus* mosquito samples were heterozygous, RS for the *Rdl* gene while only 2 (2.17 %) carried the homozygous susceptible, SS genotype (Figure 4.18).

However, when F_1 mosquitoes generated after exposure to dieldrin (15 alive and 5 dead) were genotyped for A296S-RDL mutation, there was a high presence of the mutation in resistant population [genotypic frequencies of 80 % RR (n = 12) and 20 % RS (n = 3)] with a relatively low presence in susceptible population [genotypic frequency of 20 % RS (n = 1), with the remaining mosquitoes carrying the homozygous susceptible genotype, SS for *Rdl*, (n = 4; 80 % A296)] samples (Figure 4.19). The analysis gave an allelic frequency, R (296S) of 90 % in resistant and S (A296) = 10 % in susceptible mosquito populations.

The observed genotypic frequency was shown to be at Hardy–Weinberg equilibrium (P = 0.0617). Further statistical analysis revealed that there was a significant difference (χ^2 = 16, *df* = 2, P = 0.00034) in the frequency of A296S-RDL mutation between the resistance and the susceptible populations and consequently correlation was also significant (OR = 81; P < 0.0001).

4.8 Synergist Tests with PB, STP and DM for Investigating the Involvement of Metabolic Enzymes in Insecticide Resistance of *Anopheles funestus*

There was a recovery from resistance to full susceptibility with permethrin as mosquito mortality rose from 68 to 100 % (n = 70) when permethrin was combined with the P450 inhibitor, PB (Figure 4.20). *Anopheles funestus* also recovered fully to susceptibility (100 % mortality) when permethrin was combined with STP (esterase inhibitor) and DM (GSTs inhibitor).

Table 4.2: Genetic parameters of the VGSC of *Anopheles funestus* from Akaka-Remo compared to Benin and Cameroon populations.

Locality	N(2n)	S	Pi (π)	K	h	hd	Syn	Non-syn	D	D*
Akaka-Remo	10	20	0.00524	4.75556	8	0.933	0	0	-1.54ns	-1.81ns
Kpome	22	12	0.00351	2.93939	12	0.909	0	0	-0.37ns	-0.32ns
Pahou	20	10	0.0026	2.17895	12	0.905	0	0	-0.79ns	-0.96ns
Cameroon	40	37	0.00514	4.30128	29	0.977	2	3	-1.81ns	-2.75ns

2n, number of sequences; S, number of polymorphic sites; π, nucleotide diversity; k, Average number of nucleotide difference; h, number of haplotypes; hd, haplotype diversity; syn, synomynous; Non-syn, Non-synonymous; D, Tajima's statistics; D*, Fu and Li's statistics; ns, not significant.

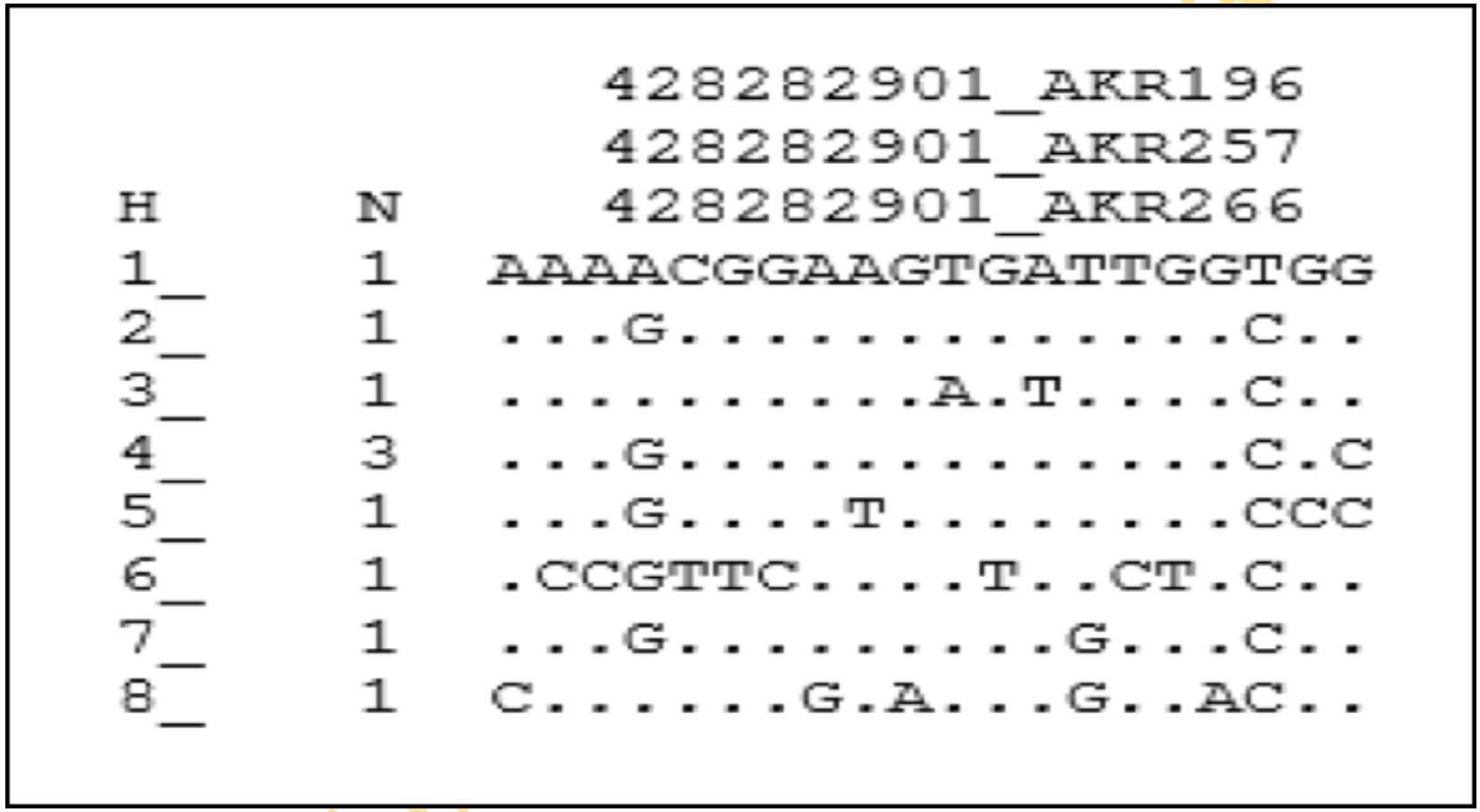


Figure 4.12: The number of haplotypes detected in *Anopheles funestus* mosquitoes sequenced for *Kdr* mutation

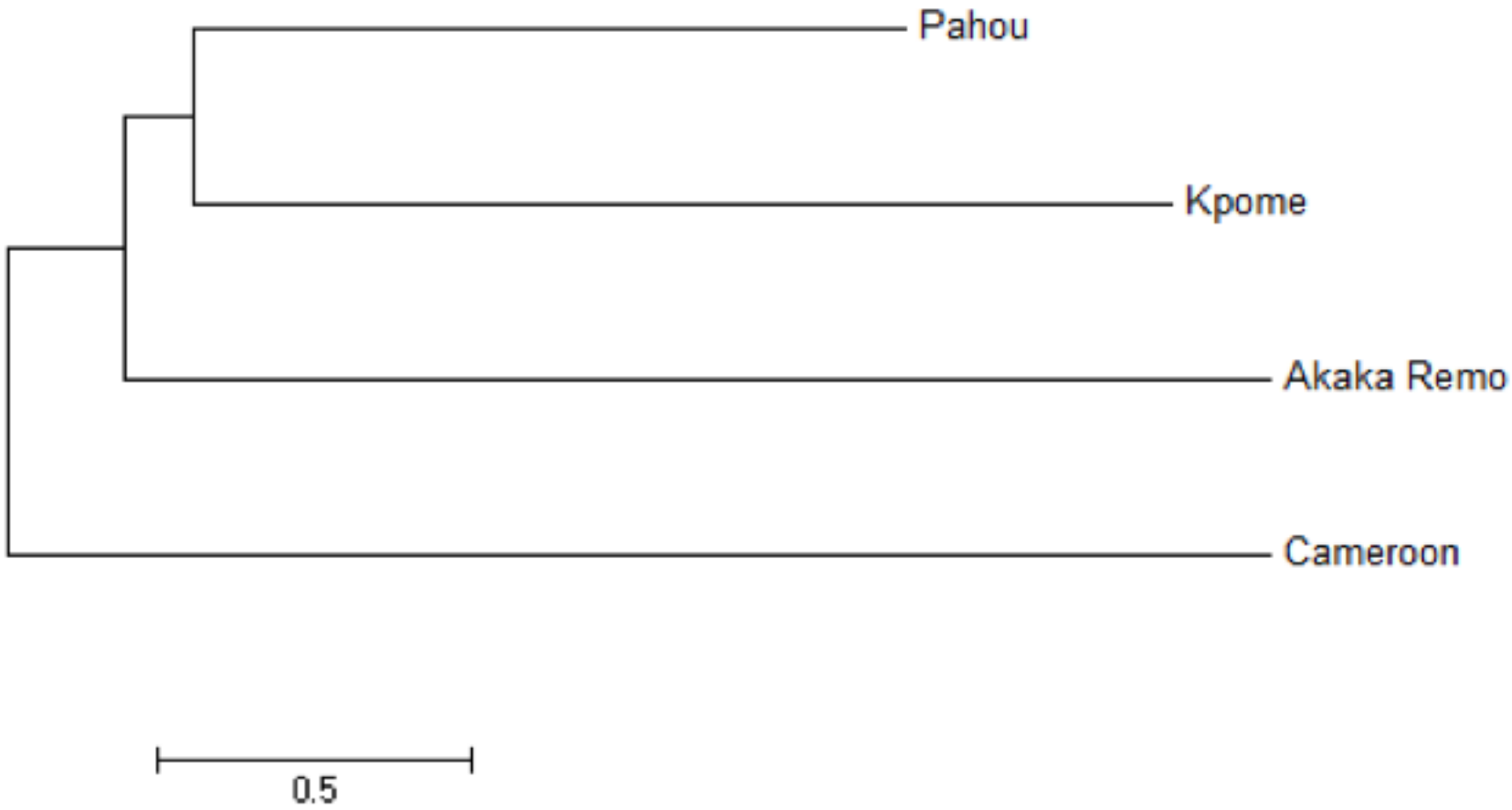


Figure 4.13: Genetic differentiation observed between four African populations of *Anopheles funestus* including Akaka-Remo based on geographical distance.

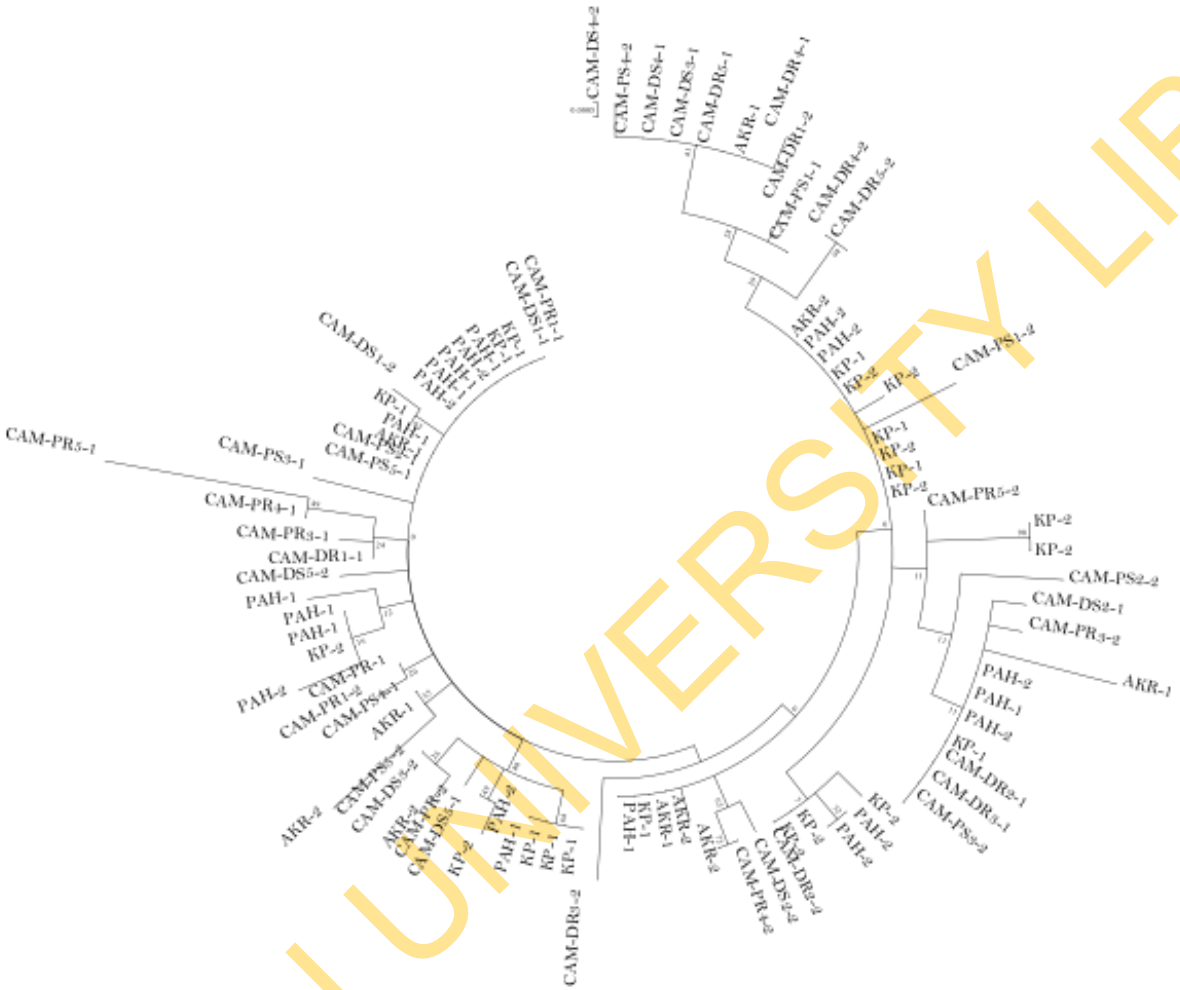
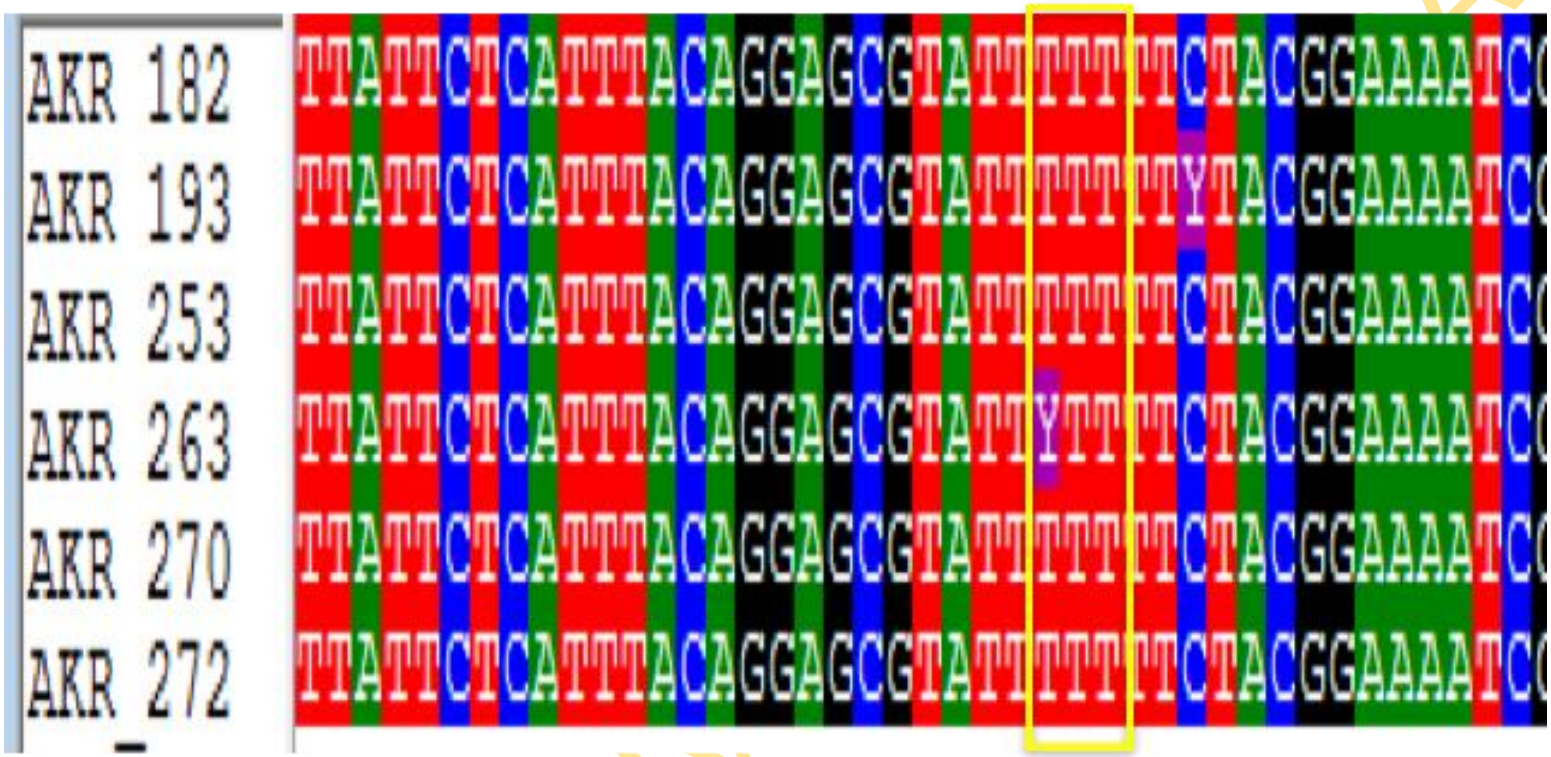


Figure 4.14: Maximum likelihood phylogenetic tree constructed from nucleotide sequences obtained from Akaka-Remo, Benin and Cameroon *Anopheles funestus*. No correlation was observed in the VGSC polymorphism of the different mosquito populations.



Wild type: Lucine, L= CTT; Mutant type: Phenylalamine, F= TTT

Figure 4.15: Sequence analysis to detect the presence of L119F mutation on the *GSTe2* gene of *Anopheles funestus*.The L119F mutation was very high and almost getting fixed mosquito population.

Table 4.3: Genetic parameters of *GSTe2* mutation in *Anopheles funestus* from Akaka-Remo

Locality	N(2n)	S	Pi (π)	K	h	hd	Syn	Non-syn	D	D*
Akaka-Remo	12	8	0.00246	1.81818	5	0.567	-	-	-1.25ns	-1.49ns

2n, number of sequences; S, number of polymorphic sites; π, nucleotide diversity; k, Average number of nucleotide difference; h, number of haplotypes; hd, haplotype diversity; syn, synomynous; Non-syn, Non-synonymous; D, Tajima's statistics; D*, Fu and Li's statistics; ns, not significant.

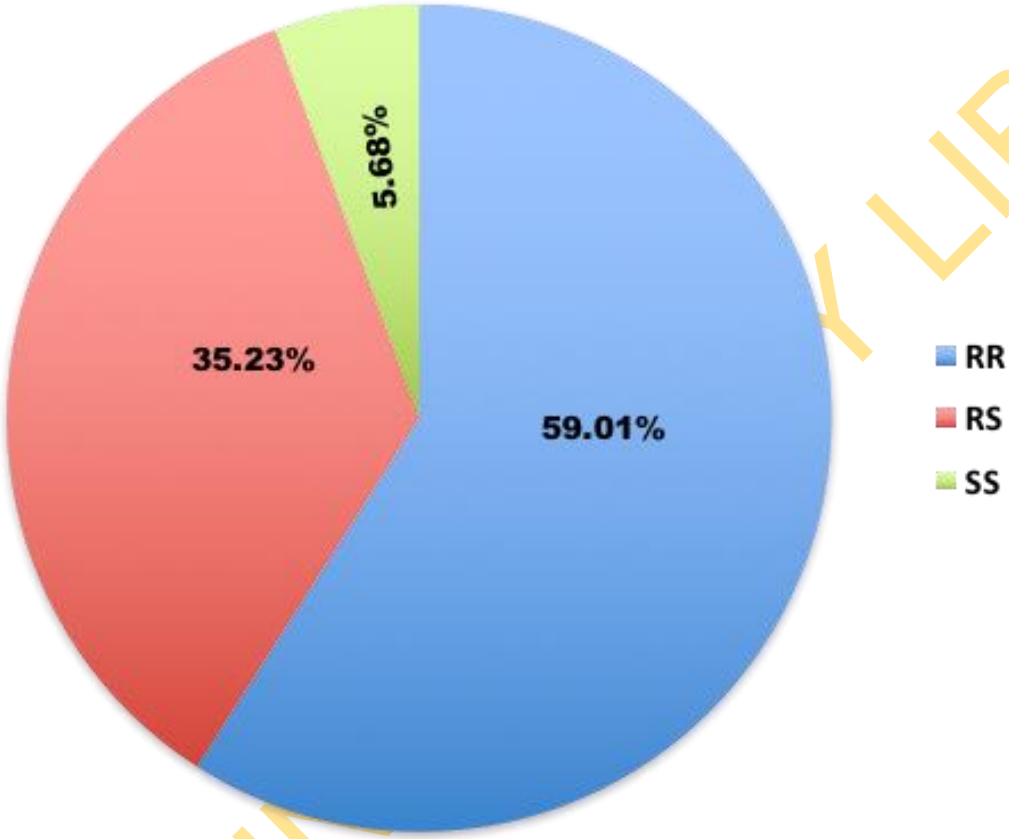


Figure 4.16: Screening for the frequency of L119F-GSTe2 mutation in wild *Anopheles funestus* (F_0) collected directly from the field. This analysis showed a high presence of RR and RS and a low presence of SS in F_0 *An. funestus* with allelic frequencies of R=77 % and S=23 %.

