

**Resting Behaviour of Endophilic Anopheline Vectors in
Three Ecological Zones of Southern Ghana and its
Implications for the use of Entomopathogenic Fungi**

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DECLARATION

I hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this dissertation is the result of research work undertaken by Michael Osae in the Department of Nuclear Agriculture and Radiation Processing, School of Nuclear and Allied Sciences, University of Ghana, under the supervision of Dr. David D. Wilson, Prof. Michael D. Wilson and Prof. Lizette L. Koekemoer. I further declare that this dissertation has not been accepted either in whole or part in any other University for the award of a degree.

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DEDICATION

This work is dedicated to the memory of my late father, Raphael Kwasi Osae for instilling in me the qualities that keep me going in life.



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LIST OF ABBREVIATIONS

AChe	Acetylcholinesterase
ANOVA	Analysis of variance
BHC	Benzene hexachloride
BHQ	Black hole quencher
<i>Bti</i>	<i>Bacillus thuringiensis</i> var israelensis
CAT	Central African Time
CB	Carbamate
CL	Confidence limit
CO ₂	Carbon dioxide
CSEZ	Coastal savanna ecological zone
CYD	Cyclodiene
CYP	Cytochrome P
DDE	Dichlorodiphenyldichloroethylene
ddH ₂ O	Double distilled water
DDT	Dichlorodiphenyltrichloroethane
DFNW	Distance from nearest wall
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FEZ	Forest ecological zone
FTEZ	Forest transition ecological zone
GABA	g-amino butyric acid
GMT	Greenwich Mean Time



GPS	Global positioning system
GSS	Ghana Statistical Services
GST	Glutathione S-transferase
HBI	Human blood index
HFG	Height from ground
HOBO [®]	Trade mark of data loggers
HSD	Highly significant difference
IPM	Integrated pest management
IRM	Insecticide resistance management
IRS	Indoor residual spraying
ITN	Insecticide treated net
ITPS	Insecticide treated plastic sheets
ITWL	Insecticide treated wall linnings
IVM	Integrated vector management
KAc	Potassium acetate
KCl	Potassium chloride
<i>kdr</i>	Knockdown resistance
LCD	Lead crystal display
LI	Light intensity
LLIN	Long lasting insecticidal net
LUBILOSA	Lutte Biologique contre les Locustes et Sauteriaux
MalERA	Malaria Eradication Research Agenda
MGB	Minor groove binder
MGBNFQ	Minor groove binder non-fluorescent quencher
MgCl ₂	Magnesium chloride

MSD	Mosquito species density
NaCl	Sodium chloride
NaVdp	Sodium-voltage-dependent
NICD	National Institute for Communicable Diseases
NMCP	National Malaria Control Program
NRD	Number of rooms sampled per day
NS	Number of the particular species
OP	Organophosphate
P450	Cytochrome P450
PCR	Polymerase chain reaction
PY	Pyrethroid
RDL	Resistance to dieldrine
RDT	Rapid diagnostic test
REDCO	Suburb of Accra where the laboratory strains originated
RH	Relative humidity
RR	Homozygous resistant
RS	Heterozygous resistant
RT-PCR	Real time polymerase chain reaction
SIT	Sterile insect technique
SPSS	Statistical Package for the Social Sciences
SS	Homozygous susceptible
<i>Taq</i>	Polymerase obtained from <i>Thermus aquaticus</i>
TE	Tris-EDTA
TMC	Total number of mosquito specimen
Tris-HCl	Tris-hydrochloride

UV	Ultraviolet radiation
VCRL	Vector Control Reference Laboratory
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
WS	Wind speed



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ABSTRACT

The aim of this study was to determine the resting behaviour and factors that influence the choice of resting sites by endophilic anopheline mosquitoes in southern Ghana. The study was carried out in six villages across three ecological zones of southern Ghana, including: the forest ecological zone (FEZ), the coastal savannah ecological zone (CSEZ) and the forest-transition ecological zone (FTEZ). For every mosquito collected, the indoor resting sites was characterised and microclimate at the actual resting site recorded using a data logger. In the laboratory, full diagnostics was carried out on a sub-sample. Resting devices constructed from different materials were tested in a screen house and in village rooms. The dominant anopheline vectors include *Anopheles gambiae* s.s., *An. coluzzii* and *An. funestus*. *An. gambiae* s.s. was the most dominant species in the villages from the FEZ and FTEZ, whereas *An. coluzzii* was most dominant in the CSEZ. *An. funestus* was present in the FEZ and CSEZ but almost absent from the FTEZ. The two *kdr* mutations (L1014F and L1014S) were present in all the ecological zones and in both *An. gambiae* s.s. and *An. coluzzii*. This is the first report of the L1014S mutation from Ghana. The populations were highly anthropophilic and *Plasmodium falciparum* was present in populations from all the villages studied. Woody materials appeared to be the most preferred resting materials for *An. gambiae*, accounting for 47 % of all the resting sites for that species. They were also found on fabrics (26 %), wall materials (12 %), and roof materials (10 %). Similarly, *An. funestus* preferred to rest on woody materials (58 %), followed by wall materials (21 %), fabrics (11 %) and roofing materials (7 %). For *An. coluzzii*, fabrics were the most preferred resting materials (38 %) followed by roof materials (21 %), wooden materials (19 %) and wall materials (9 %). All the three vector species preferred to rest higher up the room and closer to the walls, with large

proportions of *An. gambiae* s.s. (75.94 %), *An. funestus* (73.60 %) and *An. coluzzii* (58.19 %) found resting at heights above 200 cm from the floor. With reference to the nearest wall, high proportions of *An. gambiae* s.s. (68.38 %), *An. coluzzii* (80.98 %) and *An. funestus* (68.29 %) were collected within 50 cm from the closest wall. Although microclimatic conditions varied widely in the indoor environment and even at the specific resting sites, higher proportions of the endophilic anophelines were collected within narrow ranges of temperature (27-30 °C), relative humidity (65-75 %) and light intensity (0-20 Lux). Black felt and black cotton cloth were the most preferred resting devices for blood-fed females in both screen house trials and field. In general, the presence of the three most efficient vectors of malaria from indoor collections in southern Ghana and the fact that they were highly anthropophagic and effectively transmitting malaria is a high call for intensifying vector control efforts. Knowledge of the indoor resting behaviour of the major endophilic *Anopheles* vectors of southern Ghana paves the way for developing fungal spore deployment strategies for field implementation. This study has established that all the three endophilic *Anopheles* species in southern Ghana rest higher up in village rooms, prefer to rest on darker coloured materials in cool humid areas. It has further demonstrated that black felt and black cotton cloth are attractive to blood-fed females seeking resting sites to digest blood meal and develop their eggs. Guided by this knowledge, future research should focus on designing these preferred materials into resting devices that create suitable microhabitats to which endophilic anopheline mosquitoes can be attracted and contaminated with fungal spores. Further research is also needed to determine the efficacy and persistence of fungal spores on these preferred resting materials so as to determine application rates and frequency.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Insect-borne diseases are among the most devastating of all human diseases. Insects (mostly flies, fleas and lice) serve as effective vectors of disease by transmitting pathogens via taking their blood meals. Malaria, the most serious and costly of all insect-borne diseases is caused by five species of parasites of the genus *Plasmodium* that affect humans (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). Malaria due to *P. falciparum* is the most deadly form and it predominates in Africa; *P. vivax* is less dangerous but more widespread, and the other three species are found much less frequently (WHO, 2012a). Malaria parasites are transmitted to humans by the bite of infected female mosquitoes of the genus *Anopheles*. Out of 380 species of *Anopheles* only 60 transmit malaria globally (Alonso, 2006), and 7 of these (*An. gambiae* s.s., *An. arabiensis*, *An. melas*, *An. merus*, *An. funestus*, *An. nili* and *An. moucheti*) are considered dominant vector species in Africa (Sinka *et al.*, 2010). *An. gambiae* s.s. until recently was divided into two molecular forms, viz: M and S forms. These molecular forms have however been given species status, with the S form retaining the name *An. gambiae* s.s. and the M form given the name *An. coluzzii* (Coetzee *et al.*, 2013), making the dominant vector species in Africa eight instead of seven as stated earlier in Sinka *et al.* (2010). In addition to malaria, some of these species also transmit other diseases of public health importance. For example, in Ghana, *Anopheles gambiae* s.l. and *An. funestus* which are the major vectors of *P. falciparum*, have also been implicated as major vectors of *Wuchereria bancrofti*, the parasite that causes lymphatic filariasis (Boakye *et al.*, 2004). Disease vectors are thus

important public health menace that require effective control measures to reduce or eliminate their impact.

Vector control is considered an important strategy for insect-borne disease management as it breaks the transmission cycle, thereby reducing morbidity and mortality (WHO, 2013). Currently, the most effective and widely adopted vector control strategies include; insecticide-treated nets (ITNs), indoor residual spraying (IRS) and, in some specific settings, larval control (WHO, 2012a). Malaria control has seen great success in the last decade, reducing the burden by as much as 33 % in the hardest-hit region, sub-Saharan Africa, thanks to the key intervention tools such as the effective treatment drugs, preventive treatment of pregnant women, ITNs and IRS (Slutsker and Kachur, 2013). This progress appears to have slowed recently due mainly to widespread development of resistance in the mosquito vector to the major insecticides used on ITNs and for IRS (WHO, 2012b). Continued success in the fight against malaria requires persistence, cunning and most of all knowledge (Slutsker and Kachur, 2013). It is important to preserve and improve upon the utility of these insecticide-based interventions through critical insecticide resistance management (IRM) and the development of novel interventions to supplement current tools.

One novel control strategy that has gained lots of attention over the past decade is the use of entomopathogenic fungi for adult mosquito control (Hancock *et al.*, 2009; Read *et al.*, 2009; Thomas and Read, 2007; Scholte *et al.*, 2004). Two species belonging to the Hyphomycetes group (*Metarhizium anisopliae* and *Beauveria bassiana*) are the most widely used entomopathogenic fungi for insect pest control (Farenhorst, 2010). Fungal spores can be deployed against adult flying insects by applying them on the

surfaces they make contact with; A range of *M. anisopliae* and *B. bassiana* isolates have been shown to be successful in infecting and killing *Anopheles*, *Aedes* and *Culex* mosquitoes when applied on several different substrates (Scholte *et al.*, 2005, 2007, 2003a; Blanford *et al.*, 2005;). Fungi act slower than fast-acting chemical insecticides and do not offer personal protection from mosquito bites. Depending on the dose and virulence of the isolate, hyphomycetes can kill mosquitoes within several days, mostly between four and fourteen days (Mnyone *et al.*, 2009a, b; Bell *et al.*, 2009; Scholte *et al.*, 2003b) thus killing mosquito vectors before they can transmit parasites and hence reduce disease transmission (Hancock *et al.*, 2009; Scholte *et al.*, 2005). In addition to killing malaria vectors, fungi have also been shown to prevent the development of *Plasmodium* sporozoites, which could block malaria transmission altogether (Read *et al.*, 2009; Blanford *et al.*, 2005). They can also cause reductions in blood-feeding frequency (Blanford *et al.*, 2005) and reproductive fitness (Scholte *et al.*, 2006) prior to death, which could further impact on the mosquito's capacity to transmit disease (Hancock *et al.*, 2009). These hyphomycetes therefore hold great potential for integration into vector control alongside classical and other novel strategies.

For the successful development and implementation of any novel strategy (be it insecticides or biological agent based) and their integrated vector management (IVM) with conventional strategies, it is important to understand the ecology and behaviour of the target population. The importance of understanding the behavioural ecology of target species for their control has been demonstrated for several insect pest species. For tsetse control, it was demonstrated that the compound eyes of *Glossina* can perceive shape, colour and movement; moving objects are perceived at a much greater

distance. *Glossina* species also seem capable of colour discrimination; phthalogen blue being particularly attractive, whereas black and UV-reflecting white tend to stimulate landing, and yellow tends to be unattractive (Green, 1986; Green and Flint, 1986). These led to the finding that a combination of blue and black cloth efficiently attracted *Glossina* species, especially when arranged in a form such that some movement of the cloth was possible leading to the development of blue and black traps and targets, which are still relevant for *Glossina* control (Kuzoe and Schofield, 2004).

Exploiting behavioural ecology for development and implementation of control strategies has been demonstrated for mosquitoes as well. De Meillon (1934) demonstrated that the malaria vector *An. funestus* was entering houses to obtain blood meals and then remaining indoors until ready to lay eggs. Based on this understanding of the resting behaviour of *An. funestus*, adults were successfully controlled in houses by spraying pyrethrum indoors (De Meillon, 1936), laying the foundation for IRS, one of the flagship vector control strategies to date. In another example, Mnyone *et al.* (2012) exploited the behaviour of wild malaria vectors to achieve high infection with fungal biocontrol agents. In their experiment, they applied mineral oil-formulated entomopathogenic fungi using five different techniques (eave netting, eave curtain, eave curtains and panels, eave baffles and bed net strips) that each exploited the behaviour of malaria mosquitoes when entering, host-seeking or resting in experimental huts. To implement entomopathogenic fungi for vector control therefore, it is important to understand the behaviour of the target vector and exploit this to increase dissemination of fungal spores.

1.2 Rationale

Despite the successes achieved with malaria control over the last decade with current strategies, the disease still remains a problem in most parts of Africa and Asia. Most importantly, the current flagship vector control strategies of IRS and ITNs are failing in most of the worst hit areas of Africa and Asia, due mainly to the vector species developing resistance to the insecticides used on ITNs and for IRS (The malERA Consultative Group on Vector Control, 2011). This has stirred up research and development of novel control strategies such as the use of entomopathogenic fungi for adult mosquito control. Two species of hyphomycete fungi; *M. anisopliae* and *B. bassiana* have been demonstrated to hold great potential as vector control agents. However, their inclusion in the vector control mix is still remaining. Inclusion of classical vector control strategies such as IRS and ITNs into malaria control became possible only after proper understanding of the behavioural ecology of the vector populations. Thus, to effectively implement this novel strategy, it is important to further understand the behavioural ecology of the target vector species.

Current knowledge of mosquito behavioural ecology is limited to endophagy/exophagy (indoor or outdoor feeding respectively), endophily/exophily (indoor or outdoor resting respectively) with very few studies focusing on where exactly endophilic species rest indoors (Nagpal *et al.*, 2012; Ogata *et al.*, 1992; Quinones and Suarez, 1990; Roberts *et al.*, 1987; Mathis *et al.*, 1963;; Smith, 1962 a b; Smith, 1955;). Fewer still of these studies have looked at the factors that influence the choice of indoor resting sites by endophilic species, resulting in the need to carry out the current study. Furthermore, a review of the literature shows a lack of information on the indoor resting behaviour of vector mosquitoes in Ghana. Meanwhile, the distribution and behaviour depicted by vector species in any area is

influenced by the gross ecology of the terrain and the meteorological variables (Kaul and Wattal, 1968) and as such the findings on vector populations of one geographical region cannot be applied to another (Kaliwal *et al.*, 2010). Therefore, to implement entomopathogenic fungi for mosquito vector control in Ghana, it is essential to study the indoor resting behaviour of the target vector populations.

