KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI, GHANA

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PUBLIC HEALTH

DEPARTMENT OF GLOBAL AND INTERNATIONAL HEALTH

MALARIA TRANSMISSION DYNAMICS AND INSECTICIDE RESISTANCE OF MALARIA VECTORS IN THE KASSENA-NANKANA DISTRICTS OF GHANA

BY

VICTOR ASOALA (BSC. , MPH)

FERBRUARY, 2016

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A THESIS SUBMITTED TO THE DEPARTMENT OF GLOBAL AND INTERNATIONAL HEALTH, COLLEGE OF HEALTH SCIENCES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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CERTIFICATION PAGE

I here declare that this submission is my own work towards the PhD and that ,to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the university, except where due acknowledgement has been made in the text.

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Signature Date

Supervisor (s) name ……. …….

Signature Date

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Head of Dept. Name Signature Date

DEDICATION

This thesis is dedicated to my wife Maria for her tolerance of my occasional vulgar moods; a testament in itself of her unyielding devotion and love, my children Kevin Atiim and Lilian Awonniame and to all the people who never stop believing in me and who along with God, have been my „footprints in the sand"

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Their support has been unconditional all these years.

ABSTRACT

Plasmodium falciparum malaria is a serious tropical disease that causes more than one million deaths each year, mostly in Africa. It is transmitted by a range of *Anopheles* mosquitoes. Progress in global malaria control over the past decade is largely gained through investments in vector control; especially insecticide treated mosquito nets (ITNs). ITNs have been used extensively in the Kassena Nankana districts (KND) of Ghana for over two decades. This study aimed to investigate the intensity of malaria transmission and resistance status of vector populations in KND, relate these data to historical patterns of transmission intensity and determine whether the presence of insecticide resistance has an effect on malaria transmission in KND. *Anopheles gambiae* s.l. was the predominant *Anopheles* which constituted 68.82% (95% CI 68.18 - 69.45) (N=13938). *Anopheles funestus* constituted 10.97% (95% CI 10.55 – 11.41) (N=2222) whilst *An*. pharoensis and *An*. rufipes constituted the rest of 20. 21% (95% CI 19.66 - 20.77), (N=4092). Molecular analysis of the *An. gambiae* s.l. revealed only *An*. gambiae s.s. as sibling species, mainly of *Anopheles coluzzii* (M-form). *Anopheles* biting started early, peaked around 10.00pm and continued to the early hours of the morning. *Plasmodium falciparum* sporozoite infectivity results revealed active transmission by *An. gambiae* s.s.as early as 8pm and serious transmission occurred towards the early hours of the morning because of combined infective biting by both *An. gambiae* s.s and *An. funestus*. There was marked temporal variations in malaria intensity estimated as the entomological inoculation rate (EIR) with the irrigated zone experiencing the highest during the year. Compared to what was reported over a decade ago, the intensity of transmission has reduced by 66.7% from 418 to 139 infective bites/man/year. Results of the study indicated high phenotypic resistance to the insecticide classes tested. High frequencies of the Knock down resistance (kdr) and

Ace-1R alleles (responsible for pyretroid/DDT and carbamate /organophosphate resistance respectively) including the N1575Y allele (reported to enhance resistance to pyrethroids) was observed. The variations observed in biting patterns, transmission intensity and the high insecticide resistance observed in the main malaria vector has important consequences for the success of the widely used insecticide-based strategies in KND.

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ABTS	2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate
ACTs	Artemisinin-based Combination Therapies
AIDs	Acquired Immune Deficiency Syndrome
ASPCR	Allele Specific PCR
CSP ELISA	Circumsporozoite Protein enzyme-linked immunosorbent assay
DDT	dichlorodiphenyltrichloroethane,
EIR	Entomological Inoculation Rate
ETC	Exit trap catches
FRET	Fluorescence Resonance Energy Transfer
GIS	Geographical Information Systems
GPS	Geographical positioning systems
GSTs	glutathione-S-transferase enzymes
HLC	Human Landing catches
HOLA	Heated Oligonucleotide Ligation Assay
IGS-arrays	Intergenic Spacer Regions
IPT	Intermittent Preventive Treatment
IRS	Indoor residual spraying
ITNs	Insecticide Treated Nets
Kdr	Knock Down Resistance allele
KND	Kassena Nankana Districts
LLINs	Long-lasting Insecticide Treated Nets
LSTM	Liverpool School of Tropical Medicine
LTC	Light Trap Catches
Mab	Monoclonal Antibodies

MDA	Mass Drug Administration
NHDSS	Navrongo Health and Demographic Surveillance System
NHRC	Navrongo Health Research Centre
NMCP	The National Malaria Control Programme
NMIMR	Noguchi Memorial Institute for Medical Research
PCR	Polymerase Chain Reaction
PCR-RFLP	restriction fragment length polymorphisms
PEVs	Pre-erythrocytic vaccines
Pf	Plasmodium falciparum
PSC	Pyrethrum Spray Catches
rDNA	Ribosomal DNA
RM	High Resolution melt
RS	Remote Sensing
SINE200	Short Interspersed Transposable Element
SMC	Seasonal Malaria Chemoprevention
SNPs	Single Nucleotide Polymorphisms
SP	sulfadoxine-pyrimethamine
SSOP-ELISA	Sequence Specific Oligonucleotide Probe-Enzyme-Linked ImmunoSorbent Assay
ssrRNA	small subunit of ribosomal RNA
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

Malaria remains one of the most critical public health challenges accounting for about 207 million cases worldwide and 627000 deaths in 2012 despite intense national and international efforts in controlling the disease (WHO, 2013a). Morbidity and mortality are especially high among pregnant women, young children, and sick persons lacking immunity. Malaria in pregnancy causes severe anaemia and low birth weight at delivery as well as contributes to maternal deaths in malaria-endemic areas (Owusu-Agyei *et al.*, 2007). The greatest burden of malaria occurs across sub- Saharan Africa. This is because *Plasmodium falciparum*, the parasite species that causes the most severe human malaria, is dominant in this region coupled with the abundance of *Anopheles gambiae* and *An. funestus* complexes, as main vectors with greater ability towards human biting (Greenwood *et al.*, 2005) . The global spread of malaria continues to be of primary health and economic concern for over one third of the world‟s population and together with Acquired Immune Deficiency Syndrome (AIDs) and tuberculosis, one of the major communicable diseases (Lewison and Srivastava, 2008). Estimates suggest that a single bout of malaria costs the equivalent of ten working days (Liese, 1998).

In Ghana, malaria is by far the most important parasitic disease and accounts for 38 percent of all outpatient ailments, 35 percent of all admissions, and 34 percent of all deaths in children under five years (Ghana Health Service, 2011). The burden of malaria remains high in the country with about 323 per 1,000 cases reported among children under 5 years in 2008 (Asante *et al.*, 2011). Recently, total annual cost for malaria treatment of children aged 2-11 months has been valued at about 37.8 million US dollars (Sicuri *et al.*, 2013) and studies in Northern Ghana have shown that both direct and indirect costs associated with malaria

episodes burden poor households (Akazili *et al.,* 2007). Malaria is hyperendemic in the Kassena Nankana districts (KND) of the Upper East Region of Ghana and is responsible for high severe anaemia and mortality among infants (Koram *et al.,* 2003; Oduro *et al.,* 2010). Malaria transmission in KND is sharply defined by rainfall however, it is higher and perennial within the irrigated sectors (Oduro *et al.,* 2010).

As ecosystems in malaria endemic areas differ, identifying and understanding the characteristics of the local vectors is important in recognizing the pattern of malaria transmission dynamics. Many of the malaria vectors are members of species complexes and their individual contributions to malaria transmission, seasonal distribution and biting behaviour may differ (Klein *et al.,* 1992). Thus the incrimination of one species as a vector in an area may not be the same in another area hence the need for effective identification to enhance control. Molecular species identification assays developed for this have exploited species-specific polymorphisms in the ribosomal DNA gene (Scott *et al.,* 1993; Favia *et al.,* 1997; Fanello *et al.,* 2002). The *Anopheles gambiae* sensu lato (s.l.) is a species complex that comprises seven sibling species which include the most important vectors of malaria worldwide (Hay *et al.,* 2010). Members of this complex are morphologically indistinguishable but differ in their behaviour and ability as vectors of malaria. The two principal malaria vectors are *An. gambiae* s.s. and *Anopheles arabiensis*. *Anopheles melas and Anopheles merus,* also members of the complex are potential vectors but are limited by their distribution and confined to coastal regions because they can only breed in brackish water (Moreno *et al.,* 2004). *Anopheles quadriannulatus species A* and *Anopheles quadriannulatus* species *B*, recently named *Anopheles amharicus* (Coetzee *et al.,* 2013) found in Southern Africa and Ethiopia respectively are considered non malaria vectors (Coetzee *et al.,* 2000). *An. gambiae* s.s. is subdivided into reproductively isolated sub-populations of five chromosomal forms initially identified based on the patterns of

chromosome 2 inversions (Coluzzi *et al.*, 1985). Subsequent studies revealed that *An. gambiae* s.s. contains two molecular forms, M form recently named *Anopheles coluzzii* and S form, renamed as *Anopheles gambiae* s.s. (Coetzee *et al.*, 2013) which can only be recognized by the differences of their rDNA sequences, either in the intergenic spacer or in the internal transcribed spacer (della Torre *et al.*, 2001 ; Gentile *et al.*, 2002).

The biting behaviour of *Anopheles* vectors is an important component in the transmission of malaria. There have been reports indicating their tendency to bite at specific hours of the night and also whether they bite indoors (endophagic) or outdoors (exophagic), and if they rest inside (endophillic) or outside houses (exophillic) (Zimmerman *et al.*, 2013; Lindblade, 2013; Huho *et al.*, 2013). The main strategies designed to control the most efficient African *Anopheles* malaria vectors are based on these characteristics (Zimmerman and Voorham, 1997).

Several factors influence the transmission of malaria and one such factor is the vector density. To be of importance, as a vector of human disease, an insect must be relatively abundant and its habitat, adjacent to human settlement (Gillies, 1988). Human activities may play a role in the increased number of vectors in an area. For example, irrigated fields and road construction provide favourable breeding grounds to many malaria vectors. The susceptibility of the vector to infection also affects malaria transmission. The man-vector contact, measured as the frequency of biting and expressed as the man biting rate also plays an important role in transmission of malaria by the vector (Burkot and Graves, 1995). The frequency of biting also affects the rate of transmission of the disease and usually, this must be high enough to sustain transmission.

The intensity of malaria transmission by mosquitoes is important in the development of strategies aimed at controlling malaria, and several methods have been established over the

years to estimate it (Kilama *et al.,* 2014). The methods for estimating malaria transmission include traditional epidemiological estimates such as spleen rate, incidence and prevalence (Shaukat *et al.,* 2010). However, a more direct measure of transmission intensity is the entomological inoculation rate (EIR) (Kelly-Hope and McKenzie, 2009). The EIR is a parameter that associates both the behaviour and human-biting activity of malaria vectors and the risk of malaria infection (Niang *et al.,* 2013) and presents a basic and relatively simple means of quantifying levels of human exposure to infected mosquitoes (Killeen *et al.*, 2006). The EIR is estimated as the number of infective bites per person per unit time (Macdonald, 1957). It is the product of the "human biting rate" (defined as the number of bites per person per night and the proportion of biting mosquitoes that are infective referred to as the "sporozoite rate" (Birley and Charlewood, 1987). The magnitude of the EIR is influenced by the rate at which infective mosquitoes feed on humans. This is largely dependent on the mosquito density and to a great extent, the feeding habits of the vector species (Shililu *et al.,* 1998). The risk of human exposure to malaria infections in Africa is not uniform (Smith *et al.,* 2005), because variations in the abundance and dispersal of mosquitoes occur spatially and temporally in a given area (Antonio-Nkondjio *et al.,* 2006).

Measuring biting rates constitutes a very important aspect of entomological monitoring of vector control interventions, such as insecticide treated nets (Service, 1993). Several sampling methods have been used to estimate the population density of mosquitoes searching for a blood meal. The methods commonly used include the Human Landing catches (HLC), Pyrethrum spray catches (PSC), Light trap catches (LTC) and baited inverted traps. The most direct and reliable method for estimating the man biting rate is the human landing catches (Mathenge *et al.,* 2002). It is considered the most representative and usually regarded as the „gold standard", largely based on arguments that it corresponds to natural transmission dynamics as humans act as baits to attract bloodseeking mosquitoes

(Tusting *et al.*, 2014). In addition HLC gives information on the different *Anopheles* species biting people either indoors or outdoors. The biting activity throughout the night can be obtained and hence the times of maximum exposure to malaria transmission. The method can also be used to estimate the man biting rate per night and season and adults caught can be dissected to determine parity and for the calculation of sporozoite and inoculation rates (Le Goff *et al.*, 1993).

Light traps used for the light trap catches are mechanical devices that attract mosquitoes to light, a phenomenon that may not be directly associated with the act of feeding on humans (Mathenge *et al.*, 2002). These traps are usually placed indoors next to a person sleeping under a treated bed net. The trap collects mosquitoes that fail in their efforts to feed on people in the room and get attracted to the light in the trap and are sucked in by a fan. Therefore any mosquitoes captured by the trap can be taken as those that would have bitten the occupants of the room. These traps collect few mosquitoes outdoors (Chandler *et al.*, 1976).

Pyrethrum spray sheet catches *(PSC)* is used to collect indoor resting mosquitoes after knockdown by space spraying of a pyrethrum solution. The main principle of the method is that, mosquitoes tend to rest indoors after feeding and therefore any mosquitoes captured with this method would have fed on people in the room or were not able to feed in the night and were resting. This method has been used as a standard quick and easy method of collecting mosquitoes resting in huts and animal shelters (Service, 1993). It collects mosquitoes of varying physiological conditions, gives valuable information on relative changes in seasonal abundance of endophillic vectors, and provides adults for determining sporozoite and inoculation rates and information on host preferences and degree of endophilly.

The sporozoite rate concurrent with human-landing density provide data for estimating the intensity of transmission. It is usually measured as the proportion of female mosquitoes with their salivary glands infected with malaria parasites in a given sample (Mboera and Magesa, 2001 ; Mahapatra *et al.,* 2006). The understanding of the entomological aspects of the epidemiology of malaria depends in part, on the accurate assessment of this rate. Traditionally, detection of *Plasmodium* species in the mosquito is done by dissection and visual assessment of glands using a microscope (Mahapatra *et al.,* 2006). This method however requires skilled personnel, is time consuming and does not determine which *Plasmodium* species is present. This has led to the adoption of more rapid immunological and molecular approaches. One of the most widely used methods is the circumsporozoite protein enzyme-linked immunosorbent assay (CSP ELISA) (Burkot *et al.,* 1984; Wirtz *et al.,* 1987). Polymerase Chain Reaction (PCR)-based assays are usually more sensitive and specific (Barker, 1994), sensitive than ELISA and also provides information incriminating a particular vector species (Bangs *et al.,* 2002). Its application demonstrates how the prevalence of malaria parasites may be underestimated by light microscopy (Snounou *et al.,* 1993a).

Using EIR estimates have assisted in assessing the effect of vector control interventions because it quantifies the parasite- infected mosquito pool and its ability to transmit parasites to the human population (Shaukat *et al.,* 2010). The intensity and pattern of malaria transmission, and hence its epidemiology, are largely a function of the seasonality, abundance, and feeding habits of the *Anopheles*. The recognition of both the temporal and spatial variations in human biting rates and exposure dynamics has enhanced opportunities for focused malaria control (Carter *et al.,* 2000) since the variation in exposure to infected vectors is more or less the force behind focal malaria transmission (Clark *et al.,* 2008; Smith *et al.,* 2005).

In the early 1950s, the WHO launched the Global Malaria Eradication Programme based on two key tools: chloroquine for treatment and prevention and DDT for vector control. This programme had a considerable impact in some areas, particularly those with relatively low transmission rates (Brito, 2001) but never attempted to eradicate malaria in most parts of Africa, where malaria transmission was intense (Greenwood, 2008). It was abandoned in 1972 following the emergence of chloroquine-resistant *Plasmodium* parasites and DDT-resistant *Anopheles* mosquitoes (Brito, 2001). In recent times, the global community has intensified their efforts to deliver more effective interventions throughout Africa, including drug combinations with an artemisinin derivative and antivector measures leading to a decline in malaria mortality and morbidity in many areas (WHO, 2013a) and has encouraged a new call for global eradication.

In areas such as the United States of America (USA) and Europe where malaria elimination programs have been successful, vector control was a vital component (Greenwood, 2008). Current vector control strategies include Insecticide Treated Nets (ITNs)/ long-lasting Insecticide Treated Nets (LLINs), and Indoor residual spraying (IRS). The National Malaria Control Programmes (NMCP) in African countries currently relies on strategies using these vector control tools. Both methods have been shown to be very effective against *An. gambiae* and *An.funestus*; the main vectors (O‟Meara *et al.,* 2010 ; Murray *et al.,* 2012) because these feed predominantly on people at times when they are inside their houses and rest mostly indoors so that insecticidal contact is increased (Lindblade, 2013 ; Seyoum *et al.,* 2012; Huho *et al.,* 2013). As compared to IRS, ITNs are common components of malaria control programs, largely due to their ease of implementation, cost effectiveness (Becker-Dreps *et al.,* 2009; Yukich *et al.,* 2009), and impact across transmission settings As of 2010, it was estimated that 42% of households owned at least one ITN across 44 countries in sub- Saharan Africa (WHO, 2010).

Pyrethroids are a major class of synthetic insecticide widely used for controlling disease vectors because of their fast acting, high insecticidal activities and low mammalian toxicity and are the only insecticides currently available for use on bed nets (Rinkevich *et al.*, 2013). Therefore, the sustained effectiveness of ITNs depends on the continued susceptibility of malaria vectors to this class of insecticides. Four classes of insecticides (pyrethroids, organophosphates, carbamates, and organochlorines (only DDT, still available) are currently recommended for IRS in malaria control (WHO, 2012a). These four insecticide classes have been shown to share just two modes of action. The carbamates and organophosphates act by targeting the neurotransmitter

acetylcholinesterase, resulting in the accumulation of acetylcholine in synapses and this affects nerve function (Corbett *et al.,* 1984). The Pyrethroids and DDT on the other hand bind to and impede closure of neuronal sodium channels, resulting in prolonged current, which causes repetitive nerve firing and subsequently death (Davies *et al.,* 2007).

Insecticide resistance presents a serious threat to the success of the main malaria vector control strategies (IRS and ITNs) currently being deployed in malaria endemic areas (Reddy *et al.,* 2013). Insecticide resistance in *An. gambiae,* to pyrethroids, DDT, and carbamate class insecticides consist of target site resistance in specific genes that alter the sensitivity of the carrier to insecticides (Ranson *et al.,* 2000; Weill *et al.,* 2004). Molecular characterizations of the voltage-gated sodium channel (the target of DDT and pyrethroid insecticides) have shown that various mutations in the S1–S6 trans-membrane segments of domain II of this gene cause resistance in some insect species (Soderlund and Knipple, 2003). In *An. gambiae* s.s. two point mutations (L1014F and L1014S) are present at amino acid position 1014 of this gene, both of which result in an amino acid substitution conferring decreased susceptibility to pyrethroids and DDT (Reddy *et al.,* 2013). These two mutations are now referred to as knockdown resistance-west (kdr-w) or (L1014F) and kdr-east or

(L1014S) referring to where they were first detected (Coˆte d‟Ivoire in West Africa and Kenya in East Africa respectively). The L1014F and L1014S alleles result from a substitution of the leucine residue with phenylalanine and serine respectively (MartinezTorres *et al.,* 1998; Ranson *et al.,* 2000) . Later studies have indicated that these mutations can be found within *An. gambiae,* and *An. arabiensis* populations in geographically diverse areas (Pinto *et al.,* 2006 ; Verhaeghen *et al.,* 2006). Studies have established a correlation between the kdr genotype and resistance phenotype (Donnelly *et al.,* 2009). The extensive use of pyrethroids for vector control interventions and in some instances, agriculture in Africa has placed a strong selection pressure leading to increased kdr allele frequencies which may have an effect on the efficacy of pyrethroid-based vector control (Mathias *et al.,* 2011; Chouaïbou *et al.,* 2008 and N‟Guessan *et al.,* 2007). This may negatively affect the success of malaria control programs especially the use of ITNs. Other studies have however noted that correlation between kdr genotype and resistant phenotype does not necessarily imply a reduction in the impact of pyrethroid-based vector control (Protopopoff *et al.,* 2008). Several new sodium channel mutations have been identified to be associated with pyrethroid resistance in *Culex quinquefasciatus* (Rinkevich *et al.,* 2013). A new mutation, named N1575Y has recently been identified in *An. gambiae* occurring only on haplotypes associated with L*1014F*. It is an asparagine-to-tyrosine mutation at position 1575 (N1575Y) within the linker between domains III-IV of the voltage gated sodium channel (Jones *et al.,* 2012). This L1014F-1575Y has been found to compensate for the deleterious fitness effects of L1014F and/or confers additional resistance to insecticides. It has been identified in both molecular forms of *An. gambiae* in the western and central parts of Africa. In *An. coluzzii* sampled from Burkina Faso, its frequency was found to have risen significantly between 2008 and 2010 (Jones *et al.,* 2012).

Carbamates and Organophosphates provide an option to circumvent pyrethroid resistance in *An. gambiae s.s.* However resistance to these classes of insecticides has been observed in several African countries (Djogbénou *et al.,* 2008 ; Corbel *et al.,* 2007 ; Essandoh *et al.,* 2013). In laboratory and field strains of *An. gambiae* s.s., carbamates and organophosphates resistance is due to a single point mutation in the Ace-1R gene encoding acetylcholinesterase, their target binding site, leading to a substitution of the amino acid Glycine with Serine at position 119 of the encoded protein and is referred to as G119S (ACE-1R) mutation (Weill *et al.,* 2003 ; Edi *et al.,* 2012; Djogbénou *et al.,* 2008).

Insecticide resistance may also result from other physiological mechanisms such as metabolic detoxification through increased enzyme activities (monooxygenases, esterases, or glutathione- S transferases) (David *et al.,* 2013) and this is referred to as metabolic resistance. Resistance to most insecticides including pyrethroids have been linked to the action of Cytochrome P450 enzymes (P450s) (David *et al.,* 2013). Over expression of P450s have been reported in pyrethroid resistant mosquitoes in Africa (Wondji *et al.,* 2008 ; Matambo *et al.,* 2010). In some instances, esterases (Somwang *et al.,* 2011) and glutathione-S-transferase enzymes (GSTs) have also been implicated (Lumjuan *et al.,* 2011). A number of P450s linked to pyrethroid resistance in *An. gambiae* for example, the Cytochromes *CYP6P3 and CYP6M2* have been commonly reported in resistant mosquitoes in the field (Djouaka *et al.,* 2008 ; Kwiatkowska *et al.,* 2013). A study using highly DDT resistant *An. gambiae* mosquitoes in Ghana have gone further to provide further evidence that the single enzyme, CYP6M2 (already known to metabolize both type I and type II pyrethroids), confers resistance to DDT which belongs to a different class of insecticides (Mitchell *et al.,* 2012).

Mosquitoes can show multiple insecticide-resistance mechanisms (Perera *et al.,* 2008).

Studies have reported individual *An. gambiae* mosquitoes to have both the kdr and *Ace-1R* mutations (Yewhalaw *et al.,* 2011). The combined consequence of target-site insensitivity and metabolic resistance among malaria vector populations is still unclear. At present, there is no common agreement on whether the kdr genotype alone confers all the diverse resistance phenotypes (Brooke, 2008 ; Donnelly *et al.,* 2009 ; Ramphul *et al.,* 2009 ; Nwane *et al.,* 2009). There is therefore the need for additional investigations to assess the contributions of kdr mutations and other resistance mechanisms in resistance phenotypes to assist in the development of vector control strategies (Nwane *et al.,* 2013).

The widespread use of insecticides for malaria control coupled with its use in agriculture can hasten the development of insecticide resistance including resistance to multiple types of insecticides, further limiting the existing options for malaria vector control (WHO, 2012a). The impact of insecticide resistance on malaria transmission can be best examined by assessing the vector population in an area after an insecticide control strategy since a reduction in vector numbers may imply a decrease in transmission as the malaria parasite basic reproductive number is decreased (Rivero *et al.,* 2010). As IRS and to some extent the use of ITNs is to reduce the number of vectors, the emergence of insecticide resistance may however, offset this by increasing the number of mosquitoes that survive the insecticide treatment (Berticat *et al.,* 2004 ; Berticat *et al.,* 2008). There is therefore the need for the development of global resistance surveillance networks (WHO, 2012a) to enhance the development of robust resistance management strategies (Read *et al.,* 2009).

There are at least three ways of monitoring insecticide resistance in vector populations. These include (i) measures of phenotypic resistance which present a direct indication of how resistance mechanisms affect vector control activities. The limitation of this approach is that

it requires access to testing kits, insecticide impregnated papers, mosquito rearing facilities, and large numbers of mosquitoes; (ii) the frequencies of target site mutations (for example,. kdr) which can easily be ascertained. However it is still not clear how much this mechanism contributes to resistant phenotypes and (iii) measures of metabolic resistance (for example gene expression micro-arrays) considered to give strong indicators of phenotypic resistance but can be technically difficult (Mathias *et al.,* 2011).

A complimentary use of these approaches will give more accurate results.

1.1 STATEMENT OF THE PROBLEM

Insecticide treated bed nets (ITNs) have been used as the main malaria control intervention for close to two decades in the Kassena-Nankana districts (KND) of Ghana; first as the earliest experimental intervention trial followed by routine use among most community members. Prior to the bed net trial in KND, malariometric indicators did not change so much when they were compared to a previous survey in 1955 (Binka *et al.,* 1994). After the ITN trial between July 1993 and June 1995 however, it was reported that bed nets were associated with 17% reduction in mortality in children aged 6 months to 4 years (Binka *et al.,* 1996). Later after the bed net trial and following the continued use of ITNs, (Koram *et al.,* 2003) reported prevalence of malaria parasitaemia to be significantly lower among infants and young children (<2 years) who used bed nets. A study in 2010 among 2,232 pregnant women in KND indicated that more than 50% slept under an ITN and among all newborns, mother‟s use of bed net significantly reduced neonatal anemia and was useful in decreasing mean low birth weight deliveries, (Oduro *et al.,* 2010).

Currently, ITN coverage in KND is estimated to be 72% (NDSS, unpublished). The long use and relatively high coverage of bed net in KND coupled with the use of insecticides for agriculture particularly in the irrigated parts of the districts, may ultimately have affected

malaria transmission either by causing behavioural changes in the biting patterns of the vectors present (as suggested by Appawu *et al*. (2004) or resistance to commonly used insecticides as a result of selection pressure on the vectors.

In recent times, the need to protect humans from mosquito bites as a way of reducing the burden of malaria has received renewed attention. Noteworthy among many programs are the United States of America (U.S.) Presidents Malaria Initiative, for which the United Sates has pledged $1.2 billion over 5 yr to combat malaria (USAID, 2010), and the malaria control Trust of the Bill & Melinda Gates Foundation of Global Health (www.gatesfoundation.org/ topics/pages/malaria.aspx). There is only one comprehensive study that has reported malaria transmission dynamics in KND and this is well over 14 years (Appawu *et al.,* 2004) and similarly, there is only one report on the insecticide resistance status of the malaria vectors (Anto *et al.,* 2009) with no evidence of continuous monitoring of the impact of the many years of ITN deployment. There is therefore the need to measure malaria transmission indices of the district and then, subsequently monitor the level of transmission and insecticide resistance in the KND to generate information relevant to the accurate evaluation of short term interventions such as IRS and the long term use of ITNs.

1.2 RATIONALE FOR THE STUDY

Understanding the dynamics of malaria transmission in a population is critical as it provides an insight into the magnitude of the problem caused by the disease. It helps to define when and where the greatest risk occurs and guides in the development of appropriate control strategies. Furthermore, it is important to determine how the level of risk within a population may compare with surrounding populations since this may assist in the identification of key differences and similarities and to further highlight corresponding risk factors. Studies have shown that malaria transmission is varied across sites (Fontenille and Simard, 2004), and generally affected by different ecological environments to the extent that transmission

among villages separated only by short distances within the same area may differ (Dolo *et al.*, 2004 ; Okello *et al.*, 2006).

Irrigation agriculture is also known to have an effect in different settings depending on how it affects malaria transmission indicators (Appawu *et al.*, 2004 ; Hay *et al.*, 2000).

The implementation of other malaria control activities and interventions such as Artesunate Combination Therapy (ACT), Intermittent Preventive Treatment in Infants (IPTi), and Intermittent Preventive Treatment during Pregnancy (IPTp), focused antenatal care have taking place in the districts between now and then which could have influenced the dynamics of malaria transmission. The need to continuously document the transmission indicators especially when new interventions such as Insecticide Residual Spraying (IRS) are added cannot be overemphasized. Although malaria transmission is clearly not closely monitored in KND, measuring transmission and determination of vector dynamics over longer periods is still important. This will help define intra- and inter-annual variability as well as assess the impact of changes within the population as a result of the introduction of control interventions and changes in climate and land use patterns such as irrigation and urbanization.

The Tono irrigation scheme located in KND lies between latitude 10º 45‟ N and longitude 1º W. The source of water is from the river Tono. It comprises a 5 km long dam, which created an artificial lake with a surface area of 1,860 hectares. It has a water storage capacity of 93 million m^3 of water, 37 million m^3 of which could be used for irrigation.. The irrigation plots are served by two main canals with an overall length of 42 kilometres and a network of laterals and sub-laterals of a further 210 kilometres, (Asare, 2002) Insecticide use in KND for irrigation agriculture for rice, tomato and other vegetables undoubtedly can contribute to resistance of the major malaria vectors to insecticides being used for pests control in an unregulated manner (Akogbéto *et al.*, 2006). The presence of resistance can further be

enhanced by the long use of ITNs and LLNs (over two decades) as a result of widespread distribution to pregnant women and children under five, as part of the implementation of the universal coverage with LLINs by the National Malaria

Control Program (NMCP) and other organizations (Ndo *et al.*, 2011 ; Trape *et al.*, 2011 ; Corbel *et al.*, 2012). Although the only study in KND on the insecticide resistance status of the main malaria vector *An. gambiae* (Anto *et al.*, 2009) did not report any resistance to commonly used insecticides, it is important that resistance is monitored so that its emergence can be managed at the early stages using effective techniques.

This study was designed to further understand the biology and transmission dynamics of malaria as this was done over a decade ago as changes in vector behaviour and resistance status in the KND may have occurred. It is hoped that it will help design vector control strategies that may help suppress vector densities and malaria transmission in the area.

1.3 GENERAL AIMS OF THE STUDY

1. To estimate the intensity of malaria transmission in an area with intense and long use of Insecticide Treated Bed nets (ITNs) and to relate these data to historical patterns of transmission intensity

2. To assess the resistance status of vector populations in the area as a first step towards developing a resistance management strategy for the area.

1.3.1 Specific Aims:

- To determine the mosquitoes that serve as vectors of malaria in the KND map their distribution.
- To relate the distribution and frequency of the molecular forms of the main malaria vectors to varying ecological settings.

- To investigate the intensity of malaria transmission in the KND through estimation of sporozoite rate, man-biting rate and the entomological inoculation rate (EIR) of the main vectors and assess their relative seasonal changes in the KND
- To determine the current insecticide resistance status and mechanisms involved in the main malaria vector species in KND to spatially map the extent of resistance in selected areas across KND.
- To determine whether the presence of insecticide resistance has any effect on malaria transmission in the districts

CHAPTER TWO

LITERATURE REVIEW

2.1 The burden of malaria

About 3.4 billion people worldwide are at risk of malaria (WHO, 2013a). The public health impact of malaria is very high in Africa (Guerra *et al.*, 2008) as it includes areas with the highest transmission intensities (Hay *et al.*, 2010) and prevalence levels (Hay *et al.*, 2009) in the world. Malaria is one of the common causes of maternal and infant deaths in endemic areas (Greenwood, 2008). School-age children are also affected in terms of absenteeism and malaria infection may increase academic failure and drop-out rates. It has been reported that children with malaria generally have poorer nutritional status (Rowland *et al.*, 1977) and this can impair brain development (Grantham-McGregor *et al.*, 1991). Another group that is vulnerable to malaria because of diminished immunity is pregnant women. Malaria in pregnancy can cause anaemia which can result in low birth weight among babies, this being a risk factor for neurosensory, cognitive and behavioural development of children (Desai *et al.*, 2007); (McCormick *et al.*, 1992). Malaria can also interact with other infectious diseases altering susceptibility to either disease as illustrated by evidence that infection with HIV boosts the risk of uncomplicated and severe malaria (Korenromp *et al.*, 2005), whilst conversely, malaria triggers a momentary increase in viral load (Kublin *et al.*, 2005) which can foster HIV transmission.

2.2 Malaria and urbanization

It is estimated that Africa's population will almost triple by the year 2050 mainly in urban areas in West Africa, where the urban population annual growth rate is 6.3% (Donnelly *et al.* 2005). In the past, it was generally thought that urbanization will lead to low malaria prevalence because of fewer vector breeding sites (Keiser *et al.* 2004), a decrease in biting

rates because of higher ratio of humans to mosquitoes, improved access to treatment and better (mosquito-proof) housing (Warren *et al.* 1999). However, annual reports indicate increased clinical episodes of malaria in these areas. This is likely to be due mostly to the rapid development of unplanned human habitats generally associated with low income, poor education, housing and sanitation which may not experience such marked decreases in malaria transmission (Keiser *et al.* 2004). Urban malaria is likely to increase in importance as rapid urbanization will result in the majority of Africa's population living in cities in the near future (United Nations Publication 2004).

2.3 Global distribution of malaria

Malaria is not a homogeneous disease but varies from region to region, from country to country and from place to place. The disease occurs mostly in tropical and subtropical countries, particularly Africa south of the Sahara, South East Asia and the forest fringe zones in South America. The five *Plasmodium species* that infect humans are *Plasmodium falciparum* Welch, 1897, *Plasmodium vivax* Grassi & Feletti 1890, *Plasmodium malariae* Feletti & Grassi, 1889, *Plasmodium ovale* Stephens 1922 and *Plasmodium knowlesi* Sinton and Mulligan 1933. *Plasmodium falciparum* and *P. vivax* account for most of the reported malaria (Hay *et al.,* 2004). *P. falciparum* is however known to cause nearly all of the serious cases and deaths and is mostly found in sub-Saharan Africa, Southeast Asia, as well as in the Western Pacific. *Plasmoduim vivax* is widespread in most of Asia, the Eastern Mediterranean, and in most endemic countries of the Americas (Hay *et al.,* 2004). Estimates by World Health Organization indicated that there were 216 million episodes of malaria in 2010, with about 81%, occurring in Africa (and almost 91% caused by *P. falciparum*) followed by South-East Asia (13%) and Eastern Mediterranean Regions (5%)

(WHO, 2011). It is reported that more than 50% of all estimated *P. falciparum* clinical cases occurred in Nigeria, the Democratic Republic of Congo, Myanmar and India (Hay *et al.,* 2010). The global distribution of *P. falciparum* malaria is shown in figure 2.1.

2.4 Malaria in Ghana

Malaria is hyper- endemic and perennial in Ghana, with seasonal variations. In 2011, the country reported about 2.5 million cases (WHO, 2011) and *Plasmodium falciparum* accounted for about 90–98% of all infections, followed by *P. malariae* for 2–9%, and *P. ovale* for 1%. Mixed infections of *P. falciparum* and *P. malariae* are common (USAID, 2010). The Ghana Health Service reported that malaria ranked first as a cause of morbidity and mortality in children under five and accounted for 33% of hospital deaths in children under five years and about 38% of all outpatient illnesses (USAID, 2010). Figure 2.2 shows the spatial distribution of reported *P. falciparum* in Ghana. Malaria transmission occurs year-round with pronounced seasonal variations in the northern part of the country, with 3-4 months of intense malaria transmission.