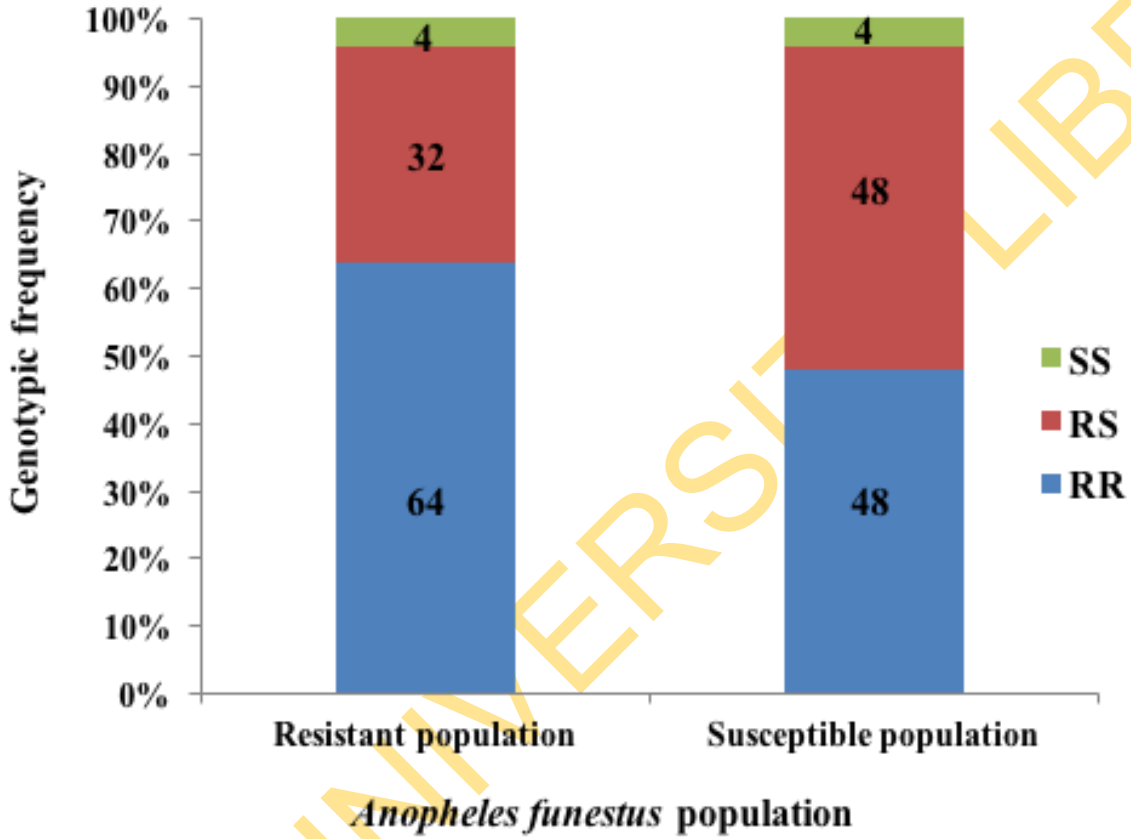


Figure 4.17: Screening for the frequency of L119F-GSTe2 mutation in resistant and susceptible *Anopheles funestus* after exposure to DDT (F_1). Allelic frequencies of 119F = 80 % in resistant and 72 % in susceptible populations; genotypic frequency is at Hardy-Weinberg equilibrium (P = 0.8935); no significant difference ($\chi 2$ = 1.37, df = 2, P = 0.5037) was observed in the frequecy of 119F; correlation was also not significant (OR = 1.56; P = 0.1859) between the two mosquito populations.

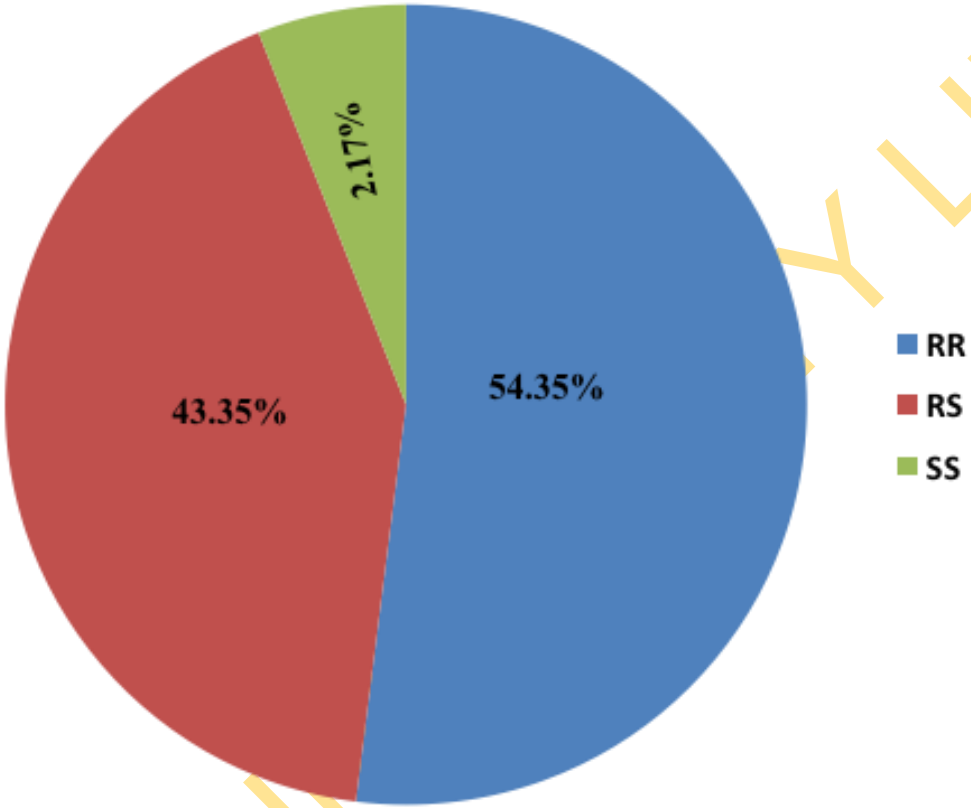


Figure 4.18: Screening for the frequency of A296S-RDL mutation in wild *Anopheles funestus* (F_0) collected directly from the field. This analysis showed a high presence of RR and RS and a low presence of SS in *An. funestus* with allelic frequencies of R = 76% and S = 24%.

Rdl: Resistance to dieldrin.

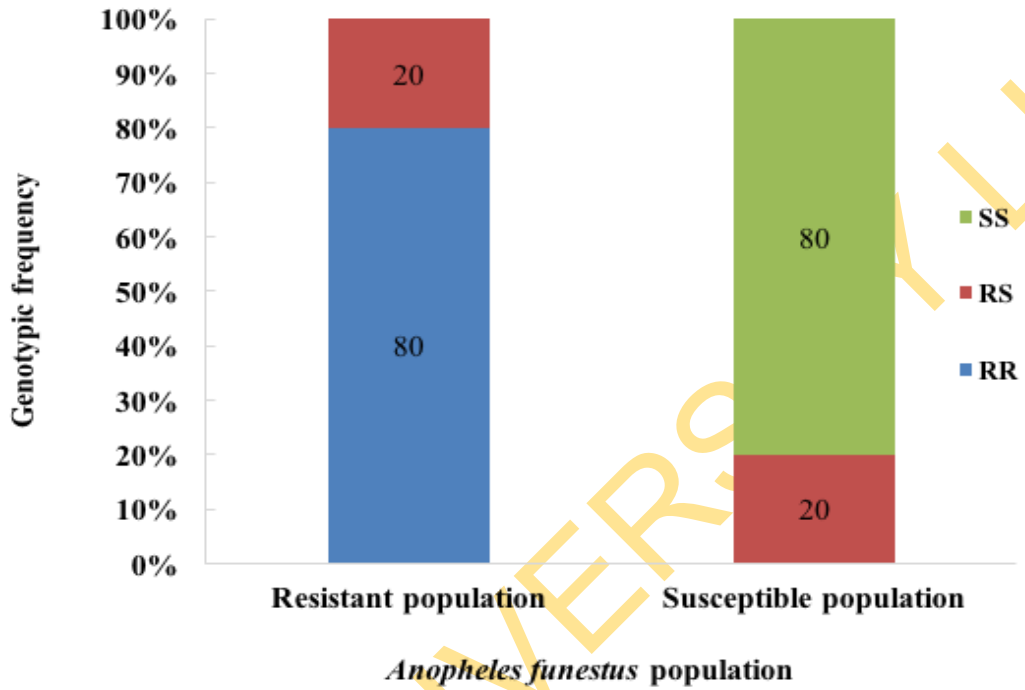


Figure 4.19: Screening for the freqency of A296S-RDL mutation in resistant and susceptible population (F_1) of *Anopheles funestus*. Allelic frequencies of 296S = 90 % in resistant and 10 % in susceptible populations; genotypic frequency is at Hardy-Weinberg equilibrium (P = 0.0617); there was a significant difference ($\chi 2$ = 16, df = 2, P = 0.00034) in the frequency of 296S; correlation was also significant (OR = 81; P < 0.0001).

The combination of DDT with PB showed a slow increase in mortality from 10 to 30 % (n = 50) (Figure 4.21). While STP plus DDT exposure resulted into 8.81 % mortality compared to the 10 % mortality recorded when mosquito was exposed to only DDT. Similarly, there was 71.42 % mortality when *An. funestus* was exposed to both DM and DDT compared to 10 % mortality when exposed to only DDT.

Similar to pyrethroids, 100 % mortality was recorded when *An. funestus* was exposed to both PB and dieldrin compared to 8 % mortality when exposed to only dieldrin (Figure 4.22). No mortality was observed in the control *An. funestus* mosquitoes; both the group exposed to control paper without insecticide treatment and the papers treated with only synergists.

4.9 Genome-wide Transcription Analysis using Microarray

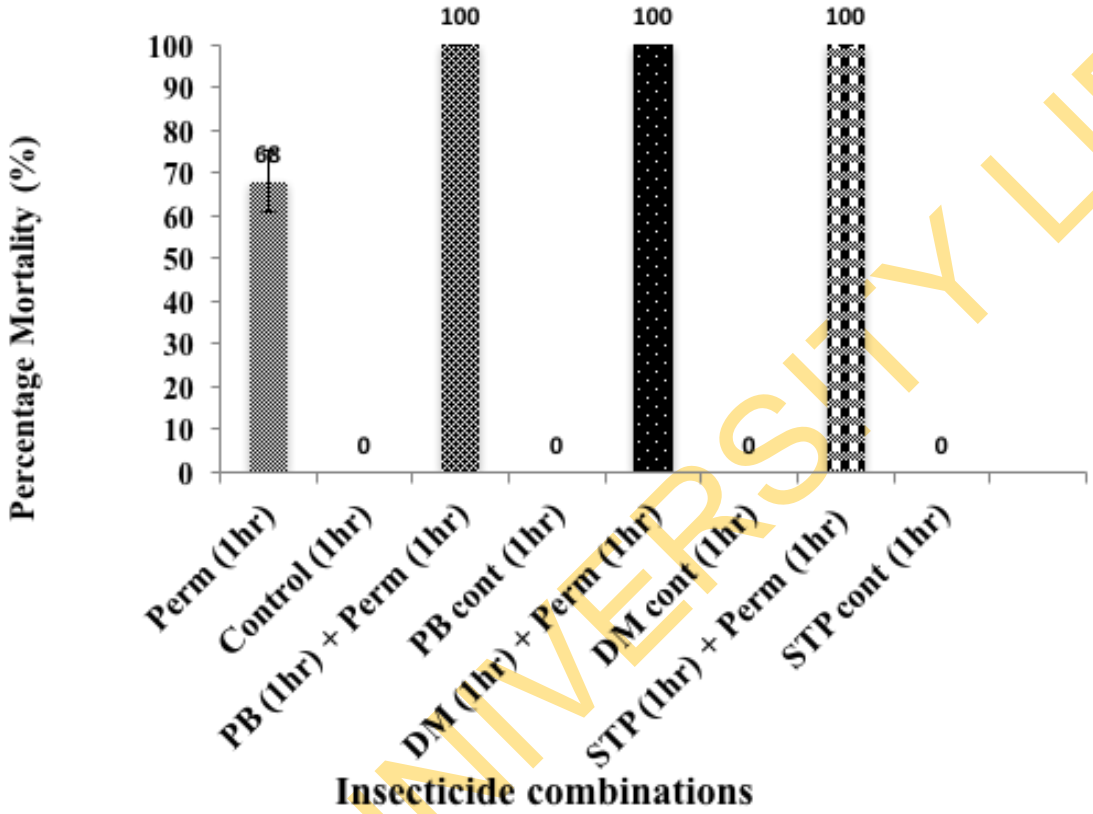
There were expressions of different gene families in all the comparisons considered. The three comparisons analysed for permethrin resistance: Rperm-S, Rperm-C and C-S and DDT resistance: R_{DDT}-S, C-S and Rperm-S produced several differentially expressed transcripts.

Overall, 1,536 (798 overexpressed) transcripts were differentially expressed in Rperm-S (Appendix 22), 1,467 transcripts (230 overexpressed) were differentially expressed in Rperm-C (Appendix 23) while the R_{DDT}-S comparison produced 664 (299 overexpressed) differentially expressed transcripts (Appendix 24), as 2473 (1093 overexpressed) transcripts were differentially expressed in C-S (Appendix 25).

4.9.1 Genome-wide transcripts Profile of Permethrin Resistance in *Anopheles funestus*

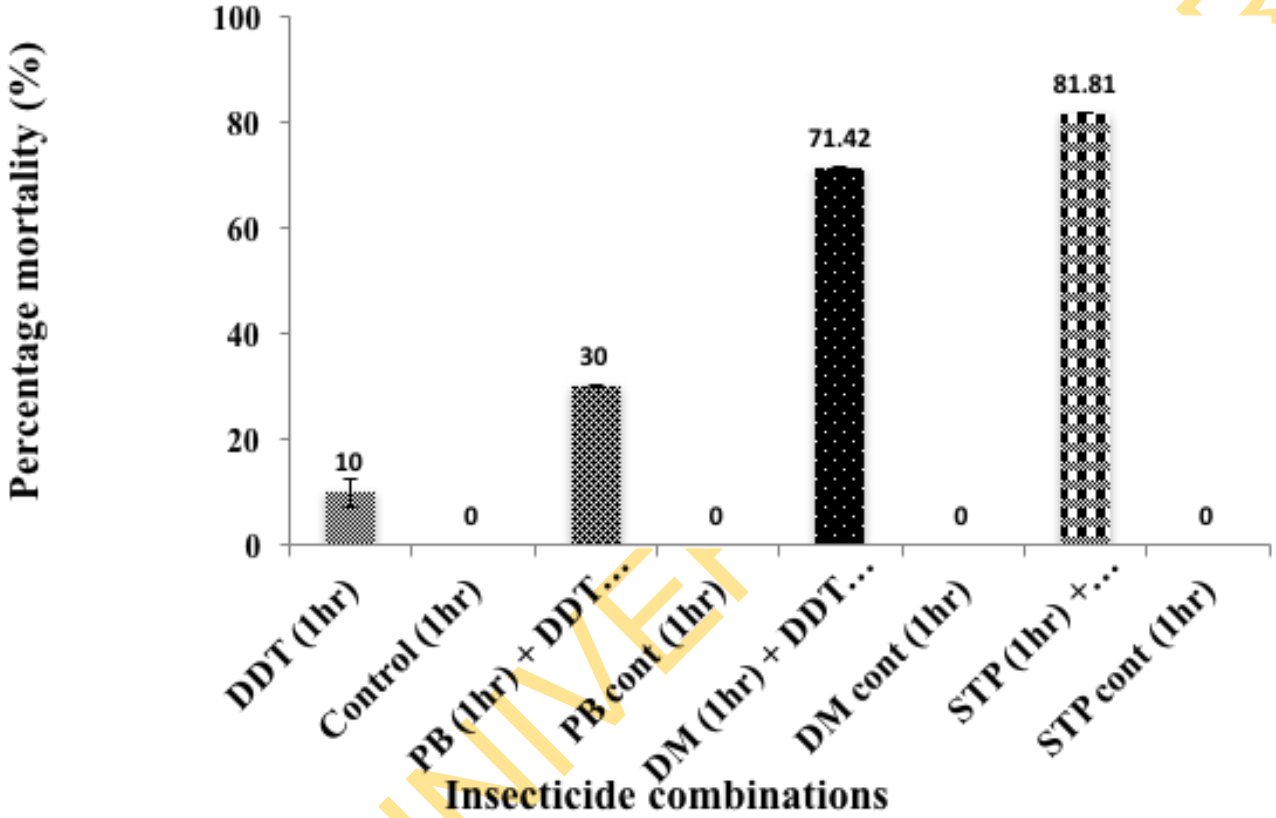
Genespring, GX 13.0 software analysis was used to identify commonly expressed transcripts in the different comparisons (Figure 4.23).

The three comparisons only produced one transcript (Afun000762) with fold changes of 5.19, 3.83 and 2.53 in Rperm-S, C-S and Rperm-C, respectively (Table 4.4). No detoxifying gene was commonly overexpressed in the 3 groups of comparisons while only the Rperm-S and C-S comparisons produced commonly overexpressed transcripts.



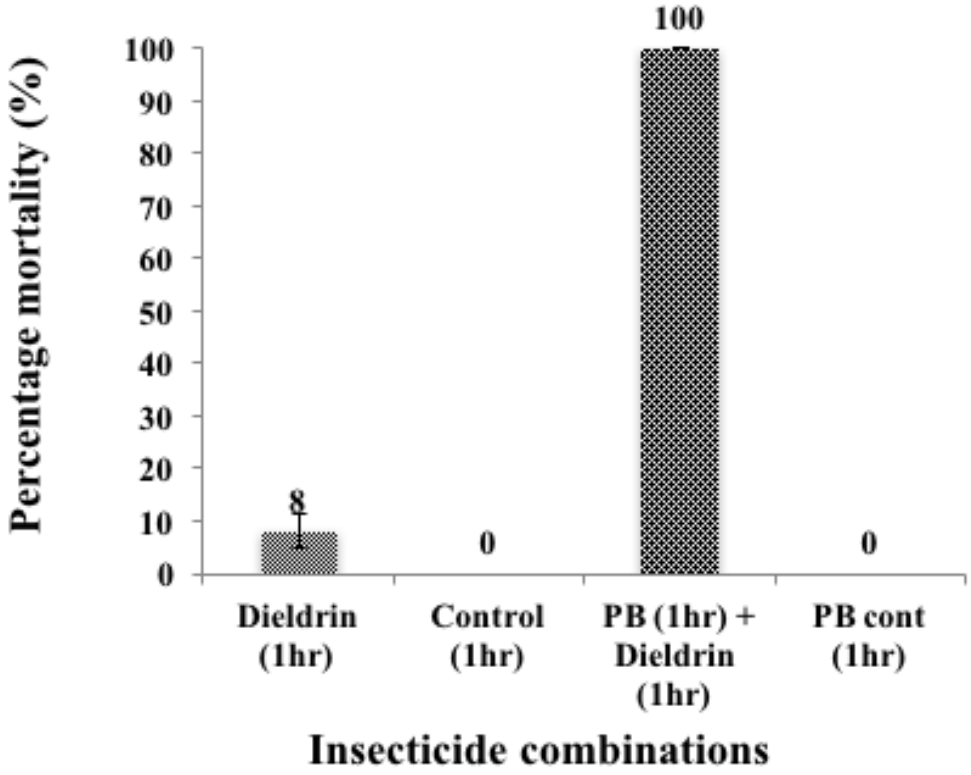
Perm: permethrin; PB: piperonyl butoxide; STP: S,S,S-tributyl phosphorotrithioate; DM: diethyl maleate; (t): Time in the bracket represents the duration of insecticide exposure

Figure 4.20: Susceptibility status of *Anopheles funestus* to permethrin after pre-exposure to synergists PB, DM and STP.