1.3 Objectives

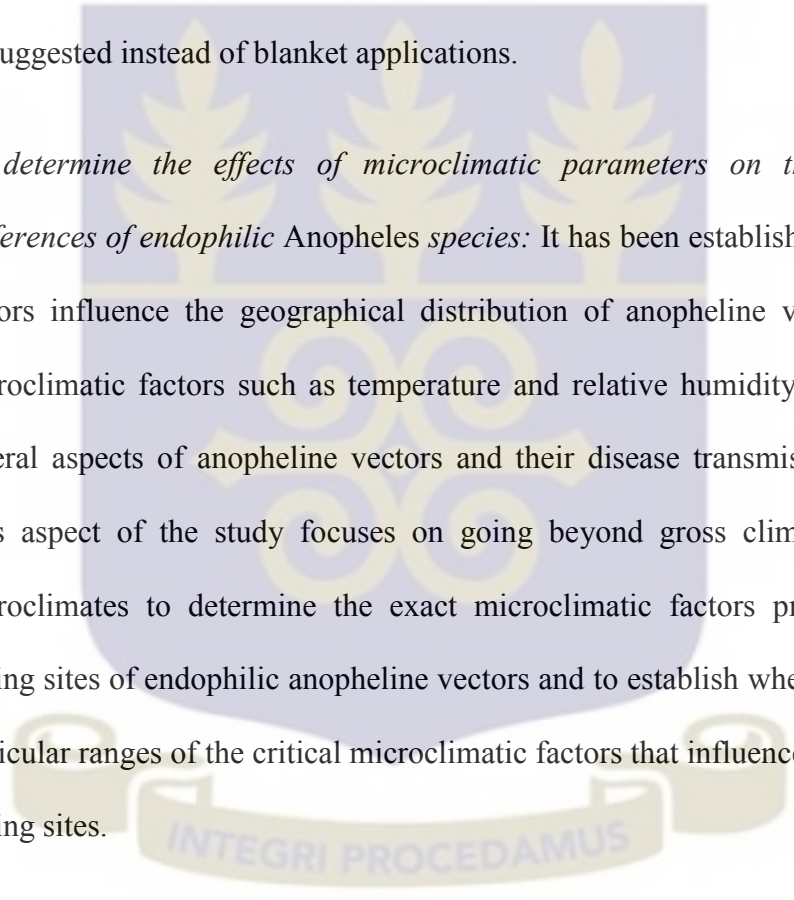
1.3.1 Main Objective

The aim of this project was to study the indoor resting behaviour of endophilic *Anopheles* mosquitoes and how this is influenced by the physical and microclimatic parameters in the indoor environment in southern Ghana.

1.3.2 Specific objectives

The set specific objectives were:

- i. *To determine the endophilic Anopheles species diversity and bionomics from the three different ecological zones of southern Ghana:* Since mosquito diversity and abundance varies in time and space, and the fact that it is critical to know the particular species of mosquitoes and their population characteristics in the target area of any control activity, this aspect of the study will focus on answering the following questions: (1) what are the major endophilic anophelines in the study area, (2) what are their status as vectors of diseases for which control is relevant and (3) what are their insecticide resistance status, as this will determine either if the current control tools of IRS and ITN are sufficient for vector control or introduction of novel strategies such as entomopathogenic fungi (which are effective even against insecticide resistant populations) is relevant.

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- ii. *To determine the indoor resting site preferences of endophilic Anopheles species in terms of location, material and colour preferences:* Understanding exactly where endophilic anopheline vectors rest is important if deployment of conventional insecticides or even novel biopesticides such as entomopathogenic fungi must be more targeted. This section of the study will focus on exactly where endophilic anophelines are found indoors so that point source dissemination of entomopathogenic fungi and even conventional insecticides can be suggested instead of blanket applications.
- iii. *To determine the effects of microclimatic parameters on the resting site preferences of endophilic Anopheles species:* It has been established that climatic factors influence the geographical distribution of anopheline vectors and that microclimatic factors such as temperature and relative humidity have effect on several aspects of anopheline vectors and their disease transmission dynamics. This aspect of the study focuses on going beyond gross climate and indoor microclimates to determine the exact microclimatic factors prevailing at the resting sites of endophilic anopheline vectors and to establish whether they prefer particular ranges of the critical microclimatic factors that influence their choice of resting sites.
- iv. *To find out the possibility of attracting endophilic Anopheles mosquitoes to resting devices constructed from different materials:* Once the indoor resting behaviour of the major anopheline vectors in the study area is understood, this aspect of the study will focus on the development and testing of resting devices that can be used as point source dissemination devices for entomopathogenic fungi.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria and Human Health

Diseases transmitted by mosquitoes and other insect vectors continue to place a critical public health burden on the world's poor, particularly in tropical and subtropical areas. Mosquitoes are responsible for the transmission of important protozoan (malaria), helminthic (lymphatic filariasis) and viral (rift valley fever, dengue, chikungunya fever and yellow fever) diseases to humans (Ratmanov *et al.*, 2013). The parasites responsible for malaria (*Plasmodium sp.*) and lymphatic filariasis (*Wuchereria bancrofti*) are transmitted by female *Anopheles* mosquitoes, while *Aedes sp.* and *Culex sp.* are responsible for the transmission of viruses that cause rift valley, chikungunya and yellow fever (Ratmanov *et al.*, 2013). Of these diseases, malaria remains the most important vector-borne disease in public health. Global spending on malaria was estimated at USD 2.3 billion in 2011 with Ghana spending in excess of USD 89.7 million on the disease (WHO, 2012a).

Five species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) are responsible for different types of malaria globally. Malaria due to *P. falciparum* is the most deadly form and it predominates in Africa; *P. vivax* is less dangerous but more widespread, and the other three species are found much less frequently (WHO, 2012a). Globally, an estimated 3.3 billion people were at risk of malaria in 2011, with populations living in sub-Saharan Africa having the highest risk of acquiring the disease: approximately 80 % of cases and 90 % of deaths are estimated to occur in Africa, with children under five years of age and pregnant women most severely affected (WHO, 2012a). In Ghana, *P. falciparum* is responsible

for hundred percent of the over 3.2 million confirmed malaria cases and 3260 deaths in 2011 with the whole population at risk (WHO, 2012a)

Out of 380 species of *Anopheles* only 60 transmit malaria globally (Alonso, 2006), and 7 of these (*An. gambiae* s.s., *An. arabiensis*, *An. melas*, *An. merus*, *An. funestus*, *An. nili* and *An. moucheti*) are considered dominant vector species in Africa (Sinka *et al.*, 2010). *An. gambiae* s.s. until recently was divided into two molecular forms, viz; M and S forms. These molecular forms have however been given species status, with the S form retaining the name *An. gambiae* s.s. and the M form given the name *An. coluzzii* (Coetzee *et al.*, 2013) making the dominant vector species in Africa 8 instead of 7 as stated in Sinka *et al.* (2010). In Ghana, *An. gambiae* s.s., *An. coluzzii* and *An. funestus* are the major vectors of *P. falciparum* but have also been implicated as major vectors of *Wuchereria bancrofti*, the causal agent for lymphatic filariasis (Boakye *et al.*, 2004). These three major vectors have been reported previously from all the ecological zones in southern Ghana (Hunt *et al.*, 2011; Coetzee *et al.*, 2006; Yawson *et al.*, 2004; Appawu *et al.*, 2001;), making their control very critical to winning the war against malaria in Ghana.

2.2 Malaria Control

Malaria is an entirely preventable and treatable disease, provided the currently recommended interventions are properly implemented. These include: (i) vector control through the use of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and, in some specific settings, larval control; (ii) chemoprevention for the most vulnerable populations, particularly pregnant women and infants; (iii) confirmation of malaria diagnosis through microscopy or rapid diagnostic tests (RDTs) for every

suspected case, and (iv) timely treatment with appropriate antimalarial medicines (according to the parasite species and any documented drug resistance) (WHO, 2012a). Vector control strategies have a proven track record of successfully reducing or interrupting disease transmission when coverage is sufficiently high. Thus, vector control has an important part to play in reducing the burden of vector-borne disease, adding resilience to the public health gains achieved through disease management and giving high priority to prevention.

2.2.1 Vector Control

Vector control has contributed significantly to reducing the burden of malaria by as much as 33 % in the hardest-hit sub-Saharan Africa region, due largely to the key intervention tools of ITN use, IRS and in some cases, larviciding (WHO, 2013; Slutsker and Kachur, 2013). These key intervention strategies of ITN and IRS rely heavily on chemical insecticides. The WHO recommends 12 insecticides from four insecticide classes (organochlorines, organophosphates, carbamates and pyrethroids) for use in IRS but only pyrethroids are recommended for use on ITNs (WHO, 2006). IRS involves the application of insecticides on internal surfaces in human or animal dwellings so that any mosquito that lands on the treated surface will pick up lethal doses of the insecticides, resulting in mortality. IRS has successfully reduced malaria cases in many areas in Africa such as the recent control campaign on Bioko Island, Equatorial Guinea (Kleinschmidt, *et al.*, 2006) and the decades-long campaign in South Africa (Hargreaves *et al.*, 2000). In Ghana, based on successful reduction in malaria incidence in the Obuasi Municipality by the Anglogold Ashanti Malaria Control Programme that depended heavily on IRS, this strategy has been expanded in

collaboration with the National Malaria Control Programme (NMCP) to cover forty other districts in Ghana (Anglogold Ashanti, 2009).

Of major importance to the Obuasi success story and for vector control globally is the use of ITNs. ITNs include nets that are treated locally by dipping in WHO recommended insecticides and long lasting insecticide nets (LLINs) made in factories using specially formulated netting material with insecticides embedded within the fibres. ITNs work by both protecting the person sleeping under the net (individual level) and by extending the effect to an entire area (community level) (WHO, 2006). Since 2007, WHO has recommended universal coverage with ITNs (preferably with LLINs), rather than a pre-determined number of nets per household or exclusively targeting household members at high risk (pregnant women and young children) (WHO, 2012a). Scaling up to universal coverage of ITNs is a high priority for Ghana's NMCP. These strategies have proved effective in most cases, however, despite the adoption of ITNs and IRS in 2004 and 2005 respectively by the NMCP as major vector control tools, malaria incidence in Ghana still remains high (WHO, 2012a). This is likely due to the widespread insecticides resistance and changes in the behaviour of the vectors (Rivero *et al.*, 2010; WHO, 2012b).

2.2.2 Insecticide resistance

Insecticides act by impairing the function of molecular targets which are crucial for life. For example, pyrethroids and DDT target the sodium-voltage-dependent (NaVdp) channel, organophosphates and carbamates target the synapse acetylcholinesterase and cyclodienes and avermectins target the GABA receptor of GABA-gated chloride channels (Bass *et al.*, 2007; Pasteur and Raymond, 1996).

Resistance to insecticides arises from one of several mechanisms including; target site insensitivity, increased detoxification, excretion, decreased penetration or behavioural avoidance of the insecticide (Wood *et al.*, 2010; Bass *et al.*, 2007; Brogdon and McAllister, 1998; Pasteur and Raymond, 1996).

Behavioural resistance has been demonstrated in insects which modify their behaviour so that they avoid the areas sprayed with the insecticide. Avoidance behaviour, also termed excito-repellency, can either be natural (protective avoidance) or developed (behavioural resistance) (Muirhead-Thomson, 1960). Protective avoidance implies that insects have an innate irritability that enables them to escape from contact with treated surfaces before they have acquired a lethal dose of the insecticide. Behavioural resistance is present where a genetic change in an insect population occurs that makes it predisposed to avoid contact with insecticides (Roberts and Andre, 1994). This change is a result of insecticide selection pressure that increases the frequency of genes conferring insecticide avoidance behaviours. The term excito-repellency is commonly used today and describes avoidance behaviours that include both contact irritancy and non-contact repellency (Roberts and Andre, 1994). Behavioural resistance is difficult to prove, because of the difficulty to ascertain if the avoidance behaviour is caused by genetic changes or through individual natural variation in the population.

Related to this and in contrast to true resistance is the concept of natural tolerance to insecticides which is caused by e.g., thicker cuticula, higher fat content, and/or larger body size. Tolerance is not based on genetic changes, but through natural variation in

individual insects to resist the effects of toxic compounds (Hoy, 1990). Tolerance may vary due to seasonal variation in physiological and morphological characteristics.

Although all the mechanisms of resistance have been described in mosquitoes (Brogdon and McAllister, 1998; Pasteur and Raymond, 1996; Lockwood *et al.*, 1984; Priester and Georghiou, 1980), target site insensitivity, especially the knockdown resistance (*kdr*) and increased detoxification are the most researched, probably due to the fact that they confer resistance to major public health insecticides such as pyrethroids (the only insecticide class recommended by the WHO for insecticide treated materials), DDT (which is being re-introduced for malaria vector control in several countries) and carbamates (WHO, 2012a; Weissmann, 2006).

2.2.2.1 Target site insensitivity

The targets of synthetic insecticides are important molecules of the nervous system. Synapse acetylcholinesterase (AChE) is the target of organophosphorus (OP) and carbamate (CB) insecticides, Na⁺ voltage- dependent (NaVdp) channel the target of DDT and pyrethroids (PY), and the GABA receptor of GABA-gated chloride channels (GABAA) the target of cyclodienes (CYD) and avermectins (Pasteur and Raymond, 1996). Alterations of amino acids responsible for insecticide binding at these sites of action cause the insecticide to be less effective or even ineffective (Brogdon and McAllister, 1998).

Insensitive acetylcholinesterase (AChE)

The neurotransmitter acetylcholine plays an important role in the transmission of the external stimuli which enables the nervous system to translate them into effective

action. When the appropriate message is successfully passed, the acetylcholinesterase (AChE) terminates the nerve impulses by catalyzing the hydrolysis of the acetylcholine (Walsh *et al.*, 2001; Mutero *et al.*, 1994). Organophosphate and carbamate insecticides target AChE by phosphorylating or carbamoylating the active-site serine and preventing it from hydrolysing the acetylcholine. Therefore, this causes the continuous action of the acetylcholine and finally the death of the insect (Vontas *et al.*, 2002).

Insensitive acetylcholinesterase was reported for the first time in spider mites (Smitssaert, 1964). Since then it has been reported from several insect species such as *Myzus persicae*, *Myzus nicotianae*, *Aphis gossypii* and *Rhyzopertha dominica* (Walsh *et al.*, 2001; Guedes *et al.*, 1997; Silver *et al.*, 1995; Moores *et al.*, 1994). In *Drosophila melanogaster* a single gene has been described (*ace-2*) with five point mutations found in three resistant strains (Mutero *et al.*, 1994). The insensitivity of AChE in *An. gambiae*, *An. albimanus* and *Culex pipiens* is due to a high level of another ace gene (*ace-1*) [Weill *et al.*, 2004].

GABA receptor mutation

The GABA neurotransmitter receptors contain five subunits found around the central ion channel (Hemingway and Ranson, 2000). Each subunit has an extracellular cysteine loop and four transmembrane domains (M1 – M4). The transmembrane domain two (M2) is the most important one because it forms the ion channel and contains the conserved alanine residue 302. Binding to its ligand GABA, the receptor increases the flow of chloride through the membrane.

Cyclodiene insecticides (e.g. dieldrin and BHC) block the GABA receptor thus preventing the inhibition of neural activity, leading to the death of the insect. Amino acid substitutions from alanine to serine or glycine within the second transmembrane region of the RDL subunit at position 302 are found associated with resistance to dieldrin in many insects (Du *et al.*, 2005; Hemingway *et al.*, 2004). An Ala → Ser substitution was found in *Drosophila melanogaster*, *D. simulans*, *Aedes aegypti*, *An. stephensi* and *An. gambiae* (Du *et al.*, 2005; ffrench-Constant *et al.*, 1993, 2000). In *An. arabiensis*, the resistance was found to be due to an alanine-glycine substitution at the same position (Du *et al.*, 2005).

Mutations in the voltage-gated sodium channel

The voltage-gated sodium channels (NaVdp) play an integral role in the transmission of nerve impulses. Pyrethroid and DDT analogs are known to be directly toxic to the nervous system, and while the two insecticides are from different groups, both target the voltage-gated sodium channel by interfering with the normal functioning of these channels (Hemingway and Ranson, 2000; Martinez-Torres *et al.*, 1997).

