MALARIA TRANSMISSION DYNAMICS AND INSECTICIDE RESISTANCE STATUS OF *ANOPHELES FUNESTUS* (DIPTERA: CULICIDAE) DURING FOUR YEARS OF INDOOR RESIDUAL SPRAYING IN NORTHERN GHANA.

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DECLARATION

I hereby certify that this thesis is the result of a research undertaken by me, Obagha Adachukwu Scholastica, 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.

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This work is dedicated to God Almighty for His mercies and to my wonderful parents and siblings for their prayers, love and support throughout this period of study.

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ABSTRACT

Malaria remains a major public health problem in Africa, with *An. funestus* as one of the principal vectors. In the control of malaria, vector control has been successfully implemented in many malaria-endemic countries with indoor residual spraying (IRS) as an existing front-line measure. IRS has been implemented in some selected sites in Northern Ghana since 2009 and its effect on the members of *An. funestus* is poorly understood. Therefore, this study was aimed at investigating the species composition, malaria transmission and insecticide resistance status of *An. funestus* Giles group in three selected sites in Northern Ghana. Samples were collected using human landing catch (HLC) and pyrethrum spray collection (PSC) techniques from two IRS areas (Tolon and Savelugu Districts) and one non-IRS area (Tamale District). Archived samples (samples from 2010, 2013 and 2014) and newly collected samples (from 2015) were analysed. A total of 688 adult females morphologically identified as *An. funestus* mosquitoes were recorded in these districts across the years. The percentage abundance of these samples per site were 85.32 %, 3.20 % and 11.48 % for Tolon, Savelugu and Tamale Districts respectively across the years. Further identification using PCR revealed that *An. funestus s.s* was the only member of the group present in these study sites. The man-biting rates of the mosquitoes collected in Tolon district was significantly higher ($P < 0.001$) as compared with the other study sites. The heads and thoraces of individual *An. funestus* samples were tested for the presence of circumsporozoite proteins of *P. falciparum* using ELISA. Sporozoite infectivity rates of 2.98 %, 0 % and 1.43 % were recorded for Tolon, Savelugu and Tamale Districts respectively. There was no statistically significant difference ($P > 0.05$) in the sporozoite rates between the study sites. Blood meal analysis of the abdomen of these mosquitoes using ELISA revealed an overall human blood index of 31.50 %, 18.18 % and 32.86 % for Tolon, Savelugu and Tamale Districts across the years and a statistically significant difference ($P < 0.001$) between the study sites. PCR detection of *kdr* gene analysed in these mosquitoes showed 38.57 % possible mutation of the target site from samples collected in Tolon District. The results from this study indicate the importance of *An. funestus s.s.* as an effective malaria vector. Entomological monitoring and complementary vector control of the species are needed in order to control the species and preserve the efficacy of IRS.

CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

°C	Degree Celsius
µl	Microliter
b/m/n	Bites/human/night
BB	Blocking Buffer
Bp	Base pair
df	Degree of freedom
µg	Microgram
µM	Micromolar
ACTs	Artemisinin-based combination therapies
CDC	Centre for Disease Control
CTAB	Cetyl Trimetyl Ammonium Bromide
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological Inoculation Rate

ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
G	Gram
GHS	Ghana Health Service
GMT	Greenwich Mean Time
GS	Grinding solution
HBR	Human biting rate
HCL	Hydrochloric acid
HLC	Human Landing Catches
IGS	Intergenic Spacer Regions
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
ITS2	Internal Transcribed Spacer Regions 2
kdr	Knockdown resistance
KND	Kassena Nankana District

L	Litre
LLIN	Long lasting insecticide-treated net
M	Milli
M	Molar
MAb	Monoclonal antibodies
MBR	Man biting rate
MPA	Mosquito/*Plasmodium* antigen
MVI	Malaria Vaccine Initiative
NaOH	Sodium hydroxide
NIAID	National Institute of Allergy and Infectious Diseases
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

POPs	Persistent Organic Pollutants
PSC	Pyrethrum spray collection
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolution per minute
s.l.	*sensu lato*
s.s.	*sensu stricto*
SddH20	Sterile double distilled water
TAE	Tris-Acetate EDTA
Tm	Melting temperature
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

Malaria is still a major public health problem in Sub-Saharan Africa and is mainly transmitted by the *Anopheles gambiae s.l.* and *Anopheles funestus* mosquitoes amongst other species (Sachs and Malaney, 2002). Recent reports show that 149 to 303 million cases of malaria occur annually, resulting in the deaths of an estimated half a million people, mostly young children (WHO, 2015). Globally, malaria has the largest disease burden (illnesses, deaths, financial costs and other indicators) of any vector-borne disease (St. Louis and Hess, 2008). It is caused by a protozoa of the genus *Plasmodium* that parasitize mammals, birds and reptiles (Vezilier *et al*., 2010).

There are approximately 3,500 species of mosquitoes grouped into 41 genera (CDC, 2012). Of these, human malaria is transmitted only by females of the genus *Anopheles*. The *Anopheles funestus* Giles group consists of at least nine species that are widely distributed throughout subtropical Africa (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968). All the nine members of the *funestus* group are difficult to distinguish morphologically: *An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. leesoni* Evans, *An. parensis* Gillies, *An. rivulorum* Leeson, *An. fuscivenosus* Leeson, *An. brucei* Service, *An. aruni* Sobtiand *An. confusus* Evans and Leeson (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

Despite the morphological similarities that exist between members of the *funestus* group, their biology and vector competence vary. *Anopheles funestus* occupies a wide range of ecologic niches,

is highly anthropophilic, and is susceptible to human malaria parasites. The vectorial capacity of *An. funestus* can frequently surpass that of *An. gambiae* in some areas (Gillies and de Meillon, 1968; Mbogo *et al*., 1999). This is attributed to the fact that the larvae of *An. funestus* develop in permanent or semi-permanent swamps or in pools along streams and river systems, as opposed to those of the *An. gambiae* complex, which prefer temporary aquatic habitats (Gillies and de Meillon, 1968), thereby allowing *An. funestus* to be less dependent on rains and become abundant during the dry seasons when *An. gambiae* densities are low (Minakawa *et al*., 2001). Thus, *An. funestus* is often considered a vector species that bridges malaria transmission during the dry season (Mbogo *et al*., 2003). Sound knowledge of vector population genetic diversity is expedient for the management of insecticide resistance and for creating innovative malaria control strategies (Crampton, 1994; Collins and Besansky, 1994).

Vector control is a fundamental intervention for global malaria control and eradication efforts. It is vital for the reduction of vector abundance and, in due course, for the interruption of malaria transmission. Currently, the two most common vector control interventions are Long-Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS). Together, these account for almost 60 % of global investment in malaria control (Roll Back Malaria Partnership, 2008). Indoor Residual Spraying (IRS) is the application of a long-lasting, residual insecticide to potential malaria vector resting surfaces such as internal walls, eaves and ceilings of all houses or structures (including domestic animal shelters) where such malaria vectors might come into contact with the insecticide (WHO, 2013a).

Indoor Residual Spraying (IRS), carried out correctly, is a powerful intervention which rapidly reduces adult mosquito vector density and longevity, consequently reducing malaria transmission. The effectiveness of IRS as a malaria control intervention arises from the fact that many important malaria vectors are endophilic (Roll Back Malaria Partnership, 2008) that is, when searching for blood meals they enter human habitations or animal shelters where they rest on the walls, ceilings and other interior surfaces before and/or after feeding on the inhabitants (Clements, 1992). When a vector comes into contact with a sprayed surface, it absorbs lethal doses of insecticide, thereby reducing its lifespan. This results in a progressive decline in vector longevity and density, especially among older female mosquitoes, reduces overall vectorial capacity and contributes to a reduction in malaria transmission. Indoor Residual Spraying is most effective against indoor feeding (endophagic) and indoor resting (endophilic) vectors (WHO, 2013a).

Insecticides have been used in different malaria control programmes such as the use of LLINs and IRS against *Anopheles* mosquitoes. These have been used for over 60 years with varying degrees of success (Park Ross, 1936; Harrison, 1978; Hargreaves *et al*., 2000, Moiroux *et al*., 2012). Insecticides that have been used widely in IRS are pyrethroids, organophosphates and Dichlorodiphenyltrichloroethane (DDT). Notwithstanding the levels of success recorded with the use of IRS in the control of malaria (Mabaso *et al*., 2004; Pluess *et al*., 2010; Najera *et al*., 2011), certain setbacks have arisen with consistent IRS in an area. These setbacks manifest in the form of resistance to the insecticides used in the spraying programme and vector biodiversity (Ferguson *et al*., 2010). The extensive use of these insecticides leading to the development of resistance in insects, is as a result of selection pressure (Bolland and Williams, 2003) and this threatens successful malaria vector control in various countries (Chaccour *et al*., 2013)

1.1 Rationale

Malaria remains a public health problem in Ghana. Selective control of malaria which involves a targeted vector control approach, is one of the main global malaria control strategy (WHO, 1995). However, the most efficient way of reducing malaria transmission is through vector control (WHO, 2001), with IRS as one of the main vector control measures currently being used (Pluess *et al*., 2010). Since the 1940's, there has been evidence of the success of IRS in reducing malaria and so many examples have been recorded (Mabaso *et al*.,2004; Pluess *et al*., 2010; Najera *et al.*, 2011).

However, the effect of IRS on the species composition of *Anopheles funestus* populations is poorly understood. Much information is available on species composition and the vectorial role of the various species of *An. gambiae* complex (Appawu *et al*., 2001, 2004; Yawson *et al*., 2004) in Ghana. But there is little information of similar nature on the different sibling species of *funestus* group that have been found in the different ecological zones of Ghana (Dadzie *et al*., 2013). At the sibling species level, *funestus* populations are more amenable to control by house spraying with residual insecticides than *Anopheles gambiae* (Kouznetsov, 1977). This is because they are highly endophilic, anthropophilic and very little information about the emergence of the *kdr* gene in *Anopheles funestus* populations is available. It is therefore important that members of the *funestus* group are accurately identified so as to understand their role in malaria disease transmission patterns, to ascertain the effect of insecticides on the group as well as formulate evidence-based control strategies to reduce malaria transmission. Indoor Residual Spraying has been on-going in Northern Ghana since 2009. This study was aimed at investigating the impact of IRS on the species composition of *funestus* population, which is one of the main malaria vectors in the area, as well

as its effect on the sporozoites infectivity of this vector during four years of the spraying programme.

1.2 Main Objective

The main objective of this study was to determine the impact of IRS on the species composition (sibling species) and sporozoite infectivity of *Anopheles funestus* populations in Northern Ghana after a six year spraying programme.

1.2.1 Specific Objectives

The specific objectives are:

1 To determine the effect of IRS on the comparative species abundance of *funestus* group in the study areas.

2. To determine the malaria transmission dynamics during the study period.
3. To determine the pyrethroid insecticide resistance status of *An. funestus* group members in the study areas.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Global Burden of Malaria

Malaria remains the most important vector-transmitted human disease. It is one of the most severe public health problems worldwide, with over 214 million people living in areas at risk of malaria (Maigemu and Hassan, 2015; WHO, 2015). It is a leading cause of morbidity and mortality in many developing countries especially in Sub-Saharan African countries where the burden of the disease is high and malaria control, one of the greatest challenges (Mabaso *et al*., 2004). Malaria has a significant impact on the health of infants, young children and pregnant women worldwide. It also contributes to malnutrition in children (National Institute of Allergy and Infectious Diseases, 2011).

Malaria transmission risks vary significantly across the continent and within countries. Malaria vector distribution, transmission intensity and disease burden can vary over short distances, between neighbouring villages and even within a single settlement (Kreuels *et al*., 2008; Mboera *et al*., 2010).

2.1.1 Malaria Situation in Ghana

Malaria is endemic and perennial in all parts of Ghana, with seasonal variations that are more noticeable in the Northern Region. Ghana's entire population of 24.2 million is at risk of malaria infection (PMI, 2014), with hyper endemicity in Northern Ghana where it peaks in the rainy season (Binka *et al*., 1994). According to Ghana Health Services (GHS) health facility data, malaria is the

number one cause of morbidity and mortality in children under five years of age, currently accounting for 33 % of hospital deaths in children under five years and about 38 % of all outpatient illnesses and 36 % of all admissions. 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 (PMI, 2014).

In terms of economic costs, malaria burden is high with treatment cost ranging between US$ 0.80 and US$ 5.30 depending on local antimalarial drug and the total cost to Africa is estimated at US$1.8 trillion per year (Sachs and Malaney, 2002).

In Africa, malaria is mainly caused by the most virulent *Plasmodium falciparum* parasite, and transmitted by efficient mosquito vectors belonging to sibling species of the *Anopheles gambiae* complex and the *Anopheles funestus* group (Sinka *et al.*, 2012; White *et al.*, 2014). The sibling species of these mosquitoes are also important vectors of the nematode parasite *Wuchereria bancrofti*, which is a widespread cause of disabling lymphatic filariasis (Simonsen *et al.*, 2014). *An. funestus* Giles mosquitoes are also widely distributed in Ghana and have been implicated as the major vectors of malaria in many areas of the country (Appawu *et al.*, 2001, 2004).

2.2 Principal Malaria Parasites and Vectors

Human malaria is exclusively transmitted by female *Anopheles* mosquitoes which are the definitive hosts of the *Plasmodium* parasites that cause the disease. More than 100 different species of *Plasmodium* exist (National Institute of Allergy and Infectious Diseases, 2007), but the species

that have been found to infect humans include; *P. falciparum, P. malariae, P. ovale, P. vivax, P. knowlesi* (Cox-Singh *et al*., 2008) and *P. cynomolgi* (Ta *et al*., 2014).

2.2.1 Taxonomic Information and Dominant Species of Malaria Vectors

Generally, mosquitoes have been grouped into 41 genera containing 3,500 species. 430 species of Anophelines have been recognized and out of these, only about 30 to 40 species transmit malaria in nature (CDC, 2012). Anophelines are found worldwide except Antarctica. Malaria is transmitted by different *Anopheles* species, depending on the region and the environment. Anophelines that can transmit malaria are found not only in malaria-endemic areas, but also in areas where malaria has been eliminated. The latter areas are thus, constantly at risk of re-introduction of the disease. In Africa, the most efficient vectors are the *Anopheles gambiae* complex, *Anopheles funestus* group, *Anopheles nili* and *Anopheles moucheti* (Sinka *et al*., 2010).

2.2.1.1 The *Anopheles gambiae s.l.* complex: Geographic Distribution and Vectorial capacity

The members of *An. gambiae* s.*l.* are important vectors of malaria, and they are a group of morphologically identical yet genetically and behaviourally distinct species that differ significantly in their ability to transmit the diseases (Coluzzi *et al*., 2002). Members of the species complex include *An. gambiae s.s.* Giles, *An. arabiensis* Patton, *An. merus* Dȍnitz, *An. melas* Theobald, *An. bwambe* White, *An. quadriannulatus* Theobald (Coetzee *et al*., 2000), and *An. amharicus* Hunt, Coetzee and Fettene (Coetzee *et al*., 2013). These sibling species vary in their vectorial ability and ecological niche, with the principal malaria vectors in the complex as *An. gambiae sensu stricto (s.s.)* and *An. arabiensis* (Coetzee *et al*., 2000).

An. gambiae s.s. is considered to be one of the most efficient malaria vectors in the world, this is due to its high vectorial capacity and known to be one of the most well studied malaria vector (Coetzee, 2004). It is a freshwater species and has the broadest geographic distribution. Its wide range, variable ecology and a combination of other traits such as anthropophily, longevity, short larval developmental period et cetera, allows it to maintain its status as one of the most efficient malaria vector in Sub-Saharan Africa (Olayemi and Ande, 2009; Sinka *et al*., 2010).

An. arabiensis on the other hand, has a wide distribution but is found predominantly in fringes of pools and highlands: Southern and Eastern Africa highlands, Sahelian areas of Western and Central Africa. In these areas, it is commonly associated with *An. funestus* and, to a lower extent, to *An. gambiae s.s.* Studies have shown that they exhibit a great deal of behavioural variability in their feeding pattern, since they can either be anthropophilic or zoophilic (Bøgh *et al*., 2001; Sinka *et al*., 2010).

An. melas and *An. merus* are both salt water breeding, restricted in distribution to brackish water along the east and west coasts respectively and consequently, the only important vectors in coastal regions (Tsy *et al*., 2003; Moreno *et al*., 2004). Although they are not as efficient as *An. gambiae* s.s and *An. arabiensis* at transmitting malaria, they are usually found in high densities, and thus, achieve the status of a dominant vector species (Temu *et al*., 1998; Cuamba and Mendis, 2009). They are therefore, considered as dominant vector species of *An. gambiae* complex.

Of the remaining members, *An. quadriannulatus* which is widespread in Southern Africa, and *An. amharicus* found in Ethiopia, are considered to be primarily zoophilic non-malaria vectors

(Coetzee, 2004; Coetzee *et al*., 2013). The final member of the complex *An. bwambae* is restricted to a region close to the Buranga hot springs in Semliki Forest National Park in Eastern Uganda (White, 1985).

Anopheles funestus Giles is acknowledged for its principal role in the transmission of human malaria (Gilles and Coetzee, 1987). Although the exact composition of *Anopheles funestus* group remains unclear (Constantini *et al*., 1999), at least nine sibling species have been recorded and they are; *An. funestus s.s.* Giles, *An. rivulorum* Leeson, *An. leesoni* Evans, *An. vaneedeni* Gillies and Coetzee, *An. parensis* Gillies, *An. confusus* Evans and Leeson, *An. aruni* Sobti, *An. fuscivenosus* Leeson, and *An. brucei* Service. They are very similar morphologically and can be distinguished morphologically only at specific stages of their development (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). More so, members of this group show significant differences in their biology and behaviour, particularly in connection to malaria, vectorial capacity and feeding preferences (Cohuet *et al*., 2003; Coetzee and Fontenille, 2004).

2.2.1.2 The *Anopheles funestus* Group: Geographic Distribution

Among the species of the *Anopheles funestus* group, *An. funestus s.s*., *An. rivulorum*, and *An. leesoni* have been found to occupy a wide geographic distribution, extending throughout sub-Saharan Africa (Gillies and Coetzee, 1987; Coetzee and Fontenille, 2004). Within these three widely distributed species, *An. funestus s.s*. is known to have the widest distribution, found nearly all across the continent including subtropical Africa and Madagascar (Gillies and Coetzee, 1987). This is because it is considered mainly as a savannah mosquito and found in lowlands and places with high altitudes such as in Central Africa 1400 m (Tchuinkam *et al*., 2010), Kenya 2000 m

(Okara *et al*., 2010) and forested areas of West and Central Africa (Oyewole *et al*., 2007; Betsi *et al*., 2010; Tanga *et al*., 2010; Adja *et al*., 2011).

An. rivulorum and *An. leesoni* are found from Ethiopia through the northern parts of South Africa and across West Africa (Gillies and Coetzee, 1987). The other species of the group are more localized or their true distribution is principally not known with *An. parensis* and *An. confusus* being found in Eastern Africa in places such as Kenya and Tanzania (Muturi *et al*., 2009), *An. aruni* in Zanzibar, *An. fuscivenosus* in Zimbabwe, *An.vaneedeni occurs* in Mpumalanga and the Northern areas of South Africa and *An. brucei* in Nigeria (Gillies and Coetzee, 1987; Cohuet *et al*., 2003).