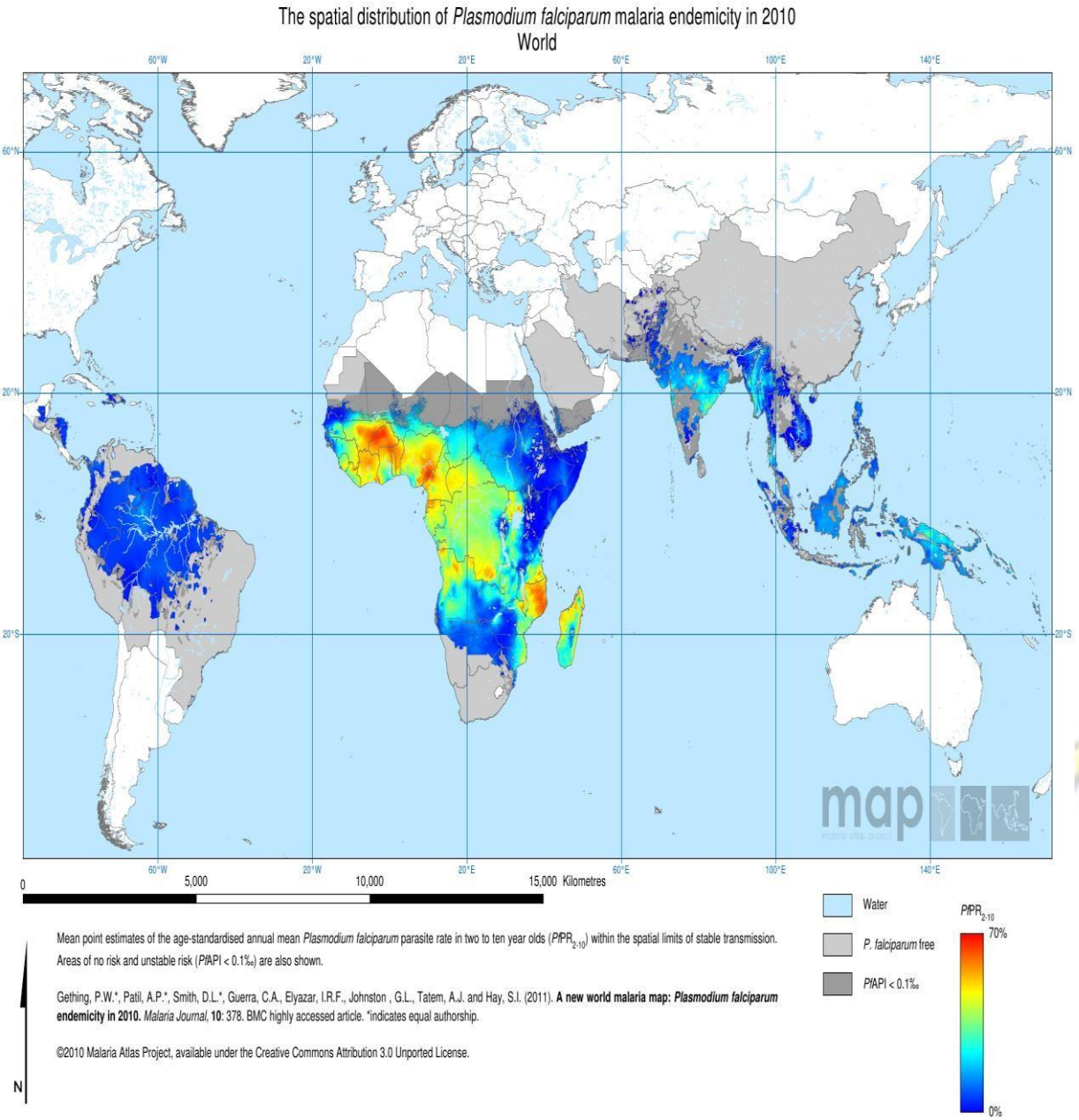


Figure 2.1 Global distribution of *Plasmodium falciparum* malaria

Source: Malaria Atlas Project by Welcome trust (www.map.ox.ac.uk/browse-resources /endemicity /Pf_mean/world

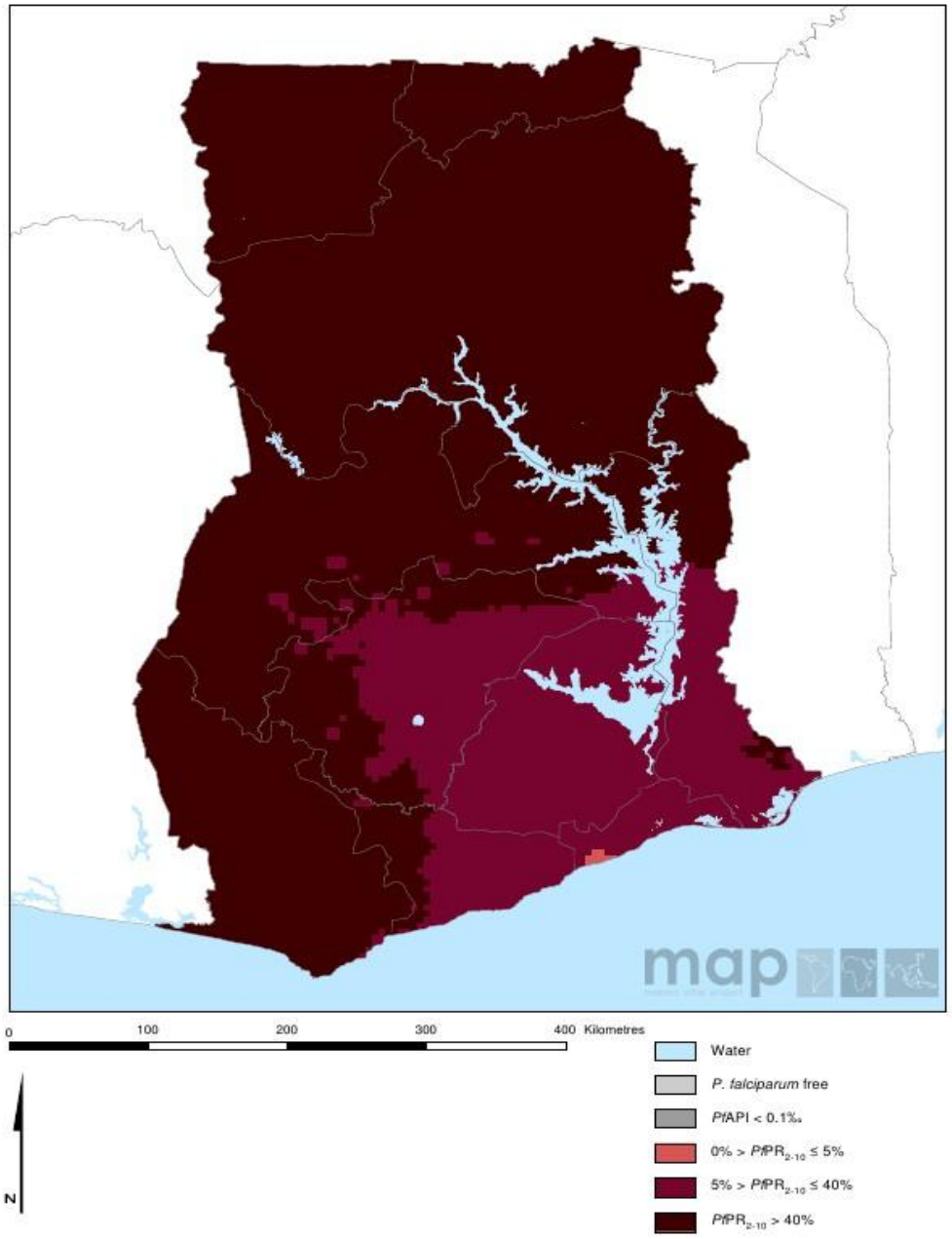


Figure 2.2 The spatial distribution of *Plasmodium falciparum* malaria stratified by endemicity class map in 2010 in Ghana

Source: http://www.map.ox.ac.uk/browse-resources/endemicity/Pf_class/GHA/.

2.5 Life cycle of malaria parasites

Briefly, the life cycle of the malaria parasite *Plasmodium*, begins in the female *Anopheles* mosquito described as the definitive host. It transmits infective forms of the parasite known

as sporozoites to a human who acts as a secondary host. A sporozoite travels through the blood vessels to the liver cells and reproduces asexually, a process known as schizogony to produce thousands of merozoites. These infect new red blood cells and undergo a series of asexual multiplication cycles (blood schizogony) to produce new infective merozoites, and eventually cause the red blood cells to burst and the infective cycle begins again (Greenwood, 2008). Some of the merozoites develop into immature sexual gametes called gametocytes and these are picked up by a mosquito when it bites an infected person. These gametocytes then mature in the mosquito gut. The male and female gametocytes fuse and form zygotes (ookinetes), which develop into new sporozoites. The sporozoites migrate to the mosquito‟s salivary glands, ready to infect a new human host. The sporozoites are injected into the skin, alongside saliva, when the mosquito takes a subsequent blood meal (Cowman *et al.*, 2012). Full details of the life cycle are depicted in figure 2.3.

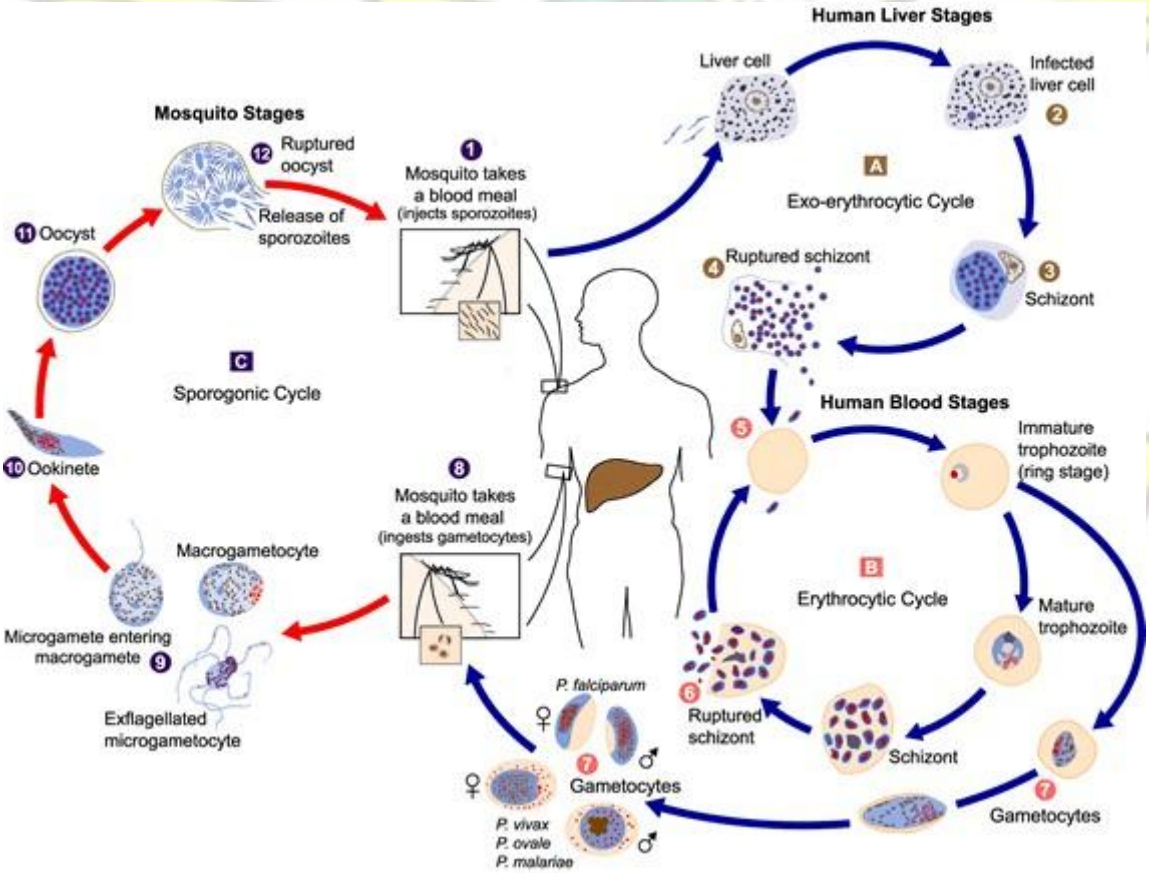


Figure 2.3 The Life cycle of malaria parasites in mosquito and man

Source: www.cdc.gov/malaria/about/biology/

2.6 The life-cycle of *Anopheles* vectors of malaria

The *Anopheles* mosquito goes through four separate and distinct stages of its life cycle: Egg, larva, pupa and adult (Service, 1993). The adult female *Anopheles* takes a blood meal approximately every two to three days. This is necessary for the development of a batch of eggs, which are normally laid before another blood meal is taken (Service, 1993). Temperature and humidity influence the time taken for a blood meal to be digested and the eggs to develop. Eggs are laid on wet mud in batches of about 100-150. The sites for egg laying vary from small amounts of residual water in places like hoof-prints and coconut husks to larger water bodies such as streams, canals, rivers, ponds and lakes depending on the species. Under favourable conditions the average life of female *Anopheles* mosquitoes is about three weeks and most lay between one and three batches of eggs (Service, 1980). The eggs usually hatch after two to three days and larvae emerge. These generally live just below the surface of water to enable them to breathe. There are four larval stages known as instars and the time required for each stage depends on the water temperature. The pupa stage lasts about two to three days at normal tropical temperatures and it is this stage that major transformation occurs to the adult stage. The pupa, which is comma shaped stays on the surface of water but does not feed and eventually its skin splits releasing the adult mosquito. Most mosquitoes mate shortly after emergence from the pupa. With a few exceptions, the female mosquitoes must bite a host and take a blood meal to obtain the necessary nutrients for the development and maturation of the eggs in the ovaries. A few species can sometimes develop at least the first batch and possibly, subsequent batches without a blood meal (Service, 1980). The speed of digestion of the blood meal depends on temperature and in most species, takes between two to three days. As the blood is digested, the white eggs in the ovaries enlarge making the abdomen become whitish posteriorly and

dark reddish anteriorly. This marks the midpoint of blood digestion and the mosquito is said to be half-gravid. When all the blood is digested and the abdomen becomes dilated and whitish carrying fully developed eggs, the mosquito is said to be gravid and searches for a suitable habitat to oviposit. The mosquito then takes another blood meal again to develop another batch of eggs. This process is repeated several times and is referred to as the gonotrophic cycle (Service, 1980). Figure 2.4 gives a generalized diagram on the life cycle of the mosquito.

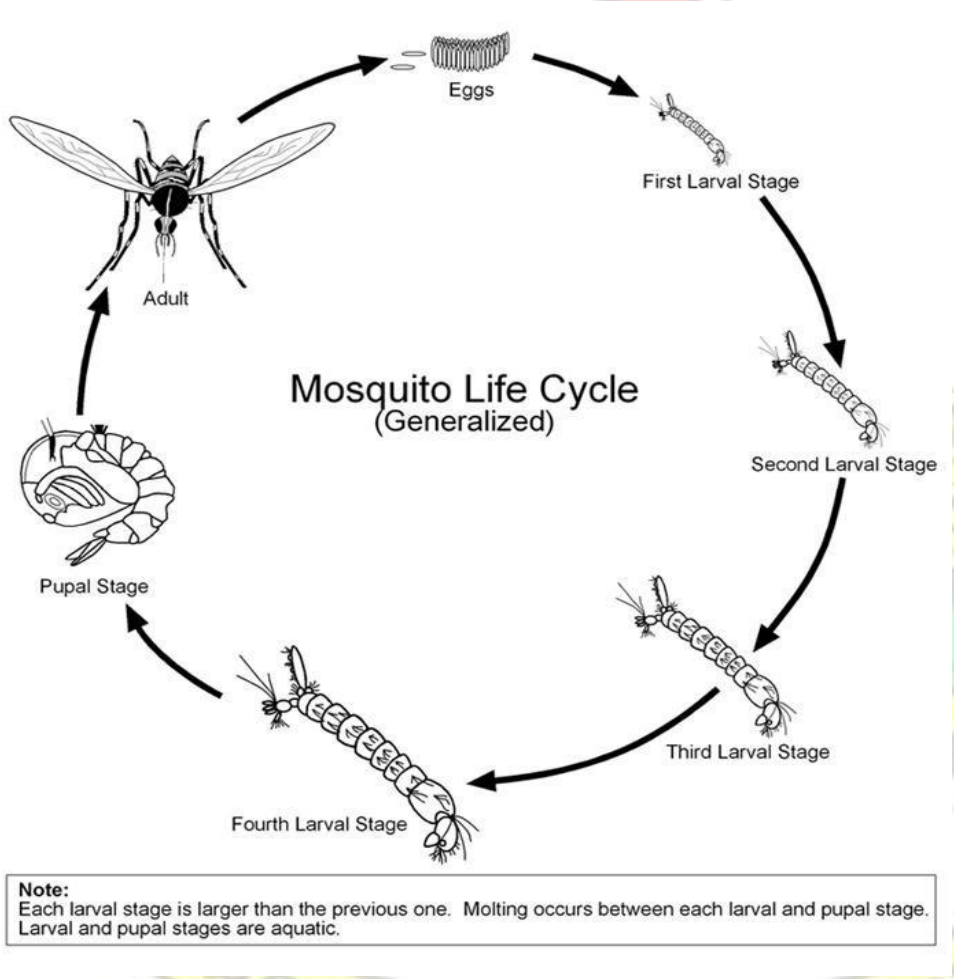


Figure 2.4 A schematic diagram showing the life cycle of the mosquito

Source: www.malaria.com

2.7 Malaria vectors in Africa

Malaria parasites can be transmitted by mosquitoes belonging to the genus *Anopheles,* which includes 465 recognised species (Harbach, 2011). About 70 of these species are known to transmit human malaria parasites (Service and Townson, 2002) and 41 are recognised to be efficient in transmitting malaria at levels of public health concern (Hay *et al.*, 2010). Africa has the most successful malaria vectors, *An. gambiae sensu stricto* subsequently referred to as „*An. gambiae'* and its sibling species, *An. arabiensis* both belonging to the *An. gambiae* complex. The other members of the complex are *An. melas* and *An. merus* (Harbach, 2004) which are often found in the western and eastern coastal areas respectively and usually considered as dominant malaria vectors (Cuamba and Mendis, 2009). The rest are either confined to well defined habitats, for example *An. bwambae,* which is found only in the geothermal springs in western Uganda (Gillies and Coetzee, 1987) do not transmit malaria but rather feed on other animals (zoophilic). Other examples are *An. quadriannulatus A* and *An. quadriannulatus B* (Coluzzi, 1984) which is now named *Anopheles amharicus* (Coetzee *et al.*, 2013). Some other *Anopheles* species like *An. funestus, An. nili* and *An. moucheti*, are considered more important vectors in some parts of Africa. There are areas where *An. funestus* has been implicated as a major vector compared to *An. gambiae* (Gillies and Coetzee, 1987 ; Coetzee and Fontenille, 2004). The heavy malaria burden in Africa is generally attributed to the relatively effective vectorial system made up of *An. gambiae, An. arabiensis,* and *An. funestus*. These vectors have the ability to exploit diverse habitats including the extensive dry savannas and semidesert areas with relatively short periods of water availability for larval development (Lehmann *et al.*, 2010).

Studies in Ghana have identified *An. gambiae s.s* and *An. funestus* as the most abundant and widespread vectors (Yawson *et al.*, 2004 ; Appawu *et al.*, 2004 ; Appawu *et al.*, 1994).

Anopheles pharoensis and *An. rufipes* are also common anophelines in northern Ghana although they have not been implicated as vectors (Appawu *et al.,* 2004).

2.8 Bionomics of the dominant malaria vectors in Africa

2.8.1 Anopheles arabiensis

An. arabiensis is a species that is generally found in the dry savannah environments and sparse woodland areas that have only just been cleared (Gillies and Coetzee, 1987). Its larvae breed in a wide range of small impermanent, sunlit, clean and shallow fresh water ponds (Himeidan *et al.*, 2008) or usually gentle flowing, partly shaded streams (Shililu *et al.*, 2007) and at times brackish habitats (Bøgh *et al.*, 2003). *An. arabiensis* breeds in irrigated rice fields but because of its preference for sun-lit areas with partial emergent vegetation, larvae are found in large numbers when the plants are still emerging (Githeko *et al.*, 1996). *An. arabiensis* exhibits variable feeding behaviour, being anthropophilic or zoophilic dependent upon host availability (Bøgh *et al.*, 2001) and its genetic makeup (White, 1974). It has also been suggested that populations found in western Africa tend to have a higher anthropophily, preferentially feeding and resting indoors, whilst those in eastern Africa exhibit greater zoophilly, bites and rest outdoors (Tirados *et al.*, 2006). with blood feeding times occurring at night, peaking at the beginning of the evening (19:00) or early hours of the morning (Oyewole and Awolola, 2006 ; Mahande *et al.*, 2007).

2.8.2 Anopheles funestus

Anopheles funestus is the only member of the *funestus* subgroup implicated as an important malaria vector (Coetzee and Fontenille, 2004). *An. funestus* larvae breed in large, stable or semi-permanent fresh water bodies usually with emergent foliage (Gillies and de Meillon, 1968), generally associated with rice cultivation and usually with its population increasing when the plants are maturing confirming its preference for shady environments (Sogoba *et*

al., 2007). *An. funestus* is highly anthropophilic (AntonioNkondjio *et al.*, 2006) usually biting during the later part of the night generally between midnight and early morning (Oyewole and Awolola, 2006 ; Oyewole *et al.*, 2007 ; Appawu *et al.*, 2004). This mosquito species is usually endophillic (Oyewole *et al.*, 2007) and has a relatively higher lifespan making it a better vector in some areas than *An. gambiae* (Coetzee and Fontenille, 2004 ; Okoye *et al.*, 2005).

2.8.3 Anopheles melas

Anopheles melas occurs on the coast of West Africa and can be a malaria vector of public health interest in coastal areas where it can occur in very high densities (Bryan, 1983). It is usually found near brackish water and can use saline water for breeding (Caputo *et al.*, 2008). They oviposit on damp ground at low tide (Giglioli, 1964), a mechanism for their eggs to avoid desiccation. Its larvae do not appear to require brackish water to develop (Giglioli, 1964). *Anopheles melas* is reported to be both anthropophilic and zoophilic, generally feeds indoors on humans but rest outdoors after feeding and is reported to feed fairly throughout the night in a continuous manner (Tuno *et al.*, 2010).

2.8.4 Anopheles merus

Anopheles merus breeds on the coast of East and South Africa both in fresh and brackish water, and is more exophillic than endophillic. It is considered as a minor malaria vector because of its inability to sustain malaria transmission alone (White, 1974). However, it was later implicated as a major malaria vector along the Tanzanian coast (Temu *et al.*, 1998) and later in Mozambique (Cuamba and Mendis, 2009). *Anopheles merus* larvae breed in shallow brackish pools and marshy areas along the coast and cannot endure high levels of salinity (Mosha and Mutero, 1982). It also feeds continuously throughout the night just like *An. melas* (Iyengar, 1962), usually found biting and resting outdoors

(Mosha and Mutero, 1982 ; Mutero *et al.*, 1984). It is reported that it‟s biting is low during early evening and then rises gradually till it peaks between midnight and 01:00 before declining to 06:00 hours (Mutero *et al.*, 1984).

2.8.5 Anopheles gambiae

The notable preference of *An. gambiae* for human blood, its capacity to feed and rest inside human houses, together with its high longevity that enables *Plasmodium* parasites to mature under different ecological settings, makes it the most successful malaria vector in the world (Simard *et al.*, 2009). It can be found in varied ecological settings within a large geographical range (Coetzee, 2004). It has chromosomal diversity presently divided into five chromosomal forms: Forest, Bamako, Savannah, Mopti and Bissau which are reproductively isolated when in sympatry (Coluzzi *et al.*, 1985). In Ghana, polytene chromosome analysis of *An. gambiae* by (Appawu *et al.*, 1994) revealed existence of three main forms characterized by different inversion frequencies: the Forest chromosomal form, typical of the moist semi-deciduous forest, the Savannah form typical of the more arid zones of the coastal and interior savannas, and the Mopti chromosomal form which is sympatric with the Savannah form in the drier areas of both coastal and interior savannas.

An. gambiae is a relatively long-lived species although shorter than *An. funestus* (Olayemi and Ande, 2009). Its larvae develop within a short period and it has been found biting and resting indoors and outdoors. Studies that have compared indoor and outdoor humanlanding catches have shown it to exhibit opportunism in blood feeding and resting locations (Appawu *et al.*, 2004 ; Oyewole and Awolola, 2006). *An. gambiae* larvae generally develop quickly in sunlit, shallow, temporary bodies of fresh water like puddles, pools and hoof prints (Mwangangi *et al.*, 2007).

2.9 Molecular forms of *Anopheles gambiae s.s*

The ability of an individual (or genotype) to produce distinct phenotypes in diverse environments is referred to as phenotypic plasticity (Agrawal, 2001) and offers a mechanism by which species can endure wide environmental disparities, and hence influence effective establishment in a new environment, before adaptive development is possible (Sexton *et al.,* 2002 ; Yeh and Price, 2004). Permanent nucleotide variations in Xlinked ribosomal DNA genes led to the description of two "molecular forms", named M form recently renamed as *Anopheles coluzzii* and S form (renamed *Anopheles gambiae* s.s*.)* (Coetzee *et al.,* 2013), among which gene flow appears highly limited and are currently recognized as incipient species (della Torre *et al.,* 2005). Both are morphologically similar and share the same resources including vertebrate hosts, adult resting sites, and freshwater larval habitats although ecological divergence have been reported between them (Simard *et al.,* 2009 ; Costantini *et al.,* 2009 ; della Torre *et al.,*

2005). Speciation of *Anopheles gambiae* s.s into these two distinct "molecular forms" is the main route of biological diversity and in the context of public health, complicates disease epidemiology as new species might change transmission patterns if they differ in traits affecting vectorial capacity (Lehmann and Diabate, 2008). There is reported ecological niche segregation as one molecular form systematically overshadows the other at the local level (Calzetta *et al.,* 2008). *Anopheles gambiae* s.s is distributed across subSaharan Africa and breeds mostly in association with rain-dependent pools and temporary puddles in West and Central Africa whilst the distribution of *An. coluzzii* overlaps with that of *An. gambiae* s.s. and is able to exploit relatively more permanent breeding sites, often closely associated with human activities, such as those created by irrigation, rice cultivation and urbanization (Costantini *et al.,* 2009 ; Simard *et al.,* 2009); (Lehmann and Diabate, 2008). This adaptation allows *An. coluzzii* to breed throughout the year, and accounts for the shift from seasonal to

year round malaria transmission in some areas (Caputo *et al.*, 2011). A study by (Yawson *et al.,* 2007) revealed greater differences between ecological zones, and between allopatric populations of a given molecular form than between *An. coluzzii* and *An. gambiae* s.s. populations. Although studies are still ongoing to fully understand the evolutionary and ecological forces that result in divergence between *An. coluzzii* and *Anopheles gambiae* s.s, Lehmann & Diabate (2008), have suggested that selection by larval predation and inter-form competition could have driven this divergence. Another study on the distribution of the molecular forms of *An. gambiae* s.s. carried out in Ghana (Yawson *et al.,* 2004) found *An. coluzzii* and *An. gambiae* s.s. to occur in sympatry in southern Ghana. This study also showed the *An. gambiae* s.s. to be predominant in the coastal savannah with the exception of a mangrove zone where rice was cultivated. *Anopheles coluzzii* was the only form collected in northern Ghana (Yawson *et al.,* 2004 ; Donnelly *et al.,* 2004). The distribution of genetic traits conferring resistance to insecticides commonly used against these vectors has also been found to differ in the two forms (Santolamazza *et al.*, 2008a).

2.10 Identification of members of *Anopheles* species complex

There are several methods for identifying the species in the *An. gambiae* complex including allozyme analysis, polytene chromosome banding patterns, high-performance liquid chromatography of cuticular hydrocarbons, and hybridization with DNA probes that are either species-specific or identify species specific restriction enzyme fragments (Collins *et al.*, 1987). However, in epidemiologic studies of transmission and in studies designed to support vector control programs, most of these methods have some limitations especially in the provision of prompt identification of the vector species within an area.

The discovery of ribosomal DNA (rDNA) sequences allowed development of probes and primers, permitting application of molecular based methods for species identification,

including DNA-DNA hybridization and polymerase chain reaction (PCR) (Scott *et al.*, 1993 ; Collins and Paskewitz, 1996 ; Rafferty *et al.*, 2002) . The PCR method has become the standard tool for species identification in this complex, but it has a disadvantage of being slow when applied to large scale field studies. The method considered to be the 'gold' standard' for species identification in this complex is the one developed by Scott and colleagues. This method is based on "specific to specific nucleotide sequences in rDNA intergenic spacers" (Scott *et al.*, 1993). There have however been reports of non-specific amplification and high rates of failures requiring repetition at times (Wilkins *et al.*, 2006). This led to the description of other methods. One such method is based on TaqMan single nucleotide polymorphism genotyping for the identification of the principal vectors in the *An. gambiae* complex members (An. *gambiae* and *An. arabiensis*) (Walker *et al.*, 2007), and this represents the first description of a 'closed tube' approach enabling the use of a single step to identify a mosquito DNA sample where single nucleotide polymorphisms (SNPs) are genotyped through PCR in a quantitative format. Two high-throughput methods based on TaqMan single nucleotide polymorphism (SNP) genotyping that require only a single step have also been described. The first assay detects and discriminates between *An. gambiae and An. arabiensis* (Walker *et al.*, 2007). It cannot however test for other members of the complex as it has been found to incorrectly identify the three species *An. quadriannulatus, An. merus* and *An. melas* as *An. gambiae*. The second TaqMan assay on the other hand identifies *An. gambiae* and *An. arabiensis* as one group and *An. quadriannulatus, An. melas* or *An. merus* as a second group (Bass *et al.*, 2007). Thus, the two assays can be used sequentially for identification. To get over this, another PCR assay known as multiplex real-time PCR assay has been developed and that requires only a single reaction to detect and discriminate the different species within the complex (Bass *et al.*, 2008b)

2.11 Identification of molecular forms of *An. gambiae*

Since the description of the two molecular forms of *An. gambiae* s.s. (della Torre *et al.*, 2001), there have been extensive studies on *An. gambiae* s.s. molecular forms focusing on their macro- and micro geographic distribution, genetics, their relative role in malaria transmission and their resistance to insecticides used in malaria vector control. In all these studies, identification of the two molecular forms is based on either direct PCRamplification of form-specific bands by allele-specific primers or by restriction digestion of PCR products (Riehle *et al.*, 2011 ; Costantini *et al.,* 2009). In recent times another PCR-method which detects the *An. coluzzii*-specific insertion of a SINE200 (short interspersed transposable element) has been developed (Santolamazza *et al.,* 2008b). *SINE200* is a ~200 bp element that is highly replicative (>3,000 copies) and extensive in the *An. gambiae* s.s. genome (Holt *et al.,* 2002). It has been suggested that SINE-PCR could be conveniently used for easy identification of *An. coluzzii* and *An. gambiae* s.s. forms without preliminary species-specific PCR identification in areas where *An. coluzzii* and *An. gambiae* s.s are not found in sympatry (Santolamazza *et al.,* 2011).

There is a general understanding that these three methods can be used interchangeably. However, this has been challenged recently (Santolamazza *et al.,* 2011) following findings of some inconsistencies from different laboratories including incorrect matching of *An. coluzzii* and *An. gambiae* s.s specific primers used in the Allele Specific PCR (AS-PCR) approach; presence of polymorphisms in the recognition sequence of restriction enzymes used in the PCR-RFLP (restriction fragment length polymorphisms) approaches; incomplete cleavage during the restriction reaction and presence of different number of copies of *An. coluzzii* and *An. gambiae* specific IGS-arrays (intergenic spacer regions) in single individuals (Santolamazza *et al.*, 2011).

2.12 Malaria Transmission

Malaria transmission dynamics in a population is very important as it provides a good understanding of the disease burden, and assists in the location of areas with the greatest risk and hence aids in development of appropriate control measures (Smith *et al.,* 2005 ; Hay *et al.,* 2008). As a result of many malaria vector control interventions and other factors such as migration, and/or changes in climate and land use patterns in many endemic areas, it will be useful to measure transmission over longer periods to determine their impact (Killeen *et al.,* 2006). Furthermore, it is important to determine how the level of risk within one community compares with other (or surrounding) communities as this will show key differences and similarities and highlight corresponding risk factors.

2.12.1 Factors affecting transmission of malaria by the vector

Several factors influence the transmission of malaria and a combination of these determine the intensity. One such factor is vectorial capacity (VC), defined as "the average number of inoculations with a specified parasite, originating from one case of malaria in unit time that the population would distribute if all the vector females biting the case become infected" (Cohuet *et al.,* 2006). and it is generally used as a convenient way to express malaria transmission risk. VC is a sequence of biological characteristics that determine the ability of mosquitoes to transmit *Plasmodium*. It measures the efficiency of vector-borne disease transmission and as such estimates the ability of the malaria vector (mechanical or biological) to transmit the parasite.

The equation used to estimate VC is

$C = ma^2p^n/-\log_e p^n$,

Where C = vectorial capacity, m = density of vectors in relation to humans, a = number of blood meals taken on humans per vector per day, p = daily survival probability of vectors

(measured in days), and *n* = incubation period in the vector (measured in days) (GarrettJones, 1964). Human malaria, vectorial systems are restricted in number as only *Anopheles* females are able to transmit the parasite to humans, added to the fact that among the more than 450 *Anopheles* species known, only 60 are considered to be definite vectors in the wild (Harbach, 2011 ; Service and Townson, 2002). VC is also limited in the sense that some species have a major role in malaria transmission, whilst others have a minor role and even in species complexes, some populations or individual mosquitoes can have different impacts on transmission (Manguin *et al.*, 2008). VC is difficult to estimate in the field but is however an essential component of mathematical models of malaria transmission as a number of assumptions must be taken into account when VC is used to either assess malaria risk or predict its evolution (Molineaux *et al.*, 1988).

Temperature, humidity and rainfall have been widely associated with the dynamics of malaria vector populations, however, there is little knowledge on how these factors affect transmission in houses where humans inhabit (Yé *et al.*, 2007). These factors have generally been used in the mapping of malaria risk areas at country and regional levels (Zacarias and Andersson, 2010). However, there have been reports of variability between the incidence of malaria and these factors at the microclimate level (Ying Zhang *et al.*, 2008). Temperature has been shown to affect both parasite and vector (Meyer, 1989). The ideal temperature for eggs of *An. gambiae* to hatch is between 24 ^{o}C and 30 ^{o}C (Impoinvil *et al.*, 2007) with an optimum temperature range of between 22^{o}C and 26^{o}C for survival and the shortest development between larvae and pupae (Bayoh and Lindsay, 2003). For *P. falciparum*, a range of 18^{o}C to 40^{o}C is important for its development in the mosquito (Cohen *et al.*, 2008) and needs only 12 days to develop at a temperature of 25^{o}C (Snow and Gilles, 2002). The presence of adult mosquito vectors can also be affected by the temperature of the microclimate (where the mosquito rests). There are suggestions that, for every 1^{o}C increase

in the average indoor minimum temperature, there is usually a 77 % increase in the number of mosquitoes near the lower limit for parasite and mosquito development (Stresman, 2010).Thus, using the mean external temperature to determine malaria risk may only be appropriate if the vector is exophillic and may not be suitable for predominantly endophillic species, meaning the resting behaviour can affect transmission estimation (Stresman, 2010). The availability of water breeding sites also affects malaria (Snow and Gilles, 2002). All year round malaria transmission is feasible in the presence of permanent water bodies such as swamps, dams and those created by irrigation (Oesterholt *et al.,* 2006); (Kreuels *et al.,* 2008) but these are dependent on the vector species and closeness to blood meals (Stresman, 2010). As extreme dry conditions tend to cause desiccation in mosquitoes, humidity can also affect transmission (Jawara *et al.,* 2008). Malaria transmission can also be affected by the water quality of breeding sites. For example, *An. gambiae* has been shown to lay fewer eggs in a habitat with a higher turbidity than the preferred cleaner water (Sumba *et al.,* 2004). There have been several studies showing an association between agriculture and malaria transmission (Packard, 1986). Some studies have reported reduction, (Ijumba *et al.,* 2002), whilst others have shown no statistically significant association (Mathenge *et al.,* 2005 ; Kerah-Hinzoumbé *et al.,* 2009 ; Wen *et al.,* 2008) and others report that agriculture increases transmission by increasing vector densities through the provision of suitable breeding sites (Sogoba *et al.,* 2007 ; Munga *et al.,* 2006).

The mosquito‟s innate immunity contributes to the intensity of transmission. This is as a result of its ability to get rid of many of the parasites (Yassine and Osta, 2010). The time taken for sporozoites to develop within a vector affects vectorial capacity as there is an increased chance of getting transmitted when they reach the salivary glands early (Cohuet *et al.,* 2006). Vector density is also important, as mosquito must be relatively abundant and have habitat adjacent to human settlement and have a high frequency of biting enough to

sustain transmission. However, this can be affected by the vector‟s host preference for human blood meals (anthropophily) and in *An. gambiae* there is a reported link between chromosomal inversions and the choice of a host (Takken *et al.*, 1998). It has also been shown that vectors prefer to bite people infected with gametocytes (Lacroix *et al.*, 2005), particularly infected pregnant women (Ansell *et al.*, 2002). Studies in Southern Ghana showed a higher transmission in an area where 93% of the mosquitoes fed on human compared to another area where about 45% of mosquitoes fed on cattle (Appawu *et al.*, 2001). Human activities may play a role in the increased number of vectors in an area. For example, irrigated fields are favourable breeding grounds to many malaria vectors. The design of a house may enable vector entry and hence affect malaria transmission. Houses that generally have screening will reduce the tendency for vectors to have contact with humans (Baragatti *et al.*, 2009). Several studies have described different house characteristics related to mosquito entry including the presence of eaves, absence of ceilings and non-existence of screening over windows (Kirby *et al.*, 2008). Mud houses with grass roofs are known to attract more mosquitoes indoors because of their cool and dark nature and have an increased risk of mosquito bites indoors (Odiere *et al.*, 2007 ; Lwetoijera *et al.*, 2013)). The attractiveness of humans are said to be another factor that draw mosquitoes into houses and therefore houses with more inhabitants will be more attractive than ones with fewer sleepers (Lwetoijera *et al.*, 2013).

2.12.2 Measurement of malaria transmission

2.12.2.1 Entomological Inoculation rate

The intensity of malaria transmission may be measured in several ways. However, compared to traditional epidemiological estimates such as spleen rate, incidence and prevalence, a more direct measure of transmission intensity is the entomological inoculation rate (EIR)

(Kelly-Hope and McKenzie, 2009).The EIR is estimated as the number of bites by infective mosquitoes per person per unit time (Macdonald, 1957). It is the product of the human biting rate (defined as the number of bites per person per day by mosquitoes and the proportion of such mosquitoes that are infective referred to as the sporozoite rate (Birley and Charlewood, 1987) and is very useful in the assessment of control interventions that are aimed at reducing human-vector contact (Drakeley *et al.,* 2003). The magnitude of the EIR is influenced by the rate at which vectors feed on humans (man biting rate), which is largely dependent on the mosquito density and to some extent, the feeding habits of the vector species (Shililu *et al.*, 1998). Measuring biting rates constitutes a very important aspect of entomological monitoring of vector control interventions, such as insecticide treated nets (Service, 1993). The sporozoite rate is another important epidemiological parameter for the assessment of malaria transmission. This is usually measured as the proportion of female mosquitoes whose salivary glands are infected with malaria parasites in a given sample (Mboera and Magesa, 2001). The understanding of the entomological aspects of the epidemiology of malaria depends in part, on the accurate assessment of this rate.