PB: piperonyl butoxide; STP: S,S,S-tributyl phosphorotrithioate; DM: diethyl maleate; (t): Time in the bracket represents the duration of insecticide exposure

Figure 4.21: Susceptibility status of *Anopheles funestus* to DDT after pre-exposure to synergists PB, DM and STP.



PB: piperonyl butoxide; (t): Time in the bracket represents the duration of insecticide exposure

Figure 4.22: Susceptibility status of *Anopheles funestus* to dieldrin after pre-exposure to synergists PB.

Common transcripts overexpressed in Rperm-S and C-S comparisons

There were 445 common transcripts overexpressed in this comparison out of the 973 transcripts that were differentially expressed. Only two of these transcripts are associated with resistance, which are the cuticle proteins (5 transcripts) and carboxylesterase (Table 4.4). Cuticle proteins had higher overexpression in this comparison with the following transcripts: CD578215.1 (Rperm-S, FC = 17.81; C-S, FC = 43.40), CD578215.1 (Rperm-S, FC = 14.75; C-S, FC = 36.74), Afun010482 (Rperm-S, FC = 18; C-S, FC = 65.91), CD577507.1 (Rperm-S, FC = 3.56; C-S, FC = 5.87) and Afun013390 (Rperm-S, FC = 4.11; C-S, FC = 3.47). Carboxylesterase produced a relatively lower fold change in Afun015266 (Rperm-S, FC = 2.88; C-S, FC = 2.88)

Common transcripts overexpressed in Rperm-S comparison

This comparison produced the *GSTU2* (FC = 4.25) transcript that belong to the GSTs family (Table 4.4). Other resistance-associated transcripts overexpressed are glucosyl glucuronosyl transferases (3 transcripts) with fold changes of 2.25, 2.44 and 2.10 and chymotrypsin 1 (Afun010134: FC = 4.75). An electron transport trans-membrane protein, cytochrome b561 (Afun008617; FC = 2.20) was also overexpressed.

Common transcripts overexpressed in C-S comparison

Four transcripts, *CYP9J3* (FC = 2.3); *CYP6P9a* (FC = 2.92); *CYP6P9b* (FC = 14.48) and *CYP6AA4* (FC = 3.49) of the cytochrome P450 monooxygenase were overexpressed (Table 4.4). *CYP6P9b* had the highest overexpression level. Other resistant-associated transcripts in this group are the short-chain dehydrogenase (AGAP001-405-RA___2R, FC = 2.42), carboxylesterase (COEAE6O, FC = 2.15); 3 transcript of trypsin (AGAP011431-RA___3L, FC = 2.02; CD578079.1, FC = 2.38 and CD578079.1 FC = 2.98); cytochrome C (BU039010.1, FC = 2.13) and 2 transcripts of the cuticle proteins (Afun008525, FC = 20.18 and CD577507.1, FC = 2.32).

Common transcripts overexpressed in Rperm-C comparison

Two transcripts of the cytochrome P450 genes were overexpressed in the Rperm-C comparison (Table 4.4). The *CYP6P9a* (FC = 2.92) and *CYP6P2* (FC = 2.47) were the

two P450 transcripts identified. The *GST* transcript (AGAP004164-RC, FC = 2.09) was also overexpressed as well as the esterase, esterase fe4 (Afun014849, FC = 2.03).

4.9.2 Genome-wide Transcripts Profile of DDT Resistance in *Anopheles funestus*

There was no common overexpressed detoxification transcript among the different groups of comparisons: R_{DDT}-S, C-S and Rperm-S; R_{DDT}-S and C-S; R_{DDT}-S and Rperm-S; Rperm-S and C-S in DDT resistance (Figure 4.24). However, there were few resistant-associated transcripts of the cytochrome P450 family, GSTs, carboxylesterase, glucosyl glucuronosyl transferases, chymotrypsin, short chain dehydrogenase, trypsin and cuticle proteins identified in other comparisons.

There was only 1 P450 transcript (*CYP6AK1*, FC = 1.69) overexpressed in R_{DDT}-S (Table 4.5). Other transcripts upregulated in the comparison are cuticle proteins (*AGAP009480-RA___3R*, FC = 1.94; *AGAP003382RA_Cuticular*, FC = 1.84), chymotrypsin 1, (combined_c3760, FC = 2.13), short-chain dehydrogenase (*CD577943.1*, FC = 1.72) and the nucleotide binding protein 2 (*AGAP011997-RA___3L*, FC = 1.64).

Transcripts of the P450s, GSTs among other detoxifying enzymes were overexpressed in the C-S comparison. Four transcripts of the P450 genes: *CYP9J3* (FC = 2.30); *CYP4AA4* (FC = 3.49); *CYP6P9b* (FC = 14.48) and *AGAP012291-RA___3L*, (FC = 2.42) and the *GSTS1* (FC=2) were overexpressed. Other detoxification transcripts overexpressed are the short-chain dehydrogenase, *AGAP001405-RA___2R* (FC = 2.42), carboxylesterase, *COEAE6O* (FC = 2.15), three transcripts of cuticle proteins (*combined_c2672*, FC=2.2; *Afun008525*, FC = 20.18; *CD577507.1*, FC = 2.32) and three transcripts of trypsin (*AGAP011431-RA___3L*, FC = 2.02; *CD578079.1*, FC = 2.7; *CD578079.1*, FC = 2.98).

The cross analysis of Rperm-S and R_{DDT}-S comparison, which was conducted to identify potential cross resistance genes associated with both DDT and permethrin resistance did not produce any transcript (Figure 4.24). However, the cross analysis produced three transcripts of glucosyl glucuronosyl transferases with fold changes of 2.25 (combined_c8336), 2.44 (Afun007571) and 2.1 (combined_c8336) for the Rperm-S comparison. In addition, there was an overexpression of *GSTU2* (FC = 4.25),

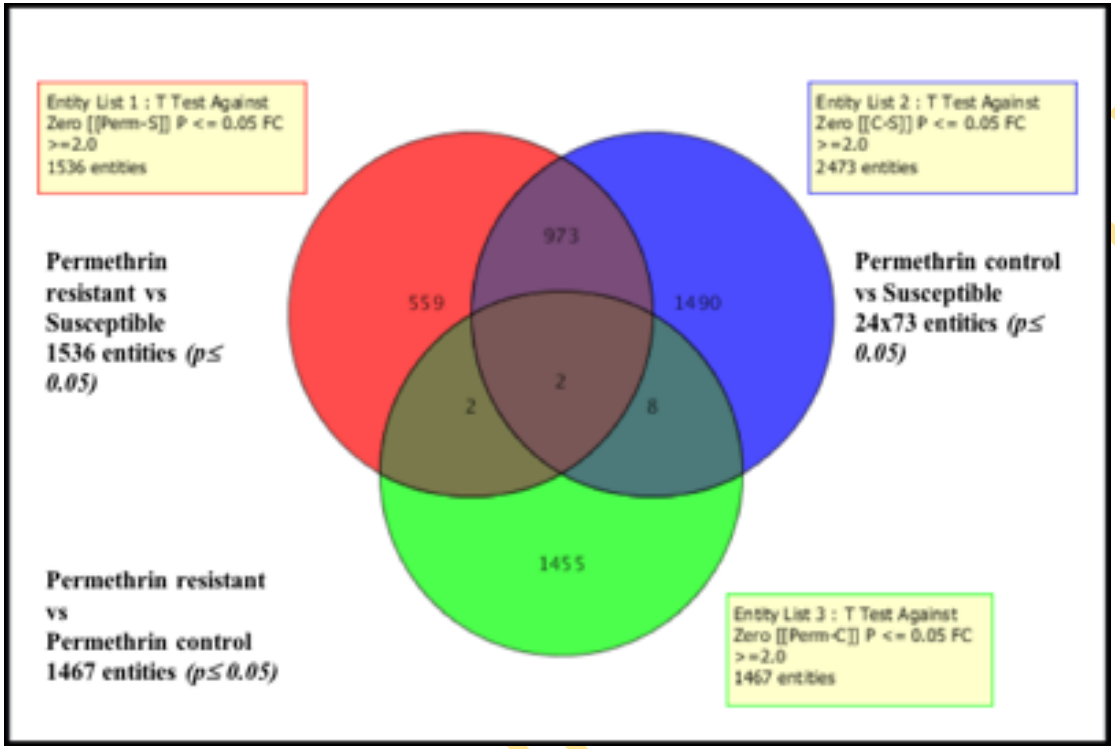


Figure 4.23: Common transcripts overexpressed in different populations of permethrin resistance analysis with microarray.

Values in the circle: Common overexpressed transcript for each comparison.

the transmembrane protein, cytochrome b561 (FC = 2.02) and the digestive enzyme, chemotrypsin 1 (FC = 4.75).

4.10 Reverse Transcriptase PCR for Investigating Target Resistance-associated Genes in Insecticide Resistance of *Anopheles funestus*

Reverse transcriptase PCR (RT-PCR) showed a significant upregulation of a key resistant-associated gene, *GSTe2* in *An. funestus* at Akaka-Remo. The delta *GSTs; GSTd3* and *GSTd1-5* were other GST genes that were overexpressed in permethrin and DDT resistant *An. funestus* compared to the control population. The two duplicate genes, *CYP6P9a* and *CYP6P9b* had a higher overexpression in DDT-resistant *An. funestus* samples compared to the permethrin-resistant population. Also, the *CYP9K1* had a higher overexpression level in DDT-resistant *An. funestus* compared to permethrin-resistant and dieldrin-resistant populations. In addition, trypsin was highly overexpressed in all resistant *An. funestus* compared to the control population.

Genes associated with Permethrin resistance

The *GSTe2* gene was upregulated in permethrin resistance (FC = 44.89) compared to the unexposed/control (FC = 22.34) population (Figure 4.25). Both *GSTd3* and *GSTd1-5* were also upregulated in resistant mosquito samples compared to the unexposed controls but with lower folds [*GSTd3*: FC = 4.27 (resistant) versus FC = 1.75 (unexposed) and *GSTd1-5*: FC = 7.1 (resistant) versus FC = 4.3 (unexposed)] compared to *GSTe2.*

None of the cytochrome P450 genes had clear expression in Rperm population over the unexposed population, except *CYP6P4a* (FC = 2.98 in resistant versus FC=1.72 in unexposed) and slightly in *CYP9K1* (FC = 2.66 in resistant versus FC = 2.44 in unexposed). *Trypsin* was the only gene that was overexpressed in Rperm population with microarray experiment (FC = 4.75) and also with a distinct upregulation in RT-PCR (FC = 2.42 in resistant versus FC = 0.31 in unexposed).

Genes associated with DDT resistance

The *GSTe2* had the highest expression in the DDT resistant population producing a fold change of 57.39 in DDT resistant samples versus FC = 22.34 in control (Figure 4.26).

Table 4.4: Detoxification genes overexpressed in permethrin resistant population of *Anopheles funestus*

S/N	Gene Name	Rperm-S [FC]	C-S [FC]	Rperm-C [FC]	Ortholog in An. gambiae	Description
1	Afun000762	5.19	3.83	2.53	AGAP006733	THO complex subunit 4
2	CD578215.1	17.81	43.40			cuticle protein
3	CD578215.1	14.75	36.74			cuticle protein
4	Afun015266	2.44	2.88		AGAP010911-PA	Carboxylesterase
5	Afun010482	18.00	65.91		AGAP008449-PA	cuticle protein
6	CD577507.1	3.56	5.87			cuticle protein
7	Afun013390	4.11	3.47		AGAP000344-PB	cuticular protein rr-1
8	combined_c8336	2.25				glucosyl glucuronosyl transferases
9	Afun007571	2.44			AGAP007920-PA	glucosyl glucuronosyl transferases
10	combined_c8336	2.10				glucosyl glucuronosyl transferases
11	Afun008617	2.02			AGAP005222-PB	cytochrome b561
12	Afun015122 (*GSTU2*)	4.25			AGAP003257-PA	glutathione-s-transferase gst
13	Afun010134	4.75			AGAP006711-PA	chymotrypsin 1
14	AGAP001405-RA___2R		2.42		AGAP001405-RA___2R	short-chain dehydrogenase
15	gb-*CYP9J3*		2.30			cytochrome p450
16	combined_c2672		2.20			cuticular protein 97eb
17	COEAE6O		2.15		AGAP002863-PA	Carboxylesterase
18	*CYP6AA4*		3.49			cytochrome p450
19	AGAP011431-RA___3L		2.02		AGAP011431-RA___3L	trypsin 5
20	CD577405.1		2.00			glutathione s-transferase
21	BU039010.1		2.13			cytochrome c
22	Afun008525		20.18		AGAP000047-PA	cuticular protein rr-1
23	CD577404.1		2.38			glutathione s-transferase
24	CD577507.1		2.32			cuticle protein
25	AGAP012291-RA___3L		2.42		AGAP012291-RA___3L	cytochrome p450
26	CD578079.1		2.70			Trypsin
27	CD578079.1		2.98			Trypsin
28	Afun010994 (CYP6P9b)		14.48		AGAP002867-PA	cytochrome p450
29	AGAP004164-RC_glutat...			2.09	AGAP004164-RC_glutat...	glutathione transferase
30	*CYP6P2*			2.47		cytochrome p450
31	Afun014849			2.03	AGAP011507-PA	esterase fe4
32	*CYP6P9a*			2.92		cytochrome p450

Also, *GSTd3* expression (FC = 5.1) was higher in DDT resistance compared to control (FC = 1.75) with almost 3 folds, while the expression of *GSTd1-5* in DDT resistant samples (FC = 5.51) was just a little more than 1 fold compared to control (FC = 4.3).

The two duplicate cytochrome P450 genes, *CYP6P9a* and *CYP6P9b* were both slightly overexpressed in DDT resistant samples compared to the control (Figure 4.26). Expression was higher in *CYP6P9a* (FC = 5.19) than *CYP6P9b* (FC = 3.91). *CYP4C27* expression was also slightly higher in DDT resistant samples (FC = 0.92) compared to control (FC = 0.65). The expression of *CYP9K1* was the highest (FC = 9.05) in all of the cytochrome P450 genes analysed compared to the control (FC=2.44).

Trypsin expression in the DDT resistant population was also significantly overexpressed (FC = 6.21) compared to control (FC = 0.31).

Genes associated with Dieldrin resistance

The GSTs genes, cytochrome P450 genes, aldehyde oxidase and trypsin were all overexpressed in dieldrin resistance samples compared to control (Figure 4.27).

The *GSTe2* expression was almost 2 folds higher in the dieldrin resistant population (FC = 41.1) compared to control (FC = 22.34). Both *GSTd3* and *GSTd1-5* genes were underexpressed in the dieldrin resistant population compared to control (*GSTd3*: FC = 0.28 vs FC = 1.75; *GSTd1-5*: FC = 0.78 vs FC = 4.30).

The duplicate genes, *CYP6P9a* and *CYP6P9b* were overexpressed in dieldrin resistant population compared to control (Figure 4.27). As observed in DDT resistant samples, *CYP6P9a* (FC = 6.51) expression was relatively higher than *CYP6P9b* (FC = 5.71). Another P450 gene duplicate, *CYP6P4a* and *CYP6P4b* were also overexpressed in dieidrin resistant samples compared to control. The expression level of *CYP6P4a* was significantly higher in dieldrin resistant population (FC = 4.75) compared to control (FC = 1.72).

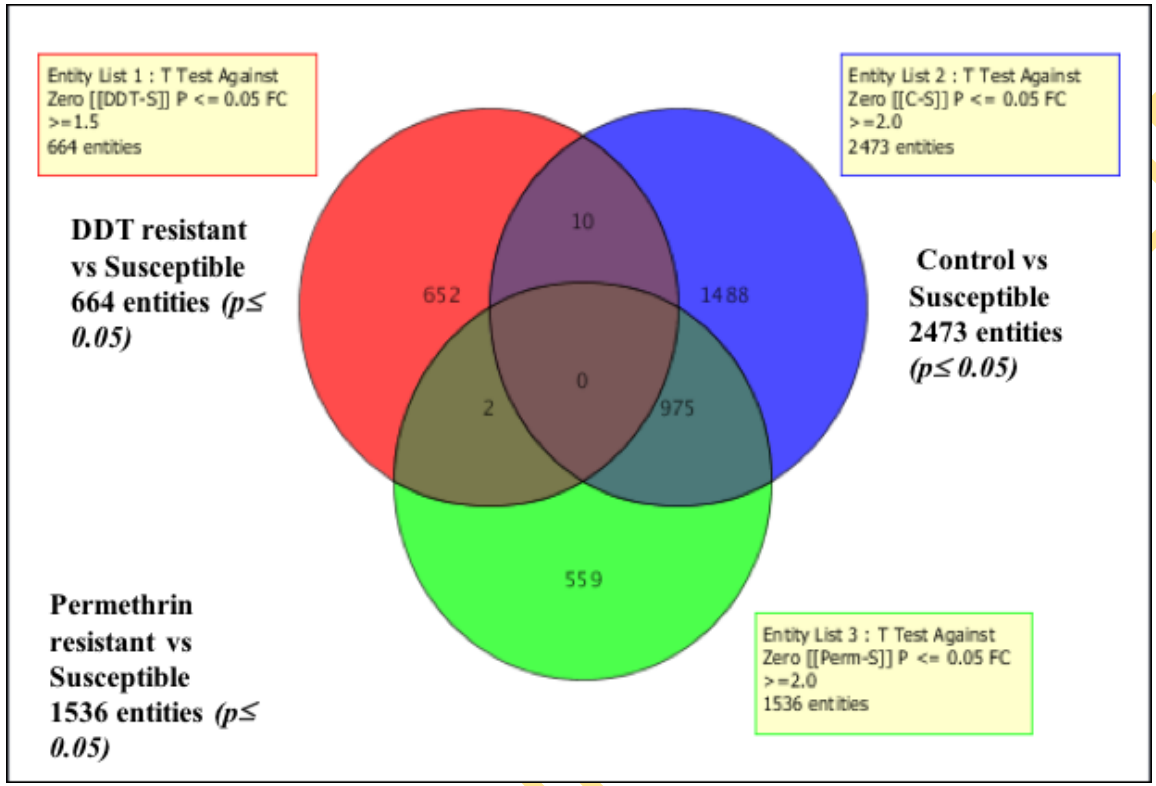


Figure 4.24: Common transcripts overexpressed in different Populations of DDT resistance analysis and the potential detection of cross-resistance genes involved in DDT and permethrin resistance with microarray.

Values in the circle: Common overexpressed transcript for each comparison.