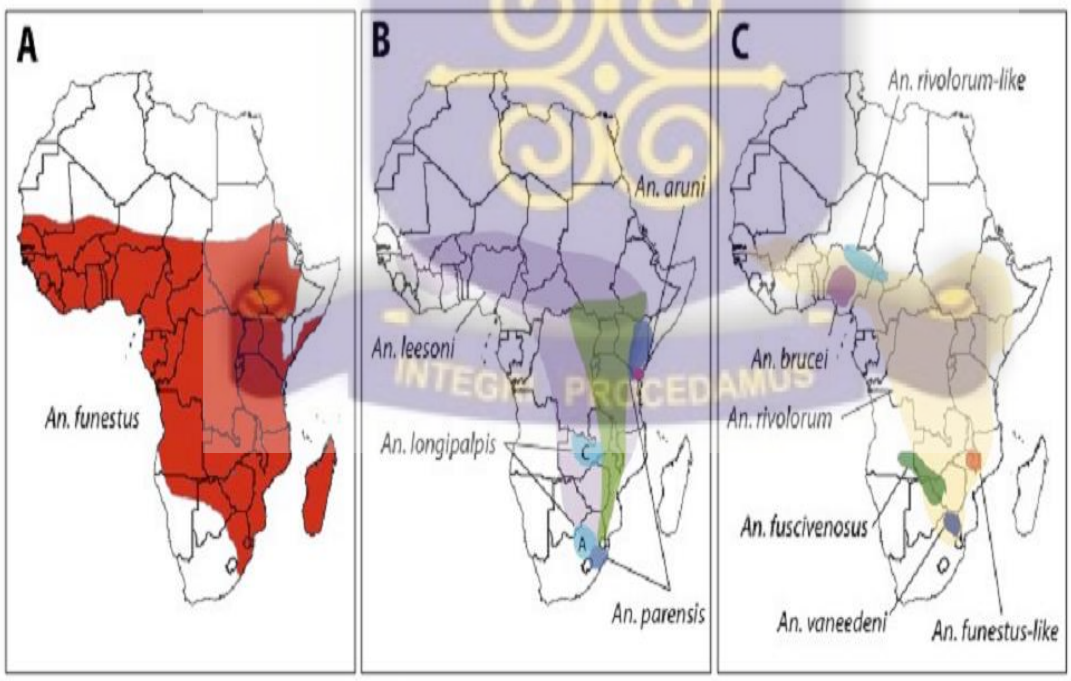

Figure 1: Map showing geographic distribution of members *An. funestus group* (Sinka *et al*., 2010; Dia *et al*., 2013)

2.2.1.3 The *Anopheles funestus* Group: Breeding Site Ecology

Anopheles funestus is typically found breeding in either natural or artificial permanent and semi-permanent water bodies with floating or emerging vegetation. Nevertheless, in areas with both vegetation types, this mosquito prefers the emerging vegetation (Gillies and De Meillon, 1968). A characteristic habitat for the larvae of *Anopheles funestus* is a large permanent or semi-permanent fresh water body with emergent vegetation in its edges such as swamps, large ponds, in weedy and grassy parts of rivers, streams, furrows and ditches. The presence of vegetation is very significant for their breeding because its aquatic stages have a pronounced selection for shaded habitats and uses emergent vegetation as refuge against predation. It can barely survive in water bodies that are directly exposed to sunlight (Gillies and De Meillon, 1968).

In some areas, artificial breeding opportunities include rice fields, wells and domestic water containers (Gillies and De Meillon, 1968). Where *An. funestus* larvae are found in rice fields, they replace *An. arabiensis* in sequence, lasting only a short time during rice plant growth and showing higher densities in older, maturing fields in opposition to the preceeding open conditions which favours *An. arabiensis* (Sogoba *et al.*, 2007; Carnevale *et al*., 1999). The principal imposing restrictions to the development of *An. funestus* are conditions such as extreme temperatures, salinity and occasionally, heavy rains.

For the other species within the *funestus* group, the biology of aquatic stages is poorly understood or not truly known. Nonetheless, studies have shown that the larva of *An. leesoni*, *An. rivulorum* and *An. vaneedeni* are often found alongside those of *An. funestus*. In places like Kenya, *An. funestus was* replaced by *An. rivulorum* in rice fields after indoor residual spraying (Abou-Nasr,

1970). The larvae of *An. rivulorum* were also found in hyacinth water protected by trees in Western Kenya (Minakawa *et al*., 2012), this confirms that the presence of vegetation is a requisite for their breeding sites and these sites are generally represented by slow moving backwaters of grassy rivers and tide pools.

Correspondingly, studies have shown that *An. parensis* develops in permanent swamps and ponds between the stalk and the developing vegetation. In spite of this, *An. parensis* is a species of stagnant water that has never been found in rivers, the larvae were always collected in marshes, temporary and permanent ponds, among stalks and developing vegetation (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968). *Anopheles aruni* also breeds in water among vegetation alongside *An. funestus s.s.* (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968), they have been found in rice fields, ponds and drains near human dwellings. For the larvae of *An. brucei*, they were found in streams of forested river beds and *An. confusus*, in contrast, breeds in the vegetation at the edges of slow flowing rivers and lake edges (Gillies and De Meillon, 1968).

2.2.1.4 The *Anopheles funestus* Group: Resting, Feeding Behaviour and Host Preference

Although morphological similarities exist between the members of the *funestus* group, their behavioural patterns differ and this has an impact on the vectorial capacity of these species. Among all the members of the *funestus* group, *An. funestus s.s.* has been extensively studied (Derua *et al*., 2015; Dadzie *et al*., 2013; Kweka *et al*., 2013; Kawada *et al*., 2012; Sinka *et al*., 2010) and has been found to be intimately associated with human habitations (Kawada *et al*., 2012).

Compared to other dominant vector species of malaria, *An. funestus* shows a consistent behaviour being highly anthropophilic as well as endophilic (Gillies and De Meillon, 1968; Awolola *et al*., 2005; Antonio-Nkondjio *et al*., 2006; Sinka *et al*., 2010). Notwithstanding this, Lochouarn *et al*. (1998) records behavioural differences amongst this species where anthropophilic behaviour was seen in Western Senegal and zoophilic behaviour in the Eastern part. Its late night biting patterns which occurs after 22.00 hr, usually between midnight and early hours of the morning makes it readily accessible to human blood without encountering undue density-dependent host avoidance (Sinka *et al*., 2010). Charlwood *et al*. (1995) records that it is probably the first species to adapt in making use of human blood as a food source.

An. funestus has a relatively high longevity. This adds to a set of other behaviours which makes it a highly efficient vector of malaria parasite (Sinka *et al*., 2010), often surpassing the efficiency of *An. gambiae* in transmitting malaria. It is regarded to as the principal malaria vector in the group with records of infection rates of up to 11 % in Tanzania (Shiff *et al*., 2005) and remarkably 50 % in Burkina Faso (Constantini *et al*., 1999). More so, it plays a major role in malaria transmission in many areas. In the Northern Ghana, *An. funestus* has been reported in the Kassena Nankana District; in Tolon, Savelugu and Tamale Districts; in Kpone-on-Sea, a fishing village in Southern Ghana (Appawu *et al*., 2004; Biloh, 2013; Tchouassi *et al*., 2012) and has been found to transmit malaria in Ghana (Dadzie *et al*., 2013). It has also been implicated as the principal reason for a major resurgence of malaria epidemics in the late 1990s in Kwazulu-Natal, South Africa (Hargreaves *et al*., 2000; Coetzee and Fontenille, 2004).

The remaining members of the *funestus* group are not restricted in their feeding behaviour, since they can feed on any available host although they are mainly zoophilic (Gillies and De Meillon, 1968). In Nigeria, *An. rivulorum* was found in proximity with human and a feeding preference for human blood meal of 40 % was recorded (Awolola *et al*., 2005) while in Kenya, *An. rivulorum* was also found in closeness with human dwellings and a remarkably higher biting and sporozoites rates in humans were recorded (Kawada *et al*., 2012). Although *An. rivulorum* is primarily zoophilic, it has been incriminated as a human malaria vector and was found naturally infected with *Plasmodium falciparum* in Tanzania (Wilkes *et al*., 1996; Temu *et al*., 2007). Because it has a lower longevity when compared with *An. funestus s.s.*, it plays a minor role in malaria transmission (Gillies and Coetzee 1987; Wilkes *et al*., 1996; Kawada *et al*., 2012).

An. vaneedeni is another zoophilic species of the *funestus* group which has been found to feed on human outdoors. Under natural conditions, it has not been found infected with *P. falciparum* although it has been found to be infected only under laboratory conditions (De Meillon *et al*., 1977). This makes its efficiency to transmit malaria parasites doubtful.

Kamau *et al.* (2003) recorded *An. parensis* in Kenya resting indoors in human habitations which had low human blood index not infected with circumsporozoite protein. Further studies in Tanzania and South Africa have shown positive specimens of *An. parensis* to *P. falciparum* indicating that they may be involved secondarily in malaria transmission (Mouatcho *et al*., 2007; Temu *et al*., 2007).

An. leesoni is a zoophilic species of the *funestus* group usually found in sympatry with *An. funestus s.s.* (Cohuet *et al*., 2003; Temu *et al*., 2007). Positive specimens of *An. lessoni* to *P. falciparum* have also been recorded (Temu *et al*., 2007) indicating that they may be involved secondarily in malaria transmission. All other members of the *funestus* group are predominantly zoophilic.

2.3 Malaria Control Interventions

Malaria is a major public health problem and this is seen in terms of lives lost and economic burden of the disease. The control of malaria has become increasingly important as it is a component of national poverty reduction strategies in most malaria endemic countries.

Therefore, the control of malaria in most endemic countries is aimed at reducing as far as possible the health impact of malaria on a population using the available resources and taking into account, other health importance (WHO, 2001). Initial attempts made by the World Health Organization in eradication of malaria was a massive worldwide campaign commenced in the mid-1950s. This program which entailed the use of insecticide spray against the malaria vector as well as chemotherapy recorded huge successes without delay, completely eradicating malaria in certain areas of the world such as the United States, India, Sri Lanka (National Institute of Allergy and Infectious Diseases, 2007). This however did not last as certain factors arose disproving the initial success recorded, thereby resulting in the resurgence of malaria in India and Sri Lanka where the disease had been eradicated.

These factors were evidently manifested in the form of *Plasmodium* parasite resistance to the antimalarial drugs administered and vector resistance to the insecticides used (National Institute

of Allergy and Infectious Diseases, 2007). Globally, there are many interventions directed towards the control of malaria. Some of the control strategies include; Chemotherapy, vaccination and vector control (Komatsu *et al*., 2007).

2.3.1 Chemotherapy

Malaria is controlled by the use of chemotherapeutic drugs against the parasite. The major class of antimalarial drugs include those that impede haem detoxification, such as chloroquine; drugs that target the metabolism of folate, such as sulphadoxine, pyrimethamine, and proguanil; and drugs that inhibit electron transport of mitochondrion, exemplified in atovaquone and artemisinins such as artemether and artesunate (Kar and Kar, 2010). Presently, the most effective antimalarial drug is artemisinin, derived from *Artemisia annua,* usually given in conjunction with one or more other drugs as artemisinin-based combination therapies (ACTs). These drugs are used in the treatment of malaria and have played a role in history in reducing the prevalence and disease burden of this infection in endemic countries (National Institute of Allergy and Diseases, 2011). In addition to these drugs used in the treatment of malaria, there are also chemoprophylactic drugs such as pyrimethamine, proguanil and atovaquone (Wells *et al*., 2009) administered to young children and pregnant women while chloroquine plus proguanil are administered to adults and non-pregnant women living in endemic areas.

However, the use of these drugs for malaria infection is faced by many challenges such as development of resistance by *Plasmodium* parasite, poor compliance to the dosages of the drugs, low efficacy of the drugs, safety issues and high cost, particularly in poorer nations (Wells *et al*., 2009). More importantly, the spread of resistance by this parasite has led to resurgence of malaria

infection in places where it was previously eliminated (National Institute of Allergy and Diseases, 2011). This led to a call for other options of control measures so as to reduce the spread and development of drug resistant malaria and increased severity of the disease.

2.3.2 Vaccination

The impact of malaria morbidity and mortality has led to a number of continuous effort to develop effective malaria vaccine candidates (Hills, 2011). Vaccines are considered the most cost effective single intervention for prevention, control and eradication of infectious diseases. Therefore, the successful development of malaria vaccine will be a huge breakthrough towards the control of malaria infection.

Efforts have been made in the development of a malaria vaccine. Several vaccines that target different stages of the lifecycle of malaria parasite are undergoing clinical trials. The most promising of these vaccine candidates is the subunit vaccine RTS,S/AS01, which generated a collaboration between Walter Reed Army Institute of Research and GlaxoSmithKline is in its Phase III clinical trials (Hills, 2011). Therefore, it is expected that sooner than later, an effective vaccine against malaria will be available for use in malaria endemic countries (Malaria Vaccine Initiative, MVI, 2015).

The current goals to tackle malaria are to reduce global malaria deaths from high levels to near-zero preventable deaths (Kar and Kar, 2010), in the long run, eradicating malaria by reducing malaria incidence to zero in malaria endemic countries. Employing other control strategies such as the use of artemisinin-based combination therapy (ACT), intermittent preventive treatment (IPT) during pregnancy and for children, long lasting insecticidal nets (LLINs) and indoor residual

spraying (IRS), as recommended by WHO (WHO, 2005; Komatsu *et al*., 2007) can be used to achieve this.

2.3.3 Vector Control

Vector control of mosquitoes is an essential part of global malaria control programs. Its aim is to reduce the contact between man and the vectors, increasing the mortality of these vectors, in so doing, malaria transmission can be reduced (Curtis and Townson, 1998). The epidemiology of malaria, the pattern and intensity of malaria transmission are primarily, a function of the vectorial capacity of the *Anopheles* vector.

Therefore, the principal goal of vector control is to reduce the vectorial capacity of the mosquito vector below the critical threshold needed to achieve a malaria reproduction number (which is defined as R_0, expected number of hosts who would be infected after one generation of the parasite by a single infectious person in a population) of less than one as quoted by Corbel *et al*. (2012). This will a go a long way in reducing the burden of malaria.

Selective vector control is one of the global malaria control strategy and it is described as the application of targeted, site-specific activities that are cost-effective (WHO, 1995). Vector control of mosquitoes can be targeted either at the larval or adult stage. Directing control measures to the adult stage of mosquitoes is more feasible and this is owed to the fact that communities and houses where these adult mosquitoes are controlled are easily identified and mapped. To an even greater degree, correlation exists between transmission intensity of malaria parasites and the mortality rate of the adult mosquito vectors (Corbel *et al*., 2012).

Current control strategies in malaria vector control interventions in endemic countries being used to reduce daily survival rates of the vector are indoor residual spraying (IRS) and insecticide treated nets (ITNs), specifically, long lasting insecticide treated nets (LLINs) (Pluess *et al*., 2010; WHO, 2014). The use of these vector control tools have proven a huge success in malaria endemic countries and WHO recommends that individuals in these countries be protected from malaria using these vector control tools singly or in combination where necessary (WHO, 2014).

Other control strategies that can be used against malaria vector include larval control, source reduction and environmental management (National Institute of Allergy and Infectious Diseases, 2011). Integrated vector control measures have been implemented to help bring down vector population. This is because mosquitoes have a very high vectorial capacity, as only a small number of them are needed to transmit the parasite. Therefore, a combination of control measures such as environmental management, the use of IRS, ITNs as well as larvicides in malaria endemic areas will help reduce the vector population thereby reducing malaria transmission in these areas.

For effective malaria control program and management, it is important to determine the spatio-temporal distribution of the vector, monitor the entomological risk factors and assess the effects of the control interventions on malaria transmission in the area (Okara *et al*., 2010)

2.3.3.1 Use of ITNs and LLINs

The use of insecticide-treated nets (ITNs) is dated as far back as the 1980s (Lindsay and Gibson, 1988). It is a control measure that prevents contact between malaria vectors and the human host, thereby reducing malaria transmission. ITNs can be categorized as conventional treated nets and long-lasting insecticidal nets. Conventional treated nets are mosquito nets that have been treated

by dipping in a WHO-recommended insecticide. In order to ensure continuous insecticidal effect of these nets, they are usually retreated at least once a year or after three washes.

On the other hand, long lasting insecticidal nets are factory-treated mosquito nets made with netting material which has insecticide incorporated within or bound around fibres. These nets retain their biological activity without retreatment for three years of recommended use under field conditions and for at least 20 WHO standard washes under laboratory conditions (WHO, 2005). This explains the efficiency of long lasting insecticidal nets over conventional treated nets, and they are the ideal type of insecticide treated nets for public health distribution programmes (WHO, 2014).

The advantaged use of long lasting insecticidal nets over conventional treated nets is also evidenced in the fact that problems associated with storage and handling of insecticides used in treating these nets by non-professionals are avoided. All the more, risks associated with environmental contamination caused by insecticides being released into water, foods and bodies are reduced (Najera and Zaim, 2002). LLINs play a significant role in the prevention of malaria in Sub-Saharan Africa (Mabaso *et al*., 2004; Lengeler, 2004). They offer personal protection to the individual users and when used by majority of the target population, provide protection for all the people in the community by reducing human-vector contact, including those who do not sleep under nets (Binka *et al*., 1996).

The efficacy of LLINs in reducing morbidity and mortality of malaria is dependent on factors such as high coverage of the distribution of nets and behaviour of the users. According to Lengeler

(2004), when full coverage is achieved, treated bed nets reduce child mortality by providing protection by an average of 20 % when compared with no nets and untreated nets. This implies that about 5.5 lives can be saved each year for every 1000 children protected with treated nets.

In Ghana, successful use of treated bed nets has been recorded in the Kassena-Nankana District (Anto *et al*., 2009). Also, in Northern Ghana, a large scale community randomized trial of treated bed nets showed a significant reduction of 17 % in all-cause child mortality among children aged 6 months to 4 years.

2.3.3.2 Indoor Residual Spraying (IRS)

Indoor residual spraying (IRS) is an effective control measure which has been used in reducing the burden of malaria worldwide (Kolaczinski *et al*., 2007). It involves the spraying of recommended insecticides inside human dwellings in a well-structured and controlled manner. The application of IRS in a targeted manner is very important in order to achieve a desired effect. Programme managers involved should make a planned decision with reference to where IRS should be carried out considering factors such as transmission ecology, malaria endemicity, cost and logistics.

The effective use of IRS as a control measure against malaria dates back to late 1930s in South Africa (Park-Ross, 1936; De Meillon, 1936) and in India (Covell *et al.*, 1938). In the 1950s and 1960s, IRS using Dichlorodiphenyltrichloroethane (DDT) became a major part of globally coordinated eradication programme. This led to a significant reduction and eradication of malaria in areas such as Asia, Europe, Latin America and Middle East (Gramiccia and Hampel, 1972;

Payne *et al*., 1976). The achievements gotten from these eradication campaigns, led to the launching of pilot projects in tropical Africa in the late 1960s up to 1970s (Garrett-Jones, 1964; Kouznetsov, 1977; Bruce- Chwatt, 1984). This proved that malaria burden and transmission in areas with high transmission can be reduced using IRS (Payne *et al*., 1976).

Presently, IRS is continually been used as a primary malaria vector control method in many areas of high transmission in Sub-Saharan Africa as an outcome of its efficacy (Skarbinski *et al*., 2012) and evidences of the successful use have been documented (Pluess *et al*., 2010; Najera *et al*., 2011; Ratovonjato *et al*., 2014). IRS is most effective against indoor resting mosquito vectors such as *An. gambiae s.s*., *An. funestus* and *An. arabiensis* (Pluess *et al*., 2010) and the insecticide used which is sprayed on the walls prevents malaria transmission by killing or repelling these adult mosquito vectors of malaria that rest on these surfaces after a blood meal (WHO, 2006). The combination of this intervention with other vector control measure such as the use of LLINs can be considered (Ngufor, 2011).

One major difference between the use of treated mosquito nets and IRS is the rate at which each intervention work in order to achieve the maximum effect. It is possible that IRS provides certain amount of protection to the individual house by repelling and reducing the abundance of mosquito vectors that fly into that house. Nevertheless, IRS has its greatest impact when the mosquito vector after feeding rests on the sprayed surface, picks up a lethal dose of the insecticide sprayed, thereby preventing the mosquito from transmitting the *Plasmodium* parasite to other inhabitants of that environs (WHO, 2006). The implication of this is that, for IRS to be effective, over 85 % of all

structures in a targeted area that are potential resting places of these vectors must be assured in order to achieve the mass effect on the vector population (WHO, 2013a).