2.12.2. Detection of sporozoite infection in malaria vectors

Traditionally, detection of *Plasmodium* species in the mosquito was carried out by visual examination of the salivary glands with the help of a microscope after dissection. This method however had a drawback of being time consuming and the need for skilled personnel coupled with the inability to identify the *Plasmodium* species present (Wirtz *et al.,* 1987). New methods based on quicker immunological and molecular approaches have now been developed. The commonest methods currently used are the circumsporozoite protein enzyme-linked immunosorbent assay known as CSP ELISA (Burkot *et al.,* 1984)). However, this method also has a few drawbacks (although it has been proven to be relatively robust and cheap) including reports that it overestimates infection rates as circumsporozoite

protein may be spread when sporozoites are shed during migration through the mosquito (Beier and Koros, 1991 ; Fontenille *et al.*, 2001). Also, mosquitoes stored in ethanol or isopropanol for later analysis can render the specimens unsuitable for ELISA testing coupled with the need to run separate assays in order to distinguish all four malaria causing *Plasmodium* species since each has specific monoclonal antibodies (Arez *et al.*, 2000). CSP ELISA may be relatively insensitive to very low-level infections and will require the use of more sensitive PCR-based assays (Arez *et al.*, 2000). PCR assays designed to detect *Plasmodium* in mosquitoes mainly utilize primers designed against either specific regions in relation to sequences encoding small subunit of ribosomal RNA (ssrRNA) which either confirm the presence of *P. falciparum* or all four *Plasmodium* species (Snounou *et al.*, 1993a ; Snounou *et al.*, 1993b ; Tassanakajon *et al.*, 1993). The

"gold standard" for PCR detection of *P. falciparum* is to use the nested approach and this comprises two rounds of PCR (Snounou *et al.*, 1993b). The nested PCR has a drawback of requiring separate reactions for the detection of each *P. falciparum* species. This has led to the development of high-throughput assays based on real-time PCR (Vo *et al.*, 2007 ; Bass *et al.*, 2008b).

2.12.3 Sampling mosquito vectors

The entomological inoculation rate requires capturing host-seeking *Anopheles* mosquitoes to determine the man biting and sporozoite rates (Beier *et al.*, 1999 ; Robert *et al.*, 2003). Several sampling methods have been used to estimate the population density of mosquitoes searching for a blood meal. The methods commonly used include the human landing catches, pyrethrum spray catches, and light trap catches.

2.14.1 Human landing catches (HLC)

Traditionally, the human landing catch (HLC) is considered the "gold standard" for estimating vector-human contact and is employed to pick mosquitoes that land on a human host to feed (Mathenge *et al.,* 2002). The principle behind the use of this method is that, humans act as baits to attract blood-seeking mosquitoes. Human landing catches give information on the different *Anopheles* mosquitoes biting people either indoors or outdoors. The biting activity throughout the night can be obtained and hence the times of maximum exposure to malaria transmission. The method can also be used to estimate the man biting rate per night and season and adults caught can be dissected to determine parity and for the estimation of sporozoite and inoculation rates (Le Goff *et al.,* 1993). Normally all night collections start at sunset and stop at sunrise. These collections are made both indoors and outdoors depending on the behaviour of the local inhabitants. In most tropical areas, the period between 18:00 hours and 06:00 hours represent the main period of biting activity and movement of majority of local vectors. The environmental conditions at the time of collection such as wind-speed, rainfall, moonlight, temperature and humidity all have influence on the movement of mosquitoes and therefore their biting activities (Service, 1976). The unique advantage of the method is that it directly samples the mosquitoes as they land to bite humans and therefore the samples obtained can be projected to represent mosquitoes for the transmission of malaria (Mathenge *et al.,* 2002). One of the disadvantages of using HLC is that, the rate at which a mosquito approaches and attacks an exposed person performing HLC may be higher than under normal conditions.

This is because, the baits in HLC are relatively more available to host seeking mosquitoes than if they were under normal circumstances, that is, either asleep (covered with bedding) or more active instead of posing as baits. This method therefore over estimates the biting

rate although it is considered as the most representative measure of man biting rates of mosquitoes (Lines *et al.,* 1991). There is also the problem of a variation in the attractiveness of the individual collectors to vectors and their ability to catch mosquitoes (Knols *et al.,* 1995). To reduce the effect of this variation, the collectors are rotated at regular intervals to include indoor and outdoor catching (Shadrawi *et al.*, 1974). Another disadvantage is that, it is labour-intensive and requires special training for collectors and extensive supervision and motivation (Knols *et al.,* 1995 ; Kelly-Hope and McKenzie, 2009). The use of HLC has reduced in recent years following ethical issues regarding potential exposure of collectors to mosquito-borne diseases (Mathenge *et al.,* 2002). Thus, HLC for large-scale monitoring of malaria transmission may not be sustained (Wong *et al.,* 2013).

2.14.2 Human Baited Traps (HBT)

One alternative to HLC is using diverse designs of bed nets that both surround and protect the human bait while passively or actively capturing the mosquitoes that come to bite (Silver, 2008). The most common human baited traps are the passive or semi passive trap designs, where a person rests or sleeps under a bed net, while mosquitoes pass through a window, a funnel, or under a gap of an outer entrapment net (Reid, 1961 ; Silver, 2008). An example is the Mbita trap that uses a funnel trap attached on top of the bed net (Mathenge *et al.,* 2002). It has been effective in estimating man biting rates in some studies (Mathenge *et al.,* 2004 ; Mathenge *et al.,* 2005), but unsuccessful in other studies (Laganier *et al.,* 2003 ; Braimah *et al.,* 2005). HBTs however have a disadvantage of allowing species that are capable of exiting even small gaps and funnel holes to escape before being captured (Charlwood *et al.,* 1986 ; Darbro and Harrington, 2006) . Another disadvantage is that, collectors take a long time to aspirate mosquitoes from the relatively large holding shelter, which can be strenuous and can lead to risk of being bitten (Govella *et al.,* 2009). The active

human bait trapping system most often attach a fan trap to the entrapment net, and usually boost the attractiveness with Center for Disease Control and Prevention (CDC) mini-light traps (LTC) hung next to the feet of a human resting under a bed net (Ndiath *et al.,* 2011 ;, Overgaard *et al.,* 2012)

2.14.3 Light trap catches (LTC)

Light traps used for mosquito sampling are mechanical devices that attract mosquitoes to light, a phenomenon that may not be directly associated with the act of feeding on humans (Mathenge *et al.,* 2002). They have however been found to sample mosquitoes successfully when hung next to a human host usually protected under a bed net (Costantini *et al.,* 1998). The main principle behind the use of light traps as a collection method is that, mosquitoes that have come into the room in search of a blood meal get attracted to the light in the trap and are sucked in by a fan. Therefore any mosquitoes captured by the trap can be taken as those that would have bitten the occupants of the room. The use of light traps for sampling mosquitoes has the advantage of trapping mosquitoes in search of a blood meal and therefore can be used to estimate man-biting rates. Light traps have however been used in the sampling of man-biting mosquitoes with varying degrees of success (Chandler *et al.,* 1975). They have been found to underestimate the relative abundance of some species of *Anopheles* (Hii *et al.,* 2000) and to have a bias in sampling older or gravid mosquitoes (Mbogo *et al.,* 1993 ; Hii *et al.,* 2000). Several studies reported close correlation between the numbers of *Anopheles* vectors captured by CDC light traps and HLC (Ndiath *et al.,* 2011 ; Sikaala *et al.,* 2013) whilst others found low catch rates (Mbogo *et al.,* 1993 ; Govella *et al.,* 2011). In addition, light traps catches have been found to overestimate EIRs as they sample *Plasmodium* infected females (Wong *et al.,* 2013).

2.14.4 Pyrethrum spray catches (PSC)

Pyrethrum spray catches are generally considered the gold-standard for sampling indoor resting mosquito. The method collects indoor resting mosquitoes after knockdown by space spraying of a pyrethrum solution. The main principle of the method is that, mosquitoes tend to rest after feeding and therefore any mosquitoes captured with this method might have fed on people in the room or were not able to feed in the night and were resting. This method has been used as a standard quick and easy method of collecting mosquitoes resting in huts and animal shelters (Service, 1993). It collects mosquitoes of varying physiological conditions, gives valuable information on relative changes in seasonal abundance of endophillic vectors, and provides adults for determining sporozoite and inoculation rates and information on host preferences and degree of exophilly. The PSC also provides unfed adult mosquitoes for dissections to determine parity and half gravids for chromosomal identifications (Appawu *et al.*, 2001 ; Service, 1976). This method has several advantages, such as collection of large numbers of mosquitoes. In addition, PSC is faster and does not require many workers and special skills. It also has the advantage of reducing the overall nuisance at least temporarily from mosquitoes and other unwanted organisms (Krajacich *et al.*, 2014). PSC has the disadvantage of being inconvenient for residents of sprayed rooms because furniture, food, cookware, animals, and water have to be removed from the dwellings before the spraying. It also samples mainly endophilic mosquitoes and is less sensitive where mosquito populations are more exophagic and exophilic (Mboera, 2005).Unlike HLC, estimation of man biting rate from PSC is done indirectly since the mosquitoes are not caught biting directly. The man-biting rate is estimated by dividing the number of blood-engorged females by the number of bedroom occupiers. What is obtained is based on the assumption that exophilly, zoophagy and exophagy have been excluded (Service, 1976). Not all the mosquitoes might have fed on humans and therefore the value

obtained is usually multiplied by a factor known as the human blood index. The human blood index is defined as the proportion of *Anopheles* that had fed on human blood. Also mosquitoes captured using the method may not represent all the mosquitoes in the room since some may escape through eaves and others may fall in places where they cannot be collected.

2.13 Malaria prevention and control

2.13.1 Malaria chemotherapy

In the past, Chloroquine was a drug that was efficient in curing malaria (Trape, 2001). Its mode of action was destroying asexual parasites in the blood (just like amodiaquine). However, this drug has lost its efficiency for *P. falciparum* treatment as a result of resistance of the parasite to the drug. There are other drugs such as primaquine that acts by destroying gametocytes of *P. falciparum* and *P. vivax* hypnozoites and thus stopping the cycle of these *plasmodium* species in the mosquito (Winstanley, 2000). In recent times, another group of drugs derived from artemisinins whose action includes the swift clearance of the reproductive blood stages as well as gametocytes of *plasmodium* have been introduced for the treatment of malaria (Dondorp and Day, 2007 ; Okell *et al.*, 2008). The WHO has recommended that these artemisinin derived drugs are used in combination with some of the other drugs such as amodiaqine (referred to as artemisininbased combination therapies or ACTs) to prevent early development of drug resistance (WHO, 2012b). Although not considered a major strategy, mass drug administration (MDA) which refers to the use of antimalarials that can both treat and act as a prophylactic, can be used in low transmission areas, to avoid re-infection and may prove useful in transmission reduction (Greenwood, 2008).

2.13.2 Preventive chemotherapy

Preventive chemotherapy refers to the routine administration of a complete treatment dose of effective antimalarials to a targeted population at risk of malaria for preventive purposes (WHO, 2012c). It is based on either Intermittent Preventive Treatment (IPT) or Seasonal Malaria Chemoprevention (SMC). IPT is usually targeted at pregnant women (IPTp) where sulfadoxine-pyrimethamine (SP) is given usually at scheduled antenatal visits; or infants (IPTi) where infants are given 3 doses of SP at specified time points (WHO, 2011) . SMC is targeted at children aged 3 to 59 months. The aim of this strategy is to sustain curative antimalarial drug concentrations in the blood during the period of high malaria risk. The WHO recommends the administration of a complete treatment dose of amodiaquine plus sulfadoxine-pyrimethamine at monthly intervals at the beginning of the transmission season, to a maximum of 4 doses (WHO, 2012c).

2.13.3 Malaria vaccines

Two main vaccine types are known and these include the Pre-erythrocytic, blood-stage and transmission blocking vaccines. The pre-erythrocytic vaccines (PEVs) are generally aimed at protecting against infection. They either seek to prevent sporozoites from invading hepatocytes or destroy them in the hepatocytes (Dubovsky and Rabinovich, 2004). However such vaccines, because of their short-lived nature may shift the occurrence of severe disease to older age groups (Ripleyballou, 2007). Thus, such vaccines will only be important in areas that have almost achieved malaria elimination and not in hyper endemic areas (Enayati and Hemingway, 2010).

The blood stage vaccines act by incapacitating the merozoites in the bloodstream or stimulate the production of antigens on the surface of red blood cells to mimic naturally

developed immunity (Sutherland, 2007) and hence can reduce the severity of disease (Good, 2001).

Another category of vaccine candidates are the transmission-blocking vaccines that need antibody and compliment to be ingested by the mosquito during blood feeding so that the antibody will prevent development of the sexual stage in the gut and hence, blocks further disease transmission (Lavazec and Bourgouin, 2008). Generally, a vaccine can only eliminate malaria if it has a 100% efficacy and covers 100% of a population at risk, but since these are not obtainable any vaccine that is developed and commercialized will need to be administered alongside the other interventions (Carter, 2001).

The increase in funding over the last 10 years has allowed over 40 vaccine projects to reach the clinical trial stage (Schwartz *et al.*, 2012). There has been indication that humans can be vaccinated against malaria from the fact that individuals who are born in endemic areas and live through the early years of exposure become resistant first to severe malaria, and then to clinical malaria (Schwartz *et al.*, 2012). It has been demonstrated that clinical protection from malaria is possible through the transfer of gamma-globulin fractions from semi-immune to naïve humans (Bouharoun-Tayoun *et al.*, 1990). The antigenically distinct multiple stages of development in both the vector and humans, has made molecular characterization of vaccine targets basis for subunit based protection for *P. falciparum* very difficult (Moorthy and Kieny, 2010). The RTS,S/AS01E vaccine is the first human anti-parasite vaccine being considered for licensure as it is going through a Phase 3 and it has demonstrated an efficacy of between 25-60% in different malaria endemic areas in Phase 2 trials (Schwartz *et al.*, 2012 ; Olotu *et al.*, 2011).

2.13.4 Malaria vector control

The most effective way of controlling malaria is to target mosquito vectors (Murray *et al.*, 2012). The main objectives of malaria vector control are to protect humans against mosquito bites and to reduce the local transmission intensity through reduction of longevity, human-vector contact and reduction of local vector populations (WHO, 2012b). Currently, vector control is the principal and vital component of all malaria control strategies. Two main interventions: long-lasting insecticidal nets (LLINs), which in recent years have largely replaced insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (Enayati *et al.*, 2009) are used. These interventions have been successful because a large population of the vectors bite indoors (endophagic), feed late at night and rest indoors after feeding (endophillic) (Bugoro *et al.*, 2011). These strategies reduce malaria transmission by killing older female mosquitoes and therefore prevents the transmission of malaria parasites by reducing the reproductive potential (WHO, 2006 ; Phillips-Howard *et al.*, 2003). These interventions have had an impact on malaria transmission reduction in parts of sub-Saharan Africa where malaria is endemic (O‟Meara *et al.*, 2010 ; Okumu and Moore, 2011 ; Zhou *et al.*, 2010).

Only four classes of insecticide: pyrethroids, organochlorines, organophosphates and carbamates have been recommended by the WHO for these interventions (WHO, 2012a). Pyrethroids are considered best as they are known to be safe and effective with little public health concerns and account for most IRS coverage and all LLINs use (WHO, 2011) . Pyrethroids have several modes of action including the opening of sodium channels which lead to continuous nerve excitation, paralysis and death of the vector (Brown, 1986). They also cause irritation, reduced feeding and landing times and hence decreases biting (WHO, 2012a). DDT is still used for malaria control because of its low cost although its application

as a pesticide in agriculture has ceased due to its decreased effectiveness and environmental persistence (Coleman and Hemingway, 2007).

The Organophosphates recommended for use for IRS are fenitrothion, Malathion and pirimiphos-methyl. These have shorter residual effects (usually 2–3 months) when compared to pyrethroids and DDT (WHO, 2009) and are more expensive.

Organophosphates act by the inhibition of acetylcholinesterase and prevent breakdown of the neurotransmitter acetylcholine, giving rise to increased stimulation of the neuromuscular junction leading to death of the vector (Brown, 1986). Carbamates are used for IRS in the form of bendiocarb and their actions are similar to the organophosphates and are more expensive than pyrethroids and DDT.

The LLINs are effective in reducing some malaria transmission but not as effective as IRS and more susceptible to insecticide resistance, as only one of the WHO recommended classes of insecticides (pyrethroids) are used for LLINs treatment (Zaim *et al.,* 2000).

2.13.5 Larval Control

Larval control can serve as a compliment to IRS and LLINs in some instances, particularly where breeding sites are not many, are permanent, and are easy to locate and treat (WHO, 2012a). This method can play an important role in malaria control where there are exophillic vectors present in an area or communities oppose IRS (Gratz and Pal, 1988). Larval control involves the use of chemicals or biological agents, however the use of the latter are technically challenging as it is expensive to raise organisms and difficult to apply in their limited aquatic sites where temperature, PH, and organic pollution may exceed the narrow requirements of the agent being used. (Ffrenchconstant, 2005 ; Rishikesh *et al.,* 1988). About 27 malaria endemic countries (including 9 from the Africa region) used larval control in specific locations where malaria transmission was high (WHO, 2012a).

2.13.6 Transgenic Mosquitoes for Malaria Control

The spread of malaria can be lessened either by decreasing the mosquito population or reducing their efficiency in transmitting the parasites (vectorial capacity). These two approaches can both be addressed through a transgenic approach: population reduction or population replacement (Boëte and Beisel, 2013). Studies are currently on-going through the use of diverse gene driving systems (Marshall, 2009) to develop *Anopheles* strains that are capable of interrupting transmission by synthesising molecules that will stop the development of parasites (Boëte and Beisel, 2013). There are suggestions of a possibility of using *Wolbachia* strains to shorten the life span of vectors in order to decrease vectorial capacity (as was done in *Aedes* mosquitoes to control dengue) (McMeniman *et al.*, 2009). It is projected that, the release of such transgenic mosquitoes into the wild could boost the mosquito‟s defence mechanisms or introduce antibodies against malaria although they still have several biological and ecological challenges to overcome including, how to spread these transgenes through wild populations (Lambrechts *et al.*, 2008), local acceptance and even international regulations on their deployment (Boëte and Beisel, 2013).

2.14 Geographical Information Systems

Geographical Information System (GIS) is an information system that is used to input, store, retrieve, manipulate, analyze and output geographically referenced data or spatial data (Saxena *et al.*, 2009). It incorporates a wide range of datasets accessible from different sources including Remote Sensing (RS) and Geographical Positioning Systems (GPS) and often referred to as the mainstay of spatial tools. GIS has the capacity to explore integrated datasets and present the results as useful knowledge to assist decision making (Saxena *et al.*, 2009). Remote sensing (RS) is the science of obtaining information about the Earth's surface. This is done by sensing and recording reflected or radiated energy and processing, evaluating, and applying that information (Reddy, 2001). Geographical positioning systems

(GPS) technology was first developed by the US Department of Defence through the Navstar satellite system. These are designed to obtain coordinate locations using hand-held GPS receivers to obtain coordinate locations from this satellite system. This is made up of over 24 satellites orbiting the earth at a distance of

20,600 km allowing for global coverage (Martin *et al.,* 2002; Boulos *et al.,* 2001). The GPS intercepts high frequency signals with time specific patterns from these satellites and by matching the signals with patterns within themselves calculate its position on earth

(latitude, longitude and altitude). A minimum of three signals enable determination of latitude and longitude but with four signals, the altitude can be included (Logsdon, 1992).

Maps produced by the GIS are useful in spatial analysis for disease control (Moore and Carpenter, 1999). Some of the distinct advantages of GIS include its power to handle repetitive tasks and rapidly evaluate spatial data from different sources and spatial areas; ability to handle huge volumes of data, indicating characteristic data, and presenting detailed cartographic output, standardisation and capacity to ask what if questions (Moore and Carpenter, 1999).

2.14.1 GIS in malaria research and control

Malaria transmission is affected by a complicated interaction of the vector, host, parasite and environment, usually directed by variable ecological and social factors (Daash *et al.*, 2009). For instance, vector bionomics is affected by climatic factors like rainfall, humidity and temperature to the extent that small changes can result in spatial variations in vector populations and hence the threat of malaria and its spread (McMichael and Haines, 1997) GIS-based malaria information system in the past was made in a make-shift manner driven by inadequate empirical data resulting in misdirection of the inadequate resources available (Snow and Marsh, 2002). The use of advanced molecular techniques such as PCR for the identification of vector species, insecticide resistance and detection of disease parasites

coupled with the evolution of technologies like GIS to support data collection, organisation, and analysis has enhanced our ability to predict, prevent, and control vector borne diseases (Eisen and Eisen, 2011). It is now possible to use GIS software such as ARC-GIS and mapping software, such as Google Earth ™ (http://earth.google.com/), to generate risk maps in different formats including overlays on satellite imagery and dynamic graphics of space-time patterns that can be viewed (Eisen and Lozano-Fuentes, 2009). GIS-based management information systems (MIS) and maps have played an important part in devising malaria insecticide and drug policies, assessing changes in malaria transmission over time, and allocating resources to control malaria (Martin *et al.,* 2002). Maps are exploratory models, used to convey and make clear data. They aid in the visualization of disparities, clustering, heterogeneity, or consistency within data. Spatial patterns can be perceived and links imagined through the use of maps. Symbols and colours are used to reveal detail or the significance of certain features (Coetzee *et al.,* 2000). GIS has enabled comprehensive studies on the relationships between the frequency of a disease and vector distribution (Saxena *et al.,* 2009).

Spatial data collection has played a major role in advancing the epidemiology of malaria. The use of GPS and RS has assisted in the provision of illustrative meteorological and geographic features (Saxena *et al.,* 2009). Remote Sensing data in GIS have been used extensively to identify, characterize and monitor breeding sites of malaria vectors and potential vectors in places like Tanzania and the Republic of Korea, Kenya and Burkina Faso (Dongus *et al.*, 2007 ; Sithiprasasna *et al.,* 2005 ; Chen *et al.*, 2006 ; Wang *et al.,* 2005) and in an integrated way to map malaria risk in several parts of Africa (Kleinschmidt *et al.,* 2001).

For effective vector control strategic planning, GIS can be used to map the distribution of malaria vectors particularly those belonging to species complexes. Examples are the work done by Coetzee *et al*. (2000) who created distribution maps for the six species of the *An. gambiae Giles* complex including climatic factors such as rainfall and temperature in Africa. Obsomer *et al.,* 2007 did the same for *An. dirus* complex distribution across Southeast Asia, and the development of a global distribution map of dominant vectors in each malaria endemic region (Kiszewski *et al.,* 2004).

Malaria control activities can also be monitored with the help of GIS. In southern Africa, the use of GIS mapping to monitor insecticide spraying operations in a continuous manner was applied (Booman *et al.,* 2003) and in Malawi, mapping of the indicators for Roll Back Malaria (RBM) enabled easy recognition of geographical inequalities of population coverage for the principal malaria control strategies (Kazembe *et al.,* 2007). One of the essential functions of GIS is the measurement of distance. In malaria risk mapping, the relationship between incidence and distance from houses to breeding sites for vectors and their roles in malaria transmission have been documented (Oesterholt *et al.,* 2006 ; Staedke *et al.,* 2003). In Sri Lanka, GIS confirmed a strong association of a maximum distance of 750 meters between a house and the breeding site for the main vector *An. culicifacies* which subsequently led to cost reduction by limiting insecticide spraying in residential areas within 750 m of streams and rivers (Konradsen *et al.,* 2003). GIS was also used in a high malaria transmission area in Equatorial Guinea to show that geographical differences in transmission parameters such as man biting rate was associated with the distance of human dwellings to the closest water body (Cano *et al.,* 2006).

Using GIS to measure distance has also revealed its effect on the influence on malaria related hospital admissions, prevalence and mortality (Ronald *et al.,* 2006 ; Schellenberg *et al.,* 1998) and in assessing the effect of ITNs on child morbidity and mortality from large-scale

trials in Ghana; it was reported that mortality rates of children living in control compounds correlated with distance from the nearest ITN compound (Binka *et al.,* 1998). It is therefore important that prior to the deployment of control inventions, malaria transmission parameters are well organised in the context of GIS (Daash *et al.,* 2009) so that appropriate spatial data is well timed for surveillance and strategic planning (Eisen *et al.,* 2011).

2.15 Insecticide resistance

Insecticide resistance is defined broadly by the World Health Organization (WHO) 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).

Global malaria indicators between the years 2000 and 2010 are reported to be declining(WHO, 2012a). It is estimated that over 254 million ITNs have been deployed between 2008 and 2010 to malaria endemic countries in Africa and this has resulted in a tremendous reduction in malaria mortality and morbidity but more than 50% of what has been achieved would be lost if the current insecticides used for vector control become ineffective (WHO, 2012a).

Currently about 40 malaria-endemic countries are reporting resistance to insecticides, mostly to pyrethroids (WHO, 2012b). Figure 2.5 below shows the distribution of pyrethroid resistance in malaria vectors in Africa.

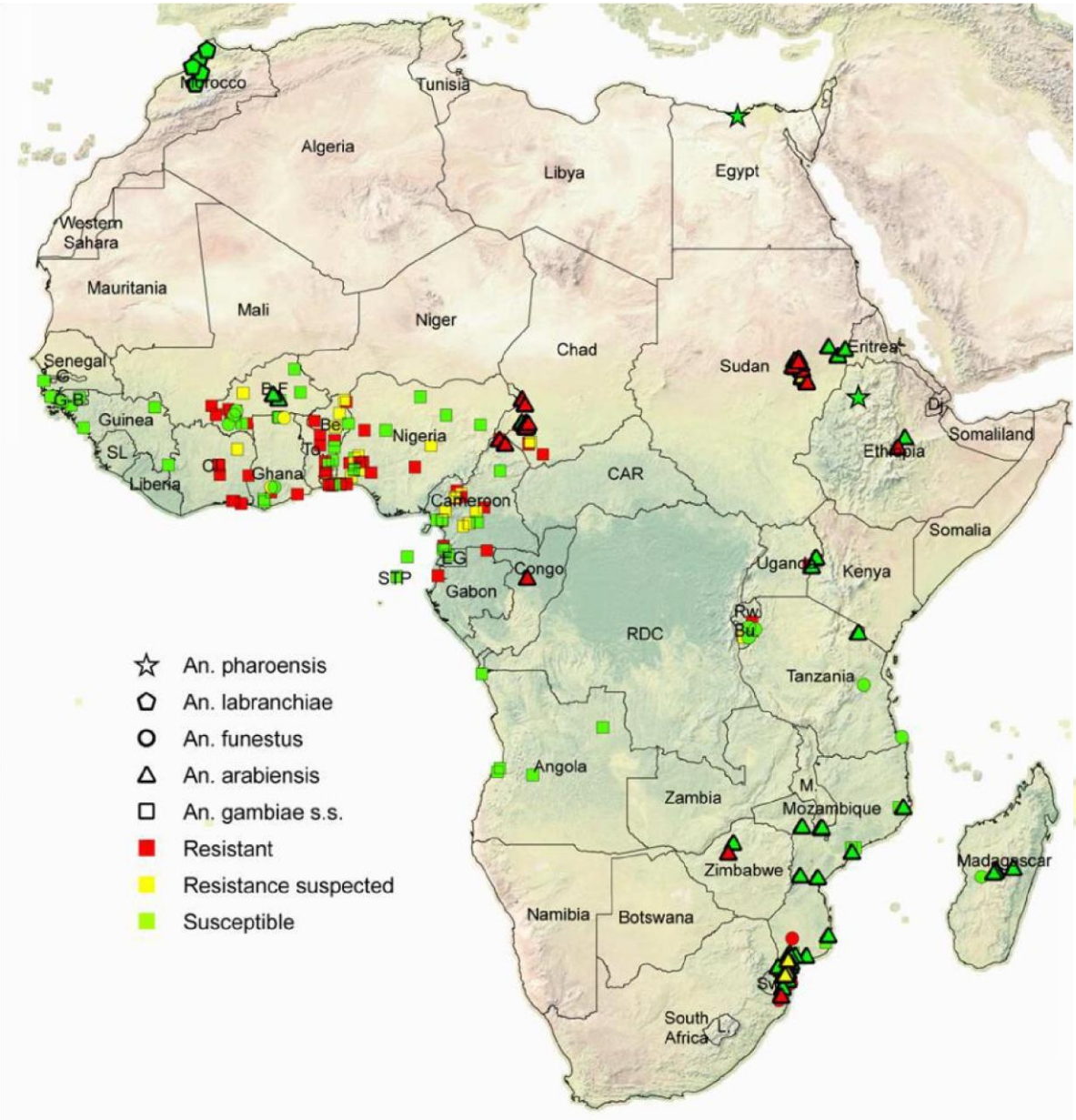


Figure 2.5 : Map of Africa showing the distribution of pyrethroid resistance in malaria vectors

Source: Ranson *et al.*, 2011

Information on insecticide resistance in Ghana is limited; studies in southern Ghana reported a high level of pyrethroids, DDT and carbamate resistance within mosquito populations (Coetzee *et al.*, 2006 ; Essandoh *et al.*, 2013). Also in northern Ghana, a study showed that *An. gambiae* and *An. funestus*, the main malaria vectors in the Kassena-Nankana district were susceptible to the insecticides being used in the treatment of bed nets however, they

emphasized the need for continuous monitoring of the pyrethroids because their efficacy was not very high (Anto *et al.*, 2009).

Phenotypic resistance is generally measured using susceptibility tests looking at vector mortality on exposure to a standard dose of the insecticide (WHO, 2012a). The WHO has defined phenotypic resistance as "development of an ability, in a strain of insects, to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (WHO, 1957). Insecticide resistance mechanisms are biological processes that are controlled genetically and under insecticide pressure insect populations produce resistant phenotypes in order to survive and reproduce (ffrench-Constant *et al.*, 2004). Resistance mechanisms can be grouped into four categories; target-site, metabolic, behavioural and cuticular resistance.

2.15.1 Insecticide Resistance Mechanisms

2.15.1.1 Metabolic Resistance

Metabolic resistance is caused by structural changes in enzymes (belonging to a small group of enzyme families) or overexpression of enzymes to enhance their ability to detoxify the insecticide (Coleman and Hemingway, 2007). These enzymes include cytochrome P450s, glutathione S-transferases (GSTs) and carboxy/cholinesterases (CCE) (Hemingway and Ranson, 2000). Metabolic resistance is more complicated although recent advances have been made in the detection of the main enzymes responsible for insecticide detoxification, enabling the creation of molecular markers for this resistance mechanism made possible by microarray- based methods used to detect detoxification genes usually over expressed in resistant mosquitoes compared to susceptible ones from the same site (David *et al.*, 2005). Resistance to most insecticides including pyrethroids have been linked to the action of P450s (David *et al.*, 2013). Over expression of P450s has been revealed in mosquitoes resistant to

pyrethroids in Africa (Wondji *et al.,* 2008 ; Matambo *et al.,* 2010). In some instances, esterases (Somwang *et al.,* 2011) and GSTs have also been implicated (Lumjuan *et al.,* 2011). A number of P450s linked to pyrethroid resistance in *An. gambiae* have been revealed using an *An. gambiae* detox-chip (David *et al.,* 2005), notably, *CYP6Z1, CYP6Z2, CYP6P3, CYP6M2* and *CYP325A3* (Müller *et al.,* 2007 ; David *et al.,* 2005).

CYP6P3 and CYP6M2 have been commonly reported in resistant mosquitoes in the field (Djouaka *et al.,* 2008; Müller *et al.,* 2008) and have been confirmed to break down Permethrin and Deltamethrin (Stevenson *et al.,* 2011). Recently, the CYP6M2 gene which had earlier shown to be over expressed in Permethrin resistant Ghanaian *An. gambiae* (Nikou *et al.,* 2003) has also been found to be over expressed in highly DDT-resistant ones from the same country (Mitchell *et al.,* 2012). This recent finding has implications on current vector control as it raises issues on earlier assumptions that cross-resistance is an attribute of only target-site mutations whilst metabolic resistance affects only a specific class of insecticides (Mitchell *et al.,* 2012).

2.15.1.2 Target-site resistance

The target site resistance mechanism is known to reduce the binding of insecticides, and enhance the rate of insecticide metabolism leading to reduction in the amount of insecticide that gets to the target site; Pyrethroids and DDT act by binding to and inhibiting the closure of neuronal sodium channels, resulting in prolonged synaptic current, which leads to repetitive nerve impulses and eventual death (Davies *et al.,* 2007). The changes in amino acids responsible for insecticide binding at its site of action can reduce the effectiveness of an insecticide. This phenomenon, referred to as knockdown resistance or *kdr*, has been shown to be the cause of DDT and pyrethroids resistance (Donnelly *et al.,* 2009). A single amino acid change (one or both of two known sites) in the axonal sodium channel insecticide-binding site is known to cause DDT-pyrethroid cross-resistance (Miyazaki *et al.,*

1996 ; Williamson *et al.,* 1996). In the case of organophosphates and carbamates, the target is acetylcholinesterase in nerve synapses. About five point mutations in the acetylcholinesterase insecticide-binding site have been shown to cause varying degrees of reduced sensitivity to organophosphates and carbamate insecticides by acting individually or together (Mutero *et al.,* 1994).

2.15.1.3 Behavioural Resistance

Insecticide exposure can result in a change in behaviour of malaria vectors, a phenomenon referred to as behavioural resistance. Behavioural resistance is defined as any modification to mosquito behaviour that facilitates eluding of insecticides (Gatton *et al.,* 2013). The contribution of behavioural resistance in agricultural pests has been long reported (Gould, 2010 ; (Sparks *et al.,* 1989). There can be a serious drawback to the current vector control interventions if malaria vectors change their behaviour by either feeding early in the evenings or feed and rest outdoors (Exophilly) (Gatton *et al.,* 2013). In addition, there are a variety of other changes in vector behaviour such as increased zoophagy that may evolve in response to intensive vector control interventions, an example is the Garki project in northern Nigeria failed partly because of exophilly even after large-scale use of IRS had reduced vectorial capacity by 90% (Molineaux and Gramiccia, 1980). There have been several reports of mosquitoes changing their behaviour following rigorous indoor use of insecticides (Bøgh *et al.,* 1998) and this could have an impact on the efficacy of current malaria vector control interventions resulting in outdoor transmission of malaria becoming increasingly important. With the renewed interest in the use of LLINs and IRS shifts in biting behaviour and increased outdoor biting by *A. gambiae* in response to LLIN use has been documented (Mbogo *et al.,* 1996) and this can affect malaria control as LLINs target indoor biting mosquitoes.

2.15.1.4 Cuticular Resistance

Reduced uptake of insecticide, often referred to as cuticular resistance, occurs as a result of modification in the insect cuticle or digestive tract linings that prevent or slow the absorption or penetration of insecticides. It is frequently described as a minor resistance mechanism (Wood *et al.,* 2010). This however is likely to be true for insects where the major route of insecticide delivery is via ingestion. However, for malaria control, where insecticides are typically delivered on bed nets or on wall surfaces, uptake of insecticides is primarily through the appendages and therefore an increase in the thickness of the tarsal cuticle, or a reduction in its permeability to lipophilic insecticides, could have a major impact on the bioavailability of an insecticide *in vivo* (Ranson *et al.,* 2011 ; Corbel and N‟Guessan, 2013). A study by Wood *et al*. (2010) reported correlation between cuticle thickness and pyrethroids resistance in *An. funestus.* Microarray studies have implicated two genes that encode cuticular proteins involved in pyrethroid resistance in *Anopheles* mosquitoes (Müller *et al.,* 2008).

2.15.2 Pyrethroid and DDT resistance in malaria vectors

Pyrethroids are synthetic structural derivatives of natural pyrethrins present in the pyrethrum extract of *Chrysanthemum* species (Elliott, 1977). The main target of pyrethroid insecticides is the voltage-gated sodium channel, which is important for the initiation and propagation of action potentials in almost all excitable cells. Pyrethroids cause prolonged opening of sodium channels which prevents channel deactivation and stabilizing the open configuration of the activation gate (Narahashi, 1996). In insects, sodium channels are large transmembrane proteins made up of four homologous domains (I to IV), with each domain consisting of six transmembrane segments (S1 to S6).

DDT has the same mode of action on the insect nervous system, targeting the neuronal voltage gated sodium ion channels. Reduced target site sensitivity, referred to as knock down resistance (*kdr*) is the mechanism shown to be linked commonly with resistance to DDT and pyrethroids (Martinez-Torres *et al.,* 1998 ; Awolola *et al.,* 2007). In *An. gambiae* two *kdr* mutations at position 1014 of the S6 transmembrane segment of the sodium channel gene have been identified; the *L1014F* mutation which induces a leucine to phenylalanine substitution and the *L1014S* mutation that induces a substitution of leucine with serine (Ranson *et al.,* 2000); (Martinez-Torres *et al.,* 1998). Both mutations in single populations have been observed through polymerase chain reaction (PCR) diagnostic assays (Pinto *et al.,* 2006). Field surveys revealed a widespread distribution of the L1014F allele in West Africa (Chandre *et al.,* 1999 ; Gentile *et al.,* 2004). In *An. gambiae,* The L1014F mutation has been observed in both *An. coluzii* (which are mostly confined to the western part of Africa) and *An. gambiae* s.s molecular forms (most widespread throughout Sub-Saharan Africa) whereas the L1014S allele is predominately found in the *An. gambiae* s.s form (Santolamazza *et al.,* 2008a) and was confined to East Africa but has also been found in *An. coluzii* in Equatorial Guinea and Cameroon (Reimer *et al.,* 2008 ; Ridl *et al.,* 2008). Investigations have also found both L1014F and L1014S alleles in parts of Gabon, Cameroon and Uganda (Pinto *et al.,* 2006 ; Verhaeghen *et al.,* 2006) raising issues about the sustainability of insecticide-based vector control programs as pyrethroids are the only insecticides recommended by the World Health Organization for insecticide-treated materials and that DDT is being re-introduced for malaria in several countries (Weissmann, 2006). Both L1014F and L1014S alleles have also been detected in *An. arabiensis* (Himeidan *et al.,* 2007).