Table 4.5: Detoxification genes overexpressed in DDT resistant population of *Anopheles funestus*

S/N	Gene Name	R_{DDT}-S [FC]	C-S [FC]	Rperm-S [FC]	Ortholog in An. gambiae	Description
1	gb-*CYP6AK1*	1.69				Cytochrome P450
2	AGAP009480-RA___3R	1.94			AGAP009480-RA___3R	cuticular protein
3	AGAP003382-RA_Cuticular	1.82			AGAP003382-RA_Cuticular	cuticle protein
4	combined_c3760	2.13				chymotrypsin 1
5	CD577943.1	1.72				short-chain dehydrogenase
6	AGAP011997-RA___3L	1.64			AGAP011997-RA___3L	nucleotide binding protein 2
7	AGAP001405-RA___2R		2.42		AGAP001405-RA___2R	short-chain dehydrogenase
8	gb-*CYP9J3*		2.30			cytochrome p450
9	combined_c2672		2.20			cuticular protein 97eb
10	COEAE6O		2.15		AGAP002863-PA	Carboxylesterase
11	*CYP6AA4*		3.49			cytochrome p450
12	AGAP011431-RA___3L		2.02		AGAP011431-RA___3L	trypsin 5
13	CD577405.1 (*GSTS1*)		2.00			glutathione s-transferase
14	Afun008525		20.18		AGAP000047-PA	cuticular protein rr-1 family
15	CD577404.1		2.38			glutathione s-transferase
16	CD577507.1		2.32			cuticle protein
17	AGAP012291-RA___3L		2.42		AGAP012291-RA___3L	cytochrome p450
18	CD578079.1		2.70			Trypsin
19	CD578079.1		2.98			Trypsin
20	Afun010994 (*CYP6P9b*)		14.48		AGAP002867-PA	cytochrome p450
21	combined_c8336			2.25		glucosyl glucuronosyl transferases
22	Afun007571			2.44	AGAP007920-PA	glucosyl glucuronosyl transferases
23	combined_c8336			2.10		glucosyl glucuronosyl transferases
24	cytochrome b561			2.02	AGAP005222-PB	cytochrome b561
25	Afun015122 (*GSTU2*)			4.25	AGAP003257-PA	glutathione-s-transferase gst
26	Afun010134			4.75	AGAP006711-PA	chymotrypsin 1

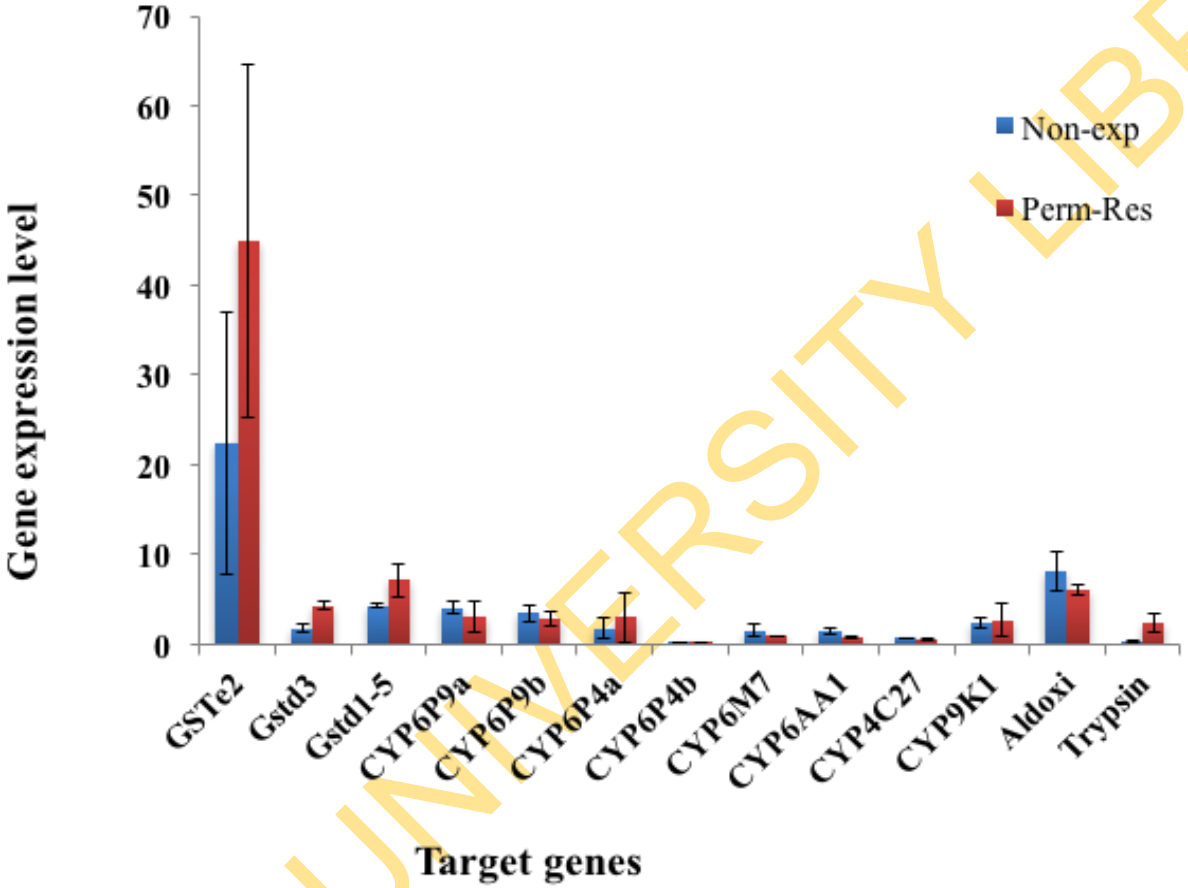


Figure 4.25: Expression level of selected metabolic genes in permethrin-resistant *Anopheles funestus* mosquitoes at Akaka-Remo.

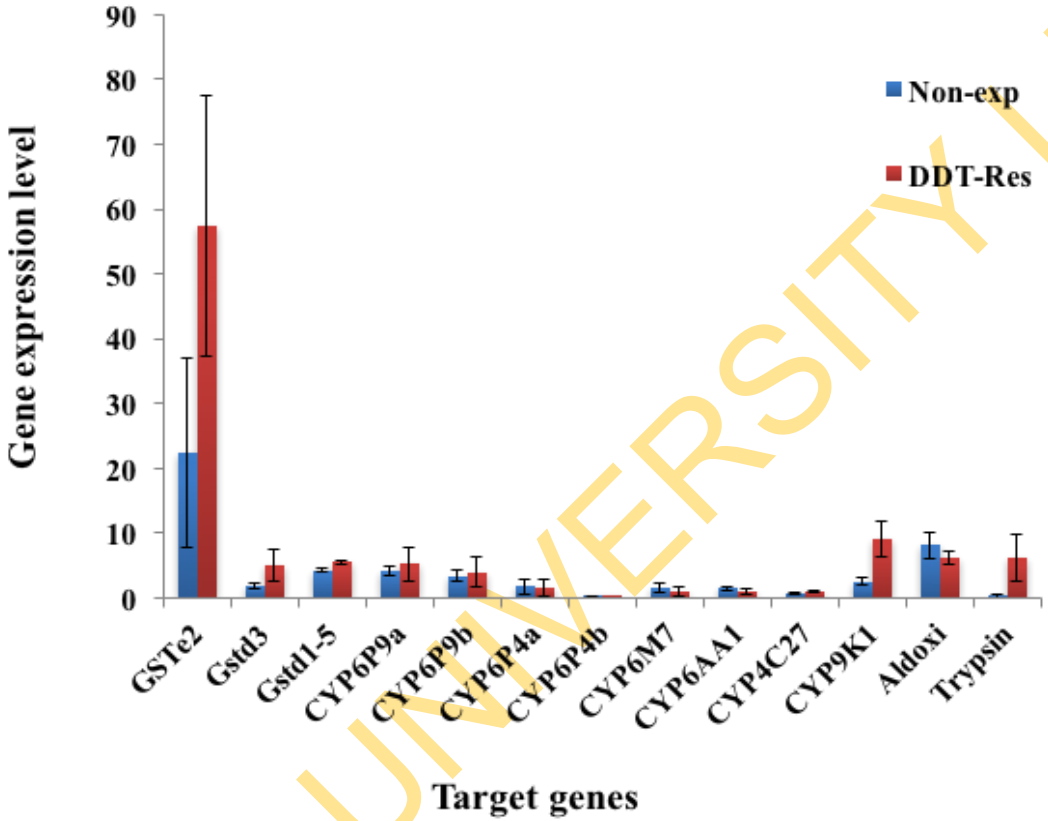


Figure 4.26: Expression level of selected metabolic genes in DDT-resistant *Anopheles funestus* mosquitoes.

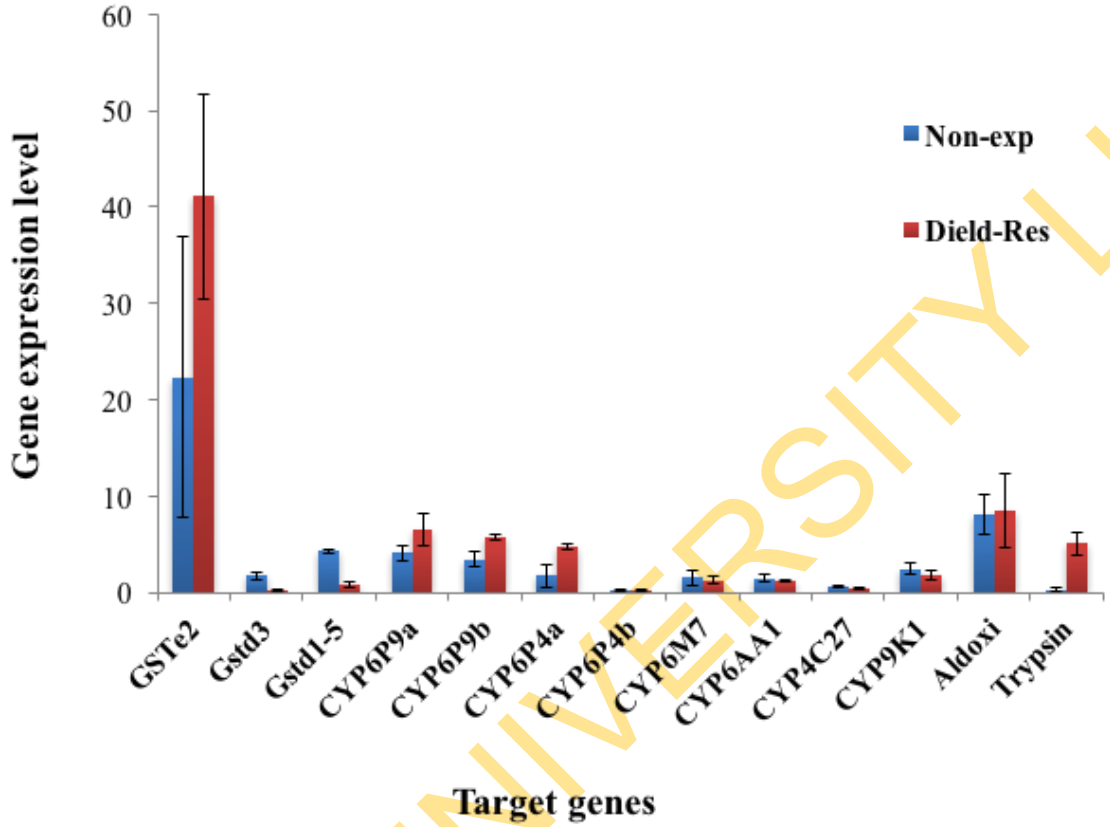


Figure 4.27: Expression level of selected metabolic genes in dieldrin-resistant *Anopheles funestus* mosquitoes.

Aldehyde oxidase was slightly overexpressed in dieldrin resistant samples (FC = 8.58) compared to control (FC = 8.12). Futhermore, *Trypsin* expression in dieldrin resistant population is similar to its expression in permethrin and DDT-resistant mosquitoes. This enzyme was overexpressed in dieldrin resistant samples (FC = 5.14) compared to control (FC = 0.31).

CHAPTER FIVE

DISCUSSION

Consistent monitoring of malaria vectors through provision of evidence-based information is key to tackle challenges facing malaria elimination in Africa. In Nigeria, the density of infected *Anopheles* mosquitoes keeps increasing and the southwestern region of the country is not an exemption (Awolola *et al.*, 2005a; Oyewole *et al.*, 2005; Oyewole and Awolola, 2006; Oduola *et al.*, 2012; Okorie *et al.*, 2015). The creation of suitable breeding environments for mosquito survival is heavily associated with the continuous growth and spread of *Anopheles* mosquitoes in this region (Adeleke *et al.*, 2013; Joseph *et al.*, 2013). High agricultural practices as a result of favorable climates, fertile soil and desired rainfall in this region have also ignorantly created more breeding sites for mosquitoes, especially in the rural and sub-urban areas (Oladepo *et al.*, 2010). As long as *Anopheles* mosquitoes breeding sites increase in our environment, malaria incidence and mortality will continue to grow. *Anopheles funestus* is not widely spread as *An. gambiae* in the southwest of Nigeria, but there are reports that it is playing a role in malaria transmission within the region (Awolola *et al.*, 2005a; Oyewole *et al.*, 2005; Oduola *et al.*, 2012).

The use of insecticides as control tools, either by spraying the walls with insecticides or sleeping under insecticide-treated nets to avoid being bitten by infected mosquitoes is promising, despite the huge challenge of resistance developed by malaria vectors (Okorie *et al.*, 2015; WHO, 2017b). Understanding the spread of infected mosquitoes in our environment, their survival rate when exposed to insecticides and the mechanisms used to survive lethal doses of insecticides are imperative to design suitable density and resistance management strategies that will be strong enough to combat malaria vectors/disease in Nigeria (Djouaka *et al.*, 2016; Ibrahim *et al.*, 2016; Menze *et al.*, 2016; Samb *et al.*, 2016).

5.1 The Role of *Anopheles funestus* in Malaria Transmission at Akaka-Remo

The dominance of *An. funestus* at Akaka-Remo over other mosquito species indicates that it could be available in all climatic season at Akaka-Remo. *Anopheles funestus*'s dominance over *An. gambiae* is also showing its importance as a potent malaria vector at Akaka-Remo. This is in contrast with the report of Oyewole *et al.* (2005), where *An. funestus* collected within two years, n = 85 was nowhere near that of *An. gambiae*, n = 500. The reason for the dominance of *An. funestus* (*An. funestus* = 315 versus *An. gambiae* = 26) at Akaka-Remo in 2015 (10 years after the first report) might not indicate that *An. funestus* is taking over as the main *Anopheles* mosquito at Akaka-Remo. It might just suggest that there are now more suitable breeding sites for *An. funestus* at Akaka-Remo. Moreover, declaring the dominance of *An. funestus* over *An. gambiae* in all climatic seasons could attract some form of sentiment, since there was no mosquito collection during the peak of the rainy season, June and September: a period that is considered as the peak of *An. gambiae* in southwest Nigeria (Uttah *et al.*, 2013; Ebenezer *et al.*, 2014).

Overall, this assessment showed that *An. funestus* could be available especially during the dry and transition from dry to rainy seasons at Akaka-Remo, a period when *An. gambiae* is rarely found in our environment (Ebenezer *et al.*, 2014). Consequently, they could be more involved in malaria transmission during these two periods when *An. gambiae* is not always available.

The 8 % *Plasmodium* infection rate observed in *An. funestus* at Akaka-Remo coupled with previous reports in Ogun (Oyewole *et al.*, 2005), Lagos (Oyewole and Awolola, 2006), Oyo and Kwara states (Awolola *et al.*, 2005a) indicates that *An. funestus s.s.* is an important malaria vector in southwest Nigeria. These reports emphasize the contribution of this vector and the threat it could pose to malaria transmission in this region. Although, the infection rate observed at Akaka-Remo is relatively low compared to other infections across Africa [such as the 50 % (Costantini *et al.*, 1999) and 20 % (Dabiré *et al.*, 2007) in Burkina Faso; the 13.6 % (Sandeu *et al.*, 2012) and 18 % (Djouaka *et al.*, 2016) in Benin and the 12.5 % in Ghana (Riveron *et al.*, 2016)], the burden of malaria is still a greater challenge in Nigeria due to high human population.

Remarkably, variations in infection rates reported in these regions above could be due to the differences in the methods used for analysis. Overall, the level of *Plasmodium* infection observed in this *An. funestus* population is a sign that they are involved in malaria transmission at Akaka-Remo. Another common member of the *An. funestus* group, *An. rivulorum* that was previously identified both indoor and outdoor at Akaka-Remo (Oyewole *et al.,* 2005) was absent in this study. This could be potentially due to a change in resting preference of this mosquito species, although more entomological studies are needed to explain this change in mosquito behaviour.

Reports on *An. gambiae* in Nigeria have shown that infection rates range mostly between 2 and 8.1% (Awolola *et al.,* 2005a; Oyewole *et al.,* 2005, 2010; Oyewole and Awolola, 2006). This observation highlights a similar malaria transmission potential of *An. funestus* compared to *An. gambiae*. As a result, *An. funestus* should be given similar medical importance accorded to *An. gambiae*, this will strengthen the efficacy of malaria control tools in Nigeria.

5.2 Multiple Insecticide Resistance of *Anopheles funestus* at Akaka-Remo

This study shows that *An. funestus s.s.* at Akaka-Remo have developed resistance to commonly used public health insecticides. Results obtained highlight the presence of multiple insecticide resistance in this malaria vector. In Nigeria, most studies of insecticide resistance have focused on *An. gambiae* with less interest on *An. funestus*, as this vector was essentially thought to be susceptible to pyrethroids (Dia and Guelbeogo, 2013). However, this study has clearly revealed that *An. funestus* at Akaka-Remo have not only developed resistant to pyrethroids but to other insecticide families.

The resistance developed against organochlorines was the highest of all the insecticide families tested with WHO bioassays. The DDT and dieldrin resistance recorded could be associated with the residual effect of the long historical usage of organochlorines in agriculture, when this sector was a key source of income in Nigeria (Ogen, 2007). This revenue generating sector later bowed to the oil boom in the 1970s, this later shifted the national attention from agriculture to the oil and gas sectors, which has become the backbone of Nigeria economy (Oil Boom, 2003).

It is possible to also argue that poor attitudes and/or ignorance of farmers towards

observing good farming practices when using this insecticide for pests control have assisted the level of observed resistance (Reid and McKenzie, 2016). During mosquito collections at Akaka-Remo, it was observed that some of the villagers used field agrochemicals to control insects at homes. Such ignorance or nonchallance could have aggravated the high level of DDT and dieldrin resistance at Akaka-Remo.

Cases of DDT resistance have also been reported in other Africa countries, such as in Uganda and Kenya with 40-42 % *An. funestus* mortality (Mulamba *et al.*, 2014) and Malawi with 69.9 % mortality (Riveron *et al.*, 2015) but none is close to the level recorded at Akaka-Remo except for the report in Benin with zero mosquito mortality (Djouaka *et al.*, 2011). High DDT resistance recorded in Benin and Nigeria in West Africa compared to a relatively lower resistance in the East and the southern Africa might be due to evolutionary changes of *An. funestus* species between regions leading to different genetic make-up. The role of genetic constitution on mosquito response to insecticide is inevitable. Generally, mutation on regions, especially the target sites of insecticides is known to influence insecticide response in insects.

The resistance recorded with dieldrin exposure is not only the highest of all the six insecticides tested but also the highest recorded in Africa until now. The 8 % mortality recorded and the 30 % mortality at Burkina-Faso (Wondji *et al.*, 2011) is an indication that dieldrin insecticide may not be effective to control *An. funestus* mosquito in West Africe. However, in southern Africa, dieldrin susceptibility have been frequently observed with *An. funestus* until a recent report of resistance in Malawi (83.9 % mortality) (Riveron *et al.*, 2015), which could be a concern for mosquito control in that region.

Overall, organochlorine resistance recorded with *An. funestus* coupled with reports of DDT resistance in *An. gambiae* mosquito populations in Nigeria (Oduola *et al.*, 2010; Okorie *et al.*, 2015) may further disapproves the re-introduction of this insecticide family as an alternative to pyrethroids for mosquito control in Nigeria. It is therefore important to determine the extent to which *An. funestus* have developed resistance to organochlorines by investigating its spread across different geographical regions in Nigeria.

Pyrethroid resistance on the other hand is high but not to the level of DDT and

dieldrin. Susceptibility test to pyrethroids is important because Nigeria depends so much on this insecticide family for malaria vector control programmes (WHO, 2016b). Pyrethroids resistance in *An. funestus* from Akaka-Remo is of great concern for malaria control programmes and there is a risk that this mosquito species would have developed resistance to pyrethroids in different regions of Nigeria due to the current use of pyrethroids both in agriculture and public health all over the country. If this happens, it will constitute more ordeals for future malaria vector control interventions through the use of pyrethroid-based insecticides.

Reports keep showing that pyrethroid resistance in *An. funestus* is spreading in West Africa. The pattern of resistance also varies in type I (permethrin) and type II (deltamethrin) pyrethroids across Africa regions. In West Africa, resistance developed against permethrin is higher than deltamethrin, suggesting a stronger effect with deltamethrin (Djouaka *et al.,* 2011; 2016). However, the pattern of pyrethroids resistance in West Africa is different in East (Mulamba *et al.,* 2014) and South Africa (Wondji *et al.,* 2012), where deltamethrin resistance is higher than that of permethrin. Just like with organochlorines, *An. funestus* from different population might be responding differently to pyrtehroids due to difference in their genetic constitutions that might not only have altered the binding site of these insecticides but have influenced the expression of key detoxification enzymes.

The level of pyrethroid resistance might be due to the fact that *An. funestus* at Akaka-Remo have developed a cross-resistance with organochlorines, a similar case between carbamates and pyrethroids in Malawi (Ibrahim *et al.,* 2016). If this is the case, then there is a call for more urgent attention on this vector, otherwise, resistance will continue to develop untill these synthetic chemicals become useless for mosquito control. The consequences will be burdensome; more resistant *An. funestus* will dominate our environments, and because they can resist commonly used insecticides, they would gain more access indoors after surviving insecticide exposure, as a result leading to increasing malaria cases in Nigeria. There is now an urgent need for further assessment to determine the extent of resistance developed against pyrethroids in Nigeria.

Bendiocarb resistance is also increasing, with carbamate-based insecticides being introduced to serve as an alternative to pyrethroids in West Africa facing huge

challenge sooner than later (Akogbéto *et al.*, 2010). Resistance already reported in Benin, Zambia, Zimbabwe and Mozambique (Cuamba *et al.*, 2010; Chanda *et al.*, 2011; Djouaka *et al.*, 2011; Choi *et al.*, 2014) is a pointer to future problems as the success of bendiocarb-based IRS already been compromised. Hence, it will be important to have more information on the potential spread of resistance across different regions in Nigeria and some underlying factors that might be responsible for resistance. This information will guide the malaria control programmes to improve her subsequent release of bendiocarb-based IRS.

