Abertay University

Investigating the role of glutathione and glutathione biosynthetic genes in the adaptation of *Anopheles gambiae* to insecticides

By

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BSc, MSc

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Science, Engineering and Technology, Abertay University Dundee, United Kingdom

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DEDICATION

This thesis is dedicated to my beloved parents;

Late Alhaji Usman Abdu

and

Late Hajiya Binta Bala,

Whose guidance and love shall never be forgotten, may their gentle souls rest in peace Ameen

AUTHOR'S DECLARATION

I, **HABIBU USMAN ABDU** declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the Abertay University, Dundee, United Kingdom. It has not been submitted before for any degree or examination in any other University.

29th day of March, 2016

(Signed)

CERTIFICATION

I certify that **HABIBU USMAN ABDU**, a PhD candidate has undertaken all the work described herein and is based on the original work done at the Abertay University, Dundee in partial fulfilment for the requirements for the award of Doctor of Philosophy in Molecular Entomology. This has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any similar title and represents an independent work on the part of the candidate.

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29th day of March, 2016

ACRONYMS/ABBREVIATIONS/SYMBOLS

AA1	Auyo agricultural 1
AA2	Auyo agricultural 2
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immune deficiency syndrome
An.	Anopheles
ANOVA	Analysis of Variance
AR	Auyo residential
ARE	Antioxidant response element
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATG	Start codon
ATP	Adenosine triphosphate
β	Beta
BA	Bichi agricultural
BD	Bendiocarb dead
BLAST	Basic Local Alignment Search Tool
BOD	Biological oxygen demand
bp	Base pair
BR	Bichi residential
BS	Bendiocarb survive
BSA	Bovine serum albumin
^{o}C	Degrees Celsius
CDC	Centre for disease control
cDNA	Complementary Deoxyribonucleic Acid
CnCC	cap 'n' collar isoform-C
DD	DDT dead
DS	DDT survive
DDT	Dichloro diphenyltrichloroethane
DMSO	Dimethyl Sulfoxide
DTNB	5, 5-Dithiobis (2-nitrobenzoic acid)
dKeap 1	Drosophila Kelch- like ECH-associated protein 1
EDTA	Ethylene diamine tetra-acetic acid
Efl	Elongation factor protein
et al.	et alia (and others)
FP	Forward PCR primer
GCLC	glutamate-cysteine ligase, catalytic subunit
GCLM	glutamate-cysteine ligase, modifier subunit
gDNA	Genomic DNA
GPS	Global positioning system
GSH	Glutathione
GSSG	Oxidized glutathione
GSTs	Glutathione-s-transferases
g/l	Gram per litre
h	Hour
IGS	Inter Genic Spacer
IRS	Indoor residual spray
ITN	Insecticide treated nets
Kb	Kilo base
Kdr	Knock down resistance
Keap1	Kelch- like ECH-associated protein 1

LLINs	Long lasting insecticide-treated nets
mg	Milligram
MGB	Minor Groove Binding
Mg/l	Milligram per litre
$MgCl_2$	Magnesium chloride
µl	Micro litre
µM	Micro molar
min	Minute
mM	Millimolar
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
Nrf2	Nuclear factor erythroid-2 related factor 2
Nf2l	Nuclear factor erythroid 2 invertebrate
NGOs	Non-Governmental organisations
nM	Nano mole
NPC	National population commission
%	Percentage
PCR	Polymerase chain reaction
QPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Reverse PCR primer
Rsp7	Ribosomal protein
s	Second (s)
SD	Standard deviation
s.l.	Sensu lato
SINE	Short interspersed element
Spp	Species
s.s.	Sensu strict
Ss	Spineless
SSA	5-Sulfosalicylic Acid
Tgo	Tango
TFBS	Transcription factor binding site
Tm	Primer melting temperature
TNB	5-thio-2-nitrobenzoic acid
TSS	Transcription start site
UBQ	Ubiquitin
USAID	United States Agency for International Development
2-VP	2-Vinylpyridine
WHO	World Health Organization
XRE	Xenobiotic response element

TABLE OF CONTENTS

CHAPTER TWO
Field Study Report

CHAPTER THREE

Susceptibility status of *Anopheles gambiae s.l.* to Insecticides

CHAPTER FOUR

Glutathione in *Anopheles gambiae s.l: Levels* and variation from agricultural and residential settings

CHAPTER FIVE

Differential expression of *Nf2e1* (*Nrf2*), *AGAP012038* (*GCLM*) and *AGAP001512* (*GCLC*) genes in selected strains of *Anopheles gambiae*

CHAPTER SIX

Discussion, Conclusions and Recommendations

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

LIST OF TABLES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

ABSTRACT

Malaria remains a serious public health challenge in the tropical world, with 584,000 deaths globally in 2013, of which 90% occurred in Africa, and mostly in pregnant women and children under the age of five. *Anopheles gambiae (An. gambiae)* is the principal malaria vector in Africa, where vector control measures involve the use of insecticides in the forms of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS). The development of insecticides resistance mitigates these approaches. Glutathione (GSH) is widely distributed among all living organisms, and is associated with detoxification pathways, especially the Glutathione S-transferases (GSTs). Its direct involvement and relevance in insecticide resistance in *An. gambiae* has not been determined. Thus, this work examines the contribution of GSH, its biosynthetic genes (*GCLM, GCLC*) and their possible transcriptional regulator *Nrf2* in insecticide resistance in *An. gambiae* sampled from agricultural setting (areas of intensive agriculture) and residential setting (domestic area). Bioinformatics analysis, W.H.O. adult susceptibility bioassays and molecular techniques were employed to investigate. Total RNA was first isolated from the adults *An. gambiae* mosquitoes raised from agricultural and residential field-caught larvae which had been either challenged or unchallenged with insecticides. Semi-quantitative RT-PCR using gel image densitometry was used to determine the expression levels of *GCLM, GCLC* genes and *Nrf2*. Bioinformatics' results established the presence of putative AGAP010259 (*AhR*) and AGAP005300 (*Nf2e1*) transcription factor binding sites in *An. gambiae GCLC* and *GCLM* promoters *in silico*. *An. gambiae s.l.* studied here were highly resistant to DDT and permethrin but less resistant to bendiocarb. Both knockdown resistance (kdr) mutation variants L1014S and L1014F that confers resistance to pyrethroid insecticides were identified in both *An. coluzzii* and *An. arabiensis* sampled from northern Nigeria. The L1014F was much associated with *An. coluzzii.* A significant positive correlation (P=0.04) between the frequency of the L1014F point mutation and resistance to DDT and permethrin was observed. However, a weak or non-significant correlation (P=0.772) between the frequency of the L1014S point mutation and resistance was also found. L1014S and L1014F mutations co-occurred in both agricultural and residential settings with high frequencies. However, the frequencies of the two mutations were greater in the agricultural settings than in the residential settings. The levels of total, reduced and oxidized GSH were significantly higher in mosquitoes from agricultural sites than those from residential sites. Increased oxidized GSH levels appears to correlate with higher DDT resistance. The expression levels of *GCLM, GCLC* and *Nrf2* were also significantly up-regulated in adults *An. gambiae* raised from agricultural and residential field-caught larvae when challenged with insecticide. However, there was higher constitutive expression of *GCLM, GCLC* and *Nrf2* in mosquitoes from agricultural setting. The increased expression levels of these genes and also GSH levels in this population suggest their roles in the response and adaptation of *An. gambiae* to insecticide challenges. There exists the feasibility of using GSH status in *An. gambiae* to monitor adaptation and resistance to insecticides.

Chapter One

General Introduction

1.0 Introduction

Malaria is the world's most important vector borne disease. Malaria is one of the most widespread infectious diseases of our time. The World Health Organization (WHO, 2013a) estimated that there were approximately 200 million clinical cases and 584,000 deaths from malaria in 2013, predominantly among children and pregnant women in sub-Saharan Africa. It is endemic in 105 countries (WHO, 1999; Breman, 2001). Malaria also poses a risk to travellers and immigrants, with imported cases increasing in non-endemic areas (WHO, 2005). Malaria parasites are transmitted by female mosquitoes belonging to the genus *Anopheles* (Onyido *et al.*, 2014).

Malaria remains one of the most critical public health challenges for Africa despite intense national and international efforts (WHO, 2012). According to the Federal Ministry of Health, Nigeria (2005), "Malaria kills more people than HIV/AIDS or any other killer disease. Malaria is endemic throughout Nigeria accounting for 25% of infant mortality". Malaria impedes on economic development not only by causing premature death but also through lost/diminished productivity, enormous medical cost and population growth (Sachs, 2002). *Anopheles gambiae (An. gambiae)* is the principal malaria vector in Africa. In most cases, particularly in highly endemic areas the ability to reduce malaria transmission will be dependent on the vector control before the focus can shift to killing the parasite in infected people. More than 60% of Nigerian population is in the north and in the northern part of Nigeria the people of Kano and Jigawa states constitute more than 30% of the northern Nigerian population (NPC, 2006b). Furthermore, the north of the country has the highest malaria burden relative to the southern region. In addition, more than 65% of arable land in Nigeria is located in the north (NPC, 2006a). The two states have the largest irrigation projects in Nigeria. Thus, intensive agriculture is the major economic activities in this region. Alongside the advantages of these dams to the development of farming and provision of food comes the disadvantage in health implication by providing suitable breeding sites for vectors

of diseases. The use and discharge of refined petroleum and other hydrocarbon products under different trade names and chemical combinations is higher in the North because of the larger population. These use and release are in the form of residual sprays (Indoor and outdoor); long lasting insecticide treated bed nets (LLINs). Therefore, contamination of the mosquito breeding sites through these human-related activities is expected to be higher in this region compared to the south. In general, insecticide usage for personal protection and for controlling crop pests in agriculture and the presence of anthropogenic pollutants in urban, agricultural or industrial areas has often been suggested as sources of selective pressures favouring insecticides resistance (Balkew *et al*., 2010; Marcombe *et al.,* 2011). Thus we hypothesized that agricultural practices constitute sources of selection pressure for the emergence of resistant *An. gambiae* population in these parts Nigeria.

1.1 Mosquito

Mosquito is a Spanish or Portuguese word meaning little fly. It belongs to the insect family Culicidae (from Latin word 'Culex' meaning midget or gnat). The scientific classification of mosquito is given below:

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Diptera
Suborder:	Nematocera
Intraorder:	Culicomorpha
Superfamily:	Culicoidea
Family:	Culicidae
Subfamily:	Anophelinae, Culicinae, Toxorlynchitinae

(Harbach, 2008)

Figure 1.1 an image of adult Anopheles mosquito showing the morphology (CDC) 2012

There are about 3, 500 species of mosquitoes of which the most well-known members belong to two subfamilies, *Anophelinae* (e.g. *Anopheles gambiae*) and *Culicinae* (e.g. *Culex quinquefasciatus*) (Briggs, 2013; Ashfaq *et al*., 2014; Khalita *et al*., 2014).

1.2 Malaria vectors

Anopheles species have a worldwide distribution, occurring in both tropical and temperate regions (Service, 1996). There are almost 500 known species of Anopheles and only about 20% of these transmit malaria, based on the essential requirements that vectors must be anthropophilic and susceptible to Plasmodium infection (Collins & Paskewitz, 1995). The major vectors of malaria in sub-Saharan Africa are found within the *An. gambiae* complex and the *An. funestus* group (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Collins & Paskewitz, 1995 and Sinka, *et al.,* 2010).

Previous studies (Merrit *et al.,* 1992; Clements, 2000 have described Anopheline mosquito larvae to possess a well- developed head with mouth brushes used for feeding, a large thorax and a segmented abdomen. They also lack any legs. Unlike other mosquito species, they possess no respiratory siphon and breathe through spiracles located on the 8th abdominal segment and, therefore, must come to the surface frequently (CDC, 2012).The larvae spend most of their time feeding on algae, bacteria, and other microorganisms in the surface microlayer. They dive below the surface only whenever disturbed. Larvae swim either by

jerky movements of the entire body or through propulsion with the mouth brushes. In contrast, Culex mosquitoes were found to hang themselves longitudinally with the head hanging downward below the air-water interface and are less active even when disturbed (Paaijmans, 2008). Mosquito larvae were reared under standard insectary conditions according to Das *et al.,* (2007) and the resulting adults identified as *An. gambiae s.l.* according to the morphological identification keys (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

The life cycle of mosquito consist of four stages; egg, larva, pupa, and adult also referred to as imago. The adult female mosquitos lay their eggs in standing water bodies such as lakes, salt marsh, water puddles, a natural reservoir, or on a plant or artificial water container. The pupa is comma shaped with the head and thorax merged into a cephalothorax with the abdomen. The pupa also floats just underneath the water surface. The pupae like the larvae also come to the surface to breathe with pair of respiratory trumpets located on the cephalothorax. The pupa does not feed during its stage of development, and few days after the formation of the pupa, it rises to the surface of the water, the dorsal surface of the cephalothorax splits and the adult, mosquito emerges. The pupal stage has less activity compared to the larval stage.

1.2.1 Transmission of malaria in man

Malaria parasites transmission in Nigeria is primarily due to the genus Anopheles, which is dominated by the *An. gambiae sensu lato (s.l.)* complex groups, eight (8) sibling species, including two of the most efficient African human malaria vectors, *Anopheles arabiensis* (Patton, 1905) and *An. gambiae sensu stricto (s.s.)* (Giles, 1902; Arnal *et al*., 2014) and the other being *An. funestus* group of mosquitoes (Mzilahowa *et al*., 2012; Alout *et al*., 2014) in Sub-Saharan Africa. The male Anopheles mosquitoes only feed on flower nectar and plant juices as sources of carbohydrate and cannot transmit malaria (WHO, 2013b). Malarial

parasites are therefore transmitted by adult female Anopheles mosquitoes (WHO, 2013c), as it is only the female that requires a blood meal in order to obtain enough protein so as to develop a batch of eggs (Artis *et al.*, 2014). *An. gambiae* reportedly takes its first blood meal 12 hours after emerging, and will take multiple meals throughout its gonotrophic cycle (Briegel and Horler, 1993; Paaijmans *et al.*, 2013). It is the female mosquito's blood-feeding habit that allows uptake of the gametocyte form of the parasite. This developmental stage of Plasmodium is the only stage infective to the Anopheline mosquito host (Smith *et al.*, 2003). Transmission to another human host occurs when an infective female Anopheles mosquito bites an uninfected person and during the blood meal, the mosquito injects Sporozoites into the blood stream and that person goes ahead to develop malaria (Figure 1.2).

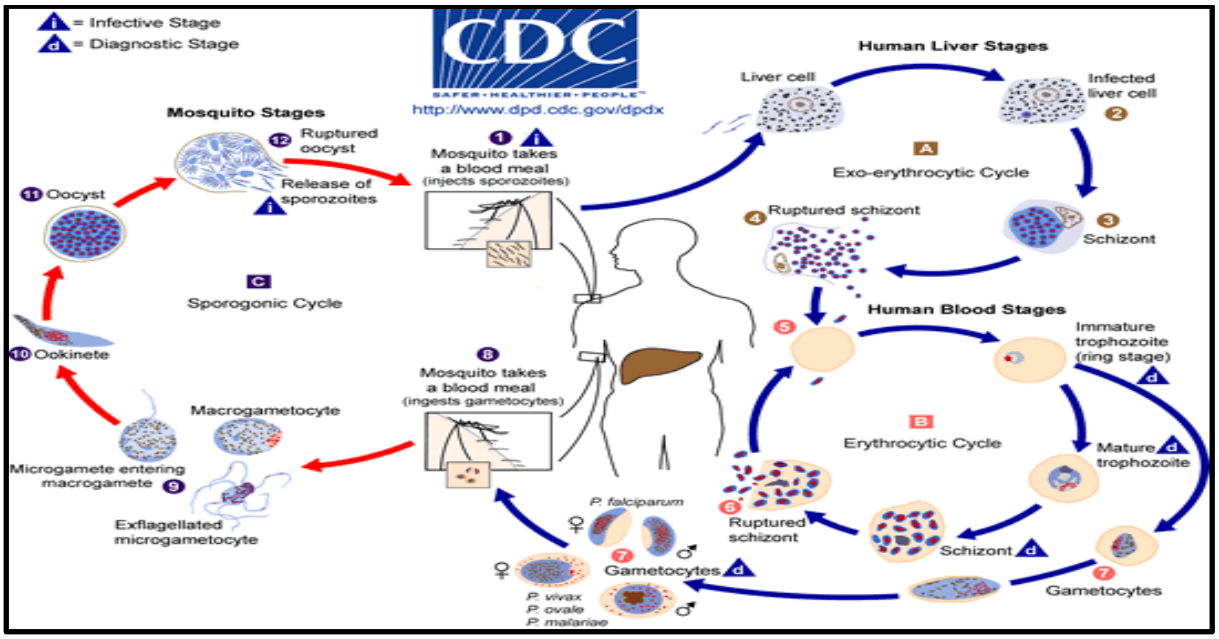


Figure 1.2 Malaria parasite transmission life cycles. The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female Anopheles mosquito inoculates Sporozoites into the human host 1. Sporozoites infect liver cells 2 and mature into schizonts 3, which rapture and release merozoites 4. After this initial replication in the liver (exo-erythrocytic schizogony A), the parasite undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rapture releasing merozoites 6. Some parasite differentiates into sexual erythrocytic stages (gametocytes) 7. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes) are ingested by an Anopheles mosquito during a blood meal 8. The parasites' multiplication in the mosquito is known as the sporogonic cycle C . While in the mosquitos' stomach, the micro gametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rapture, and release Sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the Sporozoites 1 into a new human host perpetuates the malaria life cycle. Adapted from CDC, 2012

1.2.2 Anopheles gambiae complex

The *An. gambiae* complex is a group of morphologically indistinguishable yet genetically distinct species that differ in their behaviour and vectorial capacity (Gillies & Coetzee, 1987; Hunt *et al.*, 1998). The species within the complex are *An. gambiae* Giles, *An. arabiensis* Patton, *An. quadriannulatus* Theobald species A and B, *An. merus* Donitz, *An. melas* Theobald and *An. bwambae* White. *An. gambiae* s.s. and *An. arabiensis* are major vectors; *An. bwambae, An. melas* and *An. merus* are minor malaria vectors and *An. quadriannulatus* is a non-vector (White, 1974; Coetzee *et al.,* 2000). *An. gambiae* s.s. is extremely anthropophilic (taking blood meal from humans) throughout its distribution; *An. arabiensis* is strongly anthropophilic in many parts of its distribution depending on host availability; *An. melas* and *An. merus* show intermediate anthropophily but are mainly zoophilic and *An. quadriannulatus* is zoophilic (taking blood meal from animals) (Gillies & Coetzee, 1987). *An. gambiae s.s.* comprises two incipient species named (*An. gambiae* S form and *An. gambiae* M form). The two forms are recognised by form-specific single nucleotide polymorphisms (SNPs) on the IGS and ITS regions of multi copy rDNA located on the X chromosome (Santolamazza, *et al*., 2008; Caputo *et al.,* 2011). Coetzee *et al.,* (2013) reported that based on population genomic evidences, *An. gambiae* M and S forms has recently been assigned to distinct species names. The S form conserves the *An. gambiae s.s.* name while the M form is now *An. coluzzii*.

The identification of the species provides information on the biology of the individual species which in turn determine the control measures. This is because different members of the same species complex do not necessarily share the same resistance mechanisms, and nor do they necessarily exhibit the same insecticide resistance patterns (WHO, 2013c). For example in 1977, there was an outbreak of malaria in the south-eastern Lowveld region of Zimbabwe and members of *An. gambiae* were collected in a benzene hexachloride (BHC) from organochloride-sprayed dwellings. Subsequent species identification conducted after WHO insecticide susceptibility assay show most of the survivors were members of *An.*

arabiensis while majority of the susceptible species were members of *An. quadriannulatus*. These results lead to the realisation that members of *An. arabiensis* were resistant against the insecticide used, thus resulted to a change in policy where BHC was substituted by DDT (Green, 1981). A similar situation in south Africa in1996 lead to DDT being replaced by pyrethroids as a result of environmental concern mainly due to complaints by members of the community of the objectionable build-up of DDT in the wall of their houses and increased bed bug biting activity.

Studies on behavioural and distribution pattern of mosquito species in Nigeria show there is variation in species and distribution pattern as one move from one part of the country to another. Although, other members of Anopheles species have been reported in the country, the major vectors of malaria belong to the members of *An. gambiae* and *An. funestus* complexes.

1.3 Malaria control

There are two approaches used in malaria control: chemotherapy and preventing contact between humans and vectors using interventions such as insecticides, bed nets, environmental management and biological control (Collins & Paskewitz, 1995).

1.3.1 Mosquito Vector Control

Vector control is one of the most effective measures of preventing malaria transmission which aims to prevent parasite transmission mainly through interventions targeting adult *Anopheline* vectors (Singh *et al.,* 2014). Mosquito vector control can be directed either against the adult or against the aquatic stages (WHO, 2005). The major mosquito control methods targeting the adults are indoor residual spray (IRS) and long lasting insecticide–treated bed nets (LLINs) that rely heavily on the use of insecticides (WHO, 2012; Kabula *et al.,* 2014; Yasuoka *et al*., 2014; Hemingway, 2014, Killeen *et al.,* 2014).

Insecticide application (through indoor residual spraying (IRS) and use of insecticide treated nets (ITNs)) is one of the most important components in the global control of malaria vectors (McCarroll & Hemingway, 2002; WHO, 2005). Insecticide treated nets have been shown to reduce the burden of malaria in pregnant women and young children (WHO, 2005). Indoor residual spraying and use of ITNs/LLINs has been useful in reducing malaria transmission and burden for many years (Collins & Paskewitz, 1995; WHO, 2005; Pluess *et al.,* 2010; Okuma and Moore, 2011; Overgaard *et al.,* 2012; WHO, 2012; Wondji *et al.,* 2012; Abuelmaali *et al.,* 2013).

1.3.1.1 Malaria vector control in Nigeria

Malaria is a serious health problem in Nigeria and kills more people than HIV/AIDS or any other killer disease and it is endemic throughout Nigeria accounting for 25% of infant mortality (FMoH, 2005). Malaria impedes on economic development not only by causing premature death but also through lost/diminished productivity, huge medical cost and population growth (Sachs, 2002). In 2008, the overall ownership of one LLIN coverage was 8% and reached 42% in 2010 with rural ownership higher (45%) than urban (33%) (USAID/PMI/CDC, 2011) similarly, the proportion of the vulnerable groups (children and pregnant women) sleeping under ITNs has increased from 6% in 2008 to 29% in 2010 and IRS has been piloted in selected areas in the country (NPC, 2012). In 2009 4,137,464 LLINs were freely distributed in Kano state (Zainab, 2013). Approximately, five million LLINs were distributed in Jigawa state between 2009 and 2015 (Rufa'i, 2015). As of May 2011 a total of 35.6 million LLINs had been distributed across 22 states of Nigeria, with a balance of 27.3 million to complete the remaining 15 states. This together with the World Bank supported IRS and insecticide treated nets (ITN) programs in several states in Nigeria (Seventh annual report to congress, 2013) may add to the selective pressure on malaria vectors to develop more resistance against insecticides.

1.4 Insecticides and Insecticide resistance

Insecticides are a type of pesticide that is used to specifically target and kill insects. Pesticides are chemical substances that derive their name from the French word "Peste", which means pest or plague and the Latin word "caedere", to kill (Akunyili and Ivbijaro, 2006). Pesticide therefore can be defined as any chemical substance or mixture of substances intended for preventing, destroying, repelling, or mitigating the effect of any pest of plants and animals (U.S. National Pesticide Information Centre, 2015).They include herbicides, insecticides, rodenticides, fungicides, molluscides, nematicides, repellents and attractants used in agriculture, public health, food storage or a chemical substance used for a similar purpose (NAFDAC, 1996). Both in Auyo and Bichi farmers use petrol pumps and a myriad of pesticides to protect crops. These pesticides include carbamates, organochlorine, organophosphates, and pyrethroids bearing different trade names. Application of pesticides in the form of residual sprays is the most widely adopted method of insect pest control in these areas because of their quick and effective action.

Insecticide resistance on the other hand is defined by the World Health Organisation as "the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations (Ranson *et al.,* 2011).

There are four classes of insecticides approved for public-health, namely carbamates (esters of carbamic acid), organophosphates (phosphoric acid derivatives), organochlorines (chlorinated hydrocarbons) and pyrethroids (synthetic pyrethroids) (Najera and Zaim, 2002; WHO, 2006; Kelly-Hope *et al.,* 2008; Brooke *et al*., 2013; Kabula *et al.,* 2014). However, pyrethroids are the only class of insecticide currently recommended for use on ITNs/LLINs or IRS by WHO, due to safety and cost effectiveness (WHO. 2010b; Butler, 2011; WHO, 2013c; Adams, 2014). Nevertheless, bendiocarb and dichlorodiphenyltrichloroethane (DDT) are also used in some areas for IRS (WHO, 2008; Ranson *et al.,* 2011). These four classes of insecticide share two modes of action (Ranson *et al.*, 2012).

Since the 1940s and 1950s, resistance has appeared in most major insect vectors from every genus except *Glossina* (WHO, 1992). More than 100 mosquito species including 56 species of Anopheline and 39 species of Culicine mosquitoes are known to have developed resistance to almost all classes of insecticides used for their control (WHO, 1992; Wondji *et al.,* 2012). Over the years after development of resistance to DDT, many classes of insecticides have been employed in malaria control. These include organophosphates, carbamates and most recently pyrethroids which are used as indoor residual spray (IRS) as well as insecticides treated bed nets (ITNs). Resistance have developed to most of these classes of insecticides across the sub Saharan African countries (Hemingway and Ranson, 2000). The development and rapid spread of insecticides resistance to major malaria vectors across the African countries has posed threat to effectiveness of these different mosquito control measure (Etang *et al.,* 2003; Corbel *et al.,* 2004; Ranson *et al.,* 2009; Ranson *et al.,* 2011; Kolade *et al.,* 2013). Thus constituted an impediment to vector control initiatives in African countries (Hemingway *et al.,* 2004; WHO, 2005). Massive and extensive use of insecticides in agriculture (Yadouleton *et al.,* 2010) and high ITNs and IRS coverage, or recurrent space spraying interventions (Balkew *et al.,* 2010; Marcombe *et al.,* 2011; Ranson *et al.,* 2011, Nkya *et al.,* 2014) in public health has resulted in increasing resistance among malaria vectors due to the selection pressure placed on resistance genes. The development and rapid spread of insecticides resistance to primary malaria vectors across the African countries have posed a threat to the effectiveness of these mosquito control efforts (Ranson *et al.,* 2011). Kolade *et al.,* 2013, indicated the extensive use and abuse of conventional insecticides for agriculture and personal protection has contributed immensely to the development of resistance in Anopheline mosquitoes and other insect pests. Also, lack of available alternative insecticides for vector control has also been an issue (Coleman *et al.,* 2006, Kolade *et al.,* 2013).

Pyrethroids resistance is believed to be caused mainly by high ITNs and IRS coverage, or recurrent space spraying interventions (Balkew *et al.,* 2010; Marcombe *et al.,* 2011; Nkya *et*

al., 2014). However, studies pointed out the possible role of other factors in the selection of inherited resistance mechanisms or the higher tolerance of mosquitoes to pyrethroids. Among them, insecticide use for personal protection and for controlling crop pests in agriculture and the presence of anthropogenic pollutants in urban, agricultural or industrial areas has often been suggested as additional selective pressures favouring pyrethroids resistance. Ranson *et al.,* (2000) highlighted the primary causes of insecticide resistance as alterations in the target sites and increases in the rate of insecticide metabolism. The target sites of all the major classes of insecticides have been established, and resistance-associated mutations have been identified.

On the other hand, DDT although its use has been banned, is still unofficially being used by farmers and also in homes under different trade names and chemical combinations. Awolola *et al.,* 2005, 2007 has reported the development of resistance to DDT and other classes of insecticides including organochlorine, organophosphate, carbamates and recently pyrethroid in *An. gambiae s.s. An. arabiensis* and *An. funestus* from different zones in Nigeria. In south-west Nigeria, the first case of pyrethroid resistance in *An. gambiae*, the principal malaria vector in Nigeria was documented (Awolola *et al.,* 2002) and since then the phenomenon has been well established in this region (Awolola *et al.,* 2003; Kristan *et al.,* 2003; Awolola *et al.*, 2005, 2007; Oduola *et al.,* 2010, 2012, Kolade *et al.,* 2013). Also in North-central Nigeria, Permethrin and DDT resistance in *An. gambiae s.l.* has been reported (Ndams *et al.,* 2006; Olayemi *et al.,* 2011). In North-west Nigeria, resistance to Permethrin and DDT were also reported (Ibrahim *et al.,* 2014). Particularly worrisome is recent evidences of resistance to pyrethroids in Africa malaria vector, *An. gambiae* given the recent emphasis by WHO and other international health agencies on the use of pyrethroids for impregnated bed nets in their roll back malaria campaign (Lengeler, 2004). Insecticides resistance detected in major Anopheles species to classes of insecticides commonly used includes those reported by Ranson *et al.,* 2000; Awolola, 2002; Etang *et al.,* 2003; Corbel *et al.,* 2004; Casimiro *et al.,*

2006a; Awolola, 2005, 2007; Corbel *et al.,* 2007; N'Guessan *et al*., 2007; Ranson *et al.,* 2009, 2011; Kerah-Hinzoumbe *et al.,* 2008; Djouaka, 2011, Ndiath *et al.,* 2012; Kolade *et al.,* 2013; Nwane *et al.,* 2013; Ibrahim *et al.,* 2014 and Alhassan *et al.,* 2015.

1.5 Insecticide modes of action and mechanisms of resistance

1.5.1 Insecticide modes of action

Insecticides generally target the nervous system of the insect. Organophosphate (e.g. Chlorpyrifos, Diazinon, Fenitrothion, Fenthion, Malathion and Temephos) and Carbamate (e.g. Propoxur, Bendiocarb, and Carbaryl) insecticides are acetylcholinesterase inhibitors. Cyclodiene (e.g. dieldrin) insecticides affect the chloride channel by inhibiting or antagonising the gamma amino butyric acid (GABA) receptor. Pyrethroids (e.g. Permethrin, Deltamethrin, and Cypermethrin) and DDT act on the sodium ion gated channel by prolonging sodium channel activation due to their high toxicity to insects (Chang *et al*., 2014).Thus preventing these channels from closing, resulting in continual nerve impulse transmission, tremors, and eventually death (Bloomquist, 1996; Davies *et al.,* 2007).

1.5.2 Mechanisms of insecticide resistance

Two major mechanisms have been identified to be responsible for insecticide resistance (1) an increased metabolic detoxification of insecticides through increased enzymatic activities of esterases, glutathione S-transferases and cytochrome P450 monooxygenases, as a result of their overproduction due to gene amplification (Poire *et al.,*1992, Raymond *et al.,*1989; Ranson *et al.,* 2000, 2011) and/or gene regulation (Muller *et al.,* 2008, Muller *et al.,* 2007) and (2) point mutations in the gene encoding the voltage-gated sodium channel at the target sites of insecticides, decreasing the affinity of the insecticides to its receptor. Two mutations at amino acid position 1014 of the voltage-gated sodium channel, changing either a Leucine

residue to a Phenylalanine (L1014F) [Martinez-Torres *et al.,* 1998], or a Leucine to Serine (L1014S) [Ranson *et al.,* 2000, Ranson *et al.,* 2011] have being identified in *An. gambiae* and confers knockdown resistance (kdr) to DDT and pyrethroid insecticides. Recently, a new sodium channel mutation N1575Y was found to be concurrent with the L1014F mutation in several pyrethroid resistant populations of An. gambiae. The findings of Wang, *et al.,* 2013, demonstrated that N1575Y functions as an enhancer of the L1014F/S-mediated pyrethroid resistance and provide a molecular explanation for the emerging co-occurrence of N1575Y and L1014F in pyrethroid-resistant populations in some African countries though not reported in Nigeria at the moment. On the other hand, carbamates and organophosphates share acetylcholinesterase as target site and at least two functional mutations in acetylcholinesterase 1 (ace-1) have been identified in insect species that offer reduced target sensitivity to intoxication (Alout *et al.,* 2007). One of these, ace-1R (G119S), is most commonly associated with resistance to these insecticides in *An. gambiae* (Dabire *et al.,* 2008; Djogbenou, 2008).This mutation is found in association with resistance in the M and S molecular forms (Djogbenou *et al.,* 2008). Esterase mediated sequestration of carbamates and organophosphates are documented for some insect species (Li *et al.,* 2009).

Numerous studies done over the past decades have demonstrated that multiple insecticide resistance mechanisms involving many genes exist in many insect species, including mosquitoes (Raymond *et al*., 1989; Hemingway *et al.,* 2002 & 2004; Liu and Scott, 1995, 1996, 1997 & 1998; Liu and Yue, 2000 & 2001; Ranson *et al*., 2002; Liu *et al*., 2005, 2007 & 2011; Vontas *et al*., 2005; Xu *et al*., 2005; Zhu and Liu 2008; Zhu *et al*., 2008a & 2008b).

1.5.2.1 Target Site mechanism

Resistance due to modification of neural target sites has been identified for voltage sensitive sodium channel, acetylcholinesterase, and the gamma amino butyric acid (GABA)-gated chloride channel.

1.5.2.1.1 Sodium ion channels (Knock down resistance (kdr))

The pyrethroid insecticides and the organochlorine insecticide (DDT) target the voltage-gated sodium channel on the insects' neurons (Davies *et al.,* 2007). Insecticide binding delays the closing of the sodium channel prolonging the action potential and causing repetitive neuron firing, paralysis and eventual death of the insect. Alterations in the target site that cause resistance to insecticides are often referred to as knock-down resistance (kdr). Several mutations in the sodium ion- channel have been associated with resistance to pyrethroids in a variety of insects (Davies et al., 2007). One of the most common amino acid replacements, and so far the only residue associated with pyrethroid resistance in malaria vectors, is a substitution of the leucine residue found at codon 1014 with either phenylalanine (1014F) or serine (1014S).

The *kdr* mutation has been recorded in pyrethroid resistant populations of *An. gambiae* in several West African countries including Nigeria. These include Burkina Faso (Martinez Torres *et al*., 1998; Weill *et al.,* 2000; Diabate *et al*., 2004a), Benin (Aizoun *et al.,* 2014), Cameroon (Etang *et al*., 2006; Nwane *et al.,* 2011), Cote d'Ivoire (Chandre *et al.,* 1999; Weill *et al.,* 2000) Others include Ghana (Yawson *et al*., 2004), Mali (Fanello *et al*., 2003), Nigeria (Awolola *et al*., 2002, Kolade *et al.,* 2013; Ibrahim *et al.,* 2014) and Senegal (Weill *et al.,* 2000; Ndiath *et al.,* 2012).

1.5.2.1.2 Altered acetylcholinesterase

The mode of action of organophosphates and carbamate insecticides involves alterations in acetylcholinesterase (AChE) (Hemingway, 1989; Brogdon & McAllister, 1998; Hemingway & Ranson, 2000). This resistance mechanism is due to a change in the AChE, reducing its affinity for the insecticides and, in most cases, to some extent for its normal substrate acetylcholine (Hemingway, 1989).The presence of an altered AChE gene has also been

detected at a low frequency in pyrethroid resistant populations of *An. gambiae*, *An. funestus* and *An. arabiensis* (Casimiro *et al.,* 2006a; Casimiro *et al.,* 2006b). Single nucleotide change within the gene coding for γ–gamma glutamyl receptor also appear to be responsible for resistance to dieldrin (Davies *et al*., 2007; Santolamazza *et al*., 2008). Researchers have already identified at least five point mutations in the acetylcholinesterase binding sites that may confer varying degree of resistance to organophosphorus and carbamates classes of insecticides (Heming way *et al*., 2004, Edi *et al*., 2012).

1.5.2.1.3 Gamma amino butyric acid (GABA) receptors

The gamma amino butyric acid (GABA) receptor is a chloride-ion channel in the insect's central nervous system and neuromuscular junctions (Hemingway & Ranson, 2000). Mutations of the GABA receptor are implicated as a site of action for ivermectins and cyclodienes (Hemingway & Ranson, 2000). A mutation of alanine 296 to glycine has been associated with dieldrin resistance in *An. gambiae* (Du *et al.,* 2005; Brooke *et al.*, 2006). Another mutation of alanine to serine at the same codon has also been associated with dieldrin resistance in a laboratory strain of *An. arabiensis* (Du *et al.,* 2005).

1.5.2.2 Increased metabolic detoxification

Increased metabolic detoxification is one of the most common mechanisms of insecticide resistance (Hemingway & Karunaratne, 1998 and Hemingway *et al.,* 2004). Three enzyme classes are involved in insecticide detoxification: the monooxygenases (cytochrome P450s), esterases and glutathione S-transferases (Pasteur & Raymond, 1996; Brogdon & McAllister, 1998; Hemingway & Karunaratne, 1998; Hemingway, 2000; Hemingway et al., 2004; Liu *et al*., 2006). Detoxification enzyme-based resistance occurs when increased activity of the three enzyme classes results in sequestration or detoxification of the insecticide thereby impairing the toxicity of the insecticide before it reaches its target site (Liu et al., 2006; Stradi, 2012).

