CHARACTERIZATION OF MALARIA TRANSMISSION AND INSECTICIDE SUSCEPTIBILITY STATUS OF *ANOPHELES GAMBIAE SENSU LATO* GILLES (DIPTERA: CULICIDAE) IN SHAI-OSUDOKU DISTRICT OF SOUTHERN GHANA

BY

ONONYE, NNENNA CHINYELU (B.Sc. HONS)

(10435401)

A THESIS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY (M. PHIL) DEGREE IN ENTOMOLOGY

AFRICAN REGIONAL POSTGRADUATE PROGRAMME IN INSECT SCIENCE (ARPPIS)*, UNIVERSITY OF GHANA, LEGON

JULY, 2015

***JOINT INTER-FACULTY INTERNATIONAL PROGRAMME FOR THE TRAINING OF ENTOMOLOGISTS IN WEST AFRICA.**

COLLABORATING DEPARTMENTS: ANIMAL BIOLOGY AND CONSERVATION SCIENCE AND CROP SCIENCE

DECLARATION

I hereby certify that this thesis is the result of a research undertaken by me, Ononye Nnenna Chinyelu, towards the award of Master of Philosophy in Entomology in African Regional Postgraduate Programme in Insect Science (ARPPIS), University of Ghana, Legon. This thesis has not been submitted, either in part or in full, for any other degree and all references to other people's work have been duly acknowledged.

……………………………………………………..
ONONYE, NNENNA CHINYELU
10435401
(STUDENT)

……………………………………………………..
Dr. SAMUEL KWEKU DADZIE
(SUPERVISOR)

……………………………………………………..
Dr. FRED ABOAGYE-ANTWI
(SUPERVISOR)

……………………………………………………..
Dr. ROSINA KYEREMATEN
(ARPPIS COORDINATOR)

DEDICATION

This work is dedicated to God Almighty, to my parents: Sir Chudi and Lady Caroline Ononye, and my siblings whose love and support have kept me strong throughout this study.

ACKNOWLEDGEMENTS

My profound gratitude goes to God Almighty for His wonderful gift of life and of good health to finish this research work successfully and also for His great mercies upon my life.

I am very grateful to my supervisors: Dr. Samuel K. Dadzie, of the Parasitology Department, Noguchi Memorial Institute for Medical Research, University of Ghana and Dr. Fred Aboagye-Antwi, of the Department of Animal Biology and Conservation Science, University of Ghana for their guidance, advice and support throughout the course of this study. I am also grateful to Prof. Maxwell A. Appawu, of the Noguchi Memorial Institute for Medical Research (NMIMR), for all the advice and contributions towards the progress of this study.

Special thanks to all ARRPIS lecturers, whose training and knowledge imparted on me has helped me towards the actualization of this work.

I gratefully acknowledge the support and contributions made by the staff and students of the Parasitology Department (NMIMR), particularly Mr. Kojo Sakyi Yirenkyi, Mr. Osei Kwaku Akuoko, Ms. Sellase Pi-Bansa, Ms. Rebecca Pwalia, Mr. Andy Asafu-Ajaye for their help in the field sample collections and identification; also to Mr. Kwadwo Kyeremeh Frimpong, Ms. Michelle Adimazoya, Mrs. A. Frimpong and Ms. Isabella Djameh for all the assistance during the molecular studies. Many thanks go to Prof. Kwadwo Koram, Director of the NMIMR, for the opportunity offered me to use the facilities at the Institute.

I am also thankful to colleagues and friends, with special mention of Peter Okeke, Adachukwu Obagha, Emmanuel Ottih, Yaw Assan, Lami Jinatu, Ruth Bala, Nimlin Dickson, Shaphan Yong and Franklin Ayisi, who in diverse ways helped to bring about the success of this work.

Finally to the study communities and field collectors, whose cooperation made this work possible, I say a big thank you.

This research was funded by *Deutscher Akademisher Austausch Dienst e.v.* (DAAD) in Germany and a grant from Dr. Samuel K. Dadzie.

TABLE OF CONTENTS

LISTS OF ILLUSTRATIONS

LISTS OF TABLES

LISTS OF APPENDICES

LISTS OF ABBREVIATIONS

°C	Degree Celsius
µ	Micro
µg	Microgram
µl	Microlitre
µM	Micromolar
BB	Blocking Buffer
Bp	Base pair
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DDT	Dichlorodiphenyltrichloroethane
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphates
DPBS	Dulbecco's Phosphate Buffered Saline
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene diamine tetra acetic acid
EIR	Entomological Inoculation Rate
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium Bromide
EtOH	Ethanol
G	Gram
GABA	γ Aminobutyric Acid
GNA	Ghana News Agency
GS	Grinding solution
H_2O	Water
H_2O_2	Hydrogen peroxide
HBR	Human-biting rate
HCL	Hydrochloric acid

HLC	Human landing catches
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
KAc	Potassium acetate
Kb	kilobase
Kdr	Knockdown resistance
KDT	Knockdown Time
L	Litre
LLINs	Long-lasting insecticide treated nets
m	Milli
M	Molar
MAb	Monoclonal antibodies
MBR	Man biting rate
MPA	Mosquito/*Plasmodium* antigen
Mw	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NMCP	National Malaria Control Programme
NP-40	Nonidet P-40
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline -Tween
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
PMI	President’s malaria initiative
PSC	Pyrethrum spray catch
RBM	Roll Back Malaria
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
Rpm	Revolution per minute
s.l.	*sensu lato*

s.s.	*sensu stricto*
SddH20	Sterile double distilled water
SDS	Sodium dodecylsulphate
SR	Sporozoite rate
TAE	Tris acetate EDTA
TE	Tris-EDTA
Tm	Melting temperature
Tris	2-amino-1-hydroxymethyl-1, 3-propanediol
W/v	Weight per volume
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

ABSTRACT

Malaria is a major public health problem in most developing countries. In Ghana, it is hyper-endemic with children under-five years, pregnant women and non-immune visitors being at greatest risk. The National Malaria Control Programme recognizes the use of Insecticide-Treated Nets (ITNs) as one of the multiple prevention tools in the control of the malaria vectors. But due to problems associated with ITNs which include rapid loss of bio-efficacy as a result of repeated washings and low re-treatment rates, long-lasting insecticide-treated nets (LLINs) have been developed. For new LLINs, evaluation in the field is required before they are recommended for use in malaria vector control. A site in the Shai-Osudoku district in southern Ghana has been proposed for testing new LLIN products. In preparation for such field trial, baseline data on indices of malaria transmission such as species composition, man-biting rate, sporozoite rates and entomological inoculation rates (EIR) as well as insecticide susceptibility status and resistance mechanism in the main malaria vector were investigated in some communities in Asutsuare area, a sub-district of Shai-Osudoku district. Mosquito larvae and pupae were collected using dipping method, reared to the adult stage and used for the insecticide susceptibility bioassays. Adult mosquitoes were sampled using human landing catches and pyrethrum spray catches. These were morphologically identified and then to sibling species of the *Anopheles gambiae* complex by the polymerase chain reaction (PCR) assay. Identified *An. gambiae s.s*. were further subjected to PCR assays for the identification of the molecular forms and the detection of the leucine to phenylalanine knockdown resistance (*kdr*) gene mutation. The heads and thoraces of *An. gambiae* samples were tested for the presence of circumsporozoite antigens/proteins of *Plasmodium falciparum* using the enzyme-linked immunosorbent assay (ELISA) method. The susceptibility bioassays were carried out using the standard WHO diagnostic bioassay kits and were performed on non-blood fed female mosquitoes of 2- to 5- day old. A total of 3,410 mosquitoes were collected from the four communities during the study period. 85.9% were Culicines, 12.2% *An. gambiae s.l.,* 1.2% *An. pharoensis,* 0.6% *An. coustani* and 0.2% *An. funestus*. *An. gambiae s.s*. was the only identified member of the *An. gambiae s.l*. and it consisted of both the *An. coluzzii* and S molecular forms, with *An. coluzzii* being predominant (98.9%). No hybrids of the two molecular forms were found. Higher proportion of *An. gambiae* at the study sites (with exception of Volivo/Atrobinya) were observed biting indoors relative to outdoors with peak biting activity in the second, third and fourth quarters of the night. Man-biting rate was higher in Kewum as compared with the rest of the study sites. Sporozoite rate of 0.24 % and an overall annual Entomological Inoculation Rate (EIR) of 4.9 infective bites per man per year (ib/m/y) were estimated in the study sites. The susceptibility test results showed that *An. gambiae* populations in Asutsuare are resistant to all the pyrethroids (deltamethrin, permethrin, lambdacyhalothrin, cyfluthrin and etofenprox) insecticides tested but highly resistant to permethrin. The estimated frequency of knockdown resistance (*kdr*) genotypes F(R) was 77%, which is a good criterion for testing LLINs that are effective against resistant mosquitoes under field conditions.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites of which six species of the parasite namely *Plasmodium falciparum* Welch, *P. vivax* Grassi and Feletti, *P. malariae* Laveran, *P. ovale* Stephen, *P. knowlesi* and *P. cynomolgi* are known to cause malaria in humans (Ta *et al*., 2014; Mueller *et al.*, 2007; Singh *et al.*, 2004). In Ghana, only three of the species- *P. falciparum*, *P. malariae and P. ovale* have been reported with *P. falciparum* being the main species responsible for up to 90 to 98% of the malaria cases (Asenso-Okyere and Dzatow, 1997). The parasite, with the exception of a few cases of transplacental and blood transfusion associated transmissions (Murphy and Breman, 2001), is exclusively transmitted to the human host through the bite of an infected female *Anopheles* mosquito biting mainly between dusk and dawn.

Although, there are about 400 described Anopheline mosquitoes, only 70 have been implicated as malaria vectors (Service, 1993). Members of the *Anopheles gambiae* complex and the *An. funestus* complex are the most efficient vectors in sub-Saharan Africa, including Ghana (Afrane *et al.,* 2004; Appawu, 2005). Among these complexes, the *An. gambiae* is more widespread in Ghana and throughout tropical Africa with higher vectorial capacity (Coetzee *et al.*, 2000).

The *An. gambiae* complex of sibling species comprises eight reproductively isolated species that are morphologically indistinguishable. The sibling species are *An. gambiae s.s.* Giles which is now split into *An. coluzzii* Coetzee and Wilkerson and *An. gambiae* Giles, *An. arabiensis* Patton, *An. quadriannulatus* Theobald, *An. amharicus* Hunt *et al*. (formerly designated *An. quadriannulatus* species B due to its close similarity to *An. quadriannulatus*), *An. melas* Theobald, *An. merus* Dönitz and *An. bwambae* White (Gillies and Coetzee, 1987; Coetzee *et al.*, 2013). Collectively, they are referred to as *An. gambiae sensu lato*, which means 'in the wider sense'. Among these sibling species, only *An. arabiensis*, *An. melas* and *An. gambiae s.s* have been reported in Ghana with *An. gambiae* s.s as the most predominant (Appawu *et al.*, 1994; Okoye *et al.*, 2005; de Souza *et al.*, 2010).

Malaria is present in almost one hundred countries and threatens half of the world's population especially in sub-Saharan Africa, where it remains a major cause of morbidity and mortality. According to WHO (2014) report, about 198 million cases of malaria occurred worldwide in 2013, 82 percent of which were in the WHO African region and the disease caused an estimated 584 000 deaths, mostly in children under five years of age. In Ghana, malaria accounted for 33% of hospital deaths in children under five years and about 38% of all outpatient illnesses and 36% of all admissions (PMI, 2014). In the northern part of the country, it accounted for nearly a quarter of deaths in children under five years (Binka *et al*., 1994, 1995). Owing to its hyperendemicity in the country, everyone is at risk of contracting the disease with children less than five years of age, pregnant women and non-immune visitors being at the greatest risk.

This disease representing the primary cause of mortality and morbidity in health centres caused an enormous burden to health and economy in developing countries (WHO, 2011). Its direct effects extend far beyond measures of mortality and morbidity into reducing attendance at school and productivity at work and evidence suggests that the disease can impair intellectual development (Fernando *et al*., 2003), resulting to under-development of a country. According to

Carter *et al.* (2005), economists believe that malaria is responsible for a growth penalty of up to 1.3% per year in some African countries. To this end, World Health Organization has recommended a multi-faceted strategy which aims at preventing and controlling malaria transmission. This strategy includes vector control interventions, intermittent preventive treatment, diagnostic testing and treatment with quality-assured artemisinin-based combination therapies (ACTs), as well as strong malaria surveillance (WHO, 2014). Thus, integrated approaches against both the parasites and vectors are necessary for any malaria control to be effective. Nonetheless, the spread of drug resistant parasites has shifted interest to vector control. Effort to combat malaria transmission through vector control in Africa depends mostly on interventions targeted at adult vectors using either insecticide-treated nets (ITNs) or indoor residual spraying (IRS) (Pluess *et al.*, 2010). While indoor residual spraying, wearing of protective clothing, the use of mosquito repellant creams and coils continue to play a major role in the prevention and control of malaria (Okrah *et al.*, 2002), current policy in most sub-Saharan Africa countries where malaria is endemic emphasizes the use of insecticide-treated nets due to its effectiveness in reducing childhood morbidity and mortality. For instance, large scale controlled trials of ITNs in the region during the 1990s showed significant reductions in malaria-related deaths in young children (WHO, 2003). In Ghana, however, Binka *et al.* (1996) demonstrated that the use of treated nets led to a 17 percent reduction in childhood mortality. Indeed, Lengeler (2004) has argued that about half a million child deaths in sub-Saharan Africa could be avoided each year if bednets were widely used.

However, high pyrethroid resistance, resulting from a single point mutation in the gene that encodes the sodium channel (Martinez-Torres *et al.*, 1998) have been reported in malaria vectors and this could hamper the use of treated bednets since pyrethroids are the only insecticides approved for treating bednets (Lines and Zaim, 2000). Insecticide resistance monitoring is therefore vital in order to ensure the sustainability of malaria vector control programmes that involve insecticide application because data on vector susceptibility to insecticides and their operational implications are required to guide insecticide use. Although, insecticide resistance distribution studies have been conducted in some communities in Ghana, the need to survey the other parts in the country is justified due to the focal nature of insecticide resistance (Brogdon and MacAllister, 1998), and also due to the fact that levels of insecticide resistance in the *Anopheles* vectors of malaria can differ even within comparatively small geographical scales and during different seasons of the year (Diabate *et al.*, 2004).

Apart from the development of insecticide resistance in the malaria vectors, other challenges faced during the implementation of most vector control programmes include inadequate knowledge of the vector populations, economic limitations, among others (Lounibos and Conn, 2000; Shiff, 2002). Hence, in order to effectively control malaria transmission in a particular locality using vector control interventions, adequate information on the local malaria vector ecology, distribution, transmission patterns, factors affecting transmission, as well as information on the intensity of transmission by vector populations are required (Githeko *et al.*, 1993). The intensity of transmission is a key component of epidemiologic studies of malaria and is estimated using the Entomological Inoculation Rate (EIR), an index that provides the most direct measure of the risk of human exposure to the bites of infective Anopheline vectors. This risk of human exposure to infectious bites of vectors in Africa is however, not uniform (Smith *et al*., 1995). Thus, the transmission pattern varies greatly from region to region and even from village to village in the same district (Antonio-Nkonjio *et al*., 2006). In Ghana, as in many malaria endemic countries of Africa, there is little baseline information on vector populations and variation in the intensity of malaria transmission. Where vector control programmes such as the use of ITNs or IRS are being applied, they are implemented without the knowledge of the local epidemiology of the disease, especially the relationship to vector behavior and intensity of transmission.

1.2 Justification

Efforts towards achieving malaria elimination and eradication worldwide are being put into play and such is the development of the technology of Insecticide-treated nets (ITNs) which is one of the primary interventions approved by the WHO Global Malaria Programme for effective malaria control. The importance of ITNs on malaria transmission in experimental trials in sub-Saharan Africa has been proven, as shown by reduction of various entomologic indices and more importantly, by reduction of morbidity and mortality due to malaria, especially in children under five years of age (D'Alessandro *et al.*, 1996; Binka *et al.*, 1996).

However, the rapid loss of bio-efficacy of ITNs due to washing, and low re-treatment rates limit the operational effectiveness of ITNs programme. Hence, to overcome these operational problems, some manufacturers in collaboration with WHO have developed long-lasting insecticidal-treated nets (LLINs) that will remain efficacious throughout their expected lifespan (a minimum of 3 years), with repeated washings. In 2006, the WHOPES introduced a system of time-limited (4 years) interim recommendations. After such a recommendation, the manufacturer is required to submit the candidate's LLIN for a large-scale 3-year long field evaluation in order to determine their efficacy and fabric integrity before they are recommended for use in malaria vector control, failing which interim recommendation can be withdrawn.

In preparation for such field trial, the testing sites need to be identified and characterized for malaria transmission. Shai-Osudoku district in Southern Ghana has been mapped out as a trial site. Hence, all aspects of malaria epidemiology needs to be well-understood including a better understanding of the vector transmission indices, such as the biting rate, sporozoites rate and EIR, as well as the susceptibility status of the malaria vectors to pyrethroids - the only insecticide approved for bednets, before the field trial is conducted. These will form a baseline data that would be used when assessing the impact, as well as the efficacy of such LLINs that purport to be effective against pyrethroid resistant mosquitoes.

1.3 Main Objective

The study was aimed at characterizing malaria transmission and insecticide susceptibility status of *Anopheles gambiae s.l.* in Shai-Osudoku district of southern Ghana in preparation for Phase 3 Long-lasting insecticide treated nets (LLINs) trial.

1.3.1 Specific Objectives

The specific objectives were to determine:

1. The species composition of malaria vectors in the study site.

2. The transmission indices of malaria in the study site.

3. The susceptibility status of *An. gambiae s.l.* to insecticides.

4. The insecticide resistance mechanism (knockdown resistance) in the malaria vectors.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria: The Global Impact and Burden

Malaria is present in almost one hundred countries and threatens approximately half of the world's population especially in sub-Saharan Africa, where it remains a major cause of morbidity and mortality. However, Asia, Latin America, and to a lesser extent the Middle East and parts of Europe are also affected. In all malaria endemic countries in Africa, an average of 30% of all outpatient clinic visits and between 20 - 50% of all hospital admissions are due to malaria (Snow, 1999). In 2013, about 198 million cases of malaria occurred worldwide, 82% of which were in the WHO African Region and the disease caused an estimated 584, 000 deaths, mostly in children under five years of age (WHO, 2014). According to WHO 2014 report, malaria mortality rates have fallen by 47% globally since 2000 and by 54% in the WHO African Region.

2.1.1 Malaria Burden in Ghana

Malaria is hyper-endemic and continues to be the leading cause of morbidity and mortality in children under five years of age, accounting for 33% of hospital deaths in children under five years and about 38% of all outpatient illnesses and 36% of all admissions (PMI, 2014). In the northern part of the country, the disease accounted for nearly a quarter of deaths in children under five years (Binka *et al*., 1994; 1995). Between 3.1 and 3.5 million annual cases of clinical malaria are reported in public health facilities, of which 900,000 cases are in children under five years and 3,000- 4,000 result in inpatient deaths. However, studies have shown that these reported malaria cases represent only a small fraction of the actual number of malaria episodes since roughly half of the deaths in children under five years occurred at home and are not reported to the health service (PMI, 2014). Owing to its hyperendemicity in the country, everyone is at risk of contracting the disease with children under five years, pregnant women and non-immune visitors being at the greatest risk.

2.2 Malaria Parasite: Transmission and the Symptoms

Malaria is a vector-borne infectious disease caused by protozoan parasites of the genus *Plasmodium* (phylum: Apicomplexa). Six different species of *Plasmodium*: *P. falciparum* Welch, *P. vivax* Grassi and Feletti, *P. malariae* Laveran, *P. ovale* Stephens, *P. knowlesi* and *P. cynomolgi* cause malaria in humans (Ta *et al.*, 2014; Mueller *et al.*, 2007; Singh *et al.*, 2004). *P. knowlesi* and *P. cynomolgi* are known to cause malaria in macaques but have also been reported to infect humans. *P. falciparum* is the most common cause of infection and is responsible for about 80% of all malaria cases while accounting for 90% of deaths from malaria (Mendis *et al*., 2001). *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* on the other hand cause significant morbidity but are rarely life-threatening. *P. falciparum* occurs in most malaria affected areas of the world, in tropical Africa and Asia. *P. vivax* is uncommon in sub-Saharan Africa, but common in South Asia and Central America, and is the major species in South America. *P. ovale* is found mostly in the tropical Africa with sporadic reports from other parts of the world. *P. malariae* occurs in the tropical and subtropical areas of Central and South America, Africa, and Southeast Asia. Lastly, *P. knowlesi* is absent in Africa but is mostly found in Southeast Asia particularly Cambodia, China, Indonesia, Laos, Malaysia, Myanmar, the Philippines, Singapore, Thailand and Vietnam where it accounts for up to 70% of malaria cases (McCutchan *et al.*, 2008; Cox-Singh *et al.*, 2008). Among these species, only *P. falciparum, P. malariae and P. ovale* have been reported in Ghana (Asenso-Okyere and Dzatow, 1997).

Malaria parasites are transmitted by female mosquitoes belonging to the genus *Anopheles.* Only female Anopheline mosquitoes bite and take up blood. Male *Anopheles* mosquitoes feed on plant juices and nectar, rather than blood, and therefore cannot transmit malaria. When a female mosquito bites an infected human host, it picks up the gametocytes, the sexual forms of the parasite, along with blood. Upon entering the midgut of the mosquito, the microgametocytes exflagellate by a process known as exflagellation to become microgametes and then fuse with the macrogametes to form a zygote. The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication which results in the production of sporozoites. Rupture of the mature oocyst releases the sporozoites into the mosquito's body cavity. The sporozoites then migrate to and invade the salivary glands of the mosquito (Aly *et al*., 2009). The time necessary for the development of the sporozoites varies with temperature, humidity and to a smaller extent with the species of the malaria parasite, but generally it takes about 8-15 days. The sporozoites which are now infective are injected into a human host when the mosquito takes the next blood meal. The sporozoites upon entering the bloodstream, invade the mammalian liver cells where they multiply. This multiplication takes about 7-12 days, after which the infected liver cells will rupture releasing merozoites into the bloodstream. These merozoites then invade the red blood cells where they multiply again and end up destroying the infected red cells. The released parasites invade fresh red blood cells and the cycle is repeated (Aly *et al*., 2009). Failure in the malaria parasite developmental process would prevent transmission between the mosquito and the human host.

The general symptoms of malaria include headache, nausea, fever, vomiting, chills, cough, diarrhoea, abdominal pain, muscular aching and weakness. These symptoms, however differ depending on the type of *Plasmodium* species that caused the infection and the immunity of the human host. Infection caused by *P. falciparum* normally takes about 7 to 14 days for symptoms to show, *P. knowlesi* normally takes 9 to 12 days, while *P vivax* and *P. ovale* normally takes 8 to 14 days (but in some cases may linger for months) and for *P. malariae*, the symptoms show up between 7 to 30 days (D'Avanzo *et al.*, 2002). In most severe complications, especially in young children, pregnant women and people who are immunosuppressed, other symptoms involving the brain such as cerebral malaria and those related to organ failure such as acute renal failure, pulmonary oedema, generalized convulsions, circulatory collapse, followed by coma and death may develop. In malaria endemic areas, persons repeatedly infected with malaria usually develop partial immunity, allowing asymptomatic infections to occur (WHO, 2014).

2.3 Malaria Vectors

Malaria is transmitted exclusively from an individual to another through the bites of female mosquitoes of the *Anopheles* genus (Order: Diptera, Family: Culicidae, Sub-family: Anophelinae). For any female *Anopheles* species to be able to transmit malaria, it must be able to ingest the parasites and promote their maturation until the infective stage. The mosquito must also be able to survive until the next blood meal. The genus *Anopheles* has more than 400 known taxonomic species but only about 70 of these have been incriminated as vectors of malaria (Service, 1993). Of 140 Anopheline species present in sub-Saharan Africa, only less than 20 are known to transmit malaria (Hervy *et al.*, 1998). Although only few species of female *Anopheles* mosquitoes are able to meet the requirements for being malaria vectors, the species and forms involved in malaria transmission are very diverse (Appawu, 2005). The significance of each species in malaria transmission differs by region, as does their geographical distribution. For example, *Anopheles dirus, An. minimus* and *An. sundaicus* complexes are the major malaria vectors in Southeast Asia (Trung *et al.*, 2004). In sub-Saharan Africa, the *An. gambiae* complex and the *An. funestus* complex are the most important vectors of malaria, with *An. gambiae* complex being the predominant (Gillies and Coetzee, 1987).

2.3.1 *Anopheles gambiae sensu lato*

An. gambiae Giles *s.l.* consists of eight named identical sibling species: *An. gambiae sensu stricto* Giles which comprises of *An. coluzzii* Coetzee and Wilkerson and *An. gambiae* Giles, *An. arabiensis* Patton, *An. bwambae* White, *An. quadriannulatus* Theobald, An. amharicus Hunt *et al*. (formerly designated *An. quadriannulatus* species B due to its close similarity to *An. quadriannulatus)*, *An. merus* Donitz and *An. melas* Theobald (Gillies and Coetzee, 1987; Coetzee *et al.*, 2013). These sibling species are isomorphic or morphologically indistinguishable and can only be distinguished by means of chromosome banding patters, allozyme analysis, chromatography of hydrocarbons with DNA probes and species-specific polymerase chain reaction (Gillies and Coetzee, 1987; Hunt *et al.*, 1998).

The sibling species comprising of freshwater, mineral water and saltwater breeders (Davidson *et al.*, 1967) have a wide geographical distribution and have been reported in various African countries. The freshwater breeding species include the *An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus and* An. amharicus. On the other hand, *An. bwambae* is the only mineral water breeder whereas the two saltwater breeders are the *An. merus* for the East African species and *An. melas* for the West African species. *An. melas* is confined to the coast of West Africa whereas

An. merus is confined to the coast of East Africa and islands off its coast and has also been observed in Somalia in the North to Natal in the South (Diop *et al.*, 2002). *An. quadriannulatus* was first reported in Onderstepoort, South Africa and was considered to be a highland species because of its distribution in Ethiopia (White, 1985). However, the Ethiopian population was shown to be a separate species based on cross-mating and chromosomal studies (Hunt *et al.*, 1998) and was formally assigned a new name, *An. amharicus* (Coetzee *et al.*, 2013). *An. bwambae* has been reported from the geothermal hot springs in the Semliki forest of Uganda's Bwamba County where it was found breeding in mineral water springs (White, 1985). *An. gambiae s.s.* and *An. arabiensis* are the most widespread and most efficient disease transmitting sibling species of the *An. gambiae s.l.* in Africa (Coetzee *et al.*, 2000). Their distribution and relative abundance are strongly affected by climatic factors. While *An. gambiae s.s.* is usually predominant in humid environments, *An. arabiensis* appears to be found in drier areas and predominate in arid savannas. In areas where *An. arabiensis* and *An. gambiae s.s.* co-exist, there are huge heterogeneities in densities, with the former predominating during the dry season and the later becoming more abundant in the rainy season (Coetzee *et al.*, 2000).