The organophosphate malathion is really proving to be the most reliable insecticide considering similar records of full susceptibility to *An. funestus* all over Africa (Mulamba *et al.*, 2014; Djouaka *et al.*, 2016; Menze *et al.*, 2016). This insecticide will be a good alternative to pyrethoid insecticide to manage the on-going resistance developed by different *An. funestus* populations.

In Nigeria, agrochemicals must be approved by the National Agency for Food and Drug Administration and Control, NAFDAC before they are released for commercial and domestic purposed (NAFDAC, 2004). It is possible that the mis-use and/or over use of these chemicals by farmers could be fundamental to the multi-resistance selection in this locality. The indiscriminate use of agrochemicals by farmers could have also produced high chemical residues and other environmental pollutants that are washed into the water bodies that serve as mosquito breeding sites, generating several xenobiotics that exercise a resistance selection in mosquitoes at larval stages (Akogbéto *et al.*, 2006; Antonio-Nkondjio *et al.*, 2011; Tene Fossog *et al.*, 2012; Philbert *et al.*, 2014).

Similarly, spilled petroleum products often discharged into Erititi stream at Akaka-Remo during washing of motor bikes and cars could be an intriguing factor. This activity is common in mosquito breeding sites in southwest Nigeria (Djouaka *et al.*, 2007) and might have also contributed to resistance selection of *An. funestus* at Akaka Remo through cross resistance mechanisms. Both environmental factors (generation of chemical pesticides and spillage of petroleum products) are common in Nigeria and can certainly contribute to the local selection of the observed insecticide resistance profiles. Further assessments are still needed to clearly map out the actual factor (s) contributing to the multi-insecticide resistance of *An. funestus* at Akaka-Remo.

5.3 Probable Defense Mechanisms Built by *Anopheles funestus* Against Insecticides at Akaka-Remo

Tackling the growing problems facing insecticide resistance is important to sustain the progress made so far to reduce malaria burden. Understanding the molecular basis of resistance of each insecticide class or noticing any potential cross-resistance if there is any, is cardinal for the implementation of suitable resistance management strategies of malaria vectors. Generally, target-site resistance plays a pivotal role in insecticides resistance of *An. gambiae* in Nigeria (Ibrahim *et al*., 2014). But so far, no *kdr* mutation (L1014F/S) has been reported in insecticide resistance of *An. funestus* populations (Mulamba *et al.*, 2014; Menze *et al.*, 2016; Samb *et al.*, 2016). However, other target site mutations such as N485I was recently reported to potentially drive insecticide resistance of *An. funestus* (Ibrahim *et al.*, 2016).

Till now, metabolic resistance mechanism has been the main driver of insecticide resistance in *An. funestus* (Menze *et al.*, 2016; Samb *et al.*, 2016). There has been evolvement of several metabolic enzymes that have strong capacity to detoxify synthetic insecticides in *An. funestus* (Riveron *et al.*, 2014a; Ibrahim *et al.*, 2016). Cytochrome P450 genes and Glutathione-s-transferases are the leading detoxifying enzymes that have been associated with insecticide resistance of *An. funestus* (Riveron *et al.*, 2014a; Samb *et al.*, 2016). This study has been able to explore for the first time in Nigeria, some mechanisms that could be driving the multi-insecticides resistance observed in *An. funetsus* at Akaka-Remo.

5.3.1 Analysis of the Voltage-Gated Sodium Channel Region of *Anopheles funestus*

Results obtained from sequencing the VGSC of *An. funestus* revealed that knockdown resistance is unlikely to be playing any role in the insecticide resistance of *An. funestus* at Akaka-Remo. Both L1014F and L1014S mutations that are common to *An. gambiae* in West and East Africa, respectively were absent in all the *An. funstus* mosquitoes analysed.

The VGSC polymorphisms observed at Akaka-Remo compared to Benin and Cameroon populations could be as a result of genetic evolution; either through genetic mutation, genetic drift, migration or natural selection in the different mosquito

populations (Riveron *et al.*, 2017). Generally, these genetic process could influence mosquitoes in the same population to carry different genome constitution and consequently could help some mosquitoes to survive insecticide exposure over others. Moreover, some mosquitoes also undergo multiple genetic process, which could potentially lead to extreme cases of insecticide resistance such as the zero mortality recorded against DDT in Benin *An. funestus* population (Djouaka *et al.*, 2011).

It is appeasing that the diversity of nucleotide sequence observed might not eventually have effect on the genetic determinant of *An. funestus* at Akaka-Remo. However, the potential role of *kdr* (L1014F/S) in insecticide resistance of *An. funestus* should be monitored; as it could occur suddenly, since genetic polymorphism in this mosquito species is always high. It could also help to detect other potential mutations that could drive insecticide resistance in *An. funestus* in the future (Mulamba *et al.*, 2014; Menze *et al.*, 2016).

5.3.2 Synergist Tests Successfully Implicated Key Metabolic Enzymes in Insecticide Resistance of *Anopheles funestus*

This preliminary assessment revealed the potential involvement of oxidase, esterase and GSTs in Pyrethroids and DDT resistance. The fact that *An. funestus* at Akaka-Remo showed a full susceptibility to permethrin after these enzymes were inhibited could only suggest their distinct roles in permethrin resistance. In addition, *An. funestus* at Akaka-Remo seem to always express both phase I and phase II detoxification enzymes for permethrin metabolism. This kind of scenario could only build stronger permethrin resistance for this mosquito population (David *et al.*, 2013; Mitchell *et al.*, 2014).

Simply, it will be difficult for insecticide to cross all barriers set by these metabolic enzymes and as a result will unlikely bind to the target-site. Generally, phase I metabolic enzymes (oxidase and esterase) always act first on toxic insecticides to make them water soluble before phase II (mainly GSTs) attaches its glutathione for conjugation process, to make phase I products/metabolites much more water soluble for easy excretion (Chahine and O'Donnell, 2011; Nardini *et al.*, 2012). Soluble products are always excreted from the mosquito body system using the phase II and III (mainly transport proteins) enzymes. This metabolic mechanism pattern used by *An.*

funestus at Akaka-Remo would have been key to their permethrin survival. However, some populations of *An. funestus* have built a stronger resistance strategy by over producing mutant forms of these metabolic enzymes (Riveron *et al.*, 2014a). Resistance pattern could be perculiar to different *An. funestus* population and as a result, it influences their level of resistance to insecticides.

These enzyme families are also playing a role in DDT resistance. However, esterase and GSTs are playing greater roles compared to oxidase in DDT resistance of *An. funestus*. The role of esterase in DDT resistance could be similar with the P450s and GSTs. Oxidase playing a strong role in dieldrin resistance just as in permethrin is a good bargain for *An. funestus*. It simply shows that oxidase is capable of fighting more than a family of insecticide with different target site at the same time. Before now, target-site mutation through the *Rdl* has always been the main driver of dieldrin insecticide (Wondji *et al.*, 2011). However, the involvement of oxidase in dieldrin resistance might have been responsible for the stronger resistance developed by *An. funestus* at Akaka-Remo.

5.3.3 Analysis of L119F point mutation on the *GSTe2* gene of *Anopheles funestus*

The replacement of leucine with phenylalanine on position 119 of the *GSTe2* gene in all F_0 *An. funestus* is an indication that the L119F mutation is dominant at Akaka-Remo and consequently, this mutation could be transferred to next *An. funestus* generation. The fact that L119F mutation is even close to fixation in *An. funestus* population is a big concern for malaria control programmes. DDT-based insecticide might not be the right option as an alternative to pyrethroid-based insecticides for *An. funestus* control at Akaka-Remo. This mutation alone is known to have the capacity to confer DDT resistance in *An. funestus* populations (Mulamba *et al.*, 2014; Djouaka *et al.*, 2016). As a result, unless urgent attention is given to developing stronger DDT-based insectcides, *An. funestus* might become more resistance to existing DDT insectcides at Akaka-Remo and this will further cause more problems for malaria control.

5.3.4 Genotyping Analysis of Resistant Alleles (119F and 296S) in *Anopheles funestus*

The frequency of resistant alleles gives an overall idea on the kind of mosquitoes in a

population. It provides a prior information on whether a mosquito will survivie insecticide exposure or not. Naturally, mosquitoes carrying either of this mutation are expected to survive DDT and dieldrin exposure, respectively but that is not always the case as some may die during exposure. Some other factors are responsible, such as the presence of other mechanisms playing more crucial roles in resistance.

On the other hand, this kind of mutation could make the mosquito more resistant depending on the nature of other mechanisms at work in the mosquito. Whether the presence of these resistance alleles would have grave consequence on resistance depends largely on the amount and potency of mechanisms the mosquito use to survive insecticide exposure. Genotyping analysis of the *GSTe2* and *Rdl* genes gave more information on why there was high resistance against DDT and dieldrin insecticides in *An. funestus* at Akaka-Remo.

5.3.4.1 Analysis of the L119-GSTe2 in F_0 and F_1 Population of *Anopheles funestus*

The high frequency of L119F observed in F_0 *An. funestus* is a confirmation of high mutation on this region of the *GSTe2,* indicating a high evolution of this gene in the mosquito population. The allelic frequency (R = 77%) recorded further established that this mutation is almost getting fixed in the *An. funestus* population. Also, the high frequency of the resistant allele noticed in the resistant (R = 80%) and susceptible (R = 72%) populations, suggest that if an *An. funestus* is picked at Akaka-Remo, it will likely carry the L119F mutation regardless of its DDT resistance status.

Futhermore, having such a high frequency in the susceptible population clearly showed that this resistance marker might not be good for discriminating DDT resistance in *An. funestus* at Akaka-Remo. However, the presence of high resistance allele in both the F_0 and F_1 indicates the significance role of *GSTe2* in DDT resistance of this mosquito population. This scenario has been previously observed in *An. gambiae,* where the frequency of L119F mutation was high in both resistant and susceptible population (Okorie *et al.,* 2015).

The L119F-GSTe2 mutation has also been detected in other DDT-resistant *An. funestus* populations. Cases of Benin (72.5 %), Ghana (44.2 %) and Burkina-Faso (25 %) mosquitoes in West Africa (Okoye *et al.,* 2008; Wondji *et al.,* 2011; Riveron *et al.,* 2014a); Cameroon (48.2 %) in Central Africa (Wondji *et al.,* 2011) as well as Uganda

(20.4 %) and Kenya (7.8 %) in East Africa (Mulamba *et al.*, 2014) are clear indications that this mutation has a lot to do with DDT survival in *An. funestus*. In Southern Africa (Malawi) however, L119F allele is absent despite the recent detection of DDT resistance in this region suggesting a different DDT resistance mechanism in this *An. funestus* population (Riveron *et al.*, 2015).

5.3.4.2 Analysis of the A296S-Rdl in F_0 and F_1 Population of *Anopheles funestus*

The A296S-Rdl mutation has been associated with dieldrin resistance in West Africa (Burkina Faso) and Central Africa (Cameroon) (Wondji *et al.*, 2011). The presence and frequency of this mutation at Akaka-Remo highlights how important this survival strategy is to this *An. funestus* population. The clear discrimination of this resistant marker in resistant population compared to the susceptible ones is an indication that the mutation is far from fixation, and as such could be good for discriminating dieldirn resistance in *An. funestus* at Akaka-Remo.

The presence of this mutation indicates that *An. funestus* population from Akaka-Remo adopts more than one mechanism to survive dieldrin exposure. Expressing P450s enzymes with the target site of dieldrin already been rendered insensitive is a perfect strategy for dieldrin resistance in *An. funestus*. This system will undoubtedly make the mosquito stronger to fight against dieldrin exposure. More screening of dieldrin susceptibility should be done and further studies should be conducted to determine the geographical distribution of dieldrin resistance in *An. funestus* from Nigeria.

5.3.5 Metabolic Mechanisms drive both Permethrin and DDT Resistance in *Anopheles funestus* at Akaka-Remo

The absence of the *kdr* mutation in *An. funestus* at Akaka-Remo prompted further assessments on the potential involvement of metabolic genes in pyrethroids and organochlorines resistance. These assessments revealed that *An. funestus* population uses mainly cytochrome P450 genes, esterase, GSTs, *aldehyde* oxidase and trypsin to fight the exposure of permethrin and DDT insecticides.

5.3.5.1 Metabolic Genes drive Permethrin Resistance in *Anopheles funestus*

This study provided an insight to how *An. funestus* from Akaka-Remo have been able

to withstand the lethal dose of permethrin (0.75%) insecticide. Microarray experiment produced mostly cuticle proteins in permethrin resistant mosquitoes, suggesting their prominent role in permethrin resistance. Cuticle protein functions explicitly by thickening the mosquito cuticle to reduce the amount of insecticides that will penetrate the epithelial wall of the mosquito. This mechanism could be for *An. funestus* at Akaka-Remo to survive permethrin exposure. It is therefore important to further assess the cuticles of this mosquito population to determine the specific cuticle proteins that may be playing this role.

This experiment conducted for the first time on this mosquito species population revealed the potential role of *CYP6P9b* in permethrin resistance. *CYP6P9b,* that was overexpressed only in the control versus susceptible (C-S) comparison is a duplicate gene of the *CYP6P9* class of the cytochrome P450 genes (Riveron *et al.,* 2013). Although, its role is permethrin insecticide is not definite, since it was not overexpressed in Rperm-S comparison, it could be suspected for permethrin resistance. This is so because permethrin resistance is high in this mosquito population and the implication of this is that most of the wild *An. funestus* mosquito picked in this population is likely to be resistant.

Its duplicate, *CYP6P9a* has clearly been shown through metabolism assay (Riveron *et al.,* 2014b) to metabolize both type I and type II pyrethroids. The *CYP6P9a* and *CYP6P9b* duplicates have been strongly associated with pyrethroid resistance in *An. funestus* across Africa, cases of Uganda population (Mulamba *et al.,* 2014), Malawi and Mozambique populations (Riveron *et al.,* 2013; Ibrahim *et al.,* 2016). It could be possible that both duplicate genes are playing similar roles in permethrin resistance of *An. funestus* at Akaka-Remo.

After all, the difference in their genetic make-up is just a difference of 4 % (Riveron *et al.,* 2014b), which could suggest that they may have closely related functions in insecticide resistance. Although, the fact that *CYP6P9a* was only upregulated in Rperm-C comparison and not in Rperm-S comparison could also suggest that it might not represent the best possible candidate gene for permethrin resistance of *An. funestus* at Akaka-Remo. This was supported with the RT-PCR analysis. However, the role of these duplicate genes in permethrin resistance of *An. funestus* is illustrious.

The *CYP6P2*, *CYP6AA4*, *CYP9J3*, *CYP6P4a* and *CYP9K1* are other P450 genes that their expression could be driving permethrin resistance of this mosquito population. The expression of these P450 genes showed the strength of *An. funestus* when defending itself against permethrin. Throwing all the expressed P450 enzymes at permethrin will make the mosquito to have a strong resistance fire-brick, it will always be difficult for the insecticidal compounds to escape the actions of the enzymes. Cloning and functional analysis of these specific cytochrome P450 genes are however required to understand the specific roles of each gene in resistance. Each P450 enzyme could be playing a perculiar role in resistance, if this is the case, understanding their specifc role will make us to further appreciate this resistance mechanism.

The role of epsilon (*GSTe2*) and delta (*GSTd3* and *GSTd1-5*) GST gene family in permethrin resistance was clearly displayed in the RT-PCR assessment. These genes were not implicated in microarray investigation and the possible reasons for the absence of these key resistant-associated genes in microarray could simply be as a result of biases in dyes used in microarray experiments (Yang and Speed, 2002) and cross hybridization or non-specific binding of labeled targets to array probes (Chuaqui *et al.,* 2002) that were earlier pointed out in microarray studies**.** But the fact that specific primers could amplify them in permethrin resistant mosquitoes in the RT-PCR experiment undoubtedly supports their roles in permethrin resistance of *An. funestus* at Akaka-Remo.

The *GSTe2* gene is a key detoxification gene associated with pyrethroids resistance in *An. funestus* populations. This is not only because of its elevated production in *An. funestus* when exposed to pyrethroids (Riveron *et al.,* 2013) but also its capability to metabolize pyrethroids when it has undergone genetic modifications such as L119F point mutation (Riveron *et al.,* 2014a). Elevated expression level of *GSTe2* has previously been implicated in resistance by acting as a pyrethroid-binding protein and sequestering of insecticide (Kostaropoulos *et al.,* 2001) or by protecting mosquitoes against oxidative stress and lipid peroxidation induced by pyrethroid exposure (Vontas *et al.,* 2001). In addition, a partial knockdown of an ortholog *GSTe2* in *Ae. aegypti* led to increasing mortality after exposure to deltamethrin, which also linked *GSTe2* with deltamethrin resistance in *Ae. aegypti* (Lumjuan *et al.,* 2011).

Overall, the expression of GST genes will strengthen permethrin resistance of *An.*

funestus at Akaka-Remo. The GST enzymes expression level highlighs the fact that cytochrome P450s (phase I) alone might not successfully clear off permethrin insecticide from the mosquito system without the conjugation reaction of the phase II enzymes (GSTs). Employing additional detoxification enzymes such as the phase II and other phase I enzymes such as carboxylesterases could have given more operational output to P450 enzymes against permethrin.

Elevated expression of carboxylesterases also suggests its hydrolytic or sequestering role in pyrethriods resistance of *An. funestus* population. Its expression in all the microarray comparisons showed how important it could be in building defense against pyrethroids. Trypsin is another phase I enzyme that was upregulated in permethrin resistant mosquitoes. This enzyme was overexpressed in microarray experiment and confirmed in the RT-PCR analysis. This simply suggests a high degree of its involvement in permethrin resistance of this *An. funestus* population. Trypsin has been commonly overexpressed in permethrin-resistant mosquitoes but its distinct involvement in resistance has been understudied (Nardini *et al.,* 2012; Menze *et al.,* 2016; Samb *et al.,* 2016).

The phase II detoxification gene, glucosyl glucuronosyl transferases is another common overexpressed transcript in microarray study, especially in the Rperm-S comparison. This implies it could also be playing an important role as other metabolic genes in pyrethroid resistance, as the case of its ortholog in *An. gambiae* from Ivory coast (Ingham *et al.,* 2014). Its role and that of trypsin in resistance could be clearer if specific body parts such as mid-guts and malpighian tubules of *An. funestus* are used for analysis.

5.3.5.2 Metabolic Genes drive DDT Resistance in *Anopheles funestus*

The *CYP6AK1* was the only cytochrome P450 gene overexpressed in DDT resistant mosquitoes. Similar to what was observed with permethrin resistant population, cuticle proteins were also overexpressed in R_{DDT}-S comparison, also suggesting reduced DDT penetration due to thickened walls of *An. funestus*. Trypsin was also overexpressed; it gave the highest expression (FC = 2.13) in this comparison. This digestive enzyme seems to be strongly associated with resistance regardless of the type of exposed insecticide. This observation suggests a possibility of some of this insecticidal active

ingredients crossing into the digestive tracts of the mosquito. However, the definite role of trypsin in insecticide resistance should be further studied for better understanding. This is because DDT is meant to target the neurons at the VGSC but crossing to the digestive tract is something of interest and its role should be clearly defined.

The elevated expressions of the two GST genes, *GSTS1* and *GSTU2* suggest their potential contribution to DDT resistance of *An. funestus* at Akaka-Remo. The *GSTS1* has earlier been associated with insecticide resistance in *Anopheles* mosquitoes (Ding *et al.*, 2003). In insects, sigma class of the GSTs possess a proline/alanine-rich N-terminal extension that may aid attachment to the flight muscles and make them structurally effective for metabolism (Clayton *et al.*, 1998). Although, this *GSTS* gene class shows low level activities with the typical GST substrates, their main advantage is the high affinity they have for the lipid peroxidation product, 4-hydroxynonenal (Singh *et al.*, 2011). As a result of this product, sigma GST duplicates are empowered to eliminate by-products of oxidative stress or any potential toxins seen to have invaded the mosquito system (Fang, 2012).

Although, *GSTU2* is yet to be classified into a definite family of GST, it becomes important in insecticide resistance because it shares similar phylogeny with the epsilon and delta families of GSTs, which are known resistance drivers in *Anopheles* mosquitoes (Lumjuan *et al.*, 2007; Yu *et al.*, 2008). Its fold change (FC = 2.00) in C-S comparison does not fully implicate *GSTU2* in DDT resistance. It could only suggest its potential role as time goes by since DDT resistance is very high in this mosquito population and for *An. funestus* mosquito population at Akaka-Remo to be fully resistant, it will be a matter of time if resistance is not well managed.