1.5.2.2.1 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 (Scott, 1999; Hemingway & Ranson, 2000). The P450s are heme-containing family of enzymes distributed widely in insects. They are involved in the metabolism of wide range of environmental xenobiotics including many classes of insecticides by oxidizing the insecticides. The role of P450s in the metabolism of pyrethroid insecticides has been particularly studied extensively in *An. gambiae.* Vulule *et al.,* (1999) first demonstrated the involvement of P450s in pyrethroid resistance in *An. gambiae* in Kenyan villages by displaying increased heme levels of P450s in resistant mosquitoes. The studies of Nikou *et al*., 2003; David *et al.,* 2005; Muller *et al*., 2007; Awolola *et al*, (2009); Stevenson *et al*., 2011; Silveira, 2012 further demonstrated the involvement of the family of P450s in a number of insecticides resistance in the African malaria vector.

1.5.2.2.2 Esterases

Esterases produce a broad range of insecticide resistance through sequestration of the insecticide rather than metabolizing the insecticide (Hemingway & Karunaratne, 1998; Hemingway, 1999; Hemingway & Ranson, 2000). They can also provide a narrow range of insecticide resistance through metabolism of a few insecticides with an ester bond (Hemingway & Karunaratne, 1998). In mosquitoes, esterase based resistance mechanisms are either through (a) the esterase is modified so that they metabolize insecticides more efficiently; or (b) the esterase is elevated, primarily through gene amplification (Hemingway, 1999). Esterases detoxify organophosphates and carbamates and are important to a lesser extent in resistance to pyrethroids (Pasteur & Raymond, 1996; Hemingway & Ranson, 2000).

1.5.2.2.3 Glutathione S-Transferases

Glutathione S-Transferase (GST) is another major class of detoxification enzymes system which play important role in the metabolism of many insecticides. Glutathione S-transferases (GSTs) confer resistance by conjugating reduced glutathione (GSH) to a large range of xenobiotics aiding in their detoxification and excretion (Hemingway, 1999; Hemingway, 2000; Hemingway & Ranson, 2000). Elevated levels of this enzyme have been implicated in many incidences of resistance. This increase in GST activity was believed to be due to increased level of one or more GST isoenzyme arising either through gene amplification or the most commonly increase in gene transcription. Qualitative changes in individual enzymes were earlier though to be a contributing factor, however, recent insights suggested this is not the case (Ranson and Hemingway, 2004). Elevation of the activities of GST has been confirmed as one of the major mechanisms of resistance of DDT by insects (Hemingway *et al*., 2004, Edi *et al*., 2012). GST could play a very important role in conferring insects resistance to pyrethroid by serving as a detoxification route for products of lipid peroxidation produced during the metabolism of pyrethroids (Vontas *et al*., 2001). The adaptive changes in GSH homeostasis are associated with Up-regulation of *GCLC* and *GCLM* all contributing to an adaptive response to cellular stress. Elevated GST activity has been associated with resistance to all the major classes of insecticides (Clark & Shamaan, 1984; Huang *et al.,* 1998; Vontas *et al.,* 2001; Kostaropoulos *et al.,* 2001). Insect resistance to organophosphates typically involves increase in the metabolic capabilities of detoxification enzymes as well as decreases in target site sensitivity and cuticular penetration i.e. up-regulation of esterases (Newcomb *et al.,* 1997; Li *et al.,* 2007).

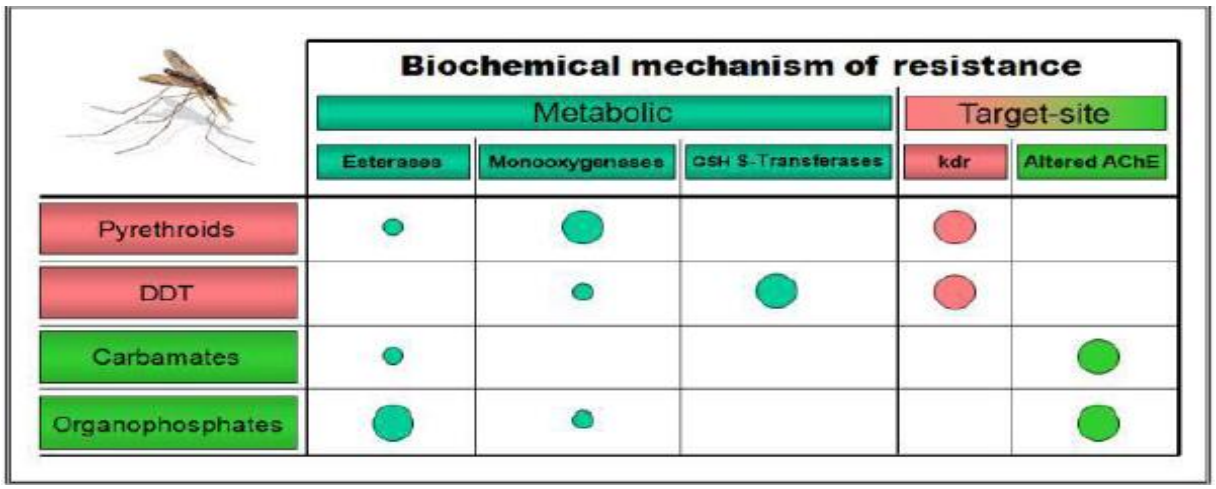


Figure 1.3 Major biochemical mechanisms conferring resistance to important classes of insecticides in adult mosquitoes (dot size gives the relative impact of the mechanism on resistance) (Oxborough, 2014)

However, control of mosquito vector remains a challenge even after continuous use of synthetic insecticides particularly pyrethroids in public health (Singh *et al*., 2014). These insecticides are the only class approved for use on insecticide treated nettings (Zaim and Guillet, 2002; Ranson *et al*., 2011) and are increasingly deployed in IRS (Indoor residual spray) programs in Africa.

1.5.3 Environmental Xenobiotics and Insecticides resistance

1.5.3.1 Xenobiotic Pollution and Insecticides Resistance in Insects

Resistance has evolved in many insect species due to long period of insecticide application for diseases control. Studies have however shown that other processes such as environmental pollution and misuses of pesticides also contribute to the evolution and continuance of insecticides tolerance. Environmental pollution resulting from industrial activities, agriculture, mining has led to change in organisms. These changes occur at various levels of organisms such as cellular level, tissue level, at the level of the organism, at population level as well as at the level of the ecosystem. As a result of pollution, insects are under pressure to respond to changes in the environment in order to survive.

These changes can take many forms, number of insect may changes as result of changes in their vital capacity and fecundity (Golutvin *et al.*, 1981; Heliovaara and Verzanen, 1994), and also population of insects resistant to insecticides may appear as a result of selection pressure posed by pollution (Lauridsen and Jersperson, 1997). According to NPC, (2006a) more than 65% of the arable land in Nigeria is located in the north. The two states Jigawa and Kano have the largest irrigation projects in Nigeria. Thus, intensive agriculture is the major economic activities in this region. Alongside the advantages of these dams to the development of farming and provision of food comes the disadvantage in health implication by providing suitable breeding sites for vectors of diseases. The use and discharge of refined petroleum and other hydrocarbon products under different trade names and chemical combinations is higher in the North because of the larger population. These use and release are in the form of Indoor residual sprays (IRS); long lasting insecticide treated bed nets (LLINs). Therefore, contamination of the mosquito breeding sites through these human-related activities is expected to be higher in this region compared to the south. In general, insecticide usage for personal protection and for controlling crop pests in agriculture and the presence of anthropogenic pollutants in urban, agricultural or industrial areas has often been suggested as sources of selective pressures favouring insecticides resistance (Balkew *et al*., 2010; Marcombe *et al.,* 2011).

One of the first organisms to develop resistance as a result of environmental pollution and insecticide pressure is the house fly *Musca domestica* (Keiding and Jespersen, 1991). Observations from other insect species have demonstrated the contribution of environmental xenobiotics to the development of resistance to insecticides used for their control. For instance, Boyer *et al.*, 2006 reported that *Aedes aegypti* larva exposed to the herbicide atrazine become tolerant to the organophosphate temephos. Similarly, exposure of *Aedes albopictus* larvae to benzothiazole and pentachlorophenol can increase their tolerance to insecticides such as carbaryl, rotenone and temephos (Suwanchaichinda and Brattsen,

2001; Suwanchaichinda and Brattsen, 2002). Induction of detoxification enzymes such as P450s GSTs and non-specific esterases in response to various environmental chemical or xenobiotics have been reported in many insect vectors. Also, studies have established correlation between increase in tolerance to various classes of insecticides in many insects and induction of detoxification enzymes as a result of prior exposure of insects to environmental chemicals (Feyereisen, 2005; Hemingway *et al*., 2002; Hemingway *et al.*, 2004). Drosophila and *Aedes aegypti* have featured more prominent than other insects' species in model studies involving induction of detoxification enzymes as a result of prior exposure to environmental xenobiotics, and incidences of tolerance to various classes of insecticides (Le Goff *et al*., 2006; Poupardin *et al*., 2008). Finally, mechanisms underlying the induction of detoxification enzyme in response to environmental xenobiotics including insecticides have been well documented (Luo *et al.*, 2004; Vontas *et al*., 2005; Backlund and Ingelman-Sundberg, 2005; Brown *et al*., 2005; Gilbert *et al*., 2002; king-Jones *et al*., 2006 and li *et al*., 2002).

1.5.3.2 Agro-allied Chemicals and Insecticides Resistance

Most of the studies on insecticides tolerance in insects have focused primary on insecticides used for public health programmes. This is understandable since the major objective is to control disease carrying vectors in order to guarantee public health safety. However, the fact that some of these insecticides or those chemically similar to them are also applied in agriculture, and the gradual emergence of agriculture as an increasingly resource intensive enterprise has necessitated a consideration of the role of agricultural practices in the evolution and development of insecticides resistance by public health vectors (Overgaard, 2006). A case for agricultural involvement in insecticides resistance was first made when resistance to DDT and the now obsolete dieldrin, appeared in Anopheline mosquitoes in Greece and Africa as a result of agricultural treatment of cotton and rice (Hamon and Garrett-Jones, 1963).

Over 90% of insecticides produced globally are used in agriculture as pesticides. Agricultural usage of insecticides can exert selection pressure on disease vectors at different stage of their development especially the larva and adult stages. Agricultural practices such as rice field and irrigation schemes create breeding sites for these vectors which are sprayed with insecticides during treatment. Wind blowing in the direction of breeding site can transfer sprayed insecticides to mosquitoes and rainfall can wash away insecticides applied on farmland into pools and water bodies where mosquitoes are breeding. Some species of mosquitoes such as *An. pharoensis* have been found to be resting on insecticides treated trees. Contamination of mosquito breeding sites by agricultural insecticides subjects mosquito larvae to selective pressure which is more likely to induce resistance more rapidly than house spraying of adult mosquitoes which reached only anthropophilic females which are generally less than 25% of the total mosquito population for semi exophilic species. Larval exposure can trigger genetic selection for resistance by the action of insecticides resistance acting at sub lethal does (Tia *et al*., 2006).

1.5.4 WHO susceptibility bioassay

According to WHO (1998, 2013c), the purpose of the susceptibility test is to detect the presence of resistant individuals in an insect population as soon as possible. The WHO insecticide susceptibility bioassay is a simple, direct response-to-exposure test. Mosquitoes are exposed to known concentrations of an insecticide for a fixed period at the end of which the number of fatalities was recorded. In its present form, the test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes. The test is used as a field, and laboratory surveillance tool with the limitation that it gives little information on the underlying mechanism(s) conferring resistance were detected (WHO, 2013c).

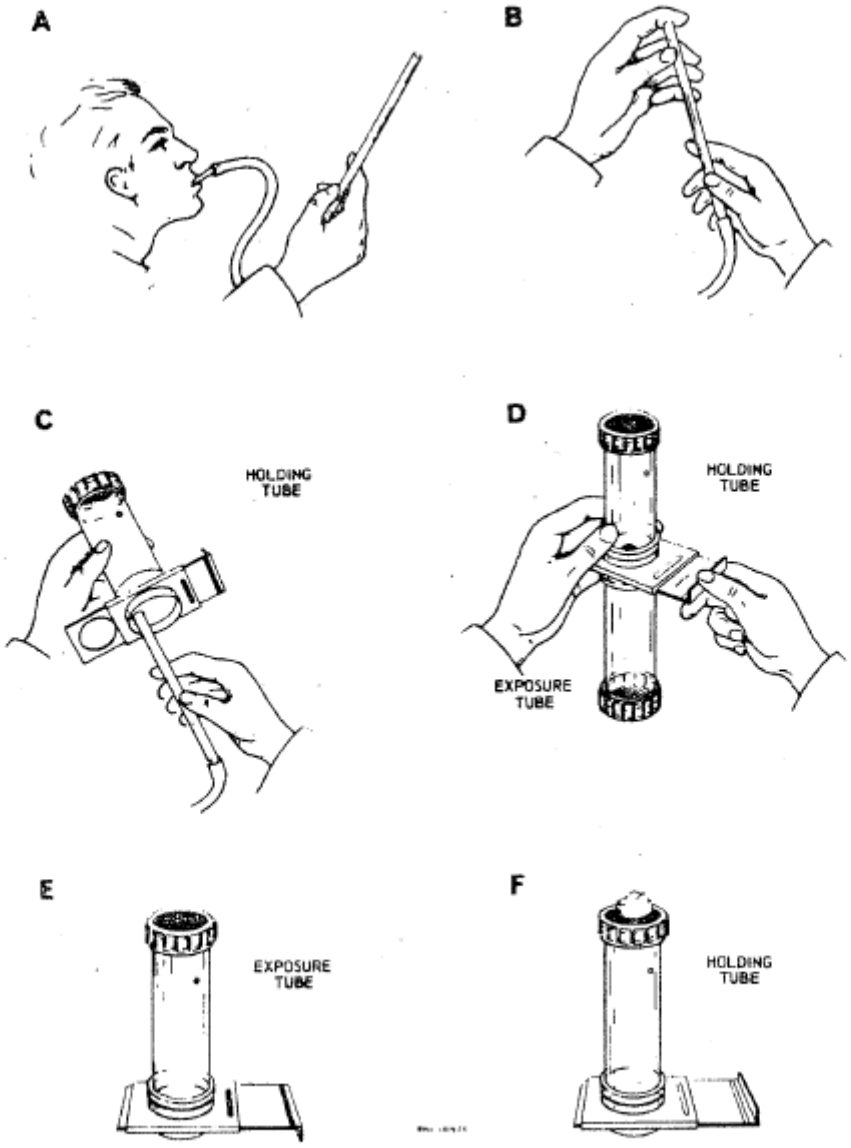


Figure 1.4 Method for determining the susceptibility or resistance of adult mosquitoes to organochlorine; organophosphate; carbamate and pyrethroid insecticides: (a) aspirator was used to collect adult mosquitoes from the cage; (b) the opening of the aspirator was blocked with a finger to avoid the escape of the mosquitoes; (c) aspirator was used to introduce adult mosquitoes into holding tube that contain untreated papers; (d) mosquitoes were gently blown into the exposure tube containing the insecticide impregnated papers; (e) mosquitoes were exposed to insecticide impregnated papers for 1h; (f) mosquitoes were transferred into holding tube and supplied with 10% sugar solution on a cotton bud. The mortality was recorded after 24 h, WHO, 1998.

1.5.5 Genetic and molecular basis of Insecticides resistance

Molecular and biochemical techniques can be used to reliably verify bioassay results and can provide valuable information on species and molecular form identities of the malaria vector. Valuable information on resistance allele frequencies and the operational mode of insecticide

resistance can be obtained as well. Polymerase chain reaction (PCR) assays were developed for the identification of species and molecular forms of the malaria vector and detection of kdr point mutations for high throughput real-time PCR (Santolamazza *et al.,* 2008; Martinez-Torres *et al.,*1998, Bass *et al.,* 2007). Initially the two kdr substitutions were referred to as kdr 'West African' (leucine-phenylalanine substitution L1014F) and kdr 'East African' (leucine-serine L1014S substitution) but recently the presence of both mutations has been confirmed throughout Africa and demonstrates the spread of the two mechanisms (Pinto *et al.,* 2006; Bass *et al.,* 2007; Badolo *et al.*, 2012; Namountougou *et al.,* 2013). The situation is complicated by the common co-occurrence of kdr and metabolic resistance (WHO, 2012). Metabolic resistance is the overexpression of enzymes that are capable of detoxifying insecticides and are found within three large enzyme families; the esterases, cytochrome-dependent P450 monooxygenases, and glutathione transferases (Matowo *et al.,* 2014b). Microarray-based molecular techniques have identified specific P450 genes that were found repeatedly overexpressed in pyrethroid resistant *An. gambiae* (Ranson *et al.,* 2011).

Over the past several years, molecular basis of metabolic resistance, involving the activities of the major detoxification enzymes has also been elucidated. Most, but not all of these metabolic based resistance mechanism involve vectors displaying increased levels or activities of detoxifying enzymes compared to their susceptible counterpart. Gene amplification and transcriptional up-regulation underlies the major molecular mechanisms of insecticide resistance resulting in both qualitative and quantitative changes in many detoxification enzymes (Hemingway *et al*., 2004). With the advent of next generation sequencing technology, several trans-acting regulatory elements upstream of many of the cloned detoxification enzymes and other candidate regulatory genes mediating the activities of several detoxification genes have been identified (Cui *et al*., 2011; Chung *et al*., 2012 Saavedra-Rodriguez *et al.,* 2012; Jaramillo-Gutierrez *et al*., 2010).

1.5.6 Role of Glutathione in insecticide Resistance

Glutathione (GSH) is a tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) that is essential to a number of cellular processes. GSH is widely distributed among all living organisms and is associated with diverse functions that include detoxification pathways, especially the Glutathione S-transferases (GSTs), antioxidant defence, maintenance of thiol status, and modulation of cell proliferation. In addition to serving as substrate for glutathione S-transferase which detoxify potentially dangerous electrophiles, GSH also provides reducing equivalents to several enzymes including ribonucleotide reductase, 3'-phosphoadesonosyl'-phosphosulfate reductase and arsenate reductase (Russell *et al.*, 1990).The glutathione molecule contributes to cysteine transport, detoxification of xenobiotics, and regulation of intracellular redox environment. It also functions as a crucial intracellular antioxidant (Meister, *et al.,* 1983; Sies, 1999; Copley and Dhillon, 2002).

Glutathione occurs in two free forms: reduced (GSH) and as glutathione disulphide or oxidized (GSSG). Also, it exists bound to thiol groups of proteins. The γ-glutamyl linkage and the presence of sulfhydryl group in GSH allow it to participate in some physiological activities. Glutathione concentrations occur in the millimolar range in cells, and the highest values have been found in hepatocytes, leukocytes, eye lens cells, and erythrocytes (Pastore, *et al.,* 2003). Under normal condition, GSH is present mostly in the reduced form but converted to the oxidized form during oxidative stress. The oxidized form can be converted back to the reduced form through the action of the enzyme glutathione reductase. Thus, the ratio of the reduced to oxidized form of GSH indicate the redox state of the cell. Under normal physiological conditions, the ratio of GSH to GSSG levels remain above 99%, but the ratio can change markedly during oxidative stress (Lu, 1999). Aerobic organisms during metabolic processes produce reactive oxygen species as by-products. Most of these reactive species are toxic to the organism and have to be eliminated from the system. To do this, the organisms synthesize intracellular thiols such as glutathione (GSH), homoglutathione, γ-

glutamyl-cysteine (γ-Glu-CYS), γ-glutamyl-cystenylserine and mycothiol (Carnegie, 1963; Newton and Javar, 1985; Klapheck *et al.,* 1992; and Newton *et al.,* 1996).

Cell stress can significantly increase reactive oxygen species (ROS) levels (Morrell, 2008; Sato *et al.,* 2014). Sato *et al.,* 2014, indicated that ROS can modify other oxygen species, proteins, or lipids, a situation often termed oxidative stress because they are highly reactive in nature. In this regard, maintaining healthy cellular ROS levels is vital to the proper physiologic function of numerous cell types in the body. Reduced GSH, the most abundant non-protein thiol antioxidant in cells, is essential for protection against oxidative injury (Valko *et al.,* 2007; Sato *et al.,* 2014). γ- Glutamylcysteine synthetase (γ-GCS) is the enzyme catalysing the first and rate-limiting step in the denovo GSH synthesis (Meister, 1983). The response of a cell to a stress often involves changes in GSH content, which may first be consumed in reactions that protect the cell by removing the deleterious compound and then restored to levels which often exceed those found before exposure to the stressor. Previous studies (Forgash, 1951; Hubatsch *et al.,* 2002; Dickinson *et al.,* 2004; Hashmat *et al.,* 2011; Khan *et al.,* 2012) have indicated that the response of a cell to stress often involves changes in GSH content, which may first be consumed in reactions that protect the cell leading to the formation of GSSG. According to Chen *et al.,* (2004), the level of total and reduced glutathione may increase, reduce or may not change significantly under conditions of oxidative stress. However, levels of GSSG and the ratio between oxidized and reduced forms of glutathione is usually used as the more accurate indicator of the redox state of a cell, particularly in situations where no apparent and significant induction of the synthesis of glutathione occurred (Chen *et al.,* 2004; Araujo *et al.,* 2008).

Observation from previous studies Lipke and Chalkley, 1962; Oeriu and Tigheciu, 1964 and Imam, 2013 have reported increased or higher GSSG levels in resistant mosquitoes sampled from sites with higher levels of environmental chemicals. Similarly, Araujo *et al.,* (2008);

Stephensen *et al.,* (2002) have established increase in oxidative stress induced by xenobiotic overload as a source of generation and accumulation of GSSG, leading to lower GSH/GSSG ratio in various organisms. Conjugation with GSH is a frequent, although not universal, aspect of both xenobiotic and normal physiological metabolism, as mentioned above, and has been thoroughly reviewed (Strange *et al.,* 2000). When glutathione conjugates are formed with small molecules they are then excreted from cells (Akerboom and Sies, 1989), which is generally considered an important detoxification mechanism, including the removal of electrophiles (Milne *et al.,* 2004). Glutathione peroxidase uses GSH as a cofactor to remove peroxides from the cell, leading to the formation of glutathione disulfide, GSSG. GSH must then be replaced through either enzymatic reduction of GSSG by glutathione reductase or de novo synthesis. Enzymatic synthesis is primarily controlled at the level of transcription of two genes, *GCLC* and *GCLM*. The regulation of these genes is predominantly mediated by the electrophile response element or EpREs. The GSH biosynthetic genes; Glutamate-Cysteine Ligase, Catalytic Subunit (*GCLC*) and γ-glutamylcyteine ligase modifier subunit (*GCLM*) are regulated by nuclear factor-like 2 (*Nrf*2) that protects the cell from oxidative stress. The GCL holoenzyme is a heterodimer composed of a catalytic subunit (*GCLC*) and a modifier subunit (*GCLM*). This official nomenclature has recently been adopted due to different names for this enzyme in the literature. While *GCLC* is itself able to synthesize γ- glutamylcysteine, interaction with the modifier (*GCLM*) subunit improves its catalytic properties by lowering the *Km* for the substrate (glutamate) and modulating the negative feedback inhibition (*Ki)* by GSH. Thus, it has been proposed that under physiological conditions, *GCLC* would not function properly without the interaction with *GCLM* (Huang, *et al.,* 1993).

GSH is essential for protection against oxidative injury, through transcription regulation. Thus, the regulation of *GCLC* and *GCLM* expression and activity is critical for GSH homeostasis. Nuclear factor erythroid 2 – related factor 2 *(Nrf*2) is a key transcription factor that plays a central role in regulating the expression of antioxidant genes (*GCLC, GCLM*)

(Kalyanaraman, 2013; Sato *et al.,* 2014). *Nrf2* is retained in the cytoplasm as an inactive complex with its cytosolic repressor, Kelch-like ECH associated protein-1 (*Keap 1*). The movement of *Nrf2* from the cytoplasm to nucleus must be preceded by its dissociation from *Keap 1* before it could bind to the DNA and activate cytoprotective genes. Studies have shown that *Nrf2/Keap1* pathway in higher mammals (Irfan and Biswas, 2009) and its ortholog *CnCC/dKeap1* in *Drosophila melanogaster* (Misra *et al.,* 2013) is involved in the expression of detoxification genes towards maintenance of intracellular GSH levels and redox homeostasis. However, it is still not known whether the orthologs of *CncC* in *An. gambiae* are involved in the maintenance of intracellular GSH levels and redox homeostasis. Since GSH is a substrate for the glutathione S-transferase system, the tripeptide may become rate-limiting when organisms are exposed to large amount of a xenobiotic. Therefore, one can assume that a lower level of GSH in particular stages of the insect would decrease the protection to poisoning afforded by a functional GSH-transferase system (Saleh *et al.,* 1978).

Previous work of Hazelton and Lang(1978), indicated marked life-span changes in GSH content with lower GSH concentration reported in the adult mosquito (*Aedes aegypti*). Possible metabolic mechanisms for this aging-specific decrease in glutathione status include GSH oxidation, utilization, and degradation. However, the work of Hazelton and Lang in 1979 and 1980 indicated that these do not account for the GSH decrease, rather the only other major possibility is GSH synthesis (Hazelton and Lang, 1983). Therefore, because of its involvement, along with glutathione S-transferases in the metabolism of a number of insecticides (Yang, 1976; Pastore *et al.,* 2003), monitoring the intracellular levels and distribution of the free forms (reduced and oxidized glutathione) is an important aspect of insect biochemistry that would help in understanding how GSH homeostasis could be affected under different conditions.

1.5.7 Regulation of gene expression

The fundamentals of the control of gene expression is attain at the transcriptional level (Bhattacharjee *et al*., 2013). This level of regulation integrates the contribution of multiple types of *cis* -acting genomic element, which are important molecular switches involved in the transcriptional regulation of a dynamic network of gene activities controlling various biological processes, including abiotic stress responses, hormone responses and developmental processes (Yamaguchi-Shinozaki and Shinozaki, 2005; Symmons and Spitz, 2013). Transcription of a gene can be regulated within a complex genomic context in which enhancers, promoters, and insulators are closely connected both along the one-dimensional linear chromosome and within the three-dimensional nuclear chromatin environment (Stees *et al*., 2012; Atkinson and Halfon, 2014; Hernandez-Garcia & Finer, 2014).

Promoter analysis is an essential step on the way to identify regulatory networks. A prerequisite for successful promoter analysis is the prediction of potential transcription

Factor binding sites (TFBS) (Cartharius, 2005, Miglani and Gakhar, 2013). Recognition of transcription factor binding sites (TFBSs) improves insights into the genes regulated by a transcription factor (TF) (Talebzadeh and Zare-Mirakabad, 2014). These target genes combined with their expression data can be used to elucidate transcriptional regulatory networks and transcription regulation mechanisms (Ernst *et al*., 2010; Won and Wang, 2010).The promoter region contains *cis*-acting elements, which are specific binding sites for proteins involved in the initiation and regulation of gene transcription (Qiu, 2003; Amit *et al.,* 2011). This transcription is controlled primarily by transcription factors (TFs) which recognize and bind to specific short DNA sequence motifs (Talebzadeh and Zare-Mirakabad*,* 2014).

Quantification of mRNA levels requires reliable methods to investigate the gene expression. There are three RT-PCR based methods for relative quantification of mRNA: semi-quantitative, competitive and real-time RT-PCR (Alizadeh *et al.,* 2011). Breljak *et al.,* 2005,

compared these methods and showed that all of them gave reliable and comparable results. Thus advocated that end-point RT-PCR such as semi-quantitative RT-PCR despite rapid advances made in the area of real-time RT-PCR may still remain useful techniques for relative mRNA quantification.

1.5.7.1 Molecular mechanisms of Nrf2 functions in the regulation of genes

Nuclear factor (erythroid-derived 2)-like 2 (*Nrf2 or NFE2L2*) is a transcription factor coded for by the *NFE2L2* gene in humans. *Nrf2* induces the expression of several antioxidant enzymes producing genes in response to oxidative stress (Kasper *et al.,* 2009).

Nrf2 targets genes involved in glutathione synthesis, elimination of reactive oxygen species (ROS), xenobiotic metabolism and drug transport (Taguchi, Motohashi and Yamamoto, 2011). Kelch-like ECH-associated protein 1 *(Keap1*) regulates the activity of *Nrf2* (Taguchi *et al.,* 2011).

1.5.7.2 Keap1 / Nrf2 signalling pathway

ARE-mediated response to oxidative stress pathway is conserved from flies to humans. In unstressed conditions, *Nrf2* (Nuclear factor erythroid 2-related Factor 2) in mammals, and *CnCC* (Cap 'n' collar C) in *Drosophila* are repressed by *Keap 1* and d*Keap1* (*Drosophila* Kelch-like ECH-Associated Protein 1) (figure 1.5), which also functions as a sensor of oxidants and electrophilic compounds (Nioi *et al*. 2003; Sykiotis & Bohmann 2008). Under normal physiological conditions, *Nrf2* is retained in the cytoplasm by the actin-binding protein *Keap1*, which also functions as an E3 ubiquitin ligase to promote *Nrf2* degradation by the 26S proteasome. Activation of this pathway through oxidative stress disrupts the *Nrf2–Keap1* interaction, allowing *Nrf2* to translocate to the nucleus, where it can heterodimerize with the small Maf (muscle aponeurosis fibromatosis) proteins and bind to antioxidant response elements (AREs) in the promoter region of the gene (Atia and Bin Abdullah, 2014). *Nrf2*,

Maf, and *Keap1* are all conserved in *D. melanogaster* and appear to exert the same regulatory interactions as described in vertebrates (Si and Liu, 2014; Dhanoa *et al*., 2013). Activation of this pathway through electrophilic xenobiotics / oxidative stress is necessary and sufficient for xenobiotic-induced transcription of a wide range of detoxification genes in *Drosophila* species (Misra *et al*., 2011; Deng and Kerpolla, 2013).

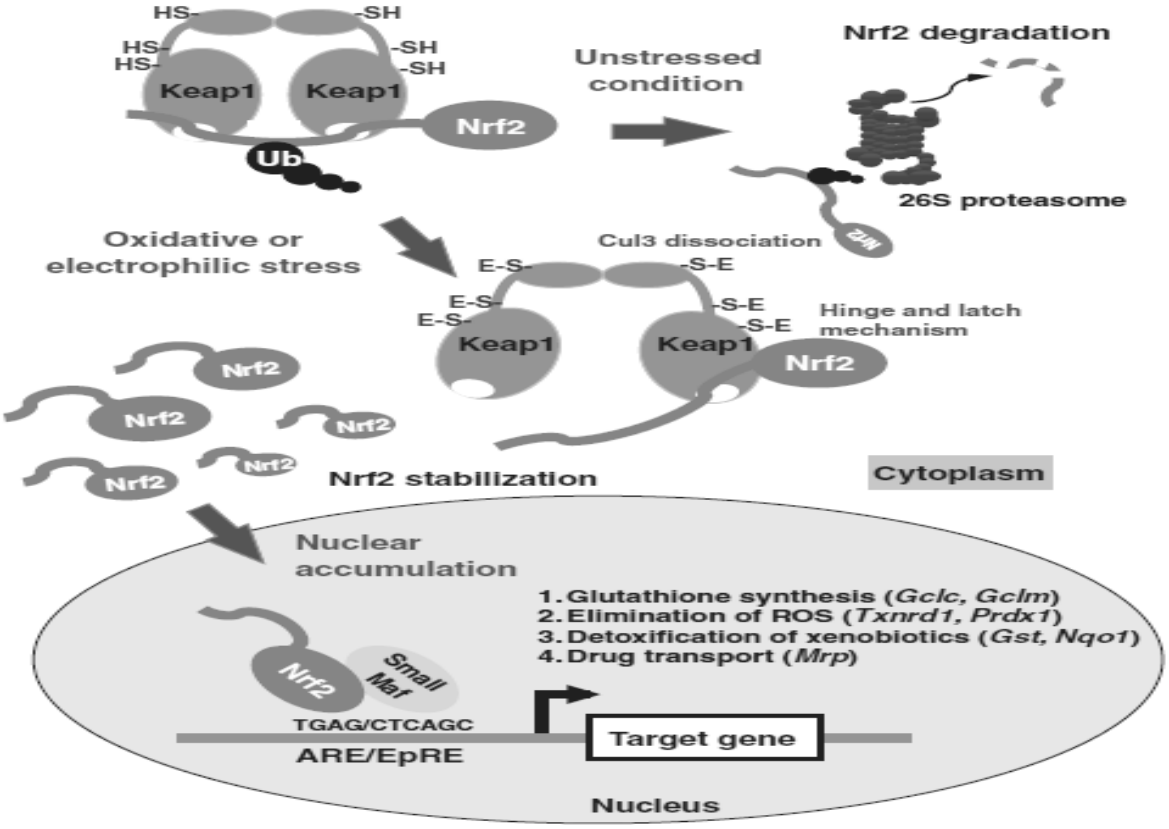


Figure 1.5 The *Keap1-Nrf2* pathway. The transcription factor *Nrf2* plays a central role in inducible expression of many cytoprotective genes in response to oxidative and electrophilic stresses. *Keap1* is a cytoplasmic protein essential for the regulation of *Nrf2* activity. Under unstressed conditions, *Nrf2* is constantly degraded via the ubiquitin–proteasome pathway in a *Keap1*-dependent manner. When oxidative or electrophilic stress inactivates *Keap1, Nrf2* is stabilized and de novo synthesized *Nrf2* translocates into nuclei. *Nrf2* heterodimerizes with small Maf proteins and activates target genes for cytoprotection through antioxidant / electrophile response element (ARE / EpRE). Target genes of *Nrf2* are involved in 1) glutathione synthesis (Glutamate-cysteine ligase, catalytic subunit (Gclc), glutamate-cysteine ligase, modifier subunit (Gclm), 2) elimination of ROS (Thioredoxin reductase 1 (Txnrd1), Peroxiredoxin 1 (Prdx1), 3) detoxification of xenobiotics (NAD(P)H dehydrogenase, quinone 1 (Nqo1), Glutathione S-transferase (GST) gene family) and 4) drug transport (Multidrug resistance-associated protein (Mrp) gene family), E, electrophile (Adapted from Taguchi *et al.,* 2011).

Overexpression of *CnCC* and depletion of *dKeap1* in *Drosophila melanogaster* activates the transcription of many genes including *GCLC, GCLM* and *GST* that protect cells from xenobiotic compounds, whereas *dKeap1* overexpression represses their transcription, indicating that the functions of these protein families in the xenobiotic response are conserved between mammals and *Drosophila* (Luchak *et al., 2007;* Duscher et al., 2014).

1.6 Research Hypothesis

Preliminary study in our laboratory indicated that likely exposure of Anopheline mosquitoes from agricultural fields to pesticides influenced GSH levels and status in the cells.

It is therefore, hypothesised that agricultural practices constitute sources of selection pressure for the emergence of insecticide resistant *Anopheles gambiae.*

1.7 Research question

Evidences have emerged that insects like other higher animals have the ability to regulate the transcription of detoxification genes in response to environmental xenobiotics. Studies have shown that *Nrf2/Keap1* pathways in higher mammals (Irfan and Biswas, 2009; Maitra *et al*., 2010) and its ortholog *CnCC/dKeap1* in *Drosophila melanogaster* (Misra *et al.,* 2011; Misra *et al.,* 2013) are involved in the expression of detoxification genes towards maintenance of intracellular GSH levels and redox homeostasis. However, it is still not known whether the orthologs of *CnCC/dKeap1* in *An. gambiae* are involved in the transcriptional up-regulation of the detoxification genes in the maintenance of intracellular GSH levels and redox homeostasis.

1.8 Research aim and specific objectives

The aim of this PhD study is to examine the molecular mechanism(s) of the role of GSH in selecting for insecticide resistance in *An. gambiae*.

To address the need for understanding the regulatory mechanism involved in the control of GSH in insecticide resistant *An. gambiae*, the study has the following specific objectives:

1. To prospect for, and conduct sampling of *An. gambiae* larva from breeding site located in different ecologies categorized and grouped into two different study zones in Jigawa and Kano States of northern Nigerian. These study zones comprise; Zone A (Intensive agricultural area) Zone B (Domestic and residential area).
2. To assess the susceptibility / resistance status of *An. gambiae* mosquito populations to main insecticides under agricultural and residential settings and the dynamics of species composition in the *An. gambiae* complex.
3. To assess the levels of the three forms of GSH (total, oxidized and reduced) in *An. gambiae* under agricultural and residential settings.
4. To identify the putative transcription factor binding site(s) present in *An. gambiae GCLC* and *GCLM*, especially with respect to *Nrf2 / Keap1 Nrf2 / ARE* axis and establish the functionality of the promoter element(s).
5. To examine the differential expression of *An. gambiae GCLC, GCLM* and *Nrf2* by insecticides by performing semi-quantitative end-point PCR
6. To use observations from 1-5 above to describe the importance of this study to the contemporary malaria management and control initiatives and to make suggestions, based on these observations, on novels strategies and approaches that could tackle the challenges facing the current malaria management programmes.