The *kdr* results from point mutations at the NaVdp. Two separate mutations at this same position have been identified, the first involving a Leucine-Phenylalanine substitution at position 1014 (L1014F) of the gene encoding the S6 transmembrane segment of domain II of the sodium channel usually called *kdr*-west (Martinez-Torres *et al.*, 1998). The second mutation is a Leucine-Serine substitution at the same codon (L1014S) also called *kdr*-east (Ranson *et al.*, 2000). The L1014F mutation was originally identified from and is widespread in West Africa and more predominant in *An. gambiae* s.s. than *An. coluzzii* (Santolamazza *et al.*, 2008; Pinto *et al.*, 2007;

Gentile *et al.*, 2004; Chandre *et al.*, 1999). The L1014S mutation on the other hand was first identified in a Kenyan specimen and was less widespread, occurring mainly in East Africa (Stump *et al.*, 2004; Ranson *et al.*, 2000). However, the L1014S mutation appears to be spreading westwards, having been reported in populations from Uganda (Verhaeghen *et al.*, 2006), Gabon (Pinto *et al.*, 2006), Cameroon (Etang *et al.*, 2006) and Equatorial Guinea (Moreno *et al.*, 2008). More recently, it has been reported in specimens from the Republic of Congo, Central Africa (Koekemoer *et al.*, 2011) and for the first time in Benin and Burkina Faso, both in West Africa (Namountougou *et al.*, 2013; Djegbe *et al.*, 2011). There has been no report of the L1014S mutation from Ghana and most of the reports of the L1014F mutation from Ghana were in *An. gambiae* s.s. and very few in *An. coluzzii* (Hunt *et al.*, 2011; Anto *et al.*, 2009; Owusu-Agyei *et al.*, 2009; Santolamazza *et al.*, 2008; Coetzee *et al.*, 2006; Yawson *et al.*, 2004). However, since the spread of both *kdr* mutations on the African continent is an ongoing process and the L1014S mutation has been reported in countries neighbouring Ghana (Santolamazza *et al.*, 2008; Namountougou *et al.*, 2013) it is important to screen for its presence in all members of *An. gambiae* complex in Ghana in order to know its presence and distribution. This knowledge is essential for vector control policy formulation.

2.2.2.2 Metabolic resistance

Metabolic resistance to insecticides occurs when the metabolic pathways of the insect become modified in ways that detoxify the insecticide or prevent the metabolism of the insecticide into its toxic form. There are three enzyme classes involved in this type of resistance: Glutathione S-transferases (GSTs), Carboxylesterase and Cytochrome

P450 monooxygenase (P450s) (Brogdon and McAllister, 1998; Pasteur and Raymond, 1996).

Glutathione S-transferase

The Glutathione S-transferases (GSTs) are a large family of detoxification enzymes found in almost all living organisms (Ranson *et al.*, 2001). They are cytosolic dimeric proteins with two subunits, consists of two domains, each containing two binding sites, the G site and the H site. The G site binds the tripeptide glutathione and the H site is the substrate binding site (Hemingway *et al.*, 2004; Ding *et al.*, 2003).

The GSTs are classified according to their location in the cell i.e. microsomal or cytosolic (Enayati *et al.*, 2005). Six classes of the insect GSTs have been identified Delta, Epsilon, Omega, Sigma, Theta and Zeta (Hemingway *et al.*, 2004). The two classes Delta and Epsilon are the most important because of their role in insecticide resistance to the major classes of insecticides (Ding *et al.*, 2003). For example, organophosphate resistance in the diamondback moth, *Plutella xylostella* is caused by elevated levels of Epsilon-GSTs (Huang *et al.*, 1998; Ku *et al.*, 1994), whereas permethrin resistance in the brown plant-hopper *Nilaparvata lugens* is associated with over expression of nIGST1-1, a class of Delta GSTs (Vontas *et al.*, 2001). GSTs, catalyse the reaction of the dehydrochlorination of DDT to the non-toxic form DDE, where the thiolate anion in the active site of the GST acts as a base and abstracts a hydrogen from DDT resulting in the elimination of chlorine to generate DDE. This type is found in *An. gambiae* and *Aedes aegypti* (Ortelli *et al.*, 2003; Ranson *et al.*, 2001). Resistance to organophosphates is due to increases in GST detoxification rates by O-dealkylation or O-dearylation reaction (Hemingway *et al.*, 2004). GSTs also

protect the insect against the toxicity of pyrethroids either by detoxification of the lipid peroxidation products induced by the insecticide or by sequestering the insecticide (Vontas *et al.*, 2002; Kostaropoulos *et al.*, 2001).

Carboxylesterase

Carboxylesterases are a large group of enzymes with different substrate specificity. According to Aldridge (1953), they are classified as A or B esterases according to their preference for the substrates α or β -naphthyl acetate. Esterases produce resistance either by rapid-binding and slow turnover of the insecticide (elevated esterase) i.e. sequestration, or metabolism of the insecticide by catalyzing the hydrolysis of carboxylic and phosphotrieste bonds in a wide range of insecticides such as organophosphate, carbamates and pyrethroids (Brogdon and McAllister, 1998; Hemingway and Karunaratne, 1998). Gene amplification has been found associated with elevated esterases that function by sequestration, for example in the aphid *Myzus persicae* and *Culex* mosquitoes, *C. quinquefasciatus* and *C. tritaeniorhynchus* (Hemingway, 2000; Claudianos *et al.*, 1999; Vaughan and Hemingway, 1995). However, studies of the elevated est α 1 in *C. pipiens* from France showed that there was no involvement of gene amplification (Raymond *et al.*, 1998). The elevated esterases of *C. quinquefasciatus* from Sri Lanka has both the sequestration and metabolic functions (Peiris and Hemingway, 1990).

Esterases which produce resistance by metabolism of the insecticide are associated with a single amino acid substitution in the structural genes (Hemingway *et al.*, 2004). This type of mechanism is often involved with malathion resistance, for example in

An. arabiensis from Sudan, *An. culicifacies* from Sri Lanka and *An. stephensi* from Pakistan (Herath *et al.*, 1987; Hemingway, 1982, 1983).

Cytochrome P450 monooxygenase (P450s)

Cytochrome P450 monooxygenases are hydrophobic, heme containing enzymes. The P450 family is one of the largest gene super-families and is found in all living organisms. The insect P450s are involved in insect growth, development, reproduction and insecticide resistance (Feyereisen, 1999). The P450s play an integral role in the metabolism of endogenous and exogenous compounds such as steroids, fatty acids and xenobiotics (Hemingway *et al.*, 2004; Rongnoparut *et al.*, 2003; Scott and Wen, 2001).

Monooxygenase enzymes are named as CYP followed by a number, a letter and a number respectively for example CYP6D1 (Scott and Wen, 2001). In insects, elevated levels of P450 results in metabolic resistance (Feyereisen, 1999). For example, the CYP6D1 is over-produced in the house fly, *Musca domestica*, which results in a high level of permethrin resistance, and a similar situation is CYP6A1 which is associated with organophosphate resistance (Hemingway *et al.*, 2004; Kasai and Scott, 2000).

In mosquitoes, elevated levels of oxidase have been reported from several species, for example, CYP6Z1 was found associated with pyrethroid resistance in *An. gambiae* from Western Kenya (Nikou *et al.*, 2003), and CYP6P5 and CYP6AA2 have been cloned from *An. minimus* resistant to deltamethrin in Thailand (Rongnoparut *et al.*, 2003). Another CYP6 gene is CYP6F1 which has been reported from a permethrin-resistant strain of *Culex quinquefasciatus* (Kasai *et al.*, 2000).

2.3 Novel Vector Control Tools

The *kdr* mutation coexists with several other mutations across West Africa, hampering vector control efforts (Santolamazza *et al.*, 2008; Namountougou *et al.*, 2013). To win the war against malaria, it is important to preserve and improve the utility of existing insecticide-based interventions through the development and implementation of a broader range of insecticides with novel modes of action (The malERA Consultative Group on Vector Control, 2011). In addition, insecticide resistance management (IRM) strategies with integrated vector management (IVM) as a key component must be implemented in regions where insecticide resistance is widespread (Beier *et al.*, 2008). This IVM must include several novel vector control strategies, most of which are still under development (Munhenga *et al.*, 2011; Farenhorst *et al.*, 2009; Scholte *et al.*, 2005; Benedict and Robinson, 2003).

The malERA Consultative Group on Vector Control (2011) has outlined five novel tools for sustainable vector control and elimination still under development. These include: 1) bio-prospecting for new insecticides and related chemical agents for environmental, dwelling and systemic application; 2) house design to impede vector access and sustainability; 3) biological agents (plants, fungi, algae, predators, niche competitors, insect viruses and other pathogens) for population suppression; 4) ecological/environmental modification (source reduction) targeting sites for breeding (oviposition), sub-adult development, and adult resting sites; 5) bio-prospecting for chemical attractant/repellent agents (synthetic and natural) for dwelling and personal applications that would target both indoor and outdoor biting vector species.

In addition to the above strategies, other tools such as genetic approaches to reduce adult longevity and targeting vector competence, biological agents targeting pathogens and the sterile insect technique are being developed for vector control (The malERA Consultative Group on Vector Control, 2011; Munhenga *et al.*, 2011). Based on technical challenges and changing vector ecology and behaviour, some of these novel strategies are more promising than others. For example, irrespective of intense bio-prospection, no new public health insecticide has been introduced in the last thirty years (Kelly-Hope *et al.*, 2008; Nauen, 2007; Zaim and Guillet, 2002; Hemingway and Ranson, 2000). On the other hand, research over the last decade has shown that entomopathogenic fungi hold great potential for vector control (Farenhorst, 2010; Hancock *et al.*, 2009; Read *et al.*, 2009; Scholte *et al.*, 2007; Thomas and Read, 2007; Blanford *et al.*, 2005; Scholte *et al.*, 2005; Scholte *et al.*, 2004; Scholte *et al.*, 2003a). The way forward will be to focus on those tools that hold greater promise for development and implementation into IVM programs.

2.3.1 Entomopathogenic fungi for vector control

Virtually all insect orders are susceptible to fungal pathogens, with over 700 species of fungi known to be pathogenic to insects and 10 have been or are presently being developed for insect control (Hajek and St. Leger, 1994). Two Hyphomycetes, *Metarhizium anisopliae* and *Beauveria bassiana* are the most widely used fungal species for insect pest control. They are ubiquitous worldwide and comprise a large number of different strains and isolates of different geographical origin and host specificity (Roberts and St. Leger, 2004; Goettel and Inglis, 1997). Under natural conditions, *Metarhizium* and *Beauveria* are found in the soil where moist conditions

allow filamentous growth and the production of infectious spores, called conidia, which infect soil-dwelling insects upon contact.

2.3.1.1 Infection and mode of action of entomopathogenic fungi

Metarhizium and *Beauveria* spores are small (2-6 μm) hydrophobic propagules that can attach to the insect cuticle through electrostatic interactions (Boucias *et al.*, 1988; Sosa-Gomez *et al.*, 1997). Under suitable (moist) conditions they can germinate and produce germ tubes (appressorium) that penetrate the insect cuticle (Fig. 2.1) using mechanical pressure and cuticle-degrading enzymes (Pedrini *et al.*, 2007; Smith and Grula, 1981; Pekar and Grula, 1979). The fungus can then enter the haemocoel (Fig. 2.1) by evading the insect's humoral immune response, which usually requires sufficient numbers of penetrating spores (Chouvenc *et al.*, 2009). Once inside the insect, the fungus produces blastospores that bud and spread through the haemolymph, then fungal hyphae will start to slowly grow, depleting nutrients, destroying insect cells, and eventually killing the insect (Gillespie and Claydon, 1989). This infection process takes several days, with the overall time to death depending mostly on fungal dose and virulence of the fungal isolate (Goettel and Inglis, 1997). When humidity is high enough, the fungus can grow out of the dead insect and produce conidia that can passively infect new insects.

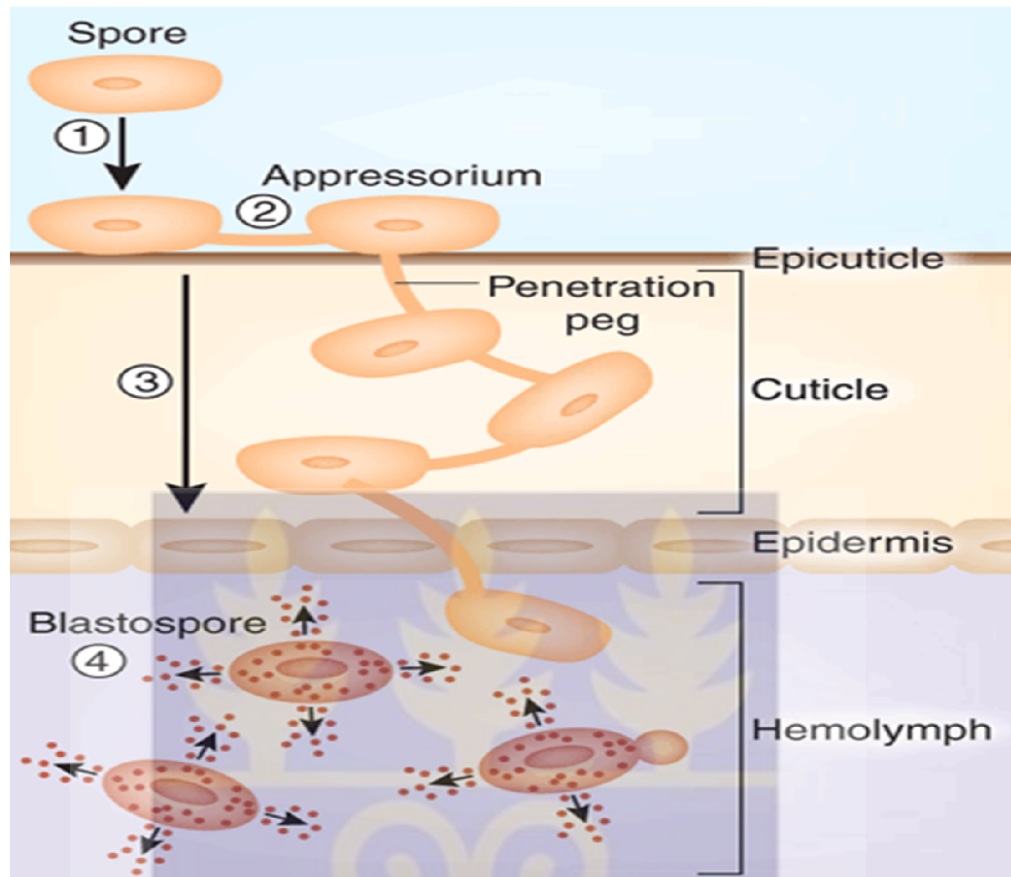


Fig. 2.1: A schematic overview of the fungal infection process. (1) recognition of the host by the fungus leads to spore germination and production of a penetration structure, the appressorium (2), which grows a penetration peg and a series of hyphal bodies to cross the cuticle and epidermis (3). Once inside the insect, the fungus produces blastospores that bud and spread through the haemolymph (4) [from Thomas & Read 2007]

2.3.2 Entomopathogens for insect control

Fungi can be deployed against pest insects outside the soil environment through so called 'inundative augmentation', which involves applying large amounts of spores for short-term insect control (Shah and Pell, 2003). Hyphomycetous conidia are relatively easy to isolate and mass-produce on artificial culture media and hence suitable for biological control (Goettel and Inglis, 1997). *Metarhizium* and *Beauveria* spores have been successfully used in pesticide formulations against insect pests such

as whiteflies, aphids, thrips, termites, locusts and beetles (Devi and Rao, 2006; Thomas and Blanford, 1998; Feng *et al.*, 1994; Zimmermann, 1993). The relative instability of fungal conidia compared to chemicals can, however, pose a practical obstacle to successful deployment. Fungal activity is, for instance, strongly influenced by humidity and temperature (Fargues and Luz, 2000; Thomas, 1997), and UV-light has a negative impact on spore viability (Fernandes *et al.*, 2007; Inglis *et al.*, 1995). Technological advances have, however, made it possible to overcome these hurdles.