Three of the sibling species namely *An. gambiae s.s.*, *An. arabiensis* and *An. melas* have been reported in Ghana, with significant variations in their ability to transmit malaria. *An. melas* is more prolific than the other members and tends to show significant outdoor feeding where people slept on mats (Tuno *et al.*, 2010). This species has been reported in the coastal zone of southern Ghana but has not been implicated in malaria transmission (Appawu *et al.*, 2001). *An. arabiensis* has been found in the western region, southern coastal zone and northern savannah zone of Ghana (Kristan *et al.*, 2003; Appawu *et al.*, 2004; Kelly-Hope *et al.*, 2006). *An. gambiae s.s.* on the other hand, has been observed in almost every ecological zone of Ghana. For example, studies by Adasi *et al.* (2000), Appawu *et al.* (2001), Kabula *et al.* (2011), Tchouassi *et al*. (2012) and Yawson *et al*. (2004) reported *An. gambiae s.s.* in the coastal savannah and coastal forest zone in the Greater Accra Region of Ghana. *An. gambiae s.s*. has also been reported from the strand and mangrove zones of the Central Region as well as the rainforest zone of the South Western Region of Ghana (Kristan *et al.*, 2003). In the rainforest middle belt or central part of Ghana, this species has been reported (Afrane *et al.*, 2004; Yawson *et al.*, 2004; Stiles-Ocran *et al.*, 2007; Dery *et al*., 2010). It is *An. gambiae s.s.* which has been found to be the most prevalent species transmitting malaria in the country (Appawu, 2005).

However, studies in West Africa have provided evidence for at least five chromosomal forms within the *An. gambiae s.s*. designated as Forest, Savanna, Mopti, Bamako and Bissau (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985, Fanello *et al.*, 2003). Three of these forms namely the Forest, the Savanna and the Mopti form have been reported in Ghana (Appawu *et al.*, 1994). Further analysis into the complexity of *An. gambiae s.s* revealed genetic differences in the non-coding sequences of the ribosomal DNA (rDNA), distinguishing between the Mopti chromosomal form and Bamako/Savanna populations (Favia *et al.*, 1997; della Torre *et al.*, 2001). Hence, the concept of molecular forms of *An. gambiae s.s.*, using the terms M and S was first introduced by della Torre *et al*. (2001) to describe such populations, with the M form coinciding with Mopti chromosomal forms and S form coinciding with the Savannah/Bamako chromosomal forms only within the boundaries of Mali and Burkina Faso while outside these boundaries, the linkage is broken. With the recent studies by Coetzee *et al.* (2013) which was based on molecular and bionomical evidence, the *An. gambiae* molecular M form is now formally referred to as *An. coluzzii* Coetzee and Wilkerson, while the S form still retains the nominotypical name *An. gambiae* Giles. In Ghana, both molecular forms have been reported, with the S form being very dominant in the middle belt, while the *An. coluzzii* (formerly M form) predominated in the northern and coastal Savannah belts (de Souza *et al.*, 2010). The S form is mostly dependent on temporary breeding sites and rainfall, whilst *An. coluzzii* is better adapted to permanent breeding sites such as rice fields and irrigation plains (Diabaté *et al.*, 2005; de Souza *et al.*, 2010).

2.3.2 *Anopheles funestus sensu lato*

An. funestus Giles is one of the three major vectors of malaria in Africa, together with *An. gambiae* Giles and *An. arabiensis* Patton of the *An. gambiae* (Coetzee *et al.*, 2000; Gillies and Coetzee, 1987). Its significance in malaria transmission is mostly observed in the dry seasons when *An. gambiae s.l.* population density declines significantly. For example, in Tanzania and Burkina Faso, *An. funestus* population have been found to increase at the end of the rainy season (Costantini *et al.*, 1999). The exact composition of the *An. funestus* complex remains unclear (Costantini *et al.*, 1999). Nevertheless, a group of at least nine morphologically similar species: *An. leesoni* Evans, *An. fuscivenosus* Leeson, *An. rivulorum* Leeson, *An. brucei* Service, *An. confusus* Evans and Leeson, *An. aruni* Sobti, *An. vaneedeni* Gillies and Coetzee, *An. funestus s.s.*, and *An. parensis* Gillies have been recognized throughout Africa (Gillies and de Mellion, 1968; Gillies and Coetzee, 1987). Of these, *An. brucei*, *An. rivulorum, An. leesoni* and *An. confusus* can be distinguished from each other based on the characters of the egg and larval features while *An. fuscivenosus* have been described based on the adult features. Other members comprising the *An. funestus* sub-group: *An. funestus s.s, An. aruni*, *An. parensis* and *An. vaneedeni* are morphologically indistinguishable in all life stages but can be differentiated by cocktail PCR assay (Koekemoer *et al.,* 2002), *An. funestus s.s*. is closely associated with human dwellings hence it plays a critical role in malaria transmission and to a lesser extent *An. rivulorum* (Kawada *et al*., 2012). The other members of the group are mainly zoophilic (Gillies and Coetzee, 1987; Fontenille *et al.,* 1997, Costantini *et al*., 1999). *An. funestus* has a wide distribution and the ability to occupy regions ranging from lowland to high altitudes, being found in almost all bio-climatic areas near swamps or rivers (Faye *et al.,* 1995). Compared to other dominant vector species in Africa, *An. funestus* shows fairly consistent behaviour, generally anthropophilic and endophilic throughout its range.

Studies in Ghana have shown the presence of *An. funestus* in some parts of the country (Dadzie *et al.,* 2012; Dery *et al*., 2010; Okoye *et al*., 2008). In the northern Ghana, *An. funestus* has been reported in the Kassena Nankana district; in Tolon, Savelugu and Tamale districts (Appawu *et al.,* 2004; Biloh, 2013). Among the members of *An. funestus* group, only the *An. funestus s.s.* has been found to transmit malaria in Ghana and it is known to be the second major malaria vector in the country (Dadzie *et al.,* 2012).

2.3.3 The *Anopheles pharoensis* Group

An. pharoensis group is made up of at least twin species and is abundant and widespread throughout the Afro-tropical region outside areas of close canopy forests (Gillies and de Meillon, 1968). They breed largely in large vegetated swamps but can also breed in irrigation ditches, rice fields, reservoirs, river edges, lake shores, small streams, ponds, pools and overgrown wells. Its role in malaria transmission in other sub-Saharan African countries is not clear in spite of several records of sporozoite-positive specimens (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987). Furthermore, reports from Senegal (Carrara *et al*., 1990; Dia *et al*., 2008) and Cameroon

(Antonio-Nkonjio *et al*., 2006), have shown that *An. pharoensis* transmit malaria in these areas. These contrasting views about the vector status of this species have been attributed to the possible existence of different sibling species within this group (Miles *et al*., 1983). In Ghana, isolated collections of *An. pharoensis* have been made since 1992 in Greater Accra, Ashanti, Central, Eastern, Western, Upper East and Upper West regions (Afari *et al*., 1995; Amuzu *et al*., 2010; Charlwood *et al*., 2011; Hunt *et al*., 2006; Dzodzomenyo *et al*., 1999; Ughasi *et al*., 2012; Dery *et al*., 2013).

2.4 The Socio-Economic Impact of Malaria

Malaria is not just a disease commonly associated with poverty but also a cause of poverty and a major hindrance to economic development particularly in malaria-endemic African countries (Sachs and Malaney, 2002). Tropical regions are mostly affected; but can also extend into some temperate zones with extreme seasonal changes and where it is widespread, the disease has been associated with major negative economic effects. The direct economic costs of malaria are enormous and weigh particularly heavily on poor countries with few resources. For example, in Africa, the economic impact of malaria has been estimated to cost $12 billion USD every year while accounting for a reduction of 1.3% of the annual economic growth rate (Gallup and Sachs, 2001). Whilst in Ghana, the annual economic burden of malaria is estimated 1-2 % of the Gross Domestic Product placing a significant financial hardships on both households and the economy. Apart from the negative effects such as impaired learning on children and loss of productivity on the major sectors of the economy, the disease has also been reported to be the leading cause of workdays lost due to illness in the country thereby contributing more to potential income lost than any other disease. At the national level, it has negative effects on the growth of tourism, investments and trade. Malaria therefore imposes a heavy cost not only on a country's current income, but also on the rate of its economic growth, which in the long run affects the level of economic development

In Ghana, studies by Asenso-Okyere and Dzator (1997) revealed that three workdays were lost by economically active patients who were ill with malaria and two workdays by the caretakers. Whereas, in another study by Asante and Asenso-Okyere (2003), nine workdays were lost by economically active patients while more than five workdays were lost by their caretakers. In terms of costs of health care, researches have shown that both direct and indirect costs associated with a malaria episode represent a substantial burden on the poorer households than on the richer ones. This agrees with studies in northern Ghana that revealed that while the cost of malaria care was just 1% of the income of the rich, it was 34% of the income of poor households (Akazili, 2002). The burden of malaria therefore is a challenge to human development manifesting itself as a cause and consequence of under-development.

2.5 Malaria: Control

The control of malaria is recognized as an important element of national poverty reduction strategies in most malaria-endemic countries because of the social and economic impacts of the disease. Hence, the goal of malaria control in most endemic countries is to reduce as much as possible the health impact of malaria on a population using resources available, and taking into account other health priorities (WHO, 2001). For effective malaria control, the World Health Organization Global Malaria Programme recommends the following primary interventions:

i. Diagnosis of malaria cases and prompt treatment with effective medicines
ii. Distribution of insecticide-treated nets (ITNs), more specifically long-lasting insecticidal nets (LLINs)
iii. Indoor residual spraying (IRS)

As these must be scaled up if countries are to move towards achieving the United Nations Millennium Development Goals by 2015. In line with this, Ghana has adopted two well proven approaches to the prevention of malaria, namely: Intermittent preventive treatment in pregnancy (IPTp) and Integrated Malaria Vector Management (IMVM) (NMCP, 2008).

2.5.1 Vector Control

Vector control remains the most generally effective measures to prevent malaria transmission and also fundamental element of the existing global strategy to fight malaria. Vector control interventions have a proven track record of successfully reducing or interrupting disease transmission when coverage is sufficiently high. It is the main way to reduce malaria transmission at the community level and the only intervention that can reduce malaria transmission from very high levels to close to zero (WHO, 2014). According to WHO malaria report (2014), the proportion of the population protected by at least one vector control method in sub-Saharan Africa has increased in recent years, with record of 48% in 2013 bringing about the recent decrease in the global malaria burden. Much of this decrease has been attributed to the use of insecticide-treated nets (ITNs), particularly long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (WHO, 2013). WHO recommends that endemic countries protect all persons at risk of malaria through LLINs or IRS, or a combination of the two interventions where it is justified (WHO, 2014). Hence, vector control strategies should be drawn up in consideration of changing insecticide resistance patterns and through an integrated vector management approach, which seeks to improve the efficacy, cost-effectiveness, ecological soundness and sustainability of disease vector control.

2.5.1.1 Use of ITNs and LLINs and the Reduction of Malaria Disease

An insecticide-treated net (ITN) is a mosquito net that repels, disables and/or kills mosquitoes coming into contact with insecticide on the netting material. There are two categories of ITNs: conventionally treated nets and long-lasting insecticidal nets.

A conventionally treated net is a mosquito net that has been treated by dipping in a WHO-recommended insecticide and to ensure its continued insecticidal effect; the net is re-treated after three washes, or at least once a year. A long-lasting insecticidal net, on the other-hand, is a factory-treated mosquito net made with netting material that has insecticide incorporated within or bound around the fibres. The net retains its effective biological activity without re-treatment for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field conditions (WHO, 2005). Hence, long-lasting insecticidal nets (LLINs) are the preferred form of Insecticide-treated mosquito nets (ITNs) for public health distribution programmes. In recent years, all distributed nets have been LLINs and between the year 2012 and 2014, over 427 million LLINs have been distributed to countries in sub-Saharan Africa by manufacturers (WHO, 2014), with the Greater Accra Region in Ghana receiving over 2 million LLINs in 2012.

LLINs play a significant role in the prevention and control of malaria. They provide personal protection, hence reducing human–vector contact and when used by a majority of the target population, can provide protection for all people in the community, including those who do not themselves sleep under nets (Binka *et al.*, 1996). Efforts to promote the use of insecticide treated bed nets as one of vector control measures have been shown to be effective in reducing transmission, clinical disease, and childhood mortality. For example, a large-scale community-randomized trial of treated bednets in Northern Ghana revealed a significant reduction in mortality by 17% in children aged 1 month to 4 years (Binka *et al.*, 1996). In Gambia, a significant decline in both mortality and morbidity associated with malaria has been attributed to the high coverage and usage of treated bednets (Ceesay *et al.*, 2008).

On the basis of five community-randomized trials, a Cochrane review concluded that when full coverage is achieved, treated bednets could reduce all-cause child mortality by an average 18% in sub-Saharan Africa (Lengeler, 2004). The general implication of this is that 5.5 lives could be saved per year for every 1000 children under 5 years of age protected.

2.5.1.1.1 Recommended Insecticides for LLINs

Although four classes of insecticides (organochlorines, organophosphates, carbamates and pyrethroids) are recommended by WHO for use against adult mosquitoes in public health programmes, modern-day malaria vector control has become highly dependent on just one class of insecticide – the pyrethroids. Pyrethroids are preferred over other classes of insecticides for the impregnation of bednets because of their fast acting effect at low doses, strong efficacy, relatively low mammalian toxicity and lesser environmental effect (Zaim *et al*., 2000). They are widely used, both in agriculture and as household pesticides; their use as larvicides is limited because of their toxicity to non-target aquatic organisms including fish. Currently, pyrethroids which include etofenprox, permethrin, cyfluthrin, deltamethrin and lambdacyhalothrin are used on all approved LLINs (WHO, 2013).

Pyrethroids are synthetic chemicals whose structure for example, the permethrin, mimic the natural pyrethrins produced by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *C. coccineum*) and constitute a major class of neurotoxic insecticides. It is estimated that over 25% of the insecticide market is dominated by pyrethroids (Georghiou, 1990). Commercially available pyrethroids insecticides now include the natural pyrethrins and the synthetic pyrethrins. These pyrethroids are nerve poisons that target the voltage-gated sodium channel in insect neurons. They do this by binding to a protein in the voltage-gated sodium channel thereby preventing it from closing. Pyrethroids affect the peripheral and central nervous system (CNS) of insect by stimulating nerve cells to produce repetitive discharges and thereby causing paralysis (Ware and Whitacre, 2004). The channels remain open after modification by pyrethroids, and delay in channel closing often results in prolonged sodium current, leading to depolarizing membrane. However, the membrane potential is shifted so that the nerve cells function in a new, and relatively stable, state of abnormal hyper-excitability. In insects this produces an incapacitating, but sub-lethal effect, known as knockdown.

2.6 Factors Involved in Malaria Transmission and Control

Understanding the biology and behaviour of *Anopheles* mosquitoes is essential to evaluating the risk of malaria transmission to humans, implementing surveillance and designing appropriate control strategies against the vectors. Although many factors are known to be involved in malaria transmission, important factors that should be taken into consideration when designing vector control programmes include the susceptibility of malaria vectors to insecticides, preferred sources for blood meals and patterns of feeding and resting of adult mosquitoes (WHO, 2003).

2.6.1 Preferred Sources for Blood Meals

One important behavioural factor is the degree to which an *Anopheles* species prefers to feed on humans (anthropophily) or animals such as cattle (zoophily). Anthropophilic *Anopheles* are more likely to transmit the malaria parasites from one person to another. Most *Anopheles* mosquitoes are not exclusively anthropophilic or zoophilic. However, the primary malaria vectors in Africa, *An. gambiae* and *An. funestus*, are strongly anthropophilic and, consequently, are two of the most efficient malaria vectors in the world.

2.6.2 Patterns of Feeding and Resting

Most *Anopheles* mosquitoes are crepuscular (active at dusk or dawn) or nocturnal (active at night). While, some *Anopheles* mosquitoes prefer to feed indoors (endophagic), others feed outdoors (exophagic). After blood feeding, some *Anopheles* mosquitoes prefer to rest indoors (endophilic) while others prefer to rest outdoors (exophilic). The highly efficient malaria vector species in sub-Saharan Africa: *An. gambiae s.s.*, *An. arabiensis* and *An. funestus s.s.* are generally considered to predominantly prefer feeding indoors at night, with peak biting activity typically occurring between midnight and the early hours of the morning, when most people in sub-Saharan Africa are asleep indoors (Gillies, 1988)

Biting by nocturnal, endophagic *Anopheles* mosquitoes can be markedly reduced through the use of insecticide-treated bed nets (ITNs) whilst endophilic mosquitoes are readily controlled by indoor spraying of residual insecticides (IRS). The success of these two interventions relies on their ability to repel or kill indoor feeding and resting mosquitoes, thus providing direct personal protection against exposure to bites, as well as reducing adult mosquito survival and human-feeding frequency (Pates and Curtis, 2005).

2.6.3 Susceptibility of Malaria Vectors to Insecticides

Insecticide-based malaria vector control interventions, more specifically Insecticide-impregnated bednets and IRS are ideal for killing indoor and outdoor feeding vectors so long as the vectors remain susceptible to the insecticides being employed in the control measures (Ranson *et al.*, 2011). However, after prolonged exposure of the malaria vectors to insecticide over several generations, insecticide resistance may develop. When controlling malaria vectors using insecticides, it is important therefore, that changes in the susceptibility level of the target vectors are monitored from time to time in order to react proactively to prevent insecticide resistance from compromising control. The standardized WHO insecticide susceptibility test which is carried out to determine the proportion of the vector population that is physiologically resistant to a particular insecticide, is a long-established method used for monitoring insecticide resistance in malaria vector mosquitoes and is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes (WHO, 2013).

In Ghana, the WHO insecticide susceptibility tests on the adult malaria vectors have been conducted across the country. In Kassena-Nankana district, susceptibility test carried out on the major malaria vectors reported 93.3% cyfluthrin susceptibility in *An. gambiae* and 94.5% in *An. funestus* (Hunt *et al*., 2011). Field-caught *An. gambiae s.l* from the rural areas in Western Region of Ghana exposed to WHO-recommended concentrations of DDT, permethrin and deltamethrin showed high levels of susceptibility to DDT (94-100%), deltamethrin (97-100%) and fully susceptible to permethrin (100%) (Kristian *et al.,* 2003), whereas within the Accra metropolis, *An. gambiae s.l.* were highly resistant to both deltamethrin and permethrin with mortality rates of 42.98-70.0% and 6.5-20.0% respectively (Kabula *et al.*, 2011). Also in the Greater Accra region, *An. gambiae s.s.* were reported to be resistant to permethrin but highly susceptible to propoxur with mortality rates of 21–92% and 92–100% respectively (Adeniran *et al*., 2009).

Elsewhere in Africa, WHO insecticide susceptibility tests have been employed in monitoring insecticide resistance. For example, in Benin, Aïkpon *et al*. (2013) reported that *An. gambiae s.l.* were susceptible to bendiocarb but fully resistant to DDT, permethrin and deltamethrin.

2.7 Malaria Transmission Indices

2.7.1 Human or Man Biting Rates of Malaria Vectors

The number of *Anopheles* mosquito vectors biting humans is one of the major determinants of malaria transmission (Beier *et al.,* 1999). The most direct way to measure human biting rate is the human landing catches (HLC) technique. This technique is important since it determines which anophelines are anthropophagic; which of the anthropophagic ones transmit malaria; human-biting rates and seasonal variations; whether the vectors are endophagic or exophagic and their peak biting time (WHO, 2003). Although the method is expensive, technically difficult to replicate and unethical in areas of drug-resistant malaria, it is special as it directly samples man-biting species (Le Goff *et al.,* 1997).

2.7.2 Detection of Sporozoites Infection Rates in Malaria Vectors

Mosquito infectivity in the field has always been estimated by determining the proportion of field collected mosquitoes carrying *Plasmodium* sporozoites. The salivary glands are examined for sporozoites and thus to determine which mosquito species carry the malaria parasite and to determine the percentage of each species that is infected. Determination of sporozoite rates is necessary to confirm the role of a particular mosquito species as a vector in order to establish the intensity of malaria transmission (inoculation rate) and assess impact of malaria control interventions. This may be done by the dissection of the salivary glands but this method has major drawbacks and so not easily applicable in the field (WHO, 2003). Wirtz *et al*. (1987) developed an enzyme-linked immunosorbent assay (ELISA) method for the detection of *Plasmodium*-specific circumsporozoite antigens (CS) from the mosquito head and thorax. The ELISA combines the specificity of antibodies with the sensitivity of enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. It can be used to test for antibodies that recognize an antigen or to detect the presence of antigens that are recognized by an antibody. The sporozoite ELISA, for example, involves the use of the monoclonal antibodies to detect circumsporozoite (CS) proteins of *P. falciparum* and the results are read using an ELISA plate reader. Sporozoite rates are then derived from the proportion of man biting *Anopheles* species that test positive for *P. falciparum* circumsporozoite protein by ELISA.

2.7.3 Entomological Inoculation Rates (EIR) of Malaria Vectors

Malaria parasite transmission intensity in the field is estimated through the determination of the entomological inoculation rate (EIR). The value of the EIR lies in the fact that it provides an estimate of the passage of malaria parasites from infective anopheline species to human populations. It is calculated as the product of the *Anopheles* mosquito biting rate and the proportion of mosquitoes carrying sporozoites in their salivary glands. Hence, can be used to estimate the level of parasite transmission to people dwelling in a given community at a given time and as a parameter to differentiate transmission intensity between communities over a period (Beier *et al.*, 1999). The entomological inoculation rate (EIR) also estimates the level of exposure of an individual to malaria-infected mosquitoes and in addition, the most favoured measure of assessing malaria endemicity and transmission intensity (Drakeley, 2003).

EIR cannot be considered an exact measure of transmission because not all bites from infected Anopheline species succeed in infecting humans. Experiments by Rickman and co-workers showed that 1 or 2 infected bites per person infected only 5 of 10 human volunteers (Rickman *et al*., 1990). There is always a strong correlation between EIR and the prevalence of malaria in a population, therefore EIR has become the most accurate measure for estimating transmission. Thus, significant reductions in the transmission intensity are necessary to reduce the prevalence of malaria infection in human populations. The EIR is expressed as the number of infective bites per person per year. It ranges from <1 bite per year in low-transmission areas to 2,979 infected bites per person per year in 1 sentinel house (Smith *et. al*., 1993).

Studies on malaria transmission conducted in Ghana have demonstrated the EIR in the northern part of the country (Navrongo), where there is an irrigation programme, to be 643 infective bites per person per year (ib/p/y) (Appawu, 2004) whereas in the southern forest (Dodowa) and coastal areas (Prampram), EIRs were 21.9 and 3.65ib/p/y respectively (Appawu *et al*., 2001). In the forest-savannah areas of the Brong Ahafo region, peak monthly EIR was estimated to be 170 infective bites per person per month (ib/p/m) while annual EIRs ranged from 490 to 866 infective bites per person per year (ib/p/y) (Abonuusum *et al*., 2011). Exceptionally high, and varied, annual EIRs have been reported at Kasena-Nankana district ranging from 157 to 1132 ib/p/y (Kasasa *et al*., 2013). EIRs in the Kintampo area ranged from 231 to 269 ib/p/y (Dery *et al*., 2010). In the Kpone-on-Sea area of coastal Ghana, annual EIR was estimated at 62.1 ib/p/y (Tchouassi *et al*., 2012).

2.8 Insecticide Resistance in Malaria Vectors

Insecticide resistance is the development of the ability in a strain of some organisms to tolerate doses of a toxicant which would prove lethal to a majority of individuals in a normal population of the same species (WHO, 2013). The use of insecticides in malaria control programmes in Africa is expanding with the extensive and rapid roll out of long lasting insecticide-treated bed nets (LLINs) and indoor residual spraying (IRS). Twelve insecticides, all belonging to just four chemical classes: organochlorines, organophosphates, carbamate and pyrethroids are approved by the World Health Organization (WHO) for use against adult mosquitoes in public health programmes. For LLINs, only one class -the pyrethroids is recommended. In Africa, the use of pyrethroids has been widely adopted in controlling agricultural pests and thus, can pose additional selection pressure on mosquitoes when insecticide contaminated ground water permeates their larval habitats (Hilary *et al*., 2009). This intensive exposure, in addition to the use of just one class of insecticides has inevitably resulted in the evolution of insecticide resistance in the malaria vectors. More than 100 mosquito species are known to have developed resistance to one or more insecticides (Hemingway and Ranson, 2000).

The standard WHO susceptibility test has long been recognized as the primary method by which insecticide resistance in malaria vector population can be detected and identified (WHO, 2013). According to the protocol, mosquito samples are exposed to a series of different insecticides using insecticide-impregnated papers with a single discriminating or diagnostic dose for each insecticide. After the exposure of mosquitoes to insecticide impregnated papers, they are held in the absence of insecticide-impregnated papers for 24 hours before mortality is recorded. The tests are performed using non-blood fed females aged 2 to 5 days post emergence either derived from larval collections or F1 progeny of wild-caught female mosquitoes. The use of males is, however, not recommended for resistance monitoring as they are usually smaller and more fragile than females, therefore tend to have higher control mortalities (WHO, 2013). This method has been widely used in the field and gives satisfactory results in detecting insecticide resistance for surveillance purposes.

Following an increase in entomological surveillance in malaria-affected regions in recent years, sufficient data have now been collected to confirm already strong suspicions that the wide-scale use of insecticide-based malaria control strategies over the past decade has been associated with the development of resistance in several important vector species, including *An. gambiae*, *An. funestus* and *An. arabiensis.* According to reports, resistance to at least one class of insecticide has been identified in 64 countries with ongoing malaria transmission, with resistance to the pyrethroids being the most common. In the 1950s and 1960s, resistance to the organochlorines such as DDT was first reported in African malaria vectors (Brown, 1958; Hamon *et al*., 1968). Pyrethroid resistance was later detected in African malaria vectors in 1993 (Elissa *et al.*, 1993). Since then there have been published reports of pyrethroid resistant populations of *Anopheles gambiae s.l.* in countries from West, Central, East and Southern Africa (Ndjemai *et al.*, 2009; Munhenga *et al.*, 2008; Protopopoff *et al.*, 2008; Awolola *et al.*, 2009). Presently, 40 malaria endemic countries have reported pyrethroid resistance (Gatton *et al*., 2013).

2.8.1 Mechanisms of Insecticide Resistance

The management of insecticide resistance is complicated by the fact that resistance takes a variety of forms. However, the mechanisms responsible for the widespread levels of resistance have been identified and they are of two types: those mediated by changes at the target site of the insecticide which is referred to as target-site resistance and those caused by increases in the rate of insecticide metabolism also referred to as metabolic resistance (WHO, 2013). Other mechanisms by which insects become resistant to insecticide include physiological modification mechanisms which permit insects to survive lethal doses of a toxicant through decreased penetration of insecticides, increased sequestration/storage of insecticides, and accelerated excretion of insecticide; and Behavioural mechanism that reduces an insect's exposure to toxic compounds or allows an insect to survive in an environment that is harmful and/or fatal to the majority of insects (Liu *et al*., 2006).