The L1014F mutation is now approaching fixation across most parts of West Africa. However, a new mutation; an asparagine-to-tyrosine mutation at position 1575 (N1575Y)

within the linker between domains III-IV of the voltage gated sodium channel on only a single long-range haplotype, also bearing L1014F has recently been identified in *An. gambiae* occurring only on haplotypes containing *1014F* (Jones *et al.,* 2012). The L1014F1575Y has been found in both *An. coluzzii* and *An. gambiae* s.s in the western and central parts of Africa and in *An. coluzzii* sampled from Burkina Faso. The frequency was found to have risen significantly from 0.053 to 0.172 between 2008 and 2010 (Jones *et al.,* 2012). These findings led to the suggestion that the N1575Y mutation compensates for deleterious fitness effects of 1014F and/or confers additional resistance to insecticides. Recent studies that have looked at correlations between response-to insecticide phenotype and *kdr* genotype in *An. gambiae* have reported association to be strongest with DDT, weaker with permethrin (type I pyrethoid) and weakest in association with deltamethrin (type II pyrethroid) (Reimer *et al.,* 2008 ; Ramphul *et al.,* 2009 ; Nwane *et al.,* 2009 ; Verhaeghen *et al.,* 2010);). In some cases other resistance factors than *kdr* have been suggested to be responsible for resistance (Nwane *et al.,* 2009 ; Verhaeghen *et al.,* 2010) possibly metabolic detoxification as has been demonstrated in *An. gambiae* populations in some countries (Djouaka *et al.,* 2008 ; Awolola *et al.,* 2009 ; Verhaeghen *et al.,* 2010).

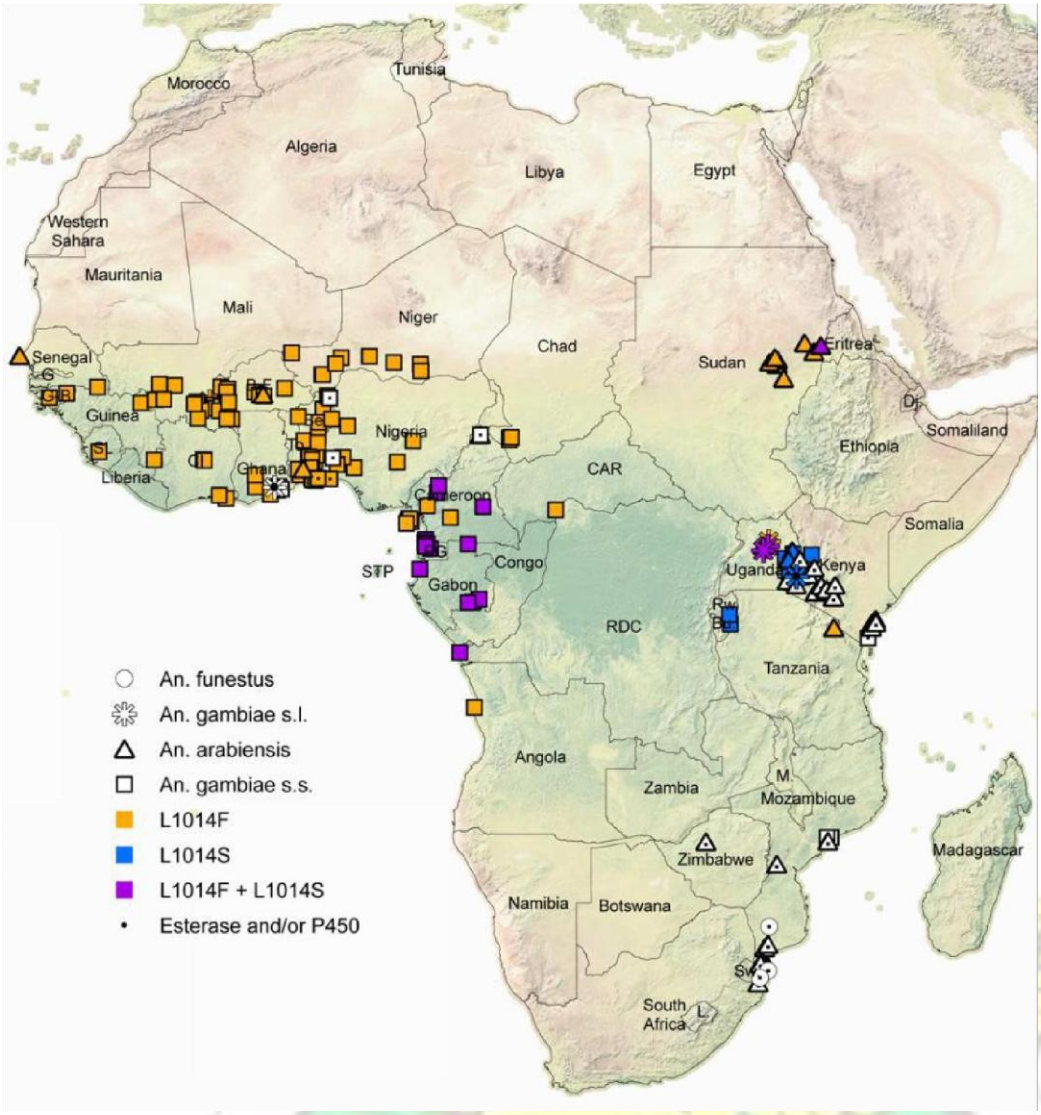


Figure 2.6 Map of Africa showing the distribution of the underlying mechanisms causing pyrethroid resistance in malaria vectors

Source: Ranson *et al.*, 2011

2.15.3 ACE-1R mutation and insecticide resistance in malaria vectors

The carbamates and organophosphates are the main alternatives to pyrethroids and DDT for vector control especially for IRS although some experimental studies on bed-nets impregnated with these insecticides either alone or in combination with pyrethroids have been reported (Fanello *et al.*, 1999 ; Guillet *et al.*, 2001). The development of resistance in

An. gambiae populations to organophosphates and carbamates could represent a major threat to their effectiveness as alternatives to Pyrethroids.

Mosquitoes are known to have two functional acetylcholinesterase genes (ACE-1R and ACE-2) however, only the former has been confirmed to be considerably associated with insecticide resistance (Weill *et al.*, 2004 ; Essandoh *et al.*, 2013)). Carbamates and organophosphates kill insects by blocking synaptic neurotransmission through inhibition of acetylcholinesterase (AChE), encoded by the ACE-1R gene in *An. gambiae*. This implies target site mutations in the VGSC gene (causing resistance to pyrethroids and DDT) will not cause cross-resistance to carbamates and organophosphates. This ACE-1R mutation is the only confirmed cause of resistance in this insecticide classes by field studies on *An. gambiae* (Ahoua Alou *et al.*, 2010 ; Edi *et al.*, 2012). Reports of a single nucleotide substitution of glycine to serine at codon position 119 (Torpedo nomenclature; G119S) in the ACE-1R gene is said to result in a major conformational changes in culicid mosquitoes resulting in resistance to insecticides (Weill *et al.*, 2003 ; Weill *et al.*, 2004) and has also been found in *An. gambiae* throughout West Africa (Djogbénou *et al.*, 2008 ; Ahoua Alou *et al.*, 2010). The knowledge of the ACE-1R effects on phenotypes of *An. gambiae* is thus also important in the determination of the resistance status of malaria vectors in an area as it will help the development of strategies on the use of organophosphates or carbamates as alternatives against pyrethroid resistant malaria

vectors in the field.

2.15.4 Detection and characterization of resistance mechanisms

2.15.4.1 WHO susceptibility assays

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 of time and then mortalities are recorded at the end (WHO, 2012a). This assay serves as the primary method for resistance surveillance. One of the limitations of the assay is that, it gives little information on the mechanism of resistance and hence requires molecular and biochemical testing to provide additional information. Diagnostic dosages are now established and has been widely adopted for use (WHO, 2012a).

2.15.4.2 CDC bottle bioassay

The CDC bottle bioassay is a complimentary method to the WHO method and is generally used for routine monitoring of mosquito resistance in vector populations. In contrast to the WHO bioassay, this assay measures the time taken to kill a sample of adult mosquitoes exposed to a known concentration of insecticide (WHO, 2012a) and involves checking the number of mosquitoes surviving after the diagnostic exposure time which is the time required to kill 100% of the original test population. Compared to the WHO tube bioassay, the CDC bottle bioassay has several advantages including, eliminating the use of preprepared test kits and insecticide-impregnated papers, greater flexibility in the type and concentration of insecticide to be tested and is relatively simple and quick to carry out. In addition, synergists can also be used together with WHO test papers to provide quick and inexpensive alternatives to more complex biochemical and molecular methods and because low discriminating dosages are used, early detection of resistance can be observed. The method however has drawbacks including difficulties in transporting bottles in the field coupled with the need to separate dead and live mosquitoes for further analysis, and also does not allow a recovery period (WHO, 2012a).

2.15.4.3 Detection of kdr mutation in *An. gambiae* s.s.

The most widely used method is based on Allele Specific PCR (ASPCR) (Ranson *et al.*,

2000); (Martinez-Torres *et al.,* 1998). Other assays that have been described more recently include the Heated Oligonucleotide Ligation Assay (HOLA) (Lynd *et al.,* 2010), the Sequence Specific Oligonucleotide Probe Enzyme-Linked ImmunoSorbent Assay (SSOPELISA) (Kulkarni *et al.,* 2006). PCR-Dot Blot (Kolaczinski *et al.,* 2000), Fluorescence Resonance Energy Transfer (FRET)/Melt Curve analysis (Verhaeghen *et al.,* 2006), PCR elongation with fluorescence (Tripet *et al.,* 2006), and the TaqMan real-time assay (Bass *et al.,* 2007). A study by Bass and colleagues (Bass *et al.,* 2008a) compared two Fluorescence-based assays based on TaqMan probes and high resolution melt (HRM) analysis (developed to detect *kdr* alleles in *An. gambiae* with AS-PCR, HOLA, SSOPELISA and PCR-Dot Blot based on performance, length and difficulty of each protocol, cost (both capital outlay and consumable cost), and safety (requirement for hazardous chemicals) and found that where maximum sensitivity and specificity are required the TaqMan real-time assay is the preferred method. They however found the cost of this assay, particularly in terms of initial capital outlay, to be higher than that of some of the other methods, but concluded that performing the TaqMan assays using a PCR machine and fluorimeter was nearly as sensitive as real-time assays and provide a cost saving in capital expenditure.

2.15.5 Insecticide Resistance Management

As insecticide resistance is becoming widespread particularly across Africa, it will be important to incorporate resistance management into vector control activities. Insecticide resistance management can be limited by logistical, financial and knowledge-gaps regarding mechanisms resulting in cross-resistance between alternative insecticides, and broadly, how high-level resistance occurs (Enayati and Hemingway, 2010). It will be important to pay particular attention to the selection of insecticides for the first rounds of spraying with the fundamental goal of maintaining vector susceptibility because of the limited classes of insecticides approved for vector control (Enayati and Hemingway, 2010). The Global Plan

for Insecticide Resistance Management (GPIRM) in malaria vectors is a strategy developed by the WHO that seeks to provide a basis for a harmonised action to sustain the success of vector control interventions and has set out five main pillars including:

i. Planning and implementing insecticide resistance management strategies in malaria-endemic countries. ii. Ensuring proper, timely, entomological and resistance monitoring and effective data management.

iii. Developing new, innovative vector control tools.

iv. Filling gaps in knowledge on mechanisms of insecticide resistance and the impact of current insecticide resistance management strategies and

v. Ensuring that enabling mechanisms (advocacy, human and financial resources) are in place (WHO, 2012a).

In recent times, there has been several reports of highly resistant phenotypes and in some cases ones with multiple resistance increasingly in populations of *An. gambiae* in West Africa (Namountougou *et al.*, 2012 ; Edi *et al.*, 2012). With the introduction of IRS in KND, information on the resistance status of the main malaria vector is urgently required.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The Kassena–Nankana district (KND) in northern Ghana lies between latitude 10.300 and 11.100 North and longitude 1.10 West close to the Ghana-Burkina Faso border (Figure 3.1). The KND has recently been split into two districts (Kassena Nankana West and Kassena Nankana East districts) but for the purpose of this study it was still considered as KND. The vegetation in these districts is dry Guinea savannah characterized by short grass and some drought-resistant trees. The climate is sub-Sahelian, with mean minimum and maximum temperatures of 20 and 40 °C, respectively. The annual rainfall ranges between 850-1000 mm in the months of May- September, followed by a prolonged dry season and during this study, the dry season was from October 2010- April 2011, whilst the wet season was from May 2011- September 2011. KND has a population of 149,653 under continuous demographic surveillance by the Navrongo Health and Demographic Surveillance System (NHDSS) based in the Navrongo Health Research Centre (NHRC) (Oduro *et al.,* 2012). Most of the people live in multi-family compounds of dispersed settlements separated from one another by farm lands. The district is divided into 5 geographical zones which are further divided into 244 clusters. About 90% of the inhabitants are subsistence farmers (Oduro *et al.,* 2012). There is a large water reservoir (Tono Dam) located in the middle of the district that provides water throughout the year mainly for irrigation. The maximum surface area of the Tono reservoir is 1860 hectares with a maximum storage of 93,106 m^3 serving 42 kilometres of main canals along the southern section of the district. The Tono irrigation scheme has provided semi-permanent water bodies, which serve as breeding sites for mosquitoes. The rice fields have proved to be

particularly well suited as breeding sites for *Anopheles gambiae* and *Anopheles funestus* the main malaria vectors in the area (Appawu *et al.,* 2004).

For the purposes of this study and to allow for comparison, the district was divided into two different micro-ecological zones (Figure 3.2).

(i) Irrigated zone, including areas within the Tono dam area and along the canals and other small water impoundments that lie south of the district.

(ii) Non-irrigated zone which includes the north-eastern end of the districts usually well drained with most rivers flooding their banks during the wet seasons and rocky highlands at the Western end of the districts which are mostly rocky and dry with some small dams dug out to provide water for livestock.

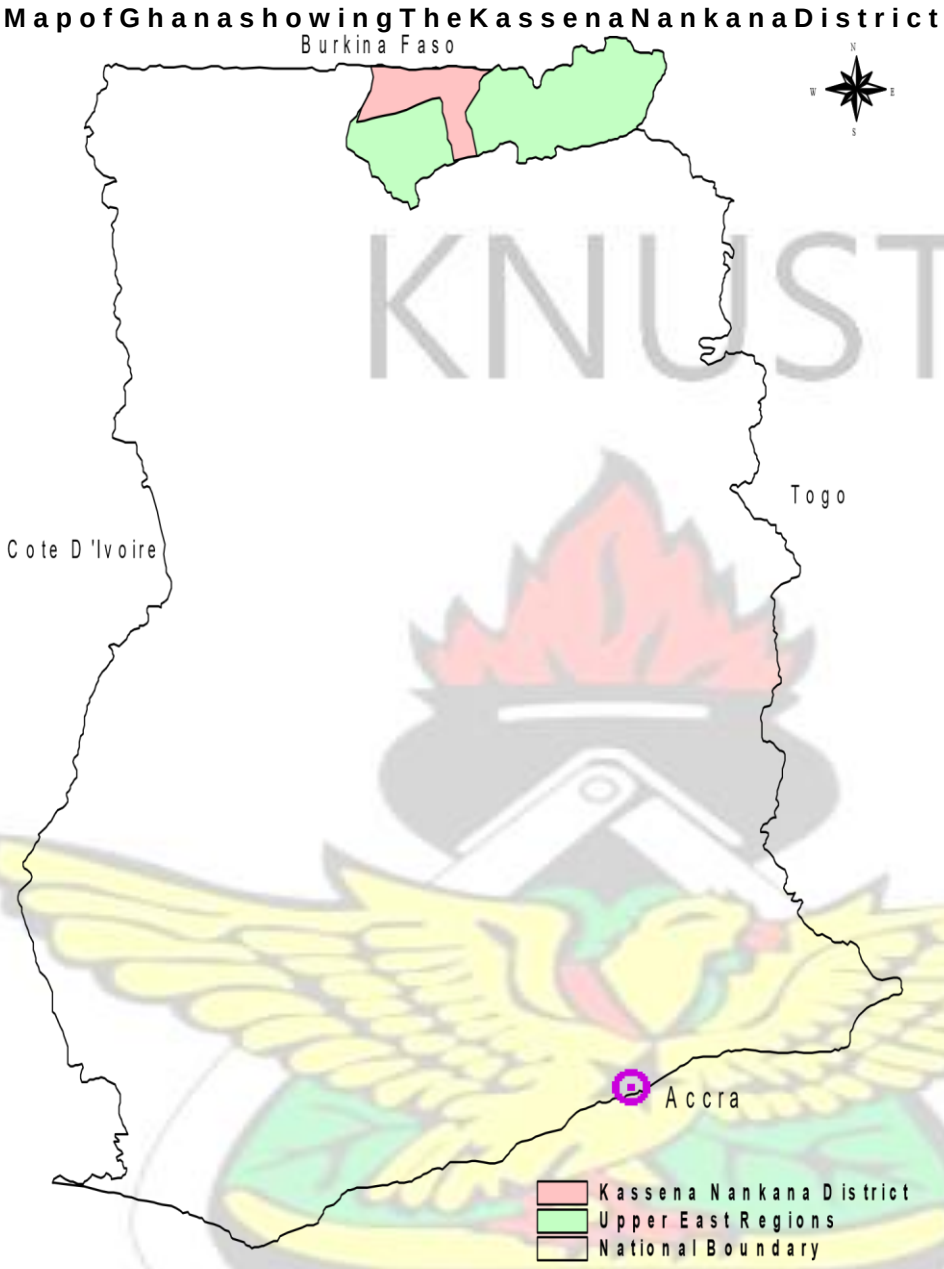


Figure 3.1 Map of Ghana showing the location of Kassena Nankana Districts

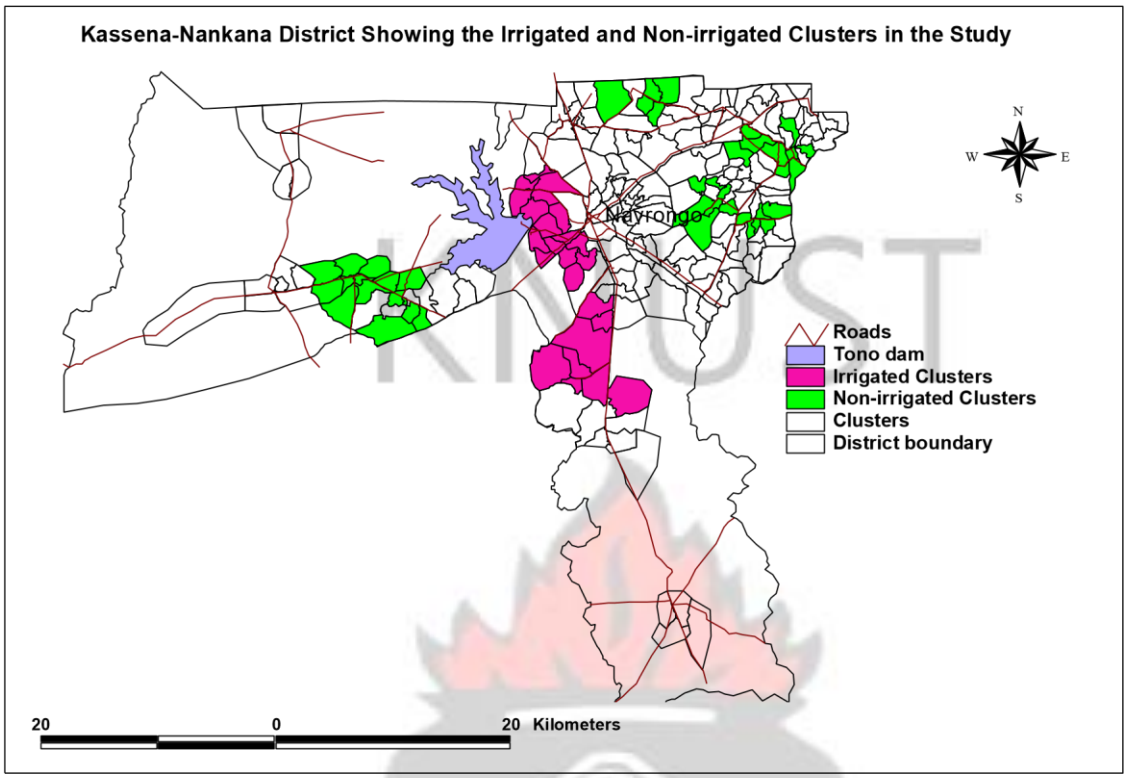


Figure 3.2. Map of the Kassena Nankana Districts showing micro-ecological zones where *Anopheles* mosquitoes were sampled

3.2 Adult mosquito collections

Sampling of adult anopheline mosquitoes was carried out weekly over a period of 52 weeks, from October 2010 to September 2011. In order that a comparison could be made with the study of Appawu *et al* in 2004, sampling was done following the same methodology. Mosquitoes were collected weekly from compounds selected randomly using the NHDSS. Two compounds referred to as the index compounds were selected per zone per week. A list of index compounds was generated from the database of the NHDSS. During each visit, the nearest compound to the index compound was selected as the second compound for the collections. Where there was a refusal by inhabitants, any nearby compound that agreed to release a room for the exercise was used. The collection methods used were all-night human landing catches and Pyrethrum spray catches following WHO standard techniques (WHO,

1975). All houses, roads and water bodies that were considered possible breeding sites were geo-referenced using a handheld GPS (eTrex, Vista, Garmin, USA).

3.2.1 Human landing collections (HLC)

Eight trained fieldworkers with a supervisor were engaged for the collection of mosquitoes twice a week from four selected compounds (two per zone). Visits to selected compounds were only made after informed consent was obtained from the heads of the compounds and room owners prior to the visits. At the selected compounds, two collectors each sat indoors and outdoors for 50 minutes of each of the hours between 1800 hours and 0700 hours to aspirate or pick with glass tubes, mosquitoes which landed on their exposed legs with the help of flashlights. . In order to compensate for differences in individual attractiveness to mosquitoes and as a precaution against dozing and inappropriate techniques, the two teams of collectors rotated between indoors and outdoors hourly after taking a ten minute break. In addition, the supervisor made surprise visits throughout the night for quality assurance and to gather the mosquitoes collected. Field workers were recruited from the communities (that are malaria endemic areas) and were not required to take any prophylaxes. They were however immediately treated according to the World

Health Organization (WHO)-recommended regimen on the basis of fever and detectable *P. falciparum* parasitemia any time they reported any suspected clinical signs of malaria . Indoor and outdoor temperatures and relative humidity were recorded during each hour of collection. Rainfall, temperature and relative humidity data for the study period were obtained from meteorological stations in Navrongo.

The captured mosquitoes were placed into paper cups covered with nets to prevent them from escaping and labelled according to the location, date of collection, site of collection (indoors or outdoors), collector‟s name and hour of collection. The mosquitoes were aspirated into paper cups and placed upright in specially made wooden boxes and transported to the NHRC

laboratory. Pieces of wet cotton wool were placed over the tops of the cups to maintain the mosquitoes at an appropriate humidity. To prevent ants from reaching the captured mosquitoes, the box containing the cups were kept far from areas where ants could be found.

3.2.2 Pyrethrum Spray Collections (PSC)

The spraying for indoor resting mosquitoes was done by PSC from different rooms in the same compound immediately after the human landing catches. Two PSC sessions were performed per visit in each compound. The inhabitants of two selected rooms were made to cover any food and stored water. The floors of the rooms were then completely covered with white cotton sheets and the rooms sprayed with the insecticide Raid™ which contained 0.15% Tetramethrin, 0.25% Allethrin, and 0.015% Deltamethrin, produced by Johnson Wax European BV Holland. One of the collectors sprayed the eaves, if any, simultaneously on the outside to prevent mosquitoes from escaping from the rooms. After 10-15 minutes the doors were opened for the knocked down mosquitoes to be collected. The white sheets were removed and the mosquitoes picked into Petri dishes lined with wet cotton wool for laboratory examination and analysis. The number of people who slept in the sprayed rooms the previous night was recorded.

3.2.3 Exit Trap Catches (ETC)

Two exit traps per site were fixed on windows in randomly selected compounds before the landing catches began in the evening and these were removed in the morning after the landing catches. Mosquitoes trapped in the cages were aspirated into paper cups covered with mesh. These paper cups were labelled accordingly and sent to the laboratory for processing.

3.3 Ethical approval

Ethical and scientific review bodies at the Kwame Nkrumah University of Science and Technology (KNUST) and NHRC reviewed and approved this study. Informed consent process started with preliminary meetings held between the study team, chiefs and community elders to outline the purpose of the survey, details of involvement, risks and benefits. Subsequently, during each visit, collections were only done after the household Head had consented freely after they were properly informed about the study. All households were randomly selected with the help of the NHDSS at the beginning of the study. To avoid excessive inconvenience to individual participants, no house was visited twice during the study period. The right to refuse or withdraw at any time was respected. Refusals were very minimal and were mainly because of unavailability of rooms. Where permission to enter a house was not given, an alternative house in the immediate proximity was selected. All field collectors in the study had consented to participate in the study following a thorough description of the benefits and risks involved. The mosquito collectors were to be offered prompt and immediate access to treatment at the district hospital in case of any infection. The study area is also endemic for filariasis and is a beneficiary of the yearly mass Albendazole and Ivermectin treatment program of the Ghana Health Service. Field workers who had not taken the drugs were made to do so before the commencement of the study.

3.4 Processing of mosquitoes

Captured mosquitoes from HLC were anaesthetized /killed with chloroform in the laboratory. All female anophelines collected were then sorted, identified morphologically to species (Gillies and de Meillon, 1968) and counted. They were classified with the help of a stereo microscope by abdominal status (unfed, blood fed, half gravid or gravid) (Gillies and

Coetzee, 1987) and stored in 1.5-ml micro centrifuge snap vials with desiccants for further laboratory analysis. These tubes were labelled, according to species, collection method and abdominal status.

3.4.1 Determination of Sporozoite Infectivity

A total of 2000 mosquitoes were randomly selected from the mosquitoes captured from HLC. All mosquitoes captured in the dry season were included in the tests whilst 20% of those captured in the rainy season were sampled because of their large numbers. The head and thorax of individual mosquitoes were separated from the abdomen and the separated parts were put into different tubes. These tubes were labelled, according to species, and collection method. Determination of the infectivity of the selected mosquitoes was performed using circumsporozoite enzyme-linked immunosorbent assay (ELISA) (Wirtz *et al.*, 1987). This method detects the presence of *P. falciparum* (Pf) circumsporozoite antigens in the captured mosquitoes. The head and thorax of individual female *Anopheles* mosquitoes were homogenized in a total of 250µl of grinding buffer (PBS, pH7.4 containing 0.5% NP-40 and 0.5% casein) using a plastic pestle. The wells of a 96microtiter plate were coated with anti-*P. falciparum* monoclonal antibodies (Mab) at 2225ºC for 30 minutes. The well contents were aspirated and blocked with blocking buffer. 50µl of homogenate (mosquito sample) was then added to each well. Positive and negative controls were also added to specific plate wells at the same time. After a 2-hour incubation period (at room temperature), the mosquito homogenates were aspirated and the wells were washed with washing buffer. Peroxidase-linked monoclonal antibodies (Mab) was then added to the wells. After 1 hour, the well contents were aspirated and the plate washed again with washing buffer. A clear ABTS (2,2'-azino-di-(3-ethylbenzthiazoline-6sulfonate) peroxidase substrate solution (100 µl) was then added to the wells and as the peroxidase enzyme reacted with the substrate, a

dark green product was observed in the wells that contained sporozoite mosquito samples as compared to the negative controls.

Positive results were recorded by visual assessment of the colour reactions.

3.4.2 Assays for susceptibility of mosquitoes to insecticides

Insecticide susceptibility tube assays were carried out using 2 to 4 days old *Anopheles* female adults, reared from larvae collected from the irrigated area (Bonia and Korania) and Kandiga in the non-irrigated communities, following the procedure described by (WHO, (1998). Insecticide treated test papers were supplied by the Vector Control Research Centre, a WHO Collaborating Centre in Penang, Malaysia and contained the following dosages: deltamethrin (0.05%), permethrin (0.75%), DDT (4.0%),

Lamdacyhalothrine (0.05%), Cyfluthrin (0.1%), Propxur (0.1%) and Bendiocarb (0.1%).

Five replicates of 20 to 25 mosquitoes each were exposed for 1 hour to each test paper. The number of mosquitoes knocked down was recorded at 10-min intervals. After exposure, mosquitoes were supplied with 10% glucose solution, and mortality was recorded at 24 hours post-exposure. Tests with untreated papers were run as controls.

Mortality rate in test samples was corrected using Abbott‟s formula (WHO, 2013b) when the mortality rate of control was between 5–20%. The resistance status was determined using the current WHO standards (WHO, 2013b). Mortality of 98-100% was indicative of susceptibility and mortality rates between 90-97% suggested increased tolerance requiring further confirmation whilst mortality of <90% indicated the existence of resistance in the test population.

All survivors and dead mosquito samples were stored on silica gel for further tests involving identification of sibling species, molecular forms and Kdr mutation.

3.4.3 Molecular analysis

Polymerase Chain Reaction (PCR) analysis of mosquito samples was done both in the Laboratories of Noguchi Memorial Institute for Medical Research (NMIMR) or Liverpool School of Tropical Medicine (LSTM). In NMIMR a sub-sample of the 2000 mosquitoes that were tested for sporozoite infectivity was randomly selected for analysis. In the LSTM, samples from the WHO susceptibility assays using bendiocarb, deltamethrin and permethrin (dead and survivors) were selected (as deltamethrin and permethrin are used for ITNs and bendiocarb is being proposed for IRS in KND). In addition, some mosquito samples from the HLC were analysed to determine allelic frequencies for the mutations that are associated with kdr and Ace-1R resistance. In order to check for any discrepancies, a sub-sample of aliquots of mosquitoes analysed in NMIMR were sent to LSTM for analysis.

3.4.3.1 DNA extraction

In the LSTM laboratory, DNA was extracted from single specimens following the DNeasy Blood and Tissue Procedure for purification of total DNA from animal tissues (QIAGEN, 2006). Mosquitoes were individually placed in 1.5 micro-centrifuge tubes. One ball bearing was then put into each tube to assist in grinding the mosquitoes. 200µl of tissue lysing buffer (180µl Qiagen Buffer ATL plus 20µl 600mAU/mg of proteinase K per sample) was added to each tube. The tubes were then sealed properly and placed in a QIAGEN TissueLyser for 5 minutes. The contents were then centrifuged at 3000 revolutions per minute (rpm) for 10 minutes to settle the contents and then incubated at 56^0C in a hybridization oven overnight. The samples were taken out and shaken vigorously for 15 seconds and 400µl of premixed buffer AL-Ethanol added to each tube, shaken vigorously again for 15 seconds and centrifuged at 3000 rpm . The contents were carefully put in a 96-well DNeasy plate and the ball bearings were then removed. Each of the DNeasy 96 plates was sealed with an air Pore Tape and centrifuged at 6000 rpm for 10 minutes followed by the addition of 500µl of wash

buffer (AW1). The contents were sealed and centrifuged at 3000 rpm for 5 minutes and 500µl of wash buffer (AW2) added and centrifuged at 6000 rpm for 15 minutes but this time, not sealed. The samples were then placed in the correct orientation on a new rack of elution micro tubes. 100µl of distilled water was added to each sample, sealed and centrifuged at 6000rpm for 2 minutes. The last step was repeated to ensure a maximum DNA yield. The DNA was then stored in a refrigerator at -20^{0}C.

3.4.3.2 Species Identification

The polymerase chain reaction (PCR) assay described by Scott *et al*. (1993) was used to identify members of the *An. gambiae* species complex. For each mosquito DNA extraction, PCR reactions (25µl) contained 1.248µl (10mM) of a universal forward primer (5' GTGTGCCCCTTCCTCGATGT), 0.624 µl (10mM) of *An. gambiae* s.s. specific reverse primer (5' CTGGTTTGGTCGGCACGTTT), 0.463 µl (10mM) of *An. arabiensis* reverse primer (5' AAGTGTCCTTCTCCATCCTA), 1.248µl (10mM) of *An. melas* reverse primer (5' TGACCAACCCACTCCCTTGA), 2.496µl (10mM) of *An. quadriannulatus* reverse primer (5' CAGACCAAGATGGTTAGTAT), 2.5 µl of Dream buffer, 0.5µl of Dream Taq, 0.5µl dNTP 1µl of DNA extract and 14.82µl nuclease free water to raise the reaction volume to 25 µl. These were then run on a G-STORM Thermal cycler manufactured by Somerton Biotechnology. The reaction program had an initial step of 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for10 minutes. The PCR products were separated by electrophoresis on

1X agarose gel, and stained with ethidium bromide. The amplicons were visualized with an ultraviolet light. The predicted DNA bands on the gel (390 bp for *An. gambiae*, 315 bp for *An. arabiensis*) were compared to a 1 Kb reference ladder.

In the case of the samples that were identified in NMIMR Laboratories, the PCR reactions (20 µl) contained 2µl of PCR buffer (10mM), 1.6 µl (25mM) of Mgcl, 0.5µl dNTp (10mM), 0.3µl (10mM) each of universal forward primer, *An. gambiae* s.s., *An. arabiensis, An. melas and An. QED* reverse primers, 11.6µl of nuclease free water, 0.1µl of Taq Polymerase and 3µl of DNA extract. These were then run on a C1000 Touch™ Thermal Cycler The reaction programme had an initial step of 94°C for 3 minutes, followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis on 1% agarose gels, and stained with 0.5 mg/ml ethidium bromide. The amplicons were visualized with a Gel Doc XR System ultraviolet gel visualize manufactured by Bio-Rad.

3.4.3.3 Identification of molecular forms of *An. gambiae* s.s

For the samples that were run in the NMIMR, molecular identification of forms within *An. gambiae s.s*. was based on the method of Fanello et al. (2003). A sub-sample of aliquots were sent to LSTM to check for any discrepancies. In the LSTM Laboratory, determination of the molecular forms was done using the SINE-200 PCR method (Santolamazza *et al.*, 2008). PCR reactions were carried out in a 25 µl reaction which contained 1µl each of 1.6a and 1.6b primers, 0.5µl of dNTP (10 mM), 0.21 µl of $MgCl_2$ (10 mM), 0.17µl of Dream Taq polymerase, 18.65µl of water and 1 µl of DNA extracted from a single mosquito. Thermal cycler conditions were 95°C for 5 minutes followed by thirty five cycles of 95°C for 30 seconds, 54°C for 30 s and 72°C for 1 min., with a final elongation at 72°C for 10 min, and a 10°C hold. The resulting products were analysed on 1% agarose gel stained with ethidium bromide and the corresponding bands read using a UV transilluminator.

3.4.3.4 Genotyping of the Pyrethroid Knockdown Resistance Mutations (kdr) & ACE-1

There are two distinct PCR assays designed to detect the *L1014F* and *L1014*S mutations in *An. gambiae*. In this study only the *L1014F* mutation, which is the more common of the two in the West **African** region was assayed. In NMIMR, detection of the standard LeuPhe, *kdr* mutation (1014F) was performed following Martinez-Torres *et al*. (1998). 1µl genomic DNA extract was combined in a 20µl total volume containing 0.2µl each of the Agd_1 (0.2µM) and Agd_3 (0.2µM), 0.25µl of Agd_2 (0.2µM) and Agd_4 (0.2µM) primers, 0.8µl of dNTP mix (0.4µM), 1.6µl of $MgCl_2$ (2mM), 2µ of PCR buffer (1X), 0.1µl of Taq Polymerase and 11.6µl of double distilled water. The PCR conditions were as follows: 3 minutes at 94^{o}C, 94^{o}C for 30 seconds, 55^{o}C for 1 minute and 72^{o}C for 1 minute for fortyfour cycles and a final extension step at 72^{o}C for 5 minutes and stored at 4^{o}C. Amplified fragments were then analysed by gel electrophoresis on a 2% agarose gel and were visualized by ethidium bromide staining under UV light.

Three different mutations: leucine - phenylalanine substitution (L1014F), asparagine-totyrosine substitution (N1575Y) and (glycine to serine substitution (ACE-1R) were determined on the samples tested in LSTM laboratory using the TaqMan assay (described by Bass *et al*, 2007 and Jones *et al.*, 2012). For each mosquito DNA extraction, PCR reactions (10 µl) contained 1 µl of genomic DNA, 5 µl of SensiMix, 0.125 µl primer probe for mutation being tested for (either ACE-1R, L1014F or 1575Y) and 3.875µl of water. Samples were run on an Agilent Technologies Stratagene MX3005P analyser. The cycling conditions varied according to the mutation that was being determined. For the L1014F and ACE-1R mutations, the samples were run at: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and then 60°C for 45 seconds. In the case of the 1575Y mutation, the samples were run at: 10 minutes at 95°C followed by 40 cycles of 92°C for 15 seconds and then 60°C for 1 minute .The increase in VIC and FAM fluorescence was monitored in real

time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 emission) of the Stratagene MX3005P.

3.5 Analysis

Data was analysed using Stata 10 and R® statistical package, version 3.1.0 data analysis and statistical software. Tests of significance between proportions and means were carried out using chi-squared and t-test analysis or Fisher‟s Exact test where appropriate from the (vasar Stats website for Statistical Computation (http://vassarstats.net/.).

The human biting rates (MBR) were calculated as the number of *Anopheles* biting per person per night (equation1below) and the sporozoite rates (SR) were inferred from the proportion of human-biting anophelines that tested positive for *Plasmoduim* circumsporozoite protein (*PfCSP*) by ELISA (equation 2 below). The EIR was calculated as the product of the human-biting rate at a given time interval and place and the sporozoite rate (equation 3 below).

$$MBR = \frac{\text{number of mosquitoes caught biting}}{\text{Number of collectors X Number of captures}} \quad \text{Equation 1}$$

$$SR = \frac{\text{No of mosquitoes positive}}{\text{Total Number Tested}} \quad \text{Equation 2}$$

$$EIR = MBR \times SR \quad \text{Equation 3}$$

Data from bioassays for each insecticide tested had their mortalities calculated as the percentage of individuals that died within 24 hours of exposure. A comparison was then made between the sub-populations from the different sites. 95% percent confidence intervals were determined. Results from the insecticide susceptibility bioassays were evaluated using the new WHO guidelines ((WHO, 2013b) where the levels of resistance were classified as follows: Those with an overall mortality ≥ 98% were considered

susceptible, those with mortality <98% but ≥ 80% were considered potentially resistant, and those with mortality <80% were strongly suspected to be resistant.