Due to the increasing global interest in reducing environmental pollution with chemical pesticides, there have been several promising developments in fungus-based insect control, particularly since the 1990s. Molecular techniques have enabled the identification of isolates and virulence factors (Ansari *et al.*, 2004; St. Leger *et al.*, 1996). Advances in spore formulations have improved fungal effectiveness under low humidity conditions and UV exposure (de Faria and Wraight, 2007; Kassa *et al.*, 2004; Alves *et al.*, 1998) and increased potential deployment options (Bateman and Alves, 2000). The development of solid-state mass-production systems has made large spore quantities available for field trials (Ypsilos and Magan, 2005; Jenkins *et al.*, 1998; Feng *et al.*, 1994). Advances in quality control, such as optimizations on the substrate, incubation temperature, harvest time and storage conditions (Jenkins and Grzywacz, 2000), have enabled the production of fungal products with standardized quality (Roberts and St. Leger, 2004).

A recent review showed that there are approximately 129 fungus-based insect control products commercially available, of which the majority is based on *Beauveria* or *Metarhizium* conidia (de Faria and Wraight, 2007). Mycotrol™, for example, is a

Beauveria-based mycoinsecticide that is commercially produced in the USA. Mycotrol formulations can be used on crops for control of grasshoppers, whiteflies, thrips and aphids, and conidia can remain infective for 12 months when stored at 25 °C (de Faria and Wraight, 2007). 'Green Muscle™ is a well-known mycoinsecticide that is commercially produced in Africa and contains spores of *M. anisopliae* var. *acridum*, which are selectively pathogenic to locusts and grasshoppers. Green Muscle was developed during a 12 year research programme called LUBILOS (Thomas, 2000), in which substantial advances in mass-production, formulation and application of *Metarhizium* were made (Kassa *et al.*, 2004; Lomer *et al.*, 2001; Jenkins *et al.*, 1998). The product can be sprayed with ultra-low-volume sprayers (Bateman and Alves, 2000), achieve 70-90 % effective coverage in the field (Lomer *et al.*, 2001) and can remain infective for >12 months when stored at 30 °C (Jenkins *et al.*, 1998; Moore *et al.*, 1996a).

More than 100 fungus-based insect control products have been registered for commercial use at the USA Environmental Protection Agency since 1995 and undergone the required stringent environmental safety tests (de Faria and Wraight, 2007; Shah and Pell, 2003). Mycotrol, for instance, was fully registered after the product was shown to induce only low, acceptable risks for non-target insects (Roberts and St. Leger, 2004). Green Muscle showed no detrimental effects on non-target organisms (Lomer *et al.*, 2001) and is now registered and recommended for locust and grasshopper control by the United Nations. These and several other environmental risk studies demonstrate that fungal biopesticides are relatively safe for the environment, especially when compared to chemical pesticides (Zimmermann, 1993).

The availability of registered products significantly boosted fungal biopesticide deployment (Shah and Pell, 2003). Use of low-tech, labour-intensive mass-production in developing countries has made the sale prices of mycoinsecticides comparable with those of chemical pesticides (Li *et al.*, 2010). Nevertheless, biopesticides still only account for approximately 5 % of all agricultural pesticides, and fungus-based products only a very small part of that (the majority of commercial biopesticides are based on microbes) [Shah and Pell, 2003; Federici, 1995]. The role of mycoinsecticides in integrated pest management (IPM) is, however, already substantial in some countries and expected to grow in the near future. In Brazil, 40 fungus-based control products were commercially produced in 2007 and approximately one million hectares was treated with *Metarhizium* for spittlebug control in 2008 (Li *et al.*, 2010). In China, half a million hectares of forest land is currently being treated with *Beauveria* annually (Li *et al.*, 2010).

2.3.3 Entomopathogenic fungi for mosquito vector control

Research on the use of entomopathogenic fungi for mosquito control started in the 1960s and several fungal species have been tested, especially for the control of mosquito larvae (Scholte *et al.* 2004). The genera *Lagenidium*, *Coelomomyces*, and *Culicinomyces* in particular showed good potential for larval control (Federici, 1995). These fungi are, however, difficult to deploy cost-effectively because they require direct larval contact and high application doses, are difficult to mass-produce, and often have short shelf-lives and low persistence after application (Scholte *et al.*, 2004). *Lagenidium giganteum* is the only registered and commercially produced aquatic mycoinsecticide for mosquito control (May *et al.* 2006). The mycelia-based product can remain effective against *Culex* and *Aedes* larvae for a whole season, but is

not suitable for *Anopheles* control or applications in organically rich water (Legner, 1995).

From 1977, the focus of larval control shifted to the use of the mosquito-pathogenic bacterium *Bacillus thuringiensis israelensis* (Bti), which was a more selective, persistent and cost-effective biological control agent (Scholte *et al.*, 2004). The increasing problems with insecticide resistance in mosquito vectors and advances in fungus production and formulation have, however, revived the interest in mycoinsecticides, and in particular those based on hyphomycetes. *Beauveria* and *Metarhizium*, for instance, were shown to have potential for effective control of *Aedes*, *Culex* and *Anopheles* larvae (Bukhari *et al.*, 2010; Alves *et al.*, 2002).

Most of the recent research efforts have focused on developing a mycoinsecticide against adult mosquitoes. Fungal spores can be deployed against these flying insects by applying them on surfaces with which they make contact. A range of *Metarhizium anisopliae* and *Beauveria bassiana* isolates have been shown to be successful in infecting and killing *Anopheles*, *Aedes* and *Culex* mosquitoes when applied on several different substrates (Scholte *et al.*, 2007, 2005, 2003a; Blanford *et al.*, 2005;). Depending on the dose and virulence of the isolate, hyphomycetes can kill mosquitoes within several days, mostly between 4 and 14 days (Bell *et al.*, 2009; Mnyone *et al.*, 2009a,b ; Scholte *et al.*, 2003b).

2.3.4 Impact of entomopathogenic fungi on vector populations

Fungi act slower than fast-acting chemical insecticides and do not offer personal protection from mosquito bites. They do, however, have potential to kill mosquitoes

before they can transmit parasites (*i.e.* < 10 days) and hence to reduce malaria transmission (Hancock *et al.*, 2009; Scholte *et al.*, 2005). Moreover, fungi have been shown to prevent the development of *Plasmodium* sporozoites, which could block malaria transmission altogether (Read *et al.*, 2009; Blanford *et al.*, 2005). Fungal infection can also cause reductions in blood-feeding frequency (Blanford *et al.*, 2005) and reproductive fitness (Scholte *et al.*, 2006) prior to death, which could further impact on the mosquito's capacity to transmit malaria (Hancock *et al.*, 2009). In addition, entomopathogenic fungi are effective against insecticide resistant mosquitoes (Farenhorst *et al.*, 2010; Howard *et al.*, 2010; Kikankie *et al.*, 2010; Farenhorst *et al.*, 2009), making them suitable for use in areas with widespread insecticide resistance.

Entomopathogenic fungi clearly hold great potential for mosquito vector control irrespective of the resistance status of the target population. With clear understanding of the mode of action, production and impact on the mosquito vectors, its implementation in the vector control mix is what is remaining. For the successful implementation of any novel strategy (be it insecticides or biological agent based) and their integrated vector management (IVM) with conventional strategies, it is important to understand the ecology and behaviour of the target population.

2.4 Exploiting the Behaviour of Insects for their Control

Effective control of most insects has been dependent on the understanding of their biology, ecology and behaviour. For example, based on the understanding of the biology and behaviour of the field cockchafer, *Melolontha vulgaris* F. (Coleoptera: Scarabaeidae) Horber (1963) was able to eradicate this pest from Switzerland. The

flight period of the cockchafer could be accurately predicted and is restricted to a few weeks every third year with males emerging before females. In 1959 and 1962, Horber (1963) conducted two field trials on *M. vulgaris* in 30 hectares of agricultural land. He collected adult males in light traps, gave them a sterilizing dose of 33 Gy from an X-ray source and released them to mate with the females that emerged later, resulting in 80 % reduction in the population in 1959 and eradication in 1962.

In another demonstration of exploiting the behaviour of insects to develop control tools, it was demonstrated that the compound eyes of *Glossina* can perceive shape, colour and movement; moving objects are perceived at a much greater distance. *Glossina* species also seem capable of colour discrimination; phthalogen blue being particularly attractive, whereas black and UV-reflecting white tend to stimulate landing, and yellow tends to be unattractive (Green, 1986; Green & Flint, 1986). These led to the finding that a combination of blue and black cloth efficiently attracted *Glossina* species, especially when arranged in a form such that some movement of the cloth was possible leading to the development of blue and black traps and targets, which are still relevant for *Glossina* control (Kuzoe and Schofield, 2004).

2.4.1 Mosquito behaviour and vector control

Exploiting behavioural ecology for development and implementation of control strategies has been demonstrated for mosquitoes as well. Early work on vector control since Ross (1897) discovered that mosquitoes transmitted malaria parasites to humans focused on larval control till De Meillon's work in the 1930s resulted in a paradigm shift (Coetzee and Hunt, 1993). De Meillon (1934) demonstrated that the malaria

vector *An. funestus* was entering houses to obtain blood meals and then remaining indoors until ready to lay eggs. Based on this understanding of the resting behaviour of *An. funestus*, adults were successfully controlled in houses by spraying pyrethrum indoors (De Meillon, 1936), laying the foundation for IRS, one of the flagship vector control strategies to date.

In another example, Mnyone *et al.* (2012) exploited the behaviour of wild malaria vectors to achieve high infection with fungal biocontrol agents. In their experiment, they applied mineral oil-formulated entomopathogenic fungi using five different techniques (eave netting, eave curtain, eave curtains and panels, eave baffles and bed net strips) that each exploited the behaviour of malaria mosquitoes when entering, host-seeking or resting in experimental huts. To implement entomopathogenic fungi for vector control therefore, it is important to understand the behaviour of the target vector and exploit this to increase dissemination of fungal spores. Entomopathogenic fungi, unlike insecticides, have delayed effect, allowing mosquitoes to feed, rest and probably lay eggs before they die. Thus if we understand their indoor resting behaviour in terms of resting site preferences, it should be possible to attract mosquitoes that enter a room to a point source where they can be contaminated with fungal spores. The use of point source dissemination will be of greater economic, social and environmental advantage as far as the use of entomopathogenic fungi is concerned. But the question that still needs to be answered is 'where exactly do mosquitoes rest, and what influences their choice of resting site'?

2.4.2 Indoor and outdoor resting

Since the work of De Meillon (1934, 1936), several people have demonstrated the resting behaviour of different species and populations of mosquitoes (Nagpal *et al.*, 2012; Ogata *et al.*, 1992; Quinones and Suarez, 1990, Roberts *et al.*, 1987, Mathis *et al.*, 1963, Smith, 1955, 1962a, b, Haddow, 1942). It has been clearly established that of the major malaria vectors in Africa, *An. gambiae* s.s., *An. coluzzii*, and *An. funestus* are highly endophilic (resting indoors) and *An. arabiensis* is highly exophilic (resting outdoors) (Sinka *et al.*, 2010). However, *An. gambiae*, *An. coluzzii* and *An. arabiensis* show more behavioural plasticity than *An. funestus*, depending on geographical location (Sharp and le Sueur, 1991; Gillies and Coetzee, 1987; White, 1974). Whether mosquitoes rest indoors or outdoors is important information for any vector control program using the currently available tools such as IRS or ITNs. However, for better efficiency of these conventional tools and proper implementation of novel strategies such as the use of entomopathogenic fungi, it is better to go beyond indoor or outdoor resting to understand exactly where these mosquitoes rest indoors and the factors that influence their choice of resting sites.

A few workers have tried to maximise the scarce resources for vector control, which has been dwindling over the years (WHO, 2011.) and optimize insecticide use by selectively applying residual sprays of insecticides to preferred indoor resting sites of mosquitoes (Corbel *et al.*, 2012; Djenontin *et al.*, 2009, Arredondo-Jimenez *et al.*, 1995). Arredondo-Jimenez *et al.* (1995) selectively applied two 1-metre wide horizontal swaths of bendiocarb insecticide to the preferred resting sites of *An. albimanus* (0.75 - 1.75 m from the base of the walls and 1-metre upwards from the base of the inner roof) to achieve the same effectiveness as full spraying for control.

Their treatment required 46 % less time and 67 % less cost than full spraying. Djenontin *et al.* (2009) achieved 80.3 % mortality in recaptured *An. gambiae* released in experimental huts treated with ITN combined with woven polypropylene treated with bendiocarb on the upper one-third of walls and 100 % for similar treatment but covering all the walls with the bendiocarb treated woven polypropylene. Similar results were obtained by Corbel *et al.* (2012). By suspending 3 m² *M. anisopliae* impregnated black cotton sheet (“targets”) on ceilings in traditional houses in a rural village in Tanzania, Scholte *et al.* (2005) were able to achieve 23 % infection of *An. gambiae* and 75 % reduction in malaria transmission.

There is thus clear evidence that mosquito behaviour provides important information for formulating vector control strategies. By evaluating their indoor resting patterns it can be verified whether mosquitoes prefer specific surfaces and whether important factors such as the surface properties or microclimatic conditions influence their choice of resting site.

2.4.3 Indoor resting behaviour of mosquitoes

Indoor resting behaviour of mosquitoes vary with several factors including: species, population, geographical location, building style and material as well as the presence of irritants such as insecticides and smoke (Nagpal *et al.*, 2012; Ogata *et al.*, 1992; Quinones and Suarez, 1990; Roberts *et al.*, 1987; Service, 1976; Smith *et al.*, 1966; Mathis *et al.* 1963; Smith, 1955, 1962a, b; Haddow, 1942). For the same species, their resting behaviour could differ across geographical locations and different species within the same geographical location may have similar resting patterns (Nagpal *et al.*, 2012; Ogata *et al.*, 1992; Quinones and Suarez, 1990; Roberts *et al.*, 1987;

Service, 1976; Smith *et al.*, 1966; Mathis *et al.* 1963; Smith, 1955, 1962a, b; Haddow, 1942).

The behaviour depicted by malaria vector species appears to vary with species and location. For example, in Columbia, *An. darlingi* and *An. marajoara* were found to have a preference of resting close to the ground whereas *An. oswaldoi* and *An. rangeli* rested higher up (Quinones and Suarez, 1990). In Brazil, Roberts *et al.* (1987) collected 59 %, 37 % and 4 % of *An. darling* on the ceiling; walls and floor respectively and in Guatemala, the greatest number (53 %) of *An. albimanus* were found resting on the under-surface of roofs (Ogata *et al.*, 1992).

In another study on *An. albimanus*, there was a higher landing frequency on interior surfaces other than walls and roofs; however, mosquitoes rested for longer periods on these two surfaces. Successive landings on walls after short flights showed that mosquitoes gradually increased their mean landing height from 1.0 to 1.4 m. Similarly, mosquitoes resting at the base of inner roofs had a successive landing height range of about 0.5 m. Nagpal *et al.* (2012) working on *An. stephensi* in India reported that this species preferred resting on hanging objects and household objects as a behavioural adaptation to avoid insecticide treated surfaces. In Tanzania, *An. gambiae* was found to prefer resting on the under-surface of thatched roofs, on mud walls and on household objects in village huts (Smith, 1955, 1962a, b). Mathis *et al.* (1963), collected 94.6 % of *An. gambiae* and *An. funestus* on ceilings in village room in Burkina Faso. Of a total of 19,354 anopheline mosquitoes, 10 % were caught on the wall and 90 % on the ceiling (Haddow, 1942). Gibbins (1933) found that *An. gambiae* and *An. funestus* were commonest on the wall and lower roof, but Garnham (1929)

working on *An. funestus* in Kisumu found that males were commonest on the walls and near the floor, while females were commonest on the roof. Haddow (1942) also found that *Culex antennatus* showed preference for the ceiling, but *Taeniorhynchus spp.* were commoner on the walls.