Thus, the major aim of IRS is to reduce, and eventually interfere with the transmission of malaria by reducing the survival of vectors, its density and human-vector contact, in a way that is safe for human health and not harmful to the environment. It does this by reducing the vector's life span to less than the time it takes for the malaria sporozoites to develop, thereby reducing the capacity of the vector to transmit *Plasmodium* parasites from one individual to the other. More so, it reduces vector density by immediate killing. In some situations, particularly with *Anopheles funestus*, IRS can lead to the local elimination of important malaria vectors. It also reduces human-vector contact through repellent effect, thereby reducing the number of mosquitoes that enter sprayed rooms.

It is important to consider areas where IRS intervention is to be carried out before selection. This selection is based on the relationship between the vector, humans and the environment, in addition to the disease transmission levels in the area under consideration. In areas with low and moderate transmission, IRS is used as a primary vector control intervention to reduce the seasonal annual peaks of malaria transmission, to prevent epidemics and to support malaria elimination. On the other hand, in areas with high transmission, IRS can be used to promptly reduce transmission of malaria to a level that can be sustained afterward with the usage of LLINs by a high proportion of the population (WHO, 2013a). While IRS has been used in some urban situations, under any circumstances it is important to take into account the biting behaviour of the vector and the actual level of malaria transmission relative to the costs of spraying a large number of structures.

In order to achieve a successful intervention using IRS, it is important to time the application of IRS. The best timing for IRS applications is to plan the completion of spray application to correspond with the build-up of the populations of vector in advance of the onset of the peak transmission season. Putting this into practice, it guarantees the fresh deposits of the insecticides used during periods of peak mosquito density, so that with timely, good quality spraying with the most recommended insecticides, effectiveness of vector control is achieved with IRS (WHO, 2013b). The IRS programme is being implemented in Ghana as one of the vector control strategies in Obuasi Municipality by the Anglo-Gold Ashanti Mining Company (Anglo-Gold Ashanti, 2007), and in Northern Ghana under the guidance of the United States Agency for International Development (USAID)'s President's Malaria Initiative (PMI) (PMI/AIRS, 2014).

2.3.3.3 Recommended Insecticides for IRS

At present, as recommended by WHO, 12 insecticides belonging to four chemical groups (one organochlorine, six pyrethroids, three organophosphates and two carbamates) can be used for IRS programme (WHO, 2013a). These insecticides have been used in a safe and effective manner to control malaria vectors around the world (WHO, 2013a).

Since the effectiveness of IRS intervention partly depends on the type of insecticide used, it is essential to consider factors such as insecticide susceptibility and vector behaviour, safety for humans and the environment and efficacy and cost-effectiveness before the choice of insecticide is made (WHO, 2006). Different insecticides have different effects on the mosquito vector and these effects could either be a repellant, an irritant or a killing one. With the purpose of maximizing the effects of insecticides on the survival of mosquito vectors and malaria transmission,

insecticides with a high killing effect are preferred to those with irritant and repellant effect (WHO, 2013b).

IRS with DDT has been an essential component of the Global Malaria Eradication Campaign and has the longest residual effect of 6-12 months compared to other insecticides (WHO, 2006). Hence, the number of application of DDT in areas with persistent malaria transmission is reduced and cost-effective. However, the use of DDT in IRS programmes became questionable as a result of its effect on humans and the environment which includes the residents in IRS-treated homes and the spray operators (Fry, 1995; Wolff *et al*., 2000; Yanez *et al*., 2002). This led to a search for alternative insecticide such as the synthetic pyrethroids which was introduced in the 1990's by National Malaria Control Program, thereby declining the use of DDT in IRS (Goodman *et al*., 2001; Zaim, 2002; Zaim and Jambulingam, 2007)

Nevertheless, the use of DDT in IRS was re-introduced for use after much consideration by the framework of the Stockholm Convention on Persistent Organic Pollutant (POPs). It was banned for use except for public health purposes (WHO, 2006) and thus, making it possible to use DDT in IRS as long as severe measures are taken to prevent misuse and leakage of this insecticide outside public health. To date, the use of DDT and pyrethroids for IRS have become the mainstay for the control of malaria vectors in various parts of the world (Ratovonjato *et al*., 2014; Skarbinski *et al*., 2012; Zhou *et al*., 2010).

Unfortunately, the emergence of resistance to these insecticides has delimited the success of their use in IRS control programmes and calls for a continuous search for alternative ways of vector control as well as alternative insecticides.

2.4 Collection of Adult Mosquitoes

In order to have a sound knowledge of *Anopheles* mosquito vectors and its role as disease transmitters in an area, it is important to collect these mosquitoes (WHO, 1995). Hence, mosquito collection is an integral part of a research and different methods are involved in collecting mosquitoes. The choice of the method to be used depends on certain factors such as the objectives of the study, the available means of collection, as well as the environment to be sampled from (Le Goff *et al*., 1993). Two methods of interest are; Human Landing Catches (HLC) and Pyrethrum Spray Collection (PSC). Other methods include exit trap collections, CDC light traps etc.

2.4.1 Human Landing Catch (HLC)

In studying the direct contact of *Anopheles* mosquitoes with humans, the human landing catch method is commonly used. This is because it gives a good measure of the human-vector contact and it is regarded to as the reference method (Ndiath *et al*., 2011). HLC is one of the most common method in collecting adult mosquitoes which are anthropophilic either feeding indoors (endophagy) or outdoors (exophagy).

HLC involves using humans as baits with the purpose of attracting and collecting the mosquito when it rests on the human collector. The effectiveness and success of this method depends largely on the attractiveness, skill and experience of the collector (Mukabana *et al*., 2004). Results gotten

from this method are used to determine the biting pattern of the *Anopheles* vector, parity rate as well as the sporozoite infectivity and rate. However, this method of *Anopheles* mosquito collection is not ethically right as these collectors are exposed to bites and possible transmission of various pathogens carried by these vectors.

2.4.2 Pyrethrum Spray Collection (PSC)

PSC is one of the commonly used methods for sampling indoor resting mosquitoes in houses and animal shelters. The mode of operation is such that the occupants, animals and easily removable objects such as small tables, chairs and exposed drinking water and food are removed from the rooms to be sprayed (Odiere *et al*., 2007). After this removal, white calico sheets are spread over the entire floor and a pyrethroid insecticide sprayed in the room. This insecticide knocks down the mosquito vector which are then collected from the spray sheets.

PSC method allows for quantitative studies to be undertaken as well as the physiological conditions of the mosquitoes collected. The physiological stages of these mosquitoes such as being young unfed, fully blood fed and half gravid, suggests their behaviour. Therefore, samples collected with this method can be used to determine the ages of the mosquito, the source of their blood meal and the infectivity rate of these resting mosquitoes.

One drawback of this method is that, it does not take into consideration the exophagic and exophilic mosquitoes, thereby avoiding the outdoor fraction of the mosquito population.

2.5 Identification of *Anopheles* Mosquito

2.5.1 Morphological Identification

In identification of mosquitoes, morphological identifications form the starting point of the process. Descriptions for *Anopheles* mosquitoes and keys for identification have been set up by Gillies and De Mellion (1968) and later improved upon by Gillies and Coetzee (1987).

All species of *Anopheles* mosquito are identified by the presence of dark and light spots on the costa margin of the wing. Whilst *An. gambiae* complex are morphologically distinguished by the presence of white patches or spots on the femur, tibiae and the first tarsal segment of all the legs with the tibia being narrowly pale apically, *An. funestus* group are distinguished by the absence of banding patterns on the dark hind tarsus.

More so, *An. gambiae* complex are distinguished by the presence of five distinctly pale spots on the costal margin of the wing, which are yellow or cream in colour, while *An. funestus* are distinguished by the presence of four pale spots on the coastal margin of the wing and the presence of dark fringe scales at the margin of the anal wing. Also, in *An. funestus* group, there is the presence of three white narrow rings on the maxillary palps.

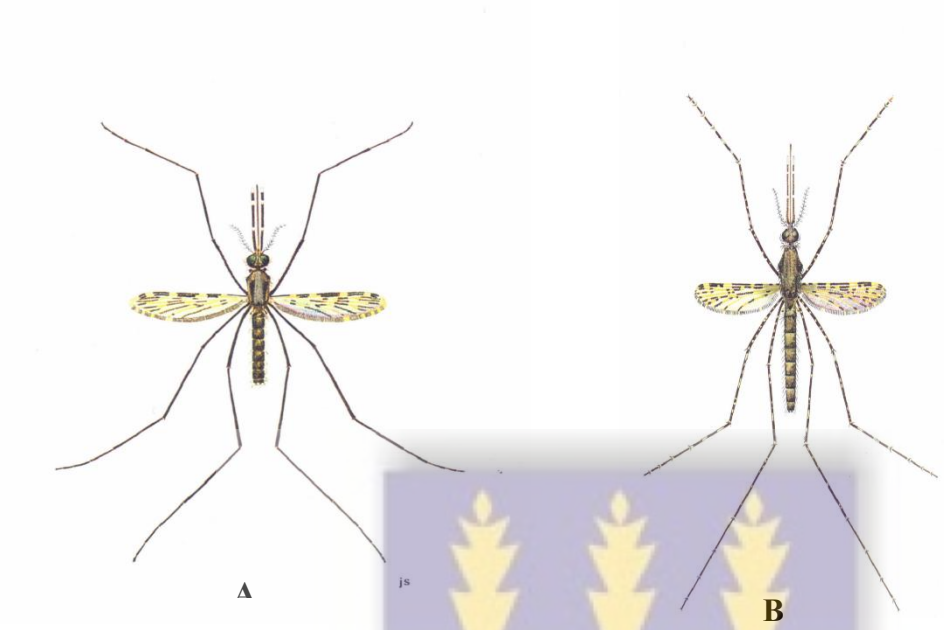

Figure 2: Picture of female *Anopheles* mosquitoes showing distinctive morphological features. (A) *An. funestus* (B) *An. gambiae.* (Gillet, 1972).

2.5.2 Molecular Techniques in Identification

Species of *Anopheles* mosquito such as *An. gambiae* group and *An. funestus* group have complexes consisting of sibling species that are morphologically indistinguishable. The striking similarity existing within these cryptic species has led to the need for other identification techniques.

Techniques such as cytotaxonomy (chromosome banding patterns), cuticular hydrocarbon analysis and allozyme analysis have been used (Baimai *et al.*, 1984; Foley *et al.*, 1993; Rasoolian *et al.*, 2008), but these techniques are associated with certain difficulties and cannot be equaled with the simple and fast method of Polymerase Chain Reaction (PCR) and DNA probe hybridization. The

integration of molecular techniques with morphological analysis helps to accurately identify and characterize members of species complexes.

2.5.2.1 Polymerase Chain Reaction (PCR)

PCR is a scientific technique that selectively amplifies a single or a few copies of DNA thereby creating thousands to millions of a particular DNA sequence. The principle of PCR is such that minute amounts of DNA can be replicated rapidly and amplified to an extent that the DNA can be easily detected and viewed. This is accomplished by the use of polymerases, which are enzymes that have the capability of stringing together individual DNA building blocks and are able to remain stable under high temperatures, in order to form long molecular strands (Joshi and Deshpande, 2010). For the polymerase to work, it requires a supply of DNA building blocks such as the adenine (A), thymine (T), cytosine (C) and guanine (G). In the presence of a small fragment of DNA known as primers, there is attachment of the building blocks to a longer DNA molecule which serves as a template in forming a new strand.

Three basic steps are involved in this technique. They include; denaturation, annealing and extension. In the first step which is the denaturation stage, the solution containing the DNA molecules to be copied, polymerases, primers and nucleotides is heated at about 95 degrees Celsius in order to denature the DNA. In the second stage, which is the annealing stage, the primers bind to the DNA at a lowered temperature of 55 degrees Celsius, priming extension. In the last stage, extension of the bound primers take place so as to create a complementary copy strand of DNA. This takes place at a temperature of 72 degrees Celsius, which is a suitable temperature for the polymerase enzyme used (Joshi and Deshpande, 2010).

The use of PCR in the study of mosquito populations is an *in vitro* method, which is developed for the synthesis of specific DNA sequences, with the presence of two nucleotide primers catalyzed by the action of the *Taq* polymerase enzyme. The primers used hybridize to the opposite strands, flanking the region of interest in the target DNA (Saiki *et al*., 1988).

2.5.2.1.1 PCR Identification of the Members of the *An. funestus* Group

Identification of the members of the *An. funestus* group is currently being done by a PCR-based method developed by Koekemoer *et al*. (2002). This method is based on species-specific primers in the Internal Transcribed Spacer Regions 2 (ITS2) region on the ribosomal DNA (rDNA), which accurately and rapidly identifies five common members of the group. The ITS2 region is a non-coding region which is separated by intergenic spacer regions (IGSs) (Paskewitz and Collins, 1997). These regions show high levels of variation within members of species complexes, thereby making it possible to easily and accurately identify members within species that are closely related using species-specific diagnostic assays.

In PCR identification of members of the *An. funestus* group, a cocktail of 20 oligonucleotide bases are used as five separate primers. These primers are used concurrently, without having any effect on amplification and can accurately identify *An. funestus s.s*., *An. vaneedeni, An. rivulorum, An. parensis* and *An. leesoni*, which are common members of the group. In Ghana, this method has been used in the study of the *An. funestus* group and only two members, *An. funestus s.s*. and *An. leesoni* were identified in Navrongo, Ejura, and Dodowa areas of Ghana (Dadzie *et al*., 2013).

2.5.2.2 The Use of Enzyme-Linked Immunosorbent Assays (ELISA)

The ELISA is an immunologic test that has been used in the molecular studies of mosquitoes. The principle of ELISA is based on the ability of specific proteins to bind to plastic surfaces and yet, maintain their antigenicity. One significant characteristic of ELISA is the immunological reaction which occurs as a result of the use of an enzyme indicator (Bangs, 1989).

ELISA technique uses highly species specific monoclonal antibodies that recognizes and binds with the antigen. The specificity of these antibodies as well as the sensitivity of the enzyme substrate adds to the unique feature of this technique. The enzyme linked to the antibody serves as a marker in detecting a specific protein, which is mostly an antibody or an antigen. There exists four types of ELISA namely; the direct, indirect, sandwich and the competitive ELISA (Aydin, 2015).

The direct ELISA as developed by Engvall and Perlmann (1971) and by Van Weemen and Schuurs (1971) led the way for other types of ELISA. The protocol of this type of ELISA is such that the antigen is coated directly to the surface of the plate, followed by a primary antibody and the enzyme. Indirect ELISA on the other hand, involves the addition of a secondary antibody before the enzyme is added thereby including an additional step to its protocol (Aydin, 2015). In sandwich ELISA which is known as the antibody screening, the primary antibody (capture antibody) is coated to the surface of the plate before the addition of the antigen, followed by the addition of the secondary antibody and then the enzyme. In competitive ELISA, more than one antigen or antibody is added to the plate and the molecules compete with each other to bind with the antigen or antibody in the well (Aydin, 2015).

The application of ELISA in mosquito studies is seen in the detection of the presence of sporozoites in mosquitoes as well as in the analysis of blood meal in mosquitoes. The sporozoite ELISA can be carried out in either freshly killed, frozen or dried mosquitoes. It involves the sandwich ELISA in which the primary monoclonal antibody (capture MAb) is bound to the wells of a plastic micro-titer plate, after which the well contents are aspirated and the remaining binding sites blocked with blocking buffer. The heads and thoraces of the mosquitoes are cut off and are ground in blocking buffer and an aliquot is added to the wells. Positive and negative controls are also added to the wells (Bangs, 1989). With the presence of CS-antigen, antigen-antibody complex will be formed with the primary antibody. This is followed by a 2-hour incubation, aspiration of the added aliquot and washing of the well.

The secondary antibody which is a peroxidase-linked MAb is added to the wells and on binding with the antigen, completes the formation of a sandwich. After 1 hour incubation, this is aspirated and the wells are washed followed by the addition of the clear peroxidase substrate (enzyme substrate) which on reaction, gives a dark green product (Bangs, 1989). Positive samples give this coloration while negative samples remain colourless. In Ghana, ELISA detection of sporozoites has been used in numerous studies and have been found positive in *An. funestuss.s.* (Appawu *et al*., 2003; Appawu *et al*., 2004; Kasasa *et al*., 2013; Dadzie *et al*., 2013).

ELISA for blood meal identification as described by Burkot *et al*. (1981) is used to determine the feeding preference of a mosquito. In Ghana, blood meal ELISA has been used in various studies (Okoye *et al*., 2005; Dadzie *et al*., 2013)

2.6 Entomological Indicators of Transmission

There are different levels of malaria transmission in different areas. In order to determine the level of transmission in an area, entomological indicators are used. Commonly employed entomological indicators include; human biting rate (HBR), human blood index (HBI), sporozoite rate (SR) and Entomological Inoculation Rate (EIR).

These indicators help to determine which mosquitoes are vectors of malaria in the area where they are found. More so, it is important in evaluating the impact of vector control measures such as IRS. Studies have shown the intensity of malaria transmission using EIRs as an indicator in Ghana (Appawu *et al*., 2004; Dadzie *et al*., 2013) and in Cameroon (Cohuet *et al*., 2004)

2.6.1 Human Biting Rate (HBR)

The average number of bites an individual receive from a mosquito species can be calculated. This is known as the human biting rate (HBR). It is an important indicator of malaria transmission (Beier *et al*., 1999) and can be measured directly from the human landing catches (HLC) made from indoor collections as well as outdoors (Garret-Jones and Shidrawi, 1969). Indirect measurement of HBR can also be calculated and this is gotten from pyrethrum spray collections of indoor mosquitoes (Garret-Jones and Shidrawi, 1969).

The importance of this indicator extends to the fact that they determine the mosquito vectors that are anthropophagic along with endophagy and exophagy (WHO, 2003). In Ghana, the man-biting rate of *An. funestus* has been studied alongside with *An. gambiae* in Kassena Nankana District (KND) of Northern Ghana in three micro-ecological zones where it was an overall rate of 36.7

bites/person/night in the irrigated area of their study sites (Appawu *et al*., 2004). Also, studies in Cameroon recorded a high human biting rate of *An. funestus* averaging 85.1 and 108.3 bites/person/night for indoors and outdoors respectively (Tanga *et al.*, 2011). It was named the most aggressive species amongst other species sampled throughout the period of study.

2.6.2 Human Blood Index (HBI)

The feeding preference of a mosquito vector species is an important parameter in determining the vectorial capacity of that species. *Anopheles* mosquitoes can either be anthropophagic or zoophagic or both, with the most efficient malaria vectors being strictly anthropophagic. This stresses the need to determine the human blood index of mosquitoes.

The HBI can be defined as the proportion of mosquitoes that has fed on human blood (Pappa *et al*., 2011). Proportion of mosquito vectors that has fed on other blood meals such as bovine, avian and ovine may also be determined and this gives an indication of the host preference of the vector. The switch in host preference of some *Anopheles* mosquito in certain areas can be explained as the response of the mosquito to the control intervention such as IRS or LLINs in that area (Teklehaimanot *et al*., 2009). The excito-repellant effect of the insecticide used in these interventions drive indoor mosquitoes seeking human hosts outdoors, where they eventually come in contact with non-human hosts and feed on them.

Blood meal analysis of *Anopheles* sp. can be done by the use of Enzyme-Linked Immunosorbent Assays (ELISAs) in order to determine the source of blood meal in the mosquito abdomen. Analysis of the sources of blood meals of mosquitoes is important in measuring the degree of

anthropophily of that particular species. Studies in Ghana and beyond have shown the HBI of *An. funestus*, affirming its anthropophilic behaviour. In Ghana, a high proportion of over 80 % of *An. funestus s.s.* sampled and tested had their source of meal as human blood (Dadzie *et al*., 2013). Also in Cameroon, on the scale of 1, the HBI of *An. funestus* sampled and tested was 0.98, indicating a highly anthropophilic behaviour of this species (Tanga *et al*., 2011).