Fisher's exact test was used to determine whether there was an association between the different genotypes and survival after exposure to the insecticides bendiocarb, deltamethrin and permethrin.

Allelic frequencies of the L1014F, N1575Y and G119S mutation among HLC samples collected from sites within KND was determined. The geographical positions of the collection sites and the percentage mortalities for the insecticide susceptibility bioassays were plotted on the map using ArcGIS 9.3 (ESRI Japan Corp, Tokyo, Japan). The total *An. gambiae* and *An. funestus* captured using HLC from the different compounds were classified into different ranges and also plotted on maps using ArcGIS 9.3 (ESRI Japan Corp, Tokyo, Japan). Similarly, maps of the extent of insecticide resistance of *An. gambiae* s.s. was done.

CHAPTER FO UR

RESULTS

4.1 MALARIA TRANSMISSION DYNAMICS

4.1.1 Mosquito Species Composition

A total of 20,252 Anopheline mosquitoes were collected by HLC over 141 man-nights. *Anopheles gambiae* sensu lato was the most abundant species, 68.88% (95% CI 68.18 - 69.45), (N=13938) although the relative abundance varied markedly in different months (Table 4.1). Other species (*An. pharoensis* and *An. rufipes*) constituted 20. 21% (95% CI 19.66 - 20.77), (N=4092) followed by An. *funestus* 10.97% (95% CI 10.55 – 11.41) (N=2222).

More *Anopheles* were captured from compounds in the irrigated area, 86.92% (95% CI 86.45 – 87.38) (17,603/20252) compared with the non-irrigated area 13.08% (95% CI 12.62 – 13.55) (2,649/20,252). *Anopheles gambiae* s.l. was the dominant species in both zones; accounting for 69.60%(95% CI 68.92 – 70.28) (12,252/17603) and 63.65% (95% CI 61.80 – 65.46) (1686/2,649 in the irrigated and non-irrigated areas respectively whilst *An. funestus* was significantly lower ($p<0.001$); 9.45% (95% CI 9.03 – 9.89) (1664/ 17603) and 21.06 % (95% CI 19.55 – 22.65) (558/2,649) in the irrigated and non-irrigated areas, respectively (Table 4.1).

In the irrigated area, 56.11% (95% CI 55.23 – 56.99) of the total *An. gambiae* captured were biting indoors (6874/12252) compared to 43.89% (95% CI 43.01 – 44.77) caught biting outdoors whilst an equal proportion 50.0% (95% CI 47.62 – 52.38) (843/1686) were caught biting both indoors and outdoors in the non-irrigated area. The proportions of *An. funestus* caught biting indoors in the irrigated and non-irrigated zones were nearly the same; 57.93%

(95% CI 55.54 – 60.28) (964/1664) and 57.71% (95% CI 53.57 – 61.74) (322/558) respectively. Similarly, outdoor biting *An. funestus* in the irrigated and nonirrigated zones constituted 42.07% (95% CI 39.72 – 44.46) (700/1664) and 42.29% (95% CI 38.26 – 46.43) (236/558) respectively (Table 4.11).

Application of species-specific PCR (TaqMan PCR) of a subset of the morphologically identified *An. gambiae* s.l. captured indicated that *An. gambiae s.s* was the only sibling species of the *An. gambiae* complex with *An. coluzzii* as the only molecular form in the study site.

Table 4.1. Species composition and abundance of human-biting Anophelines in two ecological zones in the Kassena-Nankana District, Upper East Region, Ghana

Location	IRRIGATED			Non -IRRIGATED			Total	Total		
	An.gambiae	*An. funestus*	Others	*An.gambiae*	*An. funestus*	others		*An. gambiae*	*An. funestus*	Other species
	% (No)	% (No)	% (No)	% (No)	% (No)	% (No)		% (No)	% (No)	%(No)
INDOORS	56.11(6874)	57.93(964)	28.75(1060)	50.00(843)	57.71(322)	19.51(79)	10142	76.09(7,717)	12.68(1,286)	11.23(1139)
OUTDOORS	43.89(5378)	42.07(700)	71.25(2627)	50.00(843)	42.29(236)	80.49(326)	10110	61.5 (6,221)	9.3 (936)	29.20(2953)
Total	60.50(12252)	8.22(1664)	18.21(3687)	8.33(1686)	2.76(558)	2.00(405)	20252	68.8 (13938)	11.0 (2222)	20.21(4092)

Others=*An. pharoensis* and An. *rufipes*

4.1.2 Seasonal variation and effect of climatic factors on abundance of anopheline mosquitoes

There were seasonal variations in the numbers of the two main species biting humans. In the dry season (November to April), *An. gambiae* constituted 89.8% (4766/5308) 95% CI (88.9%-90.6%), whilst in the wet season, it constituted 84.5% (9172/10852) 95% CI (83.8%-85.2%). The *An. gambiae* populations also differed seasonally in the two micro ecological zones. In the irrigated zone, it constituted 38.4% (95% CI 37.5%-39.3%) and 61.6% (95% CI 60.7%-62.5%) of the total caught biting in the dry and wet seasons respectively. In the non-irrigated zone, the species constituted 3.7% (95% CI 2.8%-4.7%) and 96.3% (95% CI 95.3%-97.2%) in the dry and wet seasons respectively. *An. funestus* caught biting also differed seasonally in both zones. The irrigated zone yielded 30.3% (95% CI 28.1%-3%) and 69.7% (95% CI67.4%-71.9%) of the total *An. funestus* captured for the dry and wet seasons respectively. In the non-irrigated area, the proportion caught biting during the dry season was 6.8% (95% CI 4.9%-9.2%) compared to 93.2% (95% CI 90.8%-95.1%) for the wet season.

Table 4.2 shows the distribution of indoor resting *Anopheles* mosquitoes and the Room index (RI), expressed as the number of mosquitoes per room (156 rooms). A total of 3697 Anophelines were collected from the two ecological zones using PSC. Out of these 1440 of *An.gambiae* and 560 *An. funestus* were collected in the wet season. This translates to a Room Index (RI) of 20.0 and 7.8 respectively The RIs were higher in the wet season than the dry season in both zones. The pattern was the same for the other *Anopheles* species resting indoors.

Higher room densities were recorded for the entire *Anopheles* species collected from the irrigated zone during the wet season.

Table 4.2. Seasonal changes in species composition and Room index of indoor resting *Anopheles* in two ecological areas in the KassenaNankana District, Upper East Region, and Ghana

Location	*An.gambiae*		*An. funestus*		others		Total
	wet	dry	wet	dry	wet	dry	
	RI(No)	RI(No)	RI(No)	RI(No)	RI(No)	RI(No)	
IRRIGATED	38.6(1236)	15.3(687)	14.1(450)	8.7(393)	4.8(154)	4.2(190)	3110
Non IRRIGATED	5.1(204)	0.9(50)	2.8(110)	0.4(20)	3.9(155)	0.9(48)	587
Total	20.0(1440)	7.3(737)	7.8(560)	4.1(413)	4.3(309)	2.4(238)	3697

Others=*An. pharoensis* and *An. rufipes*

The effect of seasonal factors (monthly mean temperature and rainfall) on the observed mosquito abundance indicated that there was positive correlation between the abundance of both *An. gambiae* and *An. funestus* and rainfall and a negative correlation between abundance and temperature across the two zones. The correlation between temperature and the abundance of *An. gambiae* and *An. funestus* were negative (-34% and -61% respectively in the irrigated area) whilst they were relatively not different in the non irrigated area (ρ= -66.1%% and -67.0% for *An. gambiae* and *An. funestus* respectively). The correlation between rainfall and the abundance was lower for *An. gambiae* compared to *An. funestus* in the irrigated zone (ρ= 22.6% and 63.0% respectively). On the other hand, in the non-irrigated area the correlation between the abundance of both species and rainfall were very high (ρ= 83.1% and 73.9% for *An. gambiae* and *An. funestus)*

respectively. The mean indoor temperature recorded during the hours when biting activity peaked was 26 ^{0}C for both *An. gambiae* and *An. funestus* (Figure 4.1). This figure also shows that the period when relatively large numbers were biting coincided with a temperature range of 24 ^{0}C -26 ^{0}C.

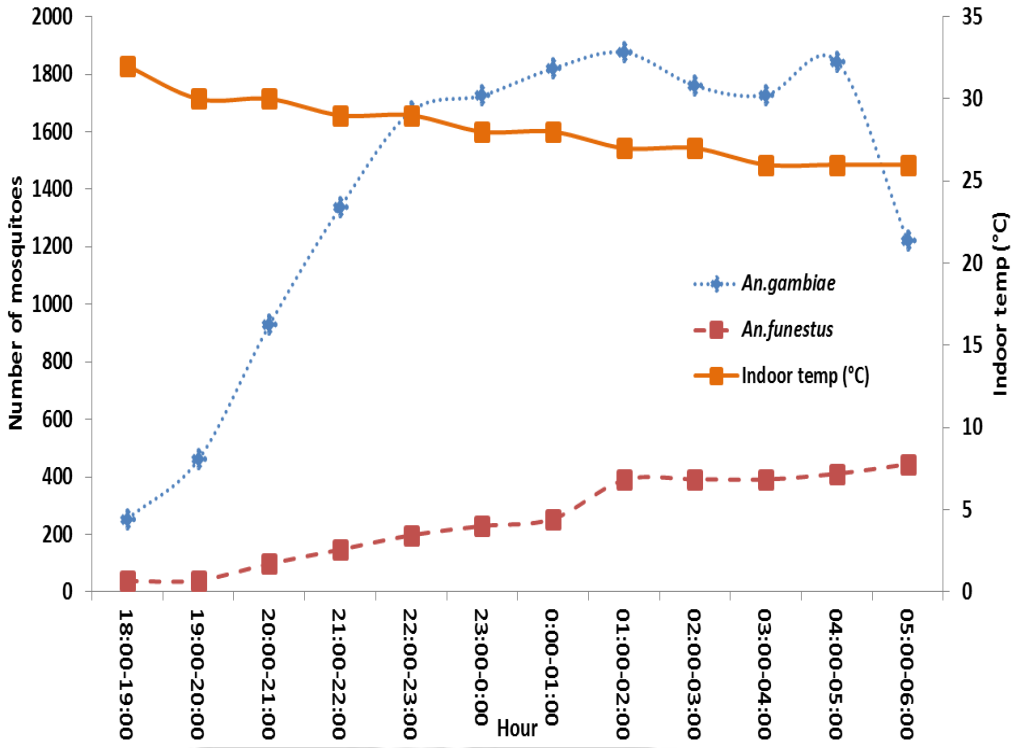
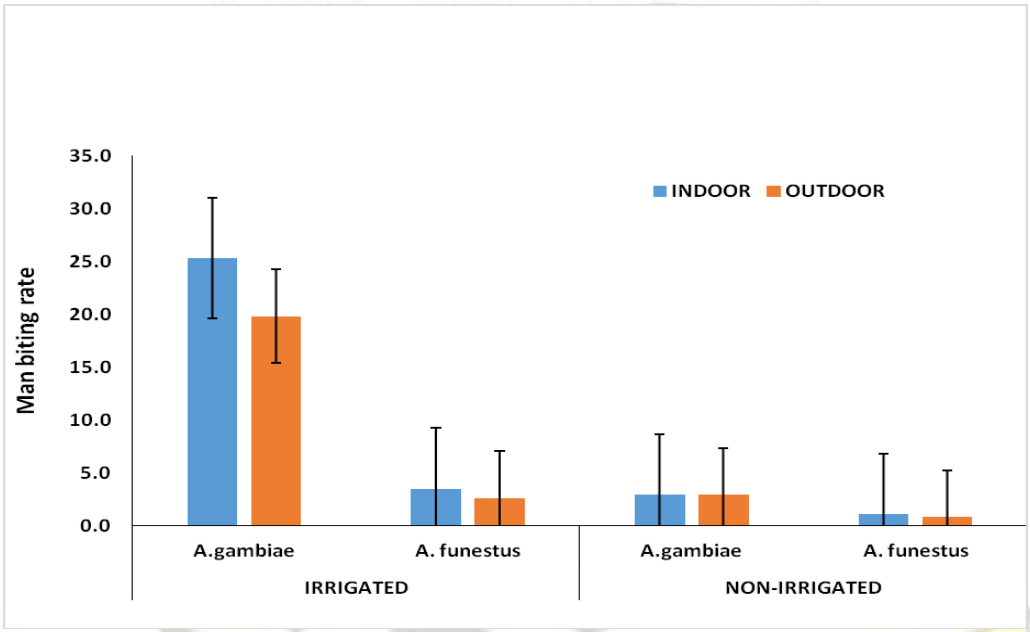


Figure 4.1 The effect of indoor temperature on the number of *An. gambiae* and *An. funestus* caught biting indoors during the night in KND, Ghana

4.1.3 Man-vector contact (Man biting rates)

The man-biting rates estimated for *An. gambiae* s.s and *An. funestus*, the two main *Anopheles* species in KND varied considerably over the duration of the study. The overall biting rate for the two species was 14.3 bites/man/night (b/m/n). *Anopheles gambiae* showed a significantly higher ($p<0.001$) biting rate in the irrigated area compared to the non-irrigated area (22.5 versus 2.9 b/m/n respectively). Similarly, *An. funestus* biting rate was significantly ($p<0.010$) higher in the irrigated than non-irrigated areas (3.1 versus 1.0 b/m/n). *An. gambiae* had indoor biting rates of 25.3 and 2.9 b/m/n for irrigated and nonirrigated areas respectively whilst *An. funestus* had 3.5 and 1.1 b/m/n for irrigated and

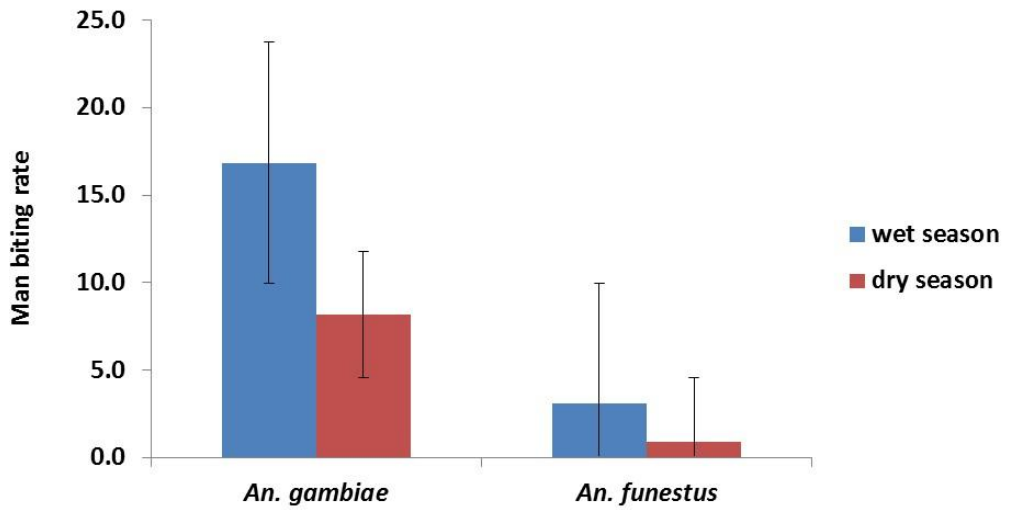
non-irrigated respectively (Figure 4.2). The outdoor biting rates for *An. gambiae* was 19.8 b/m/n compared to 2.6 b/m/n for *An. funestus* in the irrigated area whilst the biting rates were 2.9 and 0.8 b/m/n respectively in the non-irrigated areas (Figure 4.2).



Error bars = standard errors

Figure 4.2 Indoor and outdoor man biting rates of *An. gambiae* s.s. and *An. funestus* across the zones in Kassena-Nankana District, Ghana

The overall biting rate ranged from 43.97 to 167.25 b/m/n. *Anopheles gambiae* had the highest biting rate of 12.4 b/m/n whilst *An. funestus* had a relatively lower biting rate of 2.0 b/m/n during the study period. There were seasonal variations in the biting rates of the species (Figure 4.3). *An. gambiae* was the most aggressive biting species in both seasons with 16.9 b/m/n in the wet season and 8.2 b/m/n in the dry season. Similarly, *An. funestus* had a higher biting rate of 3.1 b/m/n in the wet season compared to 0.9 b/m/n in the dry season. However these differences were not significant (Figure 4.3).

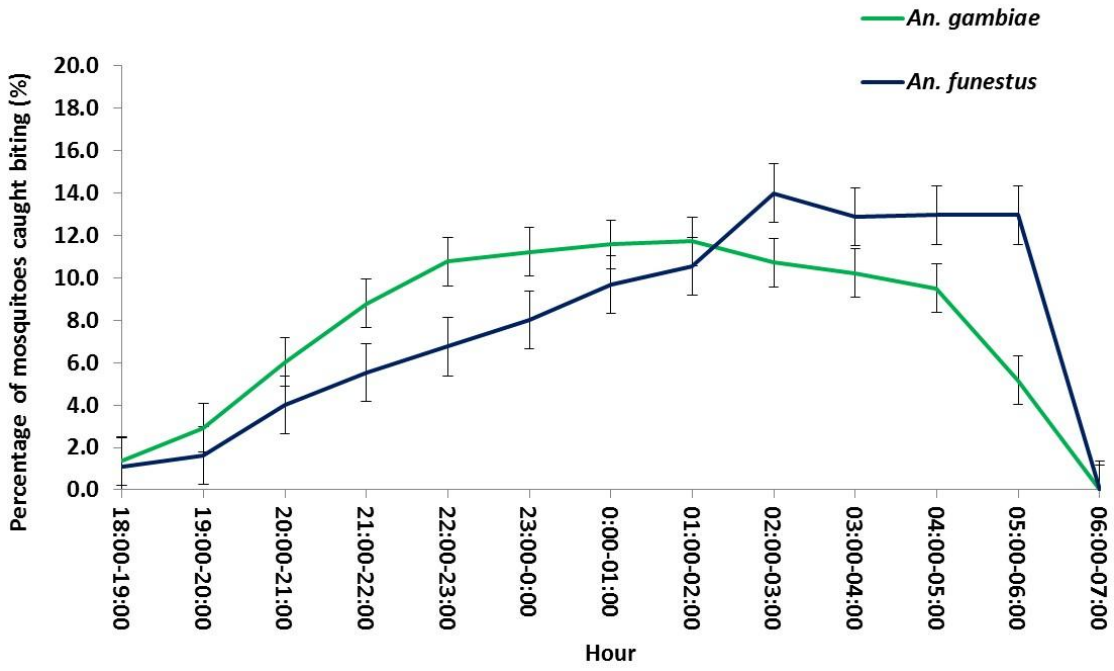


Error bars = Standard Errors

Figure 4.3. Man biting rates of *An. gambiae* s.s. and *An. funestus* during the wet and dry seasons in Kassena-Nankana District, Ghana

4.1.4 Biting cycle of Anopheline mosquitoes in Kassena-Nankana Districts

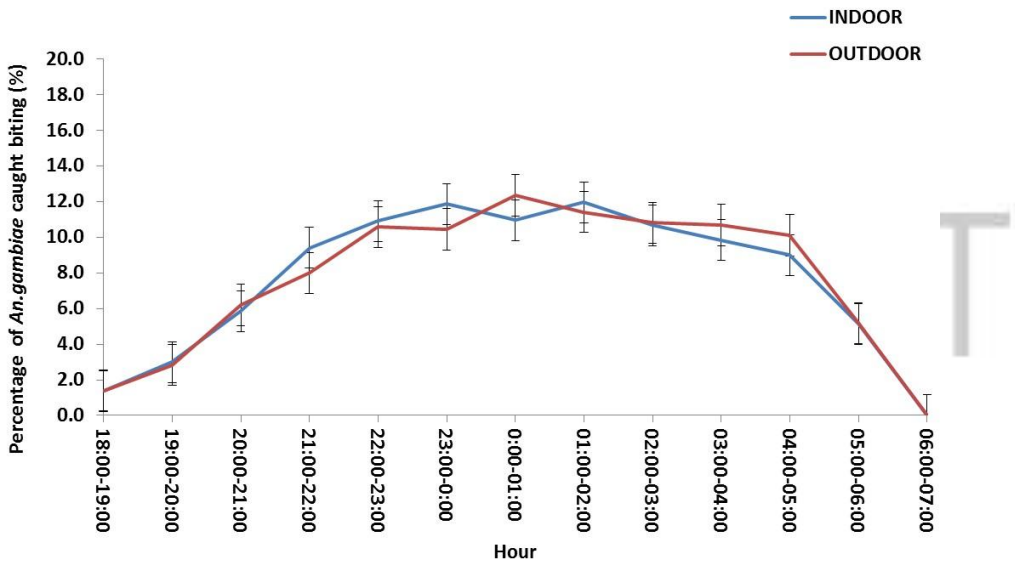
The night biting cycles of *An. gambiae* and *An. funestus* in KND are shown in Figure 4.4. The biting frequency increased steadily from 1800 hours and plateau around 0100 to 0200 hours for both species (Figure 4.4). The percentage of *An. gambiae* biting dropped after 0400 hours whilst biting was sustained by *An. funestus* up to 0500 hours.



Error bars=Standard errors

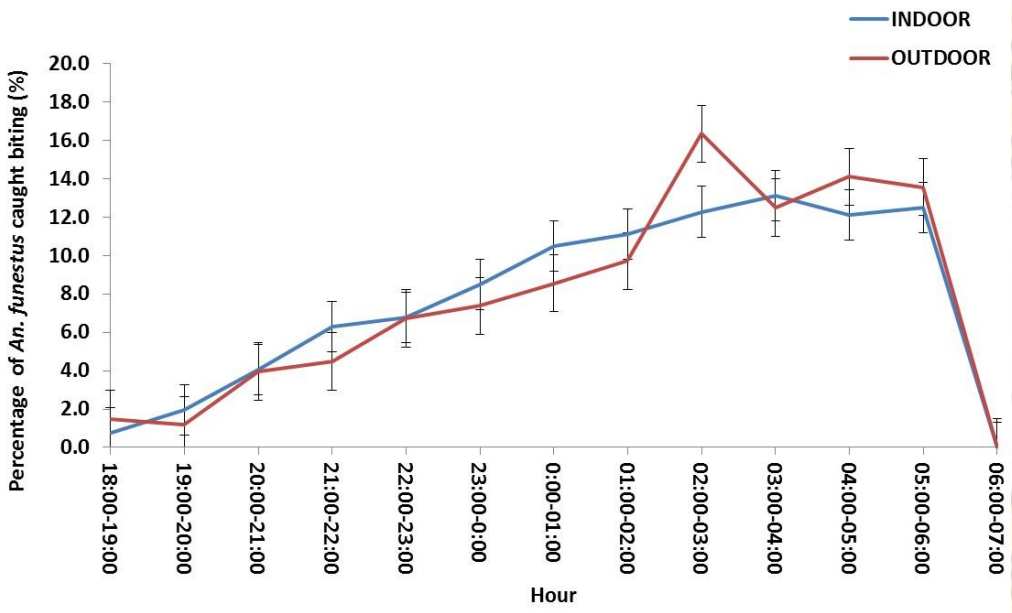
Figure 4.4 Night biting cycle of *An. gambiae* s.s. and *An. funestus* in Kassena-Nankana District, Ghana

Figures 4.5 & 4.6 show the percentages of *An. gambiae* and *An.funestus* caught biting indoors and outdoors during the night in the KND. There was a slow rise in the percentage of the species with a peak occurring around midnight for indoor and outdoor. The biting declined thereafter till morning. There was no significant variation between the numbers biting indoors and outdoors at the individual time periods. As shown in Figure 4.6, the number of *An. funestus* biting indoors increased steadily and peaked at 0300 hours whilst that for outdoor peaked much earlier at 0200 hours. There was a fluctuation in the numbers of *An. funestus* biting indoor and outdoor but the difference was not significant except at 0300 hrs.



Error bars=Standard errors

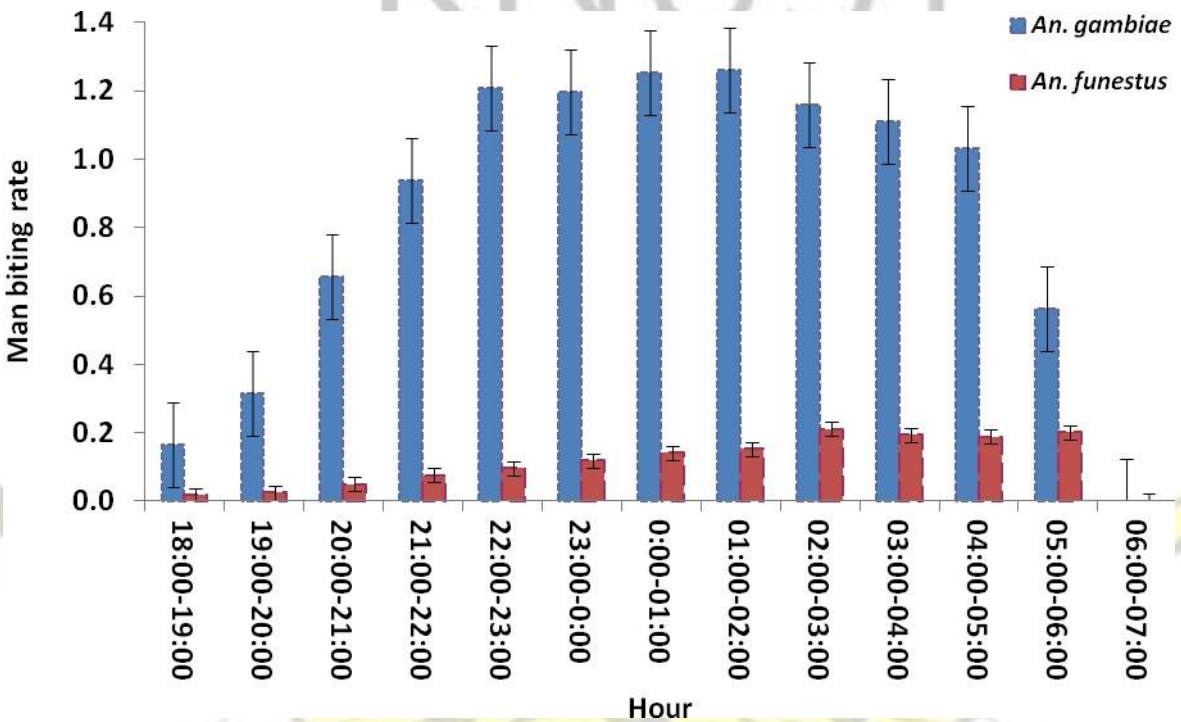
Figure 4.5 Percentages of *An. gambiae* based on total caught biting indoors and outdoors through the night in Kassena-Nankana District, Ghana



Errors bars=Standard errors

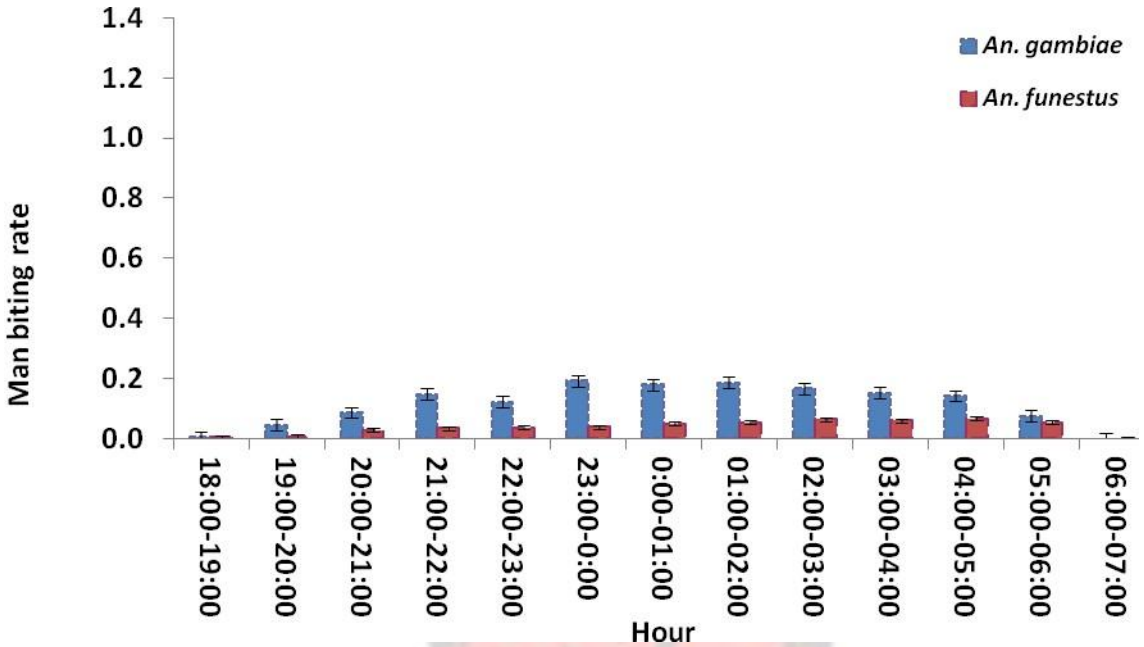
Figure 4.6 Percentages of *An.funestus* based on total caught biting indoor and outdoor through the night in Kassena-Nankana District, Ghana

Figures 4.7 and 4.8 show the hourly biting rates for the two main mosquito species (*An. gambiae* and *An. funestus*) in the irrigated and non-irrigated areas respectively. The biting rates of the two species were statistically different throughout the night in the two ecological zones, with *An. gambiae* showing relatively higher biting rates.



Error bars=Standard errors

Figure 4.7. Hourly man biting rates of *An.gambiae* and *An. funestus* caught in the irrigated zone in KND



Error bars=standard errors

Figure 4.8. Hourly man biting rates of *An.gambiae* and *An. funestus* caught in the nonirrigated zone in KND

4.1.5 Resting behaviour of anopheline mosquitoes in KND

Out of the 1321 blood-fed, mosquitoes resting indoors during the day, 71.46% and 28.54% were *An. gambiae* and *An. funestus* respectively while the gravids were 47.06% and 52.94% of *An. gambiae* and *An. funestus* respectively (Table 4.3). The overall ratio of the blood fed mosquitoes to gravid ones (F: G) was of 9.71:1, and the F: G for *An. gambiae* and *An. funestus* were 14.75:1 and 5.24:1.

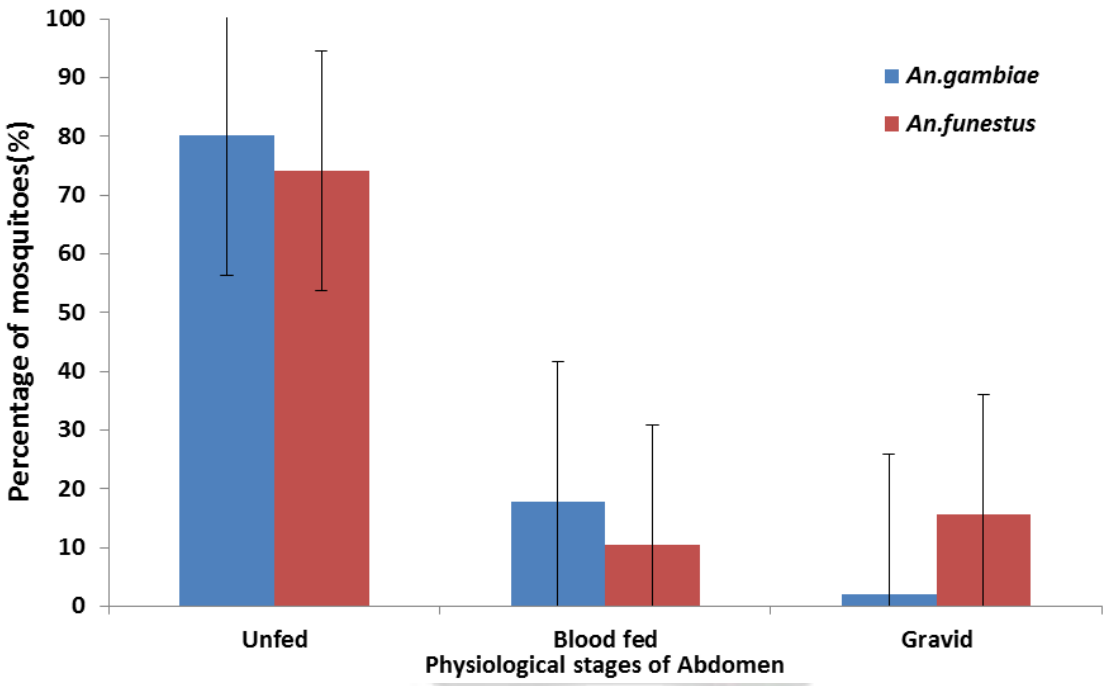
Table 4.3. Numbers and relative proportions of blood fed and gravid *An. gambiae* and *An. funestus* resting indoors in Kassena-Nankana District, Ghana

Species	BF %(No)	G %(No)	Total	%	F:G
An.gambiae s.s.	93.65 (944)	6.35(64)	1008	[a]69.18	14.75:1
An. funestus	83.96(377)	16.04(72)	449	[a]30.82	5.24:1
Total	90.67(1321)	9.33(136)	1457		9.71:1

BF = blood fed mosquitoes; G = gravid mosquitoes; BF: G = ratio of blood fed to gravid [a]

F:G ratio between *An. gambiae* and *An. funestus* statistically significant ($p < 0.001$)

The exiting behaviour of mosquitoes from dwelling rooms in compounds in the study area was investigated using exit trap collections. The physiological conditions of abdomen of mosquitoes caught exiting rooms were examined to determine their post-feeding resting behaviour. Figure 4.9 shows the proportions of the different physiological conditions of the abdomen of *An. gambiae* and *An. funestus* caught exiting from dwelling rooms. There were relatively no significant differences in the proportions of *An. gambiae* and *An. funestus* in the different abdominal conditions when considered separately. The figure indicates relatively higher proportions of gravid female of *An. funestus* than *An. gambiae* exiting whilst the opposite was found for the blood feds. There were relatively high numbers of unfed mosquitoes exiting the rooms.



Error bars=Standard errors

Figure 4.9. Abdominal conditions of *An. gambiae* and *An. funestus* caught in window exit traps of compounds in Kassena-Nankana District

A fed to gravid ratio (F:G) of 5.61:1 was obtained for the total mosquitoes captured and ratios for *An. gambiae s.s.* and *An. funestus* were 8.5:1 and 4.75:1 respectively. The ratio suggests that more blood feds tend to rest in the room immediately after feeding but a proportion of the blood feds were leaving the rooms to find another resting place to develop their eggs (Table 4.4).

Table 4.4. Numbers and relative proportions of blood fed and gravid *An. gambiae* and *An. funestus* caught in window exit traps in Kassena-Nankana District, Ghana

Species	BF	G	Total	%	F:G
An.gambiae s.s.	34	4	38	[a]35.5	8.5:1
An. funestus	57	12	69	[a] 64.5	4.75:1
Total	91	16	107		5.69:1

BF = blood fed mosquitoes; G = gravid mosquitoes; BF: G = ratio of blood fed to gravid [a]statistically significant ($p < 0.001$)

4.1.6 Spatial distribution of biting *anopheles* mosquitoes collected from study areas

GIS based maps were developed to depict the spatial distribution of *An.gambiae* and *An. funestus* in all the compounds where HLC were conducted (Figures 4.10 and 4.11). Total numbers collected from the different compounds were assigned different ranges. High numbersof *An. gambiae* were caught biting in most of the compounds located within the irrigated zone, where the numbers caught biting fell in the range of 100-841 (Figure 4.10). Relatively low numbers were caught (0-29) from compounds in the non-irrigated zones especially from the western portions of KND. Relatively lower number of *An. funestus* were caught biting in the individual compounds throughout KND, falling within the 30-99 range (Figure 4.11). The highest number of this species captured were between 100-137 and these occurred in few compounds. As shown in Figure 11, the range of the numbers of *An. funestus* captured was fairly uniform in the two micro-ecological zones although relatively few numbers were caught in the western part of the district.