This clearly shows heterogeneity in mosquito behaviour depending on ecological setting and therefore highlights the importance of understanding the indoor resting behaviour of any vector populations in a given setting before implementation of any novel or conventional malaria vector control tools. It has been established that the distribution and behaviour depicted by vector species in any area is influenced by the gross ecology of the terrain and the meteorological variables (Kaul and Wattal, 1968) and as such the findings on vector populations of one geographical region may not be applicable to another (Kaliwal *et al.*, 2010).

2.4.4 Factors influencing the choice of resting sites

The gross ecology of the terrain and the meteorological variables at any geographical location result in varied climate conditions that influence the distribution and behaviour of insects. Various studies have shown that variations between indoor and outdoor climate (Cator *et al.*, 2013; Paaajmans and Thomas, 2011; Afrane *et al.*, 2006) as well as internal variations of microclimatic factors within the indoor environment (Okech *et al.*, 2003) create mosaics of microhabitats which can be exploited by mosquitoes at different physiological stages. Afrane *et al.* (2006) found that the mean indoor temperature of houses located in the deforested areas of the Kenyan highlands were 1.2 °C higher than in houses located in the forested areas during the dry season and 0.7 °C higher during the rainy season. These variations were enough to affect

fecundity by 64.8 - 79.5 %, survival by 5 - 7 days. It also affected vectorial capacity by 106 % and 29 % in the dry and rainy seasons respectively. To determine the effect of internal variations of microclimatic factors on mosquito survival, Okech *et al.* (2003) placed *An. gambiae* at different locations within village experimental huts and monitored survival. They concluded that micro heterogeneities of temperature but not relative humidity influence survival. They also found that the side but not height within the hut at which mosquitoes were placed, influenced survival but could not be explained by either temperature or relative humidity. This observation may be due to some other factors such as airflow or light intensity heterogeneities. Indeed, Gillies and De Meillon (1968) indicated that African malaria vectors routinely aggregate at particular sites within houses such as dark crevices, the inside of the roof and under furniture.

The choice of resting site by malaria vectors, over distances as little as a few centimetres can be influenced by microclimate (Fialho and Schall, 1995). Microclimatic factors that have been identified to influence mosquitoes include: temperature, relative humidity, light intensity and wind speed (Cator *et al.*, 2013; Paaajmans and Thomas, 2011; Afrane *et al.*, 2006; Muirhead-Thomson, 1938; Shannon, 1934), of which temperature and relative humidity are the most studied.

Because mosquitoes are small ectothermic organisms, their physiology is highly influenced by the surrounding environment. Effects of temperature and relative humidity on the survival (Lyons *et al.*, 2012; Okech *et al.*, 2003; Haddow, 1942; Muirhead-Thomson, 1938) population dynamics (Parham and Michael, 2010; Paaajmans *et al.*, 2010; Afrane *et al.*, 2006) distribution (Craig *et al.*, 1999) and behaviour (Cator *et al.*, 2013; Paaajmans and Thomas, 2011; Blanford *et al.*, 2009) of mosquitoes are relatively well characterised. Muirhead-Thomson (1938), established

that *Culex fatigans* strongly avoided high temperatures. This behaviour is strongest in hungry females, less strong in those with matured ovaries and least strong in newly emerged females. Muirhead-Thomson (1938) also found that newly emerged females were more sensitive to temperature changes between 25 - 30 °C and blood fed females were sensitive to temperatures between 20 - 25 °C. Lyons (2013) found that the survival of all stages of *An. arabiensis* and *An. funestus* were highest at 32 °C and 25 °C respectively under controlled laboratory conditions with no survival at 15° C and 35 °C for both species. It has also been established that survival of *An. arabiensis* and *An. funestus* is higher at constant temperature than at fluctuating temperatures (Lyons, 2013). Other studies on the reactions of mosquitoes to relative humidity (Blanford *et al.*, 2009; Kirby and Lindsay, 2004; Muirhead-Thomson, 1938; Shannon, 1934) have clearly demonstrated that mosquitoes exhibit preferences for certain humidity ranges. According to Muirhead-Thomson (1938), the strongest humidity reaction for *Culex fatigans* is an avoidance of high humidities above 95 %, this reaction being strongest in blood-fed females. All stages of this same species showed slight but regular avoidance of low humidities and between 30 - 85 % RH, all stages were unaffected by humidity differences of as much as 40 %. Mosquitoes at low humidities risk desiccation by losing water to their surroundings and tolerance to desiccation is dependent on body water content (Gray and Bradley, 2005). Gray and Bradley (2005) found that *An. arabiensis* is more resistant to desiccation than *An. gambiae* s.s. and concluded that this physiological difference is genetic based. Lyons (2013) also established that *An. arabiensis* is more resistant to desiccation than *An. funestus*.

Since temperature and relative humidity have such great effects on various aspects of the biology of mosquitoes, it is expected that individuals will select resting locations where microclimate is favourable for optimum physiological activities. Indeed,

Blanford *et al.* (2009) indicated that, *An. stephensi* shows some capacity to behaviourally thermoregulate by resting within a narrow range of ambient temperature where body temperature is optimised for other physiological processes. However, little exists on the influence of these microclimatic parameters on the choice of resting site. It is also important to note that except for a few, studies on the effects of microclimatic factors on mosquitoes are limited to measuring general indoor climates and not microclimatic conditions at the actual resting sites of mosquitoes, making the current study very relevant.

Apart from temperature and relative humidity, the effects of other microclimatic factors such as light intensity and especially, wind speed have received very little attention. Light intensity is a key factor in *Anopheles* resting site selection (Clements, 1999). Anophelines avoid light situations during the day and the most important requirement for a resting place appears to be relative darkness, as they avoid light situations during the day (Shannon, 1934; Christophers and Missiroli, 1933). However, very few quantitative records exist on the preferred range of light intensities within which mosquitoes rest. According to Jones *et al.* (1967), light appears to inhibit activity and reset the circadian cycle of *An. gambiae*. They found that mosquitoes are moderately active in the dark, but are inactive in the light after the first half-hour and that light appears to have an inhibitory effect on mosquito activity. According to Shannon (1934), the intensity of the early morning light is an important guide in directing anophelines to their daytime resting places. When mosquitoes enter a shelter, they continue inwards until they find a suitable degree of darkness (Shannon, 1934). And for endophilic mosquitoes which have remained indoors, they frequently move to more exposed situations during the night but at dawn, seek the more protected portions of the shelter (Shannon, 1934).

Jones *et al.* (1967) suggested that the activity of mosquitoes is controlled by an endogenous rhythm which possibly controls the release of an excitatory hormone. The effect of light may be mediated through an inhibitory hormone. Though Shannon indicated that light intensity of 1 - 5 candle-foot power (10.76 - 53.83 Lux) is suitable for resting by anophelines under summer conditions in Greece, each species differs to some extent in its microclimate preference, hence the need to carry out this studies for any target population.

Similarly, most records on the effect of wind speed on mosquitoes focus on flight, dispersion, swarming and host location (Omran *et al.*, 2010; Hagemoen and Reimers, 2002; Ritchie and Rochester 2001; Hoffmann and Miller, 2001). It is known that mosquitoes appear to avoid places with high wind speeds, but exactly what range of wind speed is preferred for resting by anophelines is not known. Haddow (1942) observed that during periods of strong winds in the night, mosquito entry into huts in Kisumu diminished or even completely stopped while the wind lasted. Hoffmann and Miller (2001) indicated that wind likely affects mosquito host-seeking success via at least two mechanisms. In one situation, mosquitoes might desist from plume following because of an inability to progress up-wind at a rate sufficient to satisfy a built-in optomotor requirement (Kennedy, 1939; Klassen and Hocking, 1964; Snow, 1980; Gillies and Wilkes 1981). Another potential effect of wind is dilution of chemical attractants from the host. A given rate of emanating volatiles (such as CO₂ and lactic acid) would be expected to become less stimulatory as wind velocity increased.

The four microclimatic factors mentioned above may have been researched to varying extents in relation to mosquitoes and in most cases only one or two factors were

considered with no study looking at all four factors. Meanwhile in nature, these microclimatic factors interact with each other and with other biotic and abiotic factors to create mosaics of microhabitats. To understand how microclimate influences resting site selection therefore, it is important to take a holistic look at all the factors and determine how these interact to create suitable resting sites for the target mosquitoes.



CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

Field work was carried out in six villages in southern Ghana covering three ecological zones namely, forest ecological zones (FEZ), forest transition ecological zone (FTEZ) and coastal savannah ecological zone (CSEZ) [Fig. 3.1].

In the FEZ, mosquitoes were collected from three villages, Atatam ($06^{\circ} 17.377''$ N, $001^{\circ} 27.545''$ W), Agyenkwaso ($06^{\circ} 15.547''$ N, $001^{\circ} 26.689''$ W) and Kusa Dinkyae ($06^{\circ} 18.894''$ N, $001^{\circ} 29.622''$ W) all located in the Adansi North District of Ashanti Region (Fig. 3.2). Atatam is a village 257 m above sea level located at the foot of a hill surrounded by several streams abundant with fish. There are about 1,220 inhabitants in this village. Agyenkwaso is 223 m above sea level with a human population of about 1,300 and Kusa Dinkyae has the least population with about 800 inhabitants and lying 268 m above sea level. Atatam and Agyenkwaso are proximal to each other while Kusa Dinkyae is relatively further, isolated in the forest. There is a mountain which lies between Atatam and Kusa Dinkyae. The main economic activity in these three villages is cocoa farming with occasional coffee, oil palm and subsistent farming. All three villages have been targeted by expansion of the Obuasi malaria control program being run by AngloGold Ashanti Gold Mine because of their proximity to Obuasi Municipality.

From the CSEZ, two villages Gomoa Okyereko ($05^{\circ} 24.828''$ N, $000^{\circ} 36.284''$ W) and Gomoa Adawukwa ($05^{\circ} 24.960''$ N $000^{\circ} 36.729''$ W) were sampled (Fig. 3.2).

Okyereko and Adawukwa are located in the Gomoa East District of the Central Region of Ghana with an elevation of 19 and 22 m above sea level respectively. Okyereko is an irrigation rice farming village with about 1,500 inhabitants. Water from a dam about 500 m away from the village flows through canals to irrigate the rice fields, which are only about 100 m from the village. The irrigated rice fields are active breeding sites for different species of mosquitoes including anophelines. Cement block houses with corrugated aluminium sheets roofing dominate, and eaves are either open or closed. Adawukwa is located about 500 m from Okyereko and the two villages are separated by a river. About 500 m upstream the river is a swamp where sugarcane cultivation, the main occupation of the inhabitants takes place. There are about 1,200 people in the village, who besides sugarcane farming, are involved in maize farming and alcohol distillation from the sugarcane. There is a brick and roofing tile factory in the village and as a result, most of the houses which are either made of brick or cement blocks are roofed with brick tiles and often have open eaves.

Apart from a few households with ITNs and the use by individuals of mosquito repellent coils, there is no malaria control program running in any of the six villages. Domestic animals such as goat, sheep, chicken, dogs, cats and pigs were present in all the six villages. Cattle were not seen in any of the villages sampled. It is however noteworthy to indicate that bats were seen resting on roofing beams in rooms without ceiling in Okyereko.

The sixth village where mosquito sampling was done, Osorongma ($05^{\circ} 52.757''$ N, $000^{\circ} 06.563''$ W), lies 94 m above sea level and is located in the FTEZ. It is a peri-urban village close to Dodowa in the Dangme West District of the Greater Accra Region of Ghana with about 200 inhabitants who are mainly into cassava and mango

farming. Houses in the village are a mix of cement block and mud houses, all with corrugated aluminium sheet roofing, usually with open eaves. Demographic data were obtained from the National Analytical Report of the 2010 Population and Housing Census for Ghana (GSS, 2013).



Fig. 3.1: Map of Ghana showing sampling areas. Different coloured circles represent various ecological zones; (green circle = forest ecological zone, blue circle = forest transition ecological zone, brown circle = coastal savannah ecological zone). Map source:

http://maps.google.co.za/maps?rlz=1T4GGIE_enZA430ZA435&q=ghana+map&um=1&ie=UTF8&hq=&hnear=0xfd75acda8dad6c7:0x54d7f230d093d236,Ghana&gl=za&sa=X&ei=Un2sUebqFYOM7QaXoYAY&ved=0CCwQ8gEwAA Accessed on 03/06/13.

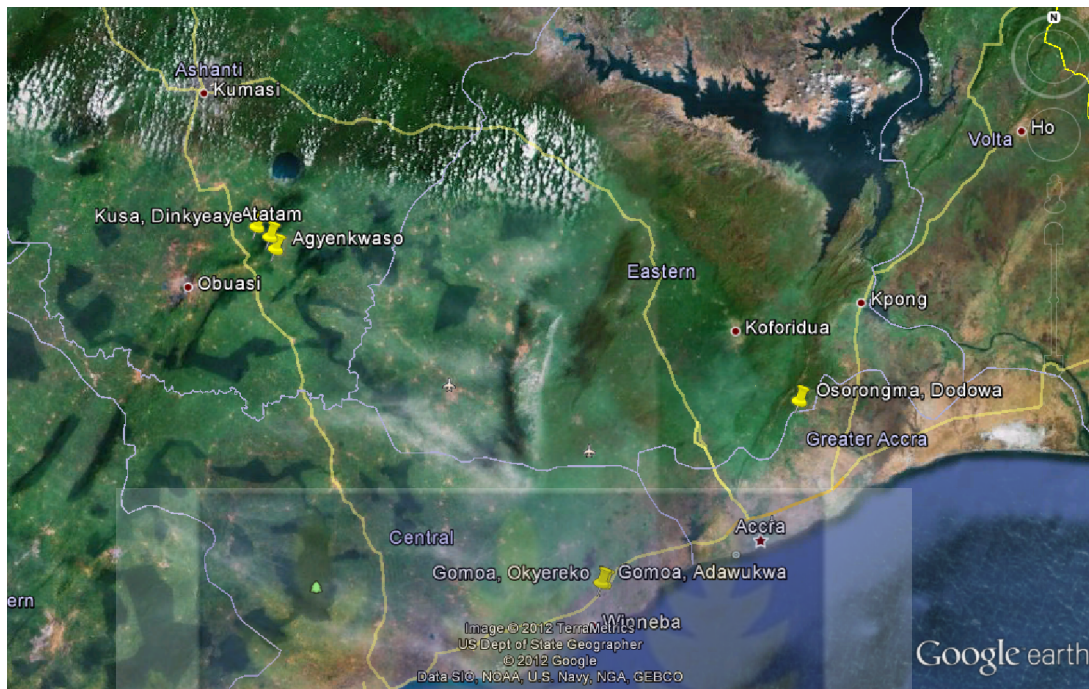


Fig. 3.2: Terrain map of part of southern Ghana showing geographical location of villages sampled (indicated by yellow pins). Map source: Google earth. Accessed at 14:32 hours CAT on 2nd May, 2013.

3.2 Mosquito Collection and Field Processing

Sampling was carried out between September, 2011 and January, 2012, spanning the minor rainy season (September - November) and the dry season (December - March) in southern Ghana. Prior to mosquito sampling, permission to gain access to each village was sought from an opinion leader such as the chief or assemblyman. In all cases, verbal informed consent was obtained from the head of each household before mosquito collections were done.

Mosquitoes were collected inside houses early in the morning between 6:00 and 10:00 hours GMT. Specifically, indoor resting mosquitoes on dark corners of walls and crevices, hanging clothes, under surfaces of furniture and roofs were searched with the aid of a flash light and aspirated into paper cups using a mechanical aspirator (sucking tube). Immediately after collection, mosquitoes were killed in a killing bottle

containing ethyl ether and individually put into 1.5 ml Eppendorf tubes containing silica gel and labelled. All specimens were transported back to the laboratory for further processing.

3.3 Field Data Collection

For each collection, site demographic data about the village was collected by interviewing either the Chief or Assemblyman. Data collected included population size, major economic activities and whether or not there was any organised vector control intervention in the village.