2.8.1.1 Metabolic Resistance

Metabolic resistance arises because of changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal, preventing the insecticide from reaching the intended site of action. In the case of malaria vectors, three enzyme systems are believed to be involved in insecticide detoxification: esterases, mono-oxygenases and glutathione *S*-transferases (WHO, 2013). Their involvement in resistance is commonly identified by increases in the characteristic metabolites they produce. Increased activities of these enzymes have been linked to resistance development due to the fact that most insecticides can be detoxified by at least one of these enzymes in insects (Vulule *et al*., 1999).

2.8.1.2 Target-Site Resistance

Target-site resistance occurs when the protein receptor that the insecticide is designed to attack is altered by a mutation; when this happens the insecticide can no longer bind to the intended target site of the receptor and thus the insect is unaffected, or less affected by the insecticide. In the case of DDT and the pyrethroids, the mutation occurs in the sodium channel receptor, conferring what is described as *kdr* or "knockdown resistance". In the case of the organophosphates and the carbamates, the mutation occurs in the protein, acetyl-cholinesterase (AChE, a neurotransmitter), conferring what is usually referred to as *Ace-1* resistance (Liu *et al*., 2006).

Knockdown resistance (*kdr*) associated with a single point mutation in the gene encoding the voltage-gated sodium channel known as the para-type sodium channel gene, is a common mechanism of resistance to both pyrethroids and DDT. This mutation results in a leucine to phenylalanine substitution (L1014F) or a leucine to serine substitution (L1014S) in the transmembrane segment 6 of domain II of the sodium channel (Williamson *et al*., 1999). In several West African countries the predominant kdr mutation in *An. gambiae* populations is the leucine to phenylalanine substitution (L1014F) and so, it is termed *kdr* west (*kdr*-w), whilst in East African populations the leucine to serine substitution (L1014S) termed *kdr* east (*kdr*-e) is more common (della Torre *et al*., 2001; Yawson *et al*., 2004; Stump *et al*., 2004). Studies have reported the existence of individuals heterozygous for both the *kdr*-w and *kdr*-e alleles (Verhaeghen *et al.*, 2006).

The knockdown resistance (*kdr*) type of the target-site insecticide resistance mechanism is known to be widespread in some parts of Ghana including the Greater Accra Region (Adasi *et al.*, 2000; Adeniran *et al*, 2009; Kabula *et al*., 2011) and the Ashanti Region (Yawson *et al*., 2004). For example, *kdr* frequencies of 88% and 91% were observed at Dodowa and Accra respectively in the Greater Accra Region (Adasi and Hemingway, 2008). Also, a *kdr* frequency of 100% was observed in the *An. gambiae* form of *An. gambiae* s.s in Kumasi in the Ashanti Region (Yawson *et al.*, 2004).

2.8.1.3 Cross and Multiple Resistance

Cross resistance arises when resistance to one insecticide confers resistance to another insecticide even where the insect has not been exposed to the latter product (insecticide). This is seen between different classes of insecticide that share the same mode of action. Thus vectors that are resistant to pyrethroids because they possess the *kdr*-resistant gene are also resistant to DDT. Likewise, Cross-resistance can also occur between organophosphate and carbamate insecticides when resistance results from an altered AChE (Daly *et al.*, 1998). The existence of cross-resistance therefore tends to restrict the choice of alternative insecticides in situations where resistance has been detected.

Multiple resistance is a common phenomenon and occurs when several different resistance mechanisms are present simultaneously in resistant insects. The different resistance mechanisms may combine to provide resistance to multiple classes of products. It is also quite common for the contribution of different mechanisms to change over time as selection processes evolve (Daly *et al.*, 1998).

2.9 Techniques in *Anopheles* species Identification

2.9.1 Morphological Identification

A number of methods have been developed which can be employed in identifying the various species of the *Anopheles*, including the unrelated species of mosquitoes with varying degrees of specificity. The methods however, are largely based on universal characters that cut across geographical zones for a particular species and are applicable in field situations where they are used in sorting out samples prior to the use of any other techniques, if needed. Established identification methods of the anopheline mosquitoes include the use of morphological characters described by Gilles and de Meillon (1968) which was later improved upon by Gilles and Coetzee (1987). These morphological keys are still the most widely used methods for taxonomic and systematic studies of anophelines, but due to the evolution of species groups and complexes within the anopheline taxa, morphological features are becoming less useful for the identification of vectors (Beebe and Cooper, 2000).

2.9.2 Molecular Tools for Distinguishing between sibling Species of *Anopheles* Mosquito Complexes

With the evolution of sibling species of anopheline mosquito complexes which are reproductively distinct but cannot be distinguished reliably using morphological procedures alone, more alternative specific method for identification is, therefore required. Techniques like chromosome banding patterns (cytotaxonomy), allozyme analysis and cuticular hydrocarbon analysis have been employed and used in the identification of the sibling species but, with a lot of limitation until the recent discovery of species-specific polymerase chain reaction (PCR) which employs the species-specific differences in the DNA sequences of the mosquito genome (Collins *et al.*, 1987).

2.9.2.1 Principles of Polymerase Chain Reaction (PCR)

The shift from the other biochemical techniques to the present DNA-based processes that make use of the PCR allows the identification of all developmental stages of insects and with the PCR, very minute amount of template DNA is needed for analysis (Sambrook *et al.*, 1989).

This DNA–based method used in studying mosquito populations is an *in vitro* method developed for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Saiki *et al.,* 1985). The reaction utilizes the DNA polymerase enzyme known as *Taq* polymerase, which is added at the beginning of the reaction to transform the PCR into a simple and robust reaction, and subsequently automated by programmable thermal cycling devices. Other reaction constituents include the deoxynucleotide triphosphates (dNTPs), two oligonucleotide primers, DNA template and the reaction buffer. All are included in the reaction mix and the amplification is carried out under certain cycling temperatures depending on the protocol employed. Notably, the PCR reaction involves three steps of repeated cycles - heat denaturation of the template DNA to separate the two strands, followed by annealing of the primers to the complementary DNA sequence at a lower temperature, and extension of the new DNA strands by the *Taq* polymerase which is done using the free nucleotides in the reaction mixture.

The specificity of PCR depends on the base pairing by the two oligonucleotide primers to the target DNA that serves as the template. These primers are short DNA sequences that bind to the two ends of the template DNA, thereby flanking the region of interest. With repeated cycles during the amplification process, billion copies of the targeted DNA are produced.

2.9.2.1.1 PCR-based Method for the Identification of *An. gambiae* complex

A PCR method developed by Scott *et al*. (1993) is currently and widely used in the identification of sibling species of the *An. gambiae* complex. The method is based on species-specific nucleotide sequences in the ribosomal DNA (rDNA) intergenic spacer regions (IGS). This intergenic spacer region of the rDNA is a non-coding region that differs significantly among the members of the

An. gambiae complex, thus can be said to be highly specific within closely related species. Hence, its use in the identification of species-hybrids as well as, any life stage or gender of individual species with only a small part of the specimen. The method uses a cocktail of 20-base oligonucleotides to identify all the sibling species of *An. gambiae s.l.* and in Ghana where only three of the sibling species (*An.gambiae s.s., An. arabiensis and An. melas*) have been reported, three of the 20-base oligonucleotides are used in the analysis.

2.9.2.2 Principles of Restriction Fragment Length Polymerization (RFLP)

Restriction Fragment Length Polymerization (RFLP) provides a means of discriminating between similar DNA samples at the species or strain level. Analysis has shown that these strains have alleles that are different due to mutations associated with restriction fragments with different sizes. Hence, its use in distinguishing the molecular forms within the *An. gambiae s.s.* which cannot be observed in the PCR-method used for identification of single species of *An. gambiae* complex.

Favia *et al.* (1997) developed a PCR-RFLP assay, which is employed in differentiating the *An. coluzzii* and S form within the *An. gambiae s.s.* specimens This method involves multiple steps where individual mosquitoes are first identified by PCR following the protocol described by Scott *et al.* (1993). In the second step, the identified *An. gambiae s.s.* specimens are further separated into their molecular forms by either of two methods, both based on the presence of two nucleotides substitutions within the amplified rDNA region. The first is based on a PCR-RFLP method, whereby the PCR amplification of a 1.3kb rDNA is followed by digestion with restriction enzymes *Hha*1 (or *Tru* 1). This restriction enzyme cuts the 1.3 kb rDNA fragment into fragments of specific band sizes for each form (Favia *et al*., 1997). However, the second method consists of a polymorphism allele-specific PCR which is based on primers specific for each of the forms. These primers amplify fragments of different sizes within the 1.3 kb rDNA region (Favia *et al*., 2001).

Recently, Fanello *et al*. (2002) developed a new method for simultaneous identification of species of the *An. gambiae* complex and molecular forms of *An. gambiae s.s.* This method, which involved the combination of the protocols described by Scott *et a.* (1993) and Favia *et al*. (1997), is based on the observation that the 5'-GCG/C-3' restriction site for *Hha* 1 enzyme lies within the *An. gambiae*-specific fragment amplified by the method of Scott *et al*. (1993) for the S form but not in *An. coluzzii* (Favia *et al*., 1997). It is therefore possible to digest this fragment to differentiate *An. coluzzii* and S forms.

2.9.2.3 Detection of Malaria Sporozoites in *Anopheles* Mosquitoes by Enzyme-Linked Immunosorbent Assay (ELISA) Technique

In determining the infection status of suspected mosquito vector species, microscopic examination and dissection have been traditionally employed. Although, this method has proven to be accurate, it is time-consuming and labour intensive (Coleman *et al*., 2000). Research efforts in recent decades have led to the development of immunological and molecular biology tools for detecting malaria sporozoites in mosquito samples (Fabre *et al*., 2004). Among these, is the use of immunological assays, in particular the circumsporozoite enzyme-linked immunosorbent assay (CS-ELISA) which has widely replaced mosquito dissection for malaria sporozoites detection in mosquitoes (Zavala *et al*., 1982; Wirtz *et al*., 1987).

Enzyme-linked immunosorbent assays (ELISAs) were developed to detect *P. falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite (CS) proteins in malaria-infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies (Mabs) used. The ELISAs detect CS proteins, which can be present in the developing oocysts, dissolved in haemolymph, and on sporozoites present in the haemocoel or in the salivary glands. In the use of ELISA, two methods are available; the sandwich method and the competitive method (Monroe, 1984).

ELISAs can be carried out on fresh, frozen, or dried mosquitoes. If specimens are to be dried, they must be processed quickly and kept dry (stored with desiccant) to prevent microbial growth that can result in high background values. In Ghana, the CS-ELISA has been used in numerous epidemiological investigations on a wide variety of Anopheline mosquitoes for plasmodium parasites and has increased our understanding of the transmission dynamics of malaria (Appawu *et al.*, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in 4 different communities: Avakpo, Kewum, Kadjanya and Volivo/ Atrobinya communities in the Asutsuare area of Shai-Osudoku district. Shai-Osudoku district is currently one of the ten districts in the Greater Accra Region located south-east of Ghana and lies between latitude 5˚ 45ˈ South and 6˚ 05ˈ North and longitude 0˚ 05ˈ East and 0˚ 20ˈ West (Figure 3.1). It is about 40.8 kilometers away from the national capital, Accra. Shai-Osudoku being one of the largest districts in Greater Accra, is spatial and has about 170 settlements with major settlements such as Asutsuare, Kordiabe, Osuwen, Ayikuma and Dodowa, the latter being the district capital.

The district is rural but is gradually catching up with the rapid urbanization of the peripheral areas surrounding the city of Accra. It has the largest land surface area of about 721 square km in the region and is bounded on the North by the Akuapim Ranges; to the South by Tema; to the East by River Volta and the West by Ga District. The land is flat and at sea level with isolated hills. In the area of demography, the district has a population of 71,520 which consist of 34,473 representing 48.2% male and 37,047 representing 51.8% females (http://www.ghanadistricts.com/pdfs/2013_district.pdf). Most of the population lives in scattered small communities of less than 2000 people. It is completely rural with wide spread poverty-subsistence farmers, fishermen and petty traders and a handful of artisans and civil servants.

The district has 22km of the Lower Volta River running through and along the Northern to Eastern boundaries. About 45,600ha of the land is currently under cultivation with about 2,200 hectares under irrigation. Irrigation agriculture is practiced on a medium scale in the district and about 15.6 percent of crop farmers are engaged in it. This medium scale irrigated crop production has developed over the last 15-20 years in the Asutsuare area, and this development is in the Volta River flood prone area. The community thus has a network of canals created for irrigation schemes. This potentially exposes inhabitants to mosquito bites all year round making the area, a site for piloting malaria related interventions since the 1990s (Akua *et al.*, 2002).

The main produce of the farmers is rice with maize and vegetables as minor crops. Average yields are around 38 bags per acre of cultivable land. Of the net irrigable area of 1,000 hectares at Asutsuare, approximately 800 hectares have been developed by 1991 and currently being worked on by 580 registered farmers.

The district usually has two rainy seasons in a year, beginning from April to July and October to December. Malaria transmission is low but perennial, and peaks slightly during and immediately after the major rainy seasons and is lowest during the dry seasons (Afari *et al*., 1995). It is estimated that individuals near the district capital are exposed to about 7.5 infective bites in the rainy season and 98% of the infections are due to *P. falciparum* (Afari *et al.*, 1995).

Figure 3.1: Map of Ghana showing the location of Shai-Osudoku District where the study was conducted (www.google.com.gh/map).

3.2 Mosquito Sampling

3.2.1 Mosquito Larval Survey

Sampling was done across the 4 communities in Asutsuare area of Shai-Osudoku district from June to November, 2014. Larvae and pupae were collected with a ladle into plastic containers using dipping method from a wide range of breeding sites (puddles, shallow wells, gutters and rice fields) but mostly from rice fields.

The *Anopheles* mosquito larvae were identified by their typical resting position, with the body parallel to the water surface and just below the surface film. Samples from each collection point were transferred into separate labeled plastic bottles and then transported to the insectary for rearing.

3.2.1.1 Insectary Rearing of Mosquitoes

Mosquito larvae collected from the breeding sites were transferred into a well-labeled mosquito rearing plastic bowls with a large surface area covered with netting material. The batches were examined and non-mosquito species were discarded with the aid of a hand-sucking pipette. In cases where larvae of culicines were present, they were identified by their angular position on the water surface and were also removed from the larval bowls. The pupae were transferred into small plastic cups and placed in labeled adult mosquito cages made of cubical metal frames and wrapped with fine mesh material for adult emergence. The larvae were maintained in water from the collection site to prevent mortalities and were fed once a day on finely ground fish meal.

Rearing conditions for the larvae and pupae were 25 ± 2 °C and 80 ± 10% relative humidity. A 12-hour photoperiod from 06:00 hour to 18:00 hour supplied by fluorescent tubes was also maintained. Pupae were picked and separated daily from the larval bowls into small plastic cups placed in adult mosquito cages for emergence. The emerged adult females of about 2-5 days old maintained in the insectary under 80 ± 10% relative humidity were transferred from the cages into small paper cups using an aspirator, and then used for the insecticide susceptibility bioassays.

Figure 3.2: Larvae collection and rearing. **A**: Rice field at Asutsuare, an ideal breeding site for mosquitoes. **B**: Plastic bowls covered with netting material for larval rearing. **C:** Mosquito cages for pupae rearing and maintaining of emerged adults.

3.2.2 Adult Mosquito Collections

3.2.2.1 Human Landing Catches (HLC)

Mosquitoes were collected for 6 nights in randomly selected compounds by a team of 2 volunteers within each study community in November, 2014. Adult mosquitoes were collected using human landing catches (HLC) method. The all night human landing catches were made inside and outside human dwelling rooms. The two-man team of trained mosquito collectors and a supervisor were located at each collection point during the collection period. Collections were made for 50 minutes each hour from 18:00-06:00 hours and mosquitoes attempting to bite the collectors within each hour were detected using flashlight, aspirated and placed in labeled paper cups covered by mesh screen (WHO, 2003). The collectors rotated between indoors and outdoors after each period of collection, to take care of differences in individual attraction or repulsion for mosquitoes. The volunteers were made to sign an informed consent form and all the participants were given prophylaxis according to the National guideline. All participants that had malaria were sent to the nearest health facility and treated for free.

3.2.2.2 Pyrethrum Spray Catches (PSC)

In addition to larvae collection and human landing catches, mosquitoes resting indoors were also collected through PSC in the early hours of 06:00 to 09:00 throughout the study period. In each of the communities**,** ten houses were randomly selected each day and were sprayed for mosquito collection. Pyrethrum Spray Catches (PSC) was performed using Raid® pyrethrum spray and white calico sheets. Occupants of the houses marked for the spraying were asked to leave, and then the floors and other belongings in the rooms were covered with the white calico sheets after which doors and windows were closed. Spraying was done in a clockwise direction in each of the rooms with more concentration on the ceilings, holes on the walls, windows and doors on both inside and outside of the rooms. About 10-15 minutes after the spray, the sheets were then carefully carried out of the room starting from the door way picking each sheet at a time by their corners. Knocked down mosquitoes found on the sheets were picked using a pair of forceps into a well labelled petri dishes lined with a layer of damped cotton wool at the bottom and filter paper on top.

3.3 Mosquito Processing

The wild mosquitoes collected were morphologically identified and preserved dry on silica gels in well-labelled 1.5 milliliters Eppendorf tubes until needed for molecular identification of sibling species complexes, molecular forms and the determination of knockdown resistance mutation as well as the *P. falciparum* parasite sporozoite rate studies.

3.4 Species Identification

3.4.1 Morphological Identification of *Anopheles* species

Mosquitoes collected were sorted out and morphologically identified using taxonomic keys as described by Gillies and Coetzee (1987) and Gillies and de Meillon (1968).

Mosquito larvae were identified as *Anopheles* mosquito species based on the fact that they lie parallel to the water surface and do not possess siphon (WHO, 2003). Adult mosquitoes were identified as *Anopheles* species using the markings on the palps, the banding and speckling on the legs and the distinctive pattern of blocks of dark and pale scales on the vein of the wings, particularly along the costa.

Anopheles gambiae s.l. were identified by the presence of five pale spots on the costal margin of the wings, anal vein coloration with three white spots, a dark apical fringe and white speckled (or spots in the median part) tibia ornamentation.

Anopheles funestus were distinguished by the presence of four pale spots on the costal margin of the wings, dark fringe scales at the margin of the anal wing vein, three white narrow rigs on the maxillary palps, and the absence of banding patterns on the dark hind tarsus.

The costal margin of the *An. coustani* wing on the other hand, had only two pale spot. Other external morphological features used to identify *An. coustani* included: the basolateral area of clypeus had a patch of dark laterally projecting scales, hind tarsomeres 4 and 5 (Ta-III4, 5) were entirely pale. Hind tarsomere 1 (Ta-III1) was broadly pale at base and apex, hind tarsomere 2 (Ta-1112) pale over approximately apical half, hind tarsomere 3 (Ta-III3) was dark at base only or entirely pale and lastly the abdominal sternum VII had a group of postero-median dark scales.

For *An. pharoensis*, the hind tarsomeres 3 and 4 (Ta-111 3, 4) were pale over apical half; hind tarsomere 5 (Ta-111 5) was entirely pale; and abdominal terga was densely covered with broad pale scale.

The morphologically identified wild-caught adult *An. pharoensis, An. funestus, An. coustani* and *An. gambiae s.l.* were preserved dry on cotton in 1.5 milliliters (ml) Eppendorf tubes containing silica gel. The Eppendorf tubes were then labelled according to *Anopheles* mosquito species, date, time and site of collection. Based on these data, each *Anopheles* species was given a special identification (ID) number, which remained constant during the study period.

3.4.2 Molecular Identification of *An. gambiae s. l.* sibling Species

3.4.2.1 Genomic deoxyribonucleic acid (DNA) extraction

Genomic DNA from *An. gambiae* mosquitoes were extracted using the Bender buffer method described by Collins *et al*. (1987) with slight modifications. The legs and abdomen of female *An. gambiae s.l.* were placed in 1.5 ml Eppendorf tube containing 100 µl Bender buffer(0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA pH 9.1, 0.5% SDS and then autoclaved). The buffer was first preheated at 65°C, then using a sterile handheld plastic pestle, the specimens were homogenized followed by incubation at 65°C for 30 minutes. Then 15 µl of pre-chilled 8 M potassium acetate was added to the homogenate and mixed well by vortexing for 5 minutes. This was followed by incubation on ice for 30 minutes after which it was centrifuged at 14,000 rpm for 10 minutes. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube and 250 µl of pre-chilled absolute ethanol added; the tube was inverted several times in order to mix well and precipitate the DNA. This was followed by incubation at -20°C for two hours. The DNA was pelleted by centrifugation at 14,000 rpm for 5 minutes and the supernatant was discarded. 200 µl of 70% ethanol was added to the pellet in order to wash the DNA, the tube gently swirled and the DNA re-pelleted by centrifugation at 10,000 rpm for 5 minutes. The supernatant was again discarded and the tube opened and inverted over a dry paper towel and left overnight to dry by evaporation. The dried DNA pellet was re-dissolved in 100 µl of sterile double distilled water and stored at -20°C until the time of use.

3.4.2.2 PCR Identification of sibling Species of the *An. gambiae s.l.*

The polymerase chain reaction (PCR) amplification protocol of Scott *et al.* (1993) designed for the species identification of single specimen of the *An.* gambiae *s.l*. was employed in the present study. The genome DNA extracted from the legs and abdomen of female mosquito samples using the process described above were used for the PCR analysis. The amplification process used one universal primer and three species-specific primers each of 20 bases (Table 3.1). The universal primer designated UN anneals to the same position on the ribosomal DNA (rDNA) of each of the five species of *An. gambiae s.l.* namely, *An. gambiae s.s.*, *An. arabiensis*, *An. melas, An. merus* and *An. quadriannulatus*. The other three primers used in the amplification process were species specific and were the reverse primer GA, which anneals to the *An. gambiae s.s* template; ME, which anneals to both the *An. merus* and *An. melas* templates; and AR, which anneals to the *An. arabiensis* template. There was another primer in the protocol of Scott *et al.* (1993) designated QD, which anneals to the *An. quadriannulatus* template. The sizes of the amplified products were 153 base pairs (bp) for *An. quadriannulatus*, 315 bp for *An. arabiensis*, 390 bp for *An. gambiae* s.s, 464 bp for *An. merus,* and 466 bp for *An. melas* sibling species. In this work, UN, GA, ME and AR primers were used as *An. gambiae* s.s, *An. arabiensis* and *An. melas* are the only *An. gambiae s.l*. reported in Ghana (Appawu *et al.*, 1994, 2004; Appawu, 2005; Kristan *et al.*, 2003; Yawson *et al.*, 2004).

Table 3.1: Species-specific primer sequences of *An. gambiae s.l.* with respective melting temperatures (Tm) and expected band sizes of amplified DNA products (Scott *et al.*, 1993).

Primer	Sequences (5' to 3')	Tm(°C)	Band size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	58.3	468
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390
ME	TGA CCA ACC CAC TCC CTT GA	57.2	464
AR	AAG TGA CCT TCT CCA TCC TA	47.4	315
QD	CAG ACC AAG ATG GTT AGT AT	42.7	153

3.4.2.3 Polymerase Chain Reaction (PCR) Amplification

Each reaction mix of 20 µl contained 1x PCR buffer (Invitrogen, USA), 1.50 mM $MgCl_2$ (Invitrogen, USA), 0.4mM each of the four (4) deoxyribonucleotide triphosphates (dNTPs) (which are the nucleotides deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP)), 10 µM each of oligonucleotide primers and 0.5 U of DNA *Taq* polymerase enzyme. 1 µl of extracted mosquito DNA was used as template in the PCR amplification. Sterile double distilled water was then added making the solution 20 µl. The reaction mix was thoroughly mixed and then centrifuged briefly for 20 seconds before being placed in a thermocycler. The temperature profile/cycling conditions for the PCR amplification were 94 °C for 3 minutes (initial melt) followed by 35 cycles of 94 °C for 30 seconds (denaturation), 50 °C for 30 seconds (annealing), 72 °C for 1 minute (extension), and a final cycle of 72 °C for 7 minutes. For each set of reactions, a negative control, which had no DNA template, was used. The amplification reactions were conducted using a polymerase chain reaction Express Thermal Cycler (MJ Research Inc., USA).

3.4.2.4 Gel Electrophoresis

Polymerase chain reaction (PCR) products were analyzed using gel electrophoresis. Here, eight microlitres (8µl) of each PCR product were added to 1µl of orange G, a gel-loading dye and electrophoresed in 2.0% agarose gel stained with 0.5 µg/ml ethidium bromide. Electrophoresis was carried out in 1x Tris Acetate EDTA (TAE) buffer at 100 volts (V) for an hour. The gel was then visualized and photographed over an ultraviolet Polaroid (UVP) dual intensity trans-illuminator at short wavelength using a Polaroid direct screen instant camera fitted with an orange filter, a hood and a Polaroid Type 667 film. The film was processed as recommended by the manufacturer (Polaroid Inc. USA). Photographs were also taken using a Kodak direct screen instant camera connected to a computer. The sizes of the PCR products were estimated by comparison with the mobility of a standard 100 bp ladder (sigma, USA) for the identification of the sibling species of the *An. gambiae s.l.*

3.4.2.5 Identification of *An. gambiae s. s.* Molecular Forms

The identification of *An. gambiae* S form and *An. coluzzii* was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocol of Fanello *et al.* (2002) with slight modifications. This protocol involves a combination of the protocols designed by Scott *et al.* (1993) and Favia *et al.* (1997). It is based on the fact that GCG^C restriction site for *Hha* 1 enzyme (Favia *et al.*, 1997) lies within the *An. gambiae s.s*. species specific fragment amplified by Scott *et al.* (1993) which makes it possible to digest this fragment directly in order to make a distinction between molecular *An. gambiae* S form and *An. coluzzii*.

The procedure is based on the PCR reaction described in sections 3.4.2.2 to 3.4.2.3 by Scott *et al.* (1993). The RFLP analysis involved the digestion of amplified PCR products from mosquito species, which have been identified as *An. gambiae s.s.* with restriction enzyme *Hha* I. The digestion was done in a total reaction mix of 15 µl volume containing 0.2 µl of *Hha* I enzyme, 1.5 µl of digestion buffer M, 12 µl of PCR product and 1.3 µl of double distilled water to make up the 15 µl volume. The incubation was done at 37 °C for five (5) hours using a PCR Express Thermal Cycler (MJ Research Inc., USA). Digested fragments were then electrophoresed using an ethidium bromide stained 2% agarose gel and photographed under ultraviolet light illumination as described in section 3.4.2.4.

The *An. gambiae* S form digestion profile is characterized by two fragments: 257 bp and 110 bp long, which results from the presence of the *Hha*1 restriction site. *An. coluzzii* does not have this restriction site and thus has a single 367 bp fragment.

3.5 Determination of the Indices of Malaria Transmission

3.5.1 Detection of *P. falciparum* Sporozoites Infectivity in *An. gambiae s. l.* using Enzyme-Linked Immunosorbent Assay (ELISA)

The head and thorax of female *An. gambiae s.l*. samples were tested for the presence of *P. falciparum* circumsporozoite protein (*Pf*CSP) following the protocol of Wirtz *et al.* (1987). For plasmodium mosquito antigen preparation, the head and thorax were homogenized in 50 µl of grinding buffer in a 1.5ml Eppendorf tube using a handheld plastic pestle after which the pestle was rinsed with 150 µl of grinding buffer into the tube making the volume of each homogenate up to 200 µl. The homogenized samples were then kept at -20°C until time of use.