2.6.3 Sporozoite Rate of *Anopheles* mosquito

Sporozoites are the infective stage of the *Plasmodium* parasite found in mosquitoes which is usually introduced into the host during blood meal. The determination of the proportion of sampled adult mosquitoes carrying *Plasmodium* sporozoites helps to measure the infectivity of these mosquitoes. The salivary glands of these mosquitoes are examined and this is necessary in determining the vectorial role of such mosquito species. It is also necessary in evaluating the impact of control interventions such as the use of IRS.

In determining the sporozoite rate of mosquitoes, methods such as dissection or the immunologic tests- Enzyme Linked Immunosorbent Assays (ELISA) are employed. Although the dissection of the salivary glands of mosquitoes for sporozoites can yield accurate results, it is very laborious and demanding, especially when working with large numbers of samples and not easily applicable in the field (Coleman *et al*., 2000). More so, freshly killed mosquitoes and a high level of skill are required including the non-specificity of the sporozoites seen (Charlwood *et al*., 2015). These are major drawbacks of this method.

Conversely, the use of ELISA as developed by Burkot *et al*. (1984) and Wirtz *et al*. (1987) has been seen as a better alternative in the detection of *Plasmodium*-specific circumsporozoite antigens from the head and thorax of mosquitoes. In doing this, monoclonal antibodies are used in order to detect the repeated amino acid sequence present in these specific proteins after which the sporozoites rate is calculated from the proportion of *Anopheles* species that test positive for *P. falciparum* circumsporozoite protein by ELISA.

2.6.4 Entomological Inoculation Rate (EIR) of *Anopheles* mosquito

In entirety, the number of infective bites which an individual receives in the night for a particular period of time, be it a month, a year or a season can be calculated. This is known as the entomological inoculation rate (EIR) and it is calculated as the product of sporozoite rate and the man biting rate, expressed as the number of infective bites per person per unit time. This helps to ascertain the intensity of malaria parasite transmission in the area under study for a particular period of time.

2.7 Pyrethroid Resistance in *Anopheles funestus*

Vector control of malaria rely heavily on the use of insecticides. Twelve insecticides, all belonging to 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 (WHO, 2013a). Commonly used among these insecticides are the pyrethroids, which are used in different forms (Zaim, 2002) such as in treating bed nets and indoor spraying. Pyrethroids are preferably used because of the rapid knockdown effect they have on mosquitoes as well as their low mammalian toxicity as compared to the other insecticide classes (Zaim, 2002).

They are also used in control of agricultural pests which in turn can produce run-offs that are insecticide contaminated and seep into the larval habitats of mosquitoes (Hilary *et al*., 2009).

All these extensive usage of the insecticide can affect its efficacy, thereby leading to the evolution of resistance of mosquitoes to this insecticide. WHO (2013a) defines insecticide resistance as the development of an 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. Resistance to pyrethroids has become widespread among different species of *Anopheles* mosquitoes in many regions of Africa (Munhenga *et al*., 2008; Protopopoff *et al*., 2008; Cuamba *et al*., 2010; Djouaka *et al*., 2011). Two major mechanisms of insecticide resistance to pyrethroids exist (Kawada *et al*., 2014). They are; target-site mutation and metabolic resistance. Other mechanisms by which insects become resistant to insecticides are; cuticle alteration and behavioural mechanism (Nkya *et al*., 2014) among others

2.7.1 Target-site Mutation

The target site for the pyrethroid insecticide is the voltage-gated sodium channel on the insect's neurons (Davies *et al*., 2007). The closing of the sodium channel is delayed by the binding of the insecticide thereby, prolonging the action potential and causing repetitive neuron firing, paralysis, and death of the insect eventually (Ranson *et al*., 2011). Changes in the target site of these insecticides confer resistance to the insect and is referred to as knockdown resistance (Ranson et al., 2011). These mutations also confer resistance to DDT which has a similar mode of action as pyrethroids. Knockdown resistance (*kdr*) to pyrethroids have been recorded in many insects (Davies *et al*., 2007). However, the only residue associated with pyrethroid knock-down resistance

(*kdr*) in malaria vectors, which is the most common amino acid replacements, is the point mutation in which the leucine residue found at codon 1014 is replaced by either phenylalanine (1014F) or serine (1014S).

In several African countries, there have been records of *kdr* mutation in *An. gambiae* populations. In West Africa, it is referred to as *kdr* west (*kdr*-w) where there is leucine to phenylalanine substitution (L1014F), whilst in East Africa, 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). In Ghana, the knockdown resistance type of target site mutation in *An. gambiae s.l* has been extensively studied and recorded (Adeniran *et al*, 2009; Kabula *et al*., 2011). *Kdr* in *An. funestus* populations has not been recorded in Ghana. However, pyrethroid resistance in *An. funestus* has been studied and recorded in Ghana (Coetzee *et al*., 2006; Okoye *et al*., 2008) as well as in other African countries (Casimiro *et al*., 2006; Wondji *et al*., 2007; Cuamba *et al*., 2010; Morgan *et al*., 2010; Djouaka *et al*., 2011). Wondji *et al*. (2012) records mutation in the target site of *An. funestus* in Malawi.

2.7.2 Metabolic Resistance

Metabolic resistance of insects to insecticides occur when elevated activities of one or more detoxification enzymes results in a sufficient proportion of the insecticide being sequestered or detoxified before it reaches the target site to impair the toxicity of the insecticide (Ranson, *et al*., 2011). The primary enzyme family responsible for pyrethroid metabolism in insects are the cytochrome P450s (Feyereisen, 2005). Among these, CYP6M2, CYP6P3 and CYP6Z2 are the three candidate P450 genes that have been identified to be repeatedly over expressed in pyrethroid

resistant populations of *An. gambiae* (Djouaka *et al*., 2008). Amongst these three candidate P450 genes, only CYP6P3 and CYP6M2 can metabolise the insecticide (Muller *et al*., 2008). Studies have identified cyp6p3 and cyp6p9 to be the supposed ortholog of *An. gambiae* in *An. funestus* as being the prime candidates of pyrethroid resistance in this species (Wondji *et al*., 2009). Other enzyme families might also play a secondary role in pyrethroid resistance (Ranson *et al*., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in three districts; Tolon, Savelugu and Tamale Districts, all in the Northern Region of Ghana (Figure 3). Within each district, three communities were selected. They were Dimabi, Gbullung and Woribogu for Tolon District; Diare, Nanton and Tarikpaa for Savelugu District and Tugu, Kulaa and Yong for Tamale District. Savelugu District has been under IRS for seven years while Tolon District was under IRS for five years as the IRS was withdrawn in this area. In this research work, Tolon and Savelugu Districts were regarded as IRS areas, while Tamale District, where no IRS has been implemented, was chosen as the control site.

The Northern Region is the largest region, in terms of land mass in Ghana, with a relatively dry climate and experiences a single rainy season from May to October. The amount of rainfall recorded annually varies between 750 mm to 1100 mm with the dry season starting from November to March/April. The maximum temperatures occur towards the end of the dry season while the minimum temperatures are in December and January. The vegetation at the area consists predominantly of grassland, especially savannah with clusters of drought-resistant trees such as baobabs, acacias, shea nut and dawadawa.

Tolon District is an irrigation site principally used for rice farming, thereby serving as breeding sites for mosquitoes with geographical coordinates 9°25′60″N, 1°4′0″W. Inhabitants of Savelugu

District mostly engage in agricultural activities such as raising livestock near the household thereby providing alternate sources of blood meal for *Anopheles* mosquitoes with geographical coordinates at 9°37′29″N, 0°49′40″W. On the other hand, Tamale District is a metropolitan district with its geographical coordinates at 9°24′0″N, 0°50′0″W. Its inhabitants also engage in agricultural activities and have standing water in burrow pits that can create breeding sites for mosquitoes.

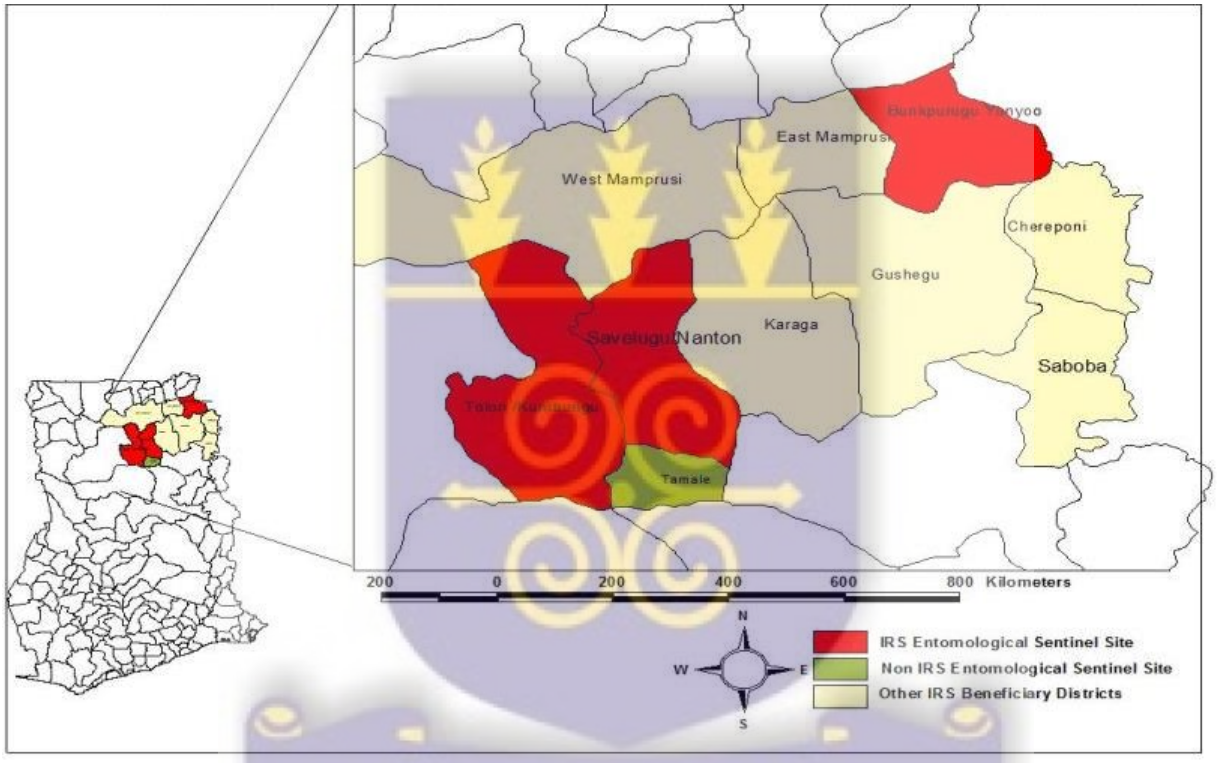

Figure 3: Map of Ghana showing the IRS Districts and entomological sentinel sites

3.2 Adult mosquito Surveys

Adult mosquitoes were collected from two randomly selected compounds for every study community in the selected districts using the human landing catch (HLC) and pyrethrum spray

collection (PSC) methods. These collections were supervised by three trained personnel from Ghana Health Services and the District Assemblies.

3.2.1 Human Landing Catches (HLC)

Adult mosquitoes were collected for four nights every month from January to December 2015, in each of the two randomly selected compounds, for every selected community in all the study districts using the HLC technique. A two-man team of four trained volunteer collectors were involved in the collection and surveying of indoor and outdoor biting mosquitoes. Collections were made from 18:00 hours (GMT) to 06:00 hours (GMT) with 10 minutes breaks for every hour. Mosquito collectors wore long sleeve shirts to ensure that only the lower legs of the collectors were exposed to blood seeking mosquitoes. Mosquitoes attempting to bite the collectors were detected with a flashlight, picked up using an aspirator and transferred into labelled paper cups covered with a mesh screen (WHO, 2003). To compensate for differential attraction of the collectors to mosquitoes, they rotated between indoors and outdoors after two hour period of collection. The duration of collection of these mosquitoes was the same used for the archived mosquito samples. The volunteers were made to sign an informed consent form indicating their willingness to participate in the study. All the collectors were given prophylaxis according to the National guideline and those that got malaria were sent to the nearest health facility and treated free of charge.

3.2.2 Pyrethrum Spray Collection (PSC)

Besides the HLC technique, indoor resting mosquitoes were also collected using the PSC technique. This was done in the early hours of the day from 06:00 to 07:00 hours GMT in the same

compounds used for HLC but in different rooms. A total of eight rooms were surveyed for each community every month for four days in a month. Pyrethrum spray collection was performed using pyrethrum spray (Raid™) and white calico sheets. Occupants of the rooms were made to leave the sample rooms. The floors and other furniture like bed as well as other belongings in the rooms were covered with white calico sheets after which the doors and the windows of the rooms were closed. Spraying of the pyrethrum in a room was done in a clockwise direction until there was a fine mist of the insecticide in the room. After about 10-15 minutes, the sheets were picked by their corners starting from the door way and carefully carried out of the room. Using a pair of forceps, knocked down adult mosquitoes were carefully picked and transferred into pre-labelled petri dishes lined with a layer of damp cotton wool covered with filter paper. These were taken to the laboratory for further processing and analysis.

3.3 Mosquito Processing

Wild adult mosquitoes collected were morphologically identified and preserved in well-labelled 1.5 milliliters microcentrifuge tubes with silica gels. These were carefully preserved at -20 °C until required for further processing and analysis. *Anopheles funestus* from archived survey samples for the years 2014, 2013 and 2010 were sorted from the cold room section of Noguchi Memorial Institute of Medical Research (NMIMR). Each of the mosquitoes collected in all these surveys were cut into different parts; legs and wings were used for molecular identification of sibling species complex using polymerase chain reaction (PCR), head and thorax for sporozoite rate determination using enzyme-linked immunosorbent assay (ELISA) and the abdomen for blood meal analysis using ELISA.

3.4 Species Identification

3.4.1 Morphological Identification

Adult mosquitoes collected after each sampling were sorted out and members of the *An. funestus* group were isolated from other anophelines using taxonomic keys described by Gillies and Coetzee (1987) and Gillies and De Meillon (1968).

Adult *Anopheles* mosquitoes were identified by the equal length of palps and proboscis, presence of markings on the palps, the banding and speckling on the legs as well as the distinctive block patterns of dark and pale scales on the vein of the wings along the costa margin. *Anopheles funestus* group were distinguished from other *Anopheles* by the presence of four pale spots on the costa margin of the wings, dark fringe scales at the margin of the anal wing vein, three white narrow rings on the maxillary palps and the absence of banding patterns on the dark hind tarsus.

3.4.2 Molecular Identification of *Anopheles funestus* Group

3.4.2.1 Genomic Deoxyribonucleic Acid (DNA) Extraction

Genomic DNA was extracted from the legs and wings of the *Anopheles* mosquito using the CTAB (Cetyl Trimethyl Ammonium Bromide) method, as described in Morlais *et al*. (2004) with a slight modification.

The extraction buffer was prepared using a mixture of 1 M Tris HCl, pH 8.0, 0.5 M EDTA, 1.4 M NaCl, 2 % cetyltrimethyl ammonium bromide (Appendix I) which were vortexed and autoclaved. Two hundred microlitre of the extraction buffer was added to each of the 1.5 ml microcentrifuge

tubes containing the legs and wings of the mosquito. This was homogenized using a motorized pestle and the homogenate was incubated at 65 °C for 5 minutes. Two hundred microliter of chloroform was then added to the mixture, mixed per inversion and centrifuged at 12,000 rpm for 5 minutes. After this, the supernatant was transferred into a new 1.5 ml microcentrifuge tubes and 200 µl of isopropanol added to the supernatant to precipitate the DNA. This was then centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded, taking care not to dislodge the pellet at the bottom. Two hundred microliter of 70 % ethanol was added to the pellet and centrifuged at 12,000 rpm for 5 minutes. Again, the supernatant was discarded and the tubes were inverted and left to dry overnight at room temperature. The DNA pellets were reconstituted in 20 µl of DNAse free water, left to stand for one hour and stored as stock at -20 °C freezer until ready to be used.

The extract was used as a DNA template for molecular species identification and the determination of *kdr* status.

3.4.2.2 PCR Identification of Members of the *Anopheles funestus* Group

The polymerase chain reaction (PCR) method as described by Koekemoer *et al.* (2002) with slight modification was used to identify members of the *An. funestus* group. The temperature conditions of the PCR were modified in order to give visible diagnostic bands for species identification. Species specific DNA sequences were amplified using seven sets of primers abbreviated as UV, FUN, VAN, RIV, RIVLIKE, PAR, LEES (Table 1). This set of primers were designed from the DNA sequences of the intergenic spacer region of the ribosomal DNA (rDNA) of the *An. funestus* group. UV is a universal primer and anneals to the same binding position on the rDNA of all the

members of the *An. funestus* group. FUN is specific to *An. funestus s.s.*, VAN is specific to *An. vaneedeni*, RIV is specific to *An. rivulorum*, RIVLIKE is specific to *An. rivulorum-like*, PAR is specific to *An. parensis* and LEES is specific to *An. leesoni*.

Table 1: Primer sequences of *An. funestus* group, expected band sizes and melting temperature (Tm) of the PCR amplified DNA products (Koekemoer *et al*., 2002).

Primers	Sequences (5'-3')	Band size (bp)	Tm (°C)
UV	TGT GAA CTG CAG GAC ACA T		55.3
FUN	GCA TCG ATG GGT TAA TCA TG	505	52.4
VAN	TGT CGA CTT GGT AGC CGA AC	587	58.0
RIV	CAA GCC GTT CGA CCC TGA TT	411	58.8
RIVLIKE	CCG CCT CCC GTG GAG TGG GGG	313	60.7
PAR	TGC GGT CCC AAG CTA GGT TC	252	60.5
LEES	TAC ACG GGC GCC ATG TAG TT	146	60.2

A master mix solution of 26 µl was prepared for each sample to be run for PCR. This mixture contained 12.5 µl of GoTaq®, 0.5 µl of 10 µm of each of the oligonucleotide primers (Eurofins Genomics) and 4 µl of the extracted DNA. The reaction mixture was topped up to 26 µl with DNase free water, after which it was placed in a thermocycler for amplification, using the following thermcycling profile; an initial denaturing step of 3 minutes at 95 °C to activate the DNA polymerase followed by 35 cycles each consisting of 30 seconds denaturation at 95 °C, 30 seconds annealing at 50 °C and 1 minute extension at 72 °C and an extension of the final cycle products for 7 minutes at 72 °C.

3.5 ELISA Technique

3.5.1 Detection of *Plasmodium falciparum* Sporozoites Infectivity in *Anopheles funestus* group using Enzyme-Linked Immunosorbent Assay (ELISA)

The head and thorax of 596 individual female *An. funestus* samples were tested for the presence of *P. falciparum* circumsporozoite protein (*Pf*CSP) using the sandwich ELISA method, following the protocol of Wirtz *et al.* (1987). In order to do this, preparation of mosquito *Plasmodium* antigen was done by homogenizing the head and thorax of individual mosquito in 50 µl of grinding buffer in a 1.5 ml microcentrifuge tube using a motorized 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 ready for 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 by putting 50 µl of the monoclonal antibodies (MAb) into each well (Appendix II). The next day, the plates were flipped empty, banged on a sterile paper towel to drain them and then incubated with 200 µl/well of the blocking buffer (BB) at room temperature for one hour. After this time, the plates were flipped empty, banged and then washed once with 200 µl/well of washing buffer (PBST). This was followed by incubation with 50 µl/well aliquots of the homogenates (test mosquito extracts and controls) at room temperature for two hours. Plates were washed twice with 200 µl/well of the washing buffer, flipped empty, banged and subsequently incubated with MAb peroxidase conjugate (0.5 µg/50 µl/well) in BB for one hour in darkness. The

plates were emptied, rinsed three times with washing buffer, banged, and then incubated with the substrate solution (Appendix II) of 100 µl/well for 30 - 60 minutes at room temperature. Reading of the plates started after 30 minutes through to the 60th minute, 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 *Pf*CSP. Those which remained colourless were regarded to be negative for *Pf*CSP. Coating of the micro-titre plate was done in such a way that wells F11 and F12 contained positive controls, wells G11 and G12 contained the blanks, wells H11 and H12 were the negative controls, and the rest of the wells contained the test mosquito extracts. 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 mosquitoes, while blocking buffer were used for the blanks. Figure 4 shows a 96 well plate showing the arrangement for ELISA.