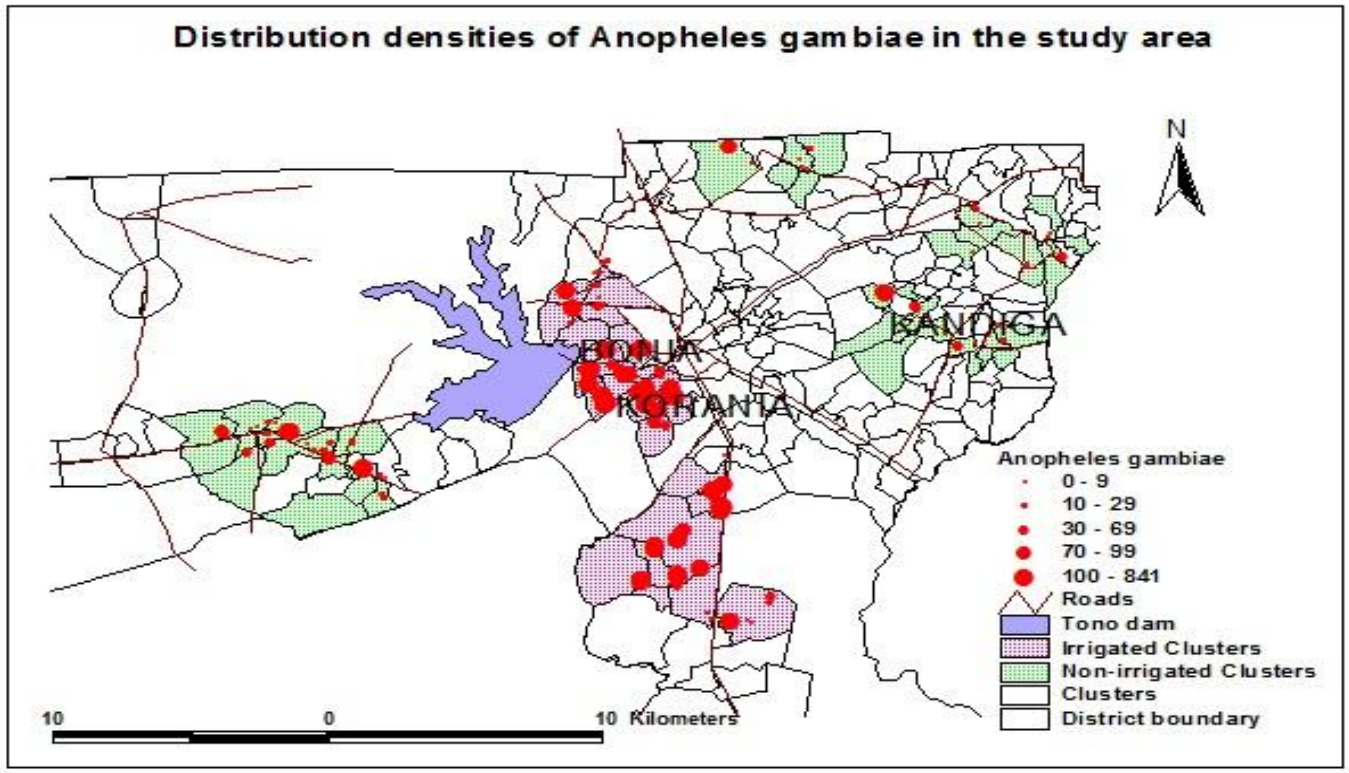


Figure 4.10. Map of KND showing the spatial distribution and ranges of numbers of *An. gambiae* caught biting within the district

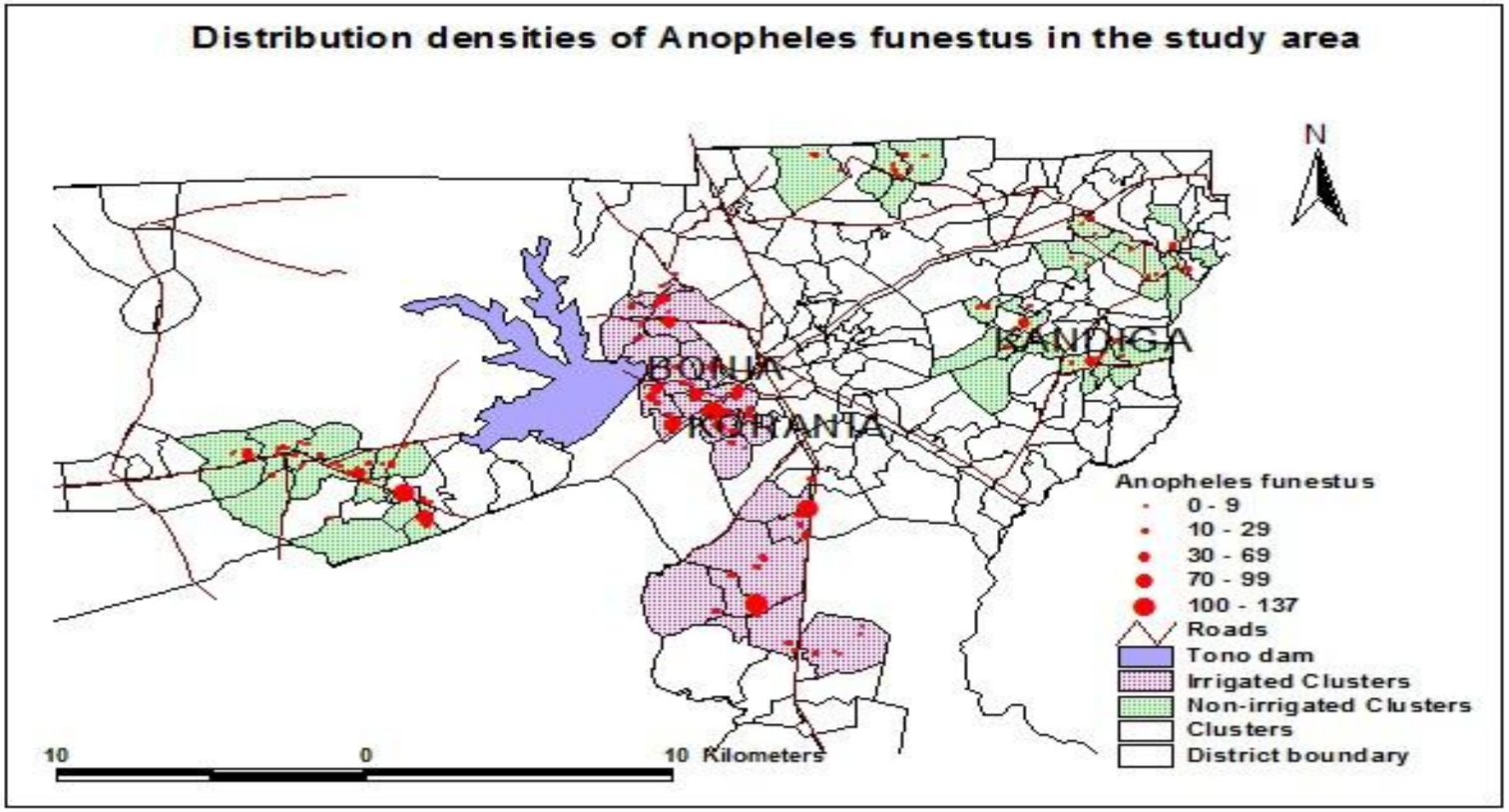


Figure 4.11: Map of KND showing the spatial distribution and ranges of numbers of *An. funestus* caught biting within the district.

4.1.7 Sporozoite infectivity (sporozoite rates) of *Anopheles* mosquitoes in KND

Table 4.5 shows the proportions of both *An. gambiae* s.s. and *An. funestus* caught biting humans during the night that were positive for sporozoite in the two ecological zones. A total of 1903 mosquitoes collected from all the sites were examined for sporozoite infectivity. Out of these, 2.6% (49/1903) of the anophelines were positive for *P. falciparum* circumsporozoite antigen. *Anopheles gambiae* had an overall (irrigated and non- irrigated) sporozoite rate of 2.66% (95% CI, 2.01%-3.52%) while *An. funestus* had 1.44% (95% CI, 0.407%5.10%). The two rates were not significantly different (p=0.366). The sporozoite rates of *An. gambiae* and *An. funestus* from the non-irrigated area were significantly higher than from the irrigated area (p<0.001). The sporozoite rate of *An. gambiae* was 1.53% (23/1503) in the irrigated and 9.20% (24/261 in the non-irrigated area; that of *An. funestus* was 0.00% (0/45) and 2.10% (2/94) in the irrigated and nonirrigated areas respectively.

Table 4.5. *Plasmodium falciparum* sporozoite infectivity of *An. gambiae* and *An. funestus* caught biting humans in 2 ecological zones in Kassena-Nankana District, Upper East Region, Ghana

	An. gambiae s.s.				*An. funestus*			
Ecological zone	No tested	No +ve	% Sp. rate	95% CI	No tested	No +ve	% Sp. rate	95% CI
Irrigated	1503	23	[a]1.53	1.0-2.3	45	0	[a]0.00	0.0-0.0
Non-irrigated	261	24	[b]9.20	6.0-13.4	94	2	[b]2.10	0.26-7.5
Total	1764	47	2.66	2.0-3.52	139	2	1.44	0.40-5.10

No +ve = number tested positive for *P falciparum* circumsporozoite antigen Sp. Rate = percentage positive [a]= SR between the two species in irrigated zone not statistically different (p=0.403) [b]= SR between the two species in irrigated zone statistically different (p=0.023)

There were variations in sporozoite infectivity for the two vector species biting indoor and outdoor in the area as shown in Table 4.6. *Anopheles gambiae* maintained a relatively similar sporozoite rate (both indoor and outdoor) in the two ecological zones. The infection rates were however significantly lower in the irrigated area; 1.94% and 1.29% for indoor and outdoor respectively and 9.16% and 9.23% respectively for indoor and outdoor in the non-irrigated area. None of the *An. funestus* from the irrigated area tested positive.

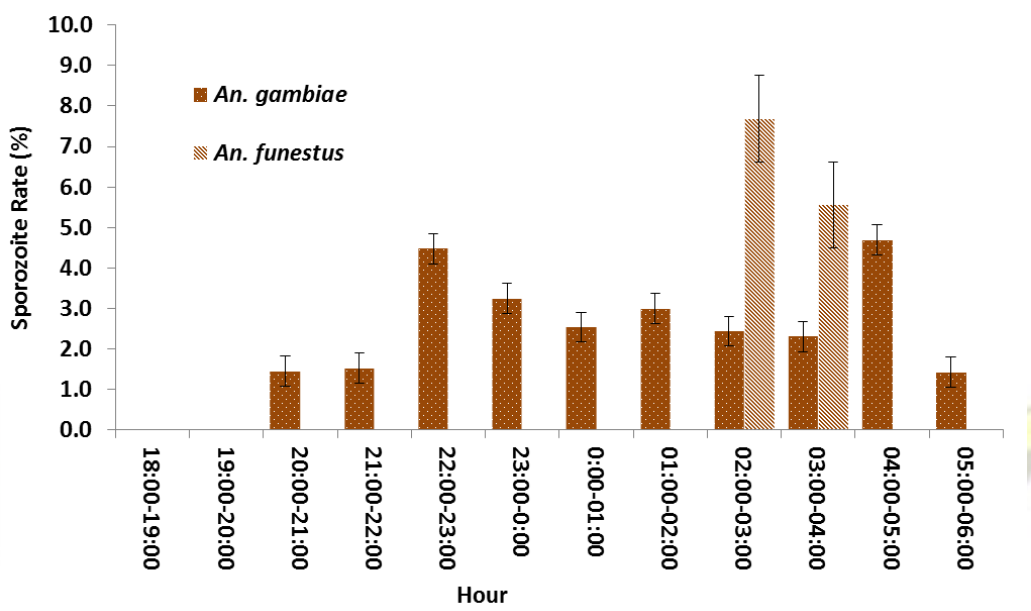
However, their sporozoite rates were 1.92% and 2.38% for indoor and outdoor respectively from the non-irrigated area (Table 4.6).

Table 4.6. *Plasmodium falciparum* sporozoite rates of indoor and outdoor biting *An. gambiae* and *An. funestus* from two ecological zones in Kassena Nankana District, Ghana

Species		Irrigated	Non-irrigated	Difference (95% CI*)
	Place of collection	SR% (tested)	SR% (tested)	
An. gambiae	Indoor	1.94(619)	9.16(131)	7.22(2.16,12.28)
	Outdoor	1.29(854)	9.23(130)	7.94(2.91,12.98)
An. funestus	Indoor	0.00(30)	1.92(52)	1.92(1.81, 5.66)
	Outdoor	0.00(15)	2.38(42)	2.38(2.23, 6.99)

95% CI*s for difference in SR between irrigated and non-irrigated

Figure 4.12 shows the sporozoite infectivity of *An. gambiae* s.s. and *An. funestus* mosquitoes captured biting during the night. Sporozoite infectivity was detected in *An. gambiae* biting after 2000 hours. A higher proportion of *An. gambiae* infective mosquitoes were collected from *22:00* to 23:00 and 04:00 to 05:00 hours. On the other hand, the only infective *An. funestus* were biting between 02:00 and 04:00 hours..



Error bars=Standard errors

Figure 4.12. Sporozoite infectivity of *An. gambiae* and *An. funestus* biting humans during the different hours of the night

There were also seasonal variations in infectivity of the vectors from the study areas. The sporozoite rates were comparable in both wet and dry season for *An. gambiae* mosquitoes sampled (2.8% and 2.7% respectively). There was however no *P. falciparum* infection detected in *An. funestus* samples from the dry season while the wet season recorded a sporozoite rate of 2.47% (Table 4.7).

Table 4.7. *Plasmodium falciparum* sporozoite infectivity of *An. gambiae* and *An.funestus* in the wet and dry seasons in Kassena-Nankana District, Upper East Region, Ghana

	An. gambiae s.s.				*An. funestus*			
Season	No tested	No +ve	% Sp. rate	95% CI	No tested	No +ve	% Sp. rate	95% CI
Wet	946	26	2.8	1.8-4.0	81	2	2.47	0.3-8.6
Dry	818	21	2.7	1.6-3.9	58	0	0	0.26-7.5
Total	1764	47	2.66	2.0-3.52	139	2	1.4	0.17-5.1

Wet = Wet season

Dry = Dry season

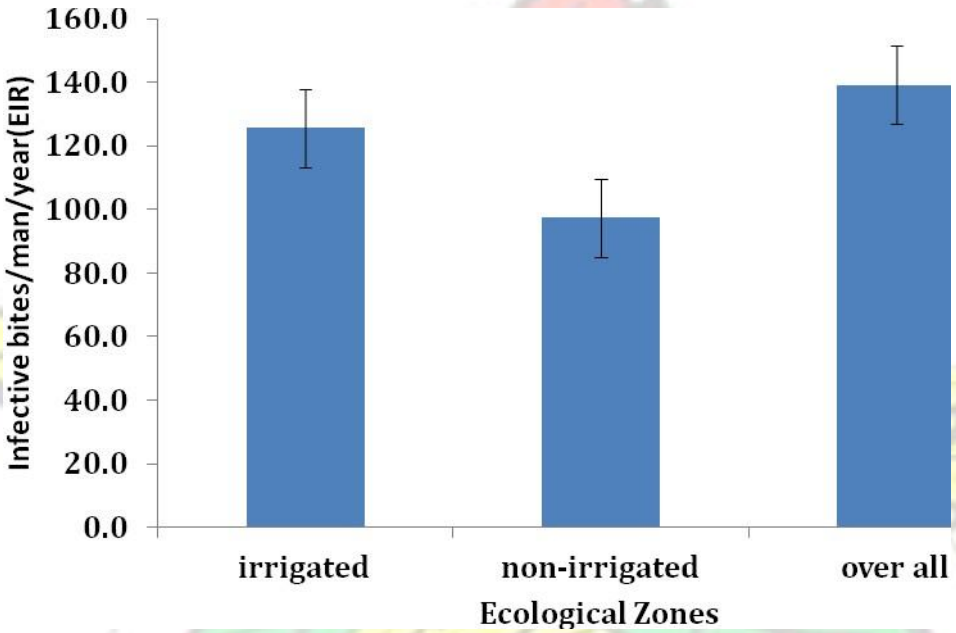
No +ve = number tested positive for *P falciparum* circumsporozoite antigen Sp.

Rate = percentage positive

4.1.8 Entomological inoculation rate (EIR) in KND

The malaria transmission intensity was estimated as EIR. The overall daily EIR for both *An. gambiae* and *An. funestus* in KND was estimated to be 0.38 infective bites/man/night (ib/m/n), whilst the daily EIRs during the wet and dry seasons were 0.46 ib/m/n and 0.21 ib/m/n respectively. In the irrigated area, the estimated daily EIR of 0.34 ib/m/n (from both *An. gambiae* and *An. funestus*) was close to the overall daily EIR (0.38) for the district. The daily EIR for the non-irrigated zone was 0.27 ib/m/n.

The overall annual entomological inoculation rates (EIR) or the intensity of transmission estimated for both *An.gambiae* and *An. funestus* vectors in KNDs was 139 infective bites / man / year (ib/m/y). Estimated EIRs across zones and seasons showed variations during the study period. The annual EIR in the irrigated and non-irrigated areas were 125.67 and 97.33 ib/m/y respectively (Figure 4.13). Transmission was shown to be seasonal, with the peak of transmission occurring in the wet season (June to October). In the wet season the EIR was significantly higher than that of the dry season (169.54 versus 6.8 ib/m/y).



Error bars=Standard errors

Figure 4.13. Entomological inoculation rates (EIR) of both *An. gambiae* s.s. and *An. funestus* in two ecological zones in Kassena-Nankana District, Ghana

The annual EIR by *An. gambiae* alone in the KND was estimated to be 122.7 infective bites/man/year (ib/m/y). Indoor and outdoor EIRs by *An. gambiae* were different in the two ecological areas with an annual value of 178.8 ib/m/y for indoor in the irrigated zone (IR) compared to 93.0 ib/m/y outdoors. The transmission levels for indoors in the nonirrigated area were 96.5 ib/m/y compared to 97.3 ib/m/y outdoors (Table 4.8). The overall EIR

estimated for *An. funestus* in KND was 10.5 ib/m/y. No EIRs were obtained in the irrigated area for both indoor and outdoor for *An. funestus* because no sporozoite infectivity was detected. However, EIR for indoor was 7.7 ib/m/y compared to 7.0 ib/m/y for outdoor in the non-irrigated area (Table 4.8).

Table 4.8. Annual entomological inoculation rates (EIR) of *An. gambiae* and *An. funestus* species captured biting indoor and outdoor from the two ecological zones in KND of Ghana

	An. gambiae s.s.		*An. funestus*	
	Indoor (ib/m/y)	Outdoor (ib/m/y)	Indoor (ib/m/y)	Outdoor (ib/m/y)[a]
Zone				
Irrigated	178.8	93.0	0.0	0.0
Non-irrigated	96.5	97.3	7.7	7.0

[a]ib/m/y= infective bites/man/year

4.2 INSECTICIDE SUSCEPTIBILITY OF MALARIA VECTORS IN KND

4.2.1 WHO Susceptibility tube Assays

A total of 6625 female unfed *An. gambiae* mosquitoes from three sites in KND were used for the bioassays. 5300 of these were exposed to insecticides belonging to the various classes of carbamates (0.1% Bendiocarb and 0.1% propoxur), organochlorine (4% DDT), and pyrethroids (0.05% deltamethrin, 0.75 % permethrin, 0.15% cyfluthrin 0.05% and Lamdacyhalothrin**)** and 1325 were used for controls. Molecular identifications found that all the sub sample of 2000 *An. gambiae* species tested were M-forms (*An. coluzzii*). *An. gambiae* showed high variability in response to the different insecticides tested (Table 4.9).

Table 4.9. Mortality (%) of *An. gambiae* s.s. collected from different sites in KND after 24hr exposure to the different insecticides

Insecticide tested	Bonia[1]			Korania[1]			Kandiga[3]		
	No. exposed	% dead (n)	95% CI	No. exposed	%.dead (n)	95% CI	No. exposed	% dead (n)	95% CI
Permethrin	400	29.0 (116)	25.0, 34.0	280	68.2 (191)	62.0, 74.0	300	60.0 (180)	54.0,.66.0
Deltamethrin	300	39.3 (118)	34.0,45.0	280	83.9 (235)	79.0, 88.0	400	51.3 (205)	46.0, 56.0
Lambdacyhalothrin	200	31.5 (63)	25.0, 38.0	280	78.2 (219)	73.0, 83.0	100	19.0 (19)	12.0, 28.0
Cyfluthrin	200	62.5 (125)	55.0,69.0	300	72.7 (218)	67.0, 78.0	100	37.0 (37)	28.0, 47.0
DDT	300	15.7 (47)	12.0, 20.0	300	40.0 (120)	34.0, 46.0	400	17.8 (71)	14.0, 22.0
Bendiocarb	200	83.0 (166)	77.0,88.0	280	63.6 (178)	58.0, 69.0	100	87.0 (87)	78.0, 93.0

[1] Korania is a community located in the irrigated area

[3]Kandiga is a community located in the non-irrigated area

[a]95% Confidence intervals calculated by the exact method

Propoxur	200	95.0 (190)	91.0, 97.0	280	91.1 (255)	87.0, 94.0	100	85.0 (85)	76.0, 91.0

[1]Bonia is a community located in the irrigated area

The mortalities for all sites ranged from 63.6-87.0% for bendiocarb; 85.0-95.0% for propoxur; 37.0-72.7% for cyfluthrin; 39.3-83.9% for deltamethrin; 19.0-78.2% for lamdacyhalothrin; 29.0-68.2% for permethrin and from 15- 40% for DDT (Table 4.9). The lowest mortality (indicating highest level of resistance) was observed for DDT at all sites (Bonia, Korania and Kandiga). In all the sites, *An. gambiae* was resistant to the pyrethroids, DDT and carbamates tested. Using GPS, all the surveyed sites were projected on a map of KND and the extent of susceptibility of *An. gambiae* to the different classes of insecticides were marked. The spatial distribution of the extent of resistance to the various insecticides tested is shown in Figures 4.14 (a-g).

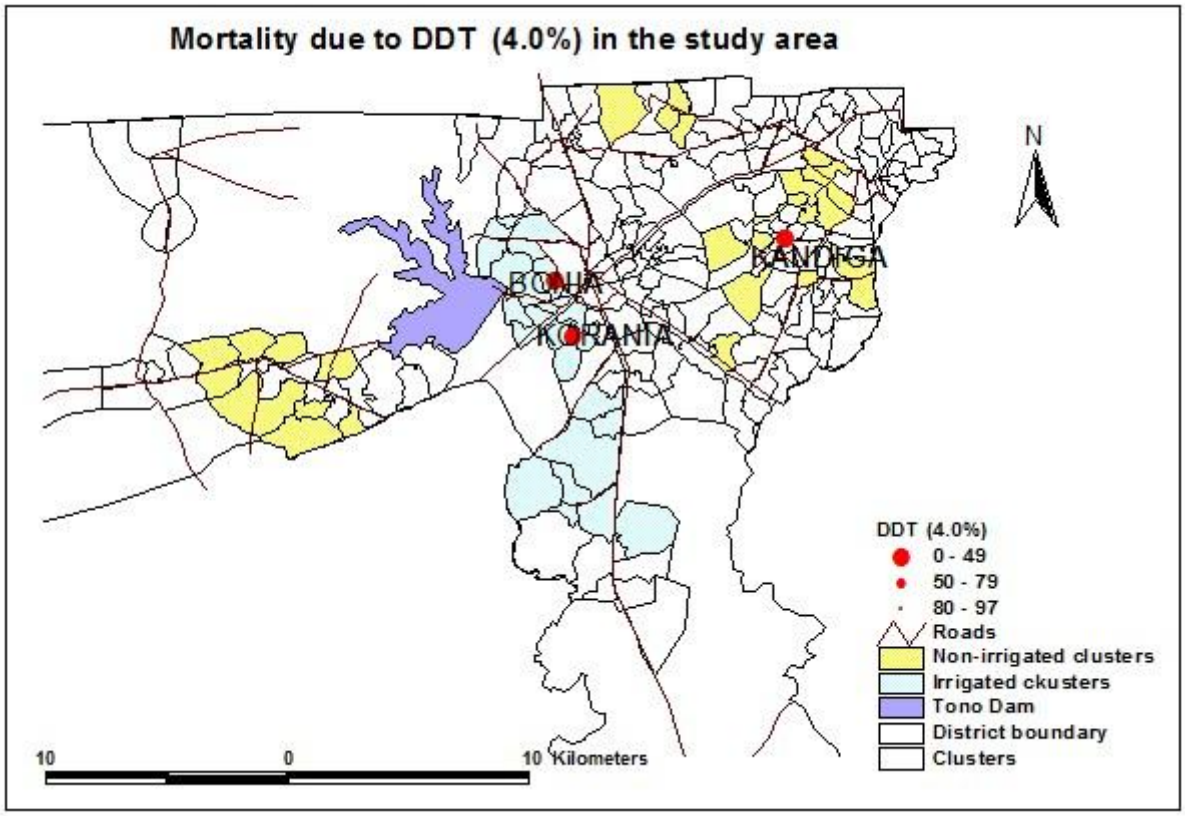


Figure 4.14 (a). A map showing the spatial distribution of the extent of DDT (4%) resistance in *An. gambiae* in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

Mortalities for the pyrethroids, deltamethrin (0.05%), lambdacyhalothrin (0.05%) and permethrin (0.1%) were very low in Bonia (39.3%, 31.5 and 29.0% respectively). Although that for cyfluthrin was relatively higher, it also indicated resistance (as shown in Table 4.9). In Kandiga, all the *An. gambiae* assayed were resistant to the pyrethroids. However cyfluthrin and lambdacyhalothrin recorded very low mortalities (37.0% and 19.0% respectively). Korania, which is quite close to Bonia, and located in the irrigated zone recorded comparatively higher mortalities (Table 4.9) for the pyrethroids and DDT although the results suggested the presence of resistance.

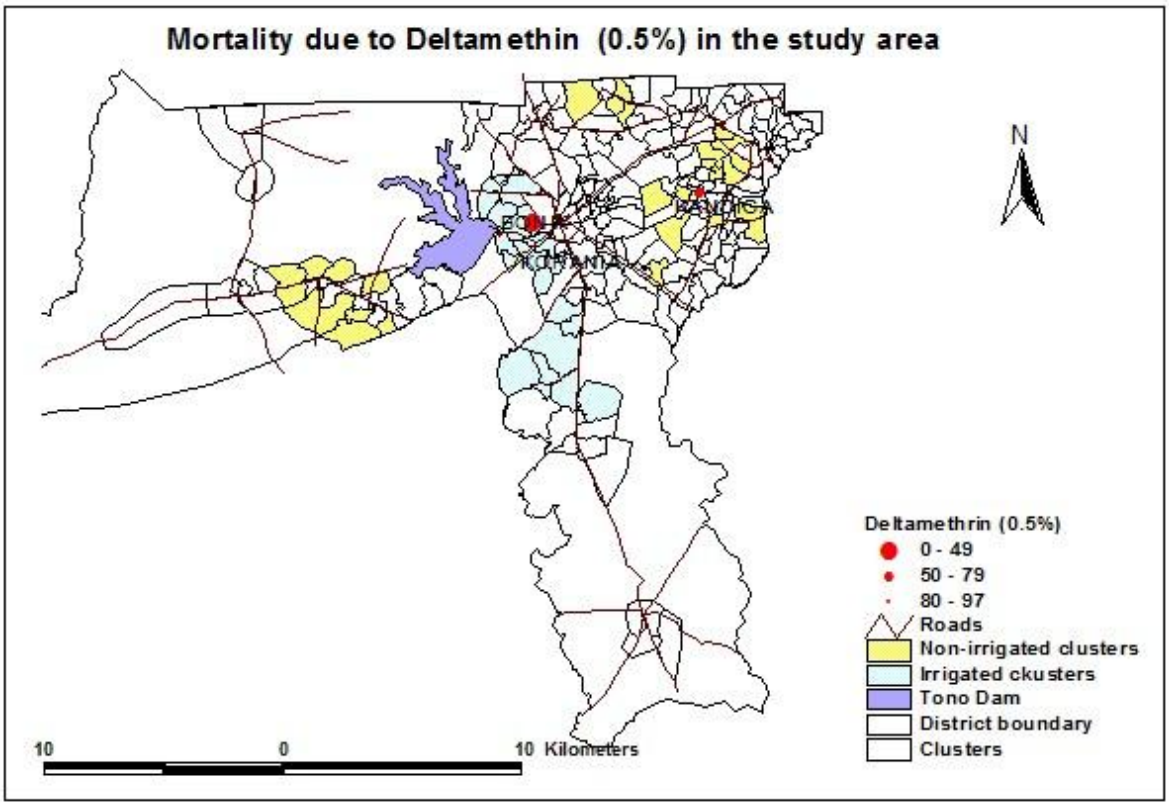


Figure 4.14 (b). A map showing the spatial distribution of the extent of deltamethrin (0.05%) resistance in *An. gambiae* in some sites in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

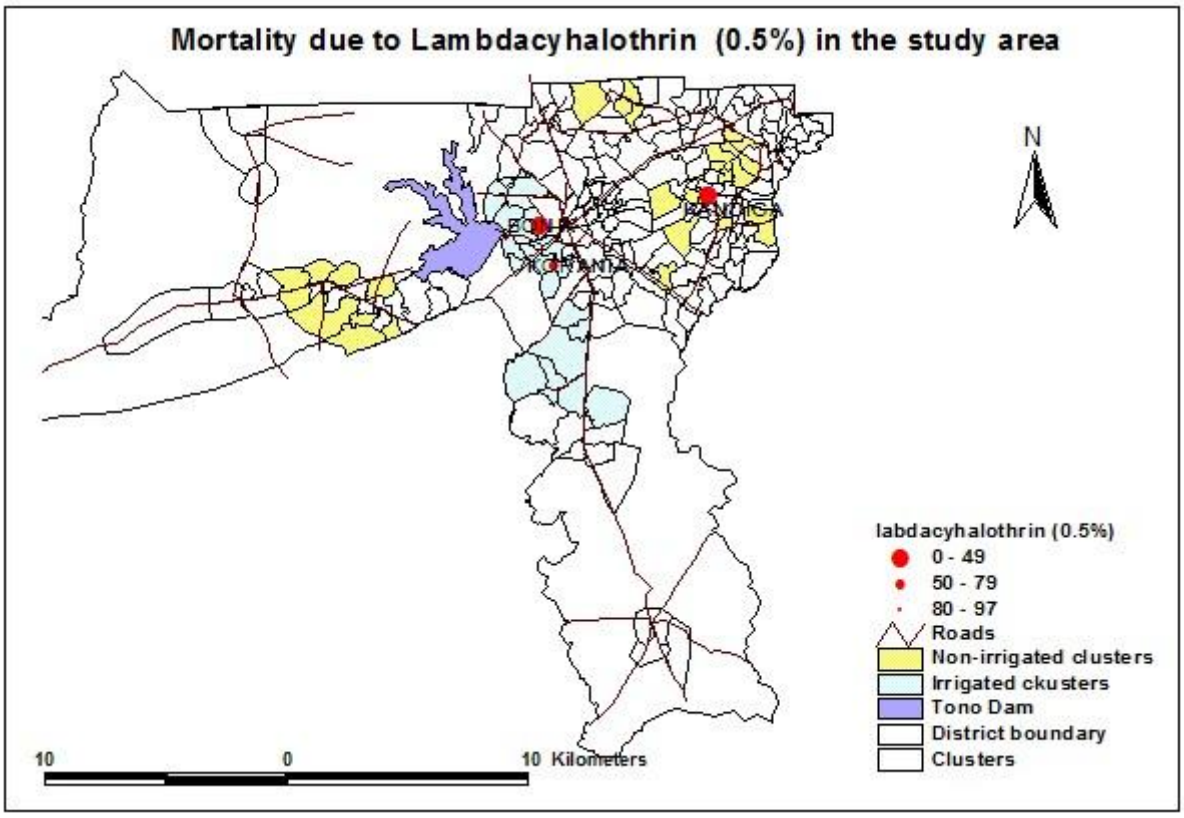


Figure 4.14 (c). A map showing the spatial distribution of the extent of lamdacyhalothrin (0.05%) resistance in *An. gambiae* in some sites in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

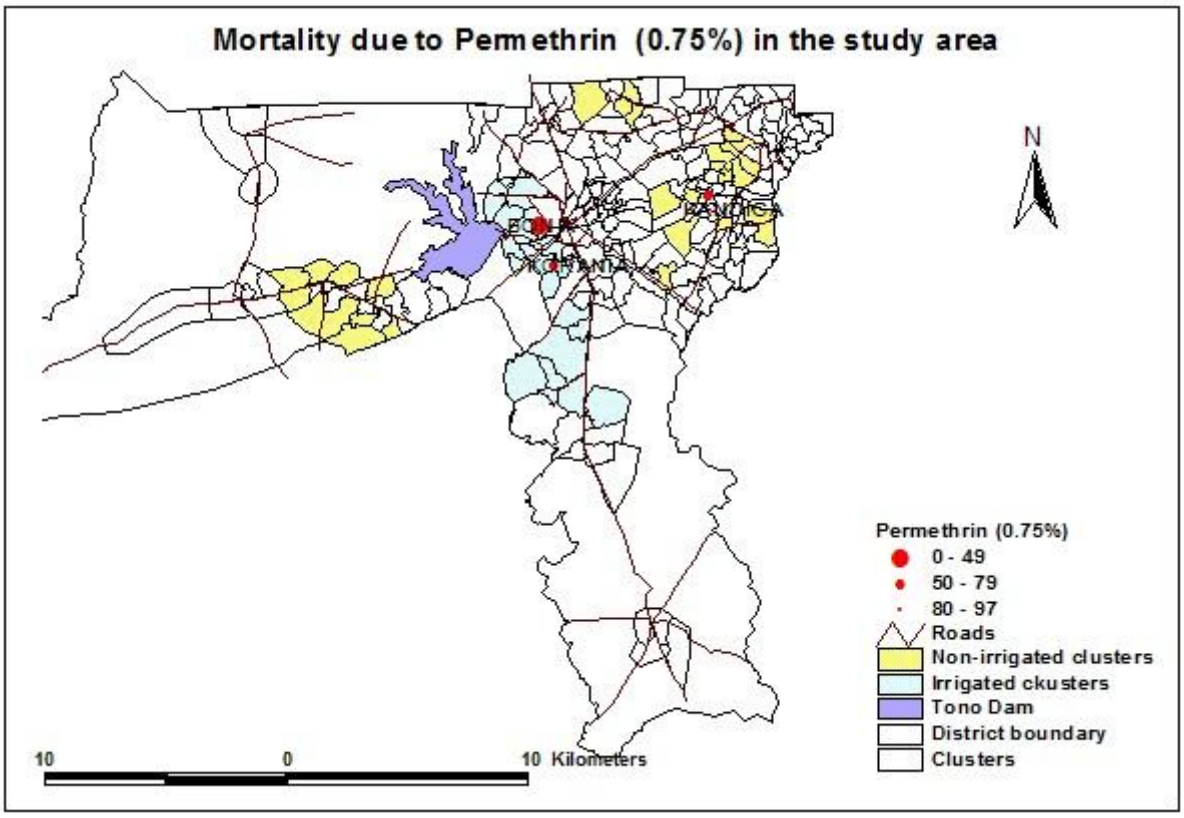


Figure 4.14 (d). A map showing the spatial distribution of the extent of permethrin (0.75%) resistance in *An. gambiae* in some sites in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

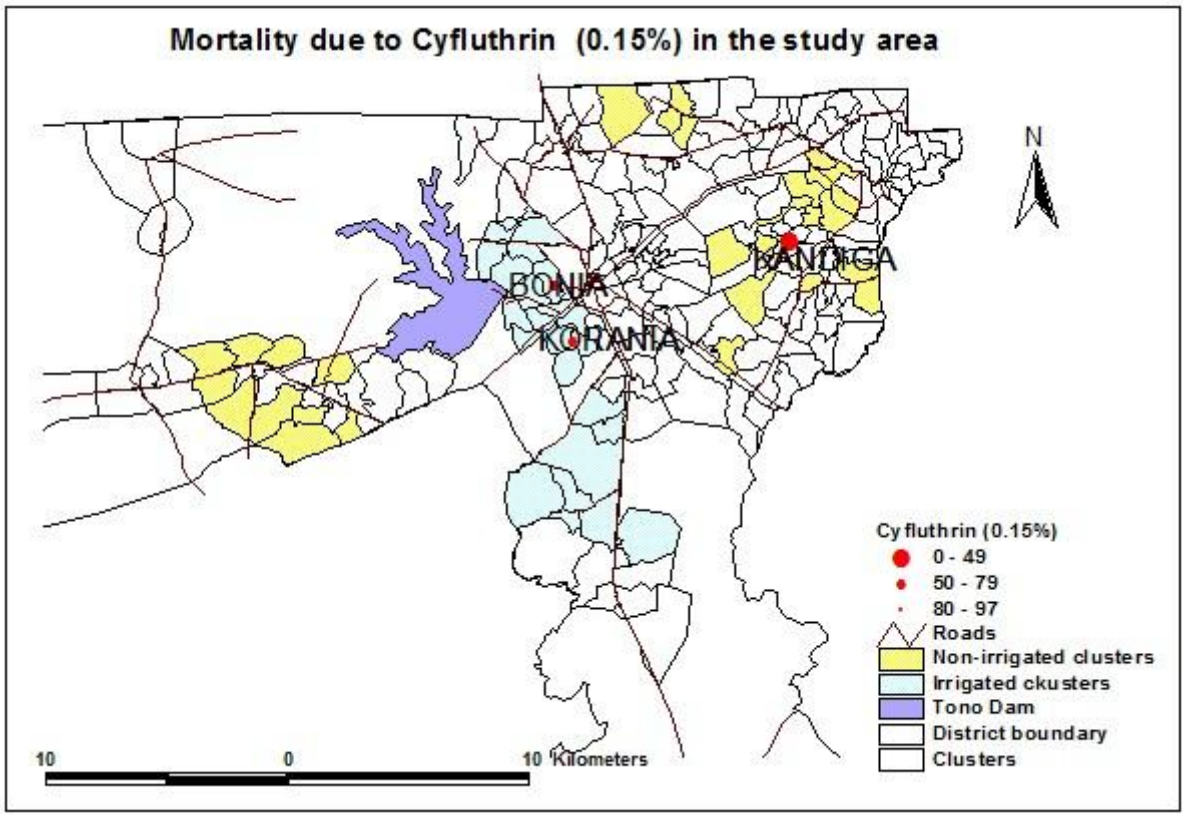


Figure 4.14 (e). A map showing the spatial distribution of the extent of cyfluthrin (0.15%) resistance in *An. gambiae* in some sites in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

Tests using the carbamates, bendiocarb and propoxur also indicated the presence of resistance. The percentage mortalities were relatively high when compared to the other insecticide classes tested. Bendiocarb recorded a range of 63.6%-87.0%, with the lowest in Korania whilst in Kandiga, propoxur susceptibility was in the range of 85.0%-95.0 %.