Following the protocol described by Wirtz *et al.* (1987), flexible 96-well micro-titre plates (Becton Dickinson and Co. Oxnard, CA, USA) were coated with captured monoclonal antibodies (Kierkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) overnight at 4°C. The coating was done such that each well contained 50 µl of the monoclonal antibodies. The plates were flipped empty, banged and then incubated at 200µl/well with blocking buffer (BB) at room temperature for 60 minutes. After this time, the plates were flipped empty, banged and then washed once at 200µl/well with washing buffer (PBS/Tween-20). This is followed by incubation with 50 µl/well aliquots of the homogenates (test mosquito extracts) at room temperature for 120 minutes. Plates were washed twice with excess washing buffer (200µl/well), flipped empty, banged and subsequently incubated with a monoclonal antibody peroxidase conjugate (0.5µg/50µl BB/well) for 60 minutes in darkness. The plates were emptied, rinsed three times with washing buffer, banged, and then incubated with the substrate solution (100µl/well) for 30-60 minutes at room temperature. Reading of the plates started after 30 minutes through to 60 minutes, following which the ELISA test results were determined visually based on colour change in the wells. Wells which changed from colourless to green were deemed positive for PfCSP. Those which remained colourless were regarded to be negative for PfCSP.

The micro-titre plate was coated such that wells F11 and F12 contained negative controls, wells G11 and G12 contained the positive controls, wells H11 and H12 were blank, and the rest of the wells contained the test samples. The positive control samples were obtained from the Parasitology/Entomology Department, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon. The negative controls on the other hand were made up of homogenates of male mosquito while for the blanks, blocking buffer were used. Figure 2.0

shows a 96 well plate showing the arrangement for ELISA test to determine the presence of *P. falciparum* circumsporozoite antigens in *An. gambiae s.s.* mosquito samples.

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

Figure 3.3: Illustration of a 96 well plate showing the arrangement for enzyme-linked immunosorbent assay (ELISA) technique to determine the presence of *P. falciparum* circumsporozoite antigens (*Pf*CSP) in *An. gambiae s.s.*

Legends: (*) represent the test samples, (+) represent the positive control samples, (-) represent the negative control samples and B represent the blanks.

3.5.2 Determination of Man biting rate (MBR) and Entomological Inoculation Rate (EIR)

The man biting rates (MBRs) were determined from mosquitoes obtained through human landing catches (HLC), as the number of *Anopheles* biting per man per night or hour as described in the protocol of the WHO (2003). The sporozoite rates were inferred from the proportion of man-biting *Anopheles* mosquito samples that tested positive for *P. falciparum* circumsporozoite protein (PfCSP) determined by the ELISA procedure. The EIRs were calculated as the product of the man-biting rates (MBRs) and the sporozoite rates at a given season and place.

3.6 WHO Insecticide Susceptibility Assays

3.6.1 Insecticide Susceptibility Test

Susceptibility tests were conducted to determine the proportion of the vector population that was physiologically resistant to 0.75 % permethrin, 0.05 % deltamethrin, 0.05 % lambda-cyhalothrin, 0.15 % cyfluthrin, and 0.5 % etofenprox pyrethroids insecticides. The susceptibility of *Anopheles* species obtained from the field-collected larvae were tested using the manual designed by the World Health Organization (2013) for testing insecticide resistance in the field.

Non-blood fed adult female *An. gambiae*, aged 2 to 5 days old were collected from mosquito cages using an aspirator and placed in paper cups covered with mesh screen. Twenty mosquitoes were transferred to a special plastic holding tube lined with insecticide free paper. A plastic tube lined with filter paper impregnated with mineral oil (used as control) was connected with the holding tube and twenty mosquitoes were transferred to the tube through a hole in the slide between the 2 tubes; also the same number of mosquitoes was transferred to plastic tube with 0.75 % permethrin-impregnated filter paper. The slide was closed and the exposure and control tubes were allowed to stand upright for 60 to 80 minutes depending on the rate of knockdown. The filter papers impregnated with mineral oil and permethrin were held in place by silver and copper rings respectively. Separate sucking tubes were used to transfer mosquitoes to the exposure and control tubes to avoid contamination. During the exposure period, the number of mosquitoes knocked down (KD) were recorded after 5, 10, 15, 20, 30, 40, 50 and 60 minutes. Mosquitoes were then transferred into the holding tube and fed on 10% glucose solution. Where the number of mosquitoes knocked down after 60 minutes were less than 80%, knocked down was recorded for further 20min (i.e. after 60thmin) in the holding tube. Mosquitoes were maintained in the holding tubes for 24 hours (the recovery period) and at the end of this period, the number of dead mosquitoes were counted and recorded. Mosquitoes were considered to be alive if they were able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they have lost legs or wings, were considered moribund and were counted as dead. The experiment was conducted at a temperature of 25°C ± 2 °C. Mosquitoes killed by contact with the permethrin insecticide impregnated paper and those killed in the control tubes at the end of the recovery period (24 hours post exposure) were counted. The experiment was replicated in order to calculate percentage (%) mortality in the exposure and control tubes. The same experimental procedure was repeated for 0.05 % deltamethrin, 0.05 % lambda-cyhalothrin, 0.15 % cyfluthrin, and 0.5 % etofenprox impregnated filter papers to determine the susceptibilities of the mosquitoes to these insecticide treated papers. Four (4) replicates were used for 0.05 % deltamethrin, 0.05 % lambda-cyhalothrin and 0.5 % etofenprox whilst three (3) replicates were conducted for 0.75 % permethrin and 0.15 % cyfluthrin due to small number of mosquito samples available.

Figure 3.4: Insecticide susceptibility tests for adult mosquitoes using the WHO protocol

3.6.2 Determination of Knockdown resistance (*kdr*) Alleles

Molecular characterization for the detection of leucine to phenylalanine knockdown resistance (*kdr)* genes was carried out on identified *An. gambiae s.s*. samples using the polymerase chain reaction (PCR) amplification method of Martinez-Torres *et al.* (1998) with slight modifications. Genome deoxyribonucleic acid (DNA) used for the PCR amplification process was extracted using the Bender buffer method described in section 3.4.2.1. Primers specific for *kdr* resistant strain of *An. gambiae s.s.* designated as Agd1 and Agd3, and those specific for susceptible strain designated as Agd2 and Agd4 were used (Table 3.2).

A 20 µl reaction mixture containing 4.1 µl of double distilled water, 12.5 µl of Go Taq, 1 µl of extracted DNA, 0.6 µl of 10 µM each of the primers Agd1, Agd2, Agd3 and Agd4 was prepared. The reaction mixture was thoroughly mixed and briefly centrifuged before being placed in the thermocycler for amplification. The PCR amplification conditions include: an initial denaturing at 94 °C for 3 minutes followed by 40 cycles of 94 °C for 30 seconds, 48 °C for 1 minute 50 seconds and 72°C for 2 minutes. A final extension cycle at 72 °C for 5 minutes was performed followed by cooling at 4 °C.

Amplified products of 8 µl were loaded onto a 2% agarose gel, stained with ethidium bromide, submerged in 1X TAE buffer and subjected to electrophoresis at 100 V for one hour after which it was visualized under UV light. The amplified fragment sizes were compared with the mobility of a standard 100 bp ladder (Invitrogen, USA) for the detection of the presence of the leucine to phenylalanine knockdown resistance (*kdr)* genes in the molecular forms of the *An. gambiae s.s*. The *kdr* genotypes of both the susceptible and resistant samples were then noted. Positive control of a known susceptible strain of *An. gambiae s.s.* was used, including a negative control that contained only the reaction mixture. Expected band sizes for susceptible, resistant and control samples were 137 bp, 195 bp and 293 bp respectively.

Table 3.2: Sequences of primers used in the *kdr* studies with their respective melting temperature (Martinez-Torres *et al.*, 1998).

Primer	Sequence (5' to 3')	Tm (°C)
Agd1(forward)	ATA GAT TCC CCG ACC ATG	64.5
Agd2 (reverse)	AGA CAA GGA TGA TGA ACC	45.6
Agd3 (reverse)	AAT TTG CAT TAC TTA CGA CA	45.2
Agd4 (forward)	CTG TAG TGA TAG GAA ATT TA	60.0

3.7 Data Analysis

Data were entered into Excel spreadsheet and imported into GENSTAT for analysis. The observed difference in man biting rate and sporozoite rate within communities were analyzed using analysis of variance (ANOVA). All statistical tests and generalizations were done by assuming 95 % confidence interval and 5 % level of significance.

Indices of malaria transmission that were determined include: the sporozoite rate - the proportion of *Anopheles* mosquitoes with sporozoites in their salivary gland detected by *Pf*CSP-ELISA; the man biting rate - the number of *Anopheles* mosquito bites per collector per night; the entomological inoculation rate (EIR) - the product of the biting rate and the sporozoite rate per unit time estimated from human-landing catches (HLC).

Insecticide susceptibility status were defined based on the WHO criteria for characterizing susceptibility, where susceptibility is defined by mortality rates greater than 98% 24 hour post-exposure and resistance is defined by mortality rates less than 80 % 24 hour post-exposure. The results of the insecticide susceptibility tests were also analyzed for dose/response relationship using regression probit analysis (Finney 1971). The linear log-time probit model was used to estimate knockdown times (KDTs), that is, time in minutes at which 50 % and 95 % of the mosquito populations assayed were knocked down. The KDT values were designated KDT_{50} and KDT_{95} respectively

CHAPTER FOUR

4.0 RESULTS

4.1 Species Composition in the Study Area

A total of 3,410 adult female mosquitoes were caught in the study area. This included 12.20 % (416) *An. gambiae s.l.*, 0.16 % (5) *An. funestus,* 1.31% (41) *An. pharoensis*, 0.61 % (19) *An. coustani*, 78.31 % (2,459) *Culex spp,* 8.50 % (267) *Mansonia spp* and 6.46 % (203) *Aedes spp.* Out of the total number of mosquitoes collected, 3,322 were caught using Human landing catches (HLC) and the remaining 88 were caught using Pyrethrum spray catches.

Table 4.1: Species Composition of Mosquitoes in the Study Sites

Species	Kewum	Kadjanya	Avakpo	Volivo/Atrobinya	Total
An. gambiae s.l.	128	99	108	81	416 (12.20 %)
An. funestus	3	2	0	0	5 (0.16 %)
An. pharoensis	0	7	12	22	41(1.31 %)
An. coustani	3	0	9	7	19 (0.61 %)
Culex spp	261	240	924	1034	2459 (78.31 %)
Mansonia spp	87	25	104	51	267 (8.50 %)
Aedes spp	20	15	77	91	203 (6.46 %)
Total	**502**	**388**	**1234**	**1286**	**3410**

Out of 502 adult female mosquitoes collected at Kewum, *Culex spp* was the dominant species accounting for 51.99 % (216), followed by *An. gambiae s.l.* 25.50 % (128). While *Mansonia spp*, *Aedes spp*, *An. coustani*, and *An. funestus* accounted for 17.33 %, 3.98 %, 0.60 % and 0.60 % respectively (Figure 4.1).

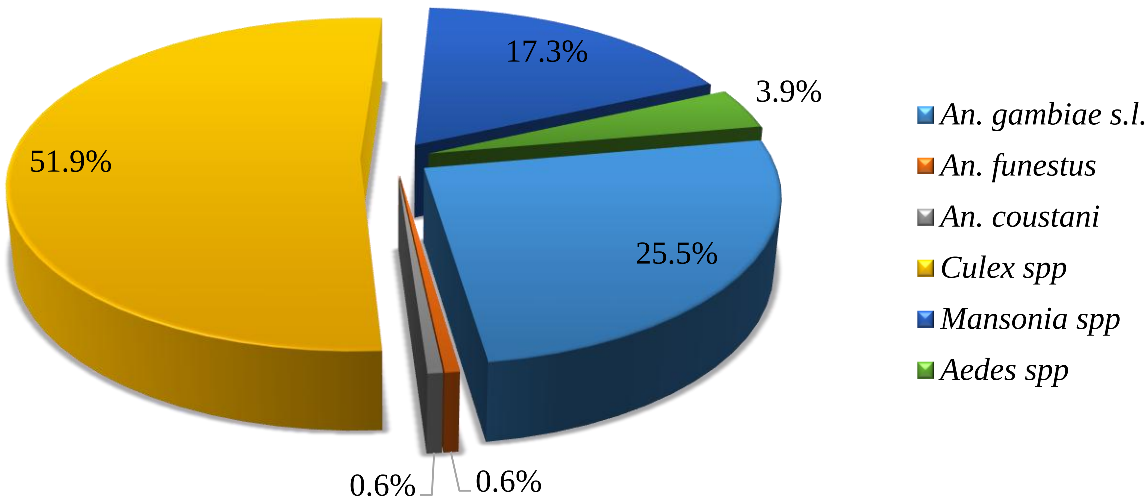


Figure 4.1: Species composition of mosquitoes collected during the sampling period at Kewum

At Kadjanya, the most abundant mosquito species was *Culex spp* which formed 61.86 % (240) out of the 388 total mosquitoes collected. This was also followed by *An. gambiae s.l.* 25.52 % (99). The rest were *Mansonia spp*, *Aedes spp*, *An. pharoensis*, and *An. funestus* forming 6.44 %, 3.87%, 1.80 % and 0.52 % respectively (Figure 4.2).

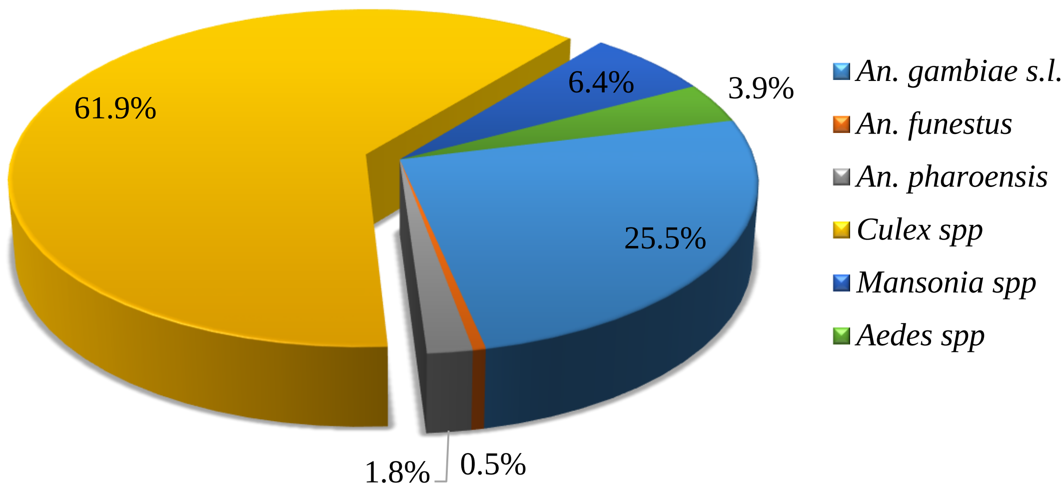


Figure 4.2: Species composition of mosquitoes collected during the sampling period at Kadjanya

At Avakpo, *Culex spp* and *An. gambiae s.l.* were the most dominant species accounting for 74.88% (924) and 8.75 % (108) respectively out of 1,234 total mosquitoes caught. This was followed by *Mansonia spp*, *Aedes spp*, *An. pharoensis*, and *An. coustani* which accounted for 8.43 %, 6.24 %, 0.97 % and 0.73 % respectively (Figure 4.3)

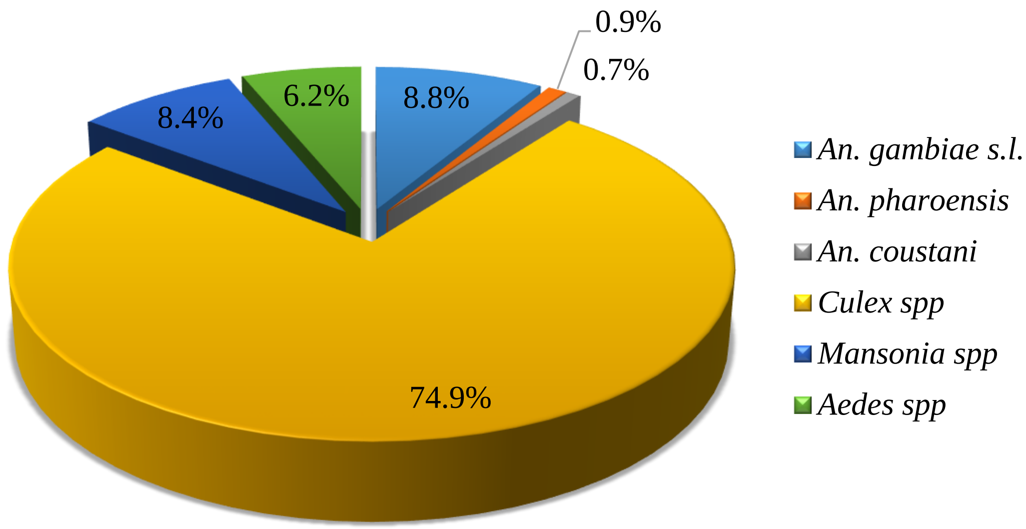


Figure 4.3: Species composition of mosquitoes collected during the sampling period at Avakpo

Out of 1,286 adult female mosquitoes caught at Volivo/Atrobinya, *An. gambiae s.l.* was the third most abundant species representing 6.30 % (81). While the most abundant was *Culex spp* 80.40 % (1034). The rest were *Aedes spp*, *Mansonia spp*, *An. pharoensis*, and *An. coustani which* represented 7.08 %, 3.97 %, 1.71 % and 0.54 % respectively (Figure 4.4).

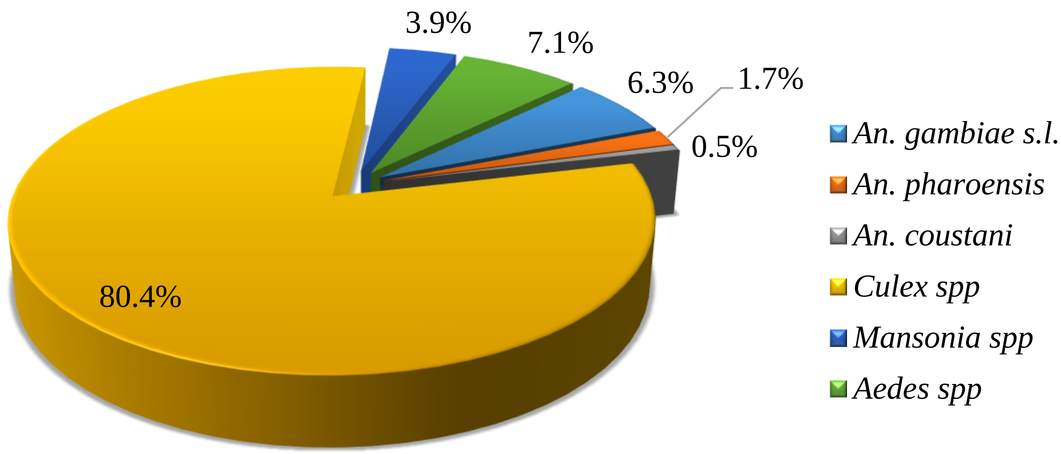


Figure 4.4: Species composition of mosquitoes collected during the sampling period at Volivo/Atrobinya

4.1.1 PCR Identification of sibling Species of *Anopheles gambiae s.l.*

In total, genomic DNAs were extracted from 185 adult female *An. gambiae s.l.* for molecular analysis. These 185 *An. gambiae* mosquitoes comprised 100 adults collected through human landing catches (HLC) and 85 adults raised in the insectary from field collected larvae. PCR amplification for species identification was successful for all the 185 *An. gambiae s.l.* processed. All successful amplicons were *An. gambiae s.s.* (length of amplified sequence was 390 bp). No other member of the *An. gambiae* species complex was detected in the study sites (Figure 4.5).

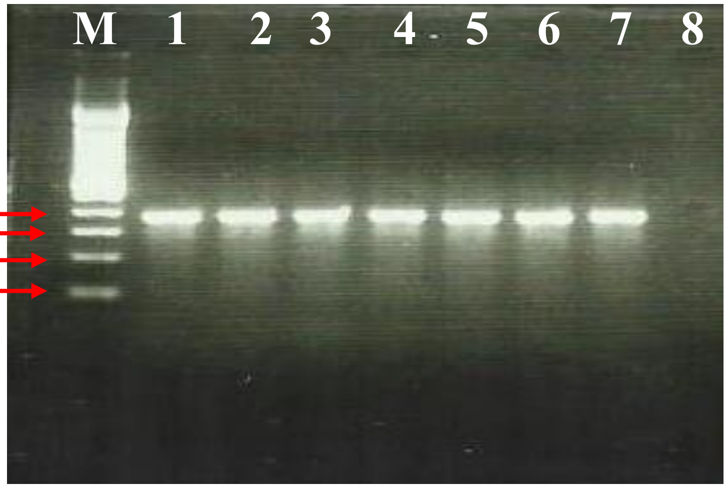
400
300
200
100


Figure 4.5: Ethidium bromide-stained 2.0 % agarose gel electrophoregram of PCR amplified rDNA_ sequences of *An. gambiae s.s.* Lane M: 100 bp ladder; lane 1: *An. gambiae s.s.* positive control; lanes 2- 4: *An. gambiae s.s*. and lane 8: negative control.

4.1.2 Identification of *Anopheles gambiae s.s.* Molecular Forms

All the 185 identified *An. gambiae s.s*. were further analyzed by PCR-RFLP to determine their molecular forms. Out of the total, 1.08 % (2) were identified as *An. gambiae* S forms while 98.92 % (183) were *An. coluzzii* forms. No hybrid was identified in the study sites. Identification was done by comparing their band sizes with that of the molecular weight 100bp ladder. *An. coluzzii* were characterized by a single fragment of 367bp while the *An. gambiae* S forms have two fragments, 110bp and 257bp (Figure 4.6).

At Kewum, 97.78 % were *An. coluzzii* while 2.22 % were S forms whereas at Kadjanya, 97.83 % were *An. coluzzii* and 2.17 % were S forms. However, at both Avakpo and Volivo/Atrobinya, all the analyzed *An. gambiae s.s*. were only *An. coluzzii* (100 %), no S forms were found (Figure 4.7).

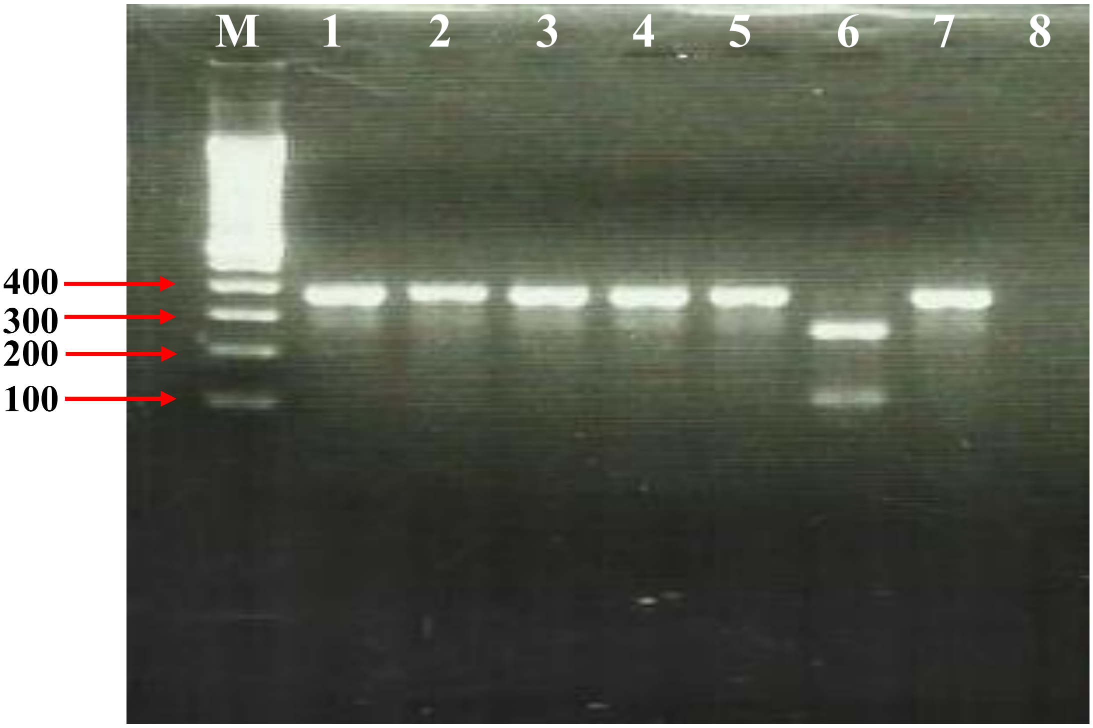


Figure 4.6: Ethidium bromide-stained 2.0 % agarose gel electrophoregram of rDNA_PCR bands produced after the identification method for *An. gambiae s.s*. Lane M: 100bp ladder; Lanes 1-5 and 7: *An. coluzzii*; Lane 7: molecular S forms of *An. gambiae* s.s; Lane 8: negative control.

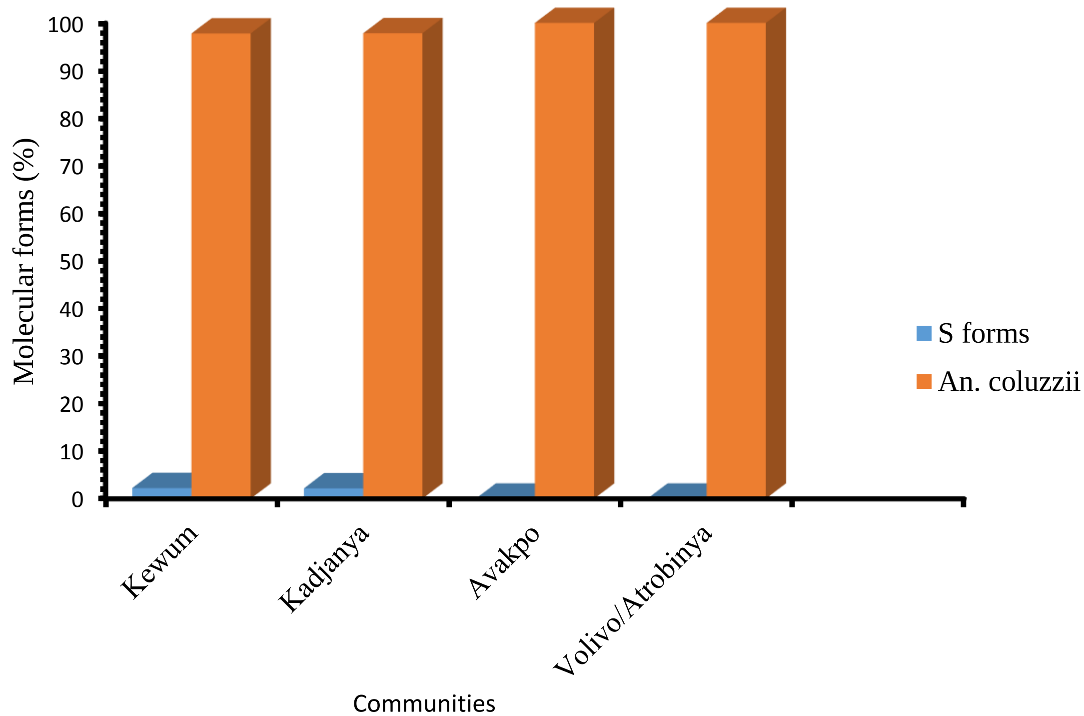


Figure 4.7: Distribution of *An. coluzzii* and *An. gambiae* S forms of *An. gambiae s.s.* across the communities

4.2 Transmission Indices

4.2.1 Man Biting Rate of *An. gambiae s.s.* in the Study Area

A total number of 380 *An. gambiae s.s.* were collected by human landing catches in the study area, with Kewum having the highest number of collection. At Kewum, the overall biting rates for *An. gambiae s.s.* collected using human landing for the entire period of study was 15.9 bites per man per night (b/m/n).