Chapter Two

Field Study Report

CHAPTER TWO

2.0 Field Study Report

2.1 Introduction

This Ph.D. study is aimed at assessing the role of glutathione and glutathione biosynthetic genes in the response and adaptation of *Anopheles gambiae (An. gambiae) s.l.* to insecticides originating from human-related activities. These particularly includes agriculture (pest control) and personal protection (against mosquitoes), as sources of selection pressure for the development and emergence of insecticide-resistant *An. gambiae* mosquitoes in northern Nigeria. Consequent to this, the identification of Anopheline breeding sites where these human-related activities serve as sources for environmental xenobiotics in the north of Nigeria was the first primary objective of this research work. Thus, field studies were conducted in towns and villages located in Jigawa and Kano states.

The field work comprises, prospecting for and identifying active breeding sites within the areas where human-related activities are taking place. Secondly, morphological identification of mosquito larvae with taxonomic keys, followed thirdly by larval sampling and rearing. Finally, during the larval sampling, a survey was carried out on each site with the aim of documenting the most commonly and widely used insecticides by conducting an interview with some farmers. Ecological parameters such as breeding site type, surrounding area type, Insecticide-treated nets (ITN) coverage within the sampling sites and coordinates of the sampling sites were also estimated. Similar studies and analyses were carried out in the residential settings. Kano and Jigawa states were chosen due to their interrelations particularly in the education, commercial and agricultural activities. They also share similar geographical settings. More than 60% of Nigerian population is in the north and in the northern part of Nigeria the people of Kano and Jigawa states constitute more than 30% of the northern Nigerian population (NPC, 2006b). Furthermore, the north of the country has

the highest malaria burden relative to the southern region. In addition, more than 65% of arable land in Nigeria is located in the north (NPC, 2006a). The two states have the largest irrigation projects in Nigeria. Thus, intensive agriculture is the major economic activities in this region. Alongside the advantages of these dams to the development of farming and provision of food comes the disadvantage in health implication by providing suitable breeding sites for vectors of diseases. The use and discharge of refined petroleum and other hydrocarbon products under different trade names and chemical combinations in the form of Indoor residual sprays (IRS); long lasting insecticide treated bed nets (LLINs) is higher in the north because of the larger population. Therefore, contamination of the mosquito breeding sites through these human-related activities is expected to be higher in this region compared to the south. In general, insecticide usage for personal protection and for controlling crop pests in agriculture and the presence of anthropogenic pollutants in urban, agricultural or industrial areas has often been suggested as sources of selective pressures favouring insecticides resistance (Balkew *et al*., 2010; Marcombe *et al.,* 2011).

Kano State (12^0 00' N, 8^0 31' E) is located within the Sudan savannah zone of West Africa about 840 kilometres from the edge of the Sahara desert (Imam and Oyeyi, 2008, Ibrahim *et al.,* 2014). Kano sees on average about 690 mm (27.2 in) of precipitation per year while Jigawa sees 600-1000 mm per year. The bulk of these falls from July through September (NIMET, 2012). Kano and Jigawa are typically scorching throughout the year though from December through February; they are noticeably cooler. Temperatures at night are cold during the months of December, January and February, with average low temperatures ranging from 11° - 14°C. Kano state borders the states of Katsina to the west, Jigawa to the East, Bauchi to the South-east Kaduna to the South-west and Niger Republic to the North. It has a population of about 13 million people, second only to Lagos state in Southwest Nigeria, and a land mass of approximately 18,684 square km. In addition, Kano city is located 481m above sea level (John, 2007). There are usually four seasons within the state; a dry, cool

season (November-February) marked by cold, dry weather with occasional haze and dust and average low temperature of between 11°C to10°C.The hot- dry season (March- May) is marked by a very hot dry weather with temperature reaching up to 44°C while the wet-warm season known in local dialect as 'Damuna' (June-October) is the proper rainy season; and lastly a dry warm season (Mid-October to mid-November) marked by high humidity and temperature. The state has the largest irrigation projects in Nigeria, with six irrigation projects and more than twenty earth dams. Alongside the advantages of these dams to the development of Agriculture and provision of food comes the disadvantage in health implication of providing suitable breeding sites for vectors of diseases. Rice paddies, in particular, have been established and increase the risk of malaria by providing suitable sites for vector development. The locality of Bichi has a broad expanse of irrigable lands where rice is the major crop grown, although other crops such as green vegetables and tomatoes are equally grown for subsistence.

Most parts of Jigawa state (12^0 00' N, 9^0 45' E) falls within the Sudan savannah vegetation region, with the occurrence of Guinea Savannah in some areas of the state especially the southern zone. Jigawa state is bordered by the state of Kano and Katsina to the west Bauchi to the east and Yobe to the northeast. It shares an international boundary with Zinder region of the Republic of Niger to the north. Jigawa has an estimated population of 5 million people on an estimated land area of 23,287 Km2 (http://en.wikipedia.org) and is the second to Kano in terms of irrigation projects. The state has one of the highest FADAMA wetlands in the country (3,437.79 square kilometres). The climate is characterized by two main season; the wet rainy season (May-September) and the dry Harmattan season (November-April) (SEEDS, 2009). Temperature ranges between 42^0C in the dry cold season, and the average annual rainfall are less than 600mm (NIMET, 2012). Irrigation farming supports the cultivation of many crops for most parts of the year, especially during the long dry season. Agriculture is an important economic activity, with subventions from the Federal

government that constitute the primary income (SEEDS, 2009). For the purpose of this study, the sites visited and sampled during the field exercise are presented in table 2.1

Table2.1 List and description of the field study sites

Study site	Nature and characteristic of study site
AA1	Areas of intensive agriculture involving use of pesticides
AA2	Areas of intensive agriculture involving use of pesticides
AR	Residential areas
BA	Areas of intensive agriculture involving use of pesticides
BR	Residential areas

2.2 Experimental approach

2.2.1Study sites and Insecticide Usage

The study localities; Bichi a town located approximately 40.5 km west of Kano city, and Auyo situated northeast of Dutse, the capital of Jigawa State (Figure 2.1) are characterized by high insecticides usage for agricultural or personal protection. (1) Bichi residential ($12^0$13' N, $8^0$15' E), an urban area with high population and a major commercial centre outside Kano metropolis, (2) Bichi agricultural ($12^0$7' N, $8^0$14' E), an area of intensive agriculture throughout the year, (3) Auyo residential ($12^0$20' N, $9^0$56' E), a market town for the vegetables and cereals produced in the irrigation area within the locality, (4) Auyo agricultural 1 ($12^0$18' N,$9^0$56 E') and (5) Auyo agricultural 2 ($12^0$21' N, $9^0$59' E) , localities with intensive farming known for their history of irrigation activities in which rice and other vegetables are produced. Both in Bichi and Auyo agricultural sites, farmers, use a large number of pesticides to protect their crops. These pesticides include; organophosphates, organochlorine, pyrethroids and carbamates under different trade names. Reliable data on

the impacts of agricultural pesticides in the wetland environments of northern Nigeria is lacking (Kimmage and Adams, 1990).

During the mosquitoes sampling, a survey was carried out on each site with the aim of documenting the most commonly and widely used insecticides. Data on pesticide usage for personal protection and agricultural practices were collected through personal interview. Farmers and/or residents were asked the following questions; (1) insecticides used (trade names, active ingredients/chemical composition, (2) cultivated crops (3) ITNs usage, (4) Mosquito Coils usage. Ecological parameters such as breeding site type, surrounding area type, within the sampling sites and coordinates of the sampling sites, were also estimated (Table 2.6).

2.2.2 Larval prospecting

Search for potential larval habitats was conducted during the rainy seasons of 2013 and 2014 (June- September). A search for the presence of mosquito larvae (Anopheline) was carried out by locating stagnant water bodies. Larval prospecting was done in each of the study zones at least once every two weeks between June and September.

2.2.3 Morphological identification of mosquito larvae and adults with taxonomic keys

After identifying active breeding habitats in all the zones visited, identification of mosquito larval species thriving in the habitats was carried out. It was carried out on the basis of morphology and behaviour following the Gillies and Coetzee (1987) morphological identification keys (morphology and horizontal position on the water surface of the *An. gambiae* larvae). The resulting adults were also identified as *An. gambiae s.l.* according to the morphological identification keys (Gillies and De Meillon, 1968, Gillies and Coetzee, 1987). Previous studies (Merrit *et al.,* 1992; Clements, 2000) have described Anopheles mosquito larvae to possess a well- developed head with mouth brushes used for feeding, a large thorax and a segmented abdomen. Anopheline larvae lack any legs. In contrast to other mosquito

species (Culicines), Anopheles larvae position themselves so that their body is parallel to the surface of the water. Unlike other mosquito species, they possess no respiratory siphon and breathe through spiracles located on the 8th abdominal segment and, therefore, must come to the surface frequently (CDC, 2012).The larvae spend most of their time feeding on algae, bacteria, and other microorganisms in the surface microlayer. They dive below the surface only whenever disturbed. Larvae swim either by jerky movements of the entire body or through propulsion with the mouth brushes. In contrast, Culex mosquitoes were found to hang themselves longitudinally with the head hanging downward below the air-water interface and are less active even when disturbed (Paaijmans, 2008).

2.2.4 Mosquito larval collection, processing, and rearing

Larval collections were carried out at the peak of the rainy season in June-September 2013 and 2014. Immature stages of *An. gambiae s.l.* were collected from the field as larvae and pupae using scoops from the five sampling sites, two from Bichi and three from Auyo in Kano and Jigawa States respectively. Larval collections in both Bichi and Auyo were conducted in residential (non-polluted) and agricultural (cultivated) sites. Anopheline larvae at each of the chosen site were collected from various natural breeding sites that include ground pools, tire tracks, and animal hoof prints. After careful larval prospection, water was scooped using a brass scoop and poured into small transparent plastic bowls. The bowls were scrutinized for the presence of unwanted organisms or predators; if any were found, a pipette was used to remove them. The coordinates of the sampling sites were taken using hand-held Global Positioning System (GPS model: Garmin eTrex 10) (John *et al.,* 2014).

Larvae were transported to the insectary at Bayero University Kano. In the insectary, any 4th instar larvae and pupae present were collected in 1.5ml Eppendorf and immediately stored in -80^{0}C freezers. The remaining larvae were maintained under standard insectary condition

(25-28 °C and ~70-80% humidity, with a 12 h day/night cycle) (Das, *et al.,* 2007) supplied with baker's yeast daily. The larvae were reared in light plastic containers covered with fine mesh mosquito nets and reared in the same water from which they were sampled to maintain the impact of the environmental xenobiotics present in the breeding habitat. The 4th instar larvae and pupae were transferred alive directly from the breeding container into 1.5ml Eppendorf tubes as they emerged. Some of the Eppendorf tubes contained RNA later (RLT) buffer (Qiagen) to protect the integrity of the genetic materials (genomic DNA and total RNA).The adults that emerged were transferred into cages from the breeding containers. Upon emergence, mosquitoes were morphologically identified as *An. gambiae s.l.* According to morphological identification keys (Gillies and De Meillon, 1968 and Gillies and Coetzee 1987).These resulting adults *An. gambiae* were maintained on 10% sugar solution until they were used for insecticide susceptibility tests.

An. gambiae s.l. larvae were collected in all the breeding sites visited in all the study localities. The sampled larvae were transported in their breeding water to the insectary. Some of the larvae were sorted out immediately and after a day or two some pupae were sorted out as well. The sorted larvae and pupae were transferred alive directly from the breeding container into 1.5 ml Eppendorf tubes as they emerged for storage. Some of the Eppendorf tubes contained RNA later (RLT) buffer (Qiagen) to protect the integrity of the genetic materials (genomic DNA and total RNA).The adults that emerged were transferred into cages from the breeding containers and fed with 10% sugar solution and randomly mixed for subsequent experiments. The adults after the susceptibility bioassay were also transferred into 1.5 ml Eppendorf tubes accordingly for storage and finally transported to the Abertay University Dundee, United Kingdom all the stored samples (larvae, pupae and adults) for further biochemical and other analyses.

2.2.5 Interviews

Interviews were conducted with the farmers during mosquito sampling in breeding sites located within and around intensive agricultural areas. The local farmers were asked to provide information on the kind of insecticides/pesticides they applied to control agricultural pest and the frequency of application. Thirty-three farmers were interviewed in each zone and their responses provided information (Table 2.3) on types and nature of agrochemicals commonly applied to farmlands located in these study areas. The residential sites were located in urban areas. The urban areas were characterized by a high degree of human activities and a high ITN coverage.

2.2.6 Physicochemical characteristics of breeding sites sampled in Kano and Jigawa

Measurements of physicochemical characteristics of breeding sites were carried out by the ministry of water resources irrigation engineering department, soil and water laboratory Kano. Parameters measured were pH, nitrates, nitrites, phosphates, Sulphates, organic Carbon all expressed in mg/l and temperature in ^{o}C. Tables 2.4 and 2.5 shows the physicochemical characteristics of *An. gambiae* breeding sites located in study zones A and B. Various ecological parameters comprising breeding site type, surrounding area type, ITN coverage around the breeding sites and use of pesticides in agriculture were estimated from each sampling site (Table 2.6).

2.3 Results

2.3.1 Responses from farmer's interview

In order to identify the most commonly and widely used insecticides by farmers which may have the potentials to select for emergence of insecticides resistance in Anopheline mosquitoes within the study areas, data on insecticide usage for agricultural practices were collected through personal interview. Results of the interview are shown in table 2.2.

Table 2.2 Insecticide usage in agricultural settings by farmers

Study site	Trade name (concentration)	Active ingredient	Class of insecticide usage frequency (%)	Cultivated crops
Bichi	Best Cypermethrin (100 g/l)	Cypermethrin	Pyrethroid	Tomato*,Rice* Pepper*,onion* Watermelon,
	Lava Force , Warrior (15g/l; 45 g/l)	Lambda cyhalothrin	(36.36)	
	DD Force, DDVP	Dichlovos		Pepper*,onion* tomato* ,Rice*
	Cygon, Cymbush (250 g/l; 400 g/l)	Dimethoate	Organophosphate	
	Term kill (600 g/l)	Chloropyrifos	(27.27)	
	2.4. D. Many	Phenoxy Acid	Phenoxy (9.09)	
	Bastion (100g/kg)	Carbofuran	Carbamate (12.12)	Cabbage, lettuce, onion* tomato* Rice*
	Thiodan, Thionex (250 g/l; 350 g/)	Endosulfan	Organochlorine (15.15)	Tomato* spinach
Auyo	DD Force , DDVP	Dichlovos	Organophosphate	Pepper*,Rice*,onion*, tomato*, cabbage
	Pyrifos, Pyrate(60 g/l)	Chlorpyrifos	(30.30)	
	2.4. D. Many	Phenoxy Acid	Phenoxy (9.09)	
	Sevin, Vet-Tek	Carbaryl	Carbamate (12.12)	Pepper*, lettuce*, Rice*
	Cypercal (12 g/l; 50 g/l; 100 g/l)	Cypermethrin	Pyrethroid	Tomato*,Pepper*, carrot, Rice*
	Cylent ear tag	Cyfluthrin	(34.85)	
	Regent (50 g/l)	Fipronil	Organochlorine (13.64)	Tomato*,cabbage

*: main cultivated crop

Table 2.2 summarizes data collected from the survey conducted in the agricultural settings of Bichi and Auyo. In these sites pyrethroids (cypermethrin, lambda-cyhalothrin and cyfluthrin) and organophosphates (dichlovos, dimethoate and chloropyrifos) were commonly used for

crop protection. In addition, organochlorine (endosulfan and fipronil) and carbamates (carbofuran and carbaryl) were also used. On the other hand, interview in Bichi and Auyo residential settings revealed Indoor Residual Sprays (IRS), Piya Piya sprays and Coils containing pyrethroid insecticides with cypermethrin, lambda-cyhalothrin and cyfluthrin as common active ingredients were mainly used for personal protection with organochlorine as well being used unofficially in local production of Piya Piya for personal protection.

2.3.2 Physicochemical characteristics of An. gambiae breeding sites

In order to assess the physicochemical characteristics of *An. gambiae s.l.* breeding sites, the levels of the physicochemical environmental factors; pH, temperature, nitrates, nitrites, phosphates, sulphates and carbon contents were determined (Tables 2.3 and 2.4), on weekly basis over the period of four weeks in the selected breeding sites and their mean distribution across the two study zones (A and B).

Table 2.3 Physicochemical characteristics of *An. gambiae s.l.* breeding sites located in study zone A

	Sampling Sites			
Environmental factor	Site1 (AA1)	Site 2 (AA2)	Site 3 (BA)	Combined sites
pH	6.70 ± 0.12[a]	7.10 ± 0.08	7.03 ± 0.09	6.94 ±0.10
Temperature (°C)	33.50 ± 0.06	33.00 ± 0.05	32.00 ± 0.06	32.83 ±0.06
Nitrate (mg/l)	6.00 ± 0.00	8.17 ± 0.06	8.90 ± 0.02	7.69 ±0.03
Nitrite (mg/l)	6.13 ± 0.12	5.73 ± 0.15	7.47 ± 0.06	6.44 ±0.11
Phosphate (mg/l)	5.37 ± 0.15	7.57 ± 0.12	8.23 ± 0.06	7.06 ±0.11
Sulphate (mg/l)	4.65 ± 0.07	5.27 ± 0.12	6.26 ± 0.12	5.39 ±0.10
Carbon content (mg/l)	1.59 ± 0.06	2.00 ± 0.00	2.85 ± 0.07	2.15 ±0.04

[a]Data presented as mean ± SD; n=3

Table 2.4 Physicochemical characteristics of *An. gambiae s.l.* breeding sites located in study zone B

	Sampling sites		
Environmental factor	Site1 (AR)	Site 2 (BR)	Combined sites
pH	6.50 ± 0.13[a]	6.70 ± 0.14	6.60 ±0.14
Temperature (°C)	34.00 ± 0.57	33.00 ± 0.78	33.5 ±0.68
Nitrate (mg/l)	2.37 ± 0.04	2.52 ± 0.04	2.45 ±0.04
Nitrite (mg/l)	1.90 ± 0.10	2.67 ± 0.13	2.29 ±0.12
Phosphate (mg/l)	1.43 ± 0.04	1.46 ± 0.10	1.45 ±0.07
Sulphate (mg/l)	2.32 ± 0.04	2.03 ± 0.08	2.18 ±0.06
Carbon content (mg/l)	2.03 ± 0.08	0.99 ± 0.13	1.51 ±0.11

[a]Data presented as mean ± SD; n=3

The results obtained (Tables 2.3 & 2.4) showed little variation in the levels of the physical environmental factors (pH and temperature) across the sampling sites in the two zones studied (A & B). However, the levels of nitrates, nitrites, phosphates, sulphates and carbon content were higher in sites located in zone A than those in zone B.

2.3.3 Correlations among physicochemical environmental factors

The results of the correlation analysis showed that pH was highly correlated with temperature, nitrate, nitrite, phosphate and sulphate but not with carbon content (Appendix III), thus pH, temperature, nitrate, nitrite, phosphate and sulphate may all be related.

A nested ANOVA was used because there are two factors: zone and site. The site is within the zone and each zone has different sites i.e. the site is nested within the zone. Overall, zones and sites are significantly different.

2.3.3.1 pH

Zone A compared to zone B is significantly different. AA1 is significantly different from BA (p= 0.000) but AA2 is not (p= 0.361). AR is significantly different from BR (p= 0.022) (Appendix IV).

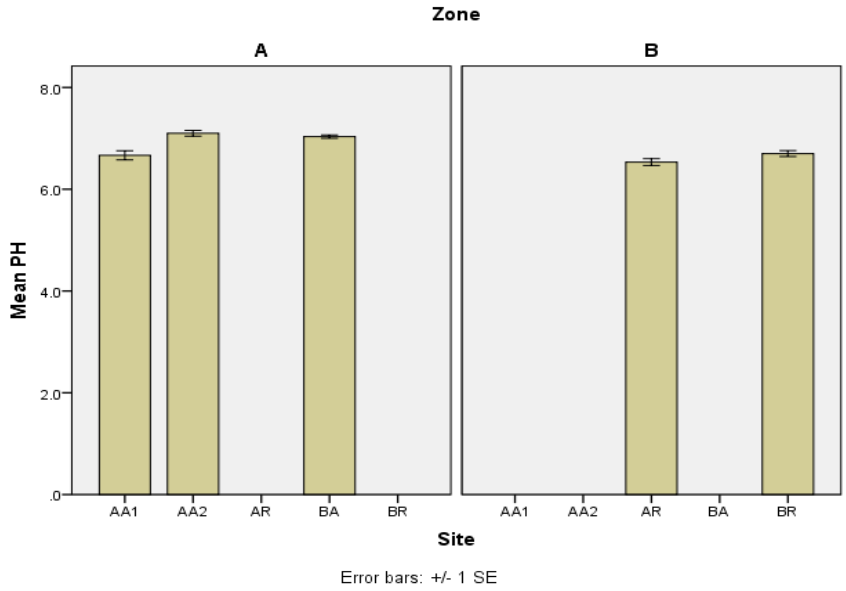


Figure 2.1 Mean pH distribution in *An. gambiae* breeding sites in Nigeria: Zone A, agricultural; zone B, residential.

2.3.3.2 Temperature

All the five sites within the two zones are significantly different statistically (p= 0.000) (appendix V).

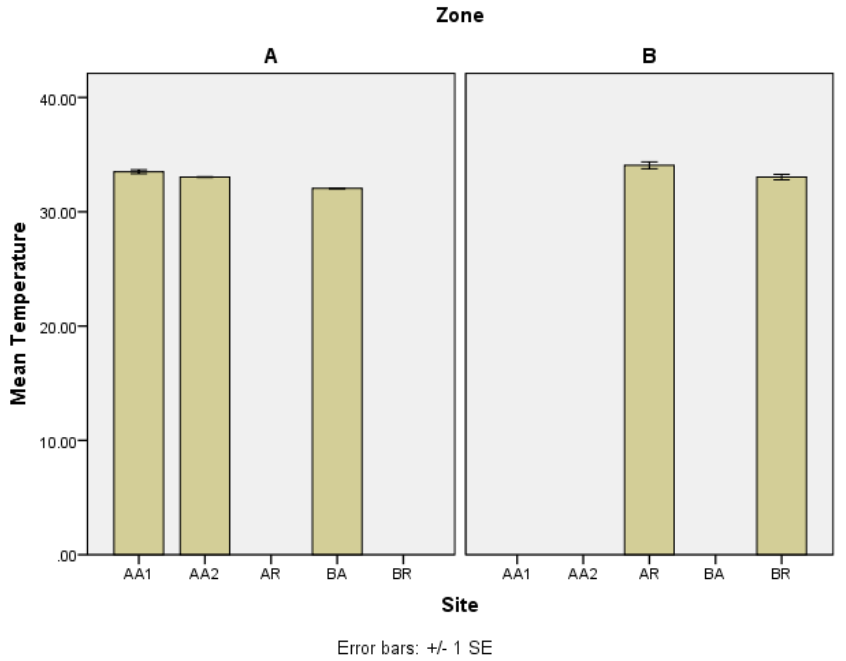


Figure 2.2 Mean temperature distribution in *An. gambiae* breeding sites in Nigeria: Zone A, agricultural; zone B, residential.

2.3.3.3 Nitrate

Overall, zone A is significantly different from zone B (p= 0.000). In zone A, AA1 and AA2 are significantly different from BA (p= 0.000) also in zone B, AR is significantly different from BR (p= 0.000) see Appendix VI. Other factors; Nitrite, Phosphate and Sulphate are related and similar with Nitrate due to high correlation (Appendix III).

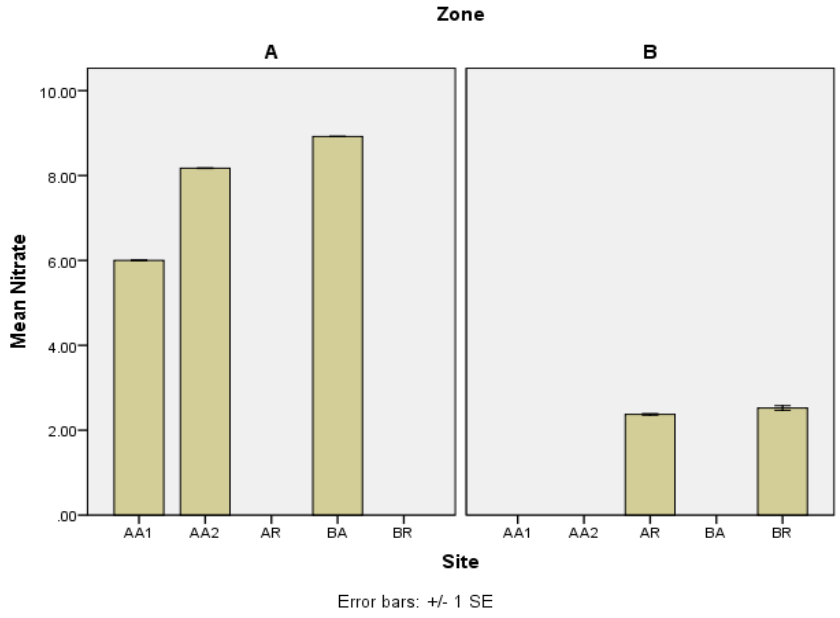


Figure 2.3 Mean Nitrate distribution in *An. gambiae* breeding sites in Nigeria: Zone A, agricultural; zone B, residential.

2.3.4 Ecological characteristics of sampled breeding sites

In order to identify the nature of Anopheles breeding ecologies and human related activities in such breeding sites in Northern Nigeria, ecological parameters and human related activities such as GPS coordinates, breeding site type, breeding site surroundings, ITN coverage and use of pesticides in agriculture and for personal protection were assessed (Table 2.5).

Table 2.5 Ecological characteristics of sampled breeding sites

Area	Site	GPS coordinates	BS type	BS surroundings	ITN coverage	Use of pesticides in agriculture
Agriculture	AA1	12°18' 5142"N 9°56' 9462"E	Rice field Irrigation channels, Hoof prints	Farms	low	Heavy
Agriculture	AA2	12^{0}21' 3879"N 9^{0}59' 8020"E	Rice field Irrigation channels, Hoof prints	Farms	low	Heavy
Agriculture	BA	12^{0}7' 8262"N 8^{0}14' 2894"E	Rice field Irrigation channels, Hoof prints	Farms	low	Heavy
Urban	AR	12^{0}20'1202"N 9^{0}56' 7242"E	Ground pools, Tyre tracts, hoof prints	Residential small farm	High	Low
Urban	BR	12^{0}13' 8782"N 8^{0}15' 9858"E	Ground pools, Tyre tracts, hoof prints	Residential Small farm	High	Low

BST: breeding site type, BS: breeding site, ITN: insecticide treated nets, AA1: Auyo agricultural, AA2: Auyo agricultural 2, AR: Auyo residential, BA: Bichi agricultural, B.R.: Bichi residential

Table 2.5 showed that in agricultural areas the breeding site types were mostly irrigation channels and hoof prints while in urban areas the breeding site types were mostly ground pools, tyre tracts and hoof prints. The surroundings of the breeding sites were large farms for agricultural areas and residential with small farms for urban areas. The agricultural areas were characterized by lower level of ITN coverage and heavy level of pesticides usage. The urban areas on the hand were characterized by high ITN coverage and low level of pesticides usage, suggesting agricultural practices as key driver for the emergence of insecticides resistance.

2.4 Discussion

It was observed from the results of the field work that, in some of the breeding sites visited, Anopheline species were found to breed exclusively alone, while in some other breeding sites there was mixed breeding where Anophelines were found to breed alongside Culicines with Anophelines predominant over the Culicines. It was further observed that, most of these breeding water bodies, where sampling was carried out, appeared relatively clean and not-polluted while others were dirty, muddy and contaminated with various materials. This finding is in contrast to that of Sattler *et al.,* (2005), that observed Anopheles mosquito to prefer relatively clean and less contaminated water bodies for breeding. However, it agrees with the finding of Imam, (2013). Close observation of the breeding sites and surrounding environments indicated that most of the contaminants were generated by human-related activities, and then washed into the mosquito breeding sites. Agriculture is one of the most important economic sectors in northern Nigeria with large areas of intensive agriculture and majority of farmers practicing small scale farming and perhaps with routine usage of pesticides. The residential areas of Auyo and Bichi were characterized by extensive domestic use of insecticides as LLINs, IRS, Piya piya sprays and coils. These activities were considered to be important in contributing to the presence of chemicals species in the mosquito breeding sites. For instance, rain water runoffs from surrounding farmlands in study zone A could bring in high amount of nitrites, nitrates, sulphates and phosphates that are usually present in fertilizers and agro-pesticides, into the mosquito breeding site (Imam and Deeni, 2014). Anopheline larvae were collected mostly from rice paddies, ground pools, tire tracks, and animal hoof prints. Most of the farmers interviewed during this field study confirmed that often they apply fertilizers (nitrate and phosphate-base) in their farms to increase the yield of their crops. The farmers further confirmed that they applied various pesticides such as Cypermethrin, Carbofuran, Cyfluthrin, Dimethoate, and Endosulfan as shown in Table 2.2 to control agricultural pest. Most of the insecticides used in agriculture are of the same

chemical classes and have the same targets and modes of action as those used for vector control (Kumar, 1984; Khambay and Jewess, 2010). Larvae from these environments are subjected to selection pressure leading to multiple insecticides resistance.

Previous studies suggested that the use of insecticides in agriculture contribute to the selection of resistance in mosquitoes. For instance, Boyer *et al.,* (2006) reported that *Aedes aegypti* larvae exposed to the herbicide Atrazine became more tolerant to the organophosphate temephos. David *et al*., (2013) also indicated that the presence of agrochemicals, urban or industrial pollutants and plant compounds in mosquito breeding sites are expected to affect pyrethroid tolerance by modulating mosquito detoxification systems. Thus, threatening the efficacy of vector control programmes (Chouaibou *et al.,* 2008; Yadouleton *et al.,* 2009, 2011).

The presence of a significant amount of *An. gambiae* larvae across the breeding environments (extensive agriculture and residential) during the sampling suggests *An. gambiae* mosquitoes are adapting or have adapted to survive over a wide range and levels of environmental xenobiotics. Previous work has already indicated that Insects including mosquitoes display functional and dynamic adaptation leading to changes in both tolerance and behaviour to environmental xenobiotics (Tauber *et al*., 1986; Imam 2013). Results (Table 2.3) showed that most of these chemicals such as nitrates, nitrites, sulphates, carbon content and phosphates were detected in higher quantities from breeding sites located around the intensive agricultural areas when compared to those around the residential areas (Table 2.4). The nitrates, nitrites, sulphates, carbon content and phosphates could be parts of the chemical insecticides or by products of its degradation are used as indicators of the presence these insecticides (Butcher, 2014). Thus have higher potential to select for resistance to these insecticides used for controlling mosquitoes.

2.5 Conclusion

The current study highlighted that the mosquito populations in these sampling sites displayed functional and dynamic adaptations leading to changes in both tolerance and behaviour to environmental xenobiotics. This study also provides eco-toxicological data supporting the role of the environment in which vectors are found in selecting for the emergence of insecticide resistant Anopheline mosquitoes. Both agricultural and residential settings are likely favouring the emergence of resistance to insecticides commonly used to control vectors (Nkya *et al.,* 2014). Further understanding of how environment moderates the selection and spread of insecticide resistance would help in improving resistance management strategies.

In order to confirm whether the human related activities: agriculture and use of insecticides for personal protection has impact in selection of resistance in mosquitoes, set of experiments were designed: Insecticides susceptibility bioassays and knockdown resistance assays to examine the levels of insecticides resistance in these mosquito populations.

Chapter Three

Susceptibility status of *Anopheles gambiae s.l.* to insecticides

CHAPTER THREE

3.0 Susceptibility status of *Anopheles gambiae s.l.* to Insecticides

3.1 Introduction

Malaria vector control programs in Africa rely heavily on the use of pesticides for insecticide-treated nets (ITNs)/long-lasting insecticide-treated nets (LLINs) and for indoor residual spraying (IRS) (WHO, 2012, Kabula *et al.,* 2014). The use of these approaches is known to contribute to the reduction of malaria transmission and burden (Pluess *et al.,* 2010 Okuma and Moore, 2011, Overgaard, *et al.,* 2012). Malaria remains one of the most critical public health challenges for Africa despite intense national and international efforts (WHO, 2012). According to the Federal Ministry of Health, Nigeria (2005), "Malaria kills more people than HIV/AIDS or any other killer disease. Malaria is endemic throughout Nigeria accounting for 25% of infant mortality". Malaria impedes on economic development not only by causing premature death but also through lost/diminished productivity, enormous medical cost and population growth (Sachs, 2002). *Anopheles gambiae (An. gambiae)* is the principal malaria vector in Africa. In most cases, particularly in highly endemic areas the ability to reduce malaria transmission will be dependent on the vector control before the focus can shift to killing the parasite in infected people.

Two primary forms of vector control; Indoor Residual Spraying (IRS) and the distribution of Long-Lasting Insecticide-Treated Nets (LLINs) have been demonstrated to reduce the number of malaria cases when properly used against insecticide susceptible mosquito populations (WHO, 2012; Wondji *et al.,* 2012). The use of both approaches has substantially increased over the years since 2000 in many malaria endemic countries with increased donor funding to achieve Roll Back Malaria targets aimed at the eradication of malaria

(WHO, 2009; Eisele *et al.,* 2012).The WHO recommends large-scale distribution of insecticide-treated nets (ITNs) to control malaria transmission. Recently, concerted international efforts have been devoted to distribute ITNs and also to increase Indoor Residual Spraying (IRS), which has contributed to a major reduction in disease burden in sub-Saharan Africa (WHO, 2012; Abuelmaali *et al.,* 2013). Further evidence of malaria burden reduction through full coverage of LLINs or coupled with IRS has been reported in some African countries (Overgaard, *et al.,* 2012; Okuma and Moore, 2011). Nigeria is currently scaling up its malaria control programme through a free mass distribution of LLINs and IRS (USAID/PMI/CDC, 2011). In 2008, the overall ownership coverage was 8% and reached 42% in 2010 (USAID/PMI/CDC, 2011). Similarly, the proportion of the vulnerable groups (children and pregnant women) sleeping under ITNs has increased from 6% in 2008 to 29% in 2010 and IRS has been piloted in selected areas in the country (NPC, 2012, Kolade *et al.,* 2013). In 2009 4,137,464 LLINs were freely distributed in Kano state (Zainab, 2013) while in Jigawa state between 2009 and 2015 approximately, five million LLINs were distributed (Rufa'i, 2015).

Four major classes of chemical insecticides (i.e. pyrethroids, organochlorines, organophosphates, and carbamates) are the mainstay of these malaria vector control strategies (Najera and Zaim, 2002; WHO, 2006; Kelly-Hope *et al.,* 2008; Kabula *et al.,* 2014). However, pyrethroids are the only class of insecticide currently recommended for use on ITNs/LLINs or IRS by WHO, due to safety and cost effectiveness (WHO, 2010b). Nevertheless, bendiocarb and dichlorodiphenyltrichloroethane (DDT) are also used in some areas for IRS (WHO, 2008; Ranson *et al.,* 2011). The development and rapid spread of insecticides resistance to major malaria vectors across the African countries has posed a threat to effectiveness of these different mosquito control measures (Etang *et al.,* 2003; Corbel *et al.,* 2004; Ranson *et al.,* 2009; Ranson *et al.,* 2011; Kolade *et al.,* 2013). Massive and extensive use of insecticides in agriculture (Yadouleton *et al.,* 2010) and high ITNs and IRS

coverage, or recurrent space spraying interventions (Balkew *et al.,* 2010; Marcombe *et al.,* 2011; Ranson *et al.,* 2011, Nkya *et al.,* 2013) in public health has resulted in increasing resistance among malaria vectors due to the selection pressure placed on resistance genes. Kolade *et al.,* 2013, indicated the extensive use and abuse of conventional insecticides for agriculture and personal protection has contributed immensely to the development of resistance in Anopheline mosquitoes and other insect pests. Also, lack of available alternative insecticides for vector control has also been an issue (Coleman *et al.,* 2006, Kolade *et al.,* 2013).