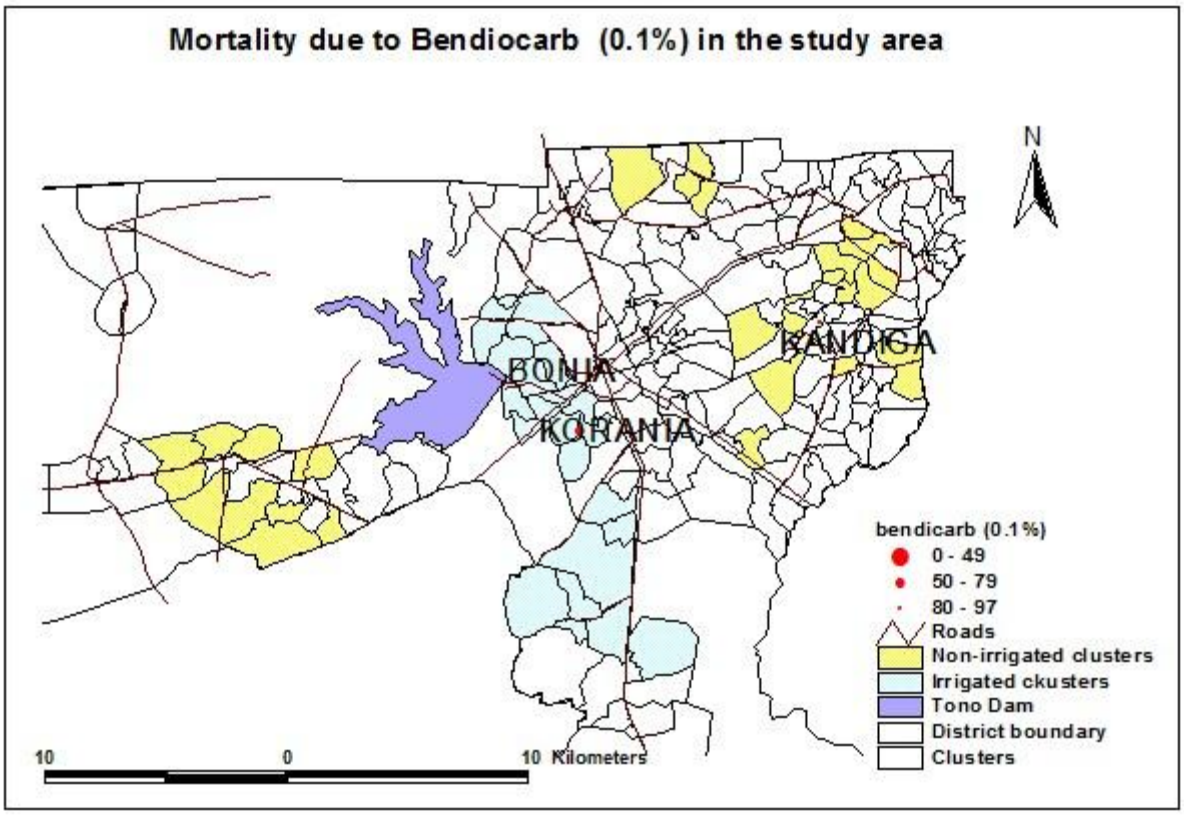


Figure 4.14 (f). A map showing the spatial distribution of the extent of Bendiocarb (0.1%) resistance in *An. gambiae* in some sites in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

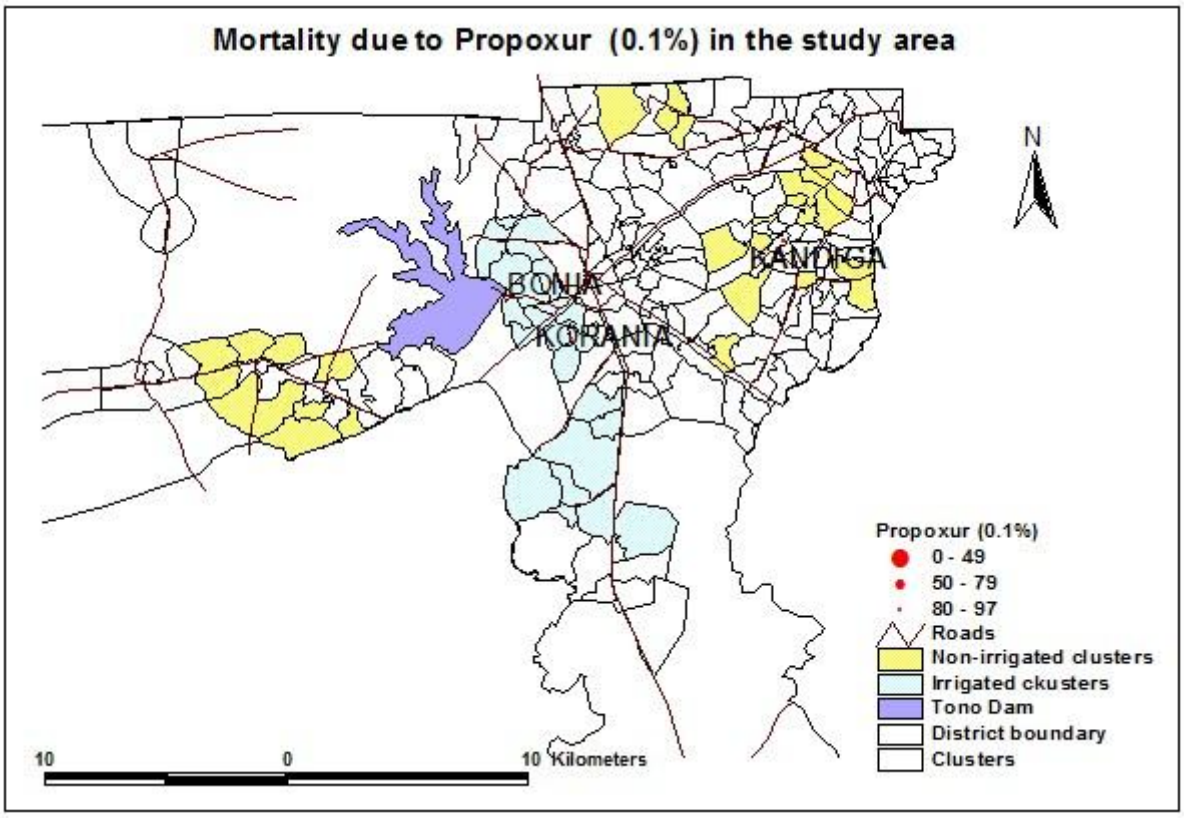


Figure 4.14 (g). A map showing the spatial distribution of the extent of propoxur (0.1%) resistance in *An. gambiae* in some sites in KND

The coloured circles depict the range of mortalities (the biggest circle= lowest mortality range as shown in the legend)

4.2.2 Frequency of kdr (L1014F) mutation in *An. gambiae* s.s.in relation to the phenotypes

To assess the role of L1014F *kdr* allele in conferring pyrethroids resistance in *An. gambiae s.s.*, the *kdr* allele was screened for 129 specimens (dead and alive) of *An. gambiae* s.s. selected from the WHO bioassays for bendiocarb, permethrin and deltamethrin. All three L1014F *kd*r genotypes (LL, LF and FF) were detected among the survivors and the dead. The L1014S allele, which is common in East Africa, was not screened. The frequencies of the L1014F *kdr* allele in *An. gambiae* mosquitoes from KND that either died or survived exposure to the insecticides deltamethrin, permethrin (pyrethroids) or bendiocarb is shown

in Table 4.10. A total of 42 (11 dead and 31 survivors) *An. gambiae* exposed to deltamethrin were screened for the L1014F allele. 36.4% (N=4) of the dead samples carried the homozygous resistant allele (FF) whilst 54,5% (N=6) carried the heterogeneous allele (LF). On the other hand, 25.8% (N=8) of mosquito samples that survived exposure to deltamethrin had the homozygous resistant allele whilst 61.3% (N= 19) were heterozygous. Allelic frequency of the L1014F in the dead samples was estimated to be 63.6% and that for the survivors was 56.5%. Fisher Exact Tests for Hardy-Weinberg equilibrium for the insecticide showed that genotype frequencies did not show any phenotype-genotype association (P>0.001) (Table 4.10).

In the case of samples of *An. gambiae* mosquitoes exposed to permethrin, a total of 53 (36 dead and 17 survivors) were screened for the presence of the L1014F alleles. For the dead samples, 52.8% (N=19) were found to carry the homozygous resistant allele whilst those carrying the heterozygous allele constituted 33.3% (N=12). For the mosquitoes that survived permethrin exposure, those carrying the homozygous resistant allele constituted 29.4% (N=9) whilst 52.9% (N=9) were heterozygous. The L1014F allele frequency in the dead mosquitoes was 69.4% compared to 55.9% for the survivors. The Fisher Exact Tests for Hardy-Weinberg equilibrium also indicated here that there was no phenotype-genotype association (P>0.001) (Table 4.10).

Samples taken from mosquitoes that were either resistant or susceptible to the carbamate insecticide bendiocarb were also screened to check whether they also carried the L1014F allele responsible for pyrethroids resistance. A total of 34 (8 dead and 26 survivors) showed varying frequencies of the L1014F alleles. They however did not indicate any phenotype-genotype association (Table 4.10).

Table 4.10. Association between L1014F genotype and mosquito survival after insecticide exposure

Insecticide	No. tested	Status	No.	No. per Genotype			1014F[2]	P-
				*LL	*LF	*FF	Frequency[1]	value[3]
Bediocarb	34	Dead	8	1	5	2	56.3	0.02
		Alive	26	0	0	26	100	
Deltamethrin	42	Dead	11	1	6	4	63.6	0.876
		Alive	31	4	19	8	56.5	
Permethrin	53	Dead	36	5	12	19	69.4	0.269
		Alive	17	3	9	5	55.9	

*LL= Wildtype; LF=1014F heterozygote and FF =1014F resistant allele[1]The frequencies were calculated for each insecticide and mosquito status (alive/dead) after insecticide exposure.

[2]1014F represent the kdr frequencies

[3]Genotypic comparisons were done using the Fisher Exact test

4.2.3 Frequency of Ace-1R mutation in mosquitoes exposed to different insecticides

To assess the role of Ace-1R allele in conferring resistance to *An. gambiae* s.s., the Ace1R genotype was screened in dead and alive mosquitoes from the WHO bioassay using bendiocarb, deltamethrin and permethrin (Table 4.11). All the Ace-1R genotypes (GG, GS and SS) were found among survivors and the dead. For *An.gambiae* samples that died

as a result of exposure to bendiocarb, a total of 35 (15 dead and 20 survivors) were screened. 66.7% (N=10) were found carrying the homozygous resistant allele (SS) whilst only 13.3% (N=2) carried the heterozygous allele (GS). In the case of the survivors only 10.0% (N=2) were carrying the homozygous allele whilst 5.0% carried the heterozygous allele. A high number (N=17) were rather found to be carrying the wild type (GG) susceptible allele. The overall frequency of the G119S alleles in survivors was 73.3 % compared to 12.5 % for the dead samples. Fisher Exact Tests for Hardy-Weinberg equilibrium showed a significant association (p-value<0.001) between the Ace-1R mutation and bendiocarb phenotypic resistance (Table 4.11).

An attempt was made to find out if the *An. gambiae* mosquitoes exposed to the pyrethroids, deltamethrin and permethrin also carried the Ace-1R alleles. For deltamethrin exposed samples, 20 dead samples were screened and of this number, 90% (N=18) were found to carry the homozygous resistant allele whilst 5% (N=1) carried the heterozygous allele. The permethrin exposed samples (56 dead and 16 survivors) did not indicate any phenotype-genotype association (p>0.001) (Table 4.11). The frequency of the G119S alleles in dead and alive samples was 50.1% and 81.2% respectively. Whilst 41.1% (n=23) of the dead samples carried the homozygous resistant allele, 81.3% (N=13) of the survivors carried this allele. Also whereas 17.9% (N=10) of the dead *An gambiae* samples exposed to permethrin were heterozygous, none of the survivors were found to carry this allele (Table 4.11).

Table 4.11. Association between genotype and mosquito survival after insecticide exposure

Insecticide	No. tested	Status	No.	NO. per Genotype	G 119S Frequency

				*GG	*GS	*SS	[1]	P-value[3]
Bediocarb	35	Dead	20	17	1	2	12.5	0.00019
		Alive	15	3	2	10	73.3	
Deltamethrin	20	Dead	20	1	1	18	92.5	#
		Alive	0	0	0	0	0	
Permethrin	72	Dead	56	23	10	23	50	0.016
		Alive	16	3	0	13	81.2	

*GG= Wildtype; *GS=G119S heterozygote and *SS = G119S resistant allele.

[1]The frequencies were calculated for each insecticide and mosquito status (alive/dead) after exposure

[2]119S represent the *ACE-1R* frequencies

[3]Genotypic comparisons were done using the Fisher Exact test

4.2.4 Allelic frequencies of kdr L1014F, N1575Y and Ace-1R mutations in night biting *An. gambiae* populations

A sub sample of *An. gambiae* caught biting in both irrigated and non-irrigated areas using HLC were genotyped for the *kdr* L1014F, N1575N and *Ace-1R* mutations. Figure 4.15 shows the distribution of allelic frequencies of the L1014F mutation in *An. gambiae s.s.* in some selected sites in KND. Biu, Bonia, Korania and Wuru are located in the irrigated zone whilst Kalvio is a community in the non-irrigated zone. A comparison of the results from Bonia and Korania was made with earlier reports to find out if there has been any change in frequency since 2004 (Yawson *et al.,* 2004). The results showed a sharp increase in L1014F frequencies from 0.58% (95% CI, 0.01%-3.2%) and 0.00% (95% CI, 0.00%-1.92%) for Bonia and Korania (Yawson *et al.,* 2004) to 69.09% (95% CI, 59.57%77.55%) for Bonia and 80.77% ((95% CI, 67.47%-90.37%) for Korania respectively

(Figure 26). The other communities also showed high frequencies of the allele; 60.00% in Biu (95% CI: 48.44%–70.80%), 86.84% in Kalvio (95% CI: 71.91%–95.59%) and 30.00% in Wuru (95% CI: 6.67%– 65.25%).

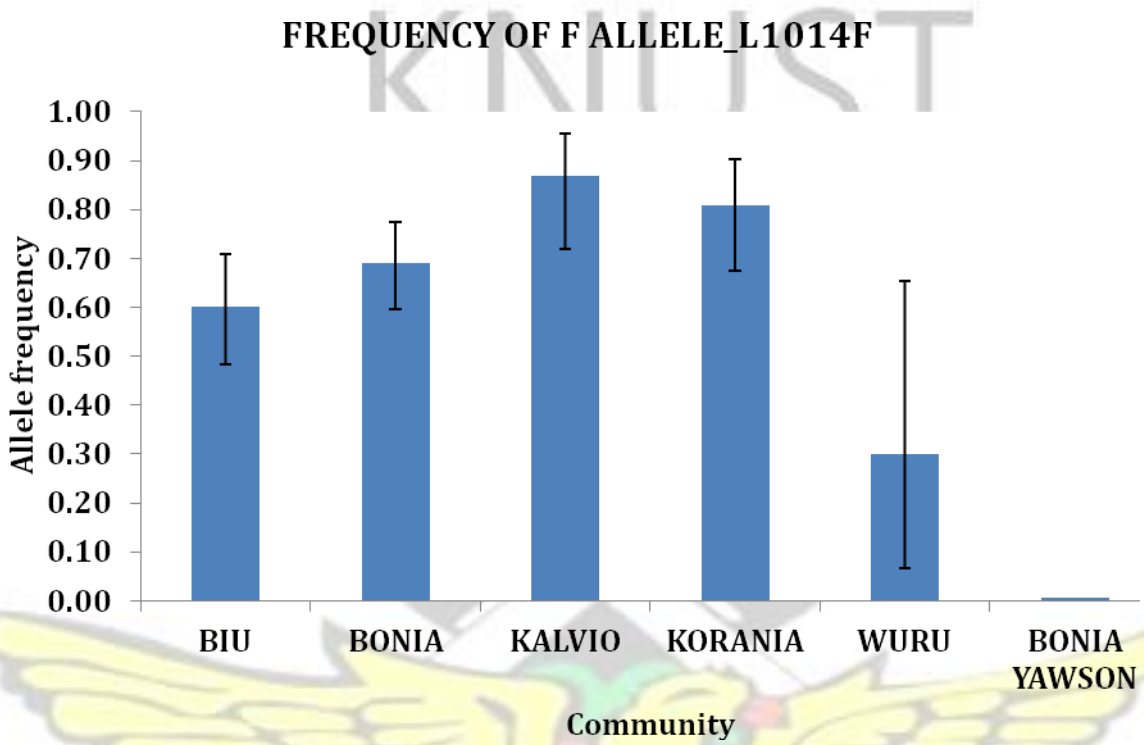


Figure 4.15. Allele frequencies of the kdr (L1014F) mutation in *An. gambiae* s.s captured by Human landing collections in some communities in KND of Ghana

Biu, Bonia, Korania and Wuru are communities in the irrigated zone. Kalvio is a community in the nonirrigated zone. Bonia Yawson and Karania Yawson denote the frequencies reported earlier (Yawson *et al.*, 2004) in these communities.

An. gambiae s.s from all the five communities which were genotyped by TaqMan PCR, also scored for the presence of the N1575Y mutation (Figure 4.16). A total of 154 *An. gambiae* were genotyped out of which 6.49% (N=10) were homozygous for the resistant mutant allele whilst 49.35% (N=76) were heterozygous and 44.16% (N=68) were the homozygous suceptibles.

The N1575Y mutation was found in *An. gambiae* s.s. with allelic frequencies of 31.03% in Biu (95% CI: 19.54%–44.54%), 42.42% in Bonia (95% CI: 30.34%–55.21%), 22.86% in Kalvio (95% CI: 13.67%–34.45%), 39.06% in Korania (95% CI: 27.10%–52.07%) and 18.00% in Wuru (95% CI: 8.58– 31.44%).

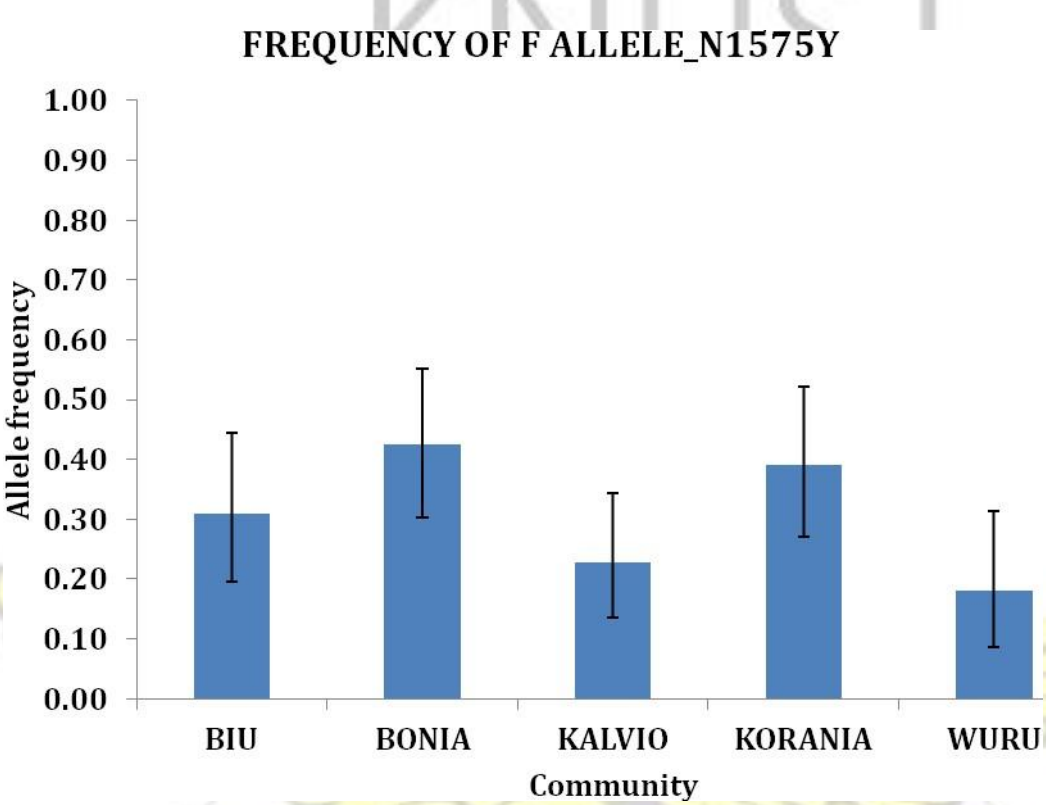


Figure 4.16. Allele frequencies of the N1575Y mutation in An. gambiae s.s captured by Human landing collections in some communities in KND of Ghana

Biu, Bonia, Korania and Wuru are communities in the irrigated zone. Kalvio is a community in the nonirrigated zone

Ace-1R G119S genotype was characterized in 186 samples of *An.gambiae* s.s.. Out of these, 18 (9.68%) were homozygous GG (wild-type), 23 (12.37%) heterozygous GS and 145 (77.95%) homozygous SS. Figure 4.17 presents the *Ace-1R* allelic frequencies observed from five sites in KND. In all the sites, there was a high frequency of the G119S allele with

the homozygote SS being the most prevalent. The G119S mutation was found at the allelic frequency of 89.29% in Biu (95% CI: 78.12%–95.97%), 83.00% in Bonia (95% CI: 74.18%–89.77%), 96.88% in Korania (95% CI: 89.16%–99.62%), 90.00% in Wuru (95% CI: 76.34%– 97.21%) and 73.21% in Kalvio (95% CI: 60.20%–81.14%).

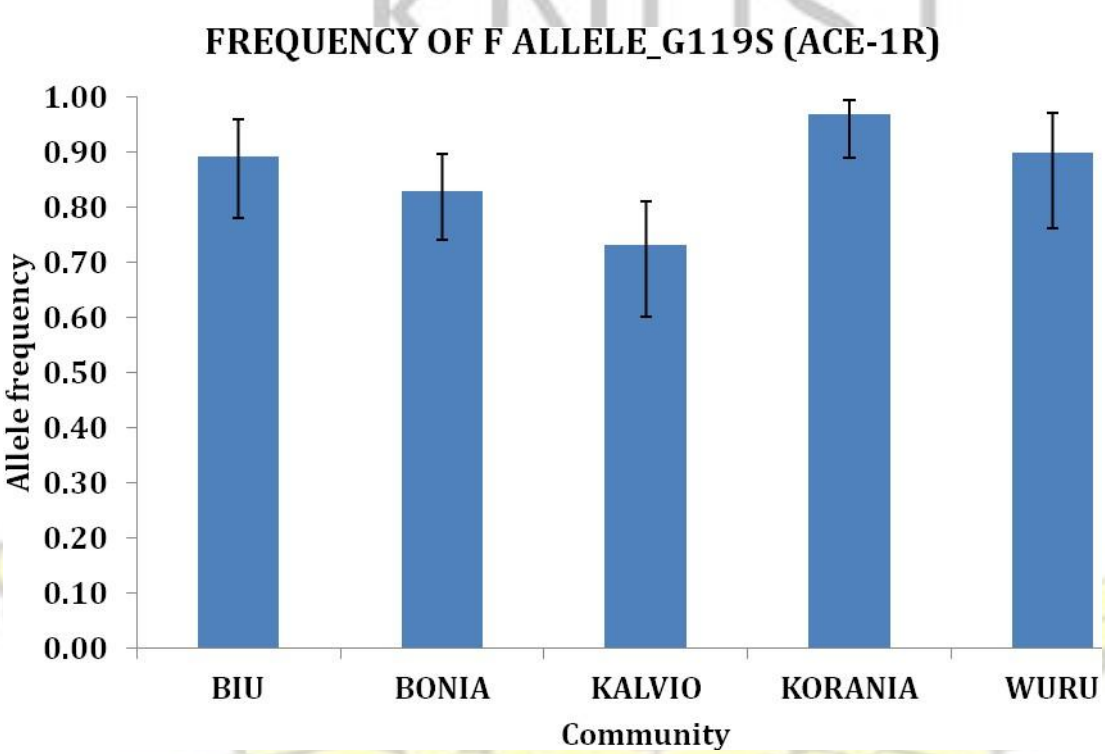


Figure 4.17. Allele frequencies of the G119S mutation in An. gambiae s.s captured by Human landing collections in some communities in KND of Ghana

Biu, Bonia, Korania and Wuru are communities in the irrigated zone. Kalvio is a community in the nonirrigated zone

4.3 Relationship between Sporozoite infection and *kdr mutation in An. gambiae*

Of the 213 HLC samples selected, a total of 49 *An. gambiae* mosquitoes were found to be infected with sporozoites of P. *falciparum.* All the 49 samples were genotyped for *kdr* L1014F. Among the sporozoite positive mosquitoes, 26.53% (N=13) were homozygous for

the *kdr* L1014F mutant allele. Among sporozoite negative mosquitoes, 26.21% (N=43) were homozygous for the *kdr* L1014F allele (Table 12). However, these percentages were not significantly different (> 0.0001). 36.73% (N=18) of the sporozoite positive samples were heterozygous whilst 4.27% (N=7) of samples that did not show positivity were heterozygous. Here also, there was no significant difference. Of the samples that exhibited sporozoite posivity, 36.73% (N=18) were found to be carrying the wildtype homozygous susceptible allele whilst 69.51%(N=114) of the sporozoite negative samples carried the wildtype homozygous susceptible allele.

Table 4.12. Kdr genotype frequencies and *P. falciparum* sporozoite infection in *An. gambiae* s.s. from KND of Ghana.

	ELISA			
GENOTYPE	%Positive(N)	%Negative(N)	TOTAL (%)	ODDS RATIO
LL	36.73(18)	69.51(114)	132 (61.97)	1
LF	36.73(18)	4.27(7)	25 (11.73)	16.30(6.07, 43.56)
FF	26.53(13)	26.21(43)	56 (26.29)	19.40(10.66, 35.44)

LL= Wildtype; LF=1014F heterozygote and FF =1014F resistant allele

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 Discussion

This study was carried out to determine current malaria transmission dynamics and insecticide susceptibility profile of malaria vectors in two different micro-ecological zones in KND.

An entomological parameter commonly used to measure the association between vectors and incidence of malaria is the abundance of Anophelines (Rubio-Palis and Curtis, 1992; Zimmerman, 1992 ; Gil *et al.,* 2003 ; Moreno *et al.,* 2007). It is therefore important to consider the relative abundance of *Anopheles* species when determining its relevance as a vector. This is because high numbers increase the chances of human–mosquito contacts and hence transmission. In this study, man biting rate (bites /person / night) and indoor resting density were used as measure of the abundance of *Anopheles* mosquitoes. Both HLC and PSC results demonstrated that *An. gambiae* s.l. was by far the most abundant *Anopheles* species biting humans in KND. This was followed by *Anopheles funestus*. Other anophelines recorded but in low densities included *An. pharoensis* and *An. rufipes.* This study confirms earlier reports that *An. gambiae s.l* is the most dominant human biting mosquito species in Ghana (Appawu *et al.,* 1994 ; Appawu *et al.,* 2001; Yawson *et al.,* 2004; Dery *et al.,* 2010; de Souza *et al.,* 2010; Tchouassi *et al.,* 2012). The reported abundance of these *Anopheles* species especially the dominance of *An. gambiae* in KND could be explained by the diverse ecological conditions of each collection site. The presence of breeding sites, animal host and suitable vegetation as a source of nectar influences the presence and abundance of mosquitoes (Wekesa *et al.,* 1997 ; Mwangangi *et al.,* 2007 ; Muturi *et al.,* 2008). The irrigated zone contains numerous suitable breeding ponds whilst

in the non-irrigated sites; mosquito breeding seems to occur in temporary rain puddles during most of the sampling periods. There are also reports of other *Anopheles* species including *An. melas*, *An. nili* Theobald, *An. hancocki* Edwards, *An. coustani* Laveran, *An. moucheti* Evans and *An. hargreavesi* Evans, found in the coastal savannah and forest zones of Ghana (Appawu *et al.,* 2001) but these were not found in this study.

An. gambiae s.s. was the only sibling species of the *An. gambiae* complex caught biting in the two micro-ecological areas of KND, unlike an earlier study in the district which reported relatively small numbers of *An. arabiensis* captured trying to bite humans at night (Appawu *et al.,* 2004). Studies in some villages in Burkina Faso, which share border with the Kassena-Nankana District and in Mali, found that populations of *An. arabiensis* were relatively high and were maintained continuously, but with seasonal variations (Taylor *et al.,* 1993). Other studies in the Garki district (Kano State, Nigeria), a typical Sudan savannah area had *An. arabiensis* outnumbering *An. gambiae* s.s. (Molineaux and Gramiccia, 1980). This study however concurs with findings in the coastal savannah areas of Ghana (Okoye *et al.,* 2005 ; Appawu *et al.,* 2001) and elsewhere in Africa (Bigoga *et al.,* 2007). All the *An. gambiae* s.s. genotyped belonged to *An. colluzzi* (M-Form). The relative dominance of *An. colluzzi* over *An. gambiae* s.s. has been found to be linked with features of breeding sites. There is evidence that the *An. colluzzi* tends to be associated with semi-permanent and man-made flooded or irrigated sites that provide permanent breeding conditions, whereas the *An. gambiae* s.s is associated with rain-dependent temporary sites ((Diabaté *et al.,* 2005 ; Touré *et al.,* 1998). The majority of breeding sites in the non-irrigated zone are rain-dependent and yet only *An. colluzzi* were identified. In

West Africa, there are reports of varying levels of hybridization between *An. colluzzi* and *An. gambiae* s.s., as a result of a process by which adaptive genes may flow from one to the other, including those conferring insecticide resistance (Fanello *et al.,* 2003 ; Yawson *et al.,*

2004). The earlier study by Appawu *et al.* (2004) found both *An. gambiae* s.s. and hybrids but the latter were at a very low level in the non-irrigated parts of KND (Rocky highlands).

This study found high numbers of *Anopheles* mosquitoes in the irrigated zone than the non-irrigated zone particularly during the dry season. No *Anopheles* mosquitoes were collected from the non-irrigated zone in the very dry months. Flooding of the fields for irrigation in the dry season however enhanced breeding and therefore ensured increased adult mosquito numbers in the irrigated zone. *An. gambiae s.l.* was more predominant than *An. funestus* in both the dry and wet seasons in the two ecological zones. *An. funestus* numbers were relatively lower than the number of *An. pharoensis* and *An. rufipes* captured put together, when compared to what was reported a decade ago by Appawu *et al*. (2004). . The reduction in the *An. funestus* population may eventually lead to its disappearance and possible replacement with non-vector species as happened in the Pare-Taveta area in Tanzania (Gillies and Smith, 1960 ; Smith, 1962) and Malindi on the coast of Kenya (Gillies and Furlong, 1964) .In Malindi, *An. funestus* was thought to have been replaced by *An. rivulorum* and *An parensis* (zoophagic, exophagic and exophillic species of the *An. funestus* group), probably due to a switch in the balance of competition for limited aquatic habitats (Gillies and Smith, 1960 ; Gillies and Furlong, 1964 ; Kawada *et al.,* 2012). *Anopheles funestus* is highly amenable to various classes of insecticides and the long use of ITNS in the area might have put more selective pressure on the populations leading to population size reduction (Wondji *et al.,* 2012). One possible explanation for the observed reduction of *An. funestus* in KND may be the high coverage and over a decade use of ITNs/LLINs and subsequent replacement with non-vector species (*An. phoerensis* and *An. rufipes*). This could be attributed to a phenomenon known as the Allee effects (Killeen *et al.,* 2013). This phenomenon refers to the situation where the fitness of individual mosquitoes are compromised at low densities (Stephens *et al.,* 1999) largely because the populations have

become so dispersed that males and females strive to find each other to mate (Stephens *et al.,* 1999 ; Gascoigne *et al.,* 2009). One can therefore imagine how the Allee effects would amplify the effects of ITNs/LLINs use in the study area especially during the dry season when *An. funestus* numbers were low as a result the shrinking of their breeding sites. Such a circumstance, offers an opportunity to eliminate them entirely using improved or additional vector control strategies (malERA Consultative Group on Vector Control, 2011 ; Ferguson *et al.,* 2010). The finding in this study is however, at variance with the study done in Kintampo in Ghana by Dery *et al*. (2010) where *An. funestus* took over as the dominant *Anopheles* mosquito in the second year of a study whilst *An. gambiae* was the dominant species in the first year. During part of larval sampling for bioassays, it was observed that the most notable *Anopheles* larval breeding habitats were poorly constructed irrigation canals (with stagnant water), and puddles in waterlogged fields, especially during the dry season. A model by Smith *et al*. (2005) showed that there is a direct link between human exposure to biting and the proximity of breeding sites to human habitation. Thus, people residing in the irrigated area especially those close to the canals face a higher risk of getting malaria due to the availability of numerous breeding sites leading to high vector abundance (Baeza *et al.,* 2013; Staedke *et al.,* 2003).

There were seasonal variations in the abundance of the two major species in the area. Majority of the mosquitoes were collected during the wet season. Mosquito numbers increased intensely when the rains started. The numbers then stabilized and then significantly reduced during the dry months. This observation is similar to other studies that show that mosquito activity is interrupted by changes in environmental conditions (Gilles and Wilkes, 1981 ; Sharp, 1983 ; Munhenga *et al.,* 2014). This study shows a similar pattern as was reported by (Appawu *et al.,* 2004) in KND and other areas (Gil *et al.,* 2003 ; Moreno *et al.,* 2007). The seasonality in spatial and temporal distributions of anopheline mosquitoes

are characteristic of the tropics (Shililu *et al.,* 2003 ; Munga *et al.,* 2006) and the high numbers of anopheline mosquitoes in the rainy season have been attributed to the increase of rain pools, the preferred breeding sites of anophelines, as well as enhanced humidity for adult mosquito survival and dispersal (Fillinger *et al.,* 2004). The significantly higher densities of anopheline mosquitoes collected during the rainy season in this study and hence high biting, to a large extent explains the similar seasonal pattern of clinical malaria episodes, with peak transmission shortly after maximum annual rainfall in KND (Koram *et al.,* 2003 ; Oduro *et al.,* 2010).

In this study, both *An. gambiae* s.s and *An. funestus* were biting inside and outside rooms. The relatively high outdoor behaviour by *An. gambiae* and *An. funestus* is similar to that reported by Appawu *et.al.* (2004) in KND. Both LLINs and IRS are principally effective at reducing the transmission of malaria because they capitalize on the indoor (endophagy) and night (nocturnality) biting and indoor resting (endophily) characteristics of *An. gambiae* and *An. funestus*. As a result, these two control strategies impart both direct personal protections against infective mosquito bites as well as indirect community protection eventually resulting in a long-term decrease in mosquito abundance. Studies have reported instances of changes in the vector host seeking behaviours attributable to

ITNs and IRS that have resulted in increased outdoor biting (White, 1974 ; Pates and Curtis, 2005) and shifts in peak biting times, (Oyewole *et al.,* 2007). This may allow malaria transmission to be maintained even after ITNs/LLINs have been fully scaled up. The potency and long use of ITNs/ LLINs in KND may in part, explain the substantial proportion of outdoor biting observed in this study. The results of this study, along with the recent study by *(*Russell *et al.,* 2010) suggest that the continual indoor application of residual insecticides results in marked shifts in outdoor feeding among malaria vector populations (Reddy *et al.,* 2011). In considering the persistence of malaria transmission in KND in the face of well-

executed scale up and the long use of LLINs, the role of outdoor biting cannot be overemphasized especially where by 21.00 hours, only few of the residents may have retired indoors. It therefore follows from this study that the addition of other preventive measures such as insecticide treated clothes (Macintyre *et al.,* 2003 ; Kimani *et al.,* 2006) and repellents delivered as topical applications or vapour-phase emanators (Achee *et al.,* 2012 ; Ogoma *et al.,* 2012 ; Dadzie *et al.,* 2013) should be considered. Also, given the widespread reliance of mosquitoes on sugar (Foster, 1995 ; Gu *et al.,* 2011 ; Huestis *et al.,* 2011), especially when infected with malaria parasites (Nyasembe *et al.,* 2014), toxic sugar baits have been found to impressively kill a wide diversity of vector species (Müller and Schlein, 2006 ; Müller and Schlein, 2008 ;Müller *et al.,* 2010 ; Killeen *et al.,* 2014) and this can be exploited as a way of killing outdoor biting mosquitoes in KND. It should be noted that Larval Control will be difficult to be used in the districts because of the unmanageable larval habitat levels. Larval control may only be possible in the dry season possibly close to broken irrigation canals that allow for smaller, discrete areas of sustained water accumulation. These may constitute hot-spots for productive *Anopheles* habitats which, given their confined spatiality, might justify localized larval control.

In malaria endemic countries, one of the essential factors of vector population vulnerability to ITNs (apart from their being endophagic or exophagic) is whether the timing of biting activity coincides with human sleeping patterns (Lindblade, 2013 ; Huho *et al.,* 2013). In this study, the proportion of *An. gambiae* and *An. funestus* biting started early. The early biting may be induced by the physiological status of mosquitoes (Clements, 1963 ; Klowden, 1994), for example, if females are unable to obtain a blood meal during the previous night, they are likely to start host seeking earlier in the night.. Also, the results showed a small proportion of mosquitoes biting earlier to be infective indicating that they are likely to be nulliparous than those biting later (Bockarie *et al.,* 1996) as adult numbers may be reduced

in areas as a result of the widespread bed net use (Magesa *et al.*, 1991). Alternatively, variations in the genetic structure of local populations may shift to those that are likely to commence feeding early However; a genetically determined behavioural change may require many years of intensive bed net use for it to occur (Mathenge *et al.*, 2001). Biting was continuous but peaked between 22:00 hours and 04:00 hour. This is the time that most people may be protected by sleeping under bed net. However, biting continued till the early hours of the morning (04:00 to 06:00 hours) and hence inhabitants who wake up before dawn (even if they slept under ITNs) or early for trade and farming activities risk getting infective bites with increased chance of getting malaria infections. The results of both indoor and outdoor biting times of the two main *Anopheles* species caught biting in KND potentially could undermine the effectiveness of ITNs/LLIN. Community members should therefore be encouraged to use topical repellents early in the evenings as protection against early biting mosquitoes. (Zimmerman and Voorham, 1997). The study found that more vectors were resting indoors after feeding. *An. gambiae* has been shown to be largely endophillic (Highton *et al.*, 1979 ; Mnzava *et al.*, 1995 ; Githeko *et al.*, 1996 ; Faye *et al.*, 1997), although there are also reports of exophilly in some areas (Bockarie *et al.*, 1994 ; Mahande *et al.*, 2007). *Anopheles funestus* is also usually classified as a more endophillic species (Mnzava *et al.*, 1995 ; Githeko *et al.*, 1996) but is also known to exhibit some exophillic behaviour (Fontenille *et al.*, 1990). This finding suggests that addition of control strategy that targets indoor resting mosquitoes such as IRS will be effective and can therefore be used to compliment ITNs which target indoor biting mosquitoes to achieve a greater effect in controlling malaria in the area.