At Kadjanya, the estimated biting rates for the entire period of study was found to be 6.9 b/m/n. This was followed by 4.2 b/m/n for Avakpo and then 3.5 b/m/n for Volivo/Atrobinya (Figure 4.8).

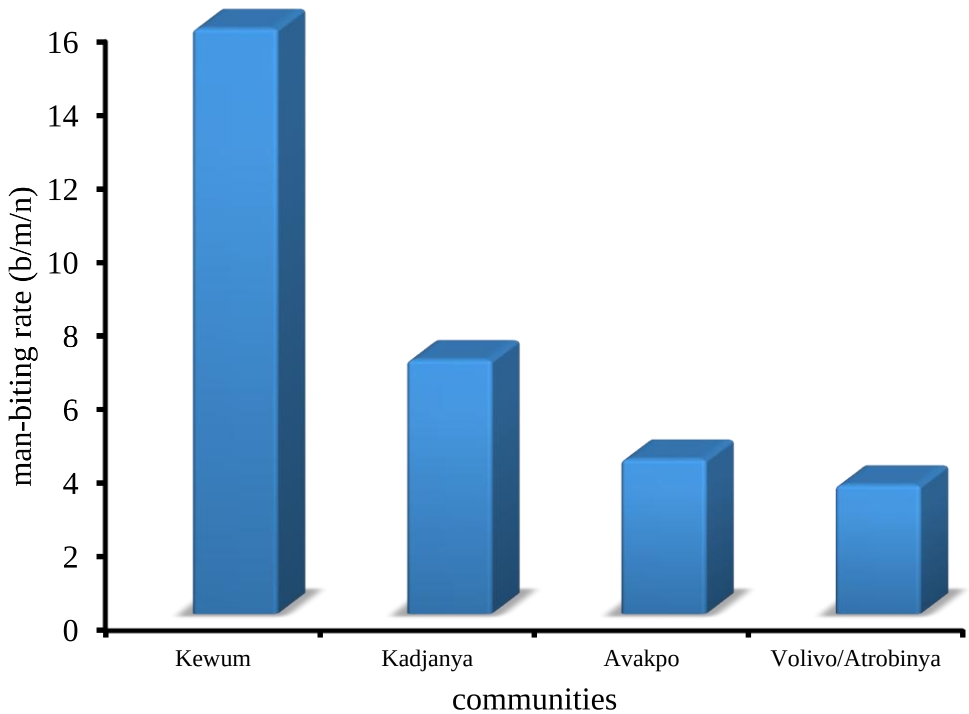


Figure 4.8: Man-biting rate of *An. gambiae s.s* during the sampling period across the communities

4.2.1.1 Indoor and Outdoor Man-Biting Rate of *An. gambiae s.s.*

With a total number of 380 *Anopheles gambiae s.s.* collected using human landing catches in the study area, 249 were collected indoor while 131 collected outdoor. Hence, biting rates of *An. gambiae s.s.* were generally higher indoor in all the study communities except for Volivo/Atrobinya where the outdoor biting rate was higher than the indoor. At Kewum, an indoor biting rate of 21.3 b/m/n was recorded. This was followed by an indoor biting rate of 8.3 b/m/n, 7.3 b/m/n, and 2.7 b/m/n for Kadjanya, Avakpo and Volivo/Atrobinya respectively.

Similarly, an outdoor biting rate of 10.5 b/m/n was recorded for Kewum, 5.5 b/m/n for Kadjanya, 1.2 b/m/n for Avakpo and 4.2 b/m/n for Volivo/Atrobinya (Figure 4.9).

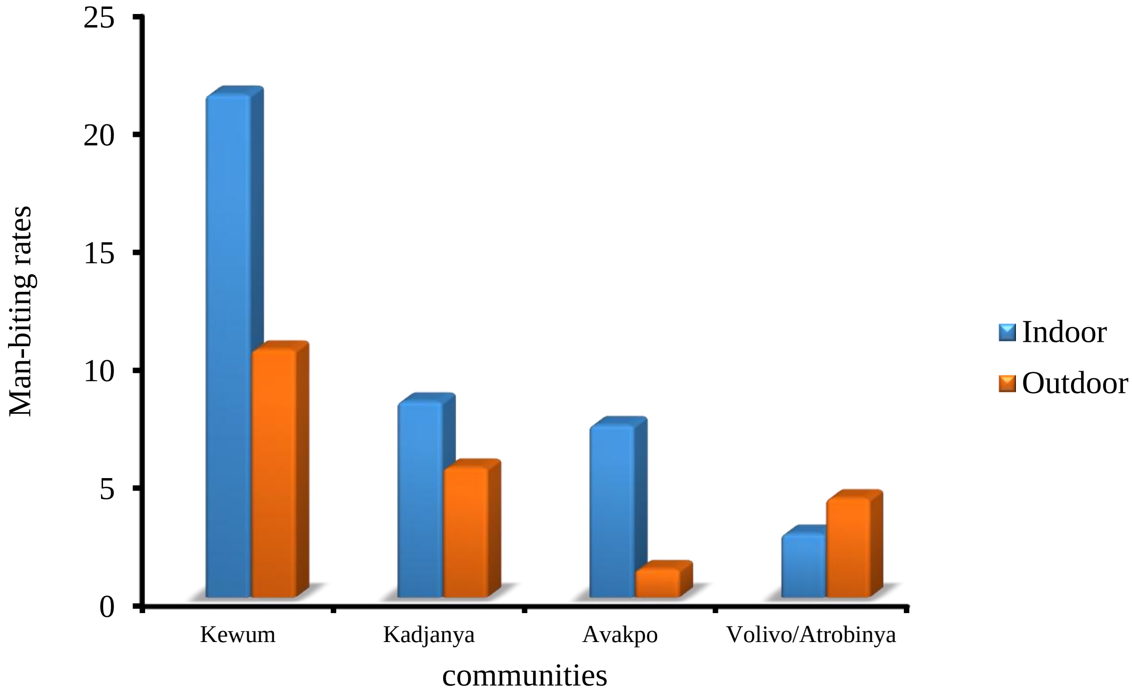


Figure 4.9: Indoor and Outdoor biting rates of *An. gambiae s.s.* across the communities

4.2.1.2 Hourly biting pattern of *An. gambiae s.s.* in the study sites

The pattern of indoor and outdoor night-biting across the four communities showed that biting rose progressively through the night and peaked during the second, third and fourth quarters of the night. In all the study sites, no biting activity was recorded in the early evening of 18:00 – 19:00 hours throughout the entire study period.

At Kewum, three major peaks were observed indoor during the second, third and fourth quarters of the night at 22:00 – 23:00, 00:00 – 01:00 and 04:00 – 05:00 hours respectively while the outdoor night-biting peaked at 23:00 – 00:00 and 03:00 – 04:00 hours with no biting activity in the early hours of the evening from 18:00 – 21:00 hours. (Figure 4.10).

At Kadjanya, two major peaks were observed during the indoor and outdoor night-biting. For indoor, biting rose progressively through the night and peaked at 00:00 – 01:00 and 02:00 – 03:00 hours. Whilst for the outdoor, peak biting was observed in the early evening at 19:00 – 20:00 hours, falling and peaking again at 01:00 – 02:00 hours (Figure 4.11).

The indoor biting at Volivo/Atrobinya rose steadily throughout the night and peaked at 03:00-04:00 hours while two major peaks were observed outdoor during the second and fourth quarters of the night at 23:00- 00:00 and 03:00- 04:00 hours respectively (Figure 4.12).

At Avakpo, the indoor biting peaked during the second and third quarters of the night at 21:00-22:00 and 02:00 – 03:00 hours. For the outdoor, no biting activity was observed in the early hours of the night till after 22:00 hours, then biting rose steadily and peaked at 04:00 – 05:00 hours (Figure 4.13).

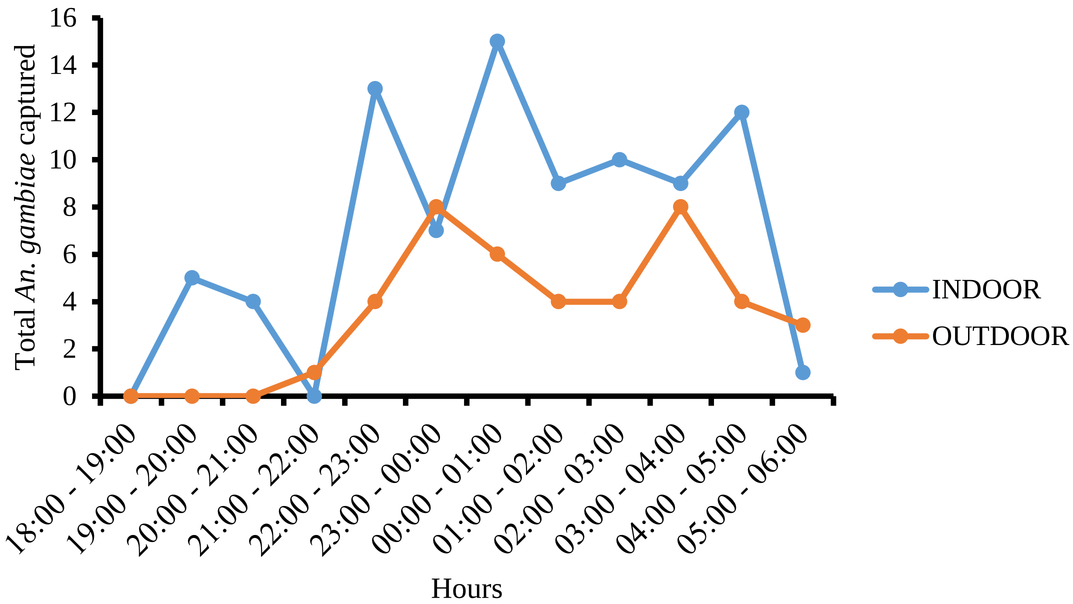


Figure 4.10: Hourly biting patterns of *An. gambiae s.s.* by Human Landing Catches during the study period at Kewum

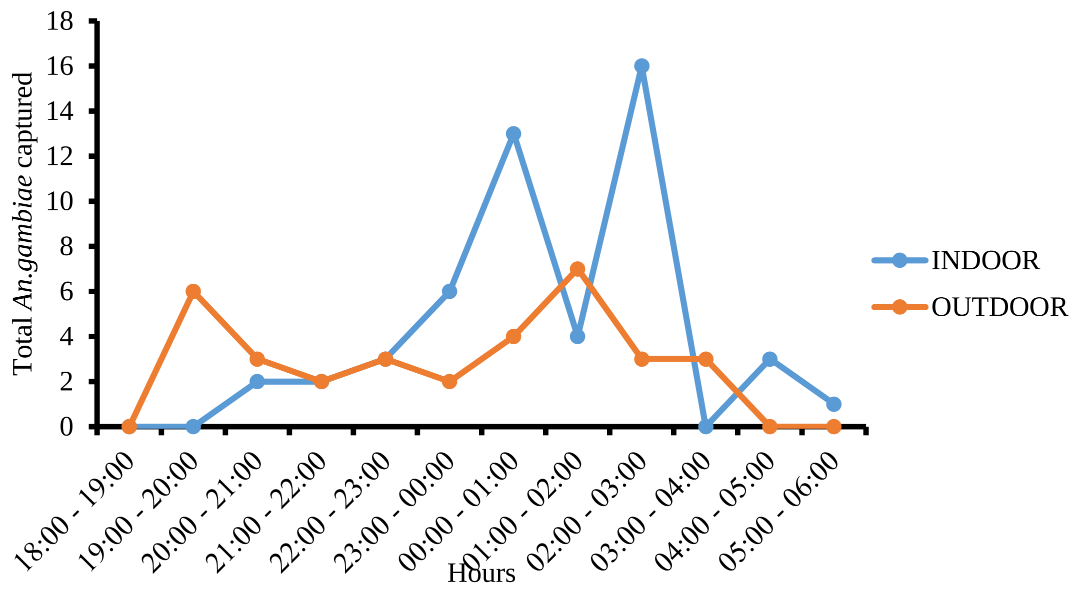


Figure 4.11: Hourly biting patterns of *An. gambiae s.s.* by Human Landing Catches during the study period at Kadjanya

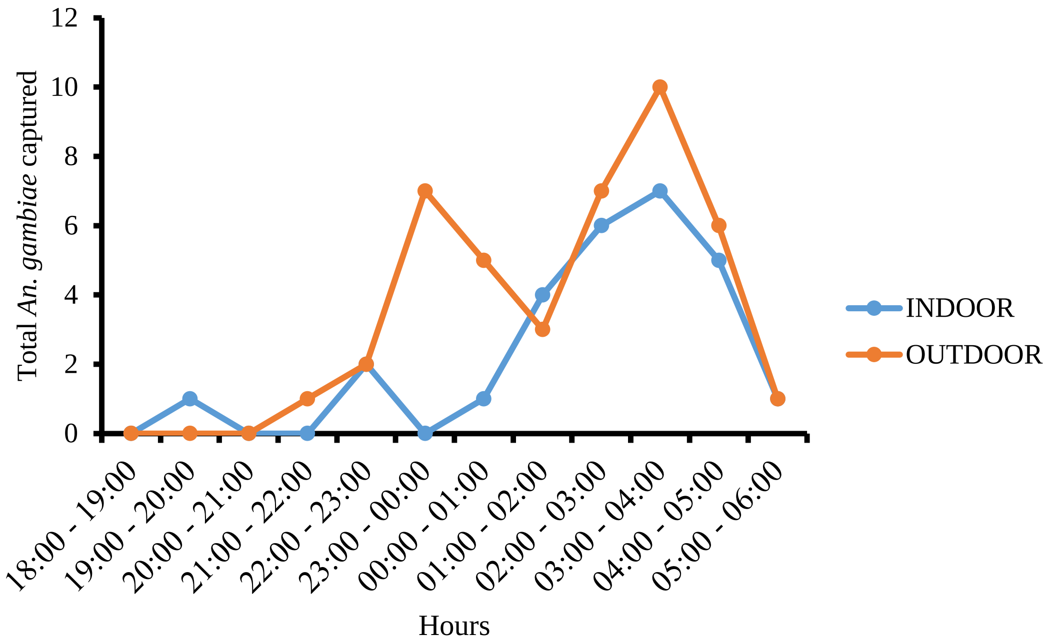


Figure 4.12: Hourly biting patterns of *An. gambiae s.s.* by Human Landing Catches during the study period at Volivo/Atrobinya

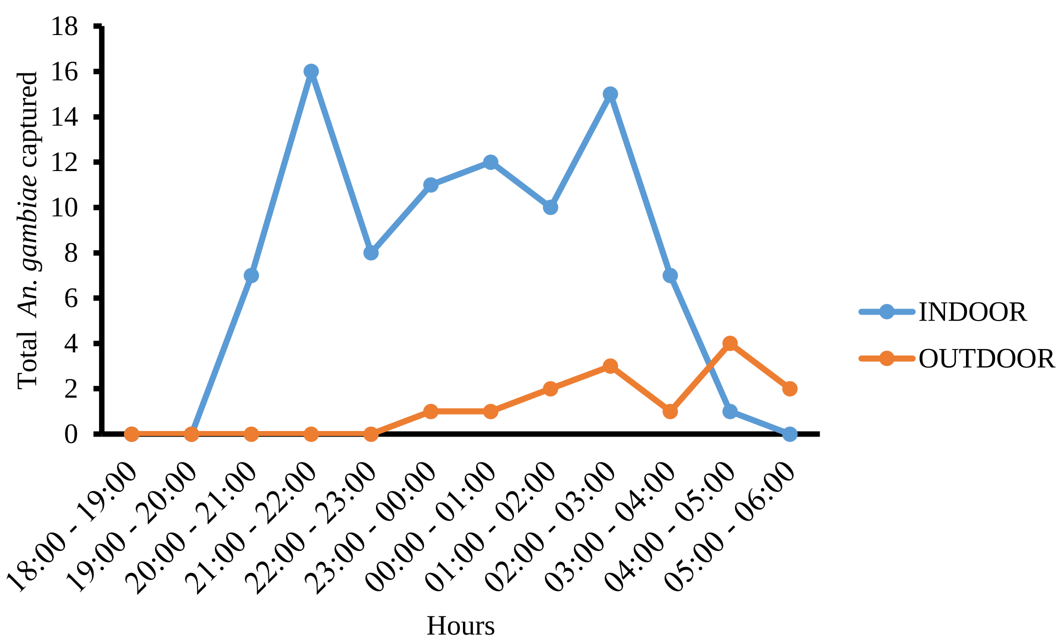


Figure 4.13: Hourly biting patterns of *An. gambiae s.s.* by Human Landing Catches during the study period at Avakpo

4.2.2 *P. falciparum* Sporozoite Rates of *An. gambiae s.s.*

On the whole, 416 *An. gambiae* mosquitoes were tested for the presence of *P. falciparum* circumsporozoite protein (*Pf*CSP) using the qualitative ELISA technique. Of the total number of mosquitoes tested, 380 and 36 were collected using human landing catches and pyrethrum spray catches respectively.

Of the 416 *An. gambiae* mosquitoes tested, only one was found positive for *Pf*CSP giving an overall sporozoite rate of 0.24% (Figure 4.14).

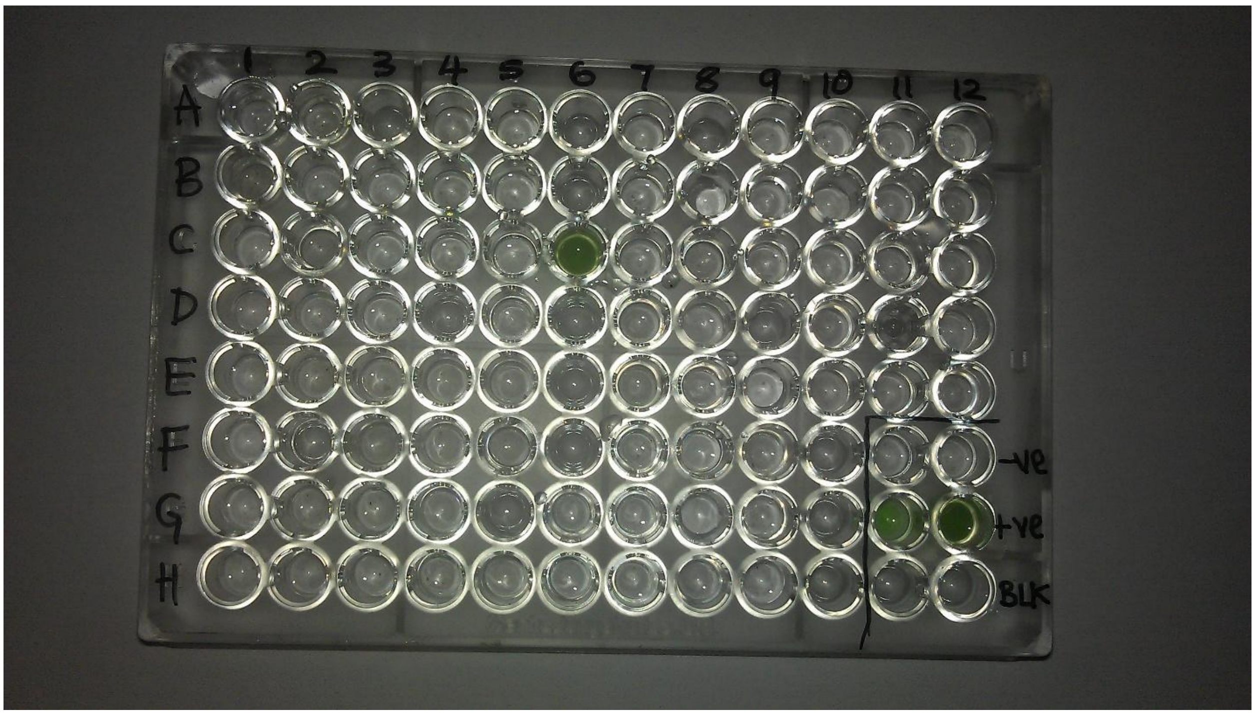


Figure 4.14: ELISA plate showing results of *Pf*CSP in tested mosquito samples. Wells F11 and F12 are negative controls, G11 and G12 are positive controls, H11 and H12 are blank, C6 is a positive sample and all other wells contained samples that are negative for *Pf*CSP.

4.2.3 Entomological Inoculation Rates.

Entomological inoculation rate (EIR) of *An. gambiae* in the study sites is shown in Table 4.2. The EIR per year was obtained by multiplying the EIR by 365 (average number of days in a year). For the entire study period, infective bites per man per night (ib/m/n) ranged from zero (0) at Kewum, Kadjanya, and Volivo/Atrobinya to ib/m/n of 0.04 at Avakpo, giving an average of 0.01 ib/m/n across the study communities. While the annual entomological inoculation rate (EIR) ranged from zero (0) infective bites per man per year (ib/m/y) for Kewum, Kadjanya, Volivo/Atrobinya to ib/m/y of 14.6 for Avakpo, giving an average EIR per year of 3.65 ib/m/y for the study area.

Table 4.2: **Number of *Pf*CSP positives, sporozoite rates, EIR in the Study Sites**

Site	Number positive	Sporozoite rate (%)	EIR	EIR per year
Kewum	-	-	-	-
Kadjanya	-	-	-	-
Avakpo	1	0.93	0.04	14.6
Volivo/Atrobinya	-	-	-	-

4.3 Insecticide Susceptibility Status of *An. gambiae s.s.* species

A total of 560 adult female *An. gambiae s.s.* of 2 to 5 days old raised in the insectary were used for the bioassay. Of these, 360 female mosquitoes were exposed to different diagnostic doses of pyrethroid insecticides while 200 female mosquitoes were used as control. The insecticides used were 0.05 % deltamethrin, 0.75% permethrin, 0.05 % lambda-cyhalothrin, 0.15 % cyfluthrin, and 0.5 % etofenprox. A total of 80 female mosquitoes were exposed to 0.05 % deltamethrin, 60 mosquitoes to 0.75 % permethrin, 80 mosquitoes to 0.05 % lambda-cyhalothrin, 60 mosquitoes to 0.15 % cyfluthrin, and 80 mosquitoes to 0.5 % etofenprox. Deltamethrin (0.05 %) had the highest knockdown rate, followed by 0.15% cyfluthrin while the insecticide with the lowest knockdown rate was 0.5% etofenprox.

4.3.1 Knockdown Times of *An. gambiae s.s.* at 60 Minutes of Exposure

The estimated times at which 50 % and 95 % of the *An. gambiae* population exposed to different concentrations of pyrethroid insecticides were knocked-down are given in Table 4.3. Deltamethrin acted faster than the other pyrethroids in knocking down 50 % and 95 % population of *An. gambiae* mosquitoes. This was followed by cyfluthrin which acted slower with 95 % population of *An. gambiae* mosquitoes as compared to lambdacyhalothrin. However, etofenprox in knocking down both 50 % and 95 % population, acted slowest than the rest of the pyrethroids. Figure 4.15 shows the average knockdown rates (%) of the mosquitoes from the study area after 5, 10, 15, 20, 30, 40, 50, 60 and 80^{th} minutes of exposure time to the diagnostic doses of each insecticide used.

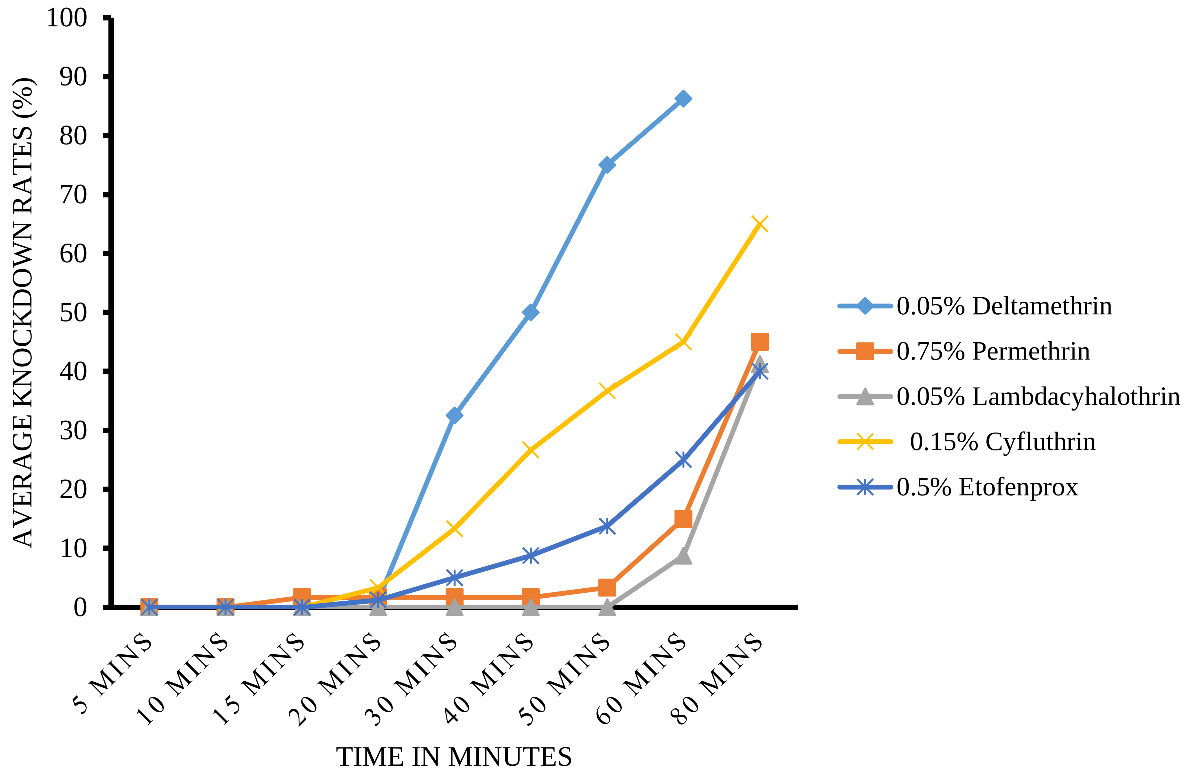


Figure 4.15: Average pyrethroid knockdown (KD) rates (%) of *An. gambiae s.s.* from the study sites

Table 4.3: KDT_{50} and KDT_{95} in minutes (with 95 % confidence intervals) of *An. gambiae s.s.* exposed to different concentrations of pyrethroid insecticides

Pyrethroids	# exposed	KDT_{50}	95% C.I.	KDT_{95}	95% C.I.
Deltamethrin	80	41.33	(34.17- 48.01)	63.27	(55.49- 76.61)
Permethrin	60	82.2	(77.99- 88.64)	112.97	(102.87-130.73)
Lambda-cyhalothrin	80	82.69	(78.73- 87.59)	106.58	(99.11- 119.68)
Cyfluthrin	60	63.61	(53.52- 74.73)	106.63	(91.93- 133.37)
Etofenprox	80	83.91	(77.98- 91.98)	133.18	(120.27- 151.72)

4.3.2 Mortality rates of *An. gambiae s.s*. after 24 hours post- exposure

Figure 4.16 shows the average mortality rates of *An.gambiae s.s* from the study sites 24 hours post-exposure to the various diagnostic concentrations of insecticides. The classification of resistance or susceptibility status of *An. gambiae* mosquitoes from the study sites for the various insecticides was based on the criteria of WHO (2013). Tested mosquitoes samples from the study area were all resistant to the pyrethroid insecticides but were highly resistant to permethrin (average mortality of 20%), as compared to the rest. No corrected mortality was made because the control mortality was below 5 % for all the various insecticides used.