Pyrethroid resistance is believed to be caused mainly by high ITNs and IRS coverage, or recurrent space spraying interventions (Balkew *et al.,* 2010; Marcombe *et al.,* 2011; Nkya *et al.,* 2013). However, studies pointed out the possible role of other factors in the selection of inherited resistance mechanisms or the higher tolerance of mosquitoes to pyrethroids. Among them, insecticide use for personal protection and for controlling crop pests in agriculture and the presence of anthropogenic pollutants in urban, agricultural or industrial areas has often been suggested as additional selective pressures favouring pyrethroid resistance. Ranson *et al.,* (2000) highlighted the primary causes of insecticide resistance as alterations in the target sites and increases in the rate of insecticide metabolism. The target sites of all the major classes of insecticides have been established, and resistance-associated mutations have been identified. The pyrethroid insecticides and the organochlorine insecticide DDT, target the voltage-gated sodium channel on the insects' neurone (Davies *et al.,* 2007); organophosphates and carbamate insecticides target the acetylcholinesterase (AChE) (Hemingway and Ranson, 2000; Edi *et al.,* 2014).

On the other hand, DDT although its use has been banned, is still unofficially being used by farmers and also in homes under different trade names and chemical combinations. Awolola *et al.,* 2005, 2007 has reported the development of resistance to DDT and other classes of

insecticides including organochlorine, organophosphate, carbamates and recently pyrethroid in *An. gambiae s.s. An. arabiensis* and *An. funestus* from different zones in Nigeria. In south-west Nigeria, the first case of pyrethroid resistance in *An. gambiae*, the principal malaria vector, in Nigeria was documented (Awolola *et al.,* 2002) and since then the phenomenon has been well established in this region (Awolola *et al.,* 2003; Kristan *et al.,* 2003; Awolola *et al.*, 2005, 2007; Oduola *et al.,* 2010, 2012, Kolade *et al.,* 2012). Also in North-central Nigeria, permethrin and DDT resistance in *An. gambiae s.l.* has been reported (Ndams *et al.,* 2006; Olayemi *et al.,* 2011). In North-west Nigeria, resistance to permethrin and DDT were also reported (Ibrahim *et al.,* 2014).

Malaria vector resistance to insecticides is conferred by two mechanisms: (1) an increased metabolic detoxification of insecticides through increased enzymatic activities of esterases, glutathione S-transferases and cytochrome P450 monooxygenases, as a result of their overproduction due to gene amplification (Poire *et al.,*1992, Raymond *et al.,*1998) and/or gene regulation (Muller *et al.,* 2008, Muller *et al.,* 2007) and (2) point mutations in the gene encoding the voltage-gated sodium channel at the target sites of insecticides, decreasing the affinity of the insecticides to its receptor. Two mutations at amino acid position 1014 of the voltage-gated sodium channel, changing either a Leucine residue to a Phenylalanine; West African mutation (L1014F) (Martinez-Torres *et al.,* 1998), or a Leucine to Serine; East African mutation (L1014S) (Ranson *et al.,* 2000) have being identified in *An. gambiae* and confers knockdown resistance (kdr) to DDT and pyrethroid insecticides. On the other hand, carbamates and organophosphates share acetylcholinesterase as target site and at least two functional mutations in acetylcholinesterase 1 (ace-1) have been identified in insect species that offer reduced target sensitivity to intoxication (Alout *et al.,* 2007). One of these, ace-1R (G119S), is most commonly associated with resistance to these insecticides in *An. gambiae* (Dabire *et al.,* 2009; Djogbenou, 2010).This mutation is found in association with resistance

in the M and S molecular forms (Djogbenou *et al.,* 2008). Esterase mediated sequestration of carbamates and organophosphates are documented for some insect species (Li *et al.,* 2009).

In light of ongoing scaling-up of control tools (LLINs and IRS) with the goal to reduce by 50% the malaria-related cases in Nigeria by 2013 (USAID/PMI/CDC, 2011), information on the susceptibility of principal malaria vectors to common insecticide used in public health and the underlying mechanism are crucial. This information will adequately inform control programs of the most suitable insecticide to use and facilitate the design of appropriate resistance management strategies (Djouaka *et al.,* 2011). However, there is the dearth of information on the insecticide resistance status of the field strain of *An. gambiae* in the northwest region of Nigeria to almost all the classes of insecticides approved by WHO for vector control.

In this chapter, we characterised two populations of *An. gambiae s.l.* sampled from northern Nigeria, established their species compositions, resistance status to pyrethroid (permethrin), organochlorine (DDT) and carbamate (bendiocarb) and genotyped for the west and east African kdr-mutations resistance markers.

3.2 Experimental approach

3.2.1 Insecticides susceptibility bioassays

According to WHO (1998, 2013c), the purpose of the susceptibility test is to detect the presence of resistant individuals in an insect population as soon as possible. To further investigate the causes and underlying mechanisms of this resistance, a subset of mosquitoes are usually screened for target site mutations (kdr, Ace-1) and /or increased activities of detoxification enzymes. So that alternative controls plans can be made to deal with the situation when the insecticide in question is no longer having the desired effect. Therefore, in this study we aimed to evaluate the susceptibility to insecticides of *An. gambiae s.l.*

mosquitoes sampled from Auyo and Bichi, in 2013 and 2014 and to report the presence of kdr mutation frequency if any.

The WHO insecticide susceptibility bioassay is a simple, direct response-to-exposure test. Mosquitoes are exposed to known concentrations of an insecticide for a fixed period at the end of which the number of fatalities was recorded. In its present form, the test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes. The test is used as a field, and laboratory surveillance tool with the limitation that it gives little information on the underlying mechanism(s) conferring resistance (WHO, 2013c).

Insecticides susceptibility tests were carried out on 2-4 days old non-blood-fed adult *An. gambiae* mosquitoes using WHO insecticide susceptibility test-kits and standard protocol for adults (WHO, 1998). Bioassay test papers impregnated with; 0.75% permethrin, 0.1% bendiocarb, and 4% DDT were obtained from the Vector Control and Research Unit, University Sains Malaysia (Penang, Malaysia). For each test, 100 mosquitoes were separated into four batches of 25 mosquitoes and exposed to impregnated filter papers while a batch of 25 mosquitoes served as control. Thus, an aspirator was used to introduce 25 non-bloods fed adult mosquitoes into five WHO holding tubes (four tests and one control) that contained untreated papers. After an hour, they were gently blown into exposure tubes containing the insecticide impregnated papers for 1h at 25 ± 4 °C and 75 to 80% relative humidity. The numbers of knockdown mosquitoes for each insecticide were recorded at every 10 minutes during the 1h exposure period. After exposure, mosquitoes were kept in observatory/recovery tube and supplied with a 10% sugar solution and allowed a 24 h recovery period. Mosquitoes exposed to untreated filter papers served as Controls (Ahoua *et al.,* 2012). The mortality was recorded after 24 h, and the susceptibility status of the population graded according to WHO recommended protocol (WHO, 2013c).

3.2.2 Mosquito species and molecular forms identification

Mosquitoes were initially identified as belonging to the *An. gambiae s.l.* complex using the morphological keys of Gillies and Coetzee, 1987. Upon completion of the susceptibility tests, random samples of 30-35 mosquitoes from bioassays batches at each study site for the control, dead and surviving specimens were subjected to genomic DNA extraction. The genomic DNA was extracted using a DNA extraction kit from Qiagen, according to manufacturer's protocol and used for polymerase chain reaction (PCR). Identification of species and molecular forms within the *An. gambiae* complex was performed using SINE-PCR (Santolamazza *et al.,* 2008).

3.2.2.1 SINE based assay

An. gambiae s.l. and *An. gambiae s.s.* populations were further identified to species and molecular form levels respectively according to the SINE PCR method (Santolamazza *et al.,* 2008). According to Santolamazza *et al.,* 2008, the PCR diagnostic approach proposed is based on the specific and irreversible insertion of a 230 bp transposable element (SINE200) in the M-form (and its absence in S-form), thus allowing an unambiguous, simple and straightforward recognition of M and S forms (even without preliminary species-specific PCR identification in areas where exclusive sympatry with *An. arabiensis* is found) (Santolamazza *et al.,* 2008). However, it is important to keep in mind that the M-form specific SINE insertion is a character linked to the IGS-SNPs defining the M-form and S-form along most of their range, but with a different evolutionary history (i.e. its origin and rapid fixation in M-form) (Santolamazza *et al.,* 2008).

It is also interesting to note that, although the S-form amplicon is identical to those of *An. melas* and *An. quadriannulatus,* the 26 bp deletion reported for *An. arabiensis* allows to propose the use of the novel approach to discriminate *An. gambiae* from *An. arabiensis* specimens without preliminary species identification in vast areas of sub-Saharan Africa

where *An. gambiae* molecular forms and *An. arabiensis* are the only species of the complex present. The S200 X6.1 locus is located only about 1 Mb from IGS region (Santolamazza, *et al.,* 2008).

Table 3.1 Reaction set up and Primers for performing SINE PCR reaction

Components	Primer sequence (5' – 3')	Quantity(µl)
Sterile water		9.0
10µM FP	TCG CCT TAG ACC TTG CGT TA	1.0
10µM RP	CGC TTC AAG AAT TCG AGA TAC	1.0
$MgCl_2$ 25µM		0.5
Taq DNA polymerase		12.5
DNA template 18ng/µl		1.0
Total		**25.0**

The reaction was set up as shown in table 3.1 in PCR tubes. PCR was carried out in Thermal cycler S1000™ Bio-Rad System. The PCR cycles comprise an initial step of 5 min at 94°C to activate the DNA polymerase. Then followed by 35 cycles, each consisting of 30 s denaturation at 94°C, 30 s annealing at 54°C and 30 s extension at 72 °C, the final cycle products are extended for 10 min at 72°C and 4°C hold. The amplicons were then analyzed as described by Santolamazza *et al.,* 2008 on 1.5% agarose gels stained with Gel red, with low and high molecular weight bands corresponding to fragments containing or lacking the targeted SINE200, respectively.

3.2.3 Knockdown resistance (kdr) assay

To investigate the causes of this resistance, we randomly screened a subset of mosquitoes for the presence of kdr 1014F and 1014S target site mutations by PCR.

3.2.3.1 Detection of knock-down resistance (kdr) alleles in An. gambiae s.l.

Mosquitoes from the control and exposed batches of the bioassays or a subset from selected bioassays were used to determine the frequency of the 1014F and 1014S alleles in the general population (Badolo *et al.,* 2012). A proportion of the dead and surviving mosquito specimen from insecticide treatments were assayed for the presence of knockdown resistance (kdr) by PCR to detect the West African (L1014F) and East African (L1014S) kdr alleles based on TaqMan assay (Bass *et al.,* 2007).

The detection of East kdr (L1014S) and West kdr (L1014F) mutations was performed using TaqMan PCR diagnostic assays described in Bass *et al.,* (2007) and a Max3000P Real-Time PCR (QPCR) system (Stratagene). According to Bass *et al.,* (2007), the assay is a PCR method employing oligonucleotide probes that are dual labelled with a fluorescent reporter dye and a quencher molecule. Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence as the reporter dye is released away from the quencher. Cleavage of allele-specific probes can be detected in a single PCR by using different reporter dyes (Livak, 1999).

Previous work characterizing the para gene region encoding domain II S4–S6 of the sodium channel from a range of insect species has shown that this region contains an intron very close to the kdr mutation site. In many insect species this intron displays a degree of variation (nucleotide substitutions or insertions /deletions) between different stains / isolates which would affect the performance of any assay that uses primer binding sites within this region. Therefore, nucleotide alignments of all the *An. gambiae* and *An. arabiensis* domain II sodium channel gene sequences available in the National Centre for Biotechnology Information (NCBI) database were compared and a region around the kdr site which was conserved in all isolates/species was selected for primer / probe design.

Table 3.2 Primers used for detection of kdr alleles

Primer name	Sequence (5'-3')	Concentration (nM)
Kdr-forward	CATTTTTCTTGGCCACTGTAGTGAT	800
Kdr-reverse	CGATCTTGGTCCATGTTAATTTGCA	800
MGB probe WT	(JOE) CTTACGACTAAATTTC (TAM)	200
MGB probe kdr-W	(6-FAM) ACGACAAAATTTC (TAM)	200
MGB probe kdr-E	(6-FAM) ACGACTGAATTTC (TAM)	200

Forward and reverse primers and three minor groove binding (MGB) probes (Applied Biosystems) were designed using the Primer Express™ Software Version 2.0(Bass *et al.,* 2007). Primers kdr-Forward and kdr-Reverse were standard oligonucleotides with no modification. The probe WT was labelled with a reporter dye 6-carboxy-fluorescein JOE at the 5' end for the detection of the wild-type allele, the probes kdrW and kdrE were labelled with 6-carboxy-fluorescein FAM for detection of the kdr-W and kdr-E alleles respectively. Each probe also carried a 3' non-fluorescent quencher 6-carboxy-tetramethylrhodamine TAMRA (TAM) and a minor groove binder at the 3' end (Perkin, 1998). The minor groove binder provides more accurate allelic discrimination by increasing the TM between matched and mismatched probes (Afonina *et al.,* 1997). The primers kdr- Forward and kdr-Reverse and the WT probe were used in one assay with probe kdrW for the kdr-W detection and in a second assay with probe kdrE for kdr-E detection. PCR reactions (25 µl) contained one µl of genomic DNA, 12.5 µl of SensiMix DNA kit (Quantace), 900 nM of each primer and 200 nM of each probe. Samples were run on a Stratagene Multiplex Quantitative PCR system (Max 3000P) using the temperature cycling conditions of 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds. Each probe consists of an oligonucleotide with a 5′ reporter dye and a 3′ quencher dye. The increase in JOE and FAM fluorescence (fluorescent dyes) 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 Max 3000P respectively.

3.3 Data Analysis / Interpretation of results

To investigate significant differences in the effect of the different insecticides on the mortality and Knockdown of *An. gambiae s.l.* for the various study areas, the probit model and mixed effect probit model were used in R statistical packages to account for the repeated measures

of knockdown over time. Interaction effects between insecticide treatments and areas were first investigated, and if significant, the impact of the insecticide treatment was analysed in each area individually. Statistical significance was determined in reference to DDT for all insecticides tested. In this study, Abbott's formula was not used for the correction of mortality rates in control tubes based on the fact that the mortality rates in all control tubes were less than 5% (Abbott, 1987). Mean percentage mortality was determined across all mosquitoes tested for a particular insecticide in a given site. WHO criteria (WHO, 2013c) for discriminating individuals for susceptibility /resistance status were applied: mortality rate between 98% - 100% indicate full susceptibility; mortality rate between 90% - 97% suspected resistance requires further investigation and mortality rates < 90% indicate the population is resistant to the tested insecticides.

To assess whether the frequency of different kdr alleles in *An. gambiae s.l.* was related to resistance SPSS 22 statistical software was used to analyse the relationships. Coefficients of correlation were calculated between the frequency of different mutations, and the corresponding mortality rates between the five sampling sites.

3.4 Results

3.4.1 Insecticides susceptibility bioassays

WHO bioassays were carried out on adult mosquito populations that were morphologically identified as *An. gambiae s.l.* offspring using Gillies and De Meillon (1968) and Gillies & Coetzee (1987) morphological keys. These mosquitoes were reared from larvae to adults from the five sampling sites. The adult mosquitoes were exposed to diagnostic doses of three insecticides; DDT (4%), permethrin (0.75%) and bendiocarb (0.1%).The results of the knockdown assessment of *An. gambiae* mosquitoes exposed to three different insecticides impregnated papers is presented in tables 3.3 and 3.4.

Table 3.3 and Table 3.4 shows the mean total number of adult *An. gambiae s.l.* knocked down at 10 min interval over the 1h exposure period to three insecticides and the mean percentage mortality recorded after 24 h recovery period. In all the sites as shown in tables 3.3 and 3.4, all the Insecticides tested shows gradual prolong knockdown abilities at various times during the 1hr exposure. None of the three insecticides recorded a complete knock down within 60 min exposure. Only bendiocarb was able to knockdown between 70 to 95% of the mosquito population tested within 60 min of exposure in all the sites.

Table 3.3 Mean number of knockdown and mean percentage mortality of *An. gambiae* population from Auyo and Bichi in Jigawa and Kano states exposed to discriminating doses of insecticides for 60 minutes in 2013

Site	Insecticide (conc.)	Mean number of knockdown (KD) mosquitoes						Mean % mortality at 24h
		10 min	20 min	30 min	40 min	50 min	60 min	
AA1	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00± 0.00	0.00± 0.00	1.00 ±1.40	2.00±1.40	2.50 ±1.30	3.25±0.96	16 ± 5.66
	Permethrin (0.75%)	0.00± 0.00	0.25± 0.50	1.25 ±1.30	2.00±1.41	2.25±1.26	3.00 ±1.20	38 ± 7.66
	Bendiocarb (0.1%)	1.25± 1.50	4.00 ± 1.83	11.00±2.58	15.50±2.40	18.25±2.75	21.25±1.50	95 ± 6.00
AA2	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.20 ±0.50	0.25±0.50	0.50±0.60	0.50 ±0.60	1.00±1.20	1.75±0.96	7 ± 3.83
	Permethrin (0.75%)	0.25±0.50	0.50±1.00	0.50±1.00	0.75±0.96	1.00±0.82	1.50±1.29	23 ± 5.22
	Bendiocarb (0.1%)	0.75±0.50	6.75±0.96	11.00±1.15	15.00±0.82	17.50±1.00	20.25±0.50	81 ± 5.03
AR	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.25 ±0.50	0.50±0.58	0.50±0.58	1.00±1.15	1.75± 0.96	2.25 ±1.50	32 ± 7.30
	Permethrin (0.75%)	0.00 ±0.00	1.00±0.00	1.00±0.00	1.50± 0.58	2.00± 0.00	2.50± 0.58	20 ± 6.53
	Bendiocarb (0.1%)	0.25 ±0.50	5.00±0.82	15.75±1.79	18.25±1.26	20.50 ±1.00	21.00±1.15	83 ± 6.83
BA	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00 ±0.00	0.00± 0.00	0.00 ± 0.00	0.25± 0.50	0.75± 0.96	1.00± 0.82	6 ± 2.31
	Permethrin (0.75%)	0.00 ±0.00	0.00±0.00	0.00± 0.00	1.00±0.00	2.50± 0.58	2.75±0.50	21 ± 6.83
	Bendiocarb (0.1%)	NT						NT
BR	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00 ±0.00	1.0± 0.00	1.2 ± 0.45	2.00± 0.70	2.20 ± 0.45	3.80 ± 0.84	27 ± 5.22
	Permethrin (0.75%)	0.00 ±0.00	0.50±0.60	2.00±1.63	2.75 ±1.71	5.00 ± 1.83	6.75± 1.26	54 ±10.07
	Bendiocarb (0.1%)	0.00 ±0.00	9.25±0.96	12.75±0.96	15.00±0.82	16.25 ±1.26	18.25 ±1.26	74 ± 5.16

NT: not tested due to insufficient mosquitoes, AA 1: Auyo Agricultural 1, AA 2: Auyo Agricultural 2, AR: Auyo Residential, BA: Bichi Agricultural, BR: Bichi Residential

Table 3.4 Mean number of knockdown and mean percentage mortality of *An. gambiae* population from Auyo and Bichi in Jigawa and Kano states exposed to discriminating doses of insecticides for 60 minutes in 2014

Site	Insecticide (conc.)	Mean number of knockdown (KD) mosquitoes						Mean % mortality at 24h
		10 min	20 min	30 min	40 min	50 min	60 min	
AA1	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	0.50± 0.71	1.00 ± 0.00	1.5 ± 0.71	14 ± 2.83
	Permethrin (0.75%)	0.00 ± 0.00	0.00 ± 0.00	1.67± 0.58	2.67 ± 0.58	4.67 ± 0.58	6.33 ± 0.58	26 ± 2.31
	Bendiocarb (0.1%)	0.00 ± 0.00	2.00 ± 0.00	12.5 ± 0.71	20.00± 1.41	20.50 ±0.71	22.5 ± 0.71	90 ± 2.83
AA2	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	1.00 ± 0.00	6 ± 2.83
	Permethrin (0.75%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	2.50 ± 0.71	10 ± 8.46
	Bendiocarb (0.1%)	2.00 ± 0.00	4.00 ± 0.00	16.50 ±2.12	18.50 ±2.12	21.50 ±2.12	22.0 ± 2.83	84 ± 5.66
AR	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00 ±0.00	0.00±0.00	0.00±0.00	1.50±0.58	3.50 ±1.00	6.50 ±1.29	35 ± 5.03
	Permethrin (0.75%)	0.00 ±0.00	0.00±0.00	0.00±0.00	0.75± 0.96	2.50± 1.29	5.00± 1.63	25 ± 6.83
	Bendiocarb (0.1%)	2.50 ±0.58	4.50±0.58	16.00±1.83	18.00±1.41	20.75 ± 1.5	21.75±1.26	88 ± 3.27
BA	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4%)	0.00 ±0.00	0.00 ±0.00	0.25 ±0.50	0.50 ±0.58	1.00 ±0.82	1.50 ±1.00	7 ± 3.83
	Permethrin (0.75%)	0.00 ±0.00	0.00 ±0.00	0.25 ±0.50	1.00 ±0.82	1.75 ±1.71	4.00 ±2.83	22 ± 5.16
	Bendiocarb (0.1%)	1.50 ±0.58	6.50 ±1.29	10.75 ±0.96	12.25 ±2.06	15.75 ±0.96	17.25 ±0.96	71 ± 5.03
BR								
	Control (0.0%)	0.00	0.00	0.00	0.00	0.00	0.00	
	DDT (4.0%)	0.00 ± 0.00	0.00± 0.00	0.00 ± 0.00	0.33 ± 0.58	1.00 ± 1.00	2.00 ± 1.00	8 ± 4.00
	Permethrin (0.75%)	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.50	0.75 ± 0.96	1.75± 1.71	4.00 ± 2.83	52 ± 8.64
	Bendiocarb (0.1%)	1.50 ± 0.71	6.50 ± 0.70	19.0 ± 1.41	22.0 ± 1.41	23.5 ± 0.71	23.5 ± 0.71	94 ± 2.83

AA 1: Auyo Agricultural 1, AA 2: Auyo Agricultural 2, AR: Auyo Residential, BA: Bichi Agricultural, BR: Bichi Residential

However, for DDT and permethrin, it took 60 min to record between 4 to 27% knockdown in all the sites. The corresponding mean percentage mortalities at each of these sites, recorded 24 h after exposure were also higher for bendiocarb than for DDT and permethrin. Mean mortality rates of *An. gambiae s.l.* in 2013 and 2014 24 h post exposure are shown in tables 3.5 and illustrated in figures 3.3, and 3.4 respectively.

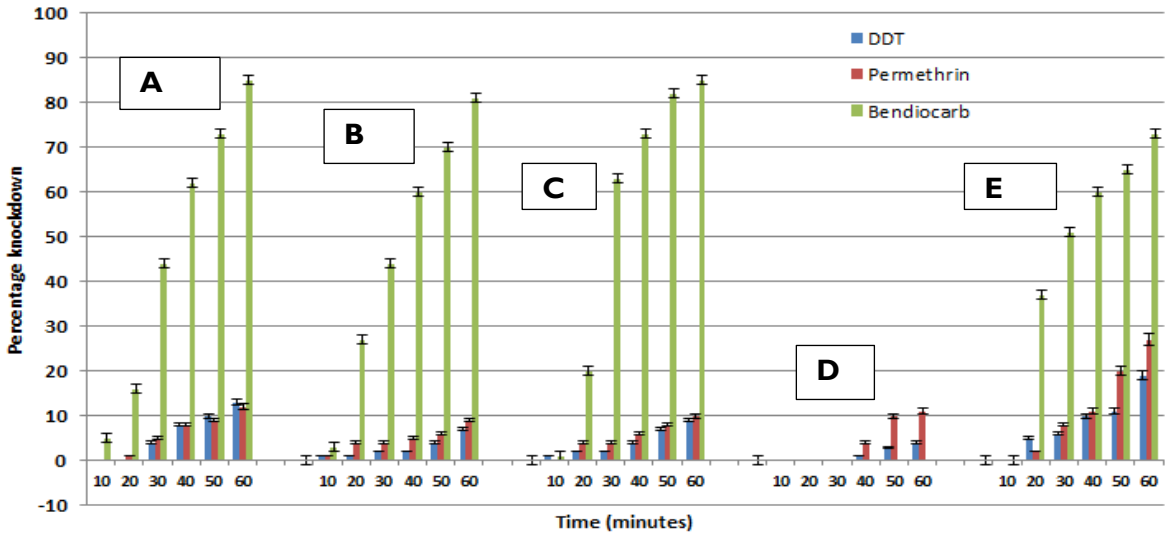


Figure 3.1 Percentage Knockdown of *An. gambiae s.l.* mosquitoes exposed to dosages of insecticides for 60 min in 2013: (A) Auyo agricultural 1, (B) Auyo agricultural 2, (C) Auyo residential, (D) Bichi agricultural and (E) Bichi residential sites

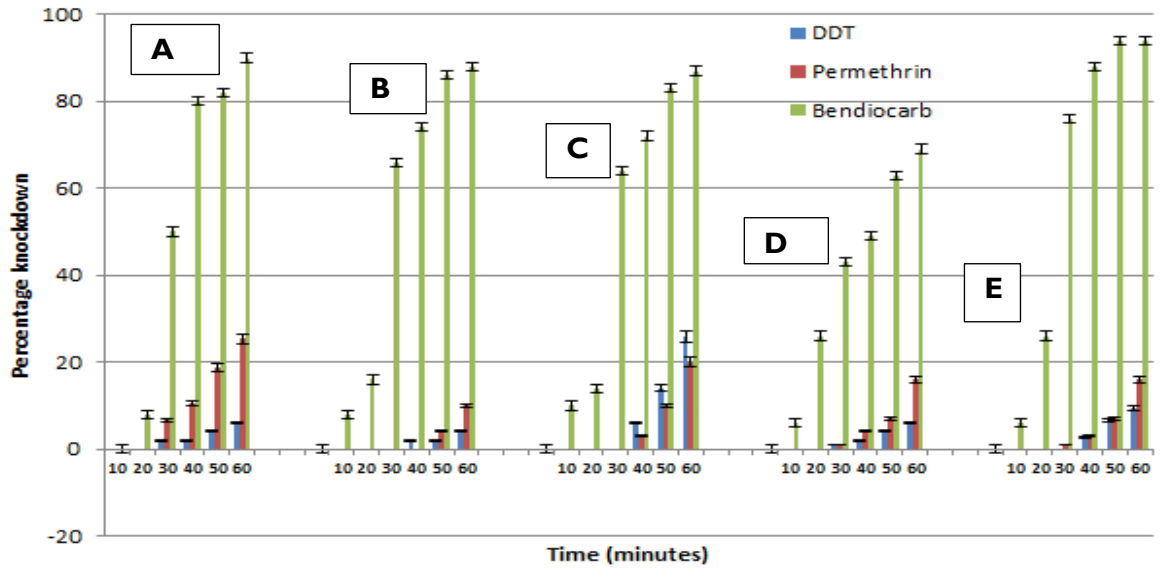


Figure 3.2 Percentage Knockdown of *An. gambiae s.l.* mosquitoes exposed to dosages of insecticides for 60 min in 2014: (A) Auyo agricultural 1, (B) Auyo agricultural 2, (C) Auyo residential, (D) Bichi agricultural and (E) Bichi residential sites

The results of the insecticides bioassays performed on adults *An. gambiae s.l.* are shown in Figures 3.3 and 3.4. Using the WHO (2013c) definitions of resistance the two populations from the Sudan savannah (Auyo and Bichi) in northern Nigeria revealed high resistance to DDT and permethrin with low prevalence of resistance to bendiocarb. In zone A comprising AA1, AA2 and BA results of the bioassays performed in 2013(Figure 3.3) showed very low mortalities with DDT and permethrin that fluctuated around 6% to 16% and 9% to 12%. Survival after exposure to bendiocarb fluctuated around 81% to 85%. *An. gambiae s.l.*

mosquitoes from zone B comprising AR and BR were also highly resistant to DDT and permethrin. The mortalities with DDT were 32% and 27% for AR and BR while for permethrin 20% and 54% mortalities were recorded respectively. Exposure to bendiocarb resulted in 83% and 74% mortalities (Figure 3.3).

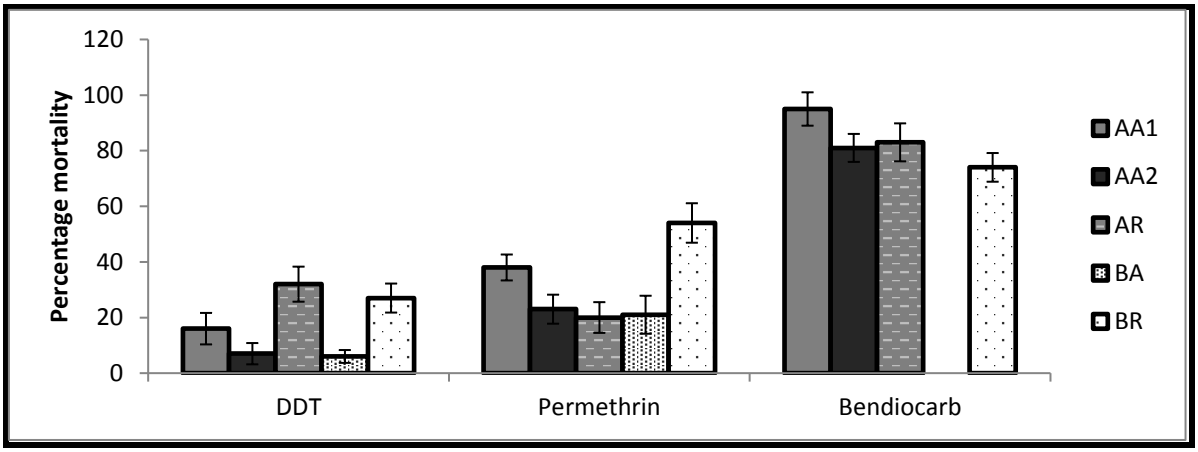


Figure 3.3 Insecticide mortality rate of *An. gambiae s.l.* 24 h post exposure in Nigeria collected in 2013 rainy season. Mosquitoes were exposed to 4% DDT, 0.75% permethrin and 0.1% bendiocarb in WHO susceptibility test at: Auyo agricultural 1(AA1); Auyo agricultural 2 (AA2); Auyo residential (AR); Bichi agricultural (BA) and Bichi residential (BR) sites

In 2014 in zone A, a similar pattern of mortality with DDT, permethrin and bendiocarb was observed. Mortality with DDT in AA1 decreased from 16% to 7% but there was no significant change in AA2 and BA. Mortality with permethrin in AA1 and AA2 also decreased from 38% to 26% and 23% to 10% respectively while for bendiocarb no significant change was recorded (21% to 22%).

DDT mortality in zone B in 2014 further revealed high resistance in these *An. gambiae* populations and was not much different from that recorded in 2013 (Figure 3.4).

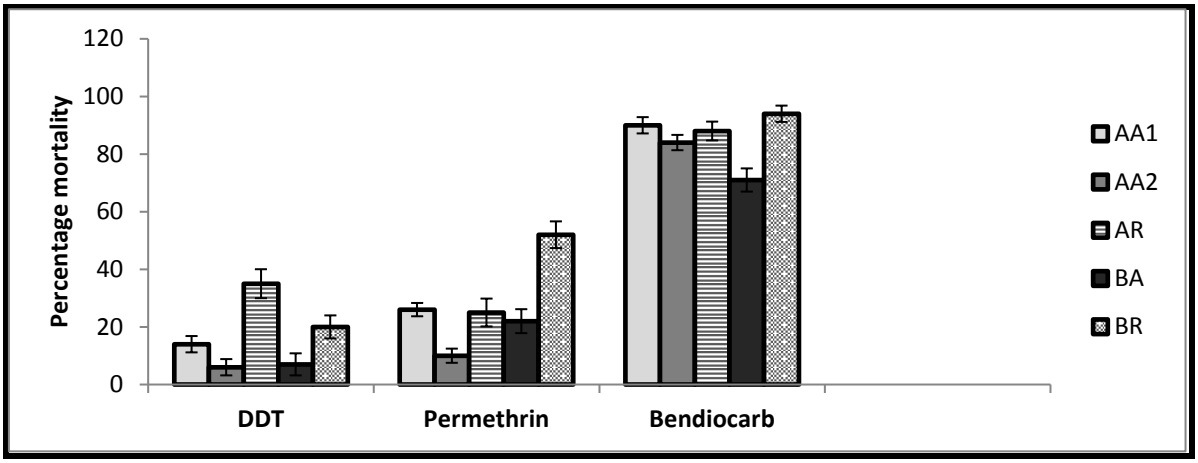


Figure 3.4 Insecticide mortality rate of *An. gambiae s.l.* 24 h post exposure in Nigeria collected in 2014 rainy season. Mosquitoes were exposed to 4% DDT, 0.75% permethrin and 0.1% bendiocarb in WHO susceptibility test at: Auyo agricultural 1(AA1); Auyo agricultural 2 (AA2); Auyo residential (AR); Bichi agricultural (BA) and Bichi residential (BR) sites

Similarly no noticeable change in mortality with permethrin was observed in zone B between 2013 and 2014. Furthermore, exposure of *An. gambiae* mosquitoes to bendiocarb in 2014 resulted in higher mortality 94% and 88% compared to 83% and 74% in 2013.

The results of the mixed effect probit model showed that only treatment with bendiocarb in zone A and B in 2013 and 2014 had significant effects on knockdowns of *An. gambiae s.l.* population exposed to these insecticides in all the study sites with P-values ($P<0.001$) in all the study sites except in 2014 at BR in zone B where the p-value was ($P<0.1$) and in 2013 at BA zone A where it was not tested. Similarly, the results of the probit model showed that bendiocarb was the only insecticide that showed significant effects on the mortality of *An. gambiae s.l.* population exposed to these insecticides in all the study sites with P-values of $P< 0.001$ in all the study sites except in 2013 at AA2 where the p-value was ($P>0.05$). However, treatment with permethrin showed varied levels of significant effects on both knockdowns and mortalities of *An. gambiae s.l.* population exposed to these insecticides compared to the baseline treatment (DDT). On knockdown, it showed a significant effect in 2013 only at BA ($P<0.01$) and in 2014 at AA1 and BA ($P<0.001$ and $P<0.1$) respectively. On mortality permethrin showed significant effect in 2013 at BA and BR ($P<0.1$and $P<0.05$) respectively

while in 2014 significant effects were recorded at AA1 and BA both with P-value (P<0.01). See appendix for statistical analyses results. Overall, in 2013 in all the sites *An. gambiae* populations demonstrated multiple resistance levels, showing reduced mortality rates to the three insecticides at the same time, although with a site- to- site variations (Figure 3.3). Resistance to DDT (4%), and permethrin (0.75%) was most widespread. Mortality rates to DDT and permethrin were below 50% in four out of the five localities investigated, reflecting high levels of resistance. However, resistance to bendiocarb was less marked with mortality rates of 95%, 81%, 83% and 74%, for Auyo agricultural 1, Auyo agricultural 2, Auyo residential and Bichi residential, respectively. The rates observed at Auyo agricultural 1 (95%) suggest reduced susceptibility that needs further monitoring in order to establish the status of resistance in the vector populations. Furthermore, in 2014 in all the sites *An. gambiae* populations demonstrated multiple resistance levels, showing reduced mortality rates to the three insecticides tested. Mortality rates recorded for DDT, permethrin and bendiocarb in the five sampling sites were: 7%, 6%, 35%, 7% and 20%; 26%, 10%, 25%, 22% and 52%; 90%, 84%, 88%, 71% and 94% for AA1, AA2, AR, BA and BR respectively.

Table 3.5 Insecticide bioassay results for *Anopheles gambiae (An. gambiae) s.l.* in 2013 and 2014 in five sampling sites in northern Nigeria

Insecticide	Site	No. exposed	No. dead	2013 mortality (%)	Susceptibility status	No. exposed	No. dead	2014 mortality (%)	Susceptibility status
DDT (4%)	AA1	100	16	16	R	50	7	14	R
	AA2	100	7	7	R	50	3	6	R
	AR	100	32	32	R	100	35	35	R
	BA	100	6	6	R	100	7	7	R
	BR	125	34	27	R	75	15	20	R
Permethrin (0.75%)	AA1	100	38	38	R	75	20	26	R
	AA2	125	29	23	R	50	5	10	R
	AR	100	20	20	R	100	25	25	R
	BA	100	21	21	R	100	22	22	R
	BR	100	54	54	R	100	52	52	R
Bendiocarb (0.1%)	AA1	100	95	95	SR	50	45	90	SR
	AA2	100	81	81	R	50	42	84	R
	AR	100	83	83	R	100	88	88	R
	BA					100	71	71	R
	BR	100	74	74	R	50	47	94	SR

AA1 = Adults Anopheline from Auyo agricultural 1; AA2 = Adults Anopheline from Auyo agricultural 2; AR = Adults Anopheline from Auyo residential; BA = Adults Anopheline from Bichi agricultural; BR = Adults Anopheline from Bichi residential; R =resistant; SR = suspected resistance.