Location of each sampled village was determined by taking Global Positioning System (GPS) coordinates at a central location using a Garmin eTrex[®]10 handheld GPS (Garmin International Inc., Kansas City, USA) [Fig. 3.3 C]. An Extech multimeter (Extech Instruments Corp., New Hampshire, USA) [Fig. 3.3 A] was used to measure outdoor climate data including; temperature (in °C), relative humidity (RH in percentage), light intensity (LI in Lux) and wind speed (WS in ms⁻¹) at an approximate central location of the village, and indoor climate at an approximate central location in each room where samples were collected. General indoor climate parameters were recorded for 8 - 20 houses per village, whereas outdoor parameters were taken once on each sampling day for each village. In addition, for each household sampled the following information was recorded: materials used for walls and roofs, presence of eaves and whether walls were painted or not.

For each household sampled, information on how many people slept in a room, whether there were adults or children and the use of any personal mosquito control measures such as aerosol insecticide sprays, mosquito repellent coils or creams or

insecticide-treated nets (ITNs) was collected by interviewing the head of the household. As much as possible rooms where mosquito control measures were being used or rooms where nobody sleeps were avoided. However, rooms where ITNs were being used were still sampled; this was based on experience from previous studies (M. Osae, unpublished data).

At each point in a room where a mosquito was collected, the resting site (roof, roofing beam, ceiling, wall, window or other household objects), resting material (wood, clay, cement, cotton, bamboo, palm or coconut frond etc), background colour and whether the resting site is movable or not was recorded. A self-retracting steel tape measure (Fig. 3.3B) was used to measure the height of resting location from the floor, and its distance from nearest wall (front back, left or right with reference to the main entrance). Microclimatic data including: temperature, relative humidity, light intensity and wind speed were recorded with an Extech multimeter. All the data related to each mosquito specimen were recorded against the specimen number for that particular mosquito on a data sheet (Appendix I)

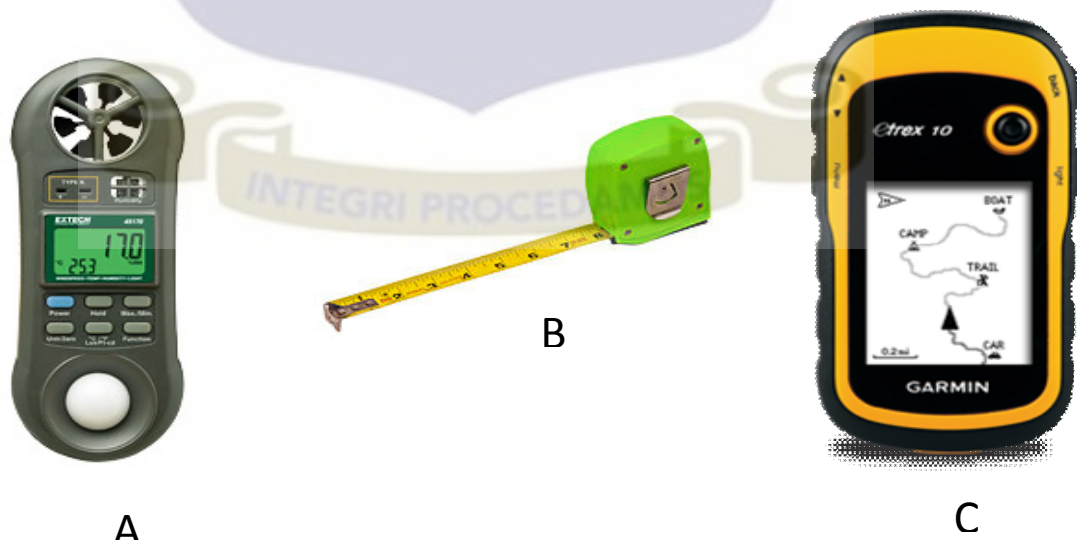


Fig. 3.3: Instruments used for field data collection. A. Extech multimeter for measuring temperature, relative humidity, light intensity and wind speed. B. Self-retracting steel measuring tape for measuring distance. C. Garmin eTrex[®]10 handheld GPS for geographic data collection.

3.4 Laboratory Processing of Field Samples

3.4.1 Morphological identification

Collected adult anophelines were identified morphologically using taxonomic keys (Gillies and Coetzee, 1987), sorted according to species groups and stored at -20 °C until required

3.4.2 DNA extraction

DNA was extracted from individual mosquitoes using the standard procedure described by Collins *et al.* (1987) [Appendix IV]. Briefly, DNA was extracted from specific parts of individual mosquitoes by grinding in extraction buffer (0.08 M NaCl, 0.16 M sucrose, 0.06 M EDTA, 0.5 % SDS 0.1 M Tris-Cl, pH 8.6) and treated with 8M potassium acetate (KAc). This was followed by precipitation of the pellet in absolute alcohol. The pellet was collected, washed in 70 % ethanol, allowed to dry and suspended in 1x TE buffer, pH 7.4 (0.1 M Tris and 0.01 M EDTA) and stored at -20 °C till ready to use.

DNA for species-specific identification, discrimination between *An. gambiae* s.s and *An. coluzzii* and *kdr* mutation detection assays was extracted from legs and wings while DNA for blood meal analysis was extracted from blood-engorged abdomens of individual mosquitoes.

3.4.3 PCR species-specific identifications

3.4.3.1 *Anopheles gambiae* complex

All specimens identified morphologically as belonging to the *An. gambiae* species complex were identified to species level using the multiplex polymerase chain reaction (PCR) assay of Scott *et al.*, (1993) [Appendix III]. The multiplex PCR

included primers specific for each of the four major members of the *An. gambiae* complex (*An. gambiae* s.s., *An. arabiensis*, *An. merus* and *An. quadriannulatus*) [Table 3.1] so that any species present could be identified in one single PCR.

Each 15 µl PCR reaction mixture contained 2.5 µl 10x PCR buffer, 2.5 µl dNTP mix, 0.5 µl MgCl₂, 0.5 µl *An. quadriannulatus* primer, 1 µl each of primers specific for *An. gambiae* s.s., *An. arabiensis* and *An. merus*, 4.9 µl deionized water, 0.1 µl *Taq* polymerase and 1 µl template DNA. Details of primer sequences and sizes are shown in Table 3.1. The PCR reaction was performed as follows; initial denaturation at 95 °C for 10 minutes, followed by 45 cycles of denaturation at 95 °C for 15 seconds, annealing at 50 °C for 20 seconds, elongation at 72 °C for 20 seconds and a final elongation of PCR products after the final cycle at 72 °C for 7 minutes. Amplification products were run in a 2.5 % agarose gel containing ethidium bromide for one hour at 110 Volts. Species identification was done based on the size of the amplicon in relation to a 1 kilobase (kb) standard molecular weight GeneRuler™ DNA ladder (Fermentas, Canada, Cat. No. SM0331) loaded on the first and last lanes of the gel.

Table 3.1: Diagnostic primer sequences and amplicon sizes for the identification of the members of the *An. gambiae* complex (Scott *et al.*, 1993)

Primer	Name Species	Primer Sequence (5' – 3')	Size (bp)
UN	Universal Reverse	GTGTGCCCCTTCCTCGATGT	-
GA	<i>An. gambiae</i>	CTGGTTTGGTCGGCACGTTT	390
ME	<i>An. merus</i>	TGACCAACCCACTCCCTTGA	464
AR	<i>An. arabiensis</i>	AAGTGTCTTCTCCATCCTA	315
QD	<i>An. quadriannulatus</i>	CAGACCAAGATGGTTAGTAT	153

3.4.3.2 Distinguishing between *Anopheles gambiae* s.s. and *Anopheles coluzzii*

Anopheles gambiae s.s. has until recently been grouped into two molecular forms (M and S forms). Each of these forms has however been given species status with the S form retaining the name *Anopheles gambiae* s.s. and the M form given the name *Anopheles coluzzii* (Coetzee *et al*, 2013). All specimens identified as *Anopheles gambiae* s.s. using the protocol of Scott *et al*. (1993) were further identified to species level using the PCR assay described by Favia *et al*. (2001) [Appendix IV]. This PCR makes use of a 2.3 kb long DNA segment derived from the 5'-most end of the 28S of the chromosomal repeat unit in order to distinguish between the M and S forms of *An. gambiae* s.s., now known as *An. gambiae* s.s. and *An. coluzzii*. The assay also allows distinguishing any hybrid between the two species.

Each 25.7 µl PCR reaction mixture contained 2.5 µl of 10x PCR buffer containing MgCl₂ (Supertherm gold™), 2.5 µl dNTP mix, 2 µl each of primers R5 (50 pmol), R3 (50 pmol), Mopint (40 pmol) and B/S int (25 pmol), 0.2 µl of *taq* polymerase (Supertherm gold™), 11.5 µl of deionized water and 1 µl template DNA. Details of primer sequences and sizes are shown in Table 3.2 below. The PCR reaction was performed as follows; an initial denaturation step of 10 minutes at 94 °C followed by 25 cycles with denaturation at 94 °C for 30 seconds, annealing at 63 °C for 30 seconds and extension at 72 °C for 30 seconds. After the final cycle, the products were extended for 7 minutes at 72 °C. Amplification products were run in a 2.5 % agarose gel containing ethidium bromide for one hour at 110 Volts. *An. gambiae* s.s. and *An. coluzzii* were identified based on the size of the amplicon in relation to a 1 kilobase (kb) standard molecular weight GeneRuler™ DNA ladder (Fermentas, Canada, Cat. No. SM0331) loaded on the first and last lanes of the gel.

Table 3.2: Diagnostic primer sequences for distinguishing between *An. gambiae* s.s. and *An. coluzzii* (Favia *et al.*, 2001)

Primer Name	Primer Sequence (5' – 3')	Size (pb)
R5	GTGTGCCCCCTTCCTCGATGT	-
R3	CTGGTTTGGTCGGCACGTTT	-
Mopint	TGACCAACCCACTCCCTTGA	727
B/S int	AAGTGTCTTCTCCATCCTA	475

3.4.3.3 *Anopheles funestus* group

Specimens morphologically identified as belonging to the *An. funestus* group were identified to species-specific level using the PCR assay as described by Koekemoer *et al.* (2002) with slight modifications (Appendix V). This method uses unique primers targeted to species-specific portions of the ITS2 region in five species (*An. funestus*, *An. lesoni*, *An. parensis*, *An. rivulorum* and *An. vaneedeni*). The assay is multiplexed such that each species identified by the assay will amplify a uniquely sized PCR fragment. Each 12.5 µl PCR reaction mixture contained: 1.25 µl of 10x PCR buffer, 1 µl dNTP mix, 0.75 µl MgCl₂, 1 µl each of primers specific for *An. funestus*, *An. lesoni*, *An. parensis*, *An. rivulorum* and *An. vaneedeni*, 0.1 µl *Taq* polymerase, 2.9 µl deionized water and 0.5 µl template DNA. Details of primer sequences and sizes are shown in Table 3.3. The PCR reaction was performed as follows: initial denaturation step at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 45 °C for 30 seconds and elongation at 72 °C for 40 seconds. After the final cycle, the PCR products are elongated at 72 °C for 5 minutes. Amplification products were run in a 2.5 % agarose gel containing ethidium bromide for one hour at 110 Volts. Samples were identified based on the size of the amplicon

in relation to a 1 kilobase (kb) standard molecular weight GeneRuler™ DNA ladder (Fermentas, Canada, Cat. No. SM0331) loaded on the first and last lanes of the gel.

Specimens that failed to be identified using the Koekemoer *et al.* (2002) method were identified using the IGS based real time PCR method for identification of *An. funestus* subgroup developed by Vezenegho *et al.* (2009). This method has been shown to be more sensitive (Vezenegho *et al.*, 2009). The method (detail protocol in Appendix VI) is based on the use of hydrolysis probes.

To perform the assay, a 20 µl reaction mixture containing: 10 µl of iQ™ Super mix; 1 µl each of forward (10 µM IGS) and reverse (10 µM UN18S IGS) primers; 0.4 µl of 10 µM preparation of probes for *An. funestus*, *An. funestus*-like and *An. parensis* labeled with reporter dyes ROX FAM and Quasar 705 respectively, as well as 0.05 µl of 10 µM probe preparation for *An. vaneedeni* labeled with the reporter dye VIC; 5.75 µl of ddH₂O and 1 µl of template DNA. Details of primer and probe sequences and sizes are shown in Table 3.4. The reaction was run using the Bio-Rad CFX96™ Real-Time PCR system with initial denaturation at 95 °C for 10 minutes, 35 cycles of denaturation at 95 °C for 15 seconds and annealing at 64°C for 60 seconds. Increase in fluorescence of each of the reporter dye ROX, Quasar 705, VIC and FAM was monitored in real-time by acquiring each cycle on the following channels: orange (560 nm excitation and 650 nm emission), purple (684 nm excitation and 730 nm emission), green (534 nm excitation and 580 nm emission) and blue (490 nm excitation and 530 nm emission) respectively. The identity of the specimens was determined based on the peaks of the reporter dyes on a plot. Any specimen that failed was deemed unidentifiable.

Table 3.3: Diagnostic primer sequences and sizes for the identification of the members of the *An. funestus* group. (Koekemoer *et al.*, 2002)

Primer Name	Species	Primer Sequence (5' – 3')	Size (bp)
UV	Universal Reverse	TGTGAACTGCAGGACACAT	-
FUN	<i>An. funestus</i>	GCATCGATGGGTAAATCATG	500
VAN	<i>An. vaneedeni</i>	TGTCGACTTGGTAGCCGAAC	550
RIV	<i>An. rivulorum</i>	CAAGCCGTTTCGACCCTGATT	400
PAR	<i>An. parensis</i>	TGCGGTCCCAAGCTAGGTTC	250
LEES	<i>An. lesoni</i>	TACACGGGCGCCATGTAGTT	153

Table 3.4: Diagnostic primers and probes used in the IGS-based real time PCR identification of *An. funestus* species

Name	Reporter dye	Primer Sequence (5' – 3')	Quencher
FUN I	-	GGCATCGATGGGTAAATCATG	-
VAN I	-	AAACCCCAAGATGTGCTCC	-
VAN 3	-	GGTTTTCAAATGAATCTC	-
N PAR	-	ATACTTGTGTGTGTGTATTG	-
RIV TM	Cy5	CTATGGCGAGACCCCGTCTAGTG	BHQ2 ^a
FUN TM	ROX	CATGGGGAAATTCAATCGAAAACCTCT	BHQ2 ^a
PAR TM	VIC	CGGAACCTAGCTTGG	MGBNFQ ^b
VAN TM	Quasar 705	CGTTGTGAAAAATGGAGATTCATTTGAAAACC	BHQ2 ^a
LEES TM	6-FAM	CCGACCGATGTACA	MGBNFQ ^b

^aBHQ2 – black hole quencher 2^bMGBNFQ – minor groove binder non-fluorescent quencher

3.4.4 Real-time PCR for detection of *kdr* mutation

The mutation associated with knockdown resistance to pyrethroids/DDT was assayed using the method described by Bass *et al.* (2007) [Appendix VII]. Knockdown resistance (*kdr*) was determined using the real-time Taqman® PCR methods developed by Bass *et al.* (2007). This method involves two separate assays for the detection of the L1014F and L1014S mutations. The reaction mixture for both assays is similar except for minor groove binding (MGB) probes specific for each mutation

type depending on which one is the target. In the present study, specimens were screened for both the L1014F and L1014S mutations. A 20 µl PCR reaction mixture contained 5.0 µl of ddH₂O; 10.0 µl of SensiMix PCR buffer (Quantace Ltd, United Kingdom); 1.6 µl of each of 800 nM forward and reverse *kdr* primers; 0.4 µl of 200 nM wild type Taqman MGB probe labelled with VIC; 0.4 µl of 200 nM L1014F or L1014S Taqman MGB probe labelled with FAM; and 1 µl DNA template. Sequences for the primers and probes are listed in Table 3.5 below. The reaction is prepared in a 96 well microtitre plate with one negative control (PCR reaction mixture without DNA template) and two each of positive wild type control (containing DNA template from a wild type mosquito) and positive homozygous mutant control (containing DNA from a homozygous mutant mosquito) and heterozygous mutant control (containing DNA from a heterozygous mutant mosquito).