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

Figure 4: Illustration of a 96 well plate showing the arrangement for enzyme-linked immunosorbent assay (ELISA) technique in determination of the presence of *P. falciparum* circumsporozoite antigens (P*f*CSP) in the *An. funestus* group. Legend: ***** represent the test samples, + represent the positive control samples, - represent the negative control samples and B represent the blanks.

3.5.2 Determination of the Human Blood Index of the *Anopheles funestus* Group

The abdomen of 600 individual female *An. funestus* mosquitoes were analyzed to identify the source of their blood meal. This was done using the direct ELISA method following the protocol of Beier *et al*. (1988) with a slight modification. Mosquito samples were prepared by grinding the abdomen of each blood fed mosquito in 50 µl of PBS (Appendix II) in 1.5 ml microcentrifuge tubes using a motorized plastic pestle in order to obtain a homogenous solution. From this solution, 1:50 dilution was done and frozen at -20 °C until ready for use.

Each well of the micro-titre plates was coated with 50 µl of the 1:50 dilution of the homogenate, loading each sample in duplicates and subsequently incubated overnight at 4 °C. The next day, the plates were flipped empty, banged and washed once with washing buffer. Two hundred microliter of blocking buffer was then added to each well and incubated at room temperature for one hour, after which the plates were flipped empty, banged and washed twice with wash buffer. Fifty microlitre of diluted host specific antibody-peroxidase conjugate (human, chicken and goat) was added to each well and incubated in the dark for one hour. Once again, the plates were flipped empty, banged and washed thrice with washing buffer. Finally, 100 µl of the substrate solution (Appendix II) was added to each well and incubated for 30 minutes at room temperature in a dark place. The micro-titre plates were read at 405 nm using the Biotek ELx800 microplate reader after 30 minutes, the plates were returned and left to incubate for another 30 minutes after which they were read using the microplate reader.

The results were analyzed by using the absorbance value of the negative control to estimate the cut off for positive samples. All test samples with absorbance greater than the sum of the mean absorbance of negative control and three times their standard deviation were considered positive for the particular host blood meal. Coating of each sample in the wells was done in duplicates. Positive and negative control coatings were done in such a way that wells G9 and G10 contained the positive controls, H9 and H10 contained the blanks, while A11 and A12, B11 and B12, C11 and C12, D11 and D12, E11 and E12, F11 and F12, G11 and G12 and finally, H11 and H12 contained the negative controls (Figure 5). Positive controls used were the blood of the expected host (human, goat and chicken) diluted in 1:2500 of PBS. On the other hand, the negative controls were homogenates of the abdomen of male mosquito and the blocking buffer were used as blanks.

The human blood index was calculated as the number of mosquitoes that tested positive for human blood per overall number of mosquitoes tested. Figure 5 shows a 96 well plate showing the arrangement for blood meal ELISA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sam1	Sam1	Sam9	Sam9	Sam17	Sam17	Sam25	Sam25	Sam33	Sam33	NEG1	NEG1
B	Sam2	Sam2	Sam10	Sam10	Sam18	Sam18	Sam26	Sam26	Sam34	Sam34	NEG2	NEG2
C	Sam3	Sam3	Sam11	Sam11	Sam19	Sam19	Sam27	Sam27	Sam35	Sam35	NEG3	NEG3
D	Sam4	Sam4	Sam12	Sam12	Sam20	Sam20	Sam28	Sam28	Sam36	Sam36	NEG4	NEG4
E	Sam5	Sam5	Sam13	Sam13	Sam21	Sam21	Sam29	Sam29	Sam37	Sam37	NEG5	NEG5
F	Sam6	Sam6	Sam14	Sam14	Sam22	Sam22	Sam30	Sam30	Sam38	Sam38	NEG6	NEG6
G	Sam7	Sam7	Sam15	Sam15	Sam23	Sam23	Sam31	Sam31	POS	POS	NEG7	NEG7
H	Sam8	Sam8	Sam16	Sam16	Sam24	Sam24	Sam32	Sam32	BLK	BLK	NEG8	NEG8

Figure 5: Illustration of a 96 well plate showing the arrangement for enzyme-linked immunosorbent assay (ELISA) technique in determination of the blood meal source in the *An. funestus* group. Legend: Sam represent the test samples, POS represent the positive control samples, NEG represent the negative control samples and BLK represent the blanks.

3.6 Determination of Man Biting Rate (MBR)

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. This was calculated using a formula by Lines *et al*. (1991):

$$MBR = \frac{\text{Number of mosquitoes collected}}{\text{Man nights}}$$

Where:

Man nights = Number of collectors × Number of nights

MBR = Man biting rate

3.7 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. funestus s.s.* samples using the slightly modified polymerase chain reaction (PCR) amplification method of Morgan *et al*. (2010) with slight modifications done by me. The volume of primers used were modified to give visible diagnostic bands for *kdr* detection. Genome Deoxyribonucleic acid (DNA) used for the PCR amplification process was extracted using the CTAB extraction method described in Section 3.4.2.1. Primers specific for detection of *kdr* mutation in *An. funestus s.s.* designated as *kdr*fun-F and *kdr*fun-R were used (Table 2). A twenty five microlitre reaction mixture containing 6.5 µl of DNase free water, 12.5 µl of GoTaq®, 4 µl of extracted DNA, 1 µl of 10 µM each of the primers *kdr*fun-F and *kdr*fun-R was prepared. The reaction mixture was thoroughly mixed and briefly centrifuged before

being placed in the thermocycler for amplification. The PCR amplification conditions used were; an initial denaturing at 95 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds and 72 °C for 1 minute. A final extension cycle at 72 °C for 10 minutes was performed followed by cooling at 4 °C. Eight microlitre of the amplified products were loaded onto a 1.5 % agarose gel, stained with ethidium bromide, submerged in 1× TAE buffer (Appendix I) 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 *An. funestus s.s.* The *kdr* status of the tested samples was noted. Expected band size for the presence of *kdr* mutation in *An. funestus* was 994 bp.

Table 2: Sequences of the *kdr* primers and their melting temperatures (Tm) (Morgan *et al*., 2010).

Primer	Sequence (5'-3')	Tm (°C)
*Kdr*fun-F	GTT CAA TGA AGC CCC TCA AA	55.3
*Kdr*fun-R	CCG AAA TTT GAC AAA AGC AAA	52.0

3.8 Data Entry and Analyses

The data collected were entered into a Microsoft Excel spreadsheet and imported into GENSTAT programme version 9 for analysis. The data on abundance, biting rates and human blood index from the IRS areas were compared with those in the control area using ANOVA, at 5 % significance level. They were log transformed to annul the effect of some deviations of the

assumptions of Analysis of variance (ANOVA). Analysis on sporozoite rates between the study areas was done using Pearson Chi-square method.

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito Abundance

A total of 688 adult female mosquitoes morphologically identified as *An. funestus* mosquitoes were caught in the IRS and non-IRS study areas in the years 2010, 2013, 2014 and 2015. This included 27.47 % (189) for 2010, 23.26 % (160) for 2013, 29.36 % (202) for 2014 and 19.91 % (137) for 2015 (Figure 6).

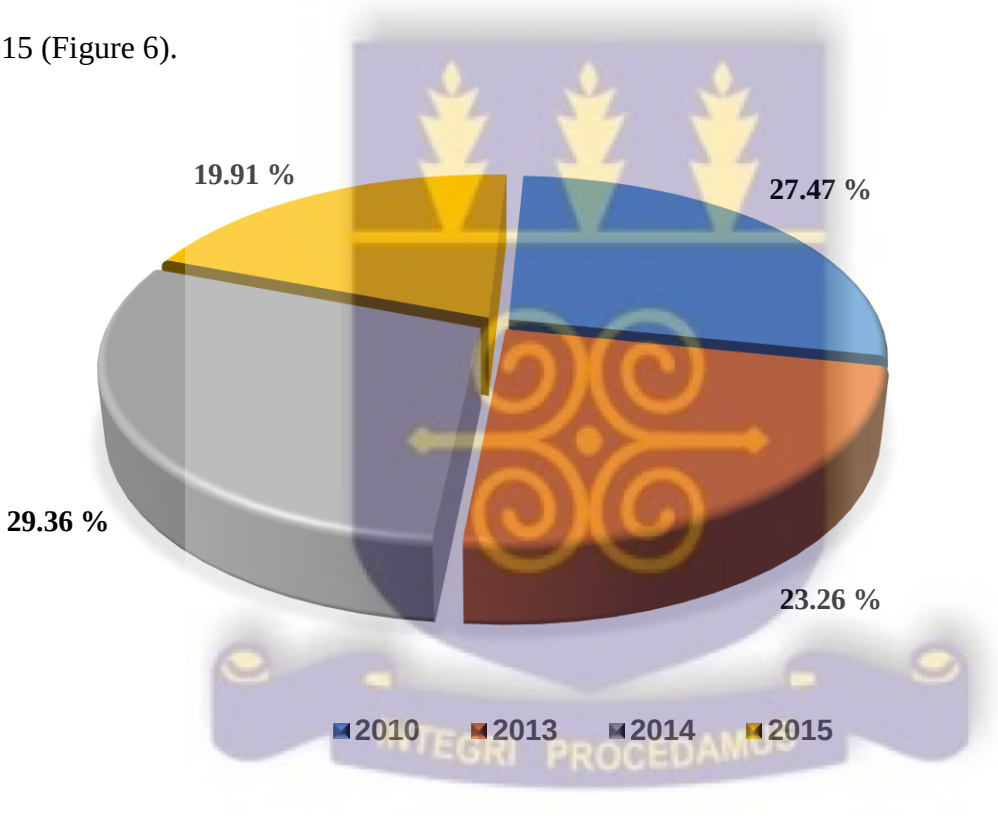

Figure 6: Overall yearly percentage composition of *An. funestus* mosquitoes collected in the study areas.

Out of the total number of mosquitoes collected, 620 were caught using indoor and outdoor Human landing catches (HLC) and the remaining 68 were caught using Pyrethrum spray collections. The indoor and outdoor human landing catches made constituted 46.66 % (321) and 43.46 % (299) respectively of the total collections as well as 9.88 % (68) for the pyrethrum spray collections across the years for both IRS and non-IRS areas (Figure 7).

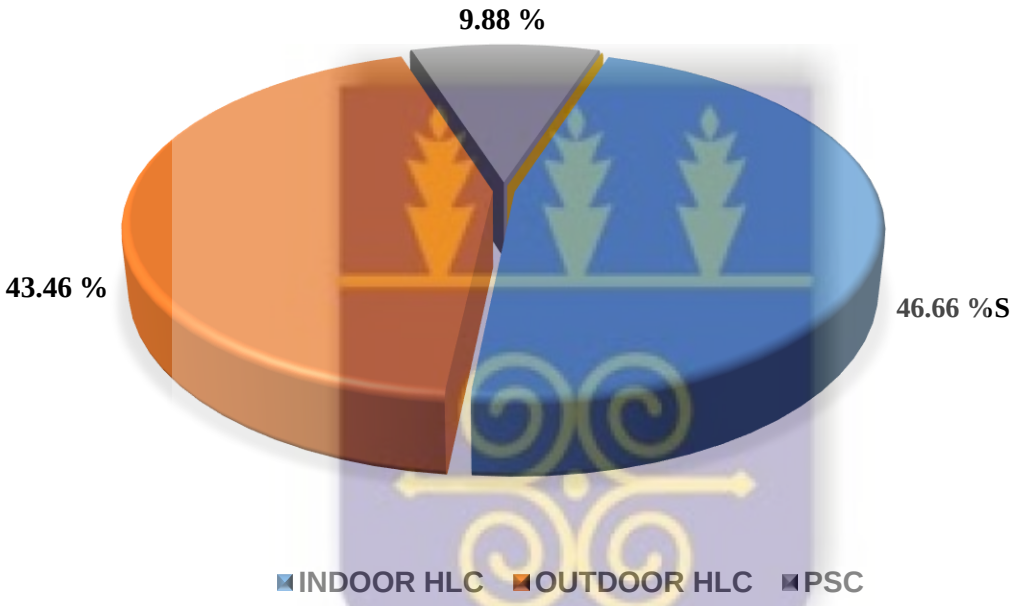

Figure 7: Percentage composition of *An. funestus* mosquitoes over the years using indoor and outdoor HLC and PSC methods in IRS and Non-IRS areas.

There was a statistically significant difference ($P < 0.001$) in the number of mosquitoes collected across the years between the IRS and Non-IRS areas (Appendix III). Further analysis showed that the number of mosquitoes collected in Tamale were significantly higher than that of Savelugu. Also, the number of mosquitoes collected in Tolon were significantly higher than that of Tamale.

4.1.1 Yearly Abundance of *Anopheles funestus* Group

The highest numbers of *An. funestus* mosquitoes were collected in Tolon District using the HLC method in the following order of years; 2014 (175), 2010 (130), 2015 (115) and 2013 (113) (Figure 8).

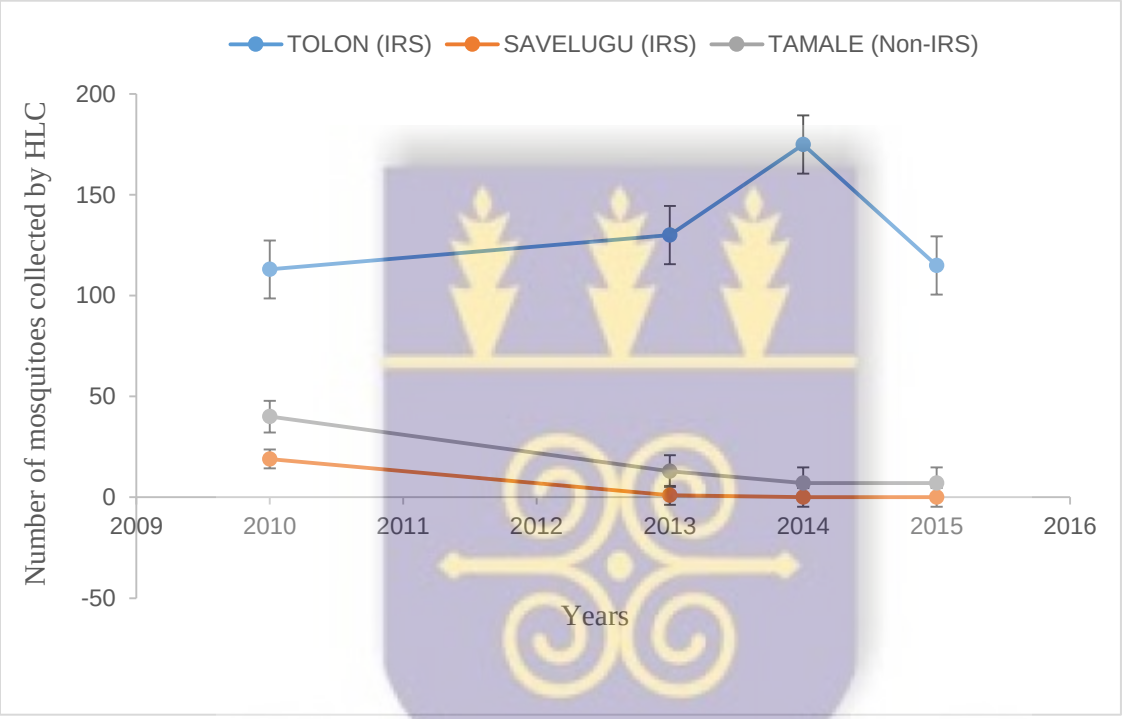

Figure 8: Yearly trends in HLC of *An. funestus* mosquitoes collected in the IRS and non-IRS areas.

Using PSC method, the highest numbers of *An. funestus* mosquitoes were also collected from Tolon District in the following order; 2014 (16), 2015 (14), 2013 (14), 2010 (10) (Figure 9)

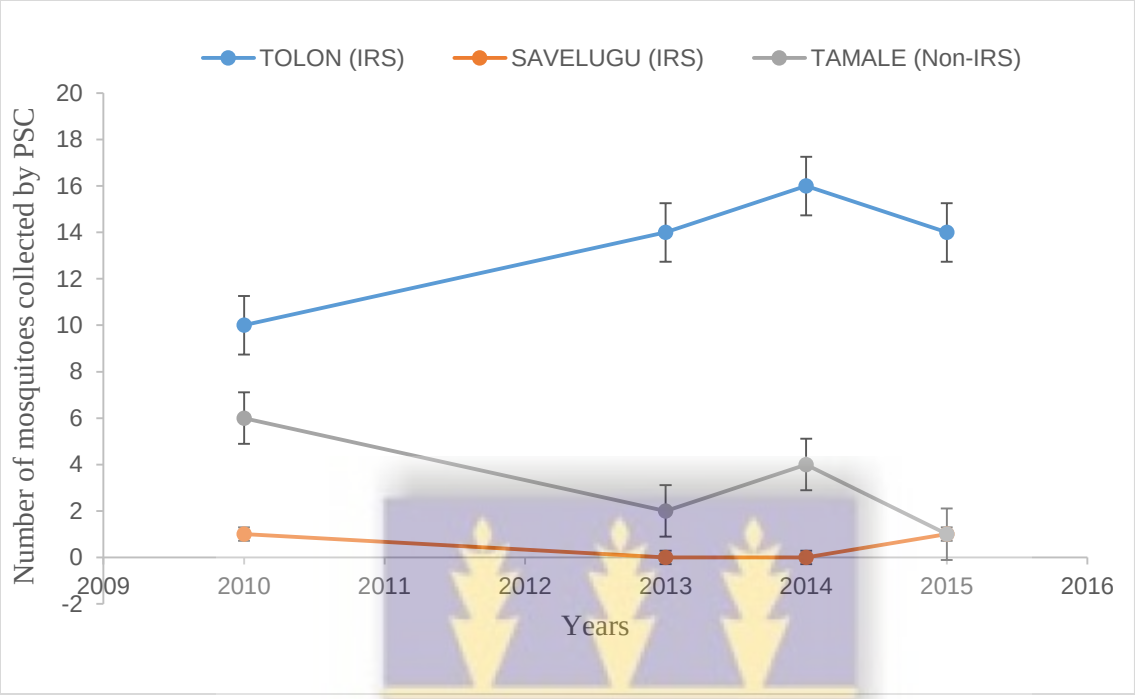

Figure 9: Yearly trends in PSC of *An. funestus* mosquitoes collected in the IRS and non-IRS areas.

4.1.1.1 Yearly Trends in Indoor and Outdoor HLC

The abundance of *An. funestus* mosquitoes collected using the Indoor HLC was at its peak in Tolon District in the year 2014 (107), followed by 2015 (61), 2013 (57) and 2010 (50). Similarly, using the outdoor HLC, the highest number of mosquitoes were collected in 2013 (73), followed by 2014 (68), 2010 (63) and 2015 (54). The least number of mosquitoes collected using indoor and outdoor HLC were gotten in Savelugu District for the respective years (Figure 10).