3.4.2 Mosquito species and molecular forms identification

A total of 1294 *An. gambiae* complex mosquitoes from Auyo and Bichi that survived exposure to DDT, permethrin and bendiocarb and 294 dead after the exposure were identified to species and molecular forms by SINE PCR according to Santolamazza, *et al.,* 2008 (Table 3.4). Among the *An. gambiae s.l.* population, approximately 75.9% were *An. coluzzii* (formerly M form) 19.4% were *An. arabiensis,* 4.5% and 0.2% were hybrid (M/S) and *An. gambiae s.s.* (formerly S form) respectively (Table 3.6).

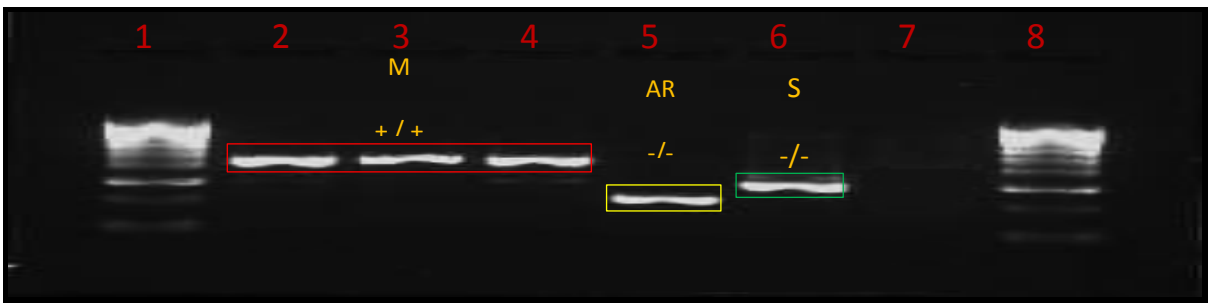


Figure 3.5 Diagnostic PCR based on S200 X6.1 in *Anopheles gambiae s.l.* indicating the presence (+) or absence (-) of insertion in *Anopheles gambiae* complex. M = *An. gambiae* M form; AR = *An. arabiensis*; S = *An. gambiae* S form. Ladder = 100 bp (Bioline hyperladder IV). Lanes 1 and 8 = 1 kb (100 bp) molecular Weight ladder; Lanes 2, 3 and 4 = M-molecular form (479 bp), lane 5 = *An. arabiensis* (223 bp) lane 6 = S Molecular form (279 bp)

Table 3.6 Species and molecular form distribution of *An. gambiae s.l.* from the five different sampling sites in northern Nigeria using SINE based assay

Site/	Molecular form of *An. gambiae*							
Year	*An. arabiensis*		M		M/S		S	
	N	%	N	%	N	%	N	%
AA1								
2013	36	30.0	64	53.0	20	17.0	0	0.0
2014	4	4.1	80	81.6	12	12.3	2	2.0
AA2								
2013	16	13.8	100	86.2	0	0.0	0	0.0
2014	8	9.1	80	90.9	0	0.0	0	0.0
AR								
2013	24	16.2	116	78.4	8	5.4	0	0.0
2014	23	16.4	110	78.6	7	5.0	0	0.0
BA								
2013	52	37.1	84	60.0	4	2.9	0	0.0
2014	40	28.6	93	66.4	7	5.0	0	0.0
BR								
2013	24	17.6	112	82.4	0	0.0	0	0.0
2014	24	14.3	144	85.7	0	0.0	0	0.0
Total	**251**	**19.4**	**983**	**75.9**	**58**	**4.5**	**2**	**0.2**

AA1= Auyo agricultural 1; AA1= Auyo agricultural 2; AR = Auyo residential; BA = Bichi agricultural; BR = Bichi residential; Sampling at AR and BA in 2014 was not done

3.4.3 Genotyping of knockdown resistance (kdr) mutations

A total of 300 identified mosquitoes from Auyo and Bichi which were exposed to DDT and permethrin were genotyped for the presence of East kdr (L1014S) and West kdr (L1014F) mutations. Of these 199 were homozygous for the susceptible wild-type, 32 were homozygous for L1014S and 69 were heterozygous for L1014S. When genotyped for L1014F, 156 were homozygous for the susceptible wild-type, 69were homozygous for L1014F and 75 were heterozygous for L1014F (Tables 3.7 and 3.8).

Table 3.7 Distribution of kdr-East (L1014S) mutation in *An. coluzzii* and *An. arabiensis* mosquitoes

	Anopheles coluzzii						*Anopheles arabiensis*					
		Genotype count			Allelic frequency			Genotype count			Allelic frequency	
Site	N	SS	SL	LL	S	L	N	SS	SL	LL	S	L
AA1	45	5	13	27	0.400	0.600	15	0	3	12	0.200	0.800
AA2	47	11	15	21	0.553	0.447	13	0	4	9	0.308	0.692
AR	40	2	9	29	0.275	0.725	20	0	0	20	0.000	1.000
BA	48	10	12	26	0.458	0.542	12	1	3	8	0.333	0.667
BR	42	3	8	31	0.262	0.738	18	0	2	16	0.111	0.889

SS, SL and LL are the three possible genotypes, where S represents the resistant L1014S allele and L represents the susceptible wild-type allele, N is number of particular species either *An. coluzzii* or *An. arabiensis*

The L1014S mutation was detected in both *An. coluzzii* and *An. arabiensis*. The L1014S mutation in agricultural setting was recorded at allelic frequencies of 40.0%, 55.3% and 45.8% (AA1, AA2 & BA) in *An. coluzzii;* and 20.0%, 30.8%, 33.3% in *An. arabiensis* respectively. The L1014S mutation was in residential setting recorded at allelic frequencies of 27.5% and 26.2% in *An. coluzzii;* and 0.0% & 11.1% in *An. arabiensis.*

Table 3.8 Distribution of kdr-West (L1014F) mutation in *An. coluzzii* and *An. arabiensis* mosquitoes

	Anopheles coluzzii						*Anopheles arabiensis*					
		Genotype count			Allelic frequency			Genotype count			Allelic frequency	
Site	N	FF	FL	LL	F	L	N	FF	FL	LL	F	L
AA1	45	10	12	23	0.489	0.511	15	0	3	12	0.200	0.800
AA2	47	20	11	16	0.659	0.341	13	2	6	5	0.616	0.384
AR	40	7	10	23	0.425	0.575	20	0	1	19	0.050	0.950
BA	48	18	14	16	0.667	0.333	12	2	2	8	0.333	0.667
BR	42	9	12	21	0.500	0.500	18	1	4	13	0.278	0.722

FF, FL and LL are the three possible genotypes, where F represents the resistant L1014F allele and L represents the susceptible wild-type allele, N is number of particular species either *An. coluzzii* or *An. arabiensis*

Similarly the L1014F mutation was detected in both *An. coluzzii* and *An. arabiensis*. The L1014F mutation was detected at allelic frequencies of 48.9%, 65.9% and 66.7% in agricultural

setting (AA1, AA2 & BA) in *An. coluzzii;* and; and 20.0%, 61.6%, 33.3% in *An. arabiensis.* While in residential setting (AR & BR) the frequencies were; 42.5% & 50.0% in *An. coluzzii;* and 5.0% & 27.8% in *An. arabiensis*. Although the two kdr mutations occurred in both *An. coluzzii* and *An. arabiensis,* the L1014F was much associated with *An. coluzzii.* A significant positive correlation (P<0.05) between the frequency of the L1014F point mutation and resistance to DDT and permethrin was observed. However, a weak or non- significant correlation (P>0.05) between the frequency of the L1014S point mutation and resistance was also found. L1014S and L1014F mutations co-occurred in both agricultural and residential settings with high frequencies. However, the frequencies of the two mutations were greater in the agricultural settings than in the residential settings.

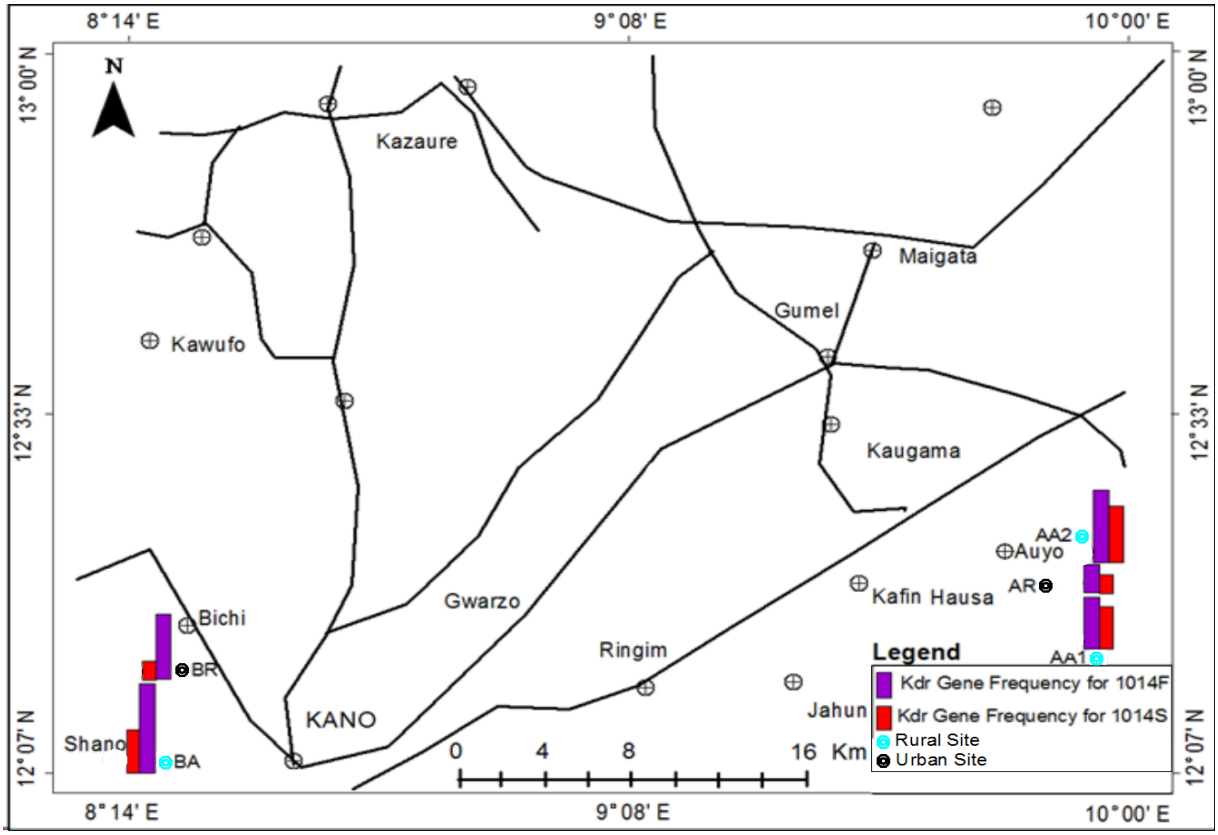


Figure 3.6 Map of the study areas in Nigeria showing the distribution of 1014F, 1014S in *An. gambiae* population.

3.5 Discussion

In this chapter, the study investigated the species composition, susceptibility status the presence and frequency distribution of east (L1014S) and west (L1014F) African kdr alleles in the *An. gambiae s.l.* complex population in the study areas (Sudan savannah of northern Nigeria) for appropriate control measures. This is because different members of the same species complex do not necessarily share the same resistance mechanisms, and nor do they necessarily exhibit the same insecticide resistance patterns (WHO, 2013c). *An. coluzzii* was predominant over *An. arabiensis* during this study. This supports previous observations that *An. gambiae s.s.* could be dominant in the Sudan savannah ecological zone compared to *An. arabiensis* that was spread across Sudan, Sahel and northern Guinea savannah ecological zones (Coluzzi *et al.,* 1979; Onyabe & Conn, 2001 and Ibrahim *et al.,* 2014). However, further collections are required to allow for the establishment of the malaria vector species distribution in this region of Nigeria.

The resistance/susceptibility status of *An. gambiae s.l.* mosquitoes to 4% DDT, 0.75% permethrin, and 0.1% bendiocarb was investigated in northwest Nigeria. Based on the conventional criteria for characterizing insecticide resistance / susceptibility (Kolade *et al.,* 2013, WHO, 2013c), where susceptibility is defined by mortality rates greater than 98% 24 h post exposure. There was evidence of high resistance among the mosquitoes tested from all the sampling sites. However, mosquitoes from agricultural sites (zone A) recorded higher insecticide resistance when compared to those from residential sites (zone B). These high levels of resistance are probably related to extensive pesticide use in the region. These different levels of insecticide susceptibility may reflect differential insecticide selection pressure exerted on field mosquito populations (Kerah-Hinzoumbe et al., 2008). Decrease in mortality rates to DDT and permethrin was associated to a significant increase in the KDT_{50} observed which were higher in agricultural sites. This is probably due to the involvement of

kdr (Bigoga *et al.,* 2014).The higher KDT_{50} recorded in this study, for DDT and permethrin is consistent with the findings of Chandre *et al.,* 1999, Ibrahim *et al.,* 2014, and Alhassan, *et al.,* 2015, indicating the involvement of kdr mechanism of resistance. This is connected to the fact that the pyrethroid (permethrin) and organochlorine insecticides (DDT) shares same mechanism of action by targeting the voltage-gated sodium channel on the insects' neurons (Davies *et al.,* 2007). The carbamate (bendiocarb) on the other hand, targets the acetylcholinesterase gene (Yewhalaw *et al.,* 2011). DDT and permethrin resistance observed in the *An. coluzzii* populations are similar to previously reported cases of DDT and permethrin resistance in the north-western, north-central and south-western Nigeria (Awolola *et al.,* 2007; Adeogun *et al.,* 2012; Ibrahim *et al.,* 2014; Alhassan *et al.,* 2015).

The massive use of pesticides in agricultural settings has been well documented as a factor contributing to the emergence of resistance in Anopheles populations (Akogbeto, 2006). As reported by Akogbeto, some populations of *An. gambiae* laid their eggs in breeding sites containing insecticides residues. Larvae from these eggs are subjected to selection pressure leading to multiple insecticides resistance. Selection for pyrethroid resistance in *An. gambiae* has been associated with the use of agricultural pesticides but not with DDT because of restricted use of DDT since it was banned (Sadasivaiah *et al.,* 2007, Adedayo *et al.,* 2010). But this was not the case in the present study, where some farmers confirmed the continued usage of DDT as pesticides and herbicides. This also suggests possible uncontrolled and illegal usage of DDT or other unspecified and unbranded locally made pesticides. The sustained usage of these pesticides in the study areas may have maintained a selection pressure for DDT resistance and possibly this is why DDT was the worst performing insecticide. The DDT and permethrin high resistance recorded in the residential areas were most likely due to increased use of household insecticides by high ITNs and IRS coverage or recurrent space spraying interventions (Balkew *et al.,* 2010; Marcombe *et al.,* 2011) and

availability of xenobiotics for larval breeding sites because agricultural practices are uncommon.

However, it was apparent there was an impact of agricultural pesticides on susceptibility of *An. gambiae* as shown by differences in the insecticides susceptibilities of these populations in agricultural and residential areas. *An. gambiae* populations from agricultural settings showed higher resistance to the insecticides tested in both 2013 and 2014. Resistance status of these *An. gambiae* populations slightly increased in 2014 as against what was recorded in 2013. Bioassay results further indicated resistance to DDT and permethrin has settled in the sampling localities. Our findings show that DDT and permethrin are unsuitable for controlling *An. gambiae s.l.* in these sampling sites. Efficient Vector control would require a different insecticide (s) or different concentrations of these insecticides. The present study reveals the co-occurrence of L1014S and L1014F mutations coupled with high insecticide resistance in the two Anopheline populations belonging to agricultural and residential settings in northwest Nigeria suggesting the spreading of the L1014S mutation gene across Africa. Agricultural activities have an effect on kdr allele's distribution compared to non–agricultural activities in the residential settings. Thus, the nature of the environment drives the resistance.

The high allelic frequencies recorded in the residential sites could be due to the increased use of pyrethroids for ITNs and IRS in public health as indicated in previous studies (Dykes *et al.,* 2015, Li *et al.,* 2015).The L1014F mutation appears to be the most significant mutation in both *An. coluzzii* and *An. arabiensis* in northwest Nigeria at present, however there exist the possibility that other mechanisms were also present and acted to confer resistance. The co-occurrence of the L1014S and L1014F mutations has already been reported in *An. gambiae s.l.* from both East and West Africa such as Kenya, Tanzania, Burkina Faso and Nigeria (Ranson *et al.,* 2000; Kabula *et al.,* 2014; Badolo *et al.,* 2012; Dabire *et al.,* 2013 and

Ibrahim *et al.,* 2014). The frequency of the L1014F mutation was positively correlated with resistance (P= 0.04) thus the higher the frequency of the L1014F mutation the higher the level of resistance to DDT and permethrin. The results also show a weak relationship between the frequency of L1014F and L1014S mutations (P=0.772). This study also reports the co-occurrence of East form of kdr and West form of kdr in *An. coluzzii* and *An. arabiensis* mosquitoes in a higher frequency than previously reported (Ibrahim *et al.,* 2014). The low but increased frequency of the East kdr indicates that its selection in this region is recent and is increasing. On the other hand, the high frequency of the West kdr observed in the *An. coluzzii* is in agreement with the observation of Ibrahim *et al.,* (2014) but contradict the observations in the south-western Nigeria (Awolola *et al.,* 2007) where the kdr mutation frequency was high in *An. gambiae s.s.* (formerly S-form) and low in *An. coluzzii* (once M-form). The very small number of the S-form (two) recorded from the field collections could be explained by the previous observation made by Coluzzi (1984) that the M-form is predominant in this type of ecological setting with irrigation systems providing ideal breeding site. Increased usage of insecticides for agricultural purposes and /or widespread of LLINs and repeated use of pyrethroids in IRS in the region could explain the high frequency of the kdr mutations.

Previous studies from this region and across some African countries have reported full susceptibility to the carbamate such as bendiocarb and malathion in *An. gambiae* mosquitoes (Ibrahim *et al.,* 2014; Kwiatkowska *et al.,* 2013 ; Antonio-Nkondijo *et al.,* 2011) thus suggesting the use of this class of insecticide as an alternative to pyrethroids and DDT in IRS. On the contrary, in this study *An. gambiae s.l.* mosquitoes tested against bendiocarb show from suspected resistance to weak resistance indicating that the *An. gambiae s.l.* mosquito population in this region have started to develop resistance against carbamate thus blocking the possibility of using it as an alternative to pyrethroids and DDT in IRS.

The occurrence of DDT, permethrin and bendiocarb resistance has not been previously reported in the Bichi residential and Bichi agricultural sites. However, similar incidents of DDT and pyrethroids resistance and bendiocarb suspected resistance in *An. gambiae s.l.* population has been reported in Auyo, northwest Nigeria (Ibrahim *et al.,* 2014, Alhassan, *et al.,* 2015), Lagos, southwest Nigeria (Awolola *et al.,* 2002; Awolola *et al.,* 2007 and Oduola *et al.,* 2010) and evidence of bendiocarb resistance in *An. gambiae* population was also reported (Oduola *et al.,* 2012).

3.6 Conclusion

This work provides a first step in detecting insecticide resistant *An. gambiae s.l.* populations so that alternative controls plans can be made to deal with the situation. The use of appropriate malaria vector control strategy requires knowledge of the distribution and composition of the primary malaria vectors and their resistance profiles.

The findings presented here provide evidence for bendiocarb, DDT and permethrin resistance as well as high frequency of L1014F kdr mutation in *An. coluzzii* and *An. arabiensis* and low frequency of L1014S kdr mutation in *An. coluzzii* and *An. arabiensis*. The high insecticides resistance observed in *An. gambiae s.l.* populations in this study particularly for pyrethroids, a pattern that likely holds true for most parts of West and Central Africa, (Awolola, 2007; Akogbeto, 2010; Ndiath *et al.,* 2012; Ibrahim *et al.,* 2014) may compromise the efficacy of LLINs and IRS on which most African countries rely on to reduce malaria transmission. Bioassay between 2013 and 2014 indicated resistance to DDT and permethrin has settled in the Sudan Savannah region of northern Nigeria. Based on the findings of this study and also of previous studies by, Corbel *et al.,* 2007, Akogbeto *et al.,* 2010 and Ibrahim *et al.,* 2014, there is a need to look into alternatives to pyrethroid insecticides to manage resistance. The two mutations L1014F and L1014S co-occurred in the five populations belonging to agricultural and residential settings in Auyo and Bichi northwest Nigeria. The

L1014F mutation was more prevalent in these populations than L1014S mutation. The frequency of L1014F mutation correlated with resistance to DDT and Permethrin. Agricultural activities have an effect on kdr allele's distribution compared to non–agricultural activities in the residential settings with higher L1014F allelic frequencies in agricultural sites than residential. Similarly, these higher L1014F allelic frequencies in agricultural sites coincide or correlate with higher insecticide resistance in agricultural sites suggesting that kdr is the primary mechanism responsible for the observed phenotypic resistance in these locations. The L1014F mutation appears to be the most significant mutation in both *An. coluzzii* and *An. arabiensis* in northwest Nigeria at present, however there exists the possibility that other mechanisms were also present and acted to confer resistance.

It is believed that the findings of this study could help and guide the choice of suitable control programme and strategy particularly in the choice of insecticide. It is therefore recommended that, there should be inter-sectoral collaboration between pest management and vector control in agricultural and public health departments respectively towards monitoring and managing the impact of the growing market and usage of agricultural pesticides on the emergence of vector resistance and also to embark on public enlightenment on the danger and repercussions of the abuse of chemical insecticides, pesticides and herbicides both in the residential and agricultural settings towards sustainable resistance management strategies. The WHO bioassays and molecular assays performed in this chapter were able detect phenotypic resistance and resistant alleles. To further unveil information on specific mechanisms responsible for the resistance recorded, these Anopheline mosquitoes were subjected to biochemical assays. All these assays are expected to provide effective vector resistance management strategies. Therefore, in chapter four of this study, biochemical assay for glutathione was employed to measure the glutathione levels in the adults *An. gambiae* raised from residential and agricultural field-caught larvae.

Chapter Four

Glutathione in *Anopheles gambiae s.l.*: Levels and variation from agricultural and residential settings

CHAPTER FOUR

4.0 Glutathione in *Anopheles gambiae s.l.:* Levels and variation from agricultural and residential settings

4.1 Introduction

Glutathione (GSH) is a tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) that is essential to a number of cellular processes. GSH is widely distributed among all living organisms and is associated with detoxification pathways, especially the Glutathione S- transferases (GSTs), antioxidant defence. The glutathione molecule contributes to detoxification of xenobiotics, and regulation of intracellular redox environment. It also functions as a crucial intracellular antioxidant (Meister, *et al.,* 1983; Sies, 1999). As a major component of phase II detoxification, the nucleophilic addition of the GSH thiol group to electrophilic centres of various endobiotic and xenobiotic substances by an extensive family of glutathione transferases render them more water soluble and thereby facilitates their excretion(Franco et al., 2007)

Glutathione occurs in two free forms: reduced (rGSH) and as glutathione disulphide or oxidized (GSSG). Also, it exists bound to thiol groups of proteins. The γ - glutamyl linkage and the presence of sulfhydryl group in GSH allow it to participate in some physiological activities. Glutathione concentrations occur in the millimolar range in cells, and the highest values have been found in hepatocytes, leukocytes, eye lens cells, and erythrocytes (Pastore, *et al.,* 2003). Under normal condition, GSH is present mostly in the reduced form but converted to the oxidized form during oxidative stress. The oxidized form can be converted back to the reduced form through the action of the enzyme glutathione reductase. Thus, the ratio of the reduced to oxidized form of GSH indicate the redox state of the cell. Under

normal physiological conditions, the ratio of rGSH to GSSG levels remain above 99%, but the ratio can change markedly during oxidative stress (Lu, 1999).

Cell stress can significantly increase reactive oxygen species (ROS) levels (Morrell, 2008; Sato *et al.,* 2014). Sato *et al.,* 2014, indicated that ROS can modify other oxygen species, proteins, or lipids, a situation often termed oxidative stress because they are highly reactive in nature. In this regard, maintaining healthy cellular ROS levels is vital to the proper physiologic function of numerous cell types in the body. Reduced GSH, the most abundant non-protein thiol antioxidant in cells, is essential for protection against oxidative injury (Valko *et al.,* 2007; Sato *et al.,* 2014). γ - Glutamylcysteine synthetase (γ - GCS) is the enzyme catalysing the first and rate-limiting step in the denovo GSH synthesis (Meister, 1983). The response of a cell to a stress often involves changes in GSH content, which may first be consumed in reactions that protect the cell by removing the deleterious compound and then restored to levels which often exceed those found before exposure to the stressor. GSH is depleted as it forms conjugates with a great variety of electrophilic compounds, primarily through the action of glutathione S-transferases (GST) (Hubatsch *et al.,* 2002). Conjugation with GSH is a frequent, although not universal, aspect of both xenobiotic and normal physiological metabolism, as mentioned above, and has been thoroughly reviewed (Strange *et al.,* 2000). When glutathione conjugates are formed with small molecules they are then excreted from cells (Akerboom and Sies, 1989), which is generally considered an important detoxification mechanism, including the removal of electrophiles (Milne *et al.,* 2004). Glutathione peroxidase uses GSH as a cofactor to remove peroxides from the cell, leading to the formation of glutathione disulfide, GSSG. GSH must then be replaced through either enzymatic reduction of GSSG by glutathione reductase or de novo synthesis.

Since GSH is a substrate for the glutathione S-transferase system, the tripeptide may become rate-limiting when organisms are exposed to large amount of a xenobiotic. Therefore, one can assume that a lower level of GSH in particular stages of the insect would decrease the protection to poisoning afforded by a functional GSH-transferase system (Saleh *et al.,* 1978). Previous work of Hazelton and Lang(1978), indicated marked life-span changes in GSH content with lower GSH concentration reported in the adult mosquito (*Aedes aegypti*). Possible metabolic mechanisms for this aging-specific decrease in glutathione status include GSH oxidation, utilization, and degradation. However, the work of Hazelton and Lang in 1979 and 1980 indicated that these do not account for the GSH decrease, rather the only other major possibility is GSH synthesis (Hazelton and Lang, 1983). Therefore, because of its involvement, along with glutathione S-transferases in the metabolism of a number of insecticides (Yang, 1976; Pastore *et al.,* 2003), monitoring the intracellular levels and distribution of the free forms (reduced and oxidized glutathione) is an important aspect of insect biochemistry that would help in understanding how GSH homeostasis could be affected under different conditions.

The objective of this chapter therefore, is to investigate the differential levels of the different forms of glutathione under agricultural and residential settings and to establish correlations between these levels and the resistance status of the *An. gambiae* sampled from agricultural and residential breeding ecologies in northern Nigeria. This will provide information on the varying levels of the three major forms of glutathione (total, reduced and oxidized) in Anopheles mosquitoes thriving in these breeding ecologies in Nigeria. The study also explores the feasibility of using GSH status in *An. gambiae* to monitor adaptation and resistance to insecticides.

4.2 Experimental approach

4.2.1 Preparation of mosquito homogenate

The experimental organism was West African malaria vector *An. gambiae s.l.*

To determine the levels of total, oxidized and reduced glutathione (tGSH, GSSG, and rGSH) in the experimental organism, homogenates of adult mosquitoes were prepared. The homogenates was prepared by homogenizing 20-30 adult mosquitoes (50-60 mg) in ice-cold potassium phosphate buffer (500 mM/5mM EDTA, pH7.2) in 1.5ml microfuge tubes with Pellet Pestle Motor (Kontes Anachem, Mettler Toledo, Luton, Bedfordshire, U.K.). The homogenization was carried out on ice. The homogenates were centrifuged at 10,000g for 10 min at 4°C using an Eppendorf centrifuge 5417R, Motor Park Way, New York, the United States (Imam and Deeni, 2014). The volume of the supernatant was measured then divided into two equal portions, one for protein assay the other for glutathione assay.

4.2.2 Protein Determination Assay

Protein standards were prepared in the same buffer as in the samples to be assayed (Abram *et al.,* 2009). A standard curve was made using Bovine serum albumin (BSA) of concentration 10mg/ml. The protein concentration of each homogenate was determined by using Bradford Reagent in 96 well plates following manufacturer's protocol. www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf).

4.2.3 Measurements of total (tGSH), oxidized (GSSG) and reduced (GSH) glutathione

4.2.3.1 Sample preparation/ deproteination

The second portion of the mosquito homogenates for glutathione assay was first deproteinized to remove interfering proteins and metabolizing enzymes. Also, the acidic environment of the deproteinized sample improves the stability of glutathione. The deproteination was done with the 5% 5-Sulfosalicylic acid (SSA) solution, centrifuged to

remove the precipitated protein. The sample was aliquot into two portions; one for tGSH and the other for GSSG assays.

4.2.3.2 Total GSH assay

In order to investigate the relationship and correlations between levels of the three forms of glutathione (tGSH, GSSG, and rGSH) and the resistance status of the *An. gambiae*, an assay of total glutathione (tGSH) and oxidized glutathione (GSSG) was carried out on mosquitoes sampled from the five sampling sites (two residential and three agricultural) using Glutathione assay kit CS0260 by Sigma-Aldrich. (www.sigmaaldrich.com/united-kingdom.html). Total GSH and GSSG were assayed by a modification of the enzymatic recycling method of Tietze (1969) using glutathione assay kit CS0260 (Sigma–Aldrich), according to manufacturer's instructions.

In order to measure the level of tGSH, any GSSG present in the sample was converted to rGSH by glutathione reductase. In this method, the rate of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) reduction is proportional to the amount of either GSH or GSSG present. The enzymatic recycling reaction was initiated by the addition of NADPH, and the rate of DTNB reduction was determined from the increase in the yellow product 5-thio-2-nitrobenzoic acid (TNB) at A_{412}. Measurement of the absorbance of the wells at 412 nm was done using a Microplate reader (Modulus microplate, Turner Biosystems, California USA).

Calculations

Glutathione concentration of the samples can be determined either by the end point method or the kinetic method. The end point method is adequate for most purposes (Merck Millipore, 2015). The end point method was used to determine the GSH concentration of the samples. One measurement at 25 minutes was taken. This rate was corrected for the reaction of DTNB with glutathione reductase without the tissue sample according to

Hazelton and Lang (1980). The full assay was performed according to manufacturer's protocol.

4.2.3.3 GSSG Assay

To measure oxidized glutathione in samples, all reduced GSH present in the samples were removed or quenched by treatment with 2-Vinylpyridine (http://www.arborassays.com/document/inserts/K006-FID.pdf) before addition of the Ellman's reagent and glutathione reductase (Griffith, 1980). A solution of ethanolic 2-Vinylpyridine was made by adding 27µl of 2-vinylpyridine (2-VP) (Sigma Catalog number 132292) to 98µl ethanol in a fume hood.

5-Sulfosalicylic acid (SSA) treated samples, and GSSG standards stocks were then treated with 1µl of the ethanolic 2-VP solution for every 50µl of the sample and incubated for 1h at room temperature. The sample (supernatant) was diluted 1: 2.5 with assay buffer. GSSG standard solutions were prepared by serial dilution of a 50µM GSSG made from the 10mM GSSG stock solution. Measurement of GSSG was performed as described for total GSH after derivatization of GSH by 2-vinylpyridine. Reduced GSH was calculated from the difference between the total glutathione and the oxidized values. The difference between total and oxidized GSH was used to obtain the levels of reduced GSH. All the GSH levels were corrected for the milligrams of protein present in the sample and expressed as nmol/mg protein. Measurement of the absorbance of the wells at 412 nm was done using a Microplate reader (Modulus microplate, Turner Biosystems, California USA). In order to assess whether the GSH levels was related to resistance, SPSS statistical package was used to calculate the Coefficients of correlation.

Chemicals

NADPH, yeast glutathione reductase (400 units/ml), 5, 5'-dithiobis (2-nitrobenzoic acid (DTNB), 5-Sulfosalicylic acid (SSA), GSH and GSSG standards, Dimethyl Sulfoxide (DMSO) and 2-vinylpyridine (2-VP) were obtained from the Sigma-Aldrich company (St. Louis, MO

63103 USA). Solutions of these reagents were prepared in 500mM potassium phosphate/ EDTA butter pH 7.2. Other chemicals were reagent grade, and sterile distilled water was used.

4.3 Results

4.3.1 Assessing the levels of the different forms of glutathione in An. gambiae from agricultural and residential zones

In order to investigate the relationship and correlation between levels of the three forms of glutathione (tGSH, rGSH and GSSG) and resistance status in *An. gambiae*, assays for tGSH and GSSG were carried out using mosquitoes sampled from agricultural and residential zones in Nigeria.

Tables 4.1 and 4.2 show the levels and the distributions of the three forms of GSH (total, reduced and oxidized) in *An. gambiae* sampled across the sampling sites in 2013 and 2014 respectively. The ratios of oxidized and reduced relative to the total GSH described. Based on the ratios obtained, more than 70% GSH is present in the reduced form across the sampling sites.

Table 4.1 Glutathione levels (nmol/mg protein) in the *An.gambiae* breeding in agricultural and residential sites in Nigeria in 2013

Site	tGSH[a] (Total)	GSSG (Oxidized)	GSH (Reduced)	GSH: tGSH[b]	GSSG: GSH[c]	GSSG: tGSH[d]	Study Zone[e]
AA1	56.4 ± 8.9	12.6 ± 0.8	43.8 ± 8.1	0.78	0.29	0.22	A
AA2	116.4 ±10.1	26.2 ± 1.0	90.3 ± 9.1	0.78	0.29	0.22	
BA	96.1 ± 7.9	16.6 ± 0.1	79.5 ± 7.8	0.83	0.21	0.17	
AR	42.5 ± 5.7	8.9 ± 1.4	33.6 ± 4.3	0.79	0.27	0.21	B
BR	41.1 ± 4.3	9.3 ± 0.9	31.8 ± 3.4	0.77	0.29	0.23	

[a]Mean ± S.D for three determinations, [b, c, d] Ratios of reduced to total; oxidized to reduced and oxidized to total glutathione; AAI = Auyo agricultural 1; AA2 = Auyo agricultural 2; BA = Bichi agricultural; AR= Auyo residential; BR = Bichi residential; [e]Zone : A = intensive agriculture; B = residential breeding ecologies

Table 4.2 Glutathione levels (nmol/mg protein) in the *An.gambiae* breeding in agricultural and residential sites in Nigeria in 2014

Sites	tGSH[a] (Total)	GSSG (Oxidized)	GSH (Reduced)	GSH: tGSH[b]	GSSG: GSH[c]	GSSG: tGSH[d]	Study Zone[e]
AA1	113.0 ± 0.3	25.4 ± 0.1	87.7 ± 0.2	0.78	0.29	0.22	A
AA2	111.3 ± 1.4	25.2 ± 0.0	86.1 ± 1.4	0.77	0.29	0.23	
BA	101.1 ± 4.5	25.3 ± 0.3	75.8 ± 4.2	0.75	0.33	0.25	
AR	114.3 ± 1.3	19.6 ± 0.1	94.7 ± 1.2	0.83	0.21	0.17	B
BR	116.0 ± 1.1	18.5 ± 0.2	97.5 ± 0.9	0.84	0.19	0.16	

[a]Mean ± S.D. for three determinations, [b, c, d] Ratios of reduced to total; oxidized to reduced and oxidized to total glutathione; AA1 = Auyo agricultural 1; AA2A = Auyo agricultural 2;; BA = Bichi agricultural; AR = Auyo residential; BR = Bichi residential; [e]Zone : A = intensive agriculture; B = residential breeding ecologies

The relative distribution of glutathione showed that the levels of tGSH and rGSH in Anopheline mosquitoes across the two study zones appeared to be similar while the levels of GSSG seemed to be higher in Anopheline mosquitoes from the study zone A (intensive agricultural study sites) compared to the study zone B (residential study sites). GSSG level in zone A in 2013 was about 2.03 fold higher than those of zone B. Similarly in Auyo GSSG level in zone A was about 2.21 fold greater than those of zone B while in Bichi GSSG level in zone A was about 1.82 fold higher than those of zone B. In 2014 the GSSG level in zone A was about 1.33 fold greater than those of zone B. In Auyo and Bichi for 2014 GSSG levels in zones A were 1.32 and 1.41 folds higher than those from zone B respectively.

To investigate the differential mean distribution of tGSH, GSSG and rGSH a one-way ANOVA in SPSS v 22 was used. The results show no significant differences ($p=0.562$ and 0.138) in the mean distribution of total and reduced glutathione levels across the study zones. However, the average distribution of oxidized glutathione across the study zones was statistically significant ($p=0.000$).