The role of *GSTe2* in DDT resistance is obvious, considering the difference in expression that was recorded in DDT resistant population (FC = 57.39) against control population (FC = 22.34) with the RT-PCR. The role of *GSTe2* in DDT resistance of other *An. funestus* population is established (Riveron *et al.*, 2014a; Djouaka *et al.*, 2016). Its elevated expression in DDT resistant mosquitoes has been key to its capacity to confer resistance in *An. funestus* (Riveron *et al.*, 2014a).

The L119F-GSTe2 mutation also detected in *An. funestus*, which is now becoming

common in different *An. funestus* populations could also influence such high *GSTe2* expression in resistant mosquitoes. In often times, mutant genes lose connections with regulatory proteins during protein expression. The consequence of this is that regulatory factors fail to bind to the promoter region, thereby leading to overproduction or underproduction of the desired proteins (Fleck *et al.*, 2016). Generally, mosquito carrying the L119F-GSTe2 mutation has an enlarged DDT binding site, which increases DDT access and eventually high DDT metabolism (Riveron *et al.*, 2014a). Remarkably, it is getting clearer that leucine (CTT) transformation to phenylalanine (TTT) on position 119 of *GSTe2* gene, which is predominant in this mosquito population, is influencing the expression level of this gene in resistant population.

However, it is necessary to conduct more *in vivo* and *in vitro* functional assays to further elucidate these findings. The delta family of the GSTs; *GSTd3* and *GSTd1-5* could also be offering vital metabolic contributions to DDT resistance. The P450 genes (*CYP6P9a/b, CYP9K1, CYP6AK1, CYP4C27, CYP9J3, CYP6AA4* and *CYP6P4a*) also had elevated expressions in DDT-resistant samples compared to control, especially in the RT-PCR analysis but because they lack the capacity to metabolise DDT (Riveron *et al.*, 2013), their roles in DDT resistance becomes trivial.

5.3.5.3 Metabolic Genes drive Dieldrin Resistance in *Anopheles funestus*

The role that *GSTe2* played in dieldrin resistance could also be very crucial just like in permethrin and DDT resistance. It gave the highest expression among all the genes analysed for potential roles in dieldrin resistance in the RT-PCR. Also, the elevated expressions recorded with P450 genes, (*CYP6P9a/b* and *CYP6P4a*), aldyhyde oxidase and trypsin suggest they might be playing diverse roles in the observed dieldrin resistance. This is the first study where metabolic mechanism (cytochrome P450s and GSTs) is implicated in dieldrin resistance of *An. funestus*.

Overall, these findings suggest that *An. funestus* population engage both target-site and metabolic mechanisms to withstand the lethal dose (4 %) of dieldrin insecticide. Further experiments to validate this observation are hereby suggested.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

In this study, the *Plasmodium* infection rate, insecticide susceptibility status and molecular basis of insecticide resistance of *An. funestus* at Akaka-Remo was investigated. This work further established the implication of *An. funestus* in malaria transmission considering the rate of *Plasmodium* infection recorded. This study is probably the first scientific report that depicts the insecticide susceptibility status and possible molecular basis of insecticide resistance of *An. funestus* in Nigeria.

Overall, WHO susceptibility test revealed the presence of multiple insecticide resistance in *An. funestus* population at Akaka-Remo. Only the organophosphate family had the capacity to fully control *An. funestus*. Molecular analysis conducted revealed that *An. funestus* population has developed multiple resistance mechanisms to withstand lethal doses of insecticides. Metabolic resistance mechanism through the overexpression of P450 genes, GSTs and esterases to fight insecticides exposure was the main driver of permethrin and DDT resistance in *An. funestus* population. Cytochrome P450 enzymes such as *CYP6P9a/b*, *CYP6P2*, *CYP6AA4* and *CYP9J3*, *CYP6P4a* and *CYP9K1* were all involved in observed resistance. Mutation on *GSTe2* that may have influenced the overproduction of the resulting enzyme have made *An. funestus* population to build a strong resistance to insecticides.

The L119F-GSTe2 is almost getting fixed in *An. funestus* population, suggesting a quick attention to this mosquito species. Dieldrin insecticide was resisted by employing detoxification enzymes such as GSTs and P450s, the first time that such will be reported in *An. funestus* mosquitoes. However, mutation on dieldrin target site (A296S-RDL), a known mechanism driving dieldrin resistance revealed that *An. funestus* population could switch or use both mechanisms to build a very strong resistance against dieldrin.

Strong resistance recorded in this study could have also been due to the involvement

of digestive enzymes like trypsin. Apart from the metabolic and target-site resistance mechanisms observed, reduced insecticide penetration through the overexpression of cuticle proteins could also be playing a crucial role in insecticide resistance.

Contribution to knowledge

1. *An. funestus* was found to be involved in malaria transmission and has developed a multiple resistance to permethrin, deltamethrin, DDT, dieldrin and bendiocarb insecticides at Akaka-Remo.

2. Knockdown resistance mechanism is absent in *An. funestus* population at Akaka-Remo.

3. Metabolic resistance mechanism drives permethrin and DDT resistance, while both target-site mutation and metabolic enzymes drive dieldrin resistance in *An. funestus* at Akaka-Remo.

4. Point mutations such as L119F on *GSTe2* and A296S on RDL are contributing to DDT and dieldrin resistance, respectively in *An. funestus* at Akaka-Remo.

Recommendation

The findings herein suggest that the implication of *An. funestus* in malaria transmission should be assessed across different regions in Nigeria. There is also a need to assess their susceptibility to commonly used insecticides in these regions for effective malaria control strategy. In reality, P450 enzymes with the carboxylesterase would have acted on exposed toxic insecticides and make them water soluble before employing the phase II gene (GSTs) to completely render the resulting metabolites harmless. However, more investigations including functional assays should be conducted to clearly ascertain the definite role of each gene in permethrin and DDT resistance.

Reduced penetration through cuticle thickening could also be contributing so much to insecticide resistance of *An. funestus* population, which also requires further validation through identifying specific cuticle proteins in resistant *An. funestus* mosquito. This is necessary because all resistant mosquitoes analysed in this study used the reduced insecticide penetration as the initial mechanism before the main mechanisms. Also, it

may be imperative to also carry out more investigations on trypsin; its overexpression in resistant mosquitoes showed that it may at some point be involved in the breakdown of insecticides, as a detoxification process.

Considering the effectiveness of the synergists in this study, there might be a need to develop improved control tools by incorporating them with insecticides as new chemical formulations in treated nets and sprays. The resistance mechanisms identified seems to be peculiar to this mosquito population, so there is need to identify the mechanisms of resistance in *An. funestus* per region for meaningful and effective improvements on malaria control interventions.

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APPENDIX

<u>Appendix 1a:</u> The livak procedure used for DNA extraction

The procedure for DNA extraction adopted from livak, 1987 is as follows:

1. Place livak buffer in heat block/water bath that has been pre-heated at 65 °C for 15 minutes and mix before use to re-dissolve precipitate.
2. Grind mosquito sample in 100 µl preheated livak grind buffer in an eppendorf tube.
3. Transfer samples at once to 65 °C heat block and incubate for 30 minutes.
4. Briefly spin samples to collect condensation.
5. Add 14 µl 8M K-acetate and mix gently through pipetting.
6. Incubate samples on ice for 30 minutes.
7. Spin samples at 13,000 rpm for 20 minutes at 4 °C.
8. Transfer supernatant carefully into a new 1.5 ml eppendorf tube. If there is a trace of debris in supernatant, it can be re-spin for 20 minutes and transfer supernatant to new tube.
9. Add 200 µl 100% ethanol to supernatant and mix gently by vortexing.
10. Spin samples at 13,000 rpm (4 °C) for 15 mins.
11. Carefully discard supernatant and make sure pellet is not dislodged.
12. Rinse the pellet with 100 µl ice cold 70% ethanol.
13. Dry pellet by leaving tubes on the bench for 1 hour, after which it can be re-suspended in 100 µl TE buffer.

Appendix 1b: Composition of the livak grind buffer used for DNA extraction

1.6 ml 5M NaCl

5.48 g sucrose

1.57 g Tris base/HCL

10.16 ml 0.5M EDTA

2.5 ml 20% SDS

Appendix 1c: Steps to prepare the livak buffer

- Add-up the volume to 100 ml (with dH2O), filter sterilise.
- Get 5 ml aliquots and store at -20 °C.
- Thaw aliquot before use and keep at 4 °C not more than 2 weeks

Appendix 2: Volume of reagents/primers used for *Anopheles funestus* species identificatio

	X1 (required volume for one sample)
Buffer A	1.5 µl
25 mM dNTPs	0.12 µl
Kapa Taq	0.12 µl
Mgcl2 25mM	0.9 µl
UV F 10mM	0.51 µl
Fun R 10mM	0.51 µl
VAN 10mM	0.51 µl
RIV 10mM	0.51 µl

PAR	10mM	0.51 µl
RIVLIKE	10mM	0.51 µl
LEES	10mM	0.51 µl
ddH20		7.79 µl
DNA		1.0 µl
Total =		**15 µl**

Appendix 3: PCR cycling reaction used for *Anopheles funestus* species identification

Step 1 94 ^{0}C – 2 min

Step 2 94 ^{0}C – 30 s

Step 3 45 ^{0}C – 30 s } X 35 cycles

Step 4 72 ^{0}C – 40 s

Step 5 72 ^{0}C – 5 min

10 ^{0}C ~ Hold

Appendix 4: Steps in Agarose gel electrophoresis

For a 1.5% gel:

- Weigh 1.5g of Agarose and transfer into a glass flask/jar
- Add 100ml TAE buffer to agarose powder
- Microwave for 1.5-2 min (until bubbling and Clear)
- Place the jar into a container with cold water to cool the outside of the jar
- Add 5 ul Ethidium Bromide/medori green to the gel and mix very well
- Pour the gel into a gel casting tray and insert the required number of combs

- Leave at room temperature for around 30 min

Running the gel:

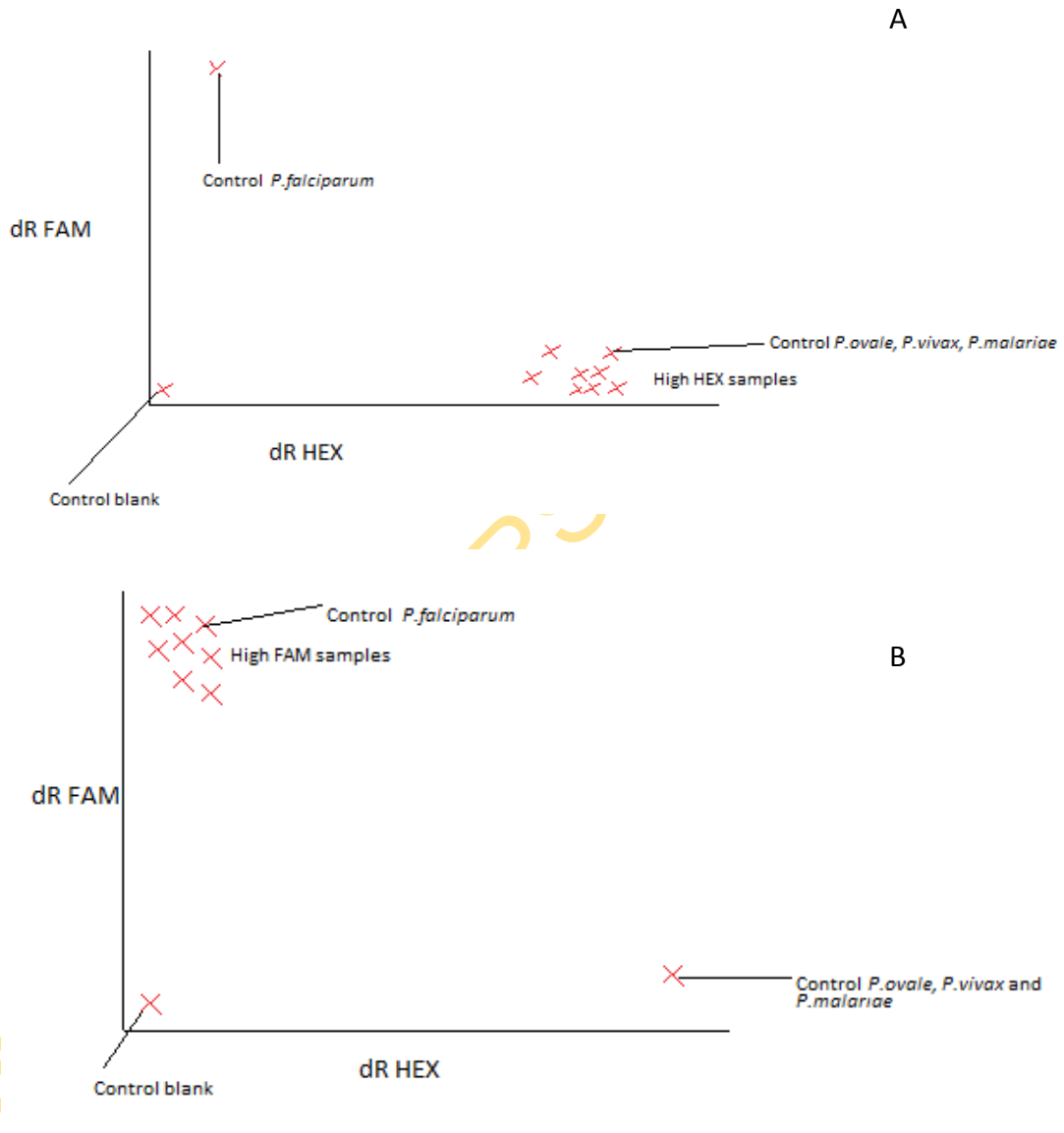
- Submerge the gel in the gel tank filled with TAE buffer
- Cut a piece of parafilm and pipette enough spots of loading dye for each sample
- Add 5ul of ladder into the end wells of the gel
- Pipette 3-6 ul of sample and mix with the loading dye spot before loading into each well
- Place the lid onto the gel tank and plug in the wires to the matching colours
- Switch on the power pack and set the voltage to 100-135V
- To confirm that the gel is running, you should see bubbles rising quickly in the gel tank at the end.
- Run gel for around 30 min

Viewing the gel (this depends of the system used for viewing)

- Switch off the power pack
- Remove the gel out of the tank
- Place the gel onto the middle of the Syngene gel documentation system G-Box
- Double click on the Syngene gel Image programme
- Click on the green button to view a live image
- You can zoom in on the image, increase the intensity at this point
- Freeze the image using the red button
- Save the image
- Print the gel image

- Close down the syngene programme on the computer but do not save changes at this point

Appendix 5: Charts showing samples that were infected with *Plasmodium* parasites



a) showing samples positive for *P. ovale, P. malariae* and *P. vivax.* b) showing samples positive for *P. falciparum.*

Appendix 6: Microarray: QIAquick OCR purification kit protocol

1. Add 5 volumes of Buffer PB to 1 volume of the PCR product and mix.

2. Check and make sure the colour of the mixture turns yellow. If the colour turns orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0 and mix.
3. Transfer PCR product into a QIAquick spin column, place column in the 2 ml collection tube provided in the kit and centrifuge for 30–60 seconds.
4. Discard flow-through and place the QIAquick column back into the same tube.
6. Add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 seconds to wash the sample.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for another 1 minute.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. For DNA elusion, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 minute.

<u>Appendix 7:</u> Microarray: Pipopure protocol for RNA extraction in mosquitoes

1. Grind mosquito sample in eppendorf tube containing 100 µl of extraction buffer (XB).
2. Incubate eppendorf tubes at 42 °C for 30 mins.
3. Centrifuge tubes at 16,000rcf (room temperature) for 2 mins.
4. Carefilly, remove the supernatant into a new tube.
5. Befrore use, make sure 250 µl conditioning buffer (CB) is added into each column onto the membrane.
6. Incubate columns at room temperature for 5 mins.
7. Centrifuge columns at 16,000 rcf for 1 min.
8. Add 100 µl of 70 % ethanol to each sample, mix gently by pipetting, and transfer to the conditioned column.
9. Spin columns at 100 rcf for 2 mins, immediately spin at 16,000 rcf for 30 secs and discard the flow-through
10. Add 100 µl of wash buffer (W1) to samples, spin at 8,000 rcf for 1 min and discard flow-through.
11. Add 40 µl of DNase mix; that is the mixture of 5 µl DNase I and 35 µl buffer RDD directly onto column.
12. Leave samples for 15 mins at room temperature.

13. Add 40 µl of W1 onto the column, spin at 8,000 rcf for 15 secs and discard flow-through.
14. Also, add 100 µl of wash buffer 2 (W2), spin at 8,000 rcf for 1 min and discard flow-through.
15. Add another 100 µl of W2, spin at 16,000 rcf for 2 min and discard flow-through.
16. Again, spin sample at 16,000 rcf for 1 min.
17. Place column into new 0.5 ml tube provided with the kit and add 30 µl elution buffer (EB) onto the membrane.
18. Leave for 1minute at room temperature, spin at 1,000 rcf for 1 min, and immediately spin again at 16,000 rcf for 1 min.
19. If RNA samples are not used immediately, label tubes clearly and store at -80 °C. Also, note that before subsequent analysis, quality and quantity of RNA should be measured by bioanalyser and NanoDrop, respectively.

<u>Appendix 8:</u> Microarray: RNA quality and quantity check

25-500 ng of the RNA sample should be used to analyse the quality by using the Agilent 2100 bioanalyzer. This is because the degradation of RNA is not detectable through NanoDrop readings.

Equipment/reagents needed:

RNA 600 Nano Kit (Agilent 5067-1511)
Agilent 2100 bioanalyzer
Ice box containing ice
microfuge
20 µl, 200 µl, 1000 µl Gilsons
RNase-free filter tips (10 µl and 200 µl)
Nuclease free water
PCR machine
Vortex mixer

Steps required to check the quality of RNA using Bioanalyser

1. Equilibrate the dye concentrates and filtered gel aliquot (65 µl) (that has been kept in the fridge) in the dark to room temperature for 30 minutes.

Note: If you notice that the gel needs filtering, add 550 µl of nano gel mix to a new spin filter column and spin at 4,000 rpm (room temperature) for 10 mins and make 65 µl aliquots.

2. Once the dye mix is equilibrated, vortex for 10 secs and spin down. Add 1 µl of dye to the 65 µl gel aliquot, mix thoroughly by vortexing and spin at 14,000 rpm for 10 mins.

Note: At this point, it is required to cover tube with alluminium foil to protect from light and store samples at room temperature until needed.

3. Get RNA aliquots and ladder aliquot that have been at -80 °C and place on ice.

Note: If it is a new kit then ladder needs to be freshly prepared, denature the whole ladder for 2 mins at 70°C and make 1.2 µl aliquots in PCR tubes to be stored at -80 °C.

4. Make sure samples are diluted with water to achieve a concentration range of 50-500 ng. Also, denature samples in a thermocycler at 70 °C for 2 mins and transfer back on ice.

Note: a single chip takes 12 samples, so aim to fill a chip to save cost.

5. Take the following over to bioanalyzer:

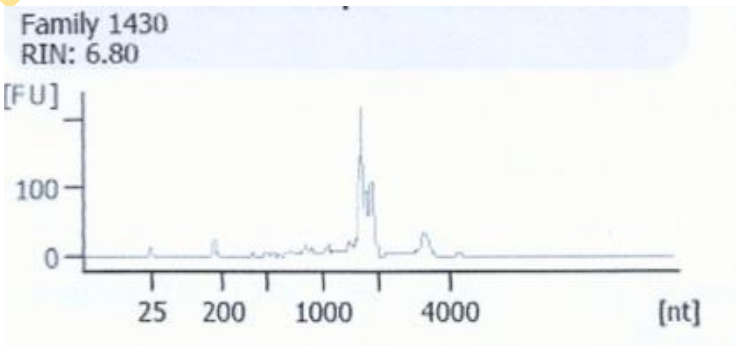
- Nano-marker
- Bioanalyzer chip
- Wash chips (x2; water & RNAse zap)
- DEPC/RNAse free water
- RNAse zap
- Gel/dye mix
- Ladder (on ice)
- Samples (on ice)
- P1000; P2; P20 & filtered tips
- Timer
- USB to save data

6. Before loading samples onto the bioanalyzer, its electrodes have to be decontaminated. This is achieved by pipetting 350 µl of RNAse Zap into the labelled wash chip, add 350 µl of RNAse free water to the other chip. Open the lid of the machine and place the chip containing RNAse Zap into the bioanalyzer for 1 min. Remove and place the other chip containing water into the machine for 10 secs. Remove chip and leave lid open for 10 secs to allow electrodes to dry.