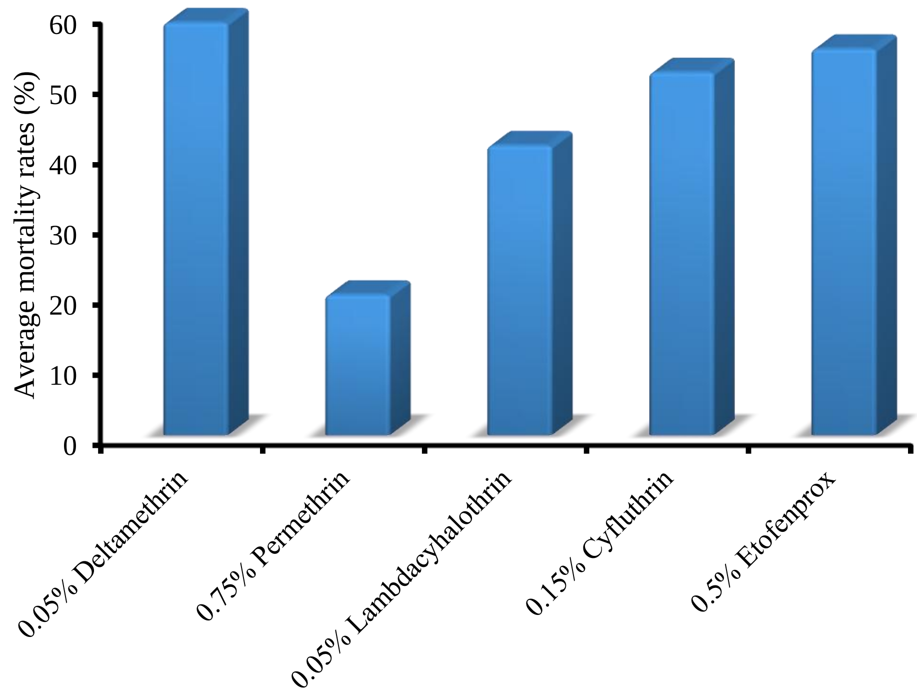


Figure 4.16: Average mortality rates of *An. gambiae s.s* from the study sites 24 hours post-exposure to the various diagnostic concentrations of insecticides

4.4 Distribution of Knockdown Resistance (*kdr*) Mutation in *Anopheles gambiae s.s.* at the Study Sites.

A total of 85 *An. gambiae s.s*. were analyzed to determine the molecular forms and *kdr* mutation. In order to have a representative sample of the population, the 85 samples included 10 survivors and 7 dead *An. gambiae* mosquitoes of each diagnostic concentration of the insecticides used in the bioassay (Table 4.4).

Of these, 91.8 % (78) were found to possess *kdr* gene mutation (53-homozygous resistant gene and 25-heterozygous) while the remaining 8.2 % (7) did not possess the *kdr* gene (susceptible gene). Figure 4.17 shows the PCR products obtained using genomic DNA of *An. gambiae s.s.* No

An. gambiae S-form was identified, hence the *kdr* mutation was present only in *An. coluzzii*. The frequency of *kdr* in *An. coluzzii* forms observed from the study site was 77%.

Table 4.4: Distribution of *Kdr* mutation in *An. gambiae* to the various insecticides

Insecticide	Bioassay phenotype (n)	RR (%)	RS (%)	SS (%)	molecular forms	
					An. coluzzii	**S forms**
Deltamethrin	Alive(10)	8 (80)	2 (20)	0	10	0
	Dead(7)	3 (42.9)	4 (57.1)	0	7	
Permethrin	Alive(10)	5 (50)	3 (30)	2 (20)	10	0
	Dead(7)	3 (42.9)	3 (42.9)	1 (14.28)	7	
Lambda-cyhalothrin	Alive(10)	6 (60)	2 (20)	2 (20)	10	0
	Dead(7)	6 (85.7)	1 (14.3)	0	7	
Cyfluthrin	Alive(10)	6 (60)	4 (40)	0	10	0
	Dead(7)	6(85.7)	1 (14.3)	0	7	
Etofenprox	Alive(10)	8 (80)	1 (10)	1 (10)	10	0
	Dead(7)	2 (28.6)	4 (57.1)	1 (14.3)	7	

Legends: RR represents the resistant gene, RS represents the heterozygous gene while SS represents the susceptible gene

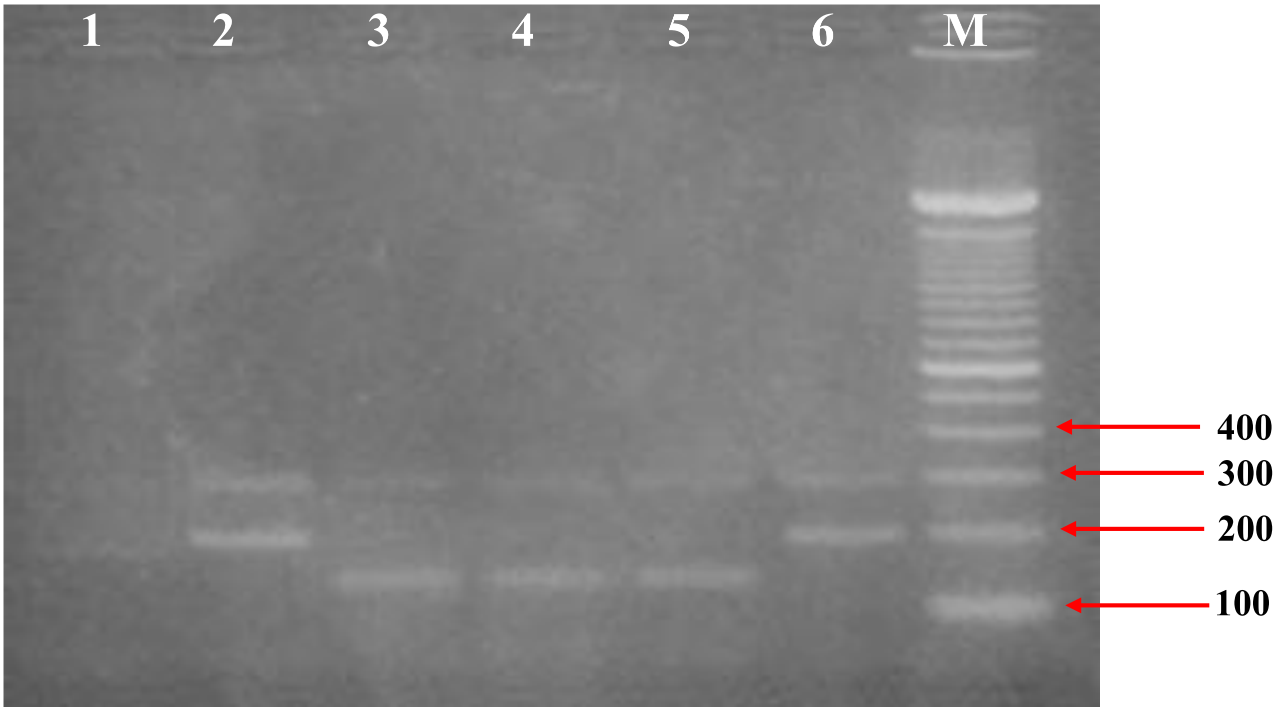


Figure 4.17: Ethidium bromide-stained 2.0 % agarose gel electrophoregram of knockdown resistance (*kdr*) mutation obtained from the analysis of *An. gambiae s.s* rDNA_PCR product. Lane 1: negative control; lane 2 and 6: Resistant *An. gambiae s.s.*; lane 3-5: Susceptible *An. gambiae s.s.* and lane M: 100bp ladder.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Species Composition and Malaria Transmission

Although seven different species of mosquitoes were collected with *Culex spp* being the most abundant; *Anopheles gambiae s.l.*, *An. funestus*, *An. pharoensis* and *An. coustani* were the only human-biting Anophelines found in all the study areas. This confirms other studies that revealed the presence of these anophelines in Southern Ghana. For example, *An. gambiae s.l.* and *An. funestus* have been reported as the most abundant and widespread vectors in the coastal savannah zones of Ghana (Appawu *et al*., 1994; Yawson *et al*., 2004), and are the major human-biting species in Dodowa in the coastal savannah zone (Appawu *et al*., 2001; Yawson *et al*., 2004). *An. gambiae* s.l, *An. funestus* and *An. pharoensis* have also been reported in Kpone-on-sea, a fishing village in Southern Ghana (Tchouassi *et al*., 2012) while *An. coustani* has been found in the coastal forest of Dodowa (Appawu *et al*., 2001).

The high proportion of *Culex* mosquitoes collected was possibly as a result of the presence of several polluted larval breeding habitats encountered in the study areas. Among the Anophelines, *An. gambiae s.l.* was found to be the leading vector from all the study sites. This is supported by other studies that indicated this species as the major malaria vector in most parts of Southern Ghana (Tchouassi *et al.,* 2012; Appawu *et al*., 1994; Yawson *et al*., 2004). The success of this species as a malaria vector includes the fact that they are highly anthropophagic and are adapted to a wide variety of micro- and macro-environmental conditions (della Torre *et al*., 2001; Kabula *et al*., 2011). The presence of *An. pharoensis* as the second dominant Anopheline caught from the study communities is similar to the situation in Prampam in the strand and mangrove zone of Southern Ghana where *An. gambiae s.l.* and *An. pharoensis* were reported as the most common biting anophelines (Appawu *et al*., 2001). Other Anophelines collected but in relatively few numbers were *An. coustani* and *An. funestus*. *An. gambiae s.l.* and *An. funestus* have been implicated as malaria vectors, however the role of *An. pharoensis* and *An. coustani* in malaria transmission in Ghana still appears to be negligible (Appawu *et al*., 2004).

Molecular analysis of *An. gambiae s.l.* found in the study areas identified *An. gambiae s.s*. as the only member of the sibling species. This observation is similar to earlier studies in some communities in the Kpone-on-sea of coastal Ghana (Tchouassi *et al*., 2012), in Kintampo in the middle belt of Ghana (Dery *et al.*, 2010) where *An. gambiae s.s* was the only sibling species of *An. gambiae s.l*. found. Elsewhere in Ghana and Africa at large, *An. gambiae s.s* has been found in sympatry with *An. arabiensis* as were the case in the Greater Accra and Volta Regions along the coast of Southern Ghana (Yawson *et al.,* 2004; Stiles-Ocran *et al.,* 2007), in the Kassena-Nankana District of Northern Ghana (Appawu *et al.*, 2004), in the Southern parts of Burkina Faso (Kelly-Hope *et al.,* 2006) and in the Southern parts of Nigeria (Kristan *et al.*, 2003). *An. gambiae s.s* also occurs in sympatry with relatively small numbers of *An. melas* in Southern Ghana (Kristan *et al*. 2003; Yawson *et al.*, 2004; Appawu *et al.*, 2001) and has been reported to be confined to the coastal areas because they prefer to breed in salt water bodies (Diop *et al.,* 2002). Therefore, the absence of *An. melas* in this present study might be attributable to the unavailability of salt water bodies in the study areas. On the other hand, the absence of *An. arabiensis* in the present study areas which are predominantly rural is consistent with earlier observation in the southern coastal zone of Ghana that *An. arabiensis* penetrates areas and tends to dominant over *An. gambiae s.s.* as a result of urbanization (Chinery, 1984).

The two molecular forms namely, *An. coluzzii* and S forms of *An. gambiae s.s* were found to be sympatric in the study communities with *An. coluzzii* being predominant. These findings are consistent with the study conducted in the Kpone-on-sea area (Tchouassi *et al*., 2012), several areas of the Greater Accra Regions in the southern part of Ghana (Kabula *et al.*, 2011) and in southern Burkina Faso (Yawson *et al.*, 2004) where both molecular forms of *An. gambiae s.s* were reported but recorded a high proportion of *An. coluzzii*. *An. coluzzii* is known to be associated with flooded/ irrigated sites, typified by extensive rice cultivation, whereas the S form is characteristically found in rain-dependent breeding sites (Diabate´ *et al*., 2005; de Souza *et al.*, 2010). Hence, *An. coluzzii* may have predominated due to the proximity of permanent breeding conditions provided by rice fields as the present study sites are irrigated sites characterized largely by extensive rice cultivation while the low occurrence of the molecular S-form is also not surprising as this form is known to be well adapted to rain-dependent breeding sites.

In the present study sites, the two molecular forms occurred in sympatry possibly as a result of the availability of both permanent and rain-dependent breeding sites since their distribution is dependent on ecological and geographical factors, however, no hybrid of both molecular forms were present. Although some studies in some parts of the country have reported the presence of this hybrid (Dery *et al*., 2010), its absence in this study agrees with some previous reports of studies conducted in several communities in Ghana (Tchouassi *et al*., 2012; Yawson *et al.*, 2004; Kabula *et al.*, 2011). The absence of the hybrid of both molecular forms in the field has been hypothesized to be due to some reproductive barriers between the molecular forms, with gene flow occurring only in some geographical locations or at some seasons (Black and Lanzaro, 2001).

The focus of this study, however, was on the *An. gambiae s.l.* and as such, the biting rates were determined only for this species. The results of the present study showed mosquitoes were biting more at Kewum and Kadjanya as compared to Avakpo and Volivo/Atrobinya, the biting rates however did not differ significantly between sites (p = 0.114). Higher proportion of the human biting *An. gambiae* mosquitoes were caught indoors than outdoors in most of the study sites but the differences were not significant (p = 0.178). The high tendency of endophagy demonstrated by *An. gambiae* is similar to earlier work in the irrigated and lowland areas of the northern savannah zone, Ghana (Appawu *et al.* 2004; Appawu, 2005) and at Dodowa in the coastal forest zone of Southern Ghana (Appawu *et al.,* 2001) where significantly higher proportion of the man-biting Anopheline mosquitoes were found biting indoors. These comparatively higher indoor biting rates observed in the present study communities implied that malaria transmission could be prevented to a significant extent if inhabitants slept under impregnated bednets, preventing human-vector contact and thus reducing the risk of infective bites.

The hourly biting pattern of the *An. gambiae* in the study communities showed mosquitoes biting throughout the night and the peak biting was observed to vary between sites, occurring during the second quarter (21:00 – 00:00 hours), third quarter (00:00 – 03:00 hours) and sometimes the fourth quarter (03:00- 06:00 hours) of the night when most of the inhabitants were in bed; hence suggesting that if inhabitants slept under impregnated bednets, human-vector contact as well as the risk of infective bites could be reduced. Low biting activity was observed mostly during the early hours of the morning (05:00 – 06:00 hours). This observation is consistent with the situation in Prampram in the coastal savannah zone and in Dodowa in the coastal forest zone of Southern Ghana (Appawu *et al.,* 2001) where significantly low biting occurred in the early mornings but contradicts with the situation in the Kassena-Nankana District of the Northern savannah zone of

Ghana (Appawu *et al.,* 2004) where significant bites occur in the early hours of the morning. However, at Avakpo, no biting activity was observed outdoor from 18:00 - 22:00 hours. Then relatively few numbers were observed from the 23:00 hours peaking after midnight in the early hours of the morning. This was probably due to the absence of power supply in the community, hence the inhabitants tend to move inside their houses early before the sun set.

The infectivity test results of the present study showed low *P. falciparum* sporozoite rates ranging from 0% at Kewum, Kadjanya and Volivo/Atrobinya to 0.93% at Avakpo in the tested *An. gambiae s.s*. and did not differ significantly between the study sites ($p = 0.50$). The observed low *P. falciparum* sporozoite rates could be possibly a result of low number of *An. gambiae s.s.* collected from the study sites. Other studies in Ghana have also reported low sporozoites rate in *An. gambiae s.s.* In the Kpone-on-sea area of coastal Ghana, Tchouassi *et al*. (2012) estimated *P. falciparum* sporozoite rates of 1.52 % while in Kintampo (middle belt), Dery *et al*. (2010) estimated *P. falciparum* sporozoite rates of 1.2 - 4.7 % in *An. gambiae s.s.* Although, low sporozoites rates have been reported in the present study and from other studies in some parts of the country, high sporozoite rates have also been reported. For example, In Kassena Nankana district (KND) of Northern Ghana, Appawu *et al.* (2004) estimated *P. falciparum* sporozoite rates of 7.2% for *An. gambiae s.s*.

The entomological inoculation rates, which refer to the product of the human biting rates and the sporozoite rates at a given time point is usually used to express the intensity of malaria transmission. It provides a standard and relatively simple means of measuring levels of exposure to infected mosquitoes and the suitability of vector control methods among others (Fontenille *et al.*, 1997). The entomological inoculation rates in the present study describe the average daily and yearly risk of an inhabitant of the study communities receiving a *P. falciparum* infected bite from *An. gambiae s.s.* The overall average inoculation rate was 0.04 infective bites per man per night (ib/m/n) during the study period, which adds up to 14.6 infective bites per man per year (ib/m/y). Previous studies have estimated EIR of 643 infective bites per man per year (ib/m/y) in the Northern part of Ghana (Navrongo), where there is an irrigation scheme (Appawu *et al.*, 2004). In the southern forest (Dodowa) and coastal areas (Prampram) of Ghana, estimated EIRs were 21.9 and 3.65 ib/m/y respectively (Appawu *et al.*, 2001). Studies undertaken in the Kpone-on-sea area of coastal Ghana also estimated EIR of 62.1 ib/m/y (Tchouassi *et al.*, 2012), while for Kintampo in the forest-savannah transitional zone of Ghana, an estimated EIR of 231-269 ib/m/y were recorded (Dery *et al.*, 2010). The finding from this study also confirms other studies in Africa, where the cultivation of irrigated rice gave rise to large numbers of potential vectors but low sporozoites and entomological inoculation rates (Ghitheko *et al.*, 1993).

5.2 Insecticide Susceptibility Status of Malaria Vectors

The insecticide susceptibility test on the populations of *An. gambiae* in the study areas showed high levels of resistance to all the pyrethroids tested. The results were based on the WHO criteria for characterizing insecticide susceptibility, where susceptibility is defined as mortality rates greater than 98 %, 24 hours post-exposure; marginal susceptibility as mortality rates between 80-97 % and resistance as mortality rates less than 80%, 24 hours post-exposure (WHO, 2013). In this study, mortality rates less than 80 % were observed in all cases. Such low mortality rates which suggest high resistance rates in the *An. gambiae* populations in the present study communities appear to be consistent with the levels of insecticide resistance reported from other communities in the Greater Accra Region of Ghana (Adeniran *et al.*, 2009; Kabula *et al.*, 2011;

Adasi and Hemingway, 2008). Adasi and Hemingway (2008) recorded mortality rates of 47.5 % and 56.4 % for *An. gambiae s.s*. exposed to 0.75 % permethrin at Dodowa and Accra respectively. Insecticide resistance in malaria vectors to pyrethroids have also been detected through bioassays outside Ghana (Akogbeto and Yakoubou, 1999). However, the result of the current study contrasts with those of Kristan *et al.* (2003) who showed that in spite of pyrethroid insecticide use for agricultural purposes in south-western Ghana, *An. gambiae s.s*. remained susceptible to deltamethrin and permethrin.

The cause of the development of insecticide resistance may be attributed to insecticide usage in the area. The present study did not establish insecticide usage pattern in the study areas but it is known that farmers who cultivate rice use pyrethroid insecticides throughout the growing season to control a wide range of agricultural pests. Studies conducted in the Greater Accra Region in southern Ghana (Adasi *et al.*, 2000) and in the Ashanti Region of the middle belt of Ghana (Stiles-Ocran *et al.,* 2007) have established a positive correlation between insecticide use and the development of insecticide resistance in *An. gambiae* species. Elsewhere in Africa, pyrethroid insecticide resistance has also been attributed to the intensive use first of DDT and, since the 1970s, of pyrethroid insecticides for crop protection purposes (Diabate *et al.*, 2004). The PCR analysis to detect the molecular basis of insecticide resistance revealed high frequency of 77% for *kdr* gene mutation. This high frequency of the *kdr* gene mutation observed is most probably accounts for the low level of mortalities observed in field populations with the pyrethroids. These results are consistent with earlier studies done in the Greater Accra Region (Adasi *et al.*, 2000; Adeniran *et al.*, 2009; Kabula *et al.*, 2011) and in the Ashanti Region (Stiles-Ocran *et al.*, 2007) which also discovered high levels of *kdr* gene mutation. For example, *kdr* gene mutation frequency of 60.5% was observed within Accra metropolis, Ghana (Kabula *et al.*, 2011). In the present study, the *kdr* gene mutation was found only in *An. coluzzii*. This high frequency of *kdr* mutations in *An. coluzzii* has been reported in previous studies in some areas in Ghana. For example, Kabula *et al.* (2011) recorded 63.60% of *kdr* mutation frequency in *An. coluzzii* (formerly M form) in one of the sampling sites in the Greater Accra Region of Ghana. Elsewhere in Africa, Corbel *et al.* (2004) recorded 78% of *kdr* mutation frequency in *An. coluzzii* (formerly M form) in the Benin Republic. On the other hand, the finding from this study contradicts previous studies in some areas in Ghana and Africa at large where high frequencies of *kdr* mutations were reported in the S form (Adeniran *et al.,* 2009; Yawson *et al.*, 2004; Diabete' *et al.,* 2004; della Torre *et al.,* 2001; Awolola *et al.,* 2003; Fanello *et al.,* 2003). The precise cause of this spatial variation in *kdr* mutation frequencies in the *An. coluzzii* (formerly M form) is not known. However, some studies have suggested that the *kdr* gene mutation reached the *An. coluzzii* (formerly M form) from the S form through genetic introgression (Diabete' *et al.,* 2004).

CHAPTER SIX

6.0 CONCLUSION, LIMITATIONS OF THE STUDY AND RECOMMENDATIONS

6.1 Conclusion

The results of the present study demonstrate that *An. gambiae s.l.* is the major human biting *Anopheles* species with the *An. gambiae s.s.* as the only sibling species of the complex. Hence, it is responsible for malaria transmission in all the study sites. Although, the two molecular forms- *An. coluzzii* (formerly M form) and S forms of the *An. gambiae s.s*. are sympatric at the study communities, *An. coluzzii* predominated. No hybrid of the molecular forms was observed.

Regarding the biting behaviour, the results indicated that a higher proportion of the *An. gambiae s.s.* tends to bite inside human dwellings. This implies that malaria parasite transmission prevention strategies which target endophagic (indoor biting) mosquitoes such as the insecticide treated bednets may be significantly useful in the present study communities.

Although a significantly higher proportion of bites occurred indoors in most of the communities, the proportion of the outdoor biting observed in Volivo/Atrobinya communities where people tend to spend long hours outdoor cannot be overlooked. Hence, public education on the outdoor feeding behaviour may also be useful in encouraging inhabitants to adopt personal protective measures that target outdoor biting such as the use of repellent and protective clothing. Also, the biting pattern of the *An. gambiae* over a night period showed variations within the study communities, with biting being higher during the second, third and fourth quarters of the night.

The results obtained in the present study show that the Asutsuare populations of *A. gambiae s.s.* are resistant to pyrethroid insecticides with a high frequency of *kdr* mutation in the mosquito vectors. The evolution of *kdr* mutation in the *An. gambiae s.s.* in these study sites may be due to the recent widespread use of pyrethroids for agricultural and domestic insect control purposes.

The malaria transmission dynamics and the insecticide susceptibility status of *An. gambiae s.s.*, the major malaria vector therefore, indicates that LLINs that are purported to be effective against resistant mosquitoes can be tested in the study area.

6.2 Limitations of the Study

- The study was done in the period when most of the rice fields were dry and hence low numbers of mosquito were collected.
- Other resistance mechanisms apart from the *kdr* mutation mechanisms were not tested.

6.3 Recommendations

The presence of other resistance mechanisms such as metabolic resistance mechanisms that might also be responsible for conferring resistance in the *An. gambiae* complex should be explored in the study area.

Future studies would be necessary to determine the association between insecticide usage patterns in the study area and the development of pyrethroid resistance in the main malaria vector *An. gambiae s.s.*

Insecticide susceptibility test which extends beyond the use of only pyrethroids into other classes of insecticides as recommended by WHO is strongly suggested. This would point out which insecticide, malaria vectors are susceptible to, hence its use in future malaria control programmes.

Future studies should also investigate the seasonal effects on malaria transmission.

REFERENCES

Abonuusum, A., Owusu-Daako, K., Tannich, E., May, J., Garms, R., Kruppa, T. (2011). Malaria transmission in two rural communities in the forest zone of Ghana. *Parasitology Research* **108**: 1465-1471.

Adasi, K. and Hemingway, J. (2008). Susceptibility to three pyrethroids and detection of knockdown resistance mutation in Ghanaian *Anopheles gambiae sensu stricto*. *Journal of Vector Ecology* **33(2**): 255- 262.

Adasi, K., Wilson, M.D. and Boakye, D.A. (2000). Studies on insecticide usage and pyrethroid resistance in populations of *Anopheles gambiae s.s*. (Diptera: Culicidae) in the Greater Accra Region of Ghana. *WHO-MIM Conference on Insecticide Resistance, Harare, Zimbabwe.*

Adeniran, T.T., Brown, C.A., Rogers, W., Wilson, M.D., Appawu, M.A. and Boakye, D.A. (2009). Susceptibility status of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to pyrethroid and carbamate insecticides in the Greater Accra region of Ghana, West Africa. *International Journal of Tropical Insect Science* **29 (3):** 124- 129.

Afari, E.A., Appawu, M.A., Dunyo, S., Baffoc-Wilmot, A. and Nkrumah, F.K. (1995). Malaria infection, morbidity and transmission in two ecologcial zones in Southern Ghana. *African Journal of Health Science* **2**: 312- 318.

Afrane, Y.A., Klinkenberg, E., Drechsel, P., Owusu-Daaku, K., Garms, R. and Kruppa, T. (2004). Does irrigated urban Agriculture influence the transmission of malaria in the city of Kumasi, Ghana? *Acta Tropica* **89**: 125- 134.