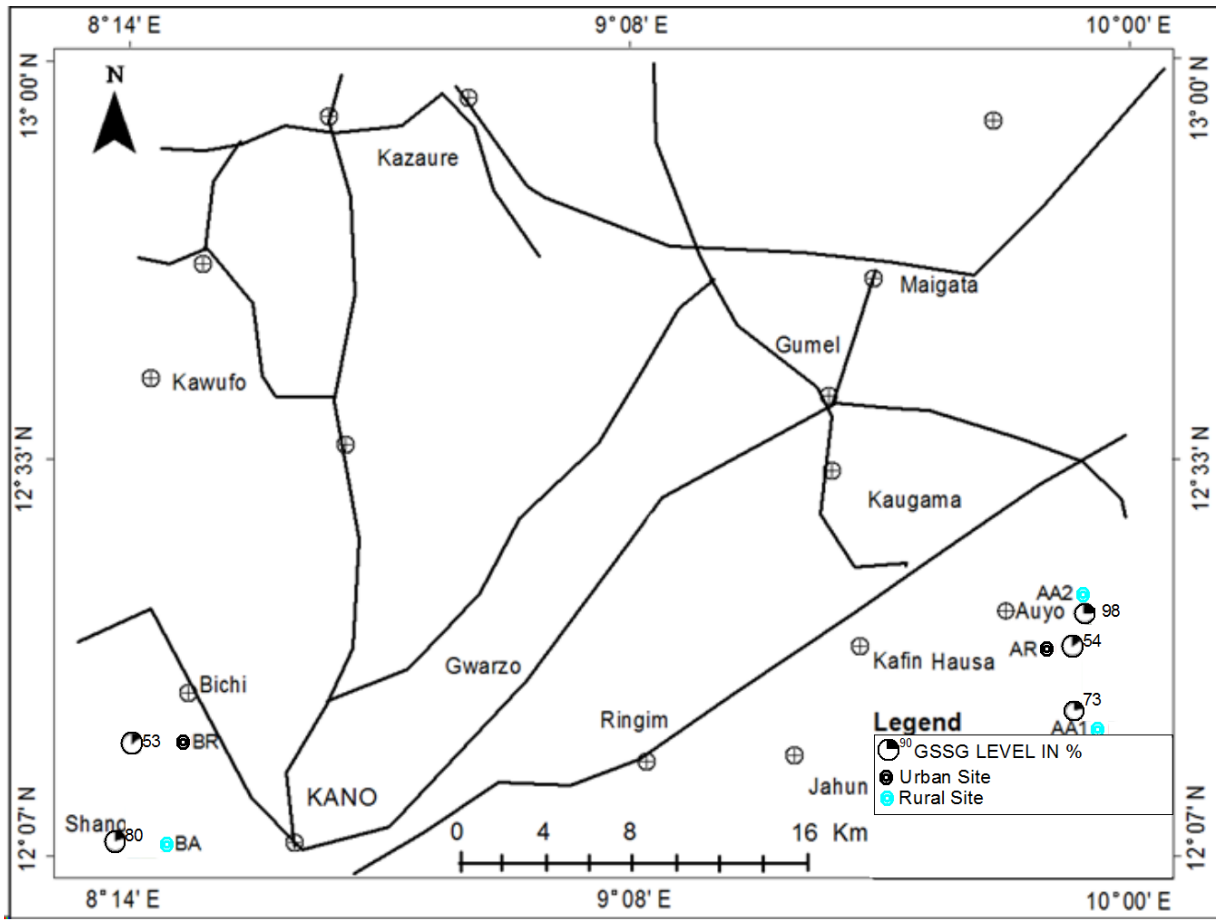


Figure 4.1 Map of the study areas in Nigeria showing the distribution of GSSG in *An. gambiae* population.

4.3.2 Relationship between levels of the three forms of glutathione and resistance status in Anopheles gambiae

To evaluate the role of glutathione in insecticides resistance and the impact of its differential levels on insecticide resistance in *An. gambiae*, the relationship and correlations between the three forms of GSH levels and the mortality rates were examined. A statistical tool in SPSS v.22 was employed to investigate these relationships.

4.3.2.1 Correlations between the three forms of glutathione

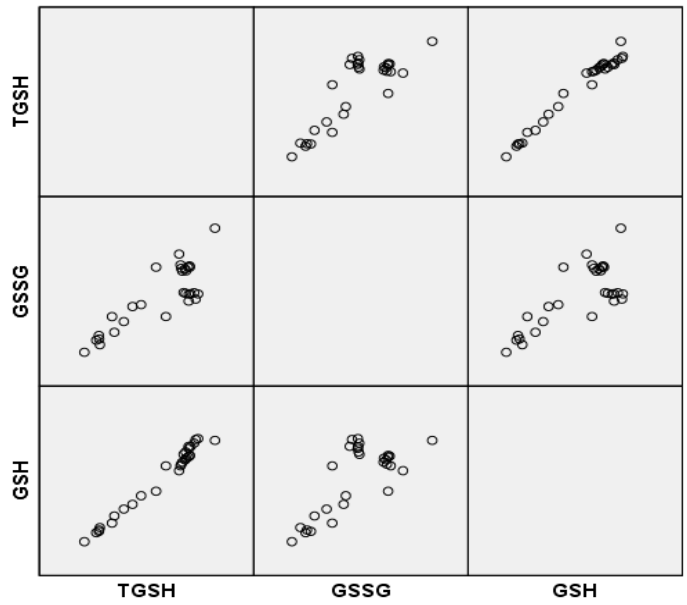
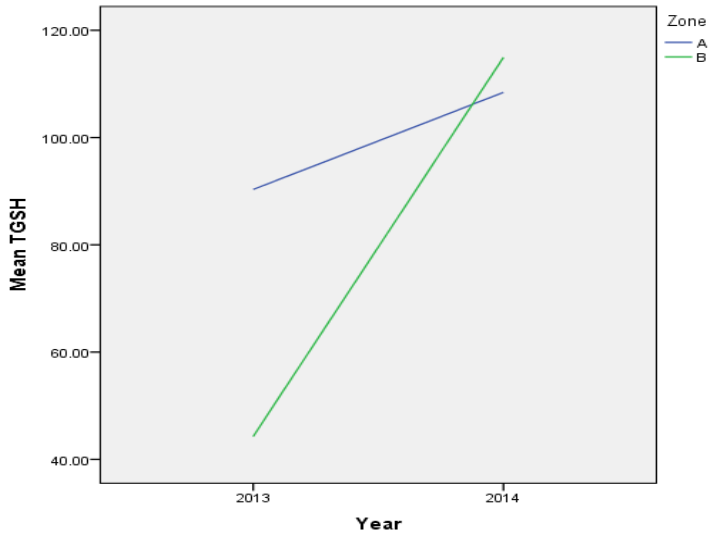


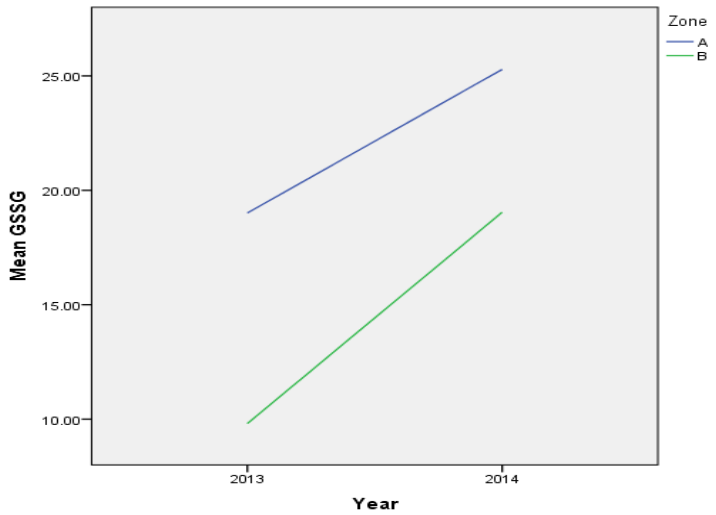
Figure 4.2 Scatter plot (matrix) of correlations between TGSH, GSSG and GSH levels. The levels of TGSH, GSSG and GSH were determined as described in sections 4.2.3.2 and 4.2.3.3 in *An. gambiae* population from northern Nigeria

The results of the correlation analysis showed overall TGSH is significantly related to GSH (p= 0.000) and GSSG (p= 0.000) in both agricultural and residential zones (See appendix VII). The results of the Pearson correlation analysis showed there were significant negative correlations between GSSG and mortality to DDT (P=0.050) in both agricultural and residential zones. Thus, increased GSSG levels observed correlate with higher insecticides resistance.

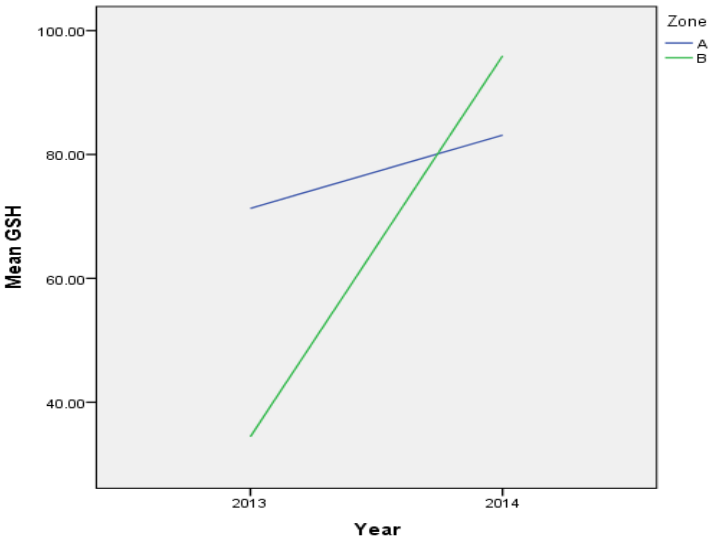
TGSH and GSH are considered one single factor because of correlation of 0.989. Interaction effect in the zones within the two years showed differences between the zones.



A



B



C

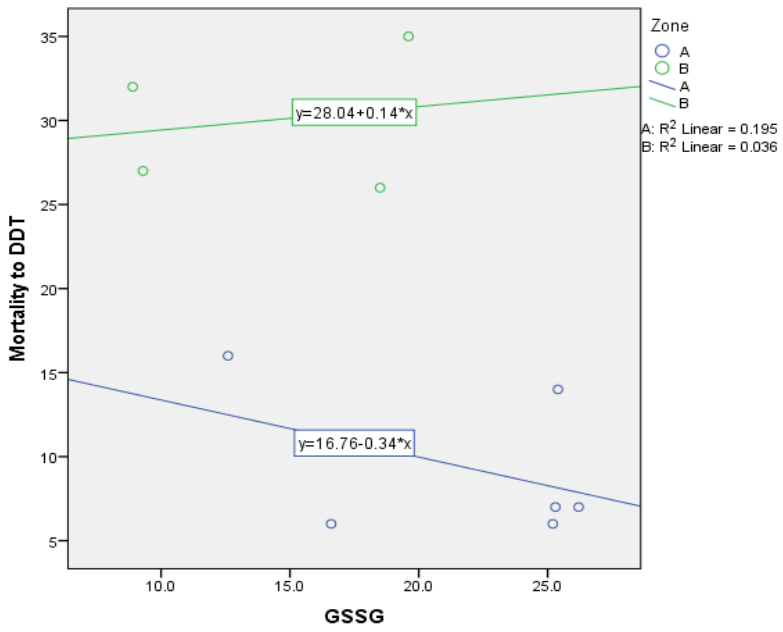
Figure 4.3 Interaction effects between zone A and B in 2013 and 2014: (A) TGSH, (B) GSSG, (C) GSH

Figure 4.2 (A) showed TGSH increased from zone A to B in 2014 and decreased from zone A to B in 2013 probably due to higher xenobiotic load from agricultural pesticides. Thus more GSH was used in zone A than in zone B. Figure 4.2 (B) showed GSSG seems to lack significant interaction while Figure 4.2 (C) showed a significant interaction similar to that in TGSH.

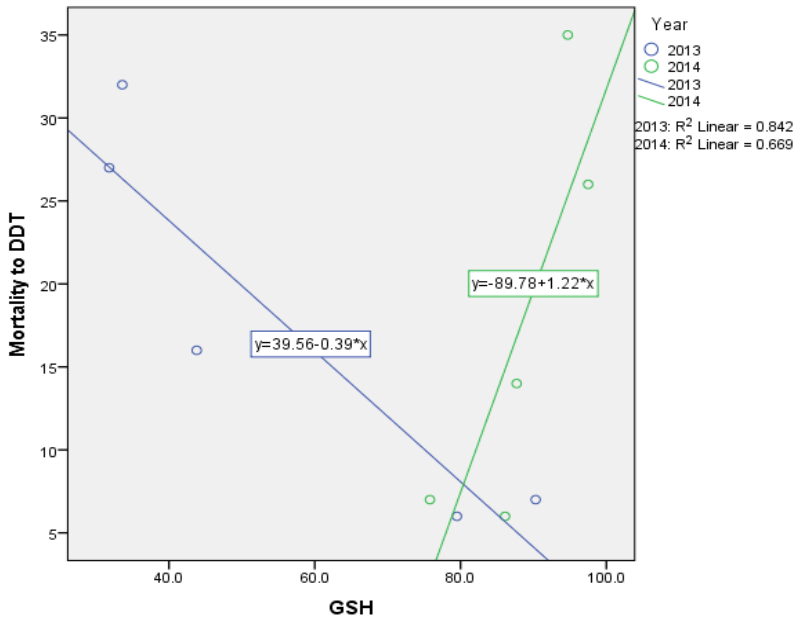
4.3.2.2 Correlations between glutathione levels and mortality to insecticides

The results of the correlation analysis showed only GSSG has significant correlation with mortality due to exposure to DDT (=0.050). See appendix VIII.

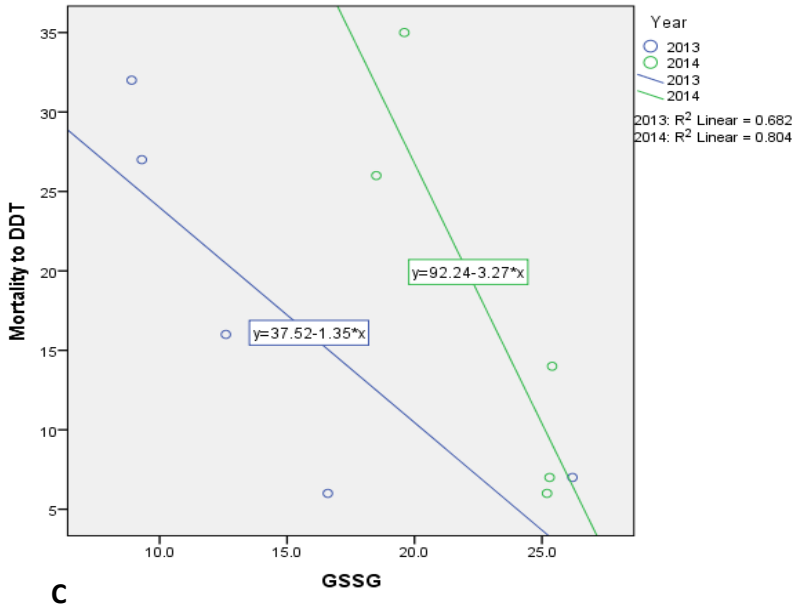
No interaction effect was observed between mortality due to DDT and glutathione levels based on zone (Figure 4.3 A) but interaction effect was observed based on year where decrease in mortality due to DDT with increase in GSH was observed in 2013 while an increase in mortality due to DDT with increase in GSH was recorded in 2014(Figure 4.3 B) and mortality to DDT decreased with increase in GSSG in 2013 and 2014 (Figure 4.3 C).



A



B



C

Figure 4.4 Interaction effect between mortality to DDT and glutathione levels: based on zone (A), based on year (B) and (C)

4.4 Discussion

The overall aim of the work presented in this chapter was to investigate the differential levels of GSSG and rGSH in *An. gambiae* under agricultural and residential settings and to establish correlations between these levels and the resistance status of the *An. gambiae* sampled from the two different breeding ecologies in northern Nigeria. The study further explores the feasibility of using GSH status in *An. gambiae* to monitor adaptation and resistance to insecticides. This study detected the presence of the two forms of glutathione; oxidized and reduced and their relationship with insecticides resistance in *An. gambiae* mosquitoes from agricultural and residential settings.

The work presented in this chapter determined the levels per mg protein of the different forms of GSH in *An. gambiae s.l.* collected from breeding sites located across the two study zones. The result obtained, shows more than 70% of the assayed GSH was present in the reduced form across the sampling sites in zones A and B. Furthermore, no significant differences ($p=0.562$ and 0.138) in the mean distribution of total and reduced glutathione levels were observed across the study zones. However, the average distribution of GSSG across the study zones was higher in study zone A compared to zone B. Pearson correlation analysis showed that the GSSG was significantly correlated with mortality ($p=0.050$).

Extensive agricultural practice is suspected as a source of selection pressure for the adaptation to insecticides in *An. gambiae*. The higher resistance to insecticides by mosquito populations in agricultural sites could be due to the impact of agricultural pesticides. This is further supported by the higher levels of GSSG recorded in study zone A that also recorded higher levels of environmental chemicals such as nitrates, nitrites, sulphates, carbon content and phosphates compared to zone B. This observation; higher GSSG concentrations is consistent with previous studies (Lipke & Chalkley, 1962; Oeriu & Tigheciu, 1964; Imam,

2013). In terms of mortality; lower mortality to insecticides implies high resistance to insecticides. The increased consumption of rGSH led to the build-up of higher levels of GSSG in zone A, which then correlated positively with resistance.

The increased oxidative stress was evidenced by the significantly higher levels of environmental chemical factors recorded in zone A compared to zone B. Observation from previous studies (Araujo *et al.,* 2008; Stephensen *et al.,* 2002) have established increase in oxidative stress induced by xenobiotic overload as a source of generation and accumulation of GSSG, leading to lower GSH/GSSG ratio in various organisms. The high levels of the oxidized form of GSH and lower reduced form of GSH recorded, and the ratio of the reduced to oxidized form of GSH indicate the redox state of the cell. It reflects the cells are under oxidative stress.

According to Chen *et al.,* (2004), the level of total and reduced glutathione may increase, reduce or may not change significantly under conditions of oxidative stress. However, levels of GSSG and the ratio between oxidized and reduced forms of glutathione is usually used as the more accurate indicator of the redox state of a cell, particularly in situations where no apparent and significant induction of the synthesis of glutathione occurred (Chen *et al.,* 2004; Araujo *et al.,* 2008). Thus, finding from this study appeared to agree with these observations. Therefore, it could be argued that while significant induction of the synthesis of glutathione may not have occurred in *An. gambiae* sampled across the two zones; there was, however, a significant increase in utilization of rGSH in mosquito samples collected from breeding sites where higher of environmental xenobiotics were recorded. The observed low changes in total and reduced glutathione contents in mosquitoes across the sampled sites despite differences in the levels of environmental chemical factors could probably be explained by the fact that glutathione is constitutively synthesized and abundantly available in all organisms (adjustable homeostatic balance). Moreover, it could be that the levels of reduced

glutathione recorded in this study represent the normal threshold levels in *An. gambiae*. This implies that despite the recorded high resistance status in mosquitoes from agricultural sites where higher levels of xenobiotics were also recorded, the levels of the glutathione was sufficient for its role in the overall detoxification process. This finding is consistent with that of Imam (2013). Lu (2009) highlighted that levels, availability and activities of glutathione responds to changes in oxidative stress induced by xenobiotic overload. It could be stated that the sources of the oxidative stress in the *An. gambiae* sampled may not be sufficient enough to cause the induction of glutathione synthesis above the threshold levels even though they were able to select for the emergence of *An. gambiae* that is highly resistant to most insecticides.

The rGSH is being converted to the GSSG form during oxidative stress and/or metabolic detoxification processes. The depletion of GSH due to oxidative stress reported in this study is consistent with previous study by Forgash (1951) in the American cockroach and similar pattern were found in mammals (Dickinson *et al.,* 2004; Hashmat *et al.,* 2011; Khan *et al.,* 2012) that the response of a cell to a stress often involves changes in GSH content, which may first be consumed in reactions that protect the cell leading to the formation of GSSG suggesting the process is highly conserved among organisms including *An. gambiae*.

It is not clear whether DDT is the only insecticide where glutathione can inform the resistance status because DDT resistance has settled in the areas under study; however the data of the bioassays shows that resistance to the other two insecticides especially Bendiocarb has not settled in these areas. So is this because resistance to these insecticides has not settled that is why significant effect of GSSG was not observed? So is it a transition or because glutathione can only tell resistance manifestation that has settled due to a particular class of insecticide like DDT? The correlation between resistance status and GSSG levels indicates resistance has settled while no correlation indicates resistance has not

settled yet. Mosquito control in areas where resistance has settled due to a particular insecticide such as DDT would not yield the desired results. Therefore, similar studies covering larger area and longer period are needed to fully establish this.

4.5 Conclusion

The study showed increased GSSG levels from mosquitoes from agricultural sites. This increase in GSSG in mosquitos from agricultural sites suggests an interaction of insecticides with GSH and a possible increase in oxidative stress induced by xenobiotic overload coming from extensive farming practices resulting to lower GSH/GSSG ratio. Thus, the study highlighted glutathione status may be mediating the response, tolerance and possibly adaptation of *An. gambiae* exposure to insecticide. The results of this study suggest a close relationship between an increase in GSSG level and resistance status of *An. coluzzii* (*An. gambiae* M-form) from agricultural sites than in *An. coluzzii* from residential sites. This study further demonstrated that GSSG levels and kdr mutations interact to enhance resistance to insecticides. This represents a significant threat to insecticide resistance management for malaria control. Methods of detecting insecticide resistance are cumbersome involving WHO bioassay, biochemical and molecular assays. Our results indicate that measuring the levels of GSH in the mosquitoes sampled could possibly be used as a tool to assess and detect the insecticide resistance status of *An. gambiae s.l.* population.

To further unveil information on specific mechanisms responsible for the resistance recorded, these Anopheline mosquitoes were subjected to molecular assays for effective vector resistance management strategies. Therefore, in chapter five of this study, semi-quantitative real-time polymerase chain reaction (Semi-quantitative RT-PCR) were employed to measure the differential *GCLM, GCLC* and (*Nf2e1*) *Nrf2* expressions in the Tiassale and Kisumu strains and uncharacterised strains of adults *An. gambiae* raised from caught larvae

from residential and agricultural fields and either when challenged or unchallenged with insecticide.

Chapter Five

Differential Expression of *Nf2e1* (*Nrf2*), *AGAP012038* (*GCLM*) and *AGAP001512* (*GCLC*) Genes in Selected Strains of *Anopheles gambiae*

CHAPTER FIVE

5.0 Differential Expression of *Nf2e1* (*Nrf2*), *AGAP012038* (*GCLM*) and *AGAP001512* (*GCLC*) Genes in Selected Strains of *Anopheles gambiae*

5.1 Introduction

Anopheles gambiae (An. gambiae) as previously mentioned is the principal malaria vector in Africa, where vector control measures involve the use of insecticides and malaria vector control programs in Africa rely heavily on the use of pesticides for insecticide-treated nets (ITNs)/long-lasting insecticide-treated nets (LLINs) and for indoor residual spraying (IRS) (WHO, 2012). Massive and extensive use of insecticides in agriculture (Yadouleton *et al.,* 2009) and high ITNs and IRS coverage, or recurrent space spraying interventions (Balkew *et al.,* 2010; Marcombe *et al.,* 2011; Ranson *et al.,* 2011) in public health has resulted in increasing resistance among malaria vectors due to the selection pressure placed on resistance genes. Misra *et al.,* (2013) reported that cross-resistance to different classes of insecticides has further complicated efforts to control insect populations. Thus, developing effective vector control strategies has become a primary focus for current research and has led to widespread efforts to understand the molecular mechanisms that underlie insect pesticide resistance. Resistance to insecticides arises mainly through target site resistance and metabolic resistance (Ffrench-Constant *et al.,* 2004; Perry *et al.,* 2011).

Insects employ an extensive array of enzymes, including cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxylesterases, which detoxify a broad range of endogenous and exogenous toxic compounds (Vontas *et al.,* 2001; Li *et al.,* 2007). These phase I and phase II enzymes can be transcriptionally activated in a constitutive manner due to mutations in either cis-acting elements or trans-acting factors, conferring pesticide resistance (Misra *et al.,* 2013). Metabolic resistance can also arise due to mutations

that increase the catalytic activity of these detoxification enzymes. In contrast to the genes involved in target site resistance, many genes associated with metabolic resistance are not vital for survival and thus tend to be more tolerant of genomic changes that alter enzyme function and/or expression. Furthermore, due to the broader spectrum of substrate specificity, cross-resistance to different classes of insecticides is more prevalent in metabolism based resistance (Misra *et al.,* 2013).

GSH is essential for protection against oxidative injury, through transcription regulation. Thus, the regulation of γ -GCS expression and activity is critical for GSH homeostasis.

The regulation of these genes is predominantly mediated by the electrophile response element or EpREs. The GSH biosynthetic genes; Glutamate-Cysteine Ligase, Catalytic Subunit (*GCLC*) and γ-glutamylcyteine ligase modifier subunit (*GCLM*) are regulated by nuclear factor-like 2 (*Nrf*2) that protects the cell from oxidative stress (Kalyanaraman, 2013; Sato *et al.,* 2014). Thus adaptive changes in GSH homeostasis are associated with Up-regulation of *GCLC* and *GCLM* all contributing to an adaptive response to cellular stress.

In *Drosophila melanogaster*, some genes involved in the metabolic activity are known to be up-regulated by the transcription factors Cap 'n' collar isoform (*CnCC*)/Drosophila Kelch-like-ECH-associated protein 1 (*dKeap 1*). These are orthologs to Nuclear factor erythroid 2-related factor 2 (*Nrf*2) / Kelch-like–ECH-associated protein 1 *(Keap 1)* signalling pathways in higher mammalians (Cao *et al.,* 2013; Misra *et al.,* 2013, Das *et al.,* 2014; Siller *et al.,* 2014). Recent studies (Misra *et al.,* 2013; Jones *et al.,* 2013; Guio *et al.,* 2014; Kuzin *et al*., 2014) have demonstrated that the evolutionarily conserved *Nrf2*/*Keap1* pathways play a central role in regulating the coordinate transcriptional response to xenobiotic compounds in *D. melanogaster*. In the absence of stress, *Nrf*2 is retained in the cytoplasm as an inactive complex with its cytosolic repressor, Kelch-like ECH associated protein-1 (*Keap 1*). The movement of *Nrf*2 from the cytoplasm to nucleus must be preceded by its dissociation from *Keap 1* through oxidative stress where it can heterodimerize with the small *Maf* (muscle

aponeurosis fibromatosis) proteins before it could bind to antioxidant response elements (AREs) in the genome (Atia and Bin Abdullah, 2014). *Nrf2*, *Maf,* and *Keap1* are all conserved in *D. melanogaster* and appear to maintain the same regulatory interactions as described in vertebrates (Misra *et al.,* 2011; Dhanoa *et al.,* 2013; Si and Liu, 2014). Activation of this pathway through electrophilic xenobiotics/oxidative stress is necessary and sufficient for xenobiotic-induced transcription of a broad range of detoxification genes in Drosophila species towards maintenance of intracellular GSH levels and redox homeostasis (Misra *et al.,* 2011; Deng and Kerpolla, 2013).

However, it is still not known whether the orthologs of *CnCC* in *An. gambiae* are involved in the maintenance of intracellular GSH levels and redox homeostasis. We, therefore, used bioinformatics and molecular biology approaches to show that *Nf2e1 / AGAP003645* pathways play central roles in the regulation of xenobiotic responses through *GCLC* and G*CLM* in *An. gambiae.* The present studies have identified *Nrf2/Keap1 (Nf2e1/AGAP003645*) pathway as active and key regulator of xenobiotic responses in both the insecticide resistant Tiassale strain, the uncharacterized insecticide selected strains and Kisumu strain of *An. gambiae.* This finding is consistent with the previous studies on the Drosophila model pathway (Misra *et al.,* 2011, 2013). These studies have implications for understanding the regulatory mechanisms of developing insecticide resistance and its impact in the control of mosquito-borne diseases.

5.2 Experimental Approach

5.2.1 Mosquito strains

Two established strains of *An. gambiae* were used (obtained from the Liverpool insect testing establishment (LITE) unit of the Liverpool School of Tropical Medicine (LSTM)). The various insecticide resistant Tiassale strain was derived from material collected in southern Ivory Coast, and the susceptible laboratory strain (Kisumu) was derived from material collected in

Western Kenya. Additionally, three other uncharacterized strains from Sudan Savannah region of northern Nigeria selected on three different insecticides according to the recommendation by the WHO (2013d).

5.2.2 WHO adult bioassay

A diagnostic test using standard WHO Test Kits tube was conducted using tarsal exposure to papers impregnated with discriminating concentrations of 4.0% DDT, 0.1% bendiocarb and 0.75% permethrin (WHO, 2013c). For each test, two to five day old female mosquitoes (n=10) each of the Tiassale and Kisumu strains and uncharacterized strains from northern Nigeria were transferred using an aspirator into a standard holding tube (containing untreated filter paper). Refer to section 3.2.1 in chapter three for detailed procedure of the adult bioassay. Cotton pads soaked in 10% sugar solution were provided during the 24 h holding period (WHO, 2013c; Vogelweith *et al.,* 2014).

5.2.3 Prediction of putative regulatory elements within GCLC and GCLM genes promoter regions

To predict the putative transcription factor binding sites (TFBS) within the 1.5kb genes promoters, an online analysis using ConSite (http://asp.ii.Uib. no:8090/cgi-bin/CONSITE/consite/) was performed (Mohammed *et al.,* 2014). The putative transcription binding sites for *AhR/ARNT* and *Nrf2/ARE* to *GCLC* and *GCLM* genes were searched using Consite website (http://consite. genereg.net/cgi-bin/Consite? rm=tin put) (Sandelin *et al.,* 2004; Miglani and Gakhar, 2013). ConSite is a user-friendly web-based interface that is used conventionally to identify cis-regulatory elements within genomic sequences.

5.2.4 Primer Design

Pairs of exon-exon boundary crossing primers for *Nf2e1 (Nrf2), AGAP012038 (GCLM)* and *AGAP001512 (GCLC)* (Table 5.1) were designed using Primer 3 online software (Broad Institute, USA) (http: //www.ncbi.nlm. nih.gov/tools/primer-blast/) against the *An.gambiae* PEST sequence.

Table 5.1 Primers used in semi-quantitative PCR (qPCR)

Gene	Accession Number	Sequence (5' – 3')	Annealing Temperature (°C)	Transcript length (bp)
NRFFP001	AGAP005300	CCGCATTTTCGCTTCTAGCC	64.7	210
NRFRP002		ATCCATTTCCTGGTGGTGGG	68.0	
GCLMFP01	AGAP012038	CAGTGACCCGCAGGAACTAC	60.5	185
GCLMRP01		TGGCAGATGTTGTCTAGCCG	59.6	
GCLCFP01	AGAP001512	GACCAGGACACACCAGGATG	61.6	286
GCLCRP01		TTGACTCCTTGTGGATCGGC	61.4	

Key: F= forward and R= reverse. The primer pairs were used on cDNA that was obtained after RNA extraction. The primers were designed using Primer 3 online software.

5.2.5 Quantification of Gene Expression

In order to measure the response of *An. gambiae* to insecticides end-point RT-PCR (semi-quantitative RT-PCR) using Gel densitometry analysis was used to quantify the extent to which GSH genes are expressed in *An. gambiae.*

5.2.5.1 Total RNA isolation

To prepare cDNA for the amplification of *Nf2e1 (Nrf2) AGAP012038 (GCLM)* and *AGAP001512 (GCLC)* primers, total RNA was isolated from Tiassale, Kisumu and uncharacterized strains of adults *An. gambiae* using RNAqueous 4PCR Kit (Cat. # AM1914, Ambion-life Technologies, Paisley, PA, UK) according to the manufacturers' recommendations. The uncharacterized *An. gambiae* strains were raised from agricultural and residential field-caught larvae challenged and unchallenged with DDT, Permethrin and

Bendiocarb insecticides. The quality and quantity of the RNA was then determined by running on 1.5% agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Oxfordshire, UK).

5.2.5.2 cDNA synthesis

2µg RNA was reverse transcribed to first strand cDNA using Superscript III reverse transcriptase (Cat. # 18080-051, Invitrogen, Life Technologies) and oligo dT20 following the manufacturer's instructions. The transcription process included incubation of the reaction mixture at 65°C for 5 min, followed by 50 min at 50°C. The cDNA was cleaned using Qiagen purification kit (Cat. # 28106, Qiagen, Valencia, CA, and USA) and quantified using Nanodrop 1000 Spectrophotometer (Nanodrop Technologies, Oxfordshire, UK). All cDNAs were diluted 10-fold with nuclease-free water, and two microliters (2µl) was utilized in each PCR reaction.

Table 5.2 Reactions set up for performing the semi-quantitative PCR reaction. PCR was carried out in Thermal cycler S1000™ Bio-Rad System

Components	**Quantity(µl)**
10 fold diluted cDNA (0.075µg)	2.0
My Taq Red Mix Polymerase	12.5
Forward primer (5µM)	1.0
Reverse primer (5µM)	1.0
RNase/DNase free water	8.5

The PCR conditions were: 95 °C for 1 min followed by 27 cycles of 95 °C for 15s, 60 °C for 15 s, 72 °C for 10 s with a final 10 min extension at 72 °C (Gaskins *et al.,* 2009). The final PCR product was resolved by electrophoresis. Images of the PCR Gel red- stained 1.5% agarose gels were acquired using Gel Doc 100 (Bio- Rad, Hercules, USA) and quantification of the bands was performed using Band scan analyzer 5.1software.

5.2.5.3 Selection of reference genes for genes quantification

The two primary methods of quantification are absolute quantification and the relative quantification (Sellars *et al.,* 2007). In absolute quantification, the exact number of copies of the gene of interest was calculated, while, in relative quantification, the expression of the gene of interest is expressed relatively to another gene. Gene expression must be normalized against a housekeeping gene (HKG) which is constitutively expressed in all cell types and tissues being used (Thellin *et al.,* 1999). The reference gene should not be regulated or influenced by the experimental conditions or between different tissues (Yilmaz *et al.,* 2012). Relative quantification is the most widely used technique. The rat

io between the amount of target gene and an endogenous reference gene, which is present in all samples were used to calculate gene expression levels (Mosquera, 2012).

Three reference genes: Ubiquitin *(UBQ),* Elongation factor protein *(Ef1),* and Ribosomal protein *(Rsp7)* (Table 5.2) were selected and used. Each gene was amplified in triplicate for the three biological repeats of the strains.

Table 5.3 Primer sequences used for normalizing genes (housekeeping genes)

Gene	GenBank	Sequence (5'-3')	References
Ubiquitin	AGAP007927-RA	ACAGACACGTTGGAAACATGC AAGGCTCGACCTCAAGTGTG	[1] [2] [6]
Elongation factor Protein	AGAP003541-RA	GGCAAGAGGCATAACGATCAATGCG GTCCATCTGCGACGCTCCG	[1] [3] [4] [6]
Ribosomal protein	AGAP010592-RA	AGAACCAGCAGACCACCATC GCTGCAAACTTCGGCTATTC	[1] [4] [5] [6]

References describe the use of individual housekeeping genes in qPCR. The numbers refer to Publications: (1) Wilding *et al.,* 2012, (2) Rebouças *et al*., 2013 (3) Matowo *et al*., 2014b, (4) Edi *et al*., 2014, (5) Mulamba *et al*., 2014 (6) Toe *et al.,* 2015

5.2.5.4 Semi-quantitative PCR (Gel densitometry analysis)

Gel densitometry analysis (Semi-quantitative PCR) was used to compare the differential expression of each of the test genes *(GCLC, GCLM* and *Nrf2)* with the housekeeping gene Ubiquitin (*UBQ*) in seven strains of *An. gambiae* using Gel DocTM imager (Life Technologies, Carlsbad, CA, USA). The primers (Table 5.1) were used to amplify the cDNA from all the *An. gambiae* strains.

For semi-quantitative analysis, the PCR must be in its exponential range providing doubling of the products amount with each cycle (Marone *et al.,* 2001; Gaskins *et al.,* 2009). The PCR products of each gene and ubiquitin were loaded on to the same gel red-stained 1% agarose gel. Stained gels were recorded and band intensity was evaluated using Bandscan analyzer 5.1software on Gel DocTM imager. Band intensity was expressed according to (Alizadeh *et al.,* 2011) as relative absorbance units. The ratio between the sample cDNA to be determined and Ubiquitin protein *(AGAP007927)* was calculated to normalize relative quantification results. Mean and standard deviation of all experiments performed were calculated after normalization to Ubiquitin. Statistical difference in expression levels was analyzed using SPSS 22 statistical package.

Statistical analysis of data using One-Way ANOVA

The One-way analysis of variance (ANOVA) was used to further statistically analyze data to establish any significance or correlation between data sets. These statistical tests were carried out using online SPSS Software version 22.