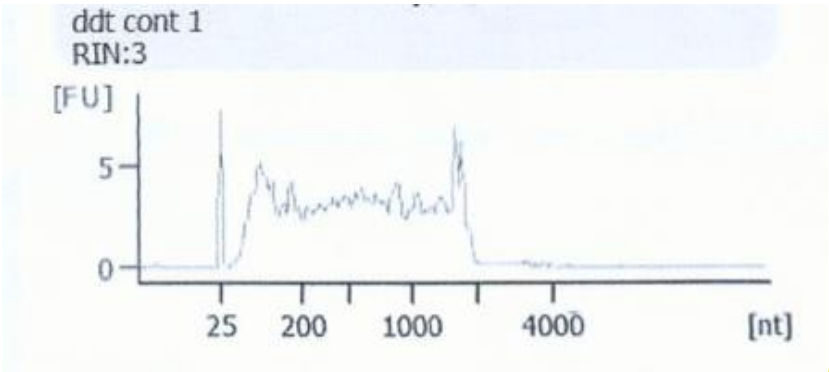
7. Switch on the bioanalyzer and the computer connected to it and launch the 2100 expert software. In the Instrument tab, click on Assays and ensure you select the Eukaryote RNA-nano assay.
8. Place a new chip into the chip priming station and pipette 9 µl of the gel-dye mix into the well marked.
9. Make sure the syringe plunger is at the 1ml position, close the station and wait for 30 secs then release the clip. Wait for another 5 secs then slowly pull the plunger back to the 1 ml position and open the priming station.
10. Pipette 9 µl of the gel dye mix into wells marked, 5 µl of the nano-marker into all 12 sample wells and into the ladder well marked, 1µl of the prepared ladder into well marked and 1 µl of each sample into wells 1-12.
11. Securely place the chip into the IKA vortexer and time it for 1 min. Vortex at 2,400 rpm for 60 secs.
12. Insert chip into the bioanalyzer and click Start. Ensure Eukaryote Total RNA nano is selected.

    Note: The chip must be run within 5 mins of preparation**.**
13. As the chip is running, sample information can be added to the data file by clicking on the Data tab and type next to the appropriate wells.
14. Once the run is finished, make sure the electrode decontamination step is repeated.
15. To export data as a PDF, click Print and select PDF option then change the file storage destination to your USB. You can also save the full file onto USB by using Save as option.

a. Below is a typical trace for good quality mosquito total RNA: the 26S is cleaved resulting in triple peaks around the 18S region, there is also a relatively flat line between 200 and 1500 nt indicating little degradation of the sample.



b. Here is an example of a badly degraded sample:



Appendix 9: Microarray: cRNA labeling

1. Thaw spike mixes A (to be labelled Cy3), B (to be labelled Cy5) and dilution buffers on ice and vortex thoroughly. Heat spike mixes at 37 °C for 5 mins, vortex again and spin down to collect contents.
2. Check the table below to establish which working dilution is required for your starting amount of RNA (i.e. 100 ng = fourth dilution).
3. Prepare the FIRST dilution of spike A and B, which can be stored at -70-80 °C for up to 2 months and freeze thawed up to 8 times. (Use RNAse free plastics and filtered tips)

a. First dillusion (1 in 20) – take 2 µl of spike stock (either A or B) to 38 µl of dilution buffer, vortex thoroughly and spin down.
b. Second dillusion (1 in 40) – take 2 µl of the first dilution and add 78 µl of dilution buffer, vortex and spin down.
c. Third dillusion (1 in 16) – take 2 µl of second dilution plus 30 µl dilution buffer
d. Fourth dillusion (1 in 2) – take 10 µl of third dilution plus 10µl dilution buffer, vortex and spin down. Keep on ice until required and discard on day of use. This is enough for 10 labelling reactions for Cy3 or Cy5, if you need to label more, increase the volumes.

Table1: Dilutions of spike A mix for cyanine 3 and spike B mix for cyanine 5 labeling

Starting Amount of RNA		Serial Dilution				Spike Mix Volume to be used in each labeling reaction (µL)
Total RNA (ng)	PolyA RNA (ng)	First	Second	Third	Fourth	
25		1:20	1:40	1:16	1:8	2
50		1:20	1:40	1:16	1:4	2
100		1:20	1:40	1:16	1:2	2
200		1:20	1:40	1:16		2
	5	1:20	1:40	1:16	1:2	2

RNA PREPARATION

Re-NanoDrop all RNA samples on the day of labelling. Make sure the solvent use to blank is the same solution the RNA was originally dissolved/eluted in. The low input labelling kit has a starting range of material from 25-200 ng. 100 ng is a good mid range amount to start with.

For 100 ng total in 1.5 µl (total starting volume of RNA), the RNA samples need to be diluted to 66.66 ng/µl with RNAse free water. This can be calculated from the NanoDrop readings.

Place 1.5 µl (100 ng) of each RNA to be labelled into PCR tubes and keep on ice until use.

HINT: To prevent mix up, place RNA to be labelled with Cy5 (fluoresces RED) into red PCR tubes and samples to be labelled with Cy3 (fluoresces GREEN) into green PCR tubes.

LABELLING REACTION

Equipment/reagents needed

1 Low Input Quick Amp Labelling Kit, two-color (Agilent 5190-2306)

2 Spike-in working dilutions

3 Nuclease free PCR and 1.5 ml tubes

4 Ice box and ice

5 microfuge

6 2 µl, 20 µl, 200 µl, 1000 µl Gilsons

7 RNase-free filter tips (2 µl, 10 µl, 200 µl, 1000 µl)

8 Nuclease free water

9 PCR machine (for all labelling incubations)

10 Vortex mixer

11 Incubator/heat block at 80 °C (to pre-warm 5x 1st strand buffer)

12 Qiagen RNeasy columns required for purification (Qiagen 74104)

- Once labelled and purified, samples are stored in 1.5 ml tubes at -80 °C until hybridization
- Store 1.5 µl in separate, labeled PCR tube for analysis on Bioanalyzer

1. Add 2 µl of either spike A fourth dilution (Cy3) or spike B fourth dilution (Cy5) to 1.5 µl of RNA in PCR tubes. Each tube should now contain 3.5 µl.
2. Prepare the T7 promoter primer as follows and add 1.8 µl to the RNA & spike mix. Each tube should now contain 5.3 µl.

Table 2: T7 Promoter Primer Mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reaction	Volume (µL) per 10 reactions
T7 Promoter Primer (green cap)	0.8	4	8
Nuclease-free water (white cap)	1	5	10
Total Volume	**1.8**	**9**	**18**

✓ Incubate primer mix at 65 °C for 10 mins to denature the template and the primer. Store on ice for 5 mins immediately following the reaction.

✓ Whilst incubating the RNA, pre-warm the 5x first strand buffer to 80 °C for 3-4 mins, vortex and spin down. If not fully re-suspended repeat heating and vortexing. Once re-suspended store at room temperature until use.

✓ Prepare the cDNA master mix at room temperature in a 1.5 ml tube (Check table 3); keep the enzyme mix (AffinityScript RNase Block Mix) on ice and add immediately prior to use. Pipette gently up and down to mix.

Table 3: cDNA Master Mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reaction	Volume (µL) per 10 reactions
5X First Strand Buffer (green cap)	2	10	20
0.1 M DTT (white cap)	1	5	10
10 mM dNTP mix (green cap)	0.5	2.5	5
AffinityScript RNase Block Mix (violet cap)	1.2	6	12
Total Volume	**4.7**	**23.5**	**47**

3 Spin the RNA samples down and then add 4.7 µl of the cDNA mix pipetting up and down. Note: Each tube should now contain 10 µl.

4 Incubate at 40 °C for 2 hrs followed by 70 °C for 15 mins. Move samples to ice for 5 mins and spin down the tubes.

5 Prepare the transcript master mix for both Cy3 (Green) and Cy5 (red) at room temperature in 1.5 ml tubes (check table 4); keep the T7 RNA polymerase blend on ice and add immediately prior to use. Protect the Cyanine dyes from light using aluminium foil and once added to the mix re-cover the tubes.

Table 4: Transcription Master Mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reaction	Volume (µL) per 10 reactions
Nuclease-free water (white cap)	0.75	3.75	7.5
5X Transcription Buffer (blue cap)	3.2	16	32
0.1 M DTT (white cap)	0.6	3	6
NTP mix (blue cap)	1	5	10
T7 RNA Polymerase Blend (red cap)	0.21	1.05	2.1
Cyanine 3-CTP or cyanine 5-CTP	0.24	1.2	2.4
Total Volume	**6**	**30**	**60**

Note: Cy3 dye (Cyanine 3-CTP) is actually Red/Pink in colour but fluoresces green while Cy5 dye (Cyanine 5-CTP) is Blue in colour but fluoresces red.

6 Add 6 µl of either Cy3 or Cy5 mix to the appropriate RNA sample and pipette up and down to mix. Protect mix and PCR tubes containing RNA and mix from light using foil. Each tube should now contain 16 µl.

7 Incubate samples at 40 °C for 2 hrs**.**

Appendix 10: Microarray: Purification of amplified cRNA

1. Transfer cRNA samples into 1.5 ml tubes
2. Add 84 µl of nuclease free water to each cRNA sample for a total volume of 100 µl
3. Add 350 µl of RTL buffer and mix by pipetting
4. Add 250 µl of molecular grade ethanol and mix by pipetting
5. Transfer the 700 µl of cRNA sample to an RNeasy spin column in a 2 ml collection tube. Spin at 13,000 rpm (4 °C) for 30 secs and discard flow through
6. Transfer column to a new collection tube and add 500 µl of buffer RPE to the column. Spin 13,000 rpm (4 °C) for 30 seconds and discard flow through
7. Add another 500 µl of buffer RPE, centrifuge at 13,000rpm (4 °C) for 60 secs and discard the flow through. If the RPE buffer remains on or near the frit of the column, centrifuge again at 13,000 rpm (4 °C) for 30 secs.
8. Transfer column to a labeled RNAse free 1.5 ml tube. Add 30 µl of RNAse free water directly onto filter membrane and incubate for 1 minute.
9. Centrifuge at 13,000 rpm (4 °C) for 30 secs to elute and store on ice before NanoDrop reading and at -80 °C after reading.

Appendix 11: Microarray: Checking the quatity of cRNA using nanodrop

1. Launch the NanoDrop software and select Microarray Measurements tab.
2. Initialize the instrument with 1-2 µl of water.
3. Select RNA-40 as the Sample type and ensure the recording button is selected so reading can be saved.
4. Add 1-2 µl of RNAse free water to blank the instrument.
5. Add 1-2 µl of cRNA sample and measure.

   Note: Make sure the baseline remains at 0 throughout measurements. If it deviates, try to re-blank the instrument.
6. Take the following records: Cy3 or Cy5 concentration (pmol/µl), RNA absorbance (260nm/280nm) and cRNA concentration (ng/µl)

7. Use the concentration (ng/µl) of cRNA to determine the sample yield using the equation below:

$$\frac{(\text{Concentration of cRNA}) \times 30\ \mu\text{L (elution volume)}}{1000} = \mu\text{g of cRNA}$$

8. You can also use the cRNA concentration (ng/µl) and Cy3 or Cy5 concentration (pmol/µl) to determine the sample specific activity using the equation below:

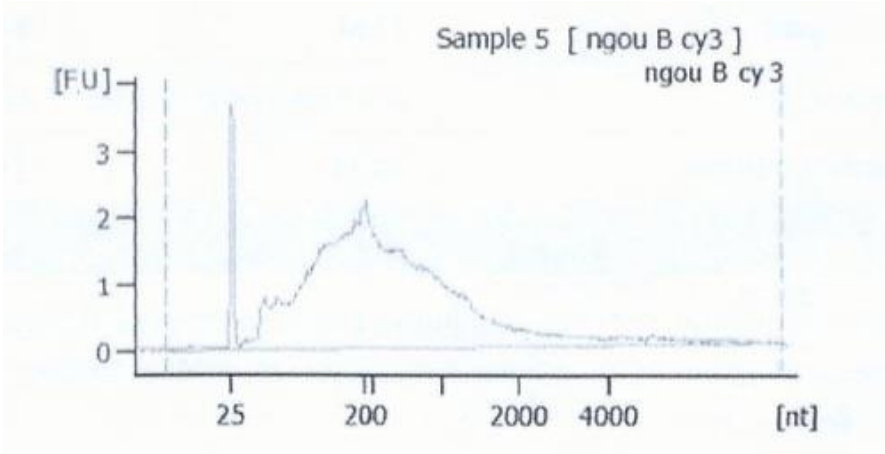
$$\frac{\text{Concentration of Cy3 or Cy5}}{\text{Concentration of cRNA}} \times 1000 = \text{pmol Cy3 per } \mu\text{g cRNA}$$

Note: If yield < 825ng and Specific Activity < 6.0pmol for either Cy3 or Cy5 per ug RNA, you have to repeat labeling. To make life easy, take 1.5µl aliquot of all labelled samples and store in a nuclease free tube at -80°C until you are ready to analyze on the Agilent Bioanalyzer. Store the rest of the cRNA in a 1.5ml tube and store at -80°C until hybridisation.

Check the quality of labeled cRNA using agilent bioanalyser

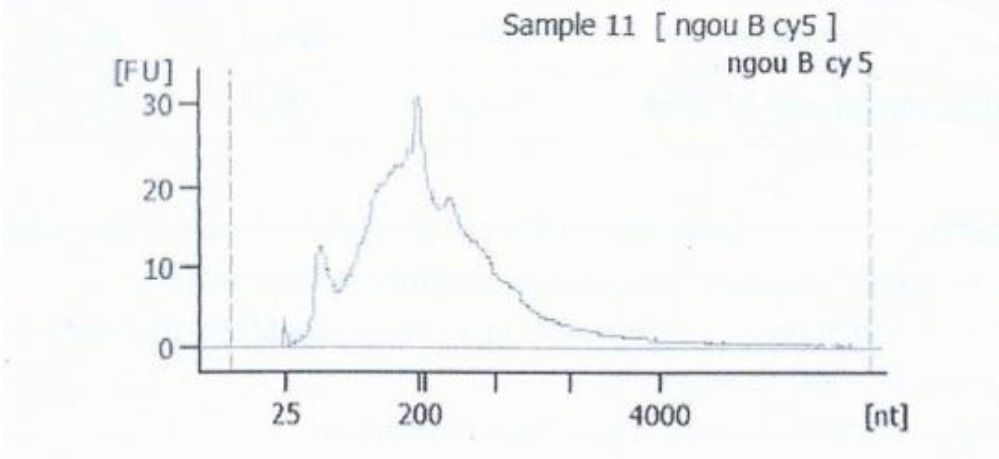
It is important to check the quality of cRNA sample before proceeding to microarray hybridisation. Follow the steps above (appendix 8) for the quality of labeled cRNA check and please note the lower quantitative range.

Below is an example of good quality cRNA labelled with Cy3:



Below is an example of the same sample labelled with Cy5

Note: Cy5 had a greater fluorescence under the bioanalyzer, that is why the fluorescence is higher on the y-axis.



Appendix 12: Microarray: Fragmentation and hybridization of target genes

Equipment/reagents needed

1. Gene Expression Hybridization kit (Agilent 5188-5242)
2. Ice box containing ice
3. Microfuge
4. Heat block
5. Nuclease free 1.5 ml tubes
6. Hybridization chambers and oven
7. Timer

8. Fragmentation should be stopped after 30 mins by placing the reaction tubes on ice for 2 mins and then add 2x GE hybridisation buffer as stated below.
9. Mix gently by pipetting, try to avoid the introduction of air bubbles and spin mixture at 13,000 rpm (room temperature) for 1 min.
10. Gather the Agilent SureHyb hybridisation chamber assembly, gasket slides and microarrays in a clean well lit area of the bench.
11. Place the hybridisation chamber base flat on the bench.
12. Peel back the plastic cover from only the gasket slides to be used and carefully remove avoiding contact with the glass within the rubber gaskets (that is handle the edges of the slide only).

Table 5: Fragmentation reaction

Components	Volume/Mass 4x44K	Volume/Mass 8x15K microarrays
cyanine 3-labeled, linearly amplified cRNA	825 ng	300 ng
cyanine 5-labeled, linearly amplified cRNA	825 ng	300 ng
10X Blocking Agent	11 µL	5 µL
Nuclease-free water	bring volume to 52.8 µL	bring volume to 24 µL
25X Fragmentation Buffer	2.2 µL	1 µL
Total Volume	55 µL	25 µL

Table 6: Hybridisation Mix

	Volumes per hybridization			
Components	1x244K	2x105K	4x44K	8x15K
cRNA from Fragmentation Mix	250 µL	125 µL	55 µL	25 µL
2x GEx Hybridization Buffer HI-RPM	250 µL	125 µL	55 µL	25 µL

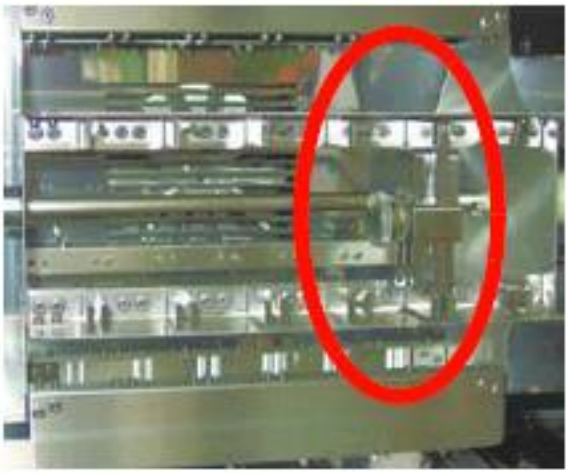
Place the gasket slide into the hybridisation chamber base so that the agilent label is facing up and is aligned with the rectangular section of the chamber base as below:

13. Slowly dispense the hybridisation mixture into the centre of the correct gasket chamber using a drag and dispense motion. Make sure the pipette do not the tip of the rubber gasket.

14. Carefully remove a new microarray slide from its box ensuring only slide edges or barcode are contacted and slowly lower onto the gasket with the agilent barcode facing down, that is Agilent -to-Agilent and the numerical barcode facing up as below. Lower parallel to the gasket slide by holding the array at either end and gently releasing when in contact with the liquid.

15. Gently place the chamber cover on top of the hybridisation sandwich then with the chamber flat on the bench slide the clamp assembly onto the chamber from the rounded (that is non-barcode) corner until it stops in the middle.

16. Firmly, tighten the clamp and rotate the array to ensure the liquid it moving only within the gaskets and all air-bubbles are mobile.
17. Place the assembly into the hybridisation oven as below and ensure that it is balanced with another hybridisation assembly.



18. Hybridise samples at 65 °C and 10 rpm for 17 hrs.
19. Place approximately 600 ml of wash buffer 2 in a sealed bottle and warm overnight at 37 °C ready for washes the following morning. Also note that if fixative solution is to be used and has formed a precipitate heat to 37 °C along with wash 2 to dissolve before use.

Appendix 13: Microarray: Washing of array

Equipment/reagents needed:

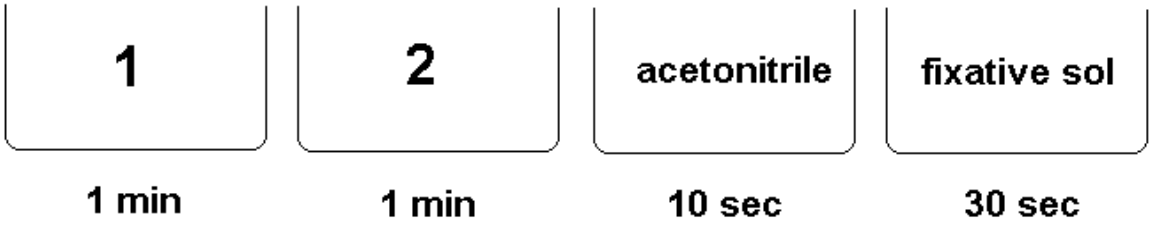
1. Five clean and dry wash glasses
2. Staining rack
3. four magnetic stir bars and at least two magnetic stir plates
4. Stop watch
5. Protective array case
6. Wash buffer 1

7. Plastic forceps

   Please, ensure that a fume hood is available for washing if acetonitrile/fixative solutions are to be used.

1. Fill two wash glasses with wash 1 and place the staining rack and a magnetic flea into one glass. Move glasses to hybridisation oven area.
2. Remove the hybridisation chamber from the oven and place it flat on the bench.
3. Carefully unscrew the clamp, use plastic tweezers to remove the slide-gasket sandwich and fully submerge in wash 1 with the gasket slide facing down. When fully submerged tease apart the sandwich with plastic tweezers (see set-up below).
4. Place the array carefully into the rack in the second wash 1 glass; ensure only the edges or the barcode area of the array should be touched.

5. Washes set-up with acetonitrile and fixative should proceed in the fume hood as below. Place stir bars into three glasses and fill one with acetonitrile and another with fixative solution (~ 600 ml needed in each glass).



6. After step 4, slowly lift the rack from the solution tilting towards the barcode end and place onto some paper towel.
7. Dry droplets from array edges on paper towel and place arrays into protective box ready for scanning.