Aïkpon, R., Ossè, R., Govoetchan, R., Sovi, A., Oké-Agbo, F. and Akogbéto, M.C. (2013). Entomological baseline data on malaria transmission and susceptibility of *Anopheles gambiae* to insecticides in preparation for Indoor Residual Spraying (IRS) in Atacora, (Benin). *Journal of Parasitology and Vector Biology* **5 (7):** 102-111.

Akazili, J. (2002). Costs to households of seeking malaria care in the Kassena-Nankana Municipality of Northern Ghana. In: Third MIM Pan-African Conference on Malaria Arusha, Tanzania, and 17-22 November 2002. Bethesda, MD, Multilateral Initiative on Malaria: Abstract 473.

Akogbeto, M. and Yakoubou, S. (1999) Resistance of malaria vectors to pyrethroids used for impregnated bednets in Benin, West Africa. *Bulletin de la Socite Pathologie Exotique* **92:** 123– 130.

Akua, I., Ansah, E., Gyapong, M., Adjei, S., Barnish, G. and Evans, D. (2002). Strategies to improve adherence to recommended chloroquine treatment regimens: a quasi-experiment in the context of integrated primary health care delivery in Ghana. *Social Science and Medicine* **55**: 2215- 26.

Aly, A.S., Vaughan, A.M. and Kappe, S.H. (2009). Malaria parasite development in the mosquito and infection of the mammalian host. *Annual Reviews of Microbiology* **63**: 195 -221.

Amuzu, H., Wilson, M.D. and Boakye, D.A. (2010). Studies of *Anopheles gambiae s.l.* (Diptera: Culicidae) exhibiting different vectorial capacities in lymphatic filariasis transmission in the Gomoa district, Ghana. *Parasites & Vectors* **3**: 85.

Antonio-Nkonjio, C., Kerah, C.H., Simard, F., Awono-Ambene, P., Chouaibou, M., Tchuinkam, T. and Fontenille, D. (2006). Complexity of malaria vectorial system in Cameroon: contribution of secondary vectors to malaria transmission. *Journal of Medical Entomology* **43**: 1215- 1221.

Appawu, M.A. (2005). Genetic diversity of the *Anopheles gambiae s.s*. (Diptera: Culicidae) and malaria transmission dynamics at selected sites in the Kasena Nankana District. *Doctor of Philosophy in Entomology Thesis, University of Ghana, Legon-Accra.*

Appawu, M.A., Owusu-Agyei, S., Dadzie, S., Asoala, V., Anto, F., Koram, K., Rogers, W., Nkrumah, F., Hoffman, S.L. and Fryauff, D.J. (2004). Malaria transmission dynamics at a site in Northern Ghana proposed for testing malaria vaccines. *Tropical Medicine and International Health* **9:** 164 - 170.

Appawu, M.A., Bosompem, K.M., Dadzie, S., Mckakpo, U.S., Anim-Baidoo, I., Dykstra, E., Szumlas ,D.E., Rogers, W.O., Kwadwo-Koram, K. and Fryauff, D.J. (2003). Detection of malaria sporozoites by standard ELISA and VecTest dipstick assay in field-collected anopheline mosquitoes from a malaria endemic site in Ghana. *Tropical Medicine and International Health* **8:** 1012–1017.

Appawu, M.A., Baffoe-Wilmot, A., Afari, E.A., Dunyo, S., Koram, K.A. and Nkrumah, F.K. (2001). Malaria vector studies in two ecological zones in southern Ghana. *African Entomology* **9**: 59- 65.

Appawu, M.A., Baffoe-Wilmot, A., Afari, E.A., Nkrumah, F.K. and Petrarca, V. (1994). Species composition and inversion polymorphism of the *Anopheles gambiae* complex in some sites of Ghana, West Africa. *Acta Tropica* **56**:15-23.

Asante, F.A. and Asenso-Okyere, K. (2003). Economic burden of malaria in Ghana. *A Technical Report Submitted to the World Health Organization (WHO), African Regional Office (AFRO).* Institute of Statistical, Social and Economic Research (ISSER), University of Ghana, Legon.

Asenso-Okyere, W.K. and Dzator, J.A. (1997). Household cost of seeking malaria care. A retrospective study of two districts in Ghana. *Social Science and Medicine* **45 (5):** 659-667.

Awolola, T.S., Oduola, O.A., Strode, C., Koekemoer, L.L., Brooke, B. and Ranson, H. (2009). Evidence of multiple pyrethroid resistance mechanisms in the malaria vector *Anopheles gambiae* sensu stricto from Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **103**: 1139- 45.

Awolola, T.S., Brooke, B.D., Koekemoer, L.L. and Coetzee, M. (2003). Absence of the *kdr* mutation in the molecular 'M' form suggests different pyrethroid resistance mechanisms in the malaria vector mosquito *Anopheles gambiae* sensu stricto. *Tropical Medicine and International Health* **8 (5):** 420-422.

Beebe, N.W. and Cooper, R.D. (2000). Systematics of malaria vectors with particular reference to *Anopheles punctulatus* group. *International Journal for Parasitology* **30**: 1- 17.

Beier, J.C., Killeen, G.F. and Githure, J.I. (1999). Short report: entomologic inoculation rates and *Plasmodium falciparum* malaria prevalence in Africa. *American Journal of Tropical Medicine and Hygiene* **61 (1):** 109-13.

Biloh, A.S. (2013). Impact of Indoor Residual Spraying on malaria transmission and insecticide resistance in *Anopheles* mosquitoes in three districts in Northern Region of Ghana. *MPhil. Entomology Thesis submitted to ARPPIS, University of Ghana, Legon-Accra.*

Binka, F.N., Kubaje, A., Adjvik, M., Williams, L.A., Lengeler, C., Maude, G.H., Armah, G.E., Kajihara, B., Adiamah, J.H. and Smith P.G. (1996). Impact of permethrin impregnated bednets on child mortality in Kassena-Nankana district, Ghana: a randomised controlled trial. *Tropical Medicine and International Health* **1**:147- 54.

Binka, F.N., Maude, G.H., Gyapong, M., Ross, D.A. and Smith, P.G. (1995). Risk factors for child mortality in Northern Ghana: a case-control study. *International Journal of Epidemiology* **24**: 127-135.

Binka, F.N., Morris, S.S., Ross, D.A., Arthur, P. and Aryeetey, M.E. (1994). Patterns of malaria morbidity and mortality in children in Northern Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88:** 381- 385.

Black, W.C. and Lanzaro, G.C. (2001). Distribution of genetic variation among the chromosomal forms of the *Anopheles gambiae s.s.*: introgressive hybridisation, adaptive inversions, or recent reproductive isolation? *Insect Molecular Biology* **10**: 3- 7.

Brogdon, W.C. and MacAllister, J.C. (1998). Insecticide resistance and vector control. *Emerging Infectious Diseases* **4**: 605- 613.

Brown, A.W. (1958). The insecticide-resistance problem: a review of developments in 1956 and 1957. *Bulletin of the World Health Organization* **18**: 309- 321.

Bryan, J.H., Di Deco, M.A., Petrarca, V. and Bullini, L. (1982). Inversion polymorphism and incipient speciation in *Anopheles gambiae s.s*. in the Gambia, West Africa. *Genetica* **59:** 167–176.

Carrara, G., Petrarca, V., Niang, M. and Coluzzi, M. (1990). *Anopheles pharoensis* and transmission of *Plasmodium falciparum* in the Senegal River Delta. *Medical and Veterinary Entomology* **4:** 421–424.

Carter, J.A., Ross, A.J. and Neville, B.G. (2005). Development impairments following severe *falciparum* malaria in children. *Tropical Medicine International Health* **10:** 3-10.

Ceesay, S.J., Casals-Pascual, C., Erskine, J., Anya, S.E., Duah, N.O., Fulford, A.J., Sesay, S.S., Abubakar, I., Dunyo, S. and Sey, O.(2008). Changes in malaria indices between 1999 and 2007 in the Gambia: a retrospective analysis**.** *Lancet* **372:** 1545-1554.

Charlwood, J.D., Tomas, E.V.E., Salgueiro, P., Egyir-Yawson, A., Pitts, R.J. and Pinto, J. (2011). Studies on the behaviour of peridomestic and endophagic M form *Anopheles* *gambiae* from a rice growing area of Ghana. *Bulletin of Entomological Research* **101**: 533- 539.

Chinery, W.A. (1984). Effect of ecological changes on the malaria vectors *Anopheles funestus* and the *Anopheles gambiae* complex of mosquitoes in Accra, Ghana. *Journal of Tropical Medicine and Hygiene* **87**: 75- 81.

Coetzee, M., Craig, L.E. and Sueur, D. (2000). Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. *Parasitology Today* **16**: 74- 77.

Coetzee, M., Hunt, R.H., Wilkerson, R., della Torre, A., Coulibaly, M.B., Besansky, N.J. (2013). *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa* **3619**: 246- 274.

Coleman, R.E., Barth, J.F., Turell, M.J., Gordon, S.W., Sattabongkot, J., Copeland, R. and Wirtz, R.A. (2000). Development and evaluation of a dipstick assay for detection of *Plasmodium falciparum* and *P. vivax* sporozoites in mosquitoes (Diptera: Culicidae). *Journal of. Medical Entomology* **37 (4**): 581-7.

Collins, F.H., Mendez, M.A., Razmussen, M.O., Mehaffey, P.C., Besansky, N.J., and Finnerty, V. (1987). A ribosomal gene differentiates members species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene* **37**: 37- 41.

Coluzzi, M.A., Petrarca, V. and Di Deco, M.A. (1985). Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. *Bolletino di Zoologia* **52**: 45- 63.

Corbel, V., Chandre, F., Brengues, C., Akogbeto, M., Lardeux, F., Hougard, J.M. and Guillet, P. (2004). Dosage-dependent effects of permethrin-treated nets on the behaviour of *Anopheles gambiae* and the selection of pyrethroid resistance. *Malaria Journal* **3:** 22.

Costantini C, Sagnon N, Ilboudo-Sanogo E, Coluzzi M, Boccolini D. (1999). Chromosomal and bionomic heterogeneities suggest incipient speciation in *Anopheles funestus* from Burkina Faso. *Parassitologia* **41**: 595-611.

Cox-Singh, J., Davis, T.M., Lee, K.S., Shamsul, S.S., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J. and Singh, B. (2008). *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clinical Infectious Diseases* **46**: 165-71.

D'Alessandro, U., Langerock P. and Bennett S. (1996). The impact of a national impregnated bednet programme on the outcome of pregnancy in primigravidae in the Gambia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**: 487–492.

Dadzie, S.K., Brenyah, R. and Appawu, M.A. (2012). Role of species composition in malaria transmission by the *Anopheles funestus* group (Diptera: Culicidae) in Ghana. *Journal of Vector Ecology* **38**: 1.

Daly, H., Doyen, J. T. and Purcell, A. H. I. (1998). Introduction to insect biology and diversity, 2nd edition. Oxford University Press. New York, New York. Chapter 14, pp 279-300.

D'Avanzo, N.J., Morris, V.M., Carter, T.R., Maillard, J.M., Scanlon, P.M., Stennies, G.M., Wilson, M., Macdonald, P.D.M. and Newman, R.D. (2002). Congenital Malaria as a

Result of *Plasmodium malariae***.** *Journal of the American Mosquito Control Association* **287:** 1520- 1521.

Davidson, G., Paterson, H.E., Coluzzi, M., Mason, G.F. and Micks, D.W. (1967). The *Anopheles gambiae* complex. *In:* Wright, J.W. & Pal, R. (Eds), *Genetics of Insect Vectors of Disease.* Elsevier Publishers, Amsterdam, pp. 211–250.

de Souza, D., Kelly-Hope, L., Lawson, B., Wilson, M. and Boakye, D. (2010) Environmental factors associated with the distribution of *Anopheles gambiae s.s.* in Ghana; an important vector of lymphatic filariasis and malaria. *PloS one* **5 (3),** pp. e9927.

della Torre, A., Fanello, C., Akogbeto, M., Dossou-Yovo, J., Favia, G., Petrarca, V, and Coluzzi, M. (2001). Molecular evidence of incipient speciation within *Anopheles gambiae s.s.* in West Africa. *Insect Molecular Biology* **10**: 9- 18.

Dery, D., Asante, K.P., Oduro, A., Asoalla, V., Owusu-Agyei, S. (2013). *Baseline entomological studies in ten districts of Ghana*. Report for the AngloGold Ashanti Company by the Kintampo Health Research Centre and the Navrongo Health Research Centre, lot number E1090, 2013.

Dery, D.B., Brown, C., Asante, K.P., Adams, M., Dosoo, D., Amenga-Etego, S., Wilson, M., Chandramohan, D., Greenwood, B. and Owusu-Agyei, S. (2010). Patterns and seasonality of malaria transmission in the forest-savannah transitional zones of Ghana. *Malaria Journal* **9**: 314.

Dia, I., Konate, L., Samb, B., Sarr, J.B., Diop, A., Rogerie, F., Faye, M., Riveau, G., Remoue, F., Diallo, M. and Fontenille, D. (2008). Bionomics of malaria vectors and relationship with malaria transmission and epidemiology in three physiographic zones in the Senegal River Basin. *Acta Tropica* **105**: 145- 153.

Diabaté, A., Dabiré, R.K., Kim, E.H., Dalton, R., Millogo, N., Baldet, T., Simard, F., Gimnig, J.E., Hawley, W.A. and Lehman, T. (2005). Larval development of the molecular forms of *Anopheles gambiae* (Diptera: Culicidae) in different habitats: A transplantation experiment. *Journal of Medical Entomology* **42 (4):** 548-553.

Diabaté, A., Brengues, C., Baldet, T., Dabiré, K.R., Hougard, J.M., Akogbeto, M., Kengne, P., Simard, F., Guillet, P., Hemingway, J. and Chandre, F. (2004). The spread of the Leu-Phe kd*r* mutation through *Anopheles gambiae* complex in Burkina Faso: genetic introgression and de novo phenomena. *Tropical Medicine and International Health* **9 (12):** 1267 - 1273.

Diop, A., Molez, J.F., Konate, L., Fontenille, D., Gaye, O., Diouf, M., Diagne, M. and Faye, O. (2002). Role of *Anopheles melas* Theobald (1930) in malaria transmission in a mangrove swamp in Saloum (Senegal). *Parasite* **9 (3):** 239-246.

Drakeley, C., Schellenberg, D., Kihonda, J., Sousa, C.A., Arez, A.P., Lopes, D., Lines, J., Mshinda, H., Lengeler, C., Schellenberg, J.A., Tanner, M. and Alonso, P. (2003). An estimation of the entomological inoculation rate for Ifakara: a semi urban area in a region of intense malaria transmission in Tanzania. *Tropical Medicine and International Health* **8**: 767- 774.

Dzodzomenyo, M., Dunyo, S.K., Ahorlu, C.K., Coker, W.Z., Appawu, M.A., Pedersen, E.M. and Simonsen, P.E. (1999). Bancroftian filariasis in an irrigation project community in southern Ghana. *Tropical Medicine and International Health* **4:** 13 - 18.

Elissa, N., Mouchet, J., Riviere, F., Meunier, J.Y. and Yao, K. (1993). Resistance of *Anopheles gambiae s.s*. to pyrethroids in Cote d'Ivoire. *Annales de la Societe Belge de Medecine Tropicale* **73:** 291-294.

Fabre, R., Berry, A., Morassin, B. and Magnaval, J.F. (2004). Comparative assessment of conventional PCR with multiplex real-time PCR using SYBR green I detection for the molecular diagnosis of imported malaria. *Parasitology* **128**: 15 - 21.

Fanello, C., Petrarca, V., della Torre, A., Santolamazza, F., Dolo, G., Coulibaly, M., Alloueche, A., Curtis, C.F., Touré, Y.T. and Coluzzi, M. (2003). The pyrethroid knock-down resistance gene in the *Anopheles gambiae* complex in Mali and further indication of incipient speciation within *Anopheles gambiae s.s*. *Insect Molecular Biology* **12(3**): 241- 245.

Fanello, C., Santomalazza, F. and della Torre, A. (2002). Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Medical and Veterinary Entomology* **16:** 461–464.

Favia, G., Lanfrancotti, A., Spanos, L., Sidén-Kiamos, I. and Louis, C. (2001). Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae s.s*. *Insect Molecular Biology* **10:** 19- 23.

Favia, G., della Torré, A., Bagayoko, M., Lanfrancotti, A., Sagnon, N.F., Toure, T.T. and Coluzzi, M. (1997). Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Molecular Biology* **6(4**): 377- 383.

Faye, O., Gaye, O., Fontenille, D., Sy, N., Konate, L., Hebrard, G., Herve, J.P., Trouillet, J., Diallo, S. and Mouchet, J. (1995). Comparison de la transmission du paludisme dans deux facies epidemiologiques au Senegal: la zone cotiere sahelienne et la zone meridonale soudaninenne. *Dakar Medical* **40**: 201-207.

Fernando, S.D., Gunawardena, D.M. and Bandara, M.R. (2003). The impact of repeated malaria attacks on the school performance of children. *American Journal of Tropical Medicine Hygiene* **69:** 582- 588.

Finney, D.J. (1971). Probit Analysis, Cambridge University press. Cambridge, United Kingdom.

Fontenille, D., Lochouarn, L., Diagne, N., Sokhna, C., Lemasson, J.J., Diatta, M., Konate, L., Faye, F., Roger, C. and Trape, J.F. (1997). High annual and seasonal variations in malaria transmission by anophelines and vector species composition in Dielmo, a holoendemic area in Senegal. *American Journal of Tropical Medicine and Hygiene* **56**: 247–253.

Gallup, J.I. and Sachs, J.D. (2001). The economic burden of malaria. *American Journal of Tropical Medicine and Hygiene* **64(1, 2):** 85-96.

Gatton, M.L., Chitnis, N., Churcher, T., Donnelly, M.J., Ghani, A.C., Godfray, H.C., Gould, F., Hastings, I., Marshall, J., Ranson, H., Rowland, M., Shaman, J. and Lindsay, S.W. (2013). The importance of mosquito behavioural adaptations to malaria control in Africa. *International Journal of Organic Evolution* **67**: 1218-1230.

Georghiou, G.P. (1990). Overview of insecticide resistance *ACS Symposium* Series **421:** 18- 41.

Ghitheko, A.K., Service, M.W., Mbogo, C.M., Atieli, F.K. and Juma, F.O. (1993). *Plasmodium falciparum* sporozoite and entomological inoculation rates at the Ahero irrigation scheme and the Miwani sugar-belt in Western Kenya. *Annals of Tropical Medicine and Parasitology* **87**: 379-391

Gillies, M.T. (1988). *Anopheles* mosquitoes: vector behaviour and bionomics. *In: Malaria. Principles and Practice of Malariology*. W.H. Wernsdorfer and Sir McGregor Eds. Churchill Livingstone City- Great Britain. pp. 453-485.

Gillies, M.T. and Coetzee, M. (1987). Supplement to the Anophelinae of Africa South of Sahara. Johannesburg: *Publication of South African Institute of Medical Research* **55:** 1-143.

Gillies, M.T. and de Meillon, B.L. (1968). *The Anophelinae of Africa South of Sahara*. Publication of the South African Institute for Medical Research, Johannesburg. 54 pp.

Hamon, J., Subra, R., Sales, S. and Coz, J. (1968). Presence in the southwestern part of Upper Volta of a population of *Anopheles gambiae* "A" resistant to DDT. *Medecine Tropicale (Mars)* **28:** 521- 528.

Hemingway, J. and Ranson, H. (2000). Insecticide resistance in insect vectors of diseases. *Annual Review of Entomology* **45:** 371-391.

Hervy, J.F., Le Goff, G., Geoffroy, B., Herve, J.P., Manga, L. and Brunhes, J. (1998). The *Anopheles* from afro-tropical region. *CD-ROM, ORSTOM editions,* Paris France.

Hilary, R., Hiba, A., Athanase, B., Wamdaogo, M. G., Clement, K.H., Elise, Y. K., Fale, N. S., Frederic, S. and Maureen, C. (2009). Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem. *Malaria Journal* **8:** 299.

Humphreys, M. (2001). Malaria: Poverty, Race, and Public Health in the United States. John Hopkins University Press. ISBN 0-8018-6637-5.

Hunt, R.H., Fuseini, G., Knowles, S., Stiles-Ocran, J., Verster, R., Kaiser, M.L., Shik Choi, K., Koekemoer, L.L. and Coetzee, M. (2011). Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa. *Parasites & Vectors* **4**: 107.

Hunt, R.H., Matambo, T.S., Koekemoer, L.L., Van Wyk, P. and Coetzee, M. (2006). Dieldrin resistance in the malaria vector *Anopheles gambiae* in Ghana. *Medical and Veterinary Entomology* **20**: 294 – 299.

Hunt, R.H., Coetzee, M. and Fettene, M. (1998). *Anopheles gambiae* complex: a new species from Ethiopia. *Transactions of Royal Society of Tropical Medicine and Hygiene* **92:** 231-235.

Kabula, B.I., Attah, P.K., Wilson, M.D. and Boakye, D.A. (2011). Characterization of *An. gambiae s.l.* and insecticide resistance profile relative to physicochemical properties of breeding habitats within the Accra Metropolis, Ghana. *Tanzania Journal of Health Research* **13:** 3.

Kasasa, S., Asoala, V., Gosoniu, L., Anto, F., Adjuik, M., Tindana, C., Smith, T., Owusu-Agyei, S., Vounatsou, P. (2013). Spatio-temporal malaria transmission patterns in Navrongo demographic surveillance site, Northern Ghana. *Malaria Journal* **12**: 63

Kawada, H., Dida, G.O., Sonye, G., Njenga, S.M., Mwandawiro, C. and Minakawa, N. (2012). Reconsideration of *Anopheles rivulorum* as a vector of *Plasmodium falciparum* in Western Kenya: Some evidence from biting time, blood preference, sporozoite positive rate, and pyrethroid resistance. *Parasites & Vectors* **5:** 230.

Kelly-Hope, L.A., Diggle, P.J., Rowlingson, B.S., Gyapong, J.O., Kyelem, D., Coleman, M., Thomson, M.C., Obsomer, V., Lindsay, S.W., Hemingway, J. and Molyneux, D.H. (2006). Short communication: Negative spatial association between lymphatic filariasis and malaria in West Africa. *Tropical Medicine and International Health* **11**: 129- 135.

Koekemoer, L.L., Kamau, L., Hunt, R.H. and Coetzee, M. (2002). A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (diptera: Culicidae) group. *The American Journal of Tropical Medicine and Hygiene* **66:** 804- 811.

Kristan, M., Fleischmann, H., della-Torre, A., Stich, A. and Curtis, C.F. (2003). Pyrethroid resistance/susceptibility and differential urban/rural distribution of *Anopheles arabiensis* and *Anopheles gambiae s.s.* malaria vectors in Nigeria and Ghana. *Medical and Veterinary Entomology* **17:** 326- 332.

Le Goff. G., Camevale, I., Fondio, E. and Robert, V. (1997). Comparison of three sampling methods of man-biting anophelines in order to estimate malaria transmission in a village of south Cameroon. *Parasite* **4:** 75- 80.

Lengeler, C. (2004). . Insecticide treated bednets and curtains for malaria control. *Cochrane Database of Systematic Reviews* 2: CD000363. 9.

Lines, J.D. and Zaim, M. (2000). Insecticide products: treatment of mosquito nets at home. *Parasitology Today* **16:** 91- 92.

Liu, N., Xu, Q., Zhu, F. and Zhang, L. (2006). Pyrethroid resistance in mosquitoes. *Insect Science* **13**: 159-166.

Lounibos, L.P. and Conn, J.E. (2000). Malaria vector heterogeneity in South America**.** *American Entomologist* **46:** 238-249**.**

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Berge J.B. and Devonshire, A.L. (1998). Molecular characterization of pyrethroid resistance in the malaria vector *Anopheles gambiae*. *Insect Molecular Science* **7:** 179- 84.

McCutchan, T.F., Piper, R.C. and Makler, M.T. (2008). Use of malaria Rapid Diagnostic Test to Identify *Plasmodium knowlesi* infection. *Emerging Infectious Diseases* **14 (11**): 1750–1752.

Mendis, K., Sina, B., Marchesini, P. and Carter, R. (2001). The neglected burden of *Plasmodium vivax* malaria. *American Journal of Tropical Medicine and Hygiene* **64 (1-2 Suppl**): 97- 106.

Miles, S.J., Green, C.A. and Hunt, R.H. (1983). Genetic observations on the taxon *Anopheles* (Cellia) *pharoensis* Theobald (Diptera: Culicidae). *Journal of Tropical Medicine and Hygiene* **86:** 153–157.

Monroe, D. (1984). *Enzyme Immunoassay in Analytical Chemistry* **56 (8),** 920A-931A.

Mueller, I., Zimmerman, P.A. and Reeder, J.C. (2007). *Plasmodium malariae* and *Plasmodium ovale*- the "bashful" malaria parasites. *Trends in Parasitology* **23 (6):** 278–83.

Munhenga, G., Masendu, H.T., Brooke, B.D., Hunt, R.H. and Koekemoer, L.K. (2008). Pyrethroid resistance in the major malaria vector *Anopheles arabiensis* from Gwave, a malaria-endemic area in Zimbabwe. *Malaria Journal* **7**: 247.

Murphy, S.C. and Breman, J.G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *American Journal of Tropical Medicine and Hygiene* **64 (1, 2):**57-67.

National Malaria Control Programme, **NMCP** (2008). *Strategic plan for malaria control in Ghana*. Ministry of Health, Republic of Ghana.

Ndjemai, H.N., Patchoke, S., Atangana, J., Etang, J., Simard, F., Bilong, C.F., Reimer, L., Cornel, A., Lanzaro, G.C. and Fondjo, E. (2009). The distribution of insecticide resistance in *Anopheles gambiae* s.l. populations from Cameroon: an update. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **103**: 1127- 38.

Okoye, P.N., Brooke, B.D., Koekemoer, L.L., Hunt, R.H. and Coetzee M (2008). Characterization of DDT, pyrethroid and carbamate resistance in *Anopheles funestus* from Obuasi, Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102**: 591- 598.

Okoye, P.N., Wilson, M.D., Boakye, D.A. and Brown, C.A. (2005). Impact of the Okyereko irrigation project in Ghana on the risk of human malaria infection by Anopheles species (Diptera: Culicidae). *African Entomology* **13 (2):** 249- 253.

Okrah, J., Traore, C., Pale, A., Sommerfeld, J. and Muller, O. (2002). Community factors associated with malaria prevention by mosquito nets: an exploratory study in rural Burkina Faso. *Tropical Medicine and International Health* **7:** 240- 248.

Pates, H. and Curtis, C. (2005). Mosquito behavior and vector control. *Annual Review of Entomology* **50**: 53-70.

President's malaria initiative, PMI (2014). *Malaria operational plan*-FY 2014. Ghana. Available at: http://www.pmi.gov/docs/default-source/default-document-library/malaria-operational-plans/fy14/ghana_mop_fy14.pdf?sfvrsn=20. Accessed 16/03/15.

Protopopoff, N., Verhaeghen, K., Van Bortel, W., Roelants, P., Marcotty, T., Baza, D., D'Alessandro, U. and Coosemans M. (2008). A significant increase in *kdr* in *Anopheles gambiae* is associated with an intensive vector control intervention in Burundi highlands. *Tropical Medicine and International Health* **13**: 1479-1487.