5.3 Results

5.3.1 Prediction of putative regulatory elements within the 1.8 kb GCLC AND GCLM genes promoters

In order to predict the putative transcription factor binding sites (TFBS) within the *GCLC* and *GCLM* gene promoters, which could improve insights into the genes regulated by a transcription factor (TF) (Talebzadeh and Zare-Mirakabad, 2014), an online analysis using ConSite was performed. Insilico analyses of *GCLC* and *GCLM* promoter regions (figures 5.1& 5.2) revealed the presence putative transcription factor binding sites for *Nrf2* and *AhR/ARNT* within the promoter regions. Refer to the genomic sequences in appendices III & IV respectively.

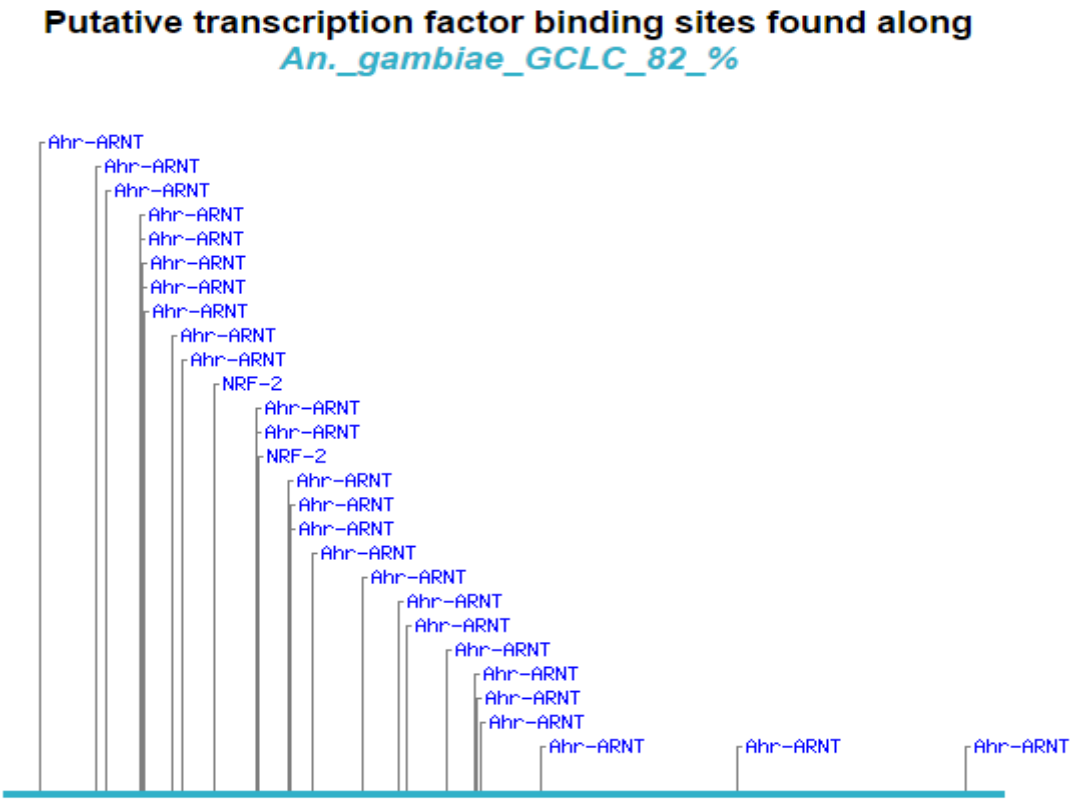


Figure 5.1 Putative transcription factor binding sites (TFBS) along *GCLC* promoter region in *An. gambiae* at 82% cut off score or stringency. The *An. gambiae* genomic Sequence 1.8 kb upstream TSS obtained from FlyBase was pasted into Consite revealed *AhR/ARNT* and *Nrf2/ARE* specifically selected TFBS. A total of 26 *AhR/ARNT* and 2 *Nrf2/ARE* putative transcription binding sites were found.

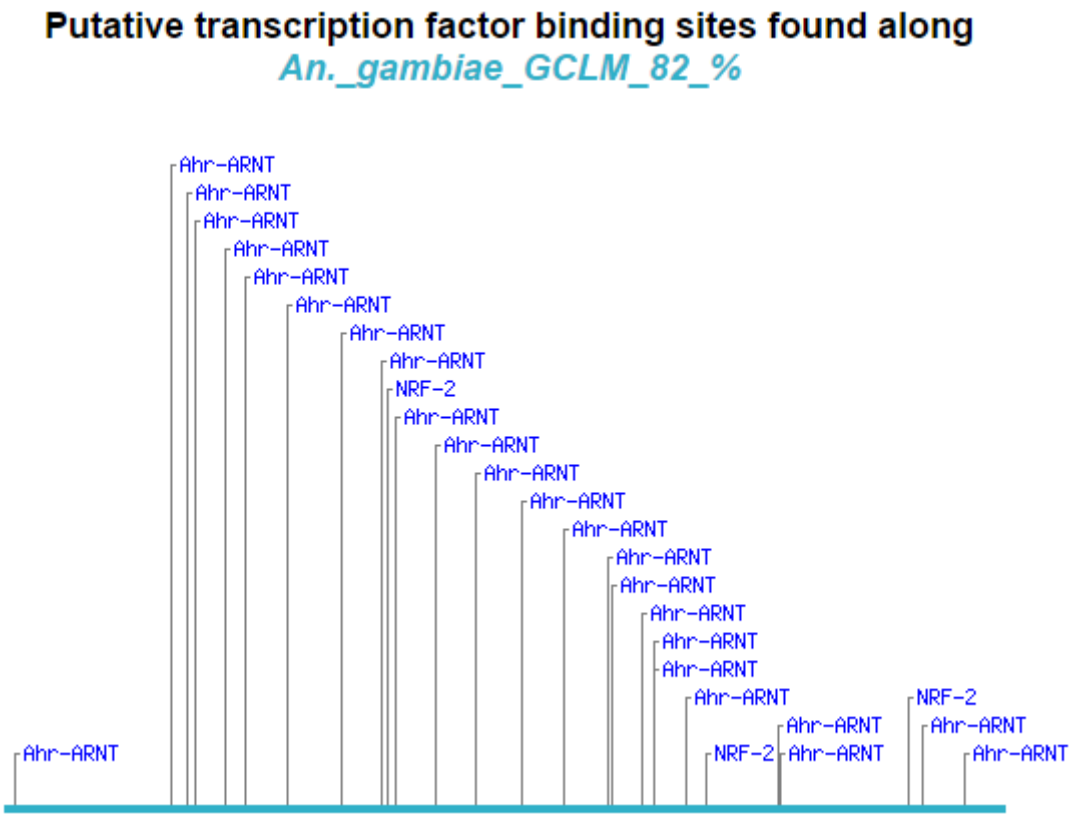


Figure 5.2 Putative transcription factor binding sites (TFBS) along *GCLM* promoter region in *An. gambiae* at 82% cut off score or stringency. The *An. gambiae* genomic Sequence 1.8 kb upstream TSS obtained from FlyBase was pasted into Consite revealed *AhR/ARNT* and *Nrf2/ARE* specifically selected TFBS. A total of 24 *AhR/ARNT* and 3 *Nrf2/ARE* putative transcription binding sites were found

5.3.2 Semi quantitative PCR (DNA quantification using Gel densitometry)

5.3.2.1 Selection of reference genes for genes quantification

To assess the differential expression levels of *GCLC, GCLM* and *Nrf2 (Nf2e1)* genes in permethrin, DDT and bendiocarb resistant and susceptible strains of *An. gambiae*, semi-quantitative end-point PCR was used to measure the expression levels of mRNA in *An. gambiae*, Figures 5.3 and 5.4 shows the bands intensities for the PCR. The expression data of *GCLC* and *GCLM* genes could be used to elucidate transcriptional regulatory networks and transcription regulation mechanisms (Ernst *et al*., 2010; Won and Wang, 2010).

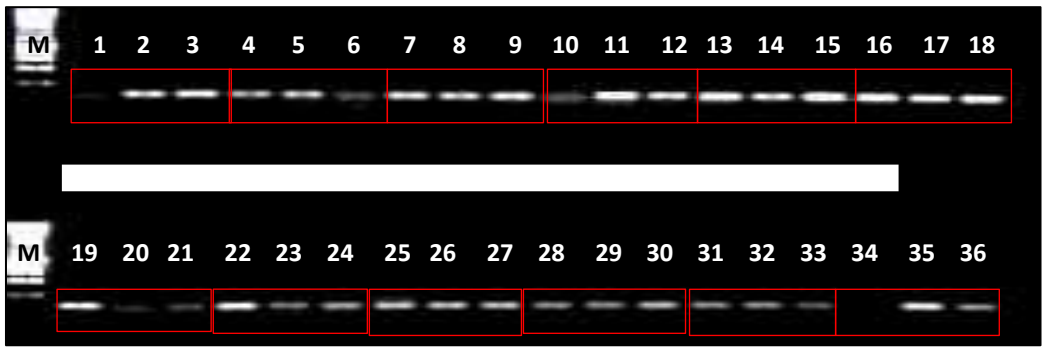


Figure 5.3 Semi-quantitative end-point PCR (DNA quantification using gel densitometry) in different strains of *An. gambiae* after exposure to insecticides. L 1-3- *UBQ*-Tias; L 4-6 –*UBQ*-Kis; L 7-9 *UBQ*-A-Perm; L 10-12-*UBQ*- A-Base; L 13-15- *Ef1*-A-Perm; L 16-18- *Ef1*-Tias; L 19-21-*Ef1*-Kis; L 22-24-*Ef1*-A-Base; L 25-27-*Rsp7*-Tias; L 28-30-*Rsp7*-A-Perm; L31-33-*Rsp7*-Kis; L34-36-*Rsp7*-A-Perm ; M is the Molecular weight ladder (Bioline).The gel was run for 40 min at 120 V.

Three genes (elongation factor protein, ribosomal protein and ubiquitin protein) were evaluated as reference genes. Of the three, only ubiquitin showed little variation in general expression levels between the seven strains of *An. gambiae* (Tables 5.5 and 5.6). Therefore, it was selected as the reference gene in this investigation.

Table 5.4 Selection of candidate reference genes in permethrin selected *An. gambiae* strains using semi-quantitative PCR

Candidate Reference gene		Tiassale (Mean ± SE)	Kisumu (Mean ± SE)	A-Perm (Mean ± SE)	A-Base (Mean ± SE)
Ubiquitin	(*UBQ*)	35.73 ± 5.05	30.06 ± 4.13	37.44 ± 2.45	40.59 ± 0.38
Elongation factor	(*EF1*)	40.13 ± 0.69	24.68 ± 7.17	31.51 ± 1.51	40.94 ± 0.00
Ribosomal Protein	(*Rsp7*)	31.27 ± 2.78	28.50 ± 3.78	23.81 ± 1.54	32.99 ± 6.97

Table 5.5 Selection of candidate reference genes in four strains of *An. gambiae* using semi-quantitative PCR

Candidate Reference gene		A-DDT (D) (Mean ± SE)	A-DDT (S) (Mean ± SE)	A-Bendi (D) (Mean ± SE)	A-Bendi (S) (Mean ± SE)
Ubiquitin	(*UBQ*)	25.92±1.16	25.06 ±0.57	24.93±1.62	24.61±0.09
Elongation factor	(*EF1*)	16.24±0.78	17.56±1.16	16.14±0.33	15.91±0.23
Ribosomal Protein	(*Rsp7*)	23.19±0.60	24.19 ±0.38	24.39±0.59	24.19± 0.38

Key: D= Dead S= Survived

5.3.3 Differential expression of detoxification genes

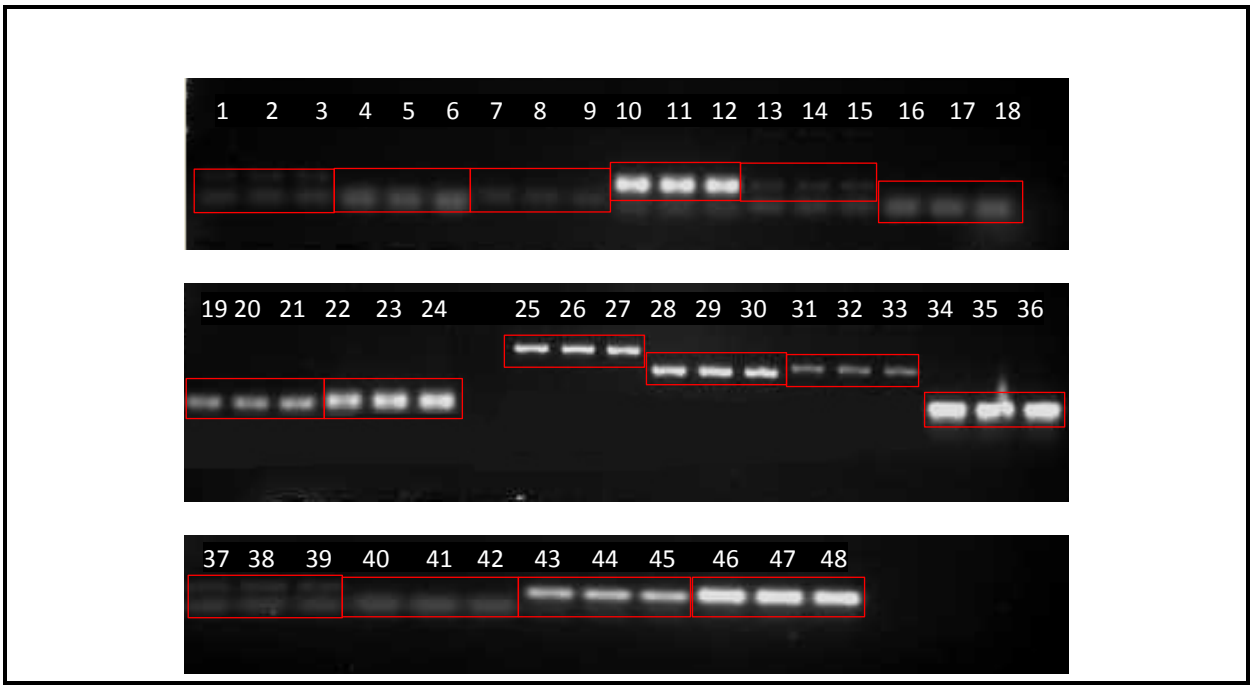


Figure 5.4 Semi-quantitative PCR (DNA quantification using gel densitometry) in different strains of An. gambiae after exposure to insecticides in agricultural zone. L1-3- *GCLC* – BD; L 4-6 –*GCLM* - BD, L 7- 9- *Nrf2*–BD; L 10-12-*UBQ*-BD; L13-15 – *GCLC*- BS; L16-18-*GCLM*- BS; L19-21-*Nrf2*-BS; L 22-24-*UBQ*-BS; L 25-27- *GCLC* Tia; L 28-30-*GCLM*-Tia, L 31-33-*Nrf2*-Tia; L 34-36-*UBQ* –Tia; L 37-39-*GCLC*-Kis; L 40-42-*GCLM*- Kis; L 43-45-*Nrf2*-Kis; L 46-48- *UBQ*-Kis

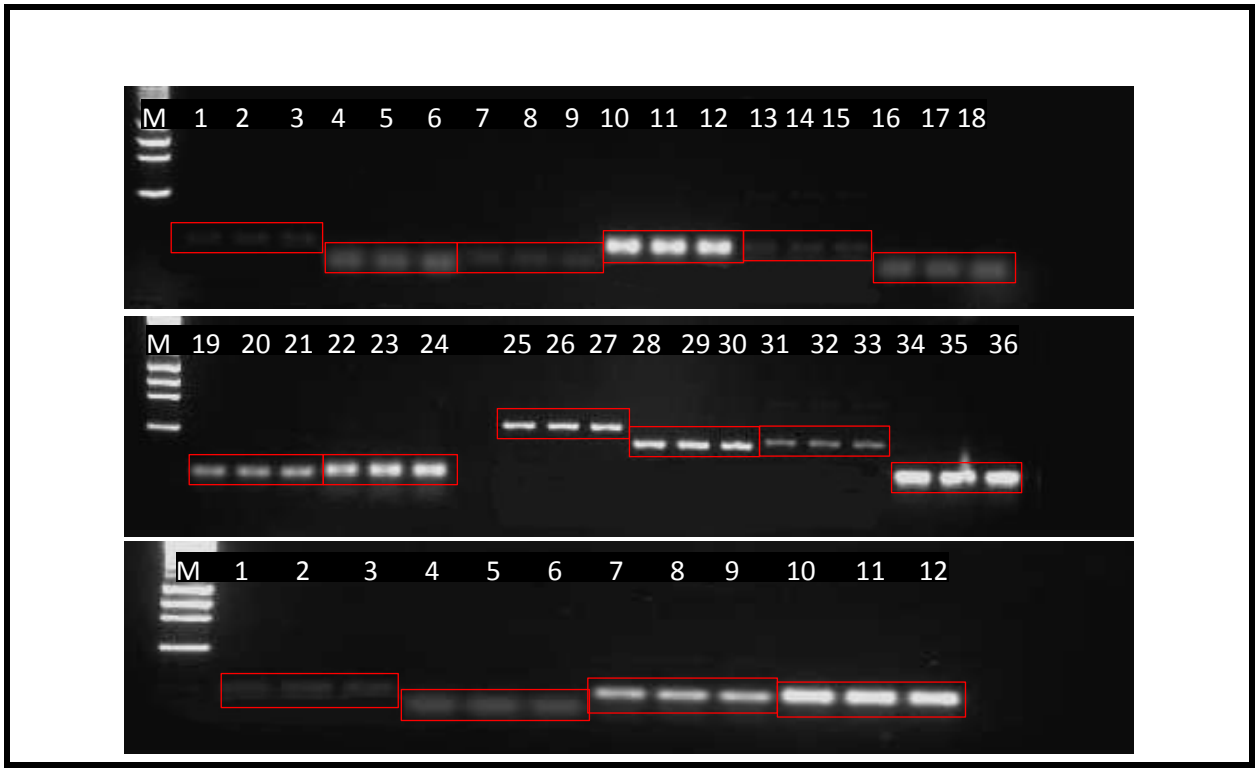


Figure 5.5 Semi-quantitative PCR (DNA quantification using gel densitometry) in different strains of An. gambiae after exposure to insecticides in agricultural zone. L1-3- *GCLC* – DD; L 4-6 –*GCLM* - DD, L 7- 9- *Nrf2*–DD; L 10-12-*UBQ*-DD; L13-15 – *GCLC*- DS; L16-18-*GCLM*- DS; L19-21-*Nrf2*-DS; L 22-24-*UBQ*-DS; L 25-27- *GCLC* A-Perm; L 28-30-*GCLM*- A-Perm, L 31-33-*Nrf2*- A-Perm; L 34-36-*UBQ*–A-Perm; L 37-39-*GCLC*-A-Base; L 40-42-*GCLM*- A-Base; L 43-45-*Nrf2*- A-Base; L 46-48-*UBQ*- A-Base

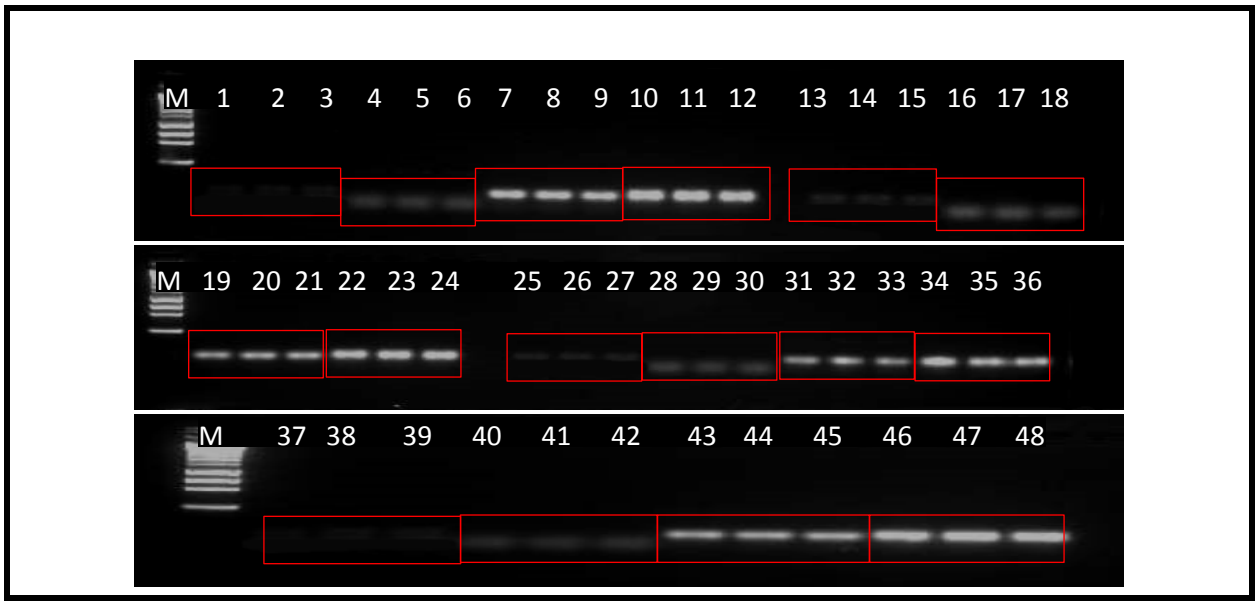


Figure 5.6 Semi-quantitative qPCR (DNA quantification using gel densitometry) in different strains of An. gambiae after exposure to insecticides in residential zone. L1-3- *GCLC* – DD; L 4-6 –*GCLM* - DD, L 7- 9- *Nrf2*–DD; L 10-12-*UBQ*-DD; L13-15 – *GCLC*- DS; L16-18-*GCLM*- DS; L19-21-*Nrf2*-DS; L 22-24-*UBQ*-DS; L 25-27- *GCLC* A-Perm; L 28-30-*GCLM*- A-Perm, L 31-33-*Nrf2*- A-Perm; L 34-36-*UBQ*–A-Perm; L 37-39-*GCLC*-A-Base; L 40-42-*GCLM*- A-Base; L 43-45-*Nrf2*- A-Base; L 46-48-*UBQ*- A-Base

The expression analysis of *GCLM, GCLC* and *Nrf2* revealed that the two genes and their transcriptional regulator were significantly up-regulated in most of the permethrin, DDT and bendiocarb resistant *An. gambiae* raised from agricultural and residential field-caught larvae while, in some, the two genes, and their transcriptional regulator were slightly up-regulated (Figures 5.7 and 5.8).

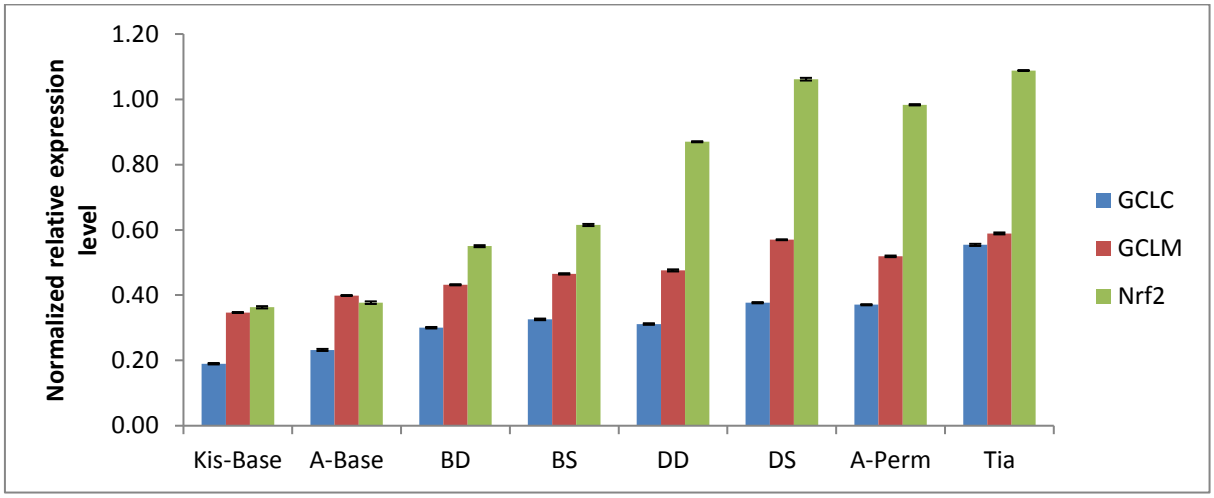


Figure 5.7 Relative expression levels of *GCLM, GCLC* and *Nrf2* genes in in seven strains of adults *An. gambiae* raised from agricultural field-caught larvae when challenged with insecticides and gene expression was analyzed via semi-qPCR. *GCLC, GCLM* and *Nrf2* values were normalized with Ubiquitin (*UBQ*). Data are presented as mean ± SE of three replicates. Gene expression values were compared by One-way ANOVA. P< 0.05 was accepted as statistically significant.

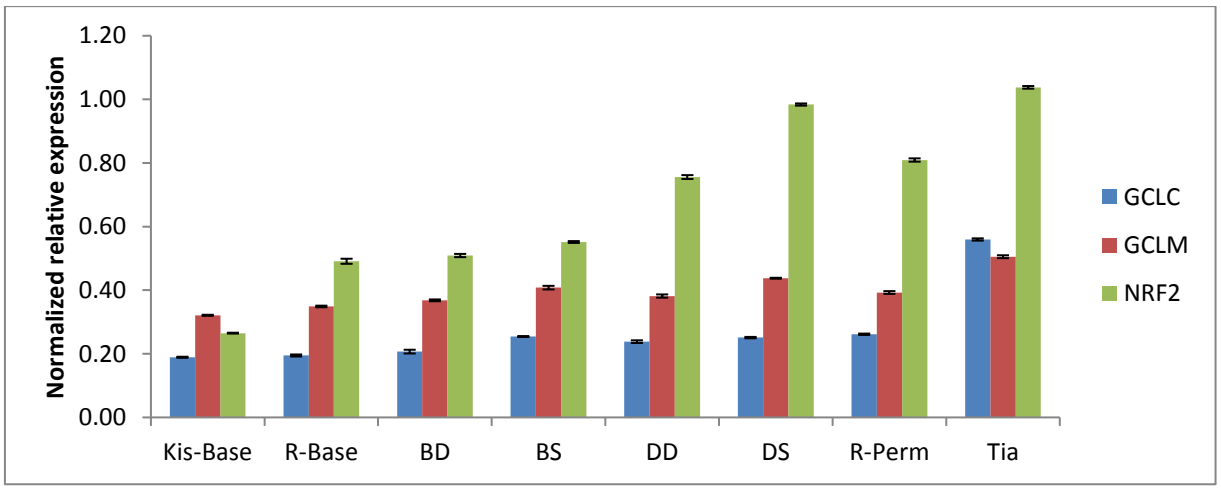


Figure 5.8 Relative expression levels of *GCLM, GCLC* and *Nrf2* genes in seven strains of adults *An. gambiae* raised from residential field-caught larvae when challenged with insecticides and gene expression was analyzed via semi-qPCR. *GCLC, GCLM* and *Nrf2* values were normalized with Ubiquitin (*UBQ*). Data are presented as mean ± SE of three replicates. Gene expression values were compared by One-way ANOVA. P< 0.05 was accepted as statistically significant.

From the data, it is clear that the levels of expression of *GCLC* and *GCLM* genes and their transcriptional regulator (*Nrf2*) particularly in the resistant strains of *An. gambiae* that survived insecticide exposure were up-regulated relative to A-Base and R-Base strains in agricultural and residential settings respectively.

Statistical analysis of data using One-Way ANOVA

The result of the one-way ANOVA on the data from the agricultural zone showed there was a significant statistical difference in the relative expression of *GCLC* gene (P≤0.001) in all the eight strains of *An. gambiae* except between strain 6 & 7 (DS & A-Perm) (P>0.05). However, there were significant statistical differences in the relative expression of *GCLM* and *Nrf2* in all the strains (P≤0.001). The increased expression levels or up-regulation of these genes and also GSSG levels in this population suggest their probable roles in the response and adaptation of *An. gambiae* to insecticide challenges from Sudan Savannah region in northern Nigeria.

The result of the one-way ANOVA on the data from the residential zone showed there was a significant statistical difference in the relative expression level of *GCLC* gene (P≤0.001) in all the eight strains except between strains 1 & 2, 4 & 6 and 4 & 7 (Kis & R-Base; BS & DS; BS & R-Perm) (P>0.05). There was also a significant statistical difference in the relative expression level of *GCLM* gene (P≤0.001) in all the eight strains except between strains 3 & 5 (BD & DD) (P>0.05). The relative expression levels of *Nrf2* showed a significant statistical difference in all the eight strains (P≤0.001). The use of Kisumu and Tiassale strains of *An. gambiae* (Figure 5.9) was to help with mapping and establishing the insecticide resistance status of *An. gambiae* from Northern Nigeria.

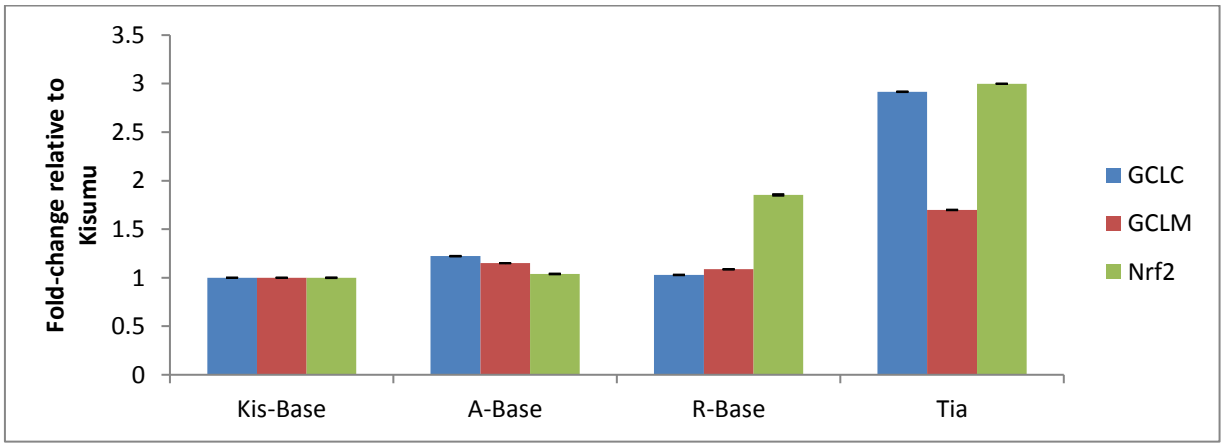


Figure 5.9 Fold-change in Tiassale and the unchallenged *An. gambiae* from agricultural (A-Base) and residential (R-Base) sites relative to Kisumu (Kis-Base)

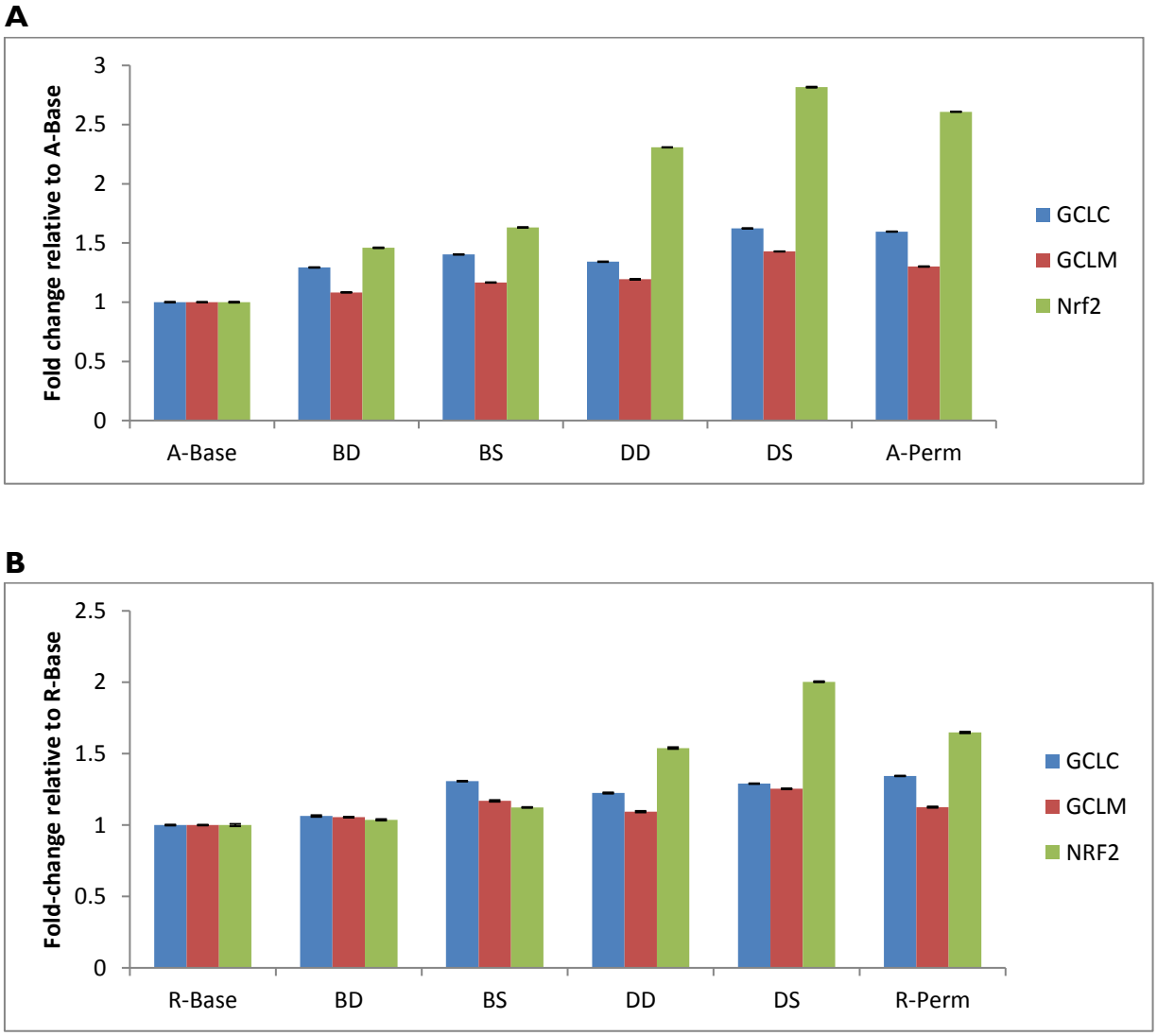


Figure 5.10 Fold-change of *GCLM, GCLC* and *Nrf2* genes in seven strains of adults *An. gambiae* raised from residential field-caught larvae when challenged with insecticides relative to A-Base (A) and R-Base (B), BD=bendiocarb dead, BS= bendiocarb survive, DD= DDT dead, DS= DDT survive, A-Perm= agricultural site permethrin, R-Perm= residential site permethrin

As earlier mentioned the Tiassale and Kisumu strains were used as reference strains thus insecticide resistance status of the uncharacterized *An. gambiae* sampled from northern Nigeria was established relative to these reference strains. The expression levels for *GCLC, GCLM,* and *Nrf2* in A-Base, R-Base and Tiassale were 1.22, 1.03, 2.92; 1.15, 1.09, 1.70 and 1.04, 1.85, 3.00-fold higher than in Kisumu respectively (figure 5.9)

The gene expression results further showed that *Nrf2* produced the highest level of expression with fold-changes 1.46, 1.63, 2.31, 2.82 and 2.61-fold higher in challenged

An.gambiae population from agricultural zone than in the unchallenged. While in the residential area, the fold-changes were 1.04, 1.12, 1.54, 2.00 and 1.65-fold higher in challenged than in the unchallenged *An. gambiae* for BD, BS, DD, DS, and A-Perm respectively.

The second highest level of gene expression was in *GCLC* with fold-changes 1.29, 1.40, 1.34, 1.62, and 1.60-fold higher in challenged than in the unchallenged in *An.gambiae* population from the agricultural zone. While in the residential area the fold-changes were 1.06, 1.31, 1.22, 1.29 and 1.34 higher in challenged than in the unchallenged *An. gambiae* for BD, BS, DD, DS, and A-Perm respectively.

Finally, *GCLM* produced fold-changes 1.08, 1.17, 1.19, 1.43, 1.30 higher in challenged than in the unchallenged *An.gambiae* population from the agricultural zone. But in the residential zone the fold-changes were 1.06, 1.17, 1.09, 1.25, 1.13-fold higher in challenged than in the unchallenged *An. gambiae* for BD, BS, DD, DS, and A-Perm respectively.