Appendix 14: cDNA synthesis

Reagent	**X 1**
Oligo(dt) 20 (50mM)	1ul
RNA in 8ul dil with water	8ul
Sterile Water	3ul
10mM dNTP mix	1ul
Total	13ul

First cycling condition = 65 ^{0}C for 5 min

Reagent	**X 1**
Add 5x first strand buffer	4ul
DTT 0.1 M	1ul
RNase Out	1ul
Superscript III RT	1.5ul
	7.5ul each in total

Total = 20.5 ul

Second cycling condition

25 ^{0}C – 5 min

50 ^{0}C – 60 min

70 ^{0}C – 15 min

Add 1ul Rnase H (E-Coli)

Third cycling condition = 37 ^{0}C for 20 min

Note: For qPCR use 1:50 dilution of cDNA

Appendix 15: Reagents/volumes needed for the Reverse Transcriptase PCR.

First, make a set of 5 dilutions from the original cDNA stock

Dilution 1 = 5ul cDNA stock plus 20ul dH2O

Dilution 2 = 10ul dilution 1 plus 40 ul dH2O

Dilution 3 = 10ul dilution 2 plus 40ul dH2O

Dilution 4 = 10ul dilution 3 plus 40ul dH2O

Dilution 5 = 10ul dilution 4 plus 40ul dH2O

Note: When using new set of primers for the first time, a standard curve needs to be done. This could be done using just one sample, technically replicated twice with all 5 dilutions plus a negative control, for each of the new primers. Reagent mix below should be considered is as follows:

Mix	**X1**
SyBr Green	10ul
10mM primer F	0.6ul
10mM primer R	0.6ul
dH2O	7.8ul
cDNA	1.0ul
Total	20ul

Appendix 16: Mx machine set up and reaction

Choose the MxPro software from the desktop

For experiment type, select SyBr green (with dissociation curve)

Plate set up screen – highlight the wells and select the well type as Standard

Collect Fluorescence data (click on the small black arrow) and select SYBR

Highlight the wells for the negative controls and select NTC for the well type and SYBR for the dye.

For the standards, the concentration needs to be set for the dilution series.

Highlight the wells for the first dilution and type 1.00e000 into the standard quality box. Then select the auto increment button and proceed to highlight the samples for the 2nd dilution, the 3rd dilution, 4th dilution and 5th dilution. Switch off the auto increment button once complete.

Highlight the wells for the same primers. And select to show well names and assign assay name, well annotation type the name of the primer/sample etc.

To label the replicates, highlight the replicates, go to replicate symbol and select 1, then switch on the auto increment and highlight the next 2 replicates 2, etc until complete then turn off the auto increment.

Thermal profile setup screen

Thermal profile design – standard

Amplification segment – fast 2 step

On the graph

95°C – 3mins

AMP 95°C – 10s } x 40 cycles

60°C – 10s END

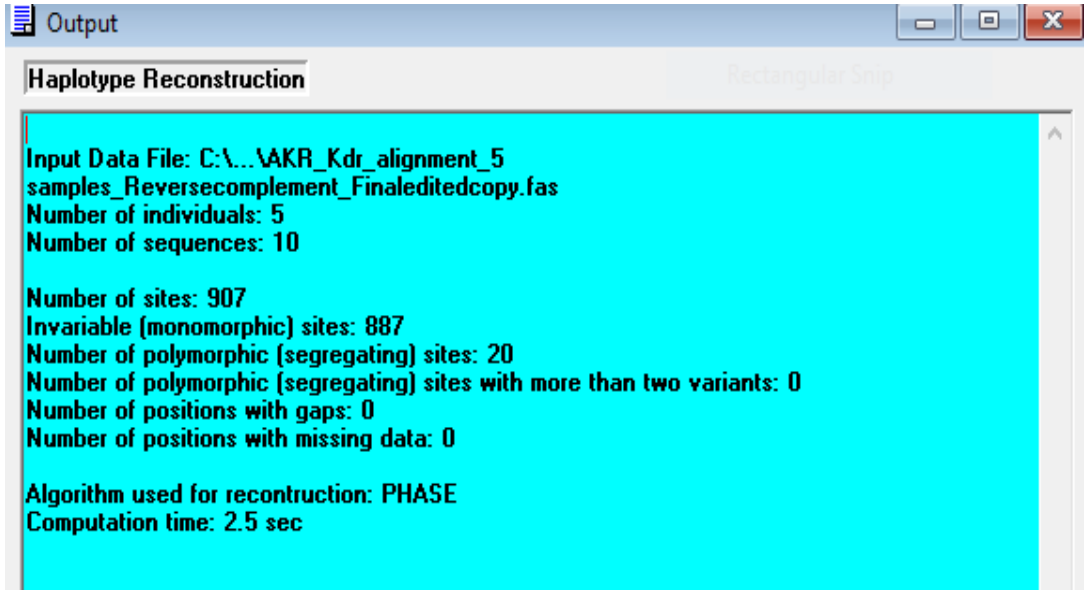
95°C – 1mins

55°C – 30s ALL

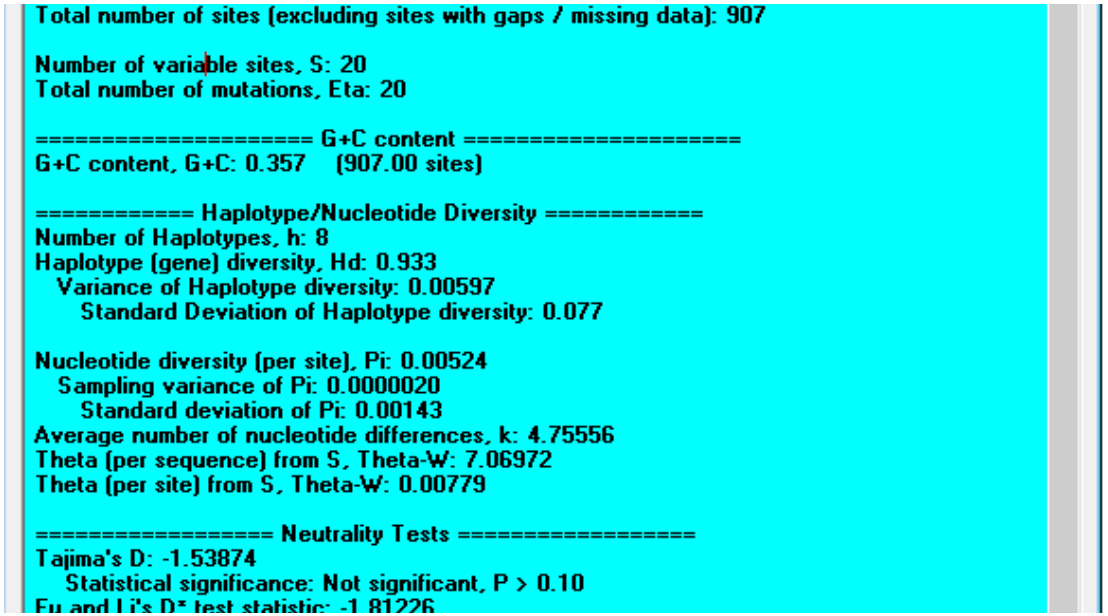
95°C – 30s

File > save as > select experiment > start run (time should be 1:12:54)

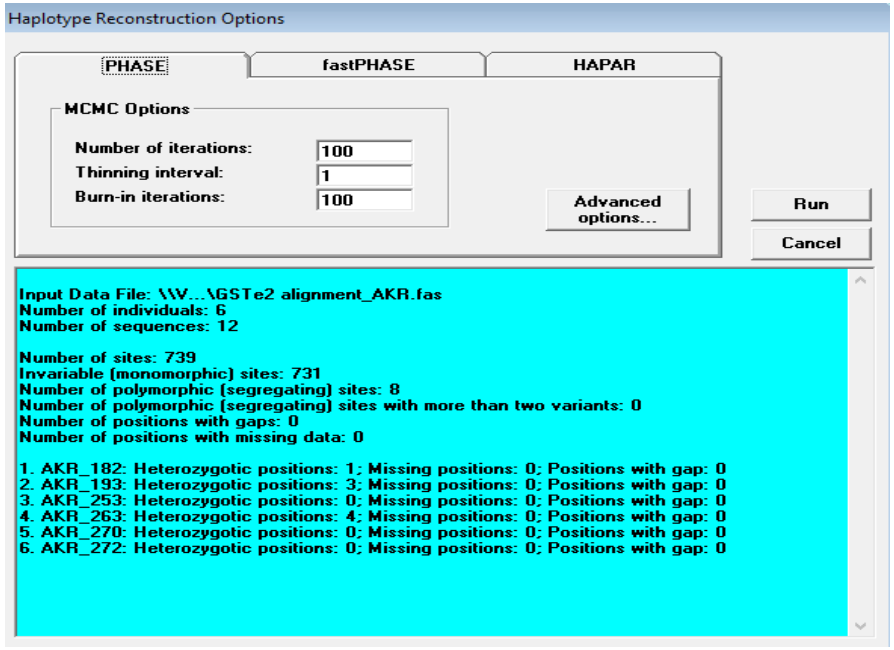
If no one is using the machine after you, tick the box to turn the lamp off.



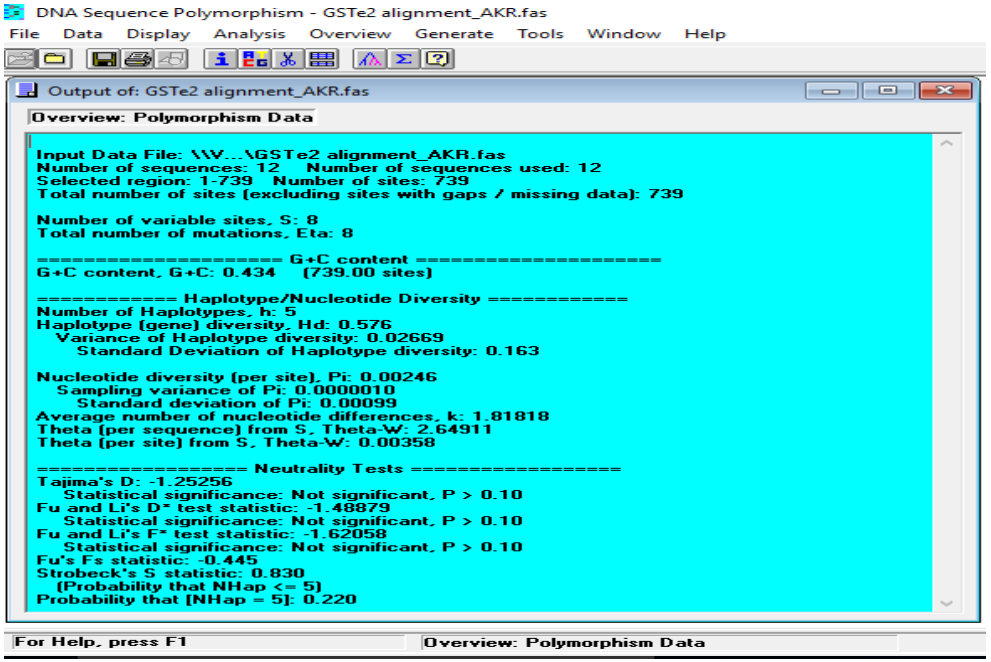
Appendix 17: Haplotype construction described from the nucleotide sequence obtained from sequencing the Voltage-Gated Sodium Channel region of *Anopheles funestus* mosquitoes at Akaka-Remo.



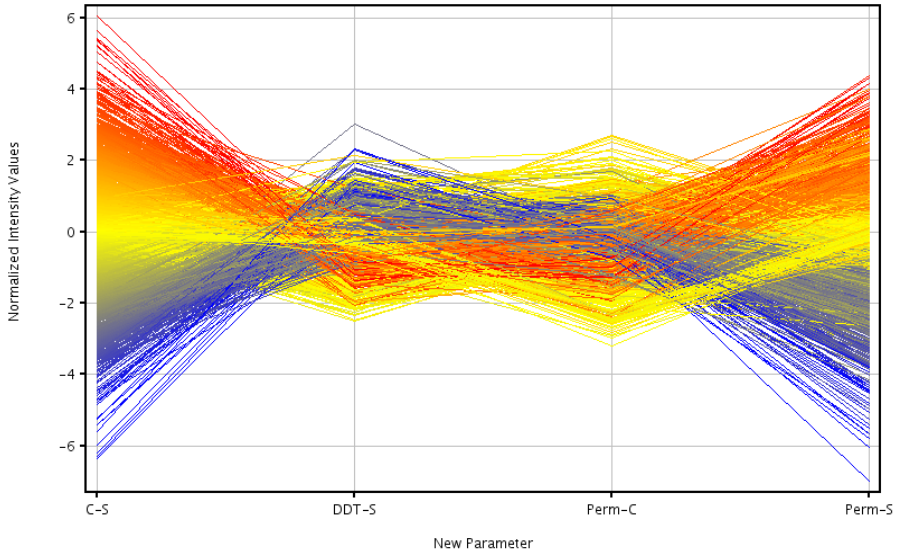
Appendix 18: Polymorphic data obtained from the sequenced VGSC of *Anopheles funestus* at Akaka-Remo



Appendix 19: Haplotype construction of L119F mutation on the *GSTe2* gene of *Anopheles funestus* mosquitoes at Akaka-Remo.

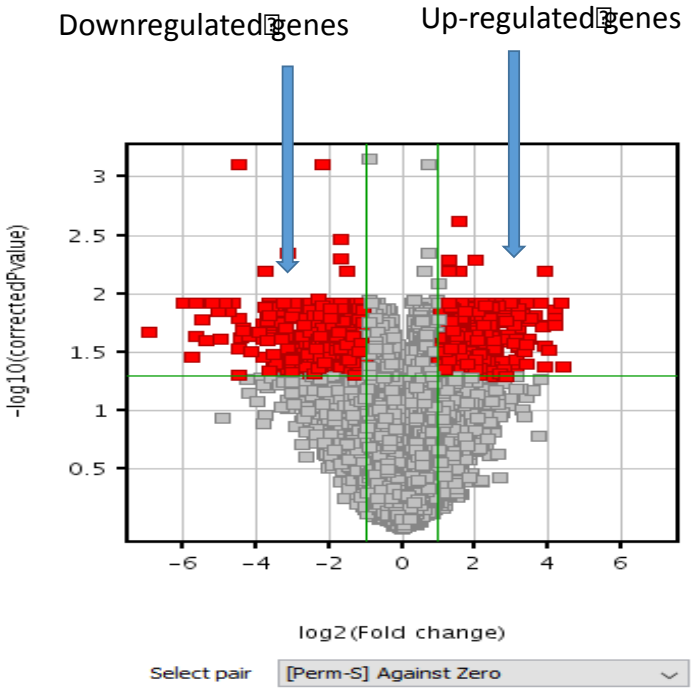


Appendix 20: Polymorphic data obtained from sequencing the L119F mutation of *Anopheles funestus* at Akaka-Remo.

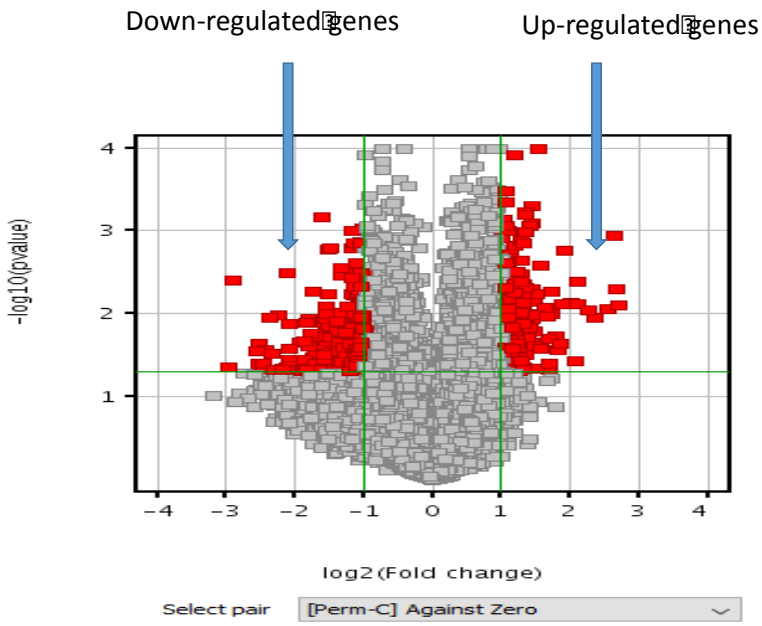


C=control population; S= susceptible population; DDT= DDT resistant Population; Perm=Permethrin resistant population

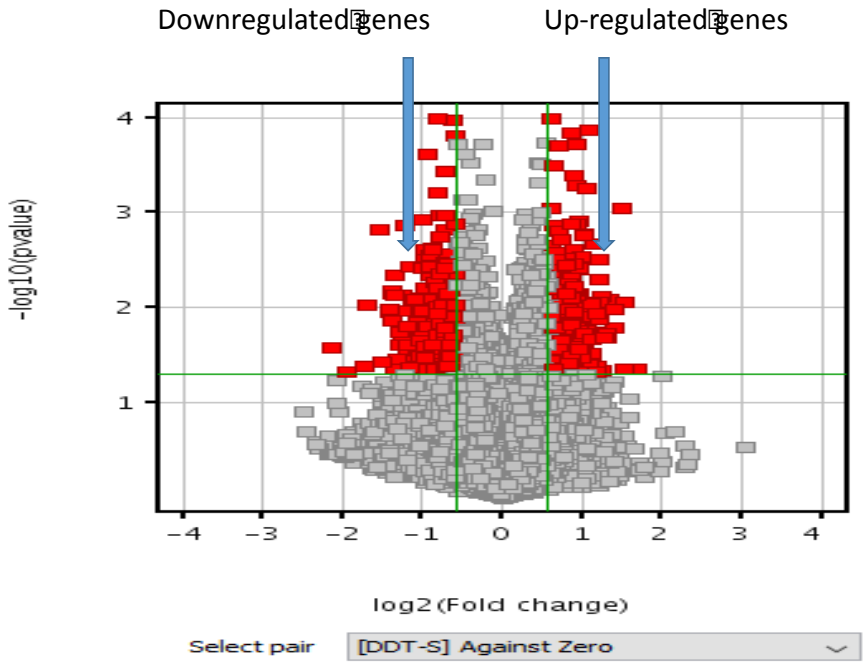
Appendix 21: Profile plot of normalized intensity values of *Anopheles funestus* mosquito population from Akaka-Remo



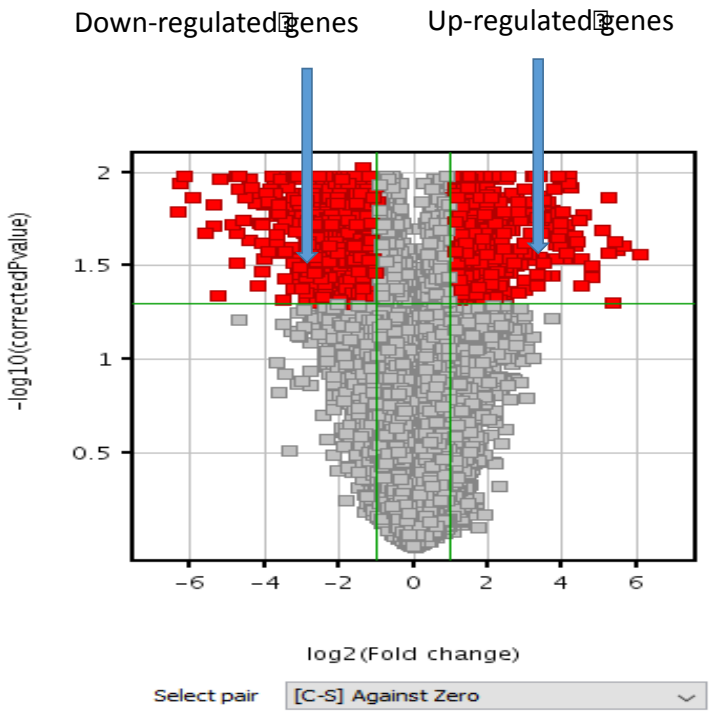
Appendix 22: Overexpressed transcripts in Perm-S comparison: T-Test against Zero, Benjamini test, cut-off 2, p≤0.05, 1536 entities out of 51090



Appendix 23: Overexpressed transcripts in Perm-C: T-Test against Zero, No correction, cut-off 2.0, p≤0.05, 1467 entities out of 51090



Appendix 24: Overexpressed transcripts in DDT-S: T-Test against Zero, No correction, cut-off 1.5, p≤0.05, 664 entities out of 51090



Appendix 25: Overexpressed transcripts in C-S: T-Test against Zero, Benjamini test, cut-off 2, p≤0.05, 2473 entities out of 51090.