Table 3.5: Diagnostic primer and probe sequences for the TaqMan RT-PCR detection of the west and east *kdr* mutations in *An. gambiae* s.s. (Bass *et al.*, 2007)

Primer Name	Primer Sequence (5' – 3')
<i>Kdr</i> -Forward	CATTTTCTTGGCCACTGTAGTGAT
<i>Kdr</i> -Reverse	CGATCTTGGTCCATGTTAATTGCA
Wild type Probe	CTTACGACTAAATTTC
<i>Kdr</i> -w Probe	ACGACAAAATTTC
<i>Kdr</i> -e Probe	ACGACTGAATTTC

The reactions were run with a Bio-Rad CFX96™ Real-Time PCR system using the following cycling conditions: initial denaturation at 95 °C for 10 minutes; 40 cycles at 95 °C for 10 seconds, 60 °C for 45 seconds and fluorescence measured at the end of each cycle. In either assay, a substantial increase in VIC fluorescence indicates a homozygous wild type (SS), a substantial increase in FAM fluorescence indicates a

homozygous mutant (RR), and a usually intermediate increase in both signals indicates a heterozygote (RS). Individuals homozygous for the *kdr-e* mutation display no increase in VIC or FAM fluorescence in the *kdr-w* assay and vice versa. Based on the above, specimens were scored for *kdr-w* and *kdr-e* as RR, RS or SS.

3.4.5 Multiplex PCR for mosquito blood meal identification

Enzyme-linked immunosorbent assay (ELISA) is used routinely for identification of blood meal source in vector studies in many laboratories including the Vector Control Reference Laboratory (VCRL) at the NICD. This method, though reliable, is expensive and time consuming, especially when tests have to be run for several blood types (Osae *et al.*, 2013). Further, under situations such as the current study where several DNA based diagnostic assays are to be run on every single sample, it becomes more prudent to use a DNA-based assay (Kent and Norris, 2005). In view of the above reasons, the method described by Kent and Norris (2005) was used in this study. As this method is not routinely used in the VCRL, it was necessary to optimise and validate it under local context and settings (Osae *et al.*, 2013). The detailed protocol used for the present study is in Appendix VIII.

In detail a 12.5 µl reaction mixture containing 5.65 µl ddH₂O, 10x PCR buffer (10 mM Tris pH 8.3, 50 mM KCl), dNTP mix (2.5 mM each dNTP), 25 nM MgCl₂, 50 mM each of primers specific for human, cow, pig, goat, dog and a universal reverse primer (Table 3.4) and *Taq* DNA polymerase (5 Uµl⁻¹); 0.5 µl template DNA were set up. Concurrently, negative control consisting of the PCR reaction mixture without template DNA and positive controls consisted of DNA extracted from human, cow, pig, goat and dog blood were also set up. Positive control for human blood was

obtained from a laboratory-reared mosquito which had been freshly fed on human volunteer arm while positives for other animal bloods were provided by the Department of Agriculture, University of Pretoria, South Africa.

The reaction was run at the following cycling conditions: initial denaturation at 95 °C for 5 minutes; 35 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute, extension at 72 °C for 1 minutes; final extension at 72 °C for 7 minutes. The PCR products were subjected to electrophoresis at 110 V, 400 mA for 1 hour 20 minutes or until proper separation of the smaller fragments of the molecular weight marker had been achieved. The gel was visualized and image captured using a Syngene G-box sydr4/115 gel documentation system. Samples were identified based on the size of the amplicon in relation to a 1 kilobase (kb) standard molecular weight GeneRuler™ DNA ladder (Fermentas, Canada, Cat. No. SM0331) loaded on the first and last lanes of the gel. The assay was used to test for the presence of human, cow, pig, goat and dog blood in the specimens. Specimens that failed to be identified were repeated once. Any specimens that gave multiple blood meals were repeated for confirmation. To confirm blood meal type, PCR products for each blood type were sequenced and checked for alignment with the respective host blood sequence.

Table 3.6: Diagnostic primer sequences and sizes for the identification of mammalian blood meals in female mosquitoes. (Kent and Norris, 2005)

Primer Name	Species	Primer Sequence (5' – 3')	Size (bp)
UNREV1025	Universal Reverse	GGTTGTCCTCCAATTCATGTTA	-
HUM741F	Human	GGCTTACTTCTCTTCATTCTCTCCT	334
COW121F	Cow	CATCGGCACAAATTTAGTCG	561
PIG573F	Pig	CCTCGCAGCCGTACATCTC	453
GOAT894F	Goat	CCTAATCTTAGTACTTGTACCCTTCCTC	132
DOG368F	Dog	GGAATTGTACTATTATTCGCAACCAT	680

3.4.6 ELISA for *Plasmodium falciparum* sporozoite detection

The head and thorax of wild *An. gambiae* and *An. funestus* female specimens were tested for *Plasmodium falciparum* infection using the ELISA method described by Wirtz *et al.* (1992) as detailed in Appendix IX. Preparations of solutions used for this method are also presented in the Appendix.

3.5 Attractiveness of Resting Devices for Endophilic Anophelines

3.5.1 Study sites

All screen house trials were carried out at the Radiation Entomology and Pest Management Centre of the Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission. Field trials were carried out in two villages in southern Ghana; Osorongma and Adawukwa where preliminary surveys showed high populations of endophilic anophelines. Detailed information on the ecology, geographical location and demography are presented in Section 3.1.

3.5.2 Mosquitoes

The *An. gambiae* s.s. colony (REDCO) which was established in 2007 from larval collections around Madina, near Accra was used for all screen house experiments. The colony is maintained under standard rearing conditions of 27 ± 2 °C; 75 ± 5 % R.H. with a natural tropical day-night cycle of 12L:12D. Larvae are maintained on Tetramin® fish food, while adults are sustained on 10 % sucrose solution. Blood feeding is done using a membrane feeding system (Hemotek® 5W1 Membrane Feeding System, Discovery Workshops, Lancashire, UK). For field experiments, wild

endophilic anopheline populations seeking resting sites inside selected houses were relied upon.

3.5.3 Resting devices

Wicker basket

These were ordered from a local cane weaver. They were made from raffia woven on a wooden frame (30 cm length x 30 cm width x 30 cm height) with one side of the square (entrance) hinged to allow opening and closing (Fig. 3.4A).

Earthenware pots

Round earthenware pots (30 cm diameter) were ordered from a local pottery. After acquisition, the exterior of each pot was painted black with emulsion paint. The point of entry of mosquitoes into the pot was 15 cm in diameter (Fig. 3.4D) and the interior resting surface remained brick red.

Resting boxes

Boxes made of wooden frames 30 cm length x 30 cm width x 30 cm height were ordered from a local carpenter. Black cotton cloth, Wood, jute or black felt, was glued to all five sides to create different resting surfaces (Fig. 3.4 B, C, E and F). In all cases mosquito net with a sleeve was glued to the hinged cover for inspection and collection.

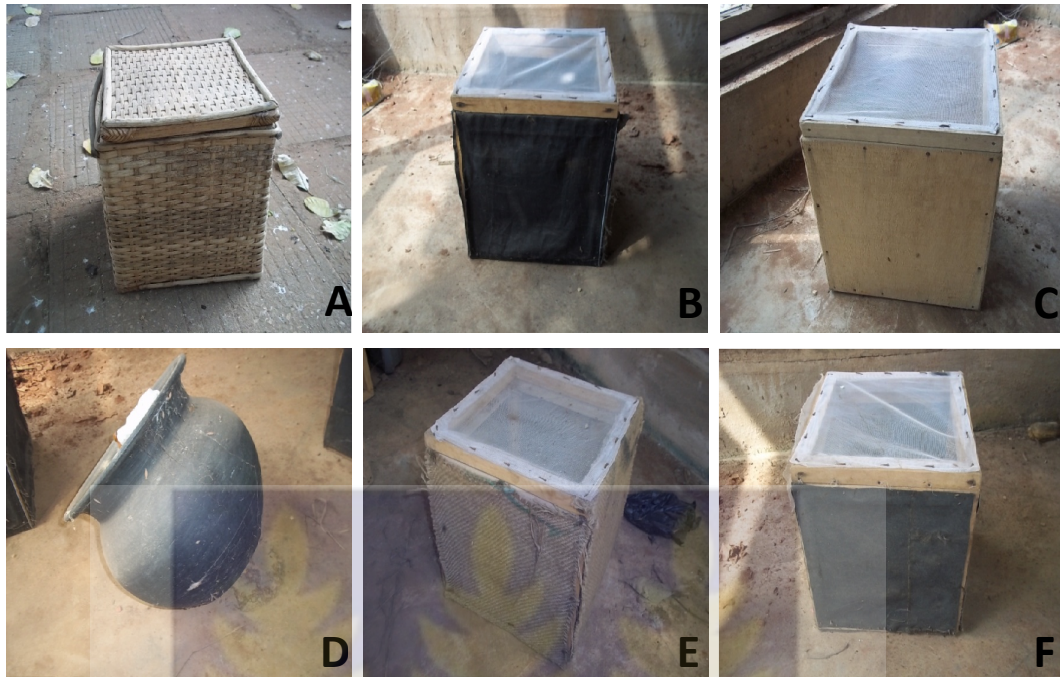


Fig. 3.4: Resting devices made from different materials. A: wicker basket, B: black cotton cloth box, C: wooden box, D: earthenware pot, E: jute box and F: black felt box

3.5.4 Experimental design

Non-choice test

Each of the six resting devices (Fig. 3.5) was randomly placed in a single 4 m (length) x 4 m (width) x 4 m (height) screen house with the cover opened to allow free access of mosquitoes. A total of 100 adult female mosquitoes per treatment arm were released at dusk (between 17:30 h GMT and 18:30 h GMT). The treatment arms consisted of; (i) Newly emerged, 3-4 days old non-blood-fed *An. gambiae* s.s colony females (ii) Blood fed, 5-6 days old *An. gambiae* s.s colony females and (iii) *An. gambiae* s.s colony females which had completed one gonotrophic cycle and were ready for the next blood meal. In all cases, mosquitoes had been pre-fed on 10 %

sucrose solution prior to release. The morning after release (between 06:00 h GMT and 07:00 h GMT), number of mosquitoes resting inside and outside of each resting device were recorded. This was replicated five times for each resting device.

Choice test

The experimental set up was similar to the non-choice test. Meanwhile in this test, all six resting devices were concurrently put into the same screen house and presented to batches of 100 adult female mosquitoes per treatment arm as described above. The number attracted to each device was recorded after one night in the screen house. This was replicated five times.



Fig. 3.5: Screen house used for choice and non-choice test of resting devices

Field trial

Field testing of the devices was carried out in Osorogma and Adawukwa. These experiments relied on the presumption of presence of natural anopheline mosquito

populations in both villages. Six houses were randomly selected based on the first six families consenting to participate in the study. Verbal informed consent was sought from the head of each family before the start of the project.

Each of the resting devices was concurrently placed in each of the six houses between 17:00 h GMT and 18:00 h GMT and rotated in a Latin Square design. The resting devices were positioned at an ideal location not directly exposed to daylight, away from the main entrance where there was minimal human interference. The resting devices were positioned in such a way that the mosquito entry points faced the centre of the room. The resting devices were left overnight and inspected the following morning, between 06:00 h GMT and 08:00 h GMT. Mosquitoes were searched with the aid of a flash light and the number of mosquitoes resting on the device counted and recorded. The device was then closed and the mosquitoes collected with an aspirator. In the case of the earthenware pot, a net was used to cover the entrance before moving it for mosquito collection. After sampling the device, indoor resting collection was done by two experienced collectors, who searched the room to collect any resting mosquitoes found till they could no longer find any. Pyrethroid spray collection was then carried out using Raid™ multipurpose insect killer (0.150 % Tetramethrin, 0.250 % Allethrin and 0.015 % Deltamethrin) with the bed, furniture and floor covered with a white cloth. Temperature and relative humidity at the time of collection were recorded using a HOBO U14 LCD Logger (Onset Computer Cooperation Inc. Southern MA, USA). The experiment was replicated six times, rotating the devices in all the rooms to ensure that each device has been tested in each room. All mosquito samples were identified morphologically and sorted into species groups using the keys of Gillies and Coetzee (1987).

3.6 Data Analysis

All statistical data analysis was done at the 0.05 confidence level using SPSS version 16 (SPSS Inc., Cary, USA) except for Chi Square test which was done online using Hardy-Weinberg equilibrium calculator (Rodriguez *et al.*, 2009). All mean analysis was done by ANOVA and Tukey's test used for mean separation. Variations in means were compared using a one way analysis of variance followed by Tukey's HSD test wherever there was significant difference for more than two means or by Student's t-test for only two means.

3.6.1 Population characteristics data

Mosquito species density was calculated as number of each species divided by total number of mosquitoes collected per room per collection date using the formula below.

$$MSD = \{(NS)/TMC\}/NRD$$

Where:

MSD = Mosquito species density

NS = Number of the particular species

TMC = Total number of mosquito specimens

NRD = Number of rooms sampled per day

Knockdown resistance (*kdr*) data was analysed using Chi Square test for goodness of fit. This test shows if the population conforms to or departs from the Hardy-Weinberg Equilibrium as well as generates the *kdr* allele frequency.

Human blood index was calculated as number of specimen positive for human blood divided by total number of specimens tested.

Sporozoite rate was calculated as number of specimen positive for *P. falciparum* divided by total number tested.

3.6.2 Resting site data

Based on population analysis of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* from the three ecological zones, which revealed that populations from the CSEZ were mainly *An. coluzzii* and *An. funestus* in similar proportions and the fact that there was no difference in prevalence of the *kdr* alleles, these were pooled together and treated as one group. Populations from FEZ and FTEZ followed the same pattern and were therefore pooled together. Furthermore, since insufficient numbers of some species were collected from all the ecological zones to allow comparisons, data was analysed per species rather than splitting into ecological zones.

Pearson's Chi-square test was used to test the relationship between mosquito frequencies and parameters evaluated including: resting site, resting site material, background colour of resting site, nearest wall to resting site, and mobility of the resting site. Pearson's Correlation was used to determine the relationship between mosquito frequencies and the height of resting site from the floor as well as distance from the nearest wall. For Chi-square tests, whenever frequencies were below five, data was transformed by adding five to each frequency in the range.

3.6.3 Microclimatic data

Resting site parameters for a total of 431 indoor-resting female anophelines were analysed. Based on the near uniformity of outdoor and indoor climate parameters across the six villages and the fact that none or very few of some species were collected from some of the villages, all data for resting site parameters were pooled together for each species across all the villages. Temperature and relative humidity data were analysed without any transformation; however, due to the large variation and number of zeros recorded for light intensity and wind speed data, these parameters were log-transformed. The proportion (%) of mosquitoes resting at varying levels of each microclimatic parameter were computed and used to plot line graphs. Pearson's correlation was used to determine the relationship between height from ground, distance from nearest wall and all resting site microclimatic parameters.

3.6.4 Resting device data

For the screen house trials, the proportion of mosquitoes resting in the devices was expressed as a percentage of the total number of mosquitoes released into the screen house. For the field trials, the proportion of mosquitoes resting in each device was expressed as a percentage of the total number of mosquitoes collected from the device plus those collected using indoor resting collection and pyrethroid spray collection for each room. All percentages were arc sine transformed and counts were transformed using natural log before analysis. Treatments were compared using ANOVA and where there was significant difference, mean separation was done using Tukey's test. All analyses were done at 95 % confidence level using the SPSS software version 16.