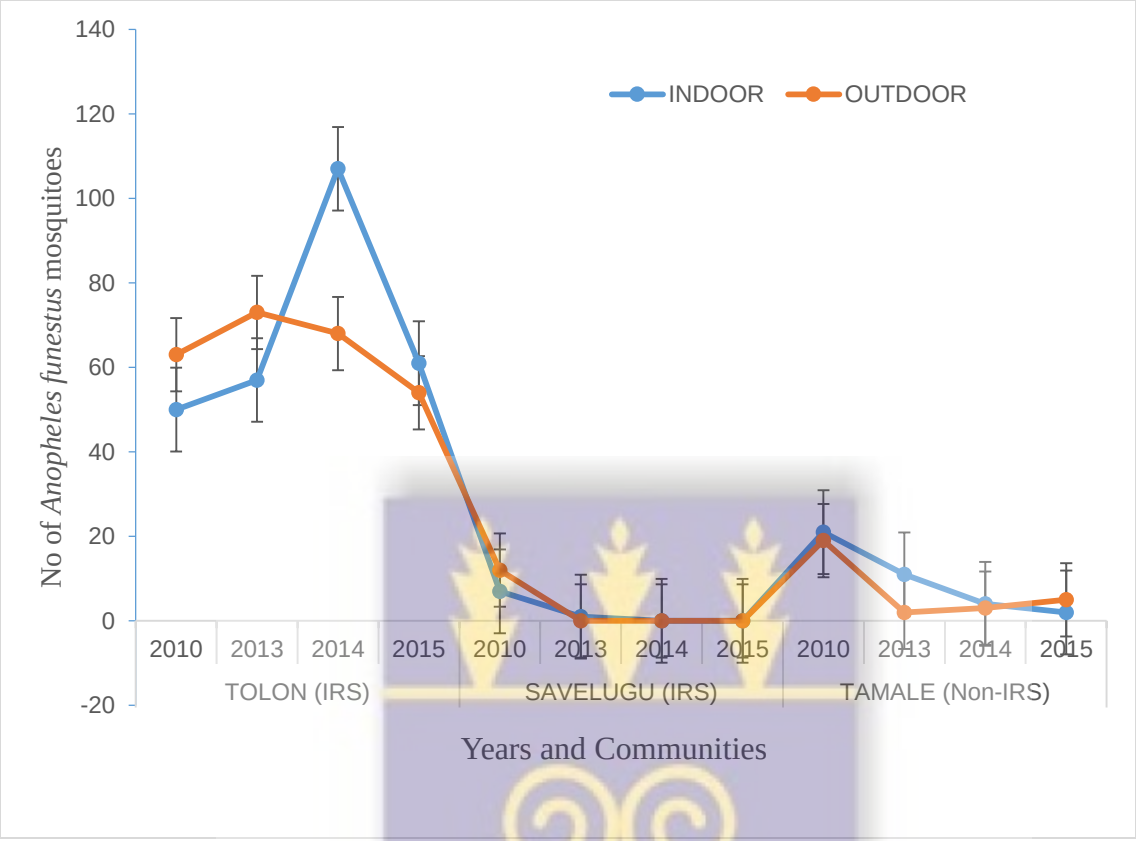

Figure 10: Yearly trends of *An. funestus* collected using indoor and outdoor HLC in IRS and Non-IRS areas.

4.2 Species Composition of the *Anopheles funestus* Group

In total, genomic DNAs were extracted from the legs and wings of 278 adult female *An. funestus* group for molecular analysis of their sibling species. This comprised of mosquito samples randomly selected from all the years; 80 (2010), 70 (2013), 98 (2014) and 60 (2015), taking into consideration the districts as well as the collection methods. All identified species were *An. funestus s.s.* based on their DNA band size which is a fragment of 505 base pairs (Figure 11).

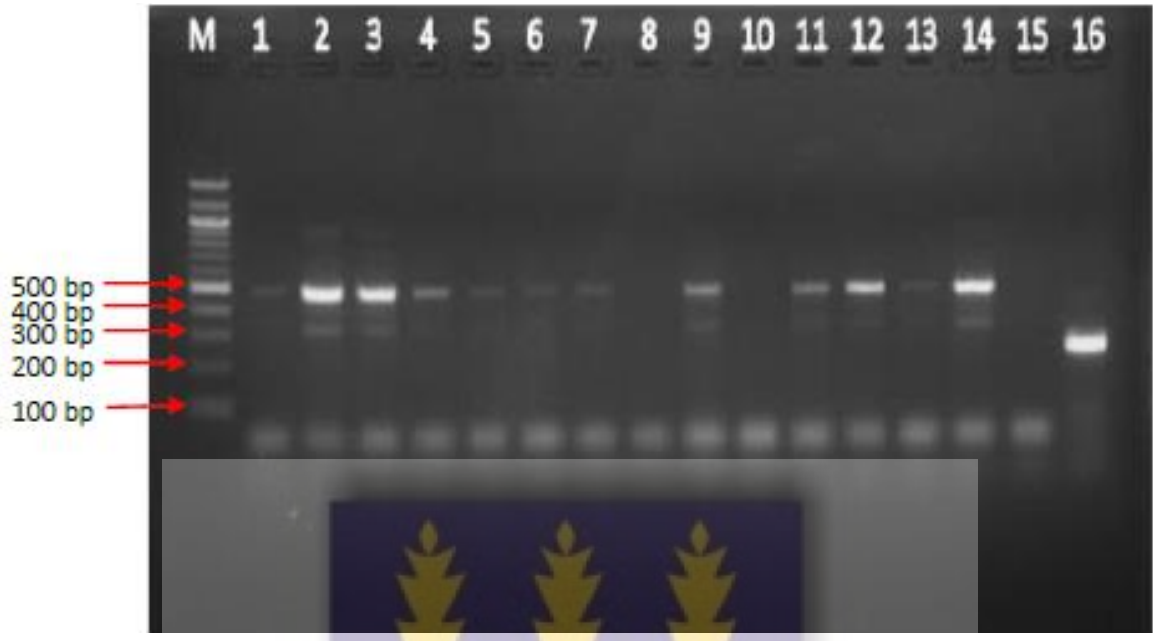

Figure 11: Ethidium bromide-stained 2.0 % agarose gel electrophoregram of PCR amplified rDNA sequences of *An. funestus s.s.* Lane M: 100 bp ladder; Lanes 1-13: *An. funestus s.s.*; Lane 14: *An. funestus s.s.* positive control; Lane 15: negative control and Lane 16: *An. parensis* positive control.

4.3 Man Biting Rate of *Anopheles funestus* in the Study Area

In each district, mosquitoes were collected by 12 collectors for 4 nights in a month for the entire sampling period. Out of the 620 *An. funestus* mosquitoes collected, 533, 20 and 67 were collected using HLC for the entire period of study in Tolon, Savelugu and Tamale Districts respectively, giving an overall biting rate of 0.69 bites per man per night (b/m/n), 0.03 b/m/n and 0.09 b/m/n for these areas respectively (Figure 12).

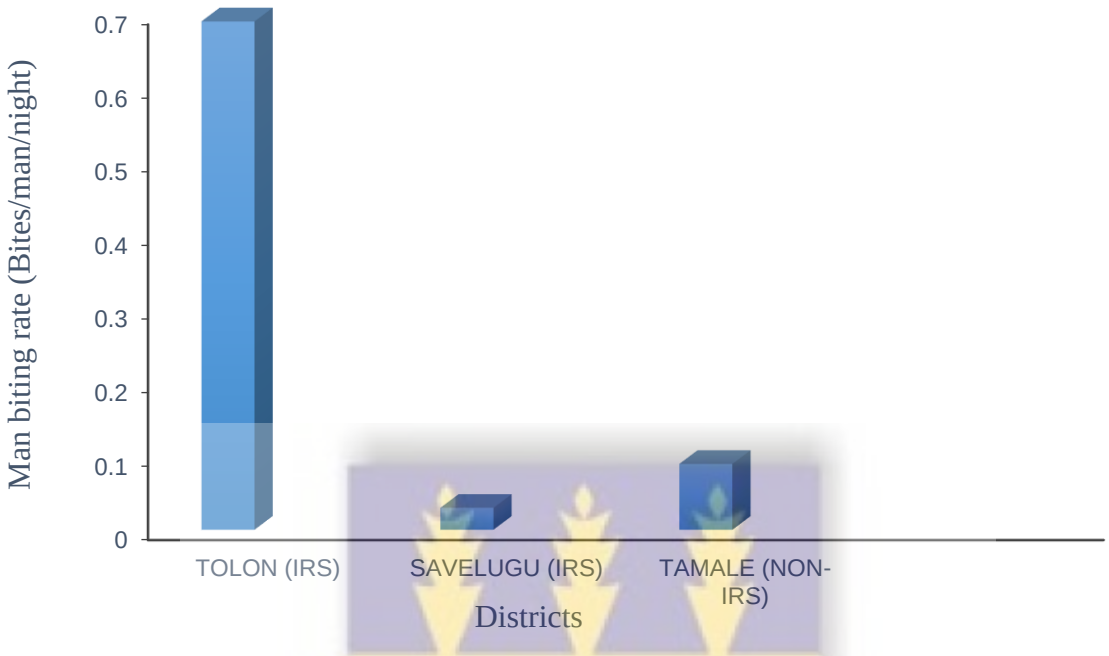

Figure 12: Overall man biting rate of *Anopheles funestus* in the study areas

4.3.1 Indoor and Outdoor Man Biting Rate of *An. funestus* Mosquitoes

With a total number of 620 *An. funestus* mosquitoes collected using HLC over the years in the study areas, 321 were collected indoor while 299 were collected outdoor. From these numbers, overall indoor biting rate of 0.36 b/m/n, 0.01 b/m/n and 0.05 b/m/n were recorded for Tolon, Savelugu and Tamale Districts respectively.

Similarly, overall outdoor biting rates of 0.34 b/m/n, 0.02 b/m/n and 0.04 b/m/n were recorded for Tolon, Savelugu and Tamale Districts respectively (Figure 13).

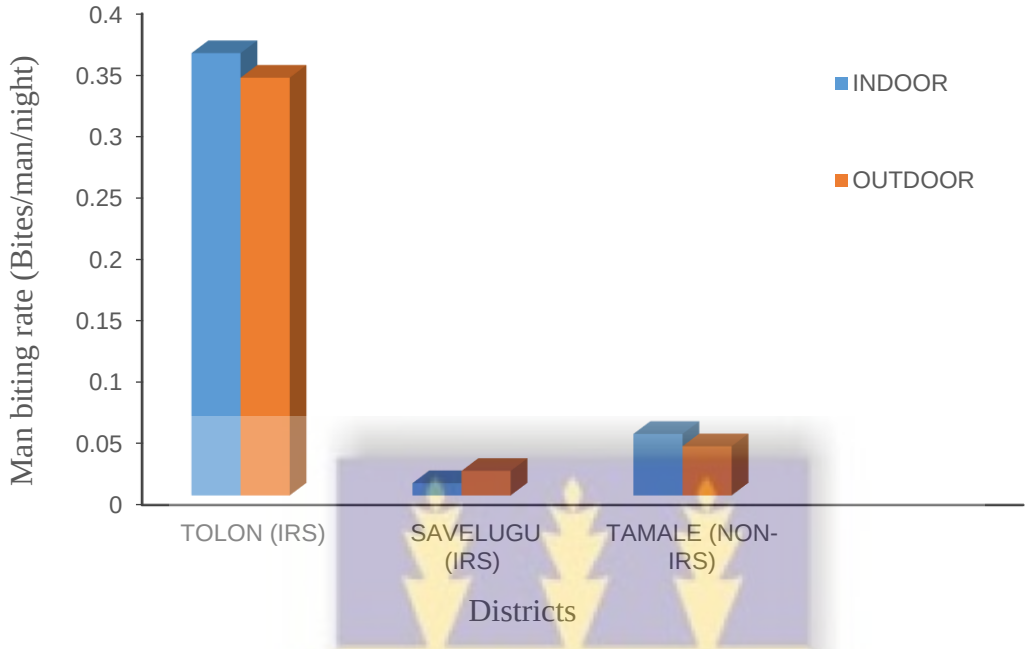

Figure 13: Overall indoor and outdoor man biting rates of *An. funestus* in the study areas.

4.3.2 Yearly Trend in Man Biting Rate of *Anopheles funestus* Mosquitoes

In the years 2010, 2013, 2014 and 2015, Tolon had the highest indoor and outdoor man biting rate recorded as 0.26, 0.33 b/m/n; 0.30, 0.38 b/m/n; 0.56, 0.35 b/m/n; and 0.32, 0.28 b/m/n respectively. On the other hand, lowest indoor and outdoor man biting rates were recorded in Savelugu for these years as 0.04, 0.13 b/m/n; 0.005, 0 b/m/n; 0 b/m/n; and 0 b/m/n respectively, while man biting rates for Tamale were recorded as 0.11, 0.1 b/m/n; 0.06, 0.01 b/m/n; 0.02, 0.02 b/m/n; and 0.01, 0.03 b/m/n in these years respectively (Figure 14).

There was a statistically significant difference ($P < 0.001$) in the biting rates of the mosquitoes collected in the IRS and Non-IRS areas across the years. Further analysis showed that the biting rate of mosquitoes in Tolon District was significantly higher than that of Tamale District.

However, there was no significant difference between Savelugu and Tamale Districts (Appendix IV).

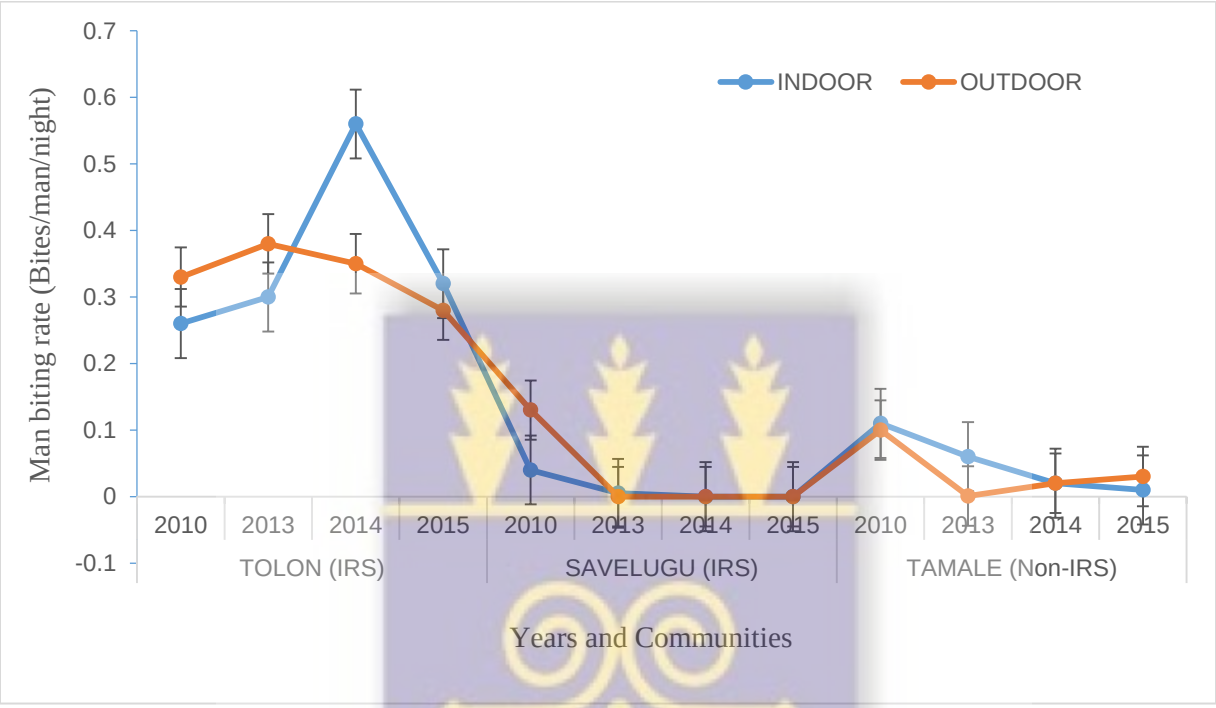

Figure 14: Yearly trend in indoor and outdoor man biting rate of *An. funestus* in IRS and non-IRS areas.

4.4 *Plasmodium falciparum* Sporozoite Infectivity Rate of *Anopheles funestus*

The heads and thoraces of 596 *An. funestus* mosquitoes were tested for the presence of *P. falciparum* circumsporozoite protein (*Pf*CSP) using the qualitative ELISA technique (Figure 15). Out of that number, 532 and 64 were collected using human landing catches and pyrethrum spray catches respectively. The number of *An. funestus* tested, number of *Pf*CSP positives and sporozoite rates at each site were as given in Table 3. Of the 596 *An. funestus* mosquitoes tested for *Pf*CSP,

16 were found positive for *Pf*CSP giving an overall sporozoite rate of 2.68 % for all the districts (Figure 15).

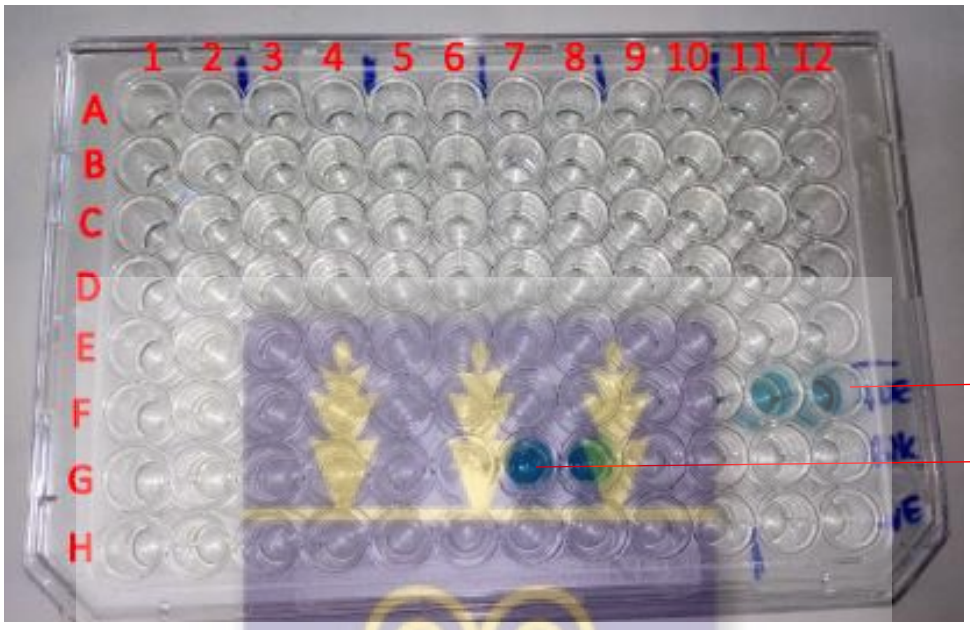

Figure 15: ELISA plate showing results of *Pf*CSP in tested mosquito samples.Wells F11 and F12 were positive controls, G11 and G12 were blank, H11 and H12 were negative controls, G7 and G8 were positive samples loaded in duplicate while all other wells contain samples that were negative for *Pf*CSP.

4.4.1 Indoor and Outdoor Sporozoite Infectivity Rates

Out of the 596 *An. funestus* mosquitoes tested for the presence of *Pf*CSP, 504 and 22 were collected from Tolon and Savelugu Districts respectively, while 70 were collected from Tamale District. Sixteen samples were found positive for *Pf*CSP. Out of this, 8 were collected using indoor HLC, 4 using outdoor HLC and the remaining 4 using PSC.

No indoor and outdoor sporozoite rates were recorded for Savelugu District across the years while the indoor and outdoor sporozoite rates recorded were 2.99 % (1/234) and 1.82 % (4/220) for

Tolon District, 3.13 % (1/32) and 0 % for Tamale District over the years (Figure 16).

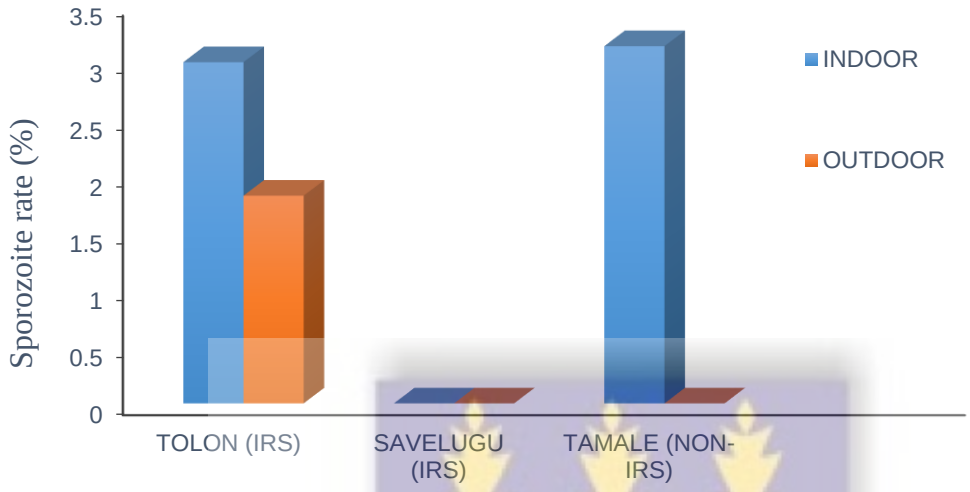

Figure 16: Indoor and outdoor trends in sporozoite rates in the IRS and Non-IRS areas

These sporozoite rates were recorded in all the years with 2014 being the year with the highest sporozoite rate in Tolon District (Figure 17). However, there was no statistically significant difference ($P > 0.05$, $df = 6$, $\chi^2 = 9.708$) in the sporozoite rate between the IRS and Non-IRS areas across the years (Appendix V).