The *P. falciparum* infectivity results showed that *An. gambiae s.s.* is the main vector of malaria in the study area with *An. funestus* serving as a secondary vector. This finding is similar to earlier studies in the KND (Appawu *et al.*, 2004) and other parts of Ghana

(Appawu *et al.*, 2001 ; Okoye *et al.*, 2005; Dery *et al.*, 2010). However, the sporozoite infectivity of 2% estimated for *An. funestus* in this study was too high possibly because of the low numbers tested. This is at variance with a report in some parts of Ghana where as *An. funestus* did not carry any sporozoites (Tchouassi *et al.*, 2012). *Anopheles gambiae* mosquitoes caught as early as 20:00 hours were infective and also recorded two peaks of intense infectivity and this has implications on malaria transmission. The early infective biting indicates that community members have a high risk of infection since most of them would not be in bed even if they intend to sleep under bed-nets later in the night. A higher proportion of *An. funestus* caught biting at dawn were infective. However, the combined infective biting by both Species at dawn makes the intensity of transmission high. This together with the reported continuous biting till the early hours of the morning may put people who wake up early at risk as infective biting still take place at 05:00 hours. Children whose mothers raise their bed-nets on waking up would also be exposed to infective bites.

Sporozoite rates for both *An. gambiae* and *An. funestus* species were lower in the irrigated than the non-irrigated zone This appears to be a trend in irrigated areas across Africa, where sporozoite infectivity in vectors are usually lower. This could be due to the large numbers of nulliparous individuals, from the breeding sites provided by the irrigation all year round (Lemasson *et al.,* 1997 ; Ijumba and Lindsay, 2001 ; Fontenille *et al.,* 1997 ; Robert *et al.,* 1998).

Given that the very high temperatures in the dry season which compel inhabitants of KND to sleep outdoors, it may be necessary to introduce new control tools such as repellents, since sporozoites were detected in outdoor biting *An. gambiae* s.s. and *An. funestus* in the dry season.

Anopheles gambiae is the main malaria vector driving the levels of malaria transmission in KND as it recorded high EIRs in both ecological zones during the wet and dry seasons whilst *An. funestus* served as a secondary vector with relatively low estimated EIRs. The comparatively low EIR for *An. funestus* is a reflection of the low densities of the species in KND. The magnitude of the EIR is affected by the rate at which vectors feed on humans which is largely dependent on the mosquito density and to some extent the feeding habits of the vector species. It should however be noted that even the low levels of transmission by *An. funestus* may still contribute significantly in levels of malaria in a population (Mbogo *et al.*, 1995). Studies in sub-Saharan Africa, have reported that villages only a few kilometres apart such as in this study can have differing EIRs and that such differences can profoundly affect the epidemiolog,y of malaria in these areas including the prevalence of infection, and symptomatic presentation of clinical disease (Breman, 2001). Malaria transmission was highly seasonal and overall estimated transmission intensity showed each resident in the districts will be exposed to 0.38 infective bites/man/ night, which implies an individual, is likely to get approximately one infective bite every three nights if unprotected. Compared to the estimates that were reported a decade ago by Appawu *et al*. (2004), the intensity of transmission in KND reduced by 66.7% from 418 to 139 ib/m/y. This reduction could be attributed to the fact that the past two decades has seen some changes in malaria control. For instance, first-line treatment has changed from chloroquine through to artemisinin-based combination therapies (ACTs) as a result of drug resistance

(Oduro *et al.*, 2010). In addition increased funding has enhanced high coverage of LLINs. This study further confirms previous reports of a general reduction in malaria transmission in other African countries, (O"Meara *et al.*, 2010 ; Ceesay *et al.*, 2010 ; Drakeley and Lines, 2014). This similarity further confirms the reductions in reported clinical malaria cases observed over time following the introduction of ITNs/LLINs in the study area (Koram *et al.*, 2003). These rates are however higher than in neighbouring Benin where inoculation

rates ranged from 9.62 to 21.65 infected bites of *An. gambiae* per person per year (Sovi *et al.,* 2013). Indeed, the EIR in this study is closer to those recorded in the middle belt of Ghana where 231 infective bites/ man/ year and 269 infective bites/ man/ year were recorded for two conservative years (Dery *et al.,* 2010). Although there has been a reduction of EIR with time, transmission is still intense and hence the need for additional vector control tools as LLINs are not enough to eradicate malaria in KND. Additionally, a high level of human adherence to using these tools may be required.

A survey of annual *P. falciparum* EIRs across Africa revealed malaria transmission intensity to be highly variable with ranges from < 1 to > 1,000 infective bites per person per year (Beier *et al.,* 1999 ; Fontenille and Simard, 2004), and generally affected by local land-use (Hay *et al.,* 2000). Several studies have consequently compared transmission among different ecological zones and even among villages separated only by short distances within the same area (Dolo *et al.,* 2004 ; Okello *et al.,* 2006). This study found marked temporal and spatial variations in malaria transmission intensity, with the irrigation zone experiencing the highest EIR throughout the year. This could be explained by the environmental differences in the two zones, and more so the availability of favourable breeding sites in the irrigated areas and hence the large population of *An. colluzzi*. Irrigation has been shown to affect EIRs depending on the area. It has been shown to increase in some areas (Dolo *et al.,* 2004 ; Koudou *et al.,* 2005 ; Appawu *et al.,* 2004) and have little or decreased overall influence (Robert *et al.,* 1985 ; Githeko *et al.,* 1993 ; Dossou-yovo *et al.,* 1994). The noticeable variation in EIRs within the two ecological zones in KND may explain the different malaria epidemiological conditions that may exist within a small area (Shililu *et al.,* 1998). Findings of this study together with the other studies mentioned above support the evidence that malaria transmission risk varies even at micro-ecological levels. Since each ecological zone presents different transmission intensities as shown by the

present study, an understanding of local vectors, their bionomics and factors that affect transmission intensity are vital for successful malaria control. Seasonal transmission was observed in the two micro-ecological zones, with the greatest EIRs recorded at the end of the long rains in October which recorded an EIR of 207.76 ib/m/y. Transmission was relatively higher in the wet season than in the dry season. In other parts of Africa, irrigation has been confirmed to change the transmission pattern from seasonal to perennial especially during the dry season presumably because mosquito numbers are not directly linked with rainy season (Sissoko *et al.,* 2004; Dolo *et al.,* 2004 ; Ijumba *et al.,* 2002). In non-irrigated semi-arid areas malaria transmission usually falls below detection level during the dry season and rather increases in the wet season (Dolo *et al.,* 2004). This study showed a similar pattern with a marked effect of the rainy season in the non-irrigated zone. This increase may be as a result of an increase in mosquito breeding sites (Oesterholt *et al.,* 2006).

Insecticide susceptibility bioassay results from this study showed high resistance levels of *An. gambiae* s.s. to all the insecticide classes including pyrethroids and most significantly to DDT. These finding is at variance with a study by Anto and others (Anto *et al.,* 2009) which reported susceptibility of *An. gambiae* to the pyrethroids used for ITN treatment in KND six years before the current study. This indicates that resistance had developed in the area over the years.

Using the standard WHO criteria for characterizing insecticide resistance, this study found evidence for phenotypic resistance in *An. gambiae* in all the selected sites for the insecticides tested. However, relatively high mortalities of *An. gambiae* s.s. from all the sites were detected when exposed to Propoxur. This indicates that in the presence of pyrethroid resistance, this insecticide could be an alternative insecticide. The high occurrence of insecticide resistance in the *An. gambiae* s.s. population of KND may be attributed to the frequent use of insecticides for ITNs and LLNs for personal protection and to some extent,

Agriculture. The use of insecticides for Agriculture has been proposed to be one of the major causes of insecticide resistance in malaria vectors (Chouaïbou *et al.,* 2008 ; Diabate *et al.,* 2002 ; Akogbéto *et al.,* 2006). There is evidence that insecticide selection pressures as a result of considerable use of LLINs can lead to the development of insecticide resistance (Trape *et al.,* 2011 ; Corbel *et al.,* 2012). The long use of ITNs (over two decades) and widespread distribution of LLINs to pregnant women and children under five, as part of the implementation of the universal coverage with LLINs by the NMCP (Ghana Health Service, 2011) might have increased insecticide selection pressure on mosquitoes in the area (Ndo *et al.,* 2011). Additionally, irrigation in KND which has undoubtedly led to increased rice and tomato and other vegetable farming, is often linked to intensive and uncontrolled insecticide use and this can be presumed to lead a marked effect on the ecology and resistance levels in malaria vectors in the districts.

This study found that resistant kdr L1014F and Ace-1R 119S alleles occurred within the same mosquito , suggesting the presence of multiple resistance mutations in *An. gambiae* s.s. individuals similar to what has been reported in neighbouring Burkina Faso (Dabire *et al.,* 2012 ; Namountougou *et al.,* 2013).

The L1014F allele responsible for kdr mutation which results in pyrethroid and DDT resistance is the commonest in West Africa. Yawson *et al*, (2004) reported the occurrence of the gene in population of *An. gambiae* from the same area, although in much lower frequency. The first description of the presence of L1014F in *An. coluzzi* was from Benin in 1998 (Akogbéto and Yakoubou, 1999), where it was reported to have introgressed from sympatric S-form populations (Weill *et al.,* 2000 ; Weetman *et al.,* 2010) and is said to have increased in frequency (Corbel *et al.,* 2007 ; Lynd *et al.,* 2010). Subsequently, it has been postulated that this allele is spreading to other parts of West Africa (Weill *et al.,* 2000

; Santolamazza *et al.,* 2008a ; Etang *et al.,* 2009). There was no association between the resistance phenotypes and the L1014F genotypes as shown by Hardy Weinberg equilibrium for the pyrethroids. However, there was a positive association between the Ace-1R genotype and the resistant phenotype after exposure to bendiocarb. The lack of association for the pyrethroids, however, has been reported by Matambo et al.(2007) in a laboratory colony of *An. arabiensis* from Sennar, and also in other mosquito species such as *Culex quinquefasciatus* and *Culex pipiens* (Liu *et al.,* 2006). The lack of association between the *kdr* resistant alleles and the phenotype may suggest the involvement of other resistant mechanisms such as metabolic resistance (Coleman and Hemingway, 2007). Cytochrome P450 genes have been reported to be overexpressed in some pyrethroid resistance associated with *An. gambiae* s. s., sometimes in association with the L1014F kdr allele (Corbel *et al.,* 2007 ; Djouaka *et al.,* 2008 ; Mitchell *et al.,* 2012 ; Müller *et al.,* 2008).

The study also looked at the frequency of the L1014F kdr mutation in *An. gambiae* sampled when biting humans in KND and found high frequencies of the gene among the *An. gambiae* s.s. populations; as compared to the very low frequencies of this L1014F allele found in *An. gambiae* resting in dwellings in two communities (0.58% and 0.00% for Bonia and Korania respectively) a decade ago (Yawson *et al.,* 2004). Dramatic increase in the L1014F allele frequency in these sites was found in the current study.

However, direct comparisons with their populations are confounded by factors such as the method of collection and the fact that larvae collected in the previous study might not be from areas directly exposed to insecticides. The high frequencies of *kdr* gene estimated in this study are similar to those reported in other parts of Africa (Czeher *et al.,* 2008 ; Dabire *et al.,* 2012 Reddy *et al.,* 2013). Other studies have however found relatively low frequencies in some areas (Fanello *et al.,* 2003 ; Awolola *et al.,* 2003). The presence of high frequencies of L1014F alleles in *An. gambiae* s.s populations from the KND may have implications for

the effectiveness of the current vector control strategy which is based on pyrethroid insecticides. However, it is essential to note that high frequencies of *kdr* genotype may not necessarily mean reduced impact of pyrethroid-based vector control as evidenced by studies in Burundi, where pyrethroids based vector control method significantly reduced malaria transmission notwithstanding the high frequency of L1014S (Protopopoff *et al.*, 2008), and also in Cote d"Ivoire, where pyrethroids treated bed nets provided a protective effect even when the L1014F allele frequency was > 80% in *An. gambiae s.s.* (Martinez-Torres *et al.*, 1998).

The Ace-1R G119S mutation, which confers resistance to carbamates and organophosphate insecticides, has been reported in *An. gambiae* populations in Africa (Djogbénou *et al.*, 2008 ; Dabiré *et al.*, 2009 ; Ahoua Alou *et al.*, 2010). In this study the Ace-1R G119S mutation was found to be very strongly associated with phenotypic resistance to bendiocarb. The allele was also found in high frequency among population of biting mosquitoes. This is similar to a study in southern Ghana (Essandoh *et al.*, 2013) that found the gene to occur in individual mosquitoes that were already carrying the resistant kdr L1014F allele. The presence of both resistance mechanisms (*kdr* and *Ace-1R*) in the major malaria vector in KND may enable them to be simultaneously resistant to pyrethroids, carbamates, and organophosphates (Chouaïbou *et al.*, 2008 ; Corbel *et al.*, 2007). This has the potential to greatly undermine malaria control efforts in the district, especially when the NMCP is rolling out IRS. There will therefore be the need for further studies to ascertain the phenotypic effects, especially when the two mutations occur concurrently, taking note of the presence of any metabolic-based resistance. Additionally, the operational impact of these mutations on the efficacy of pyrethroids, organophosphate and carbamate based vector control strategies need to be investigated.

The N1575Y mutation which was originally detected in the *VGSC* strain of *An. gambiae* s.s. S form from Burkina Faso was genotyped in samples from HLC. This gene functions as an enhancer of the L1014F/S-mediated pyrethroid and DDT resistance in *An. gambiae* s.s. (Jones *et al.*, 2012). Frequencies ranging from 18.00%-42.42% were recorded and mostly found occurring with the *kdr* L1014 mutation. Two communities in the irrigated area, Bonia and Korania recorded the highest frequencies (42.42% and 39.06% respectively). Detection of this mutation in *An. gambiae* populations in KND serves as a baseline and a perfect opportunity to continuously monitor its spread in the field and if possible, forecast the extent and assess the selection coefficients through modelling studies (Barbosa *et al.*, 2011).

Attempt was made in this study to determine whether insecticide resistant *An. gambiae* s.s from KND collected using HLC were more likely to be carrying the malaria parasite and ultimately whether the high frequencies of the Kdr L1014F mutations observed had any effect on malaria transmission in the districts. The results indicated that *An. gambiae* s.s mosquitoes with the resistant allele recorded lower sporozoite infectivity compared to their susceptible counterparts suggesting that the resistant *An. gambiae* s.s. in KND may be transmitting less malaria than their susceptible counterparts. Presumably, the high frequency of *kdr* gene reported in the districts may not affect the effectiveness of vector control interventions such as ITNs and LLNs. Notwithstanding the high level of pyrethroids resistance observed in the area, EIR had declined from 418 infective bites/man/year in 2004 to 139 bites/man/year. The findings in this study is similar to observations in Bioko, the main island of Equatorial Guinea (Hemingway *et al.*, 2013) where it was also reported that pyrethroid resistant *kdr* homozygotes in M (*An. coluzzii*) and S forms of *An. gambiae* had lower sporozoite positivity compared to their susceptible counterparts and was concluded that *kdr* status alone did not operationally reduce the effectiveness of pyrethroid IRS. Also in a multi-village randomized control trial in Cote d"Ivoire, (Henry *et al.*, 2005) found that

pyrethroid-treated LLIN efficacy was not negatively affected by the presence of *kdr*, although other studies have reported otherwise. For instance a study by Verhaeghen *et al.* (2010), found that 70% *P. falciparum* infective mosquitoes responsible for malaria transmission in the dry season carried the L1014S *kdr* allele. whilst another study in Senegal suggested kdr had an effect on increased malaria transmission (Trape *et al.*, 2011). However the presence of other insecticide resistance mechanisms in addition to the *kdr* mutation can affect the malaria control efforts in KND.

The findings of the current study have shown the increasing spate of insecticide resistance in KND. In this area, there is little, if any follow-up monitoring for insecticide resistance organized by the agencies responsible for the deployment of ITNs and LLNs. This study also provides information on the frequency and distribution of three target-site resistance mutations (Kdr L1014F, Ace-1RR 119S and N1575Y) in *An. gambiae* populations in KND. This may constitute a significant risk to malaria control and if new strategies are not taken in time, resistance may compromise the malaria control effort as has been reported in other places (N‟Guessan *et al.*, 2007 ; Asidi *et al.*, 2012).

The dispersal of malaria vectors and variations in the characteristic malaria risk is not homogeneous in any given area and therefore requires much effort towards determining local spatial spread of the disease (Hay *et al.*, 1996) for the effective deployment of control strategies. GIS has potential applications in this respect. This study mapped the spatial distribution of *An. gambiae* s.s and *An. funestus* (the main vectors of malaria) captured from KND using HLC and also depicted the insecticide resistance status of *An. gambiae* s.s. to the different classes of insecticides commonly used for vector control by using GIS applications. These maps could provide baseline information for malaria programme managers to effectively direct interventions against the vectors. This can eventually be

extended to the nearby districts where malaria is also endemic to improve the tracking of entomological indicators such as species composition and insecticide resistance status, and ultimately the mapping of high-risk areas of malaria transmission and extent of insecticide resistance of vectors to commonly used insecticides for control. The use of GIS applications in such a manner will facilitate decision making and realistic utilization of the scarce resources in a cost-effective manner.

5.2 Conclusions

This study has shown that there are variations in abundance and spatial dynamics of the malaria vectors (*An. gambiae* s.s. and *An. funestus*), responsible for maintaining the status quo of malaria in the study site during the study period. This study confirmed that *An. gambiae* s.s is the only member of *An. gambiae* complex present and *An. coluzzii* being the only molecular form present in KND. The plasticity noticed in biting patterns, especially the almost equal outdoor and indoor biting behaviour of the vectors has important consequences for the success of the widely used ITNs and LLINs as the main malaria control strategy in KND. and therefore the importance of close monitoring of the local malaria epidemiology as it relates to vector behaviour cannot be overemphasized.

There will also be the need to add on control strategies that target outdoor biting vectors.

The EIR or the level of exposure of inhabitants of KND to *P. falciparum* sporozoite infective inoculations was high with periods of intense EIRs coinciding with periods of increased vector density (biting rates). Malaria transmission in KND is highly seasonal with the peak during the wet season and is maintained by *An. gambiae* with *An. funestus* as a secondary vector. Overall transmission has however reduced greatly when compared to the EIR reported over a decade ago by Appawu *et al*. (2004). *Anopheles gambiae* has two peaks of intense infectivity and this coupled with the combined infective bites of both species from 02:00 hours to 05:00 hours increases the risk of transmission for inhabitants who are not

protected. EIR in both dry and wet seasons is comparatively higher in areas where irrigated farming takes place suggesting that the irrigated areas are more favourable for the breeding and activity of malaria vector mosquitoes. The findings of this study support the evidence that malaria transmission risk varies between regions and even between neighbouring communities because of micro-ecological differences. This further stresses the fact that assessing malaria transmission risk within micro- and agro-ecological zones provides a detailed picture of the spatial distribution to enhance specific area control strategies.

The insecticide resistance data presented in the study showed that resistance to the different classes of insecticides especially to the pyrethroids used for the ITNs and LLINs treatment as measured by conventional bioassays is clearly widespread in *An gambiae*, the main malaria vector in KND. This highlights the threat to the effectiveness of the current vector control strategies. The results indicated resistance to be conferred by target-site insensitivity such as *kdr* and *ace-1R*, with a possibility of other metabolic mechanisms or a combination of all in the same populations of *An. gambiae* s.s. It can therefore be concluded that future vector control efforts in the districts should include monitoring of metabolic resistance by characterizing the biochemical interactions between insecticides and resistant target sites. The high frequencies of L1015F and Ace-1R alleles (responsible for pyrethroids/DDT and carbamate/organophosphate resistance respectively) and N1575Y allele (known to enhance pyrethroids resistance) in *An. gambiae* samples leads to a conclusion that there is increased selection pressure on malaria vectors, caused at least in part by the scale up of ITN coverage and probably insecticide use for agriculture. (The role of agriculture in the selection of resistance in natural mosquito‟s populations however needs to be clarified, both in terms of insecticides usage and quantity as it was not done during the current study). *Anopheles gambiae* populations in KND may be primed for further increases in pyrethroids resistance to a point where ITNs/LLNs begin to lose their effectiveness. In this study the presence of

the kdr mutation did not correlate with high sporozoite infectivity and hence it can be concluded that the high frequency of this mechanism may not have affected malaria transmission. However this study demonstrates that the *An. gambiae* population in KND may contain a variety of resistant mechanisms and this should be worrying. The presence of multiple resistance mechanisms may constitute an obstacle for the future success of malaria control programmes based on ITNs or IRS with pyrethroids or organophosphates/ carbamates in the area. This highlights the urgent need to continue monitoring insecticide resistance in malaria vectors, given the importance of the vector control against the disease.

The results of this study are of great importance in the assessment of the dynamics of malaria transmission and insecticide resistance in KND following over two decades of ITN use by the population. It provides information for evidence-based planning and implementation of malaria control activities targeting vectors.

5.3 Recommendations

1. Surveillance on seasonal population dynamics of malaria vectors in the different ecological zones in KND to ascertain mosquito abundance is recommended.
2. There is evidence that *An. colluzzi* (previously referred to as M-form) may more often be found in semi permanent and man-made breeding sites. The results of this study found only this form in KND. It is therefore recommendered that consideration should be given to suitable environmental modifications to reduce mosquito breeding in the types of water bodies where *An. colluzzi* predominates, larviciding of breeding habitats (to reduce larval density) or environmental management coupled with the proper maintenance of *irrigation* channels, with regular clearing of vegetation on the margins, and regular repair of any cracks or damages on the walls would form a useful adjunct to ITNs.

3. There is need to explore methods of managing and controlling outdoor biting vectors with the use of repellents.
4. There should be continuous evaluation of the effectiveness of the existing malaria control interventions through field and laboratory monitoring of insecticide resistance in order to provide evidence and develop appropriate resistance management strategies.

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APPENDICES

Appendix 1. Hardy-Weinberg equilibrium

A mathematical description of the fact that the relative frequencies of 2 or more alleles in a population do not change because of mendelian segregation; alleles and genotype frequencies remain constant in a random-mating population in the absence of inbreeding, selection or other evolutionary forces (i.e. if there is no mutation, migration or selection). In a large population in which there is random mating and in the absence of forces that change the proportions of the alleles at a given locus, the original proportions of the genotypes will remain constant from generation to generation. The genotypes are said to be in HW equilibrium. For example, for a gene with alternative alleles A and a, if frequency of (the more common) allele A is p and frequency of less common allele a is q, then genotype frequencies after one generation of random mating will always be $(p+q)^2 = P^2$ (A) +2pq (A & a) $+q^2$ (a). Factors that can affect or alter allele frequencies to produce significant deviation from the proportions predicted by HW principle are: mutation, migration, (immigration-emigration), genetic drift (random loss of alleles, more likely in small populations), non-random mating and selection (can produce adaptive evolutionary change because it depends on nature of the environment). Non random mating: - inbreeding does not change frequency of alleles but rather increases the proportion of individuals that are homozygous (i.e. promotes occurrence of double recessive combinations).

Appendix 2 Solutions and buffers for ELISA

Phosphate Buffered Saline (PBS), pH 7.2: 1 bottle of Dulbecco"s PBS was added to 1 Litre distilled water (dH_2O), mixed and adjusted to pH 7.2 and stored at 4^oC

Blocking Buffer (BB): Contains 0.5% Casein, 0.1N NaOH and PBS, pH 7.4 To

900ml of PBS was added 1 litre of 5g Casein and 100ml of NaOH.

Grinding Buffer-Blocking Buffer with a detergent, Nonidet-40 (BB: NP-40)

To 1ml BB was added 5□l NP-40 and mixed well to dissolve the NP-40 in the BB and stored at 4^{o}C

Wash Solution (PBS-Tween): PBS plus 0.05% Tween-20. 0.5ml of Tween-20 was added to 20 to 1 litre of PBS mixed well and stored at 4^{o}C

Substrate: 2,2‟-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) at a concentration of 0.3g/L in a glycine citric buffer. The concentration of the H_2O_2 is 0.01%.

Appendix 3. Solutions and buffers for PCR

The following standard solutions were prepares using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121ib/sq in for 15 minutes in an Eyela Autoclave (Rikikkaki, Tokyo).

Solutions for DNA extraction

TE (pH 8.0) 10mM Tris-HCl (pH), 1mM EDTA (pH8.0). Stored at room temperature. Ethidium Bromide (10mg/ml) -1g of Ethidium Bromide was completely dissolved in 100ml ddw and stored in the dark at room temperature.

Solutions for electrophoresis

Agarose gels

10X TAE buffer 242 g Tris base, 57.1 ml glacial acetic acid, 100ml 0.5M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with ddw.

0.5 M EDTA (pH 8.0) 186g of EDTA, dissolved in 800ml ddw, pH adjusted with NaOH pellets, the volume made to 1000 ml with ddw and stored at room temperature.

Gel loading buffers

5X orange G 20% (w/v) Ficoll, 25mM EDTA, 2.5% (w/v) orange G. Stored at 4°C. 100 bp molecular weight marker. The first band is 100 bp, the subsequent ones measure 200,300….1000 bp.

Appendix 4. 1014F TaqMan Assay Method (kdr West)

Equipment

Pipette (1000 µl, 20 µl, 10 µl)

Pipette tips (1000µl, 20µl, 10µl)

Optical 96 well plate or optical tubes

Optical caps

Tube centrifuge

Plate centrifuge

Desktop mixer/vortexer

Wipes and Ethanol (or other suitable cleaning agent)

Procedure

1. Put on the correct PPE i.e. gloves and a lab coat.
2. Clear a sufficient work area and wipe work bench down with 90% Ethanol (9parts Ethanol to 1 part distilled water or sterile water) or a similar cleaning agent.
3. Obtain the reagents from -20°C:

   Sensimix

Primer/Probe*

IMPORTANT - *Obtain the correct primer/probe for 1014F assays

4. Allow reagents to thaw for approximately 5 minutes. Protect the primer/probe from light as much as possible by covering it while it thaws.
5. The following equations are to work out the volume of each reagent needed in the master mix:

Volume of **Sensimix for 1 well/tube = 5 µl**

5 X the number of wells/tubes to run (+10%*) = volume of Sensimix to add

Volume of **Primer/Probe for 1 well/tube = 0.125 µl**

0.125 X the number of wells/tubes to run (+10%*) = volume of Primer/probe to add

Volume **of Water for 1 well/tube = 3.875 µl**

3.875 X the number of wells/tubes to run (+10%*) = volume of water to add

IMPORTANT – *Add an additional 10% worth of reactions e.g. 105 reactions worth of master mix for 96 reactions should be made to account for loss of liquid in the pipette tips and tubes. A blank control using just the master mix should always be run to control for contamination. It is best practice to also run positive controls.

6. Prepare the mastermix in a 1.5 ml eppendorf tube. If running entire plates of 96 wells, use the volumes shown in Table 1. Otherwise, adjust accordingly.. **Table 1 – Volumes to add**

Reagents	Volume (x105)
Sensimix	525.00 µl
Primer/Probe	13.125 µl
Autoclaved water	406.875 µl
	Total = 945.00 µl

Calculation check – <u>Total volume (µl)</u> = 9 (µl)

No. Of wells

7. Mix the contents of the tube for approximately 5 seconds using e.g. a desktop mixer/vortexer and centrifuge tube for approximately 10 seconds at 2000 rpm.
8. Add 9ul of this master mix to the correct number of wells of an optical 96 well plate or optical tubes.
9. Add 10 µl of the master mix to one of the last wells and this will be one of the blank controls.. NTC‟s could also be added which is 9 µl master mix and 1 µl water.
10. Add 1ul of the DNA template (or positive control DNA) to the appropriate well and record details of sample numbers in a similar template below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTE – Slowly draw the liquid back up into the pipette tip to rinse the tip and dispense as much DNA as possible.

11. Seal the wells of the plate/tubes with optical caps.
IMPORTANT - Ensure a tight fit of the optical cap or they may come off because of the high temperature the instrument reaches and the samples may evaporate.

12. Centrifuge the plate at 2000 rpm for 15 seconds to collect the samples at the bottom of the well.
13. Now the plate/tubes is/are ready to be run on suitable instrumentation.

Running the plate

Run the plate on suitable instrumentation under the standard programme or specify the settings favourable for optimum results. The following settings are for an Agilent qPCR instrument:

1. Turn on the computer linked up to the instrument.
2. Turn on the instrument.
3. Start up the software MXPro – MX3005P.
4. Select „New“ in the toolbar menu.
5. Select Allele Discrimination – SNP“s (or suitable programme)
6. Highlight turn on lamp for warm up as this will take approximately 20 minutes.

On plate set up section:

7. Highlight specific wells or click „ALL“ for whole plate analysis.
8. Go to „well type“ and in the dropdown menu select unknown.
9. Go to „Collect fluorescence data“ and highlight FAM and VIC (could also be called HEX or IPC)

On thermal profile set up:

10. Go to „Thermal profile design“ tab and highlight standard
11. Go to „Pre-melt/RT segments“ tab and click „1 plateau“
12. Go to „Amplification segments“ tab and click „2 fast step“. The 2 fast step settings should be displayed.

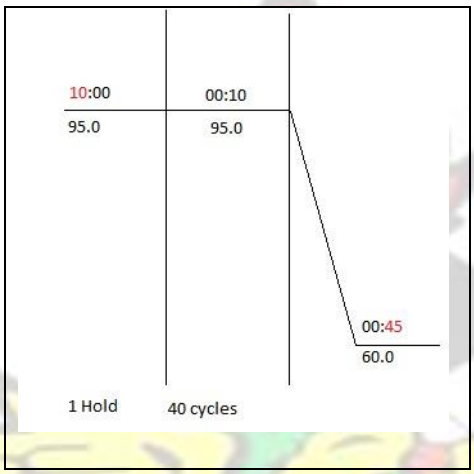
13. Run the plate at the following temperature settings:

    1 Hold: 95°C for 10 minutes (change from the standard 15:00 minutes)

    40 cycles of: 95°C for 10 seconds

    60°C for 45 seconds (change from the standard 30 seconds)

    Should look similar to below:



NOTE - the time changes are highlighted in red.

14. Save the plate template to a specific drive naming it with test name, sample names and the date for easy recall. For example:

    KdrWest_A1-H12_DATE

15. Lift the outer door and then the inner lid of the instrument by pulling it outwards then upwards and place the plate/tubes in the correct orientation.

    IMPORTANT - well A1 should be in the top left hand corner.

16. Make sure the plate is secure and correct before closing the lid.

17. Click the START button or RUN button

18. An „estimated time of completion“ should come up to ensure that the instrument, computer and template are all successfully linked and ready to run.
19. For this programme the run time should be approximately 1:18:
20. Complete use log book

Interpretation of results

1. Once the run is completed, click on the results tab and go to the „area to analyse“. Highlight each section to see the results displayed differently or highlight „consolidate report“ to see all.
2. Figure 1 is an example of a scatter plot that shows samples that express the wild type or susceptible allele (Leucine).

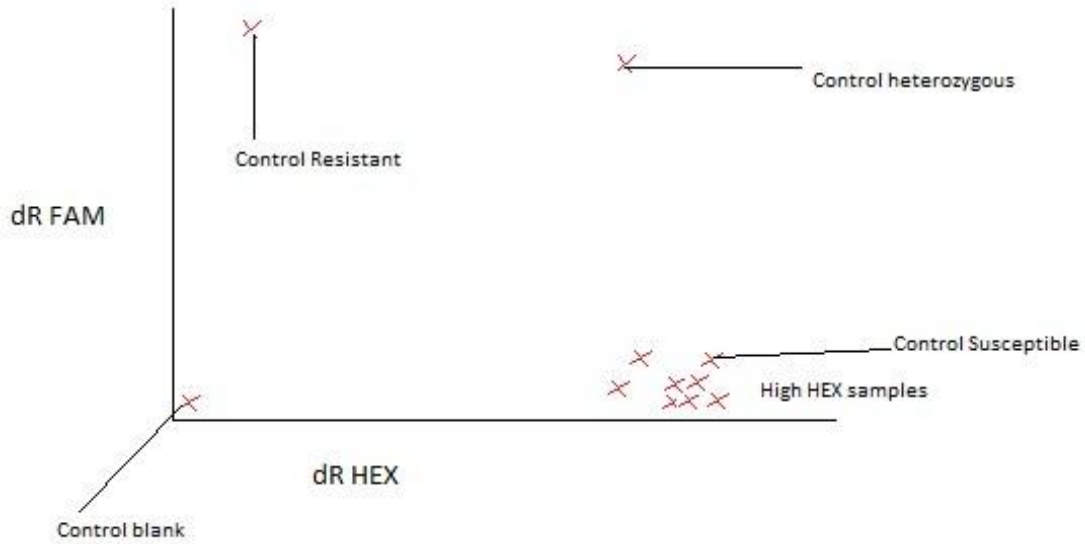


Figure 1. Example of a graph showing samples that express the wild type or susceptible allele.

3. Figure 2 is an example of a scatter plot that shows samples that are homozygous for the1014F allele.

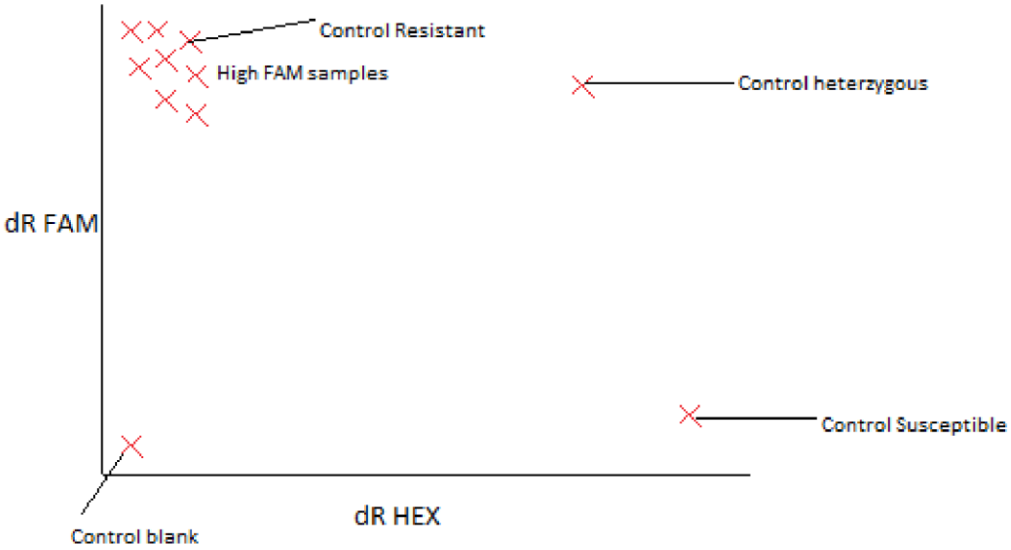


Figure 2. Example of a graph showing samples that express the mutant or resistant allele.

4. Figure 3 is an example of a scatter plot that shows samples that contain both the 1014F kdr allele and the susceptible allele

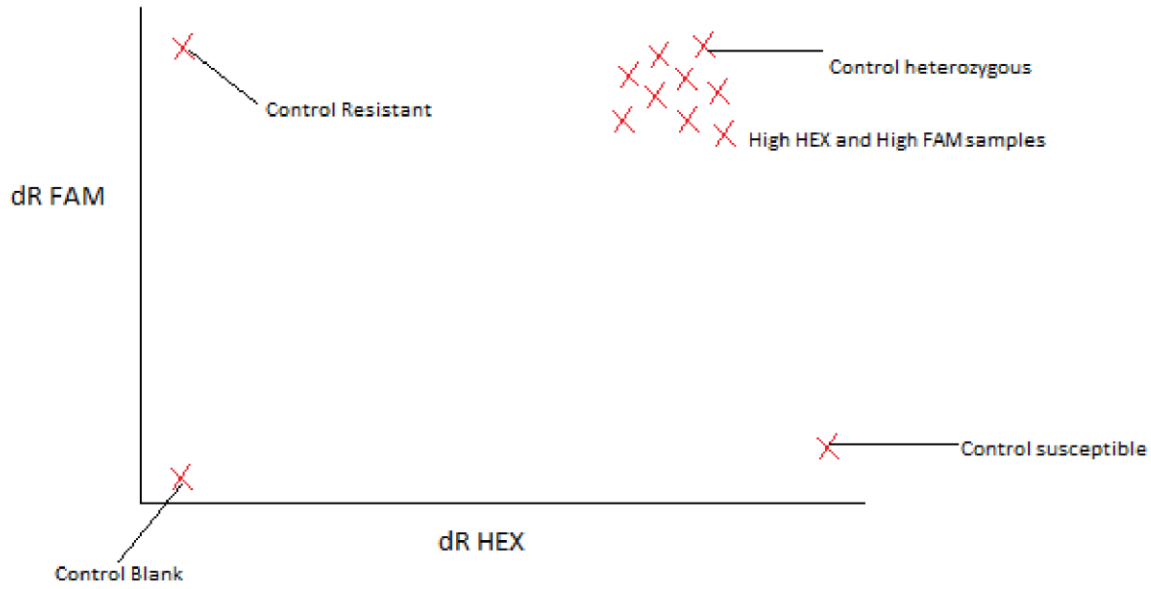


Figure 3. Example of a graph showing samples that are heterozygous or expressing both the mutant and the susceptible allele.

5. Record the results and date of the test in a lab book to keep an accurate record.