CHAPTER FOUR

INDOOR RESTING MOSQUITO DIVERSITY, RESISTANCE AND VECTORIAL CAPACITY IN THREE ECOLOGICAL ZONES OF GHANA

4.1 Background

In Ghana, four dominant malaria vector species are responsible for human malaria transmission including: three members of the *An. gambiae* complex; *An. gambiae* Giles *sensu stricto*, and *An. arabiensis* and *An. coluzzii* as well as *An. funestus* s.s (WHO, 2012a). *An. gambiae sensu lato* and members of the *An. funestus* group are sympatric over much of their range in Africa (Sinka *et al.*, 2010). Studies in southern Ghana have found that *An. gambiae* s.s., *An. coluzzii* and *An. funestus* are the dominant malaria vector species (Appawu *et al.*, 2001; Yawson *et al.*, 2004; Coetzee *et al.*, 2006; Hunt *et al.*, 2011). However, this information alone is general and cannot be used to extrapolate effective vector control strategies against these species under the diverse ecological conditions experienced in Ghana. It has been established that occurrence, population density and vectorial capacity of *An. gambiae* s.l. and *An. funestus* vary geographically and are influenced by a number of environmental conditions such as climate, and vegetation (Joshi *et al.*, 1975; Lindsay *et al.*, 1998; Appawu *et al.*, 2001; Sinka *et al.*, 2010) as well as activities which influence their breeding. It is therefore essential to understand the distribution and population dynamics of these anophelines at any ecological settings before implementing appropriate malaria vector control interventions.

In Ghana, the national vector control programme spearheaded by the Ghana National Malaria Control Program (NMCP) among other malaria control initiatives prioritized the use of ITNs and IRS as the main malaria vector control tools (WHO, 2012a). These strategies heavily rely on few insecticides approved by WHOPES (WHO,

2006). Unfortunately, there is overwhelming evidence that mosquito populations in Ghana have developed resistance to insecticides being used for malaria vector control (Yawson *et al.*, 2004; Coetzee *et al.*, 2006; Owusu-Agyei *et al.*, 2009; Hunt *et al.*, 2011). This development, therefore calls for a shift to an integrated vector management (IVM) approach. There are several approaches available including but not limited to; insecticide treated wall linings (ITWL), entomopathogenic fungi, larviciding and the development and implementation of novel strategies to counter this problem. In addition, insecticide resistance management (IRM) plans need to be implemented to prolong effectiveness of the insecticides currently available for malaria vector control (WHO, 2012b). For effective implementation of IVM and IRM strategies, it is important to understand the biology and ecology of the target population. It is against this background that the studies reported in this chapter focused on understanding the bionomics and susceptibility levels of vector populations from the different ecological settings in Ghana.

With the ultimate objective of implementing entomopathogenic fungi as a malaria vector control option, this study focused on understanding vector behaviour in relation to deployment of entomopathogenic fungi. The first step towards this is to know the bionomics of the target populations. This chapter reports studies on the indoor resting populations of anophelines in two ecological zones of Ghana.

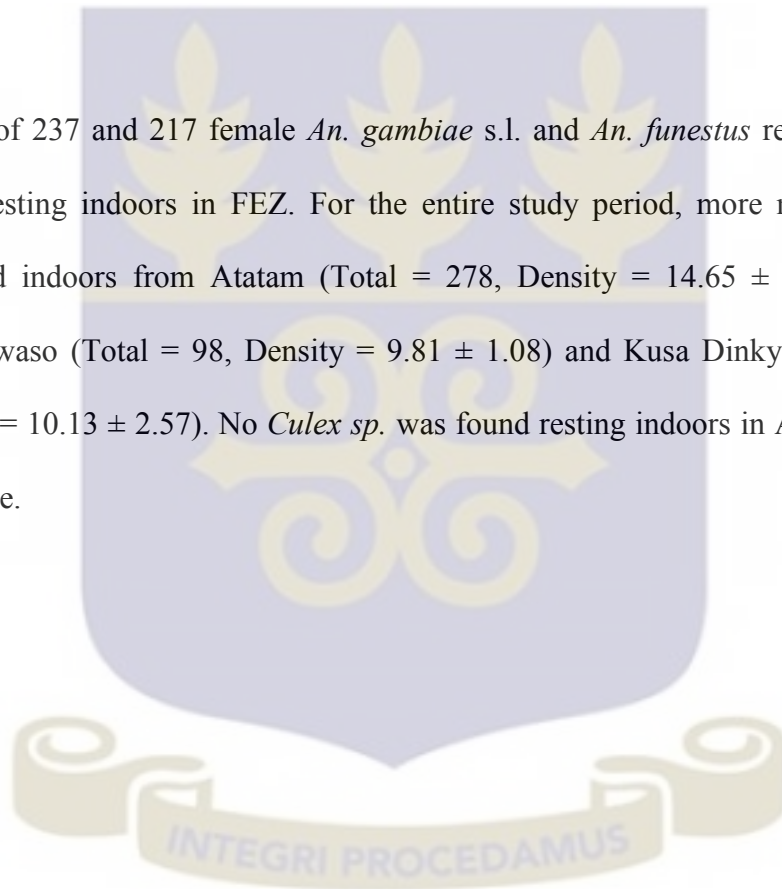
4.2 Results

4.2.1 Indoor resting mosquito density

All mosquitoes collected were morphologically identified followed by molecular identification of a sub-sample of 30 or all (where less than 30 specimens were

collected) specimens per village per collection date to group them into *An. gambiae* complex or *An. funestus* group using PCR (Figs. 4.1 and 4.2). A total of 925 indoor resting mosquitoes comprising; 640 (68 %) female *An. gambiae s.l.*, 252 (27 %) female *An. funestus*, 34 (4 %) male anophelines and 9 (1 %) *Culex sp* were collected from the six villages during the study period. Of 892 female anophelines collected, 431(49 %) sub-sampled for molecular diagnostics, resulted in; 308 (71 %) and 123 (29 %) *An. gambiae s.l.* and *An. funestus* respectively.

A total of 237 and 217 female *An. gambiae s.l.* and *An. funestus* respectively, were found resting indoors in FEZ. For the entire study period, more mosquitoes were collected indoors from Atatam (Total = 278, Density = 14.65 ± 1.90) than from Agyenkwaso (Total = 98, Density = 9.81 ± 1.08) and Kusa Dinkyeae (Total = 78, Density = 10.13 ± 2.57). No *Culex sp.* was found resting indoors in Atatam and Kusa Dinkyeae.



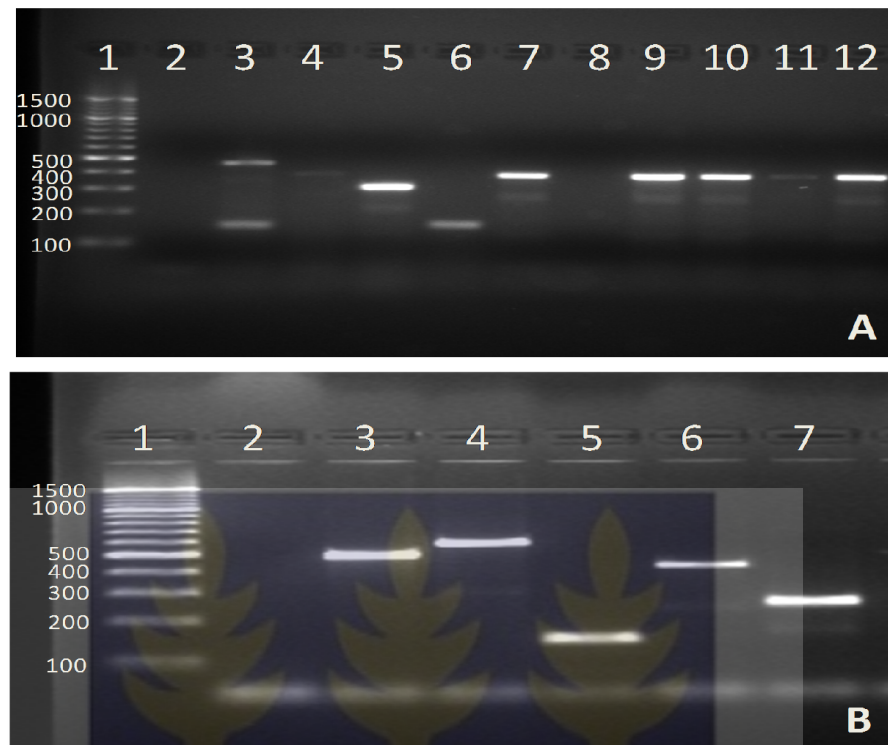


Fig 4.1: A 2.5 % agarose gel stained with ethidium bromide for visualising species-specific DNA amplicons for: A – *An. gambiae* complex: lane 1, 100 base pair molecular weight marker; lane 2, negative control; lanes 3, 4, 5 and 6, positive controls for *An. merus*, *An. gambiae* s.s., *An. arabiensis* and *An. quadriannulatus* respectively; lanes 7 – 12, field collected samples identified as *An. gambiae* s.s. (subject to further discrimination between *An. gambiae* s.s. and *An. coluzzii*) except lane 8 which failed to amplify. B – *An. funestus* group: lane 1, 100 base pair molecular weight marker; lane 2, negative control; lanes 3, 4, 5, 6 and 7, positive controls for *An. funestus*, *An. vaneedeni*, *An. leesoni*, *An. rivulorum* and *An. parensis* respectively.

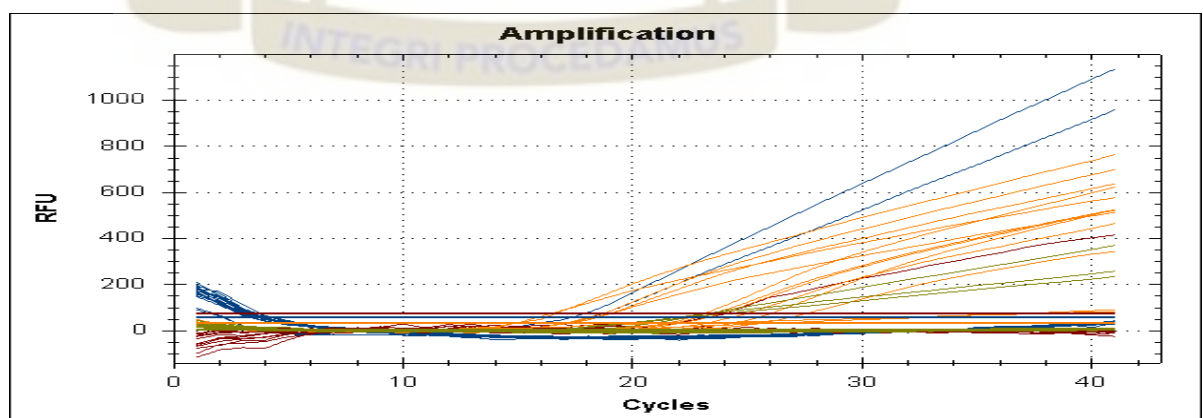


Fig. 4.2: Identification of members of the *An. funestus* sub-group using the IGS based real time PCR assay. *An. funestus* (Orange trace), *An. parensis* (purple trace), *An. vaneedeni* (green trace) and *An. funestus*-like (blue trace).

Table 4.1: Indoor resting mosquito density in six villages across three ecological zones of Ghana

Village	Mean Number of Mosquitoes / Room $\pm$ S.E.			
	<i>An. gambiae</i> s.l.	<i>An. funestus</i> group	<i>Culex</i> sp.	Total
FEZ				
Atatam	7.20 $\pm$ 1.59 ^b	7.45 $\pm$ 1.46 ^b	0.00 $\pm$ 0.00 ^c	14.65 $\pm$ 1.90 ^a
Agyenkwaso	4.36 $\pm$ 1.15 ^b	5.18 $\pm$ 1.33 ^{ab}	0.27 $\pm$ 0.14 ^c	9.81 $\pm$ 1.08 ^a
Kusa Dinkyae	8.63 $\pm$ 2.82 ^a	1.50 $\pm$ 0.76 ^b	0.00 $\pm$ 0.00 ^c	10.13 $\pm$ 2.57 ^a
FTEZ				
Osorongma	10.00 $\pm$ 2.49 ^a	0.00 $\pm$ 0.00 ^b	0.00 $\pm$ 0.00 ^b	10.00 $\pm$ 2.49 ^a
CSEZ				
Adawukwa	14.75 $\pm$ 2.60 ^a	4.38 $\pm$ 2.78 ^b	0.38 $\pm$ 0.38 ^c	19.51 $\pm$ 1.15 ^a
Okyereko	12.13 $\pm$ 2.19 ^a	0.13 $\pm$ 0.13 ^b	0.33 $\pm$ 0.33 ^b	12.39 $\pm$ 2.13 ^a
Total	9.18 $\pm$ 0.91^b	3.54 $\pm$ 0.65^c	0.11 $\pm$ 0.05^d	12.83 $\pm$ 0.90^a

Means in the same row with different letters as superscripts are significantly different at the 0.05 confidence level by one way ANOVA and Tukey's test.

For *An. gambiae* s.l., 130, 41 and 66 females were collected from Atatam, Agyenkwaso and Kusa Dinkyae respectively, whereas, for *An. funestus*, 148 females were collected from Atatam, 57 from Agyenkwaso and 12 from Kusa Dinkyae. Table 4.1 presents indoor resting densities for the different species in FEZ. During the sampling period, Atatam recorded a mean mosquito density of 7.20 $\pm$ 1.59 (95 % CI [3.87, 10.53]) and 7.45 $\pm$ 1.46 (95 % CI [4.40, 10.50]) for *An. gambiae* s.l. and *An. funestus* respectively, which were not significantly different from each other ($F_{(3,76)} = 35.32$, $p = 0.829$). In Agyenkwaso, there was significant difference in the indoor resting densities of the three mosquito species collected ($F_{(3,40)} = 19.20$, $p = 0.0001$). Tukey post-hoc comparisons of the three species indicate that the mean densities of *An. gambiae* s.l. (4.36 $\pm$ 1.15, 95 % CI [1.81, 6.92]) and *An. funestus* (5.18 $\pm$ 1.33, 95 % CI [2.23, 8.14]) did not differ significantly from each other ($p = 0.769$) but both

were significantly higher ($P < 0.05$) than that of *Culex sp.* (0.27 ± 0.14 , 95 % CI [-0.04, 0.59]). In Kusa Dinkyae however, *An. gambiae* s.l. had higher indoor resting density (8.63 ± 2.82 , 95 % CI [1.95, 15.30]) but this was not significantly different ($F_{(3,28)} = 12.47$, $p = 0.052$) from that of *An. funestus* (1.50 ± 0.76 , 95 % CI [-0.29, 3.29]).

In the CSEZ, a total of 318 female anophelines found resting indoors, comprised: *An. gambiae* s.l. (300), *An. funestus* (13) and *Culex sp.* (5). In Adawukwa, the species distribution was 134, 10 and 3 for *An. gambiae* s.l., *An. funestus* and *Culex sp.* respectively and in Okyereko it was 166, 3 and 3 for *An. gambiae* s.l., *An. funestus* and *Culex sp.* respectively. Data for mosquito indoor resting densities for CSEZ are summarised in Table 4.1. Collectively, indoor resting densities for anophelines was 19.50 ± 1.15 (95 % CI [16.78, 22.22]) in Adawukwa and 12.40 ± 2.13 (95 % CI [7.83, 16.97]) in Okyereko. Results show that in Adawukwa the mean indoor resting densities of the three species were significantly different ($F_{(3,28)} = 22.15$, $p = 0.0001$). Tukeys post-hoc test indicated that the mean density was significantly highest for *An. gambiae* s.l. (14.75 ± 2.60 , 95 % CI [8.61, 20.89]), followed by *A. funestus* (4.38 ± 2.78 , 95 % CI [-2.21, 10.96]) and *Culex sp.* (0.38 ± 0.38 , 95 % CI [-0.51, 1.26]). Similar significant difference in the indoor resting density was observed