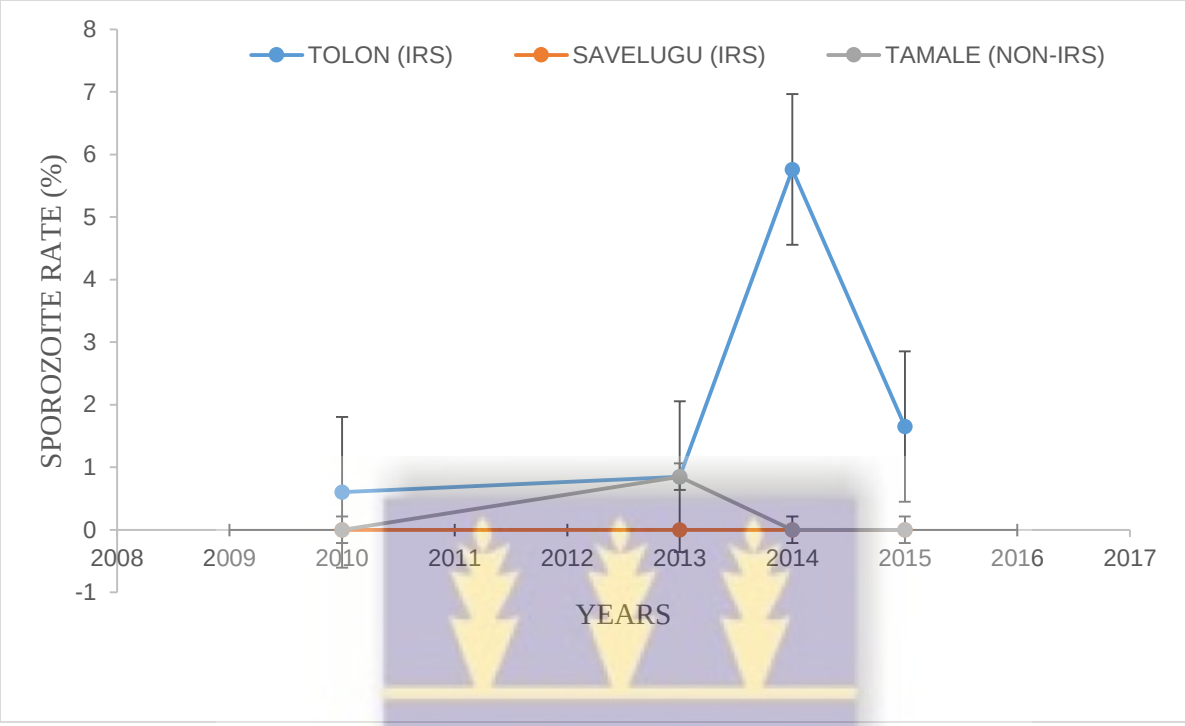

Figure 17: Yearly trends in sporozoite rates in IRS and non-IRS areas.

4.4.2 Entomological Inoculation Rate

Entomological inoculation rate (EIR) of *An. funestus* in the study sites is shown in Table 3. The EIR per year was obtained by multiplying the EIR by 365 (average number of days in a year). For the entire study period, the annual entomological inoculation rate (EIR) ranged from zero (0) infective bites per man per year (ib/m/y) for Savelugu District, 1.83 ib/m/y for Tamale District, to 20.44 ib/m/y for Tolon District in 2014.

Table 3: Annual sporozoite rates and EIR of *An. funestus* in the study sites

Indices	Tolon District				Savelugu District				Tamale District			
	2010	2013	2014	2015	2010	2013	2014	2015	2010	2013	2014	2015
No. tested	109	103	180	112	20	1	0	1	37	14	11	8
SR (%)	0.92	0.97	6.11	1.79	0	0	0	0	0	7.14	0	0
MBR	0.59	0.68	0.91	0.60	0.134	0.005	0	0.005	0.21	0.07	0.04	0.04
EIR/year (ib/m/y)	1.97	2.41	20.44	4.02	0	0	0	0	0	1.83	0	0

4.5 Human Blood Index (HBI)

A total of 600 *An. funestus* female mosquitoes were analyzed for source of blood meal using quantitative ELISA technique (Figure 18). Of this number, 31.17 % (187) tested positive for human blood while 9.5 % (57) tested positive for animal blood (chicken and goat) for the entire study period.

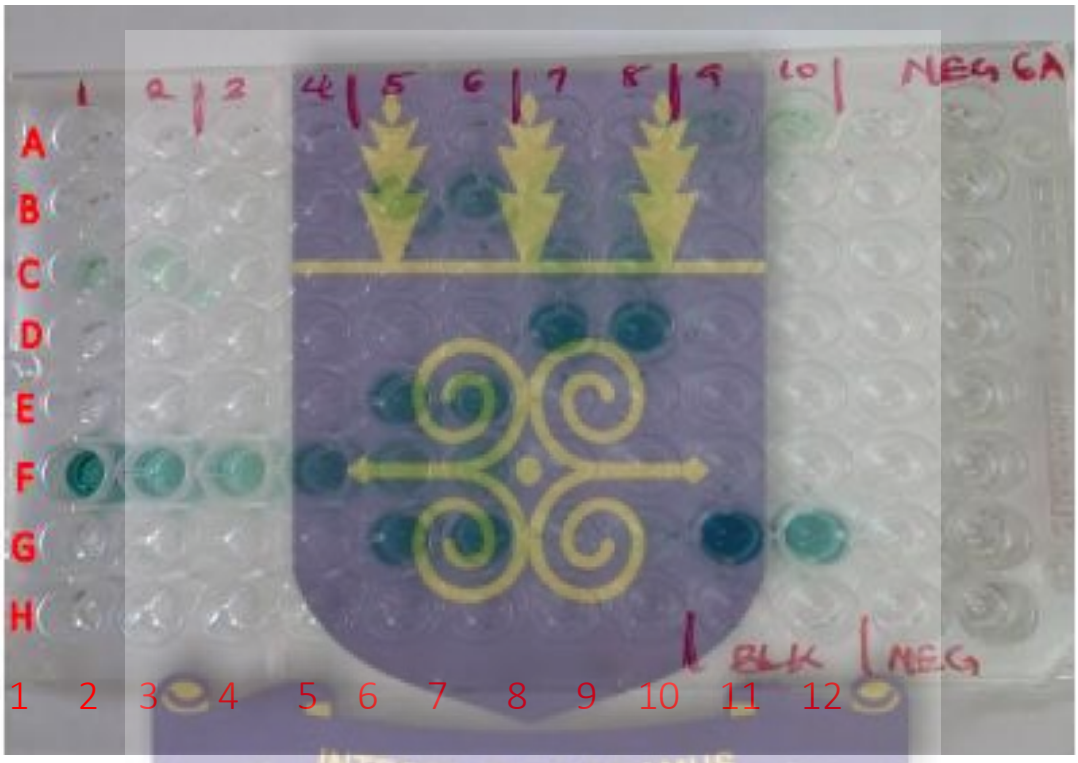

Figure 18: ELISA plate showing results of blood meal source in tested mosquito samples. Wells A11 and A12, B11 and B12…H11 and H12 are negative controls, G9 and G10 are positive controls, H9 and H10 are blank, F1 and F2, F3 and F4, B5 and B6, E5 and E6, F5 and F6, G5 and G6, B7 and B8, C7 and C8, D7 and D8 and A9 and A10 are positive samples of human IgG. All other wells contained samples that are negative for human IgG.

Out of the 600 *An. funestus* mosquitoes analyzed, cumulative totals of 508, 22 and 70 were collected from Tolon, Savelugu and Tamale Districts respectively across the years. Table 4 shows the number of *An. funestus* mosquitoes tested for each year in these districts and number of positives for human and animal blood. There was a statistically significant difference ($P < 0.001$) in the HBI of *An. funestus* collected in the study areas across the years. Further analysis showed that the HBI of Tamale District was significantly higher than that of Savelugu District and the HBI of Tolon District was significantly higher than that of Tamale (Appendix VI).

Table 4: Yearly human blood indices of *An. funestus* from the study sites

Indices	Tolon District				Savelugu District				Tamale District			
	2010	2013	2014	2015	2010	2013	2014	2015	2010	2013	2014	2015
No. tested	109	103	184	112	20	1	0	1	37	14	11	8
No. positive for human blood (%)	18.35	36.89	36.96	30.36	20.00	0	0	0	24.32	57.14	27.27	37.50
No. positive for animal blood	7.34	16.50	7.61	4.46	10.00	0	0	0	16.22	21.43	9.09	12.50

4.6 Distribution of knockdown Resistance (*kdr*) Mutation in *Anopheles funestus s.s.* at the Study Sites.

A total of 70 adult *An. funestus s.s.* mosquitoes were analyzed to determine their *kdr* status. In order to have a representative sample of the population, 50 samples were chosen from Tolon District while 7 and 13 samples were chosen from Savelugu and Tamale Districts respectively. The identification of the gene was done based on the presence of the band size of 994 base pair (Figure 19). Molecular analysis done showed that 27 (38.57 %) of the mosquitoes examined possessed the resistant gene (*kdr*).

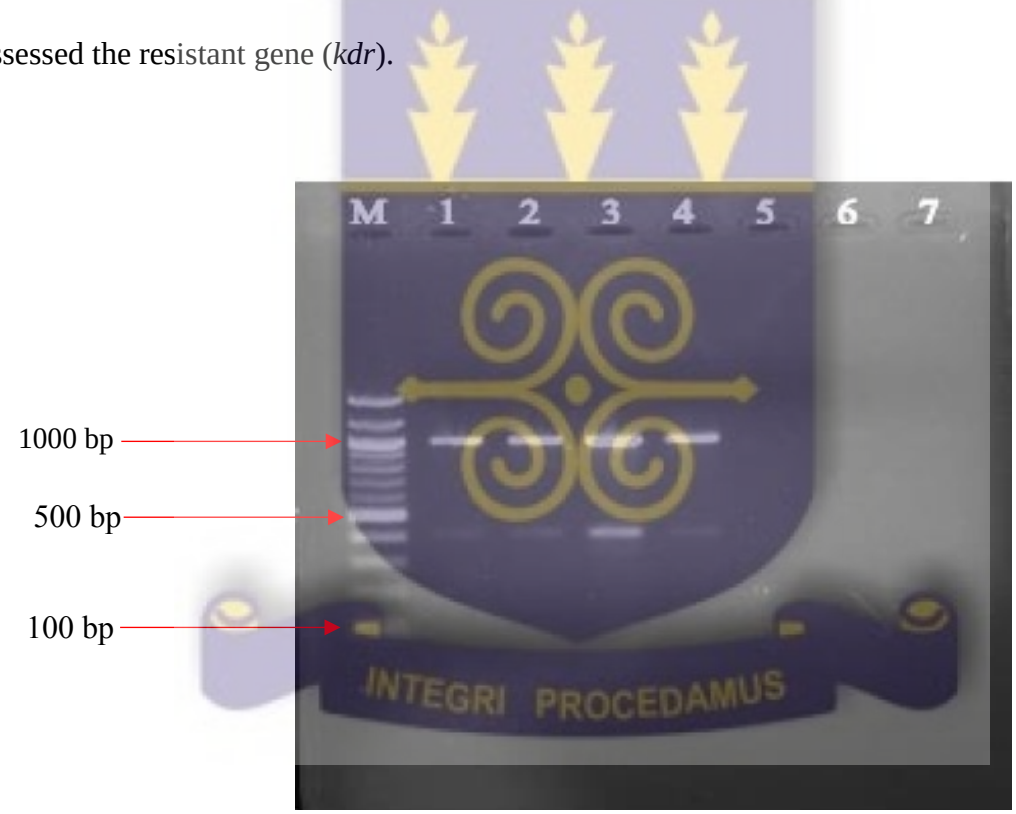

Figure 19: Ethidium bromide-stained 1.5 % agarose gel electrophoregram of knockdown resistance (*kdr*) mutation obtained from the analysis of *An. funestus s.s.* rDNA PCR product. Lane M: 100bp ladder. Lanes 1, 2, 3, 4: Positive *An. funestus s.s.* samples for *kdr* mutation.

Figure 20 shows the distribution of *kdr* gene in *An. funestus s.s.* across the years. *Kdr g*ene was present only in samples collected from Tolon Districts occurring in 2013, 2014 and 2015.

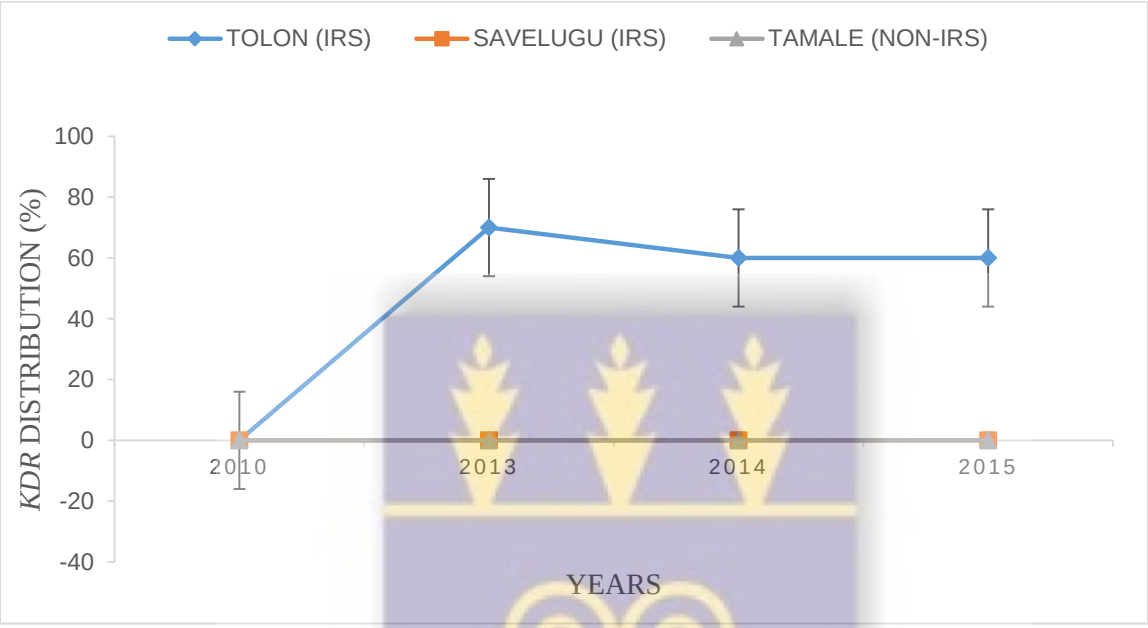

Figure 20: Yearly distribution of *kdr* mutation in the *Anopheles funestus s.s.* from the study sites.

CHAPTER FIVE

5.0 DISCUSSION

Anopheles funestus group include mosquitoes that are important vectors of malaria in Ghana as well as other parts of Sub-Saharan Africa (Appawu *et al*., 2004; Mouatcho *et al*., 2007; Dadzie *et al*., 2013; Kweka *et al*., 2013). Malaria, one of the most severe public health problems, is endemic in these areas (Maigemu and Hassan, 2015) and is a leading cause of morbidity and mortality. Control measures set in place are aimed at reducing malaria transmission. Much of the successes recorded in this regard is largely due to vector control strategies exemplified by the use of insecticide-treated bed nets (ITNs) and indoor residual spraying (WHO, 2014).

Indoor residual spraying (IRS) is continually used as a primary vector control strategy in many areas of Sub-Saharan Africa (Skarbinski *et al*., 2012) and effective at mostly indoor resting mosquitoes such as *An. gambiae s.s.* and *An. funestus* (Pluess *et al*., 2010). The present study was part of an ongoing IRS project in Northern Ghana which was aimed at studying the impact of IRS on the species composition, malaria transmission and insecticide resistance status of *An. funestus* during four years of the spraying programme in the IRS areas and a control area where no IRS has been implemented. The availability of archived mosquitoes collected from 2010, 2013 and 2014, provided a distinctive opportunity to compare the past species composition, sporozoite infectivity and *kdr* mutation in members of the *An. funestus* group to what was obtained in freshly collected specimens in 2015 in IRS areas (Tolon and Savelugu Districts) and Non-IRS area (Tamale District).

5.1 Species Composition and Man Biting Rates (MBR).

An. funestus group were collected in the study sites in the years 2010, 2013, 2014 and 2015 constituting the sampling period. Although other species of mosquitoes were also collected alongside *An. funestus*, they were not of interest in this study and therefore, were not contained within the analysis of this study. The presence of *An. funestus* in these study sites confirms the findings of other studies that recorded their presence in many parts of Ghana (Appawu *et al*., 2004; Tchouassi *et al*., 2012; Biloh, 2013, Dadzie *et al*., 2013), living in close association with humans and co-habiting with other *Anopheles* mosquitoes (Appawu *et al*., 2001; Yawson *et al*., 2004).

The abundance of *An. funestus* mosquitoes in the study sites were significantly at its peak in Tolon District as compared to the other two sites. This can be explained by the fact that *An. funestus* has a preference for breeding in semi-permanent water bodies with emerging vegetation (Gillies and De Meillon, 1968), which is typical of conditions present in Tolon District as irrigation and rice farming sites. Another factor that could have contributed to their high abundance in this area is the withdrawal of IRS from 2013 to 2015 leading to an increasing trend in its abundance from 2010 to 2015. Also, the abundance of the species in this area was significantly higher than the control district (Tamale) and this, calls for serious intervention in order to reduce their abundance and consequently, malaria transmission.

Conversely, the lowest abundance of *An. funestus* were encountered in Savelugu District with no record of this species in 2014 and only one (1) in 2015 indicating a decline in the abundance of the species in this area as the years progressed. This can be attributed to the implementation of IRS in the area for the past seven years which has had a positive impact on the control of the species.

In addition, *An. funestus* is known to be amenable to control by IRS (Kouznetsov, 1977) due to its anthropophilicity and endophilicity.

Molecular analysis of *An. funestus* group revealed that *An. funestus s.s.* was the only member of the group found in the three study sites. At the time of this study, *An. funestus s.s.* could probably be the only member of this group present in Northern Ghana as its presence had earlier been recorded in studies across Ghana (Appawu *et al.*, 2004; Tchouassi *et al*., 2012; Biloh, 2013; Dadzie *et al.*, 2013) as well as other African countries (Oyewole *et al*., 2007; Tchuinkam *et al*., 2010; Okara *et al*., 2010 and Adja *et al*., 2011). *Anopheles leesoni* which has been previously recorded in Navrongo, Ejura and Dodowa areas of Ghana by Dadzie *et al*. (2013) was not identified by PCR in this present study.

Man biting rate (MBR) of the *An. funestus* in Tolon District was significantly higher relative to the other study sites. This may be due to the presence of irrigation sites in this area which provided suitable breeding sites for the species. This is similar to the findings of Appawu *et al*. (2004) where the MBR of the species were higher in an irrigated area in Kassena Nankana District (KND) of Northern Ghana as compared to non-irrigated ones in KND. Dadzie *et al*. (2013) also recorded a relatively high biting rate of this species in the Sahel Savannah area where there was an irrigation scheme. However, there was no significant difference in MBR of this species collected in Savelugu and Tamale Districts.