5.4 Discussion

In Africa, vector control measures involve the use of chemical insecticides. Chemical pesticides play a vital role in vector control. However, the continuous and indiscriminate use of insecticides in a population has led to the development of insecticides resistance to all the WHO recommended insecticides that mitigate these approaches. Consequently, a concerted effort has been aimed at understanding the regulatory mechanisms by which mosquitoes acquire insecticide resistance. Insecticide resistance can be due to the selection of changes in insect enzyme systems, leading to rapid detoxification or sequestration of insecticide or due to alterations of the insecticide target site preventing the insecticide-target site interaction. Increased metabolic capacity is usually achieved by increased activity of monooxygenases, GSTs or esterases (Devika *et al.,* 2008). Increased enzyme activity can be brought about by gene amplification, up-regulation, coding sequence mutations or by a combination of these

mechanisms. P450s can mediate resistance to all classes of insecticides. GSTs can mediate resistance to organophosphates, organochlorines, and pyrethroids. Esterases can provide resistance to organophosphates, carbamates and pyrethroids that are rich with ester bonds (Kostaropoulos *et al.,* 2001; Li *et al.*, 2007; Devika *et al.,* 2008). Reports correlating the elevated levels of GST with resistance to pyrethroids do exist for the red flour beetle *(Tribolium castaneum)* (Reidy *et al.,* 1990; Papadopoulos *et al.,* 2001) and *Aedes aegypti* (Grant and Matsumura 1989).

The bioinformatics analysis performed revealed putative transcription factor binding site for *Nrf*2 along 1.5 kb *GCLC* and *GCLM* promoter regions. This potentially suggests that *GCLC and GCLM* are under the control of *Nrf*2 through *ARE* in the 5'–flanking region. It is therefore proposed that *Nrf2/Keap* 1 pathway may be involved in the cellular network that maintains redox homeostasis in order to protect cells from oxidative stress induced by their respective ligands. The present results clearly suggest the differential effects of DDT, permethrin and bendiocarb insecticides on *An. gambiae* populations belonging to two different habitats (agricultural and residential). The increased expression levels of *GCLC* and *GCLM* genes and also GSH levels in this population suggest some roles of these genes and GSH in the response to and adaptation of *An. gambiae* to insecticide challenges. Thus, the study provides some evidence that GSH is playing a role in the possible adaptation to insecticides resistance by mosquito. Results presented in the present study indicate that differential expression of the *GCLC* and *GCLM* through possible transcriptional control by *Nf2e1/Keap 1* pathway is potentially a key to the overexpression of *GCLC, GCLM,* and may be other detoxifying genes to overcome insecticides and to confer resistance to permethrin, DDT and bendiocarb in *An. gambiae* strains. Thus consistent with previous studies (Misra *et al.,* 2011, 2013) that *CnCC/dKeap 1* pathway plays a key role in the coordinated induction of detoxification gene expression in response to xenobiotic treatment. And ectopic activation of this pathway is sufficient to confer resistance to Malathion. Although, the results suggest

that the *Nf2e1/AGAP003645* pathway is necessary for the overexpression of the detoxification genes, activation of this pathway is unlikely to be the only factor that contributes to insecticide resistance in these strains.

The finding of this study is consistent with the previous findings of Ffrench-Constant *et al.,* 2004; Li *et al.,* 2007; David *et al.,* 2010; Cassone *et al.,* 2014 which reported that wild population and laboratory-selected strains of insecticide-resistant *An. gambiae* overexpress some detoxifying genes demonstrating a correlation between their resistance and detoxification gene expression. The association of overexpressed detoxification genes with insecticide resistance connects these genes to metabolic resistance (Cassone *et al.,* 2014). The results of the semi-quantitative PCR further support the susceptibility bioassay results. The susceptibility bioassay revealed higher insecticide resistance in the agricultural zone which relates to a higher expression of the *GCLC* & *GCLM* genes and vice versa. The up-regulation of the detoxification genes in resistant insects has previously been reported (Diabate *et al.,* 2002; Muller *et al.,* 2008; Jones *et al.,* 2012).The present studies in respect of bioassay and biochemical estimations have revealed the potential mechanism developed by the local malaria vectors to oppose the insecticides. Further, the study of enzymes involved in the detoxification mechanism will help us to introduce appropriate control measures, such as combinations of insecticides and synergists for a better and effective control program of malaria (Vanlalhruaia *et al.,* 2014). More so, subsequent wet-lab experiments such as Dual luciferase assay and real-time quantitative polymerase chain reaction (RT-qPCR) to establish the functionality of the promoter element(s) and analyze the regulation of *An. gambiae* detoxification genes in this case *GCLC and GCLM* by insecticides are needed.

5.5 Conclusions

In conclusion, in this study we conducted a series of experiments employing biochemical and molecular approaches to characterize the mechanism of insecticide resistance in *An. gambiae* population sampled from two different ecologies in the northern Nigeria. Overall, the results indicate that the high phenotype resistance recorded in susceptibility bioassay in these mosquito populations was most probably due to target site resistance mechanism. Thus the GSSG levels, kdr mutations and overexpression of the *GCLC* and *GCLM* genes recorded in the present study interact to enhance resistance to insecticides. Thus represents a considerable threat to insecticide resistance management for malaria control. However, this knowledge could significantly be utilized in understanding the molecular mechanisms that underlie insect pesticide resistance towards developing effective vector control strategies

Chapter Six

Discussion, conclusion and Recommendations for Future Research

Chapter Six

6.0 Discussion and Conclusion

6.1Discussion

This research work investigated the role glutathione and glutathione biosynthetic genes in the response and tolerance of *An. gambiae* to chemical insecticides in northern Nigeria. This research also investigated the dynamics of species composition in the *An. gambiae* complex and their susceptibility/resistance status to commonly used insecticides in northern Nigeria. The research work furthermore investigated whether the phenotypic resistance observed in the susceptibility bioassays was linked to knockdown resistance (kdr) and the levels of glutathione. Overall, this study examined the molecular mechanism(s) of the role of GSH in selecting for insecticide resistance in *An. gambiae* the principal malaria vector in Africa. The working hypothesis set out at the beginning of this study was that agricultural activity/practices constitute sources of selection pressure for the emergence of insecticide resistant *An. gambiae.* To allow us to test this hypothesis, bioinformatics, bioassays, biochemical and molecular techniques were used to investigate.

Previous studies revealed that glutathione and its biosynthetic genes are associated with detoxification pathways, especially the Glutathione S- transferases (GSTs) which have been implicated in the development of insecticide resistance through the detoxification of xenobiotics including insecticides used in the control of *An. gambiae*. However, evidences have emerged that insects like other higher animals have the ability to up-regulate the transcription of detoxification genes in response to environmental xenobiotics by (*CnCC*) / (*dKeap* 1) and or *Spineless* (*Ss*) / *Tango* (*tgo*) signalling pathways in *Drosophila melanogaster* (Irfan and Biswas, 2009; Misra et al., 2011; Misra et al., 2013; Kuzin *et al*., 2014). However, it

is still not known whether the orthologs of *CnCC* in *An. gambiae* are involved in the maintenance of intracellular GSH levels and redox homeostasis.

Accordingly, the work presented here aims to develop knowledge of involvement and possible role of glutathione and its biosynthetic genes in selecting for insecticide resistance in *An. gambiae.*

To achieve this aim, the following specific objectives were therefore designed;

1. To prospect for, and conduct sampling of *An. gambiae* larva from breeding site located in different ecologies categorized and grouped into two different study zones: Zone A (Intensive agricultural area) Zone B (Domestic/residential environments).
2. To assess the susceptibility/resistance status of *An. gambiae* mosquito populations to DDT, permethrin and bendiocarb insecticides under agricultural and residential settings and the dynamics of species composition in the *An. gambiae* complex.
3. To assess the levels of the three forms of GSH (total, oxidized and reduced) in *An. gambiae* in agricultural and residential zones.
4. To identify the putative transcription factor binding site(s) present in *An. gambiae GCLC* and *GCLM*, especially with respect to *Nrf2 / Keap*1 *Nrf2 / ARE* axis and establish the functionality of the promoter element(s).
5. To examine the differential expression of *An. gambiae GCLC, GCLM* and *Nrf2* by insecticides by performing semi-quantitative RT-PCR (Gel densitometry).

Subsequently, these specific goals were addressed in four distinct but interrelated chapters. Chapter two presented the detailed description of the study areas; highlights the physicochemical characteristics of breeding sites sampled, insecticide usage, mosquito larval collection, processing, and rearing as well as morphological identification of mosquito larvae

performed with aid of taxonomic keys. Chapter three investigated the susceptibility status and the dynamics of species composition in the *An. gambiae* complex in northern Nigeria. Additionally genomic DNA was used for genotyping of the sodium channel gene to detect L1014F and L1014S mutations (kdr West Africa and kdr East Africa respectively). Correlation analysis of resistance and the different mutations was carried out. Chapter four investigated the differential levels of the different forms of glutathione under agricultural and residential settings and correlated these levels to the resistance status of the *An. gambiae* sampled from the two different breeding ecologies in northern Nigeria. The chapter also explores the feasibility of using GSH status in *An. gambiae* to monitor response and adaptation to insecticides. Chapter five investigated the differential expression of the *GCLC, GCLM* and AGAP005300 (*Nf2e1*), in adults *An. gambiae* raised from agricultural and residential field-caught larvae challenged and unchallenged with insecticides using end-point semi-quantitative RT-PCR.

Overall the work in this thesis has shown the following:

6.2 Field study

The results and observations recorded in this research work proved within the limits of all the experimental conditions, the working hypothesis of this study: that agricultural activity/ practices constitute sources of selection pressure for the emergence of insecticide resistant *An. gambiae*. *Anopheles gambiae* mosquitoes were sampled from different breeding ecologies defined by marked differences in environmental xenobiotic concentrations in northern Nigeria. The types and concentrations of these environmental xenobiotics and factors were a function of the different human related activities taking place within and around the mosquito breeding sites. A significant number of *An. gambiae* larvae were recorded in breeding sites with comparably high levels of these xenobiotics. This finding suggests *An.*

gambiae mosquitoes are adapting or have adapted to survive over a wide range and levels of environmental xenobiotics in their breeding ecologies. Most of the insecticides used by farmers in agriculture are of the same chemical classes and have the same targets and modes of action as those used for vector control that include Organochlorines, Organophosphates, Pyrethroids and Carbamates (Kumar, 1984; Khambay and Jewess, 2010).

6.3 WHO susceptibility / resistance status of An. gambiae

WHO susceptibility assays performed revealed high resistance among the mosquitoes tested from all the sampling sites. However, mosquitoes from agricultural sites (zone A) recorded higher insecticide resistance when compared to those from residential sites (zone B). This decrease in mortality rates to DDT and permethrin was associated to a significant increase in the KDT_{50} observed which were higher in agricultural sites suggesting the involvement of kdr, this is consistent with the findings of Chandre *et al.,* 1999; Bigoga *et al.,* 2014; Ibrahim *et al.,* 2014, and Alhassan, *et al.,* 2015, indicating the possible involvement of kdr mechanism of resistance. The co-occurrence of L1014S and L1014F mutations coupled with high insecticide resistance in the two populations belonging to agricultural and residential settings in northwest Nigeria suggest the spreading of the L1014S mutation gene across Africa. The nature of the environment drives the resistance as agricultural activities have an effect on kdr allele's distribution compared to non–agricultural activities in the residential settings. The high allelic frequencies recorded in the residential sites could be due to the increased use of pyrethroids for ITNs and IRS in public health as indicated in previous studies (Dykes *et al.,* 2015, Li *et al.,* 2015).The L1014F mutation appears to be the most significant mutation in both *An. coluzzii* and *An. arabiensis* in northwest Nigeria at present,

however there exists the possibility that other mechanisms were also present and acted to confer resistance.

On the contrary, in this study *An. gambiae s.l.* mosquitoes tested against bendiocarb show from suspected resistance to weak resistance indicating that the *An. gambiae s.l.* mosquitos' population in this region has started to develop resistance against carbamate and thus blocking the possibility of using this class of insecticide as an alternative to pyrethroids and DDT in IRS. Agricultural activities have an effect on kdr allele's distribution compared to non-agricultural activities in the residential settings with higher L1014F allelic frequencies in agricultural sites than residential. Similarly, these higher L1014F allelic frequencies in agricultural sites coincide or correlate with higher insecticide resistance in agricultural sites suggesting that kdr is the primary mechanism responsible for the observed phenotypic resistance in these locations.

6.4 Glutathione levels and distribution

The biochemical assays allowed us to determine the levels of the three forms of glutathione (total, reduced and oxidized glutathione) in *An. gambiae* sampled from the two study zones. The results obtained indicates that although significant induction in the synthesis of glutathione may not have occurred in response to the varying concentrations of xenobiotic over the two studied zones, there was however, a significant increase in the utilization of rGSH. The increased utilization of rGSH characterized by significant differences in the levels of GSSG was significantly associated with DDT resistance of the *An. gambiae* population studied which was higher in mosquitoes sampled from zone A compared to those sampled from zone B. Our results further, demonstrated that increased GSSG levels and the

presence of the kdr mutations especially in agricultural zone interact to enhance resistance. Therefore, these findings suggest that measuring the levels of GSH in mosquitoes sampled could possibly be used as a tool to assess, monitor and detect insecticide resistance status in *An. gambiae s.l.* population.

6.5 Establishing putative transcription binding sites within the 5' region upstream of GCLC and GCLM

A literature review indicated that there was no previous data on the identification of location of putative transcription factor binding sites (TFBS) for (*AGAP005300*) (*Nf2e1*) (*Nrf2*) and (*AGAP010259*) (*AhR*) within the 5' upstream region of *GCLC* and *GCLM* in *An. gambiae*. In this study therefore, 24 *AhR* (*AGAP010259*) and 4 *Nrf2* (*Nf2*e1) and 26 *AhR* (*AGAP010259*) and 3 *Nrf2* (*Nf2*e1) TFBS were established within the 5' 1.5 kb regions upstream of *GCLC* and *GCLM* respectively. This potentially suggests that *GCLC* and *GCLM* are under the control of *Nrf2* through *ARE* and or *AhR* through Xenobiotic response element (XRE) in the 5'–flanking region. Binding of *AGAP010259* and/or *Nf2*e1 genes at the TFBS could drive the transcription of *GCLC* and /or *GCLM* through their respective signalling pathways thereby influencing the metabolism of insecticides in *An. gambiae* as discussed in Chapter One (section 1.5.2) and Chapter Five (Section 5.2.3) of this study.

To establish the functionality of the promoter element(s) and analyze the regulation of *An. gambiae GCLC* and *GCLM* by insecticides, subsequent wet-lab experiments such as Dual luciferase assay and / or real-time quantitative polymerase chain reaction (RT- PCR) would be of interest. This is in order to reveal the expression profile of the *GCLC and GCLM* gene through *Nrf2* transcription factor.

6.6 Differential expression of GCLC, GCLM and Nf2e1 (Nrf2) in selected strains of Anopheles gambiae

The experiments described in Chapter Five (Sections 5.2.4) of this study were performed in order to examine the expression and regulation of *GCLC, GCLM* and *Nf2e1* in the Tiassale, Kisumu and seven strains of *An. gambiae.* This study was the first to report the expression of *GCLC, GCLM* and *Nf2e1* genes using semi-quantitative RT-PCR in *An. gambiae*. The data gathered served to highlight the differences in the expression levels of *GCLC, GCLM* and *Nf2e1* between the Tiassale resistant, Kisumu susceptible, agricultural and residential-challenged as well as the unchallenged strains of *An. gambiae* from Sudan savannah of northern Nigeria. The *An. gambiae* strains from Nigeria were also challenged with WHO recommended doses of the following insecticides: 0.1 % bendiocarb, 4% DDT and 0.75% permethrin to allow us address the research hypothesis raised in this study on the possible up-regulation of the detoxification genes through *Nrf2* transcription in *An. gambiae* in the presence of oxidative stress.

Despite the fact that malaria burden is high in Nigeria, information on the principal malaria vectors such as species compositions, the impact of environmental factors in deriving the genetics to selection pressure for vector adaptation and resistance profiles is grossly lacking particularly in the Sudan savannah region of northern Nigeria. With the exception of Ibrahim *et al*., (2014) and Alhassan *et al.,* (2015) who reported the exposure of the agricultural strains of *An. gambiae* to DDT & lambda-cyhalothrin, and DDT & bendiocarb respectively no other studies were reported before. The data from Chapter Five (Section 5.4.2.2) of this work suggests that *GCLC, GCLM* and (*Nf2e1*) *Nrf2* were expressed in all the selected strains of *An. gambiae*. Comparison between the Kisumu, Tiassale, agricultural base and residential base strains indicated that the level of expression of *GCLC, GCLM* and *Nf2e1* in all the strains of *An. gambiae* were similar and significant in the Kisumu strain ($P< 0.001$).

The differential expressions of these genes in all the selected strains of *An. gambiae* therefore provide a vital understanding into the potential roles of *Nf2e1 / dKeap* 1 signalling pathways in insecticide resistance in *An. gambiae*. The over-expression of *Nf2e1* and depletion of *AGAP003645* is protective in *An. gambiae* to insecticide challenge that potentially activates the transcription of *GCLC* and *GCLM* genes leading to more GSH synthesis and protect cells from xenobiotic compounds, whereas *AGAP003645* overexpression potentially represses their transcription, indicating that the functions of these protein families in the xenobiotic response are conserved between mammals, *Drosophila melanogaster* and *An. gambiae.* The differential expression of *Nf2e1* in the selected insecticide resistant Tiassale, agricultural and residential challenged strains of *An. gambiae* in particular therefore has revealed a connection between *Nf2e1 / AGAP003645* signalling pathways and *GCLC* and *GCLM* up-regulations in insecticide resistance.

This research is the first study of its kind to look at the role of glutathione and its biosynthetic genes in *An. gambiae* resistance to insecticides. The study revealed increased expression levels of these genes and also GSH levels in *An. gambiae* population suggesting their roles in the response and adaptation of *An. gambiae* to insecticide challenges. Thus, the study provides evidence that GSH is playing a role in the tolerance and adaptation to insecticides resistance. Results also indicated that differential expression of the *GCLC* and *GCLM* through *Nf2e1/Keap 1* pathway is potentially a key to the overexpression of detoxifying genes in insecticide resistant *An. gambiae* strains challenged with permethrin, DDT and bendiocarb in both agricultural and residential zones in Nigeria.

Key Findings

- The current study highlighted that the mosquito populations in these study sites displayed functional and dynamic adaptations leading to changes in both tolerance and behaviour. This study also provides physicochemical data supporting the role of the environment in which vectors are found in selecting for the emergence of insecticide resistant Anopheline mosquitoes.
- The predominance of *An. coluzzii,* its resistance profile to major insecticides and co-occurrence of the east and west African kdr mutations reported in this study can help and guide the implementation of suitable vector control strategy especially in the choice of insecticide for vector control in the current trends of multiple insecticides resistance in this region of the Sudan savannah where such information was unavailable.
- The results further suggest a close relationship between an increase in GSSG level and DDT resistance in *An. coluzzii* (*An. gambiae* M-form) from agricultural sites than in *An. coluzzii* from residential sites. This study further demonstrated that GSSG levels and kdr mutations interact to enhance resistance to insecticides. Thus, the study highlighted glutathione status may be mediating the response and possibly adaptation of *An. gambiae* exposure to insecticide.
- The relative quantification analysis of the *GCLC* and *GCLM* genes expression using semi-quantitative RT-PCR method demonstrates a correlation with resistance (bioassay data) of the *An. gambiae* strains. The high level of resistance to DDT and Permethrin correlated with high GSSG levels that also correlated with elevated levels of *GCLC, GCLM* and *Nrf2* gene expression. The correlation between the detoxification genes in this case *GCLC* and *GCLM* expression and high resistance enable the prediction of resistance using a molecular method. It was clear that the

levels of these genes were up-regulated as compared to the baselines strains. The quantitative PCR analysis has helped us understand the possible reason behind the different degree of resistance exhibited by these strains of *An. gambiae* due to different expression levels.

6.7 Conclusions

The findings from this PhD research has demonstrated that agricultural activities through the use of wide array of chemical pesticides not targeted at malaria vectors however, acts to select for the development of insecticides resistance in *An. gambiae*. Findings from the current study suggest that xenobiotic characteristics of some *An. gambiae* breeding ecologies could select *An. gambiae* for resistance to insecticides even before exposure to insecticides. Subsequently, *An. gambiae* emerging from such ecologies might have developed appropriate mechanism (s) to tolerate any insecticides used to control them. The findings from this PhD study demonstrated that the high resistance to insecticides recorded was likely due to genetic (target site mutation) resistance mechanism and probably metabolic (enzyme detoxification) mechanism.

Results of this study also suggest the possibility that the *Nf*2e1 / (*AGAP003645*) pathway is potentially a key to the overexpression of *GCLC, GCLM* and possibly other detoxifying genes in the challenged and unchallenged Tiassale, Kisumu and strains of *Anopheles gambiae* from agricultural and residential sites. Inhibition of the *Nf*2e1/*dKeap* 1 as suggested by Mohammed, (2014) in particular may potentially improve the efficacy of insecticides and development of methods to knockdown or inhibit these pathways may prove fruitful. This work has increased our knowledge of the role of glutathione and its biosynthetic genes in insecticide resistance in *An. gambiae*. These findings have implications in the ability to control the spread of malaria due to the reduction in insecticide resistance in *An. gambiae*.

6.9 Recommendations

While the research activities reported in this thesis have addressed a number of critical issues relating to role of glutathione and its biosynthetic genes in *An. gambiae* resistance to insecticides in Nigeria, it is imperative to identify some key areas of research that would complement and progress the findings of the study. Consequently, the following recommendations are made for future research work;

- There is need to carry out investigation on the activities of any other environmental xenobiotic compounds other than the ones studied here- that may be present in *An. gambiae* breeding habitats with the view to examining their impact, if any , on the growth, development and behaviour of the mosquitoes emerging from such ecologies, especially towards any control initiatives.
- More so, subsequent wet-lab experiments such as Dual luciferase assay and real-time quantitative polymerase chain reaction (RT-qPCR) to establish the functionality of the promoter element(s) and analyze the regulation of *An. gambiae* detoxification genes by insecticides are needed.

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Appendix I Conference Abstract published from the thesis 2014

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Investigating the role of glutathione and glutathione biosynthetic enzymes in the adaptation of *Anopheles gambiae* to insecticides

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Glutathione (GSH) plays a central role in the detoxification of xenobiotics. The GSH biosynthetic gene γ-glutamylcyteine ligase modifier subunit (*GCLM*) is regulated by nuclear factor-like 2 *(Nrf2)* that protects cell from oxidative stress. To examine the molecular mechanism and the role of GSH in the adaptation of *Anopheles gambiae* mosquito to insecticides, bioinformatics analyses of *GCLM* promoter region was performed. Also bioassay carried out on mosquito larvae collected from north western Nigeria where insecticides are used for agriculture or against mosquitoes. Two to five day old adult mosquitoes were tested for susceptibility to insecticides using standard WHO procedures. GSH levels were determined. Insilico analyses of GCLM promoter region revealed several putative transcription factor binding sites, including *Nrf2* binding sites, which may be involved in its transcriptional regulation. Results indicate 6–54% (DDT and pyrethroids), 74–95% (Bendiocarb) mortality rates among the mosquitoes tested. Reduced GSH levels were found to be lower in mosquitoes from agricultural sites than those from residential sites. The decreased reduced GSH in mosquitoes from agricultural sites indicates an interaction of insecticides with GSH. Extensive agricultural practice is suspected as a source for selection pressure for the adaptation to insecticides in *Anopheles gambiae*.

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A role for glutathione and its biosynthetic genes in *Anopheles gambiae* insecticide resistance

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Anopheles gambiae (An. gambiae) is the principal malaria vector in Africa, where vector control measures involve the use of insecticides. The development of insecticides resistance mitigates these approaches. Glutathione (GSH) is widely distributed among all living organisms and is associated with detoxification pathways, especially the Glutathione S-transferases (GSTs). Its direct involvement and relevance in insecticide resistance in *An. gambiae* has not been determined. Thus, this work examines the contribution of GSH, its biosynthetic genes (*GCLM, GCLC)* and their possible transcriptional regulator (*Nrf2*) in insecticide resistance in *An. gambiae*. We use bioinformatics, bioassay and molecular techniques to investigate. *An. gambiae* s.l. studied here were highly resistant to DDT and permethrin but less resistant to bendiocarb. The levels of total, reduced and oxidized GSH were higher in mosquitoes from agricultural sites than those from residential sites. Increased oxidized GSH levels relate with higher insecticides resistance. The expression of *GCLM, GCLC* and *Nrf2* were up-regulated in adults *An. gambiae* raised from agricultural field-caught larvae when challenged with insecticide. The increased expression levels of these genes and also GSH levels in this population suggest their roles in the response and adaptation of *An. gambiae* to insecticide challenges. There exists the feasibility of using GSH status in *An. gambiae* to monitor adaptation and resistance to insecticides.

*Corresponding author.

Appendix III

Correlations

		PH	Temperature	Nitrate	Nitrite	Phosphate	Sulphate	Carbon Content
PH	Pearson Correlation	1	-.560*	.815**	.681**	.809**	.744**	.491
	Sig. (2-tailed)		.030	.000	.005	.000	.001	.063
	N	15	15	15	15	15	15	15
Temperature	Pearson Correlation	-.560*	1	-.655**	-.652**	-.632*	-.620*	-.422
	Sig. (2-tailed)	.030		.008	.008	.012	.014	.117
	N	15	15	15	15	15	15	15
Nitrate	Pearson Correlation	.815**	-.655**	1	.947**	.999**	.988**	.676**
	Sig. (2-tailed)	.000	.008		.000	.000	.000	.006
	N	15	15	15	15	15	15	15
Nitrite	Pearson Correlation	.681**	-.652**	.947**	1	.947**	.968**	.579*
	Sig. (2-tailed)	.005	.008	.000		.000	.000	.024
	N	15	15	15	15	15	15	15
Phosphate	Pearson Correlation	.809**	-.632*	.999**	.947**	1	.989**	.670**
	Sig. (2-tailed)	.000	.012	.000	.000		.000	.006
	N	15	15	15	15	15	15	15
Sulphate	Pearson Correlation	.744**	-.620*	.988**	.968**	.989**	1	.719**
	Sig. (2-tailed)	.001	.014	.000	.000	.000		.003
	N	15	15	15	15	15	15	15
Carbon Content	Pearson Correlation	.491	-.422	.676**	.579*	.670**	.719**	1
	Sig. (2-tailed)	.063	.117	.006	.024	.006	.003	
	N	15	15	15	15	15	15	15

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix IV

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		
			Lower	Upper	Wald Chi-Square	df	Sig.
(Intercept)	6.700	.0516	6.599	6.801	16833.750	1	.000
[Zone=A]	.333	.0730	.190	.476	20.833	1	.000
[Zone=B]	0[a]	.	.	.	.	.	.
[Site=AA1]([Zone=A])	-.367	.0730	-.510	-.224	25.208	1	.000
[Site=AA2]([Zone=A])	.067	.0730	-.076	.210	.833	1	.361
[Site=BA]([Zone=A])	0[a]	.	.	.	.	.	.
[Site=AR]([Zone=B])	-.167	.0730	-.310	-.024	5.208	1	.022
[Site=BR]([Zone=B])	0[a]	.	.	.	.	.	.
(Scale)	.008[b]	.0029	.004	.016			

Dependent Variable: PH

Model: (Intercept), Zone, Site(Zone)

a. Set to zero because this parameter is redundant.

b. Maximum likelihood estimate.

Appendix V

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		
			Lower	Upper	Wald Chi-Square	df	Sig.
(Intercept)	33.033	.1523	32.735	33.332	47067.592	1	.000
[Zone=A]	-.990	.2153	-1.412	-.568	21.138	1	.000
[Zone=B]	0[a]	.	.	.	.	.	.
[Site=AA1]([Zone=A])	1.457	.2153	1.035	1.879	45.762	1	.000
[Site=AA2]([Zone=A])	.990	.2153	.568	1.412	21.138	1	.000
[Site=BA]([Zone=A])	0[a]	.	.	.	.	.	.
[Site=AR]([Zone=B])	1.033	.2153	.611	1.455	23.029	1	.000
[Site=BR]([Zone=B])	0[a]	.	.	.	.	.	.
(Scale)	.070[b]	.0254	.034	.142			

Dependent Variable: Temperature

Model: (Intercept), Zone, Site(Zone)

a. Set to zero because this parameter is redundant.

b. Maximum likelihood estimate.

Appendix VI

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		
			Lower	Upper	Wald Chi-Square	df	Sig.
(Intercept)	2.523	.0209	2.482	2.564	14618.597	1	.000
[Zone=A]	6.397	.0295	6.339	6.455	46971.441	1	.000
[Zone=B]	0[a]	.	.	.	.	.	.
[Site=AA1]([Zone=A])	-2.920	.0295	-2.978	-2.862	9787.959	1	.000
[Site=AA2]([Zone=A])	-.747	.0295	-.805	-.689	640.000	1	.000
[Site=BA]([Zone=A])	0[a]	.	.	.	.	.	.
[Site=AR]([Zone=B])	-.150	.0295	-.208	-.092	25.829	1	.000
[Site=BR]([Zone=B])	0[a]	.	.	.	.	.	.
(Scale)	.001[b]	.0005	.001	.003			

Dependent Variable: Nitrate

Model: (Intercept), Zone, Site(Zone)

a. Set to zero because this parameter is redundant.

b. Maximum likelihood estimate.

Appendix VII

Correlations

		TGSH	GSSG	GSH
TGSH	Pearson Correlation	1	.841**	.989**
	Sig. (2-tailed)		.000	.000
	N	30	30	30
GSSG	Pearson Correlation	.841**	1	.752**
	Sig. (2-tailed)	.000		.000
	N	30	30	30
GSH	Pearson Correlation	.989**	.752**	1
	Sig. (2-tailed)	.000	.000	
	N	30	30	30

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix VIII

Correlations

		TGSH	GSSG	GSH	Mortality to DDT	Mortality to Permethrin	Mortality to Bendiocarb
TGSH	Pearson Correlation	1	.886**	.992**	-.386	-.335	.198
	Sig. (2-tailed)		.001	.000	.271	.345	.609
	N	10	10	10	10	10	9
GSSG	Pearson Correlation	.886**	1	.822**	-.616	-.489	-.065
	Sig. (2-tailed)	.001		.004	.050	.151	.867
	N	10	10	10	10	10	9
GSH	Pearson Correlation	.992**	.822**	1	-.310	-.281	.262
	Sig. (2-tailed)	.000	.004		.383	.431	.497
	N	10	10	10	10	10	9
Mortality to DDT	Pearson Correlation	-.386	-.616	-.310	1	.458	.227
	Sig. (2-tailed)	.271	.050	.383		.183	.558
	N	10	10	10	10	10	9
Mortality to Permethrin	Pearson Correlation	-.335	-.489	-.281	.458	1	.161
	Sig. (2-tailed)	.345	.151	.431	.183		.679
	N	10	10	10	10	10	9
Mortality to Bendiocarb	Pearson Correlation	.198	-.065	.262	.227	.161	1
	Sig. (2-tailed)	.609	.867	.497	.558	.679	
	N	9	9	9	9	9	9

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix IX

AGAP001512_RA_GCLC SEQUENCE

.....gaggttaacagctctagcagcgagaagaacgcgcgttagtatttagtaaa

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GCACGAACGAGGTCGTCTACGCTTGCTTCCCGAAAGGAACCGACTCCCGAAACTCCCAAA

gtgcgttgagtattttcgttggtgtgtgtgtgtgtctgttcctggttccattcccgct
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ACACCAGG**ATG**GGTTTGCTAAGCGAAGGCAGTCCTCTGACGTGGGATGAAACGAAGGCGC
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TCGACGATAAGCAGCGCGCAGTCCAGGTATCGCTGCGGGCGCAAGAAATTCTGGCCAAGC
TGAACGAAAAGGAAGCGGCCGATCCACAAG

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cctttccag

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CGCGGCTGGGATGTCCGCGCTTCACGTTCCCACCGGCGGTACCGACGCCGGACGATGAGG
CGTGCGCGGCCCGGTCGAACTTTTTCCCCGACGAAGCCATCTTCCCGGGCCATCCGCGGT
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CGATCTACCCGGATCGTGATACGCAAACGCCGGTCGAGGGCAGCATACCGTCCCACCCGA
GCCACGTGCACATGGACGCGATGGGCTTCGGTATGGGTTGCTGCTGTTTGCAGCTCACGT
TCCAGGCGTGTAATATTAGCGAGGCGCGCACACTGTACGACCAGCTGACGCCGATGTGCC
CTATAATGCTCGCGCTGACGGCGGCCAGTCCGGCGTACCGTGGCTTTCTGACCGATGTGG
ACTGCCGGTGGAACGTGATCTCGGCCTCGGTCGACTGCCGCACGCGGGAGGAGCGTGGCG
AGGAGCCGCTGAAGAACGATCGGTTCCGCATTTACAAGTCACGCTACGATTCGATCGATT
CTTACCTCTCGCCGGCAGGAGAGAA

Key: Exons / Introns

- **Translated sequence**
- **Flanking sequence**
- **UTR**
- **Transcribed sequence is in capitals while non-transcribed DNA is in lower case.**

Appendix X

AGAP012038_RA_GCLM SEQUENCE

.....taggcttcaattggcagcaaaaggaagaacagcagcacgacaaaagcaac

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CTGACGGACTGTCTCCGCTCGACGTTCGGCGAAGCGGACGTAACCGAGCTGCCCCCGAAC
CGGCGGCTCATCACACGCCGCAACAACGATCTGCTGGAAAAGGTGAAGGAGCATCCAAGG
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GGGAAGATTGGCCAGCTCGGCATTGCCGATCTGGATGCGGACTCGCTCAAGGAGCTGTAC
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CCGCCGCAGCTGCAGGCGTACTGCAATCAGAACGAAATTCAGCTACTAACGCACAGTGAC
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gtgagtgtttgacgctgtctagagattgcctttgatgctttataatgtccgttttattct
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AACTACTCCCACGGGAAGTGCTGGACGAGCTGGACCTGGCAAGCTACGGGGCCGGCTGGA
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TAC

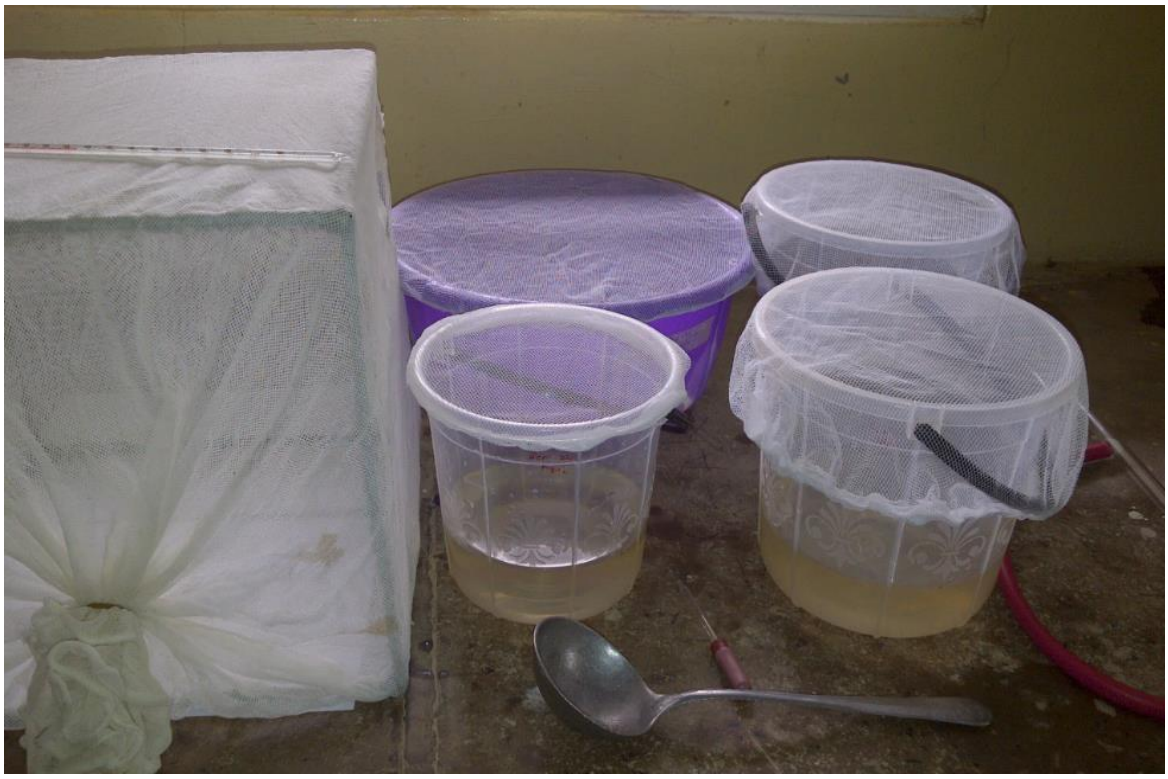
agaaaaacaaaacttaacaaaagcaacagcaacaaatgcacttaacagaa.........

Key: Exons / Introns

- **Translated sequence**
- **Flanking sequence**
- **UTR**
- **Transcribed sequence is in capitals while non-transcribed DNA is in lower case.**

Appendix XI Selected Field study Photos for mosquito larval collections

Appendix XII Adult mosquitoes rearing



Appendix XIII WHO adult susceptibility bioassay