Ranson, H., Nguessan, R., Lines, J., Moiroux, N., Nkuni, Z. and Corbel, V: (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends in Parasitology* **27**: 91–98.

Rickman, L.S., Jones, T.R., Long, G.W., Paparello, S., Schneider, I., Paul, C.F., Beaudoin, R. and Hoffman, S.L. (1990). *Plasmodium falciparum* infected *Anopheles stephensi* inconsistently transmit malaria to humans. *American Journal of Tropical Medicine and Hygiene* **43:** 441- 445.

Sachs, J.D. and Malaney, P. (2002). The economic and social burden of malaria. *Nature* **415:** 680-685.

Saiki, R.K., Scharf, S., Faloona, F., Horn, G.I., Mullis, K.B., Erlich, H.A. and Arndeim, N. (1985). Enzymatic amplification of beta-globin genomic sequence and restriction analysis of sickle cell anaemia. *Science* **230:** 1350-1354.

Sambrook, J., Fritsch, F. and Maniatis, T. (1989). *Molecular Cloning:* A Laboratory Manual. 2 Edn. Cold Spring Harbour Laboratory, New York.

Scott, J.A., Brogdou, W.G. and Collins, F.H. (1993). Identification of single specimens of the *Anopheles gambiae. American Journal of Tropical Medicine and Hygiene* **49**:520–529.

Service, M.W. (1993) The *Anopheles* vector. In: Gilles H.M. Warrell D.A. (eds) Bruce-Chwatt's Essential Malariology. 3rd edition. Boston: Edward Arnold, pp. 97-123.

Shiff, C. (2002) A call for integrated approaches to controlling malaria. *Parasitology Today* **3 (3)**: 125.

Singh, B., Kim Sung, L. and Matusop, A. (2004). A large focus of naturally-acquired *Plasmodium knowlesi* infections in human beings. *Lancet* **363 (9414**): 1017–24.

Smith, T., Charlwood, J.D., Kihonda, J., Mwankusye, S., Billingsley, P., Meuwissen, J., Lyimo, E., Takken, W., Teuscher, T. and Tanner, M. (1993). Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. *Acta Tropica* **54:** 55–72.

Smith, T., Charlwood, J.D., Takken, W., Tanner, M. and Spiegelhalter, D.J. (1995). Mapping the densities of malaria vectors within a single village. *Acta Tropica* **59**: 1- 18.

Snow, R. W., Craig, M., Deichmann, U. and Marsh, K. (1999). Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. *Bulletin of the World Health Organization* **77:** 624- 640.

Stiles-Ocran, J.B., Wilson, M.D., Frimpong, M.T., Owiredu, W.K. and Boakye, D.A. (2007). Studies on insecticide usage pattern and resistance status of *Anopheles gambiae s.s.* in the Ashanti Region of Ghana. *American Journal of Tropical Medicine and Hygiene* **77**: 170-257.

Stump, A.D., Atieli, F.K., Vulule, J.M. and Besansky, N.J. (2004). Dynamics of the pyrethroid knockdown resistance allele in Western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. *American Journal of Tropical Medicine and Hygiene* **70**: 591-596.

Ta, T.H., Hisam, S., Lanza, M., Jiram, A.I., Ismail, N. and Rubio, J.M. (2014). First case of a naturally-acquired human infection with *Plasmodium cynomolgi*. *Malaria Journal* **13**: 68.

Tchouassi, D.P., Quakyi, I.A., Addison, E.A., Bosompem, K.M., Wilson, M.D., Appawu, M.A., Brown, C.A. and Boakye, D.A. (2012). Characterization of malaria transmission by vector populations for improved interventions during the dry season in the Kpone-on-Sea area of coastal Ghana. *Parasites & Vectors* **5:** 212.

Tuno, N., Kjaerandsen, J., Badu, K. and Kruppa T (2010). Blood-Feeding behavior of *Anopheles gambiae* and *Anopheles melas* in Ghana, Western Africa. *Journal of Medical Entomology* **47**: 28-31.

Ughasi, J., Bekard, H.E., Coulibaly, M., Adabie-Gomez, D., Gyapong, J., Appawu, M.A., Wilson, M.D. and Boakye, D.A. (2012). *Mansonia africana* and *Mansonia uniformis* are vectors in the transmission of *Wuchereria bancrofti* lymphatic filariasis in Ghana. *Parasites & Vectors* **5**: 89.

Verhaeghen, K., Van Bortel, W., Roelants, P., Backeljau, T. and Coosemans, M. (2006). Detection of the East and West African *kdr* mutation in *Anopheles gambiae* and *Anopheles* *arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis. *Malaria Journal* **5**:16.

Vulule, J.M., Beach, R.F., Atieli, F.K., Mount, D.L., Roberts, J.M. and Mwangu, R.W. (1999). Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin impregnated bednets and curtains in Kenya. *Medical and Veterinary Entomology* **8:** 71–75.

Ware, G.W. and Whitacre, D.M. (2004). The Pesticide Book, 6th Ed. 496 pp. Meister Media Worldwide, Willoughby, Ohio. (ISBN 1892829-11-8).

White, G.B. (1985). *Anopheles bwambae* n. sp., a malaria vector in the Semliki Valley, Uganda, and its relationships with other sibling species of the *An. gambiae* complex (Diptera: Culicidae). *Systematic Entomology* **10:** 501–522.

Williamson, M.S., Martinez-Torres, D., Hick, C.A. and Devonshire, A.L. (1996). Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. *Molecular and General Genetics* **252**: 51-60.

Wirtz, R.A., Burkot, T.R., Graves, P.M. and Andre, R.G. (1987). Field evaluation of enzyme-linked immunosorbent assays for *Plasmodium falciparum* and *Plasmodium vivax* sporozoites in mosquitoes (Diptera: Culicidae) from Papua New Guinea. *Journal of Medical Entomology* **24 (4):** 233- 237.

World Health Organization (2014). *World malaria report 2014.* Available at http://who.int/malaria/publications/world_malaria_report_2014/en/pdf. Accessed 20/03/15.

World Health Organization (2013). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization

World Health Organization (2011). *World malaria report 2011*. Geneva, World Health Organization**.**

World Health Organization (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets.* Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11). Available at http://whqlibdoc.who.int/hq/2005/WHO_CDS_WHOPES_GCDPP_2005.11.pdf

World Health Organization (2003). Malaria entomology and vector control. Learner's Guide. HIV/AIDS, Tuberculosis and Malaria, Roll Back Malaria. WHO/CDS/CPE/SMT/2002.18 Rev.1

World Health Organization (2001). Malaria vector control, insecticides for indoor residual spraying. World Health Organization communicable disease control, prevention and eradication. WHO pesticide evaluation scheme (WHOPES). 69- 89.

Yawson, A.E., Mccall, P.J., Wilson, M.D. and Donnelly, M.J. (2004). Species abundance and insecticide resistance of *Anopheles gambiae* in selected areas of Ghana and Burkina Faso. *Medical and Veterinary Entomology* **18:** 372-377.

Zaim, M., Aitio, A. and Nakashima, N. (2000). Safety of pyrethroid-treated nets. *Medical and Veterinary Entomology* **14:** 1- 5.

Zavala, F., Gwadz, R.W., Nussenzweig, R.S. & Nussenzweig, V. (1982). Monoclonal antibodies circumsporozoite proteins identify the species of malaria parasite in infected mosquitoes. *Nature* **299**: 737-738.

APPENDICES

Appendix I: Molecular Biology Techniques

Preparation of Standard Solutions Used in Molecular Biology Studies

Standard solutions used were prepared using sterile double distilled water (sddH$_2$0) and autoclaved where appropriate at 121 1b/sq. for 15 minutes (Hirayama autoclave, Tokyo). The solutions include:

a. Solutions for deoxyribonucleic acid (DNA) extraction

Bender buffer: 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA pH 9.1, 0.5% SDS. The solution was autoclaved and then stored at 4°C.

8 M Potassium Acetate: 39.26g of Potassium acetate was dissolved in 50 ml of sterile double distilled water and then stored at room temperature.

70% alcohol: 70 ml of absolute alcohol was added to 30 ml of sterile double distilled water making a volume of 100 ml. The solution was then stored at room temperature.

b. Solutions for electrophoresis

10 X TAE buffer: 242 g Tris Base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH was adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with sddH$_2$0.

Ethidium bromide, EtBr (10 mg/ml): 1 g of EtBr was completely dissolved in 100 ml sddH$_2$0 and stored in a dark bottle in the dark at room temperature.

Gel loading **buffer** (5X orange G): 20 % w/v Ficoll, 25 mM EDTA, 2.5 % (w/v) orange G. Stored at room temperature.

DNA molecular weight size marker

The 100 bp molecular weight size marker was obtained from sigma and was diluted according to the manufacturer's recommendations and used as standard for the experimentation. For the 100 bp ladder, the first band size is 100 bp, the next ones measure 200, 300, 400, 500, 600, 700, 800, 900 and 1000 base pairs.

Appendix II: Biochemical Method

Solutions Used in ELISA Studies

a. Mosquito/*Plasmodium* Antigen (MPA) preparation

Grinding solution (GS)

Blocking buffer (BB) and Nonidet P–40 (NP – 40) were mixed in the ratio of 5 µl NP – 40: 1 ml BB. The resulting solution was then stored at stored at 4°C until it was required for use.

MPA Preparation

Test mosquito sample was put in a pre-labelled 1.5 ml eppendorf tube containing 50 µl GS. It was then crushed with a pestle to obtain a homogenous suspension. The pestle was rinsed with more 150 µl GS into the suspension to obtain a total of 200 µl of MPA suspension. MPA extract was then tested immediately.

b. Dulbecco's Phosphate Buffered Saline (DPBS) Preparation

Calibration of flask

The flask was calibrated by pouring 1 litre of tap water into the flask, a stirring rod was gently slided alongside the flask and the final volume of the water in the flask was marked.

Preparation of buffer

The tap water in step I. above was discarded and the flask rinsed with distilled water. 750ml of distilled water was poured into the calibrated flask. The stirring rod was rinsed with distilled water and slid into the flask. The flask was placed on an electronic magnetic stirrer and stirred. A bottle of DPBS powder was emptied into the flask while the content was still stirring. The bottle was rinsed thoroughly and the content was added to the flask. The pH of the solution was adjusted to 7.2. More distilled water was added to the content of the flask to the level of the calibrated mark. The solution was stored at room temperature.

c. Washing Buffer [PBST (tween)] Preparation

The washing buffer was made up of PBS and 0.05 % Tween 20. The washing buffer was prepared following the method for the preparation of PBS above to the stage of pH adjustment. Zero point 5ml of Tween 20 was added to 1litre PBS while the content was being stirred. The Tween 20 was released slowly, a litre at a time, but continuously into the content of the flask to ensure prompt dissolution. More distilled water was added to the level of the calibrated mark.

d. Blocking buffer (BB) preparation

5g of casein was suspended in 0.1 M NaOH and the resulting solution brought to a boil. After the dissolution of the casein, 900 PBS was slowly added to the content and was allowed to cool. The pH adjusted to 7.2 with hydrochloric acid and stored at 4 ^{o}C.

e. Substrate Solution Preparation

Two solutions, namely, solution A, which consists of KP&LABTS and solution B, which consists of hydrogen peroxide, were mixed in a ratio of 1:1 immediately before use.

f. Preparation of Solution for coating one micro-titre plate

The stock captured monoclonal antibody (Mab) was 0.5 μg/μl. The coating rate was 0.1 μg/50 μl/well. One micro-titre plate contains 96 wells. This was approximated to 100 wells, the extra 4 wells being provision against spillage. 10 (100 x 1) μg Mab was required to coat one plate. Since the stock captured Mab was 0.5 μg/μl, 10 μg Mab was contained in 10 x 1/ 0.5 = 20 μl solution. Since the coating rate of the MAb was 0.1 μg/50 μl/well, 5000 (50 x 100) μl of Mab solution was needed to coat one micro-titre plate. 4, 980 μl of PBS was added to 20 μl (10 μg Mab) solution. The plate was coated immediately and incubated at 4°C overnight and used the following day. Each well was coated with 50 μl (0.1 μg Mab).

Appendix III: Indoor and Outdoor Human Landing Catches Data

Indoor Human Landing Catches during the Sampling period at Kewum

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	0	0	0	0	0	0	0
19:00 - 20:00	10	5	0	4	0	1	0
20:00 - 21:00	22	4	0	12	0	6	0
21:00 - 22:00	7	0	0	5	0	1	1
22:00 - 23:00	23	13	1	6	0	2	1
23:00 - 00:00	21	7	0	10	2	2	0
00:00 - 01:00	17	15	0	0	0	2	0
01:00 - 02:00	12	9	0	0	0	3	0
02:00 - 03:00	16	10	0	4	0	2	0
03:00 - 04:00	22	9	0	2	0	6	5
04:00 - 05:00	15	12	2	0	0	1	0
05:00 - 06:00	3	1	0	1	0	1	0
Total	**168**	**85**	**3**	**44**	***2***	**27**	**7**

Outdoor Human Landing Catches during the Sampling period at Kewum

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**0**	0	0	0	0	0	0
19:00 - 20:00	**22**	0	0	16	0	4	2
20:00 - 21:00	**67**	0	0	59	0	5	3
21:00 - 22:00	**50**	1	0	39	0	*10*	0
22:00 - 23:00	**38**	4	0	17	0	*17*	0
23:00 - 00:00	**29**	8	0	14	0	*5*	2
00:00 - 01:00	**25**	6	0	14	0	*5*	0
01:00 - 02:00	**27**	4	0	17	0	*4*	2
02:00 - 03:00	**23**	4	0	13	1	*3*	2
03:00 - 04:00	**24**	8	0	12	0	*2*	2
04:00 - 05:00	**11**	4	0	5	0	*2*	0
05:00 - 06:00	**12**	3	0	6	0	*3*	0
Total	**328**	**42**	**0**	**212**	**1**	**60**	**13**

Indoor Human Landing Catches during the Sampling period at Avakpo

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. pharoensis*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**3**	0	0	2	0	0	1	0
19:00 - 20:00	**15**	0	0	12	0	0	2	1
20:00 - 21:00	**43**	7	0	24	1	1	7	3
21:00 - 22:00	**60**	16	0	24	3	0	8	9
22:00 - 23:00	**51**	8	0	36	1	1	3	2
23:00 - 00:00	**43**	11	0	25	0	1	5	1
00:00 - 01:00	**44**	12	0	29	0	0	1	2
01:00 - 02:00	**39**	10	0	26	0	0	1	2
02:00 - 03:00	**38**	15	0	15	0	1	5	2
03:00 - 04:00	**31**	7	0	18	1	0	4	1
04:00 - 05:00	**34**	1	0	32	0	0	1	0
05:00 - 06:00	**16**	0	0	11	1	0	3	1
Total	**417**	**87**	**0**	**254**	**7**	**4**	**41**	**24**

Outdoor Human Landing Catches during the Sampling period at Avakpo

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. pharoensis*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**14**	0	0	7	0	0	1	6
19:00 - 20:00	**46**	0	0	35	0	0	8	3
20:00 - 21:00	**108**	0	0	83	4	0	6	15
21:00 - 22:00	**145**	0	4	118	0	1	14	8
22:00 - 23:00	**94**	0	0	81	0	0	10	3
23:00 - 00:00	**81**	1	0	70	0	3	2	5
00:00 - 01:00	**80**	1	0	68	0	0	7	4
01:00 - 02:00	**67**	2	0	56	1	1	5	2
02:00 - 03:00	**51**	3	0	41	0	0	3	4
03:00 - 04:00	**58**	1	0	52	0	0	3	2
04:00 - 05:00	**36**	4	0	29	0	0	2	1
05:00 - 06:00	**26**	2	0	22	0	0	2	0
Total	**806**	**14**	**4**	**662**	**5**	**5**	**63**	**53**

Indoor Human Landing Catches during the Sampling period at Volivo/Atrobinya

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. pharoensis*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**1**	0	0	1	0	0	0	0
19:00 - 20:00	**16**	1	0	14	0	0	0	1
20:00 - 21:00	**57**	0	0	48	1	1	1	6
21:00 - 22:00	**59**	0	0	51	0	0	2	6
22:00 - 23:00	**65**	2	0	56	0	0	2	5
23:00 - 00:00	**35**	0	0	32	0	0	0	3
00:00 - 01:00	**48**	1	0	45	0	0	2	0
01:00 - 02:00	**34**	4	0	24	1	0	2	3
02:00 - 03:00	**51**	6	0	33	0	0	1	11
03:00 - 04:00	**47**	7	0	38	2	0	0	0
04:00 - 05:00	**41**	5	0	32	0	0	0	4
05:00 - 06:00	**24**	1	0	23	0	0	0	0
Total	**478**	**27**	**0**	**397**	**4**	**1**	**10**	**39**

Outdoor Human Landing Catches during the Sampling period at Volivo/Atrobinya

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. pharoensis*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**0**	0	0	0	0	0	0	0
19:00 - 20:00	**35**	0	0	26	0	0	4	5
20:00 - 21:00	**84**	0	0	63	5	4	1	11
21:00 - 22:00	**76**	1	0	65	0	0	3	7
22:00 - 23:00	**60**	2	0	48	4	0	4	2
23:00 - 00:00	**92**	7	0	79	0	2	2	2
00:00 - 01:00	**85**	5	0	71	0	0	3	6
01:00 - 02:00	**78**	3	0	63	3	0	4	5
02:00 - 03:00	**55**	7	0	40	2	0	5	1
03:00 - 04:00	**109**	9	0	85	2	0	3	10
04:00 - 05:00	**59**	7	0	48	1	0	2	1
05:00 - 06:00	**46**	1	0	38	1	0	4	2
Total	**779**	**42**	**0**	**626**	**18**	**6**	**35**	**52**

Indoor Human Landing Catches during the Sampling period at Kadjanya

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. pharoensis*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**0**	0	0	0	0	0	0	0
19:00 - 20:00	**7**	0	0	7	0	0	0	0
20:00 - 21:00	**10**	2	0	8	0	0	0	0
21:00 - 22:00	**28**	2	0	22	0	0	3	1
22:00 - 23:00	**20**	3	0	16	0	0	1	0
23:00 - 00:00	**14**	6	0	8	0	0	0	0
00:00 - 01:00	**19**	13	0	6	0	0	0	0
01:00 - 02:00	**7**	4	0	2	0	0	0	1
02:00 - 03:00	**21**	16	0	3	0	0	0	2
03:00 - 04:00	**14**	0	0	13	1	0	0	0
04:00 - 05:00	**12**	3	1	6	0	0	0	2
05:00 - 06:00	**1**	1	0	0	0	0	0	0
Total	**153**	**50**	**1**	**91**	**1**	**0**	**4**	6

Outdoor Human Landing Catches during the Sampling period at Kadjanya

HOUR	Number caught	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*An. pharoensis*	*An. coustani*	*Mansonia spp*	*Aedes spp*
18:00 - 19:00	**0**	0	0	0	0	0	0	0
19:00 - 20:00	**18**	6	0	10	0	0	1	1
20:00 - 21:00	**17**	3	0	9	0	0	5	0
21:00 - 22:00	**27**	2	0	21	1	0	2	1
22:00 - 23:00	**34**	3	0	28	1	0	2	0
23:00 - 00:00	**12**	2	0	8	0	0	0	2
00:00 - 01:00	**19**	4	0	11	1	0	2	1
01:00 - 02:00	**18**	7	0	7	2	0	1	1
02:00 - 03:00	**17**	3	0	11	0	0	3	0
03:00 - 04:00	**15**	3	0	12	0	0	0	0
04:00 - 05:00	**14**	0	0	13	1	0	0	0
05:00 - 06:00	**2**	0	0	2	0	0	0	0
Total	**193**	**33**	**0**	**132**	**6**	**0**	**16**	**6**

Appendix IV: Data on Pyrethroid Spray Catches (PSC)

Pyrethroid Spray Catches at Kewum

Date	House number	Number of rooms sprayed	Number collected	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*Mansonia spp*
7/10/2014	AS/KE/012A	1	2	2	0	0	0
7/10/2014	AS/KE/012B	1	0	0	0	0	0
7/10/2014	AS/KE/009	2	3	0	0	3	0
7/10/2014	AS/KE/011	1	0	0	0	0	0
8/10/2014	AS/KE/012C	1	2	0	0	2	0
8/10/2014	AS/KE/014	1	0	0	0	0	0
8/10/2014	AS/KE/017	1	0	0	0	0	0

Pyrethroid Spray Catches at Avakpo

Date	House number	Number of rooms sprayed	Number collected	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*Mansonia spp*
7/10/2014	AS/AV/009A	2	1	1	0	0	0
8/10/2014	AS/AV/009B	2	1	1	0	0	0
8/10/2014	AS/AV/009C	2	2	1	0	1	0
8/10/2014	AS/AV/010	2	5	0	0	5	0
9/10/2014	AS/AV/009D	1	3	1	0	2	0

Pyrethroid Spray Catches at Kadjanya

Date	House number	Number of rooms sprayed	Number collected	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*Mansonia spp*	*Aedes spp*
10/10/2014	AS/KA/004B	2	1	1	0	0	0	0
10/10/2014	AS/KA/044	1	0	0	0	0	0	0
10/10/2014	AS/KA/005	2	5	3	1	1	0	0
10/10/2014	AS/KA/008A	1	0	0	0	0	0	0
10/10/2014	AS/KA/008B	3	0	0	0	0	0	0
11/10/2014	AS/KA/007	2	2	2	0	0	0	0
11/10/2014	AS/KA/031A	2	0	0	0	0	0	0
11/10/2014	AS/KA/031B	3	2	1	0	1	0	0
11/10/2014	AS/KA/006	2	0	0	0	0	0	0
11/10/2014	AS/KA/045A	2	4	1	0	3	0	0
12/10/2014	AS/KA/031C	1	3	0	0	0	0	3
12/10/2014	AS/KA/045B	2	10	8	0	2	0	0
12/10/2014	AS/KA/034	2	15	0	0	10	5	0

Pyrethroid Spray Catches at Volivo/Atrobinya

Date	House number	Number of rooms sprayed	Number collected	*An. gambiae s.l*	*An. funestus*	*Culex spp*	*Mansonia spp*
7/10/2014	AS/AR/082A	2	1	1	0	0	0
7/10/2014	AS/AR/082B	2	1	1	0	0	0
7/10/2014	AS/AR/077	1	0	0	0	0	0
7/10/2014	AS/AR/078	2	5	1	0	0	4
7/10/2014	AS/AR/079	2	2	0	0	2	0
9/10/2014	AS/VG/049B	1	0	0	0	0	0
9/10/2014	AS/VG/049C	2	0	0	0	0	0
9/10/2014	AS/VG/049D	1	1	0	0	1	0
9/10/2014	AS/VG/049A	2	0	0	0	0	0
10/10/2014	AS/VG/032A	1	0	0	0	0	0
10/10/2014	AS/VG/032B	2	0	0	0	0	0
10/10/2014	AS/VG/032C	2	0	0	0	0	0
11/10/2014	AS/VG/053	3	0	0	0	0	0
11/10/2014	AS/VG/052	2	0	0	0	0	0

11/10/2014	AS/VG/055A	1	0	0	0	0	0
11/10/2014	AS/VG/055B	1	0	0	0	0	0
11/10/2014	AS/VG/069	3	9	2	0	7	0
12/10/2014	AS/VG/046A	1	0	0	0	0	0
12/10/2014	AS/VG/046B	1	0	0	0	0	0
12/10/2014	AS/VG/074A	1	2	0	0	1	1
12/10/2014	AS/VG/074B	1	0	0	0	0	0
12/10/2014	AS/VG/074C	3	5	5	0	0	0
12/10/2014	AS/VG/074A	2	1	0	0	0	1

Appendix V: Data on Pyrethroid Insecticide Susceptibility in *Anopheles gambiae s.s* from the Study Area

Data on 0.05% Deltamethrin Insecticide susceptibility

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. exposed	20	20	20	20	20	20

Number of knocked down (KD) mosquitoes after exposure in minutes

Time(Minutes)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
5	0	0	0	0	0	0
10	0	0	0	0	0	0
15	0	0	0	0	0	0
20	1	0	0	0	0	0
30	9	7	7	3	0	0
40	13	9	11	7	0	0
50	15	14	16	15	0	0
60	18	17	17	17	0	0

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. dead	12	12	9	14	0	0
No. alive	8	8	11	6	20	20
% mortality	60	60	45	70	0	0

Data on 0.75% Permethrin Insecticide Susceptibility

	Replicate 1	Replicate 2	Replicate 3	Control 1	Control 2
No. exposed	20	20	20	20	20

Number of knocked down (KD) mosquitoes after exposure in minutes

Time(Minutes)	Replicate 1	Replicate 2	Replicate 3	Control 1	Control 2
5	0	0	0	0	0
10	0	0	0	0	0
15	0	0	1	0	0
20	0	0	1	0	0
30	0	0	1	0	0
40	0	0	1	0	0
50	0	1	1	0	0
60	1	5	3	0	0
80	12	9	6	0	0

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Control 1	Control 2
No. dead	2	7	3	0	0
No. alive	18	13	17	20	20
% mortality	10	35	15	0	0

Data on 0.05% Lambda-Cyhalothrin Insecticide Susceptibility

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. exposed	20	20	20	20	20	20

Number of knocked down (KD) mosquitoes after exposure in minutes

Kd Time(Minutes)	REP1	REP2	REP3	REP4	CONTROL	CONTROL
5	0	0	0	0	0	0
10	0	0	0	0	0	0
15	0	0	0	0	0	0
20	0	0	0	0	0	0
30	0	0	0	0	0	0
40	0	0	0	0	0	0
50	0	0	0	0	0	0
60	1	2	2	2	0	0
80	8	7	9	9	0	0

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. dead	12	12	9	14	0	0
No. alive	8	8	11	6	20	20

Data on 0.15% Cyfluthrin Insecticide Susceptibility

	Replicate 1	Replicate 2	Replicate 3	Control 1	Control 2
No. exposed	20	20	20	20	20

Number of knocked down (KD) mosquitoes after exposure in minutes

Kd Time(Minutes)	REP1	REP2	REP3	CONTROL	CONTROL
5	0	0	0	0	0
10	0	0	0	0	0
15	0	0	0	0	0
20	0	0	2	0	0
30	3	1	4	0	0
40	6	4	6	0	0
50	7	6	9	0	0
60	8	10	9	0	0
80	13	11	15	0	0

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Control 1	Control 2
No. dead	11	8	12	0	0
No. alive	9	12	8	20	20
% mortality	55	40	60	0	0

Data on 0.5% Etofenprox Insecticide Susceptibility

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. exposed	20	20	20	20	20	20

Number of knocked down (KD) mosquitoes after exposure in minutes

Time(Minutes)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
5	0	0	0	0	0	0
10	0	0	0	0	0	0
15	0	0	0	0	0	0
20	0	1	0	0	0	0
30	1	2	0	1	0	0
40	1	2	2	2	0	0
50	4	2	3	2	0	0
60	6	5	5	4	0	0
80	12	11	5	4	0	0

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. dead	17	19	2	6	0	0
No. alive	3	1	18	14	20	20
% mortality	85	95	10	30	0	0