5.2 Sporozoite Infectivity Rate and Human Blood Index (HBI)

The present study analysed the infectivity rate of the species using circumsporozoite enzyme-linked immunosorbent assays (CS-ELISAs). The results recorded sporozoites rates in tested *An. funestus* samples. From 2010 to 2015, 0 % sporozoite rate was recorded in Savelugu District while in Tolon District, positive results of sporozoite rates were recorded with an increasing trend ranging from 0.92 % in 2010 to 1.79 % in 2015, with the highest rate of 6.11 % recorded in 2014. The positive results of sporozoite rates in Tolon District which was recorded in this area despite the use of the spraying programme can be explained by the emerging insecticide resistance seen in the tested mosquito samples collected in this area. In Tamale District, positive sporozoite rate of 7.14 % was recorded for only 2013 while other years had 0 % rates. However, there were no significant differences in the sporozoite rates of the species in the three study sites.

The results of this analysis showed that *An. funestus s.s.* is an efficient vector of malaria in the Northern Region of Ghana. This is consistent with the findings of Appawu *et al.* (2004) who reported a sporozoite rate of 7.1 % in *An. funestus* mosquitoes found in Kassena Nankana District (KND) of Northern Ghana. Dadzie *et al.* (2013) also recorded an overall sporozoite rate of 7.6 % in Navrongo, Ejura and Dodowa areas of Ghana. Similar findings was also seen in other African countries such as in La Côte d'Ivoire (Adja *et al.*, 2011), where a 5.0 % sporozoite rate was recorded in *An. funestus s.s.* found in Gbatta and Kpèhiri forested areas of the country.

The entomological inoculation rates (EIR) which is a measure of infective bites per man at a given point in time was assessed for the different sites and years. This is used to measure the levels of exposure to infected mosquitoes, the intensity of malaria transmission, as well as the suitability of

vector control methods. In the present study, average EIR of 7.3 infective bites per man per year (ib/m/y) was recorded in Tolon District throughout the 4 years with an increasing trend as the years progressed. In Savelugu District, no infective bites of this species was recorded while in Tamale District, an average of 5.11 ib/m/y was recorded throughout the years.

The implication of these results is that the inhabitants of Tolon District were at a greater risk of malaria transmission as compared to the control site - Tamale. This could be as a result of the irrigation scheme in this area which is consistent with the findings of Appawu *et al*. (2004) where high EIR of 630 infective bites was recorded in the irrigated areas of KND in Northern Region of Ghana, although the EIR was based on both the *An. gambiae* and *An. funestus* sampled during the study. Also, Dadzie *et al*. (2013) recorded a high EIR of 255.5 infective bites in the irrigated areas of Sahel Savannah in Northern Ghana. Other African countries such as Cameroon records a high intensity of malaria transmission of 172 infective bites due to *An. funestus s.s.* in the savannah forested area throughout the study period (Cohuet *et al*., 2004). The EIR recorded in this study was considerably low as compared to the previous studies, this may be due to the small sample size encountered in this study. Nonetheless, the present study confirms the efficiency of *An. funestus s.s.* as a malaria vector in these districts as well as in Ghana.

Comparing the EIR recorded in Savelugu District with that of Tamale District, it remained constant throughout the study period unlike what was obtained in Tolon District. It can be deduced that the indoor residual spraying in Savelugu District had a positive impact on the infectivity rate of the species. This is in line with the findings of Chanda *et al*. (2012) where an intervention effect of IRS was pronounced on *An. funestus s.s*. indicating the amenability of this species to control by

IRS. However, with the irrigation scheme in Tolon District, high human blood index and emergence of resistance in mosquito samples in this area, the EIR increased as the years progressed.

Analysis by ELISA for the detection of blood meal source in the species was done and this was used in the calculation of Human Blood Index (HBI) for the tested samples. The results showed an HBI of 31.5 % and animal blood index (ABI) of 8.66 % in Tolon District, HBI of 18.18 % and ABI of 9.09 % in Savelugu District while HBI of 32.86 % and ABI of 15.71 % in Tamale District. In this regard, there was a high preference for human blood over animal blood for this species indicating a degree of high anthropophagy amongst them. This is consistent with the findings of Osae *et al*. (2015) which recorded an anthropophagic behaviour in *An. funestus* sampled in Southern Ghana. Studies by Dadzie *et al*. (2013) also recorded a high proportion of the species (over 80 %) fed on human blood in samples collected from Navrongo, Ejura and Dodowa areas of Ghana.

Accordingly, Sinka *et al*. (2010) indicates the consistent behaviour of anthropophily and endophily in *An. funestus* mosquitoes. The results in this present work as well as past studies (Sinka *et al*., 2010; Dadzie *et al*., 2013 and Osae *et al*., 2015) highlight one factor that contributes to their high vectorial capacity which in turn, is used to measure the rate at which a vector population transmits malaria (Garrett-Jones, 1964). Taking this into account, the high degree of human feeding could be a factor contributing to the positive results of *Plasmodium falciparum* Circumsporozoite Protein as was seen in the present study. In Savelugu District, there was a significantly lower difference with the HBI found in this area as compared with Tamale District. This may be attributed to the

efficacy of the long term intervention used in this area. Although there were lower degrees of HBI recorded in the present study as compared to other previous studies (Osae *et al*., 2015; Dadzie *et al*., 2013), there was no entire shift away from humans in host selection for *An. funestus* mosquitoes collected even with the implementation of IRS in Tolon and Savelugu Districts. This emphasizes the consistent anthropophilic behaviour of this species and the need for additional vector control methods in order to sustain the impact of IRS recorded in Savelugu District as well as control interventions in Tolon District.

5.3 *Kdr* Mutation in *An. funestus s.s.*

Insecticide resistance of mosquitoes is a threat to effective malaria control. The *kdr* gene mutation which is associated with pyrethroid resistance as well as cross resistance with DDT in mosquitoes was assessed using molecular analysis. Out of 70 samples tested, 25 (35.71 %) showed that a possible mutation in the *kdr* gene of the species. This result was detected in only samples from Tolon District in the years 2013, 2014 and 2015 giving a 50 % (25 out of 50 tested) result. The implication of this result is that a form of mutation in the *kdr* gene which confers resistance to pyrethroids is emerging in the populations of this species present in Tolon District. Factors such as the pyrethroid insecticide used in the IRS programme as well as residues from agricultural activity in this area, given that irrigated rice production rely heavily on insecticide usage, could contribute to emerging resistance in *Anopheles* mosquito species.

Although insecticide susceptibility tests which is used to check other mechanisms of resistance were not done in this work due to lack of colonies, studies on other mechanisms of resistance to pyrethroids by this species has been recorded in Ghana (Coetzee *et al*., 2006; Okoye *et al*., 2008)

as well as in other African countries (Casimiro *et al*., 2006; Wondji *et al*., 2007; Cuamba *et al*., 2010; Morgan *et al*., 2010; Djouaka *et al*., 2011). A similar kind of mutation was also detected in the *An. funestus* samples collected in Malawi by Wondji *et al*. (2012). The absence of other resistance profiling assays in this work impede any definitive assurance of this mechanism of resistance in the tested samples. Therefore, further analysis of different mechanisms of resistance, sequencing and pyrosequencing of the *kdr* gene should be carried out in this species in order to monitor emerging resistant populations and effective vector management systems proposed.

CHAPTER SIX

6.0 CONCLUSION, LIMITATIONS OF THE STUDY AND RECOMMENDATIONS

6.1 Conclusion

The present study focused on the *An. funestus* group by analysing newly collected and archived mosquito specimens. The result of this research demonstrates that *An. funestus s.s.* was the only member of the *An. funestus* group present in the study areas (both IRS and Non-IRS areas). As regards the biting rate, the results indicated that their rates differed in these districts in this ascending order; Savelugu, Tamale and Tolon Districts indicating the impact of IRS in Savelugu District. There was a decline in the *An. funestus* numbers over the years gotten in Savelugu district and this may be due to the effect of the IRS in this area, since *An. funestus* is known to be amenable to control by IRS.

Furthermore, the results obtained indicate the vectorial importance of *An. funestus s.s.* in the transmission of malaria given that positive results of *P. falciparum* sporozoite tests were gotten in Tolon and Tamale Districts. It is also important to note the possible emergence of *kdr* gene mutation in the populations tested.

6.2 Limitations and Recommendations of the Study

In the samples analysed, samples for 2011 and 2012 were not available at the time of this study, and so, there was no room for evaluating what happened in these years and comparing it with 2015 samples. Also, *An. funestus* samples collected from the sites were few. Therefore, further research should expand on the sample size. Other resistance mechanisms associated with pyrethroid

resistance was not tested in this research also due to lack of colonies of the species. This necessitates the importance of further research on resistance profile of the species.

With the introduction of IRS in these areas, it is recommended that insecticide resistance in *An. funestus* should be carefully monitored. This will improve the implementation and management of future control measures and serve as a basis for evidence-based alternative control measures.

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APPENDICES

Appendix 1

Molecular Biology Techniques

Preparation of Standard Solutions used in Molecular Biology Studies.

Standard solutions used were prepared using sterile double distilled water (sddH20) and autoclaved where appropriate at 121 1b/sq. for 15 minutes (Hirayama autoclave, Tokyo). The solutions include:

a. Solutions for Deoxyribonucleic Acid (DNA) Extraction

Extraction Buffer: 1 M Tris HCl, pH 8.0, 0.5M EDTA, 1.4 M NaCl, 2 % cetylltrimethyl ammonium bromide.

Chloroform: Stock solution was used as ordered from Sigma-Aldrich USA.

Isopropanol: Stock solution was used as ordered from Sigma-Aldrich USA.

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.

PCR Primers

Primers used were diluted as specified by the Manufacturers, Eurofins Genomics, USA.

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 sddH20.

Ethidium bromide, EtBr (10 mg/ml): 1 g of EtBr was completely dissolved in 100 ml sddH20 and stored in a dark bottle in the dark at room temperature.

2 % Agarose Gel

This was prepared by dissolving 1.2 g of the Agarose powder in 60 ml 1× TAE buffer and microwaved to dissolve the powder and then cooled under running tap water. Then 2.0 µl (10 mg/ml) EtBr was added and the beaker stirred to mix thoroughly. The solution was then poured in a gel mould (Mini gel system, BIORAD, USA) with combs of <1 mm and left for about 15 minutes to solidify. The combs were removed and wells created in which samples were loaded.

DNA molecular weight size marker: The 100 bp molecular weight size marker was obtained from sigma, USA 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 Sporozoite and Blood meal 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 4 °C until it was required for use.

MPA Preparation

Test mosquito sample was put in a pre-labelled 1.5 ml microcentrifuge 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 stored frozen at -20 °C until time of use.

b. Phosphate Buffered Saline (PBS) Preparation

Calibration of flask

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

Preparation of Buffer

The tap water in the step above was discarded and the flask rinsed with distilled water. 800ml 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. Four (4) tablets of PBS were put 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 7.4 by adding acid or alkaline and measured using a pH meter. 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. Preparation of Antigen from Abdomen for Blood Meal ELISA

Test mosquito sample was put in a pre-labelled 1.5 ml microcentrifuge tube and 50 µl of PBS was added to it. It was then crushed using a sterile plastic pestle to obtain a homogenous suspension. 1:50 dilution was prepared from the homogenous solution by taking 10 µl of the homogenous solution and adding it to 490 µl of PBS. The homogenate was then stored frozen at -20 °C until time of use.

d.Washing Buffer [PBST (Tween 20)] 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.

e. Blocking Buffer (BB) Preparation

5 g of casein was suspended in 0.1 M NaOH and the resulting solution brought to a boil. After the dissolution of the casein, 900 ml 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 °C.

f. Conjugate Diluent Preparation

25 µl of Tween 20 was added to 100 ml of BB in order to obtain the conjugate diluent. The conjugate diluent was used to prepare the host specific antibody-peroxidase conjugate by adding one part of the conjugate to 2000 parts of the conjugate diluent.

g. 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.

h. 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/50µl/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 (1 0 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

Analysis of Variance for Mosquito Abundance in IRS and Non-IRS areas

Variate: %_log_transformed

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
YEAR stratum	3	1.4401	0.4800	3.42	0.030
YEAR.*Units* stratum					
COMMUNITY	2	14.6592	7.3296	52.18	<.001
Residual	30	4.2139	0.1405		
Total	35	20.3132			

Tables of means

Grand mean 0.764

COMMUNITY	Savelugu	Tamale	Tolon
	0.033	0.673	1.588

Least significant differences of means (5 % level)

Variance = 0.1405 with 30 degrees of freedom

Fisher's Protected Least Significant Difference test
Experimentwise error rate = 0.0500

F value is 52.18 on 2 and 30 degrees of freedom
Overall F test is significant, pairwise testing proceeds.

Comparisonwise error rate = 0.0500

Mean	vs Mean	t	significant
Savelugu	Tamale	-4.183	Yes
Savelugu	Tolon	-10.163	Yes
Tamale	Tolon	-5.980	Yes

Identifier	Mean
Savelugu	0.0328 a
Tamale	0.6728 b
Tolon	1.5877 c

RAW DATA FOR MOSQUITO ABUNDANCE IN THE STUDY AREAS

COMMUNITY	YEAR	COLLECTION METHOD	YIELD
TOLON	2015	Indoor HLC	61
		Outdoor HLC	54
		PSC	14
SAVELUGU	2015	Indoor HLC	0
		Outdoor HLC	0
		PSC	1
TAMALE	2015	Indoor HLC	1
		Outdoor HLC	5
		PSC	1
TOLON	2014	Indoor HLC	107
		Outdoor HLC	68
		PSC	16
SAVELUGU	2014	Indoor HLC	0
		Outdoor HLC	0
		PSC	0
TAMALE	2014	Indoor HLC	4
		Outdoor HLC	3
		PSC	4
TOLON	2013	Indoor HLC	57
		Outdoor HLC	73
		PSC	14
SAVELUGU	2013	Indoor HLC	1
		Outdoor HLC	0
		PSC	0
TAMALE	2013	Indoor HLC	11
		Outdoor HLC	2
		PSC	2
TOLON	2010	Indoor HLC	50
		Outdoor HLC	63
		PSC	10
SAVELUGU	2010	Indoor HLC	7
		Outdoor HLC	12
		PSC	1
TAMALE	2010	Indoor HLC	21
		Outdoor HLC	19
		PSC	4

Appendix IV

Analysis of Variance for Man Biting Rate in IRS and Non-IRS areas

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
YEAR stratum	3	0.02217	0.00739	0.57	0.653
YEAR.*Units* stratum					
Community	2	1.07334	0.53667	41.67	<.001
Residual	6	0.07728	0.01288		
Total	11	1.17279			

Tables of means

Grand mean 0.273

COMMUNITY	Savelugu	Tamale	Tolon
	0.035	0.090	0.695

Least significant differences of means (5 % level)

All pairwise comparisons are tested.

Variance = 0.0129 with 6 degrees of freedom

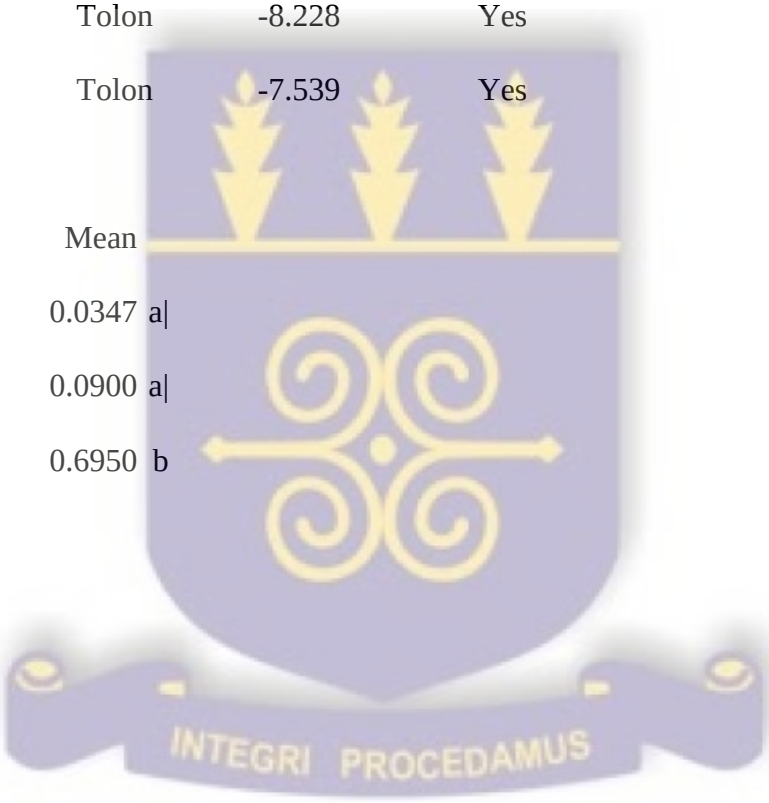
Fisher's Protected Least Significant Difference test

Experiment wise error rate = 0.0500
F value is 41.67 on 2 and 6 degrees of freedom
Overall F test is significant, pairwise testing proceeds.

Comparison wise error rate = 0.0500

Mean	vs Mean	t	significant
Savelugu	Tamale	-0.688	No
Savelugu	Tolon	-8.228	Yes
Tamale	Tolon	-7.539	Yes

Identifier	Mean	
Savelugu	0.0347	a|
Tamale	0.0900	a|
Tolon	0.6950	b



Appendix V

Chi-square of independence for Sporozoite Rate in the Study areas

COMMUNITY	YEAR	CSP RATE
Tolon	2015	1.79
	2014	6.11
	2013	0.97
	2010	0.92
Savelugu	2015	0
	2014	0
	2013	0
	2010	0
Tamale	2015	0
	2014	0
	2013	7.14
	2010	0
χ^2		**9.708**
Df		**6**
Significance		**P > 0.05**

Contingency Table

Observed CSP Rates	Expected CSP Rates	Chi square test statistic
0.92	0.532	0.283
0.97	4.690	2.950
6.11	3.533	1.879
1.79	1.035	0.551
0	0.000	0.000
0	0.000	0.000
0	0.000	0.000
0	0.000	0.000
0	0.000	0.000
7.14	3.420	4.045
0	0.000	0.000
0	0.000	0.000
χ^2		**9.708558**
P-value		**0.137474**

Appendix VI

Analysis of Variance for Human Blood Index in IRS and Non-IRS areas

Variate: %_log_transformed

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
YEAR stratum	3	0.51713	0.17238	2.19	0.191
YEAR.*Units* stratum					
COMMUNITY	2	5.31547	2.65774	33.72	<.001
Residual	6	0.47289	0.07882		
Total	11	6.30550			

Tables of means

Grand mean 0.751

COMMUNITY	Savelugu	Tamale	Tolon
	-0.063	0.749	1.568

Least significant differences of means (5 % level)

All pairwise comparisons are tested.

Variance = 0.0788 with 6 degrees of freedom

Fisher's Protected Least Significant Difference test

Experimentwise error rate = 0.0500
F value is 33.72 on 2 and 6 degrees of freedom
Overall F test is significant, pairwise testing proceeds.

Comparisonwise error rate = 0.0500

Mean	vs Mean	t	significant
Savelugu	Tamale	-4.087	Yes
Savelugu	Tolon	-8.212	Yes
Tamale	Tolon	-4.126	Yes

Identifier	Mean
Savelugu	-0.0625 a
Tamale	0.7488 b
Tolon	1.5677 c

RAW DATA FOR HUMAN BLOOD INDEX AND BITING RATE IN THE STUDY AREAS

COMMUNITY	YEAR	HUMAN BLOOD INDEX	BITING RATE
TOLON	2015	34	0.60
	2014	68	0.91
	2013	38	0.68
	2010	20	0.59
SAVELUGU	2015	4	0
	2014	0	0
	2013	0	0.005
	2010	0	0.134
TAMALE	2015	9	0.04
	2014	8	0.04
	2013	3	0.07
	2010	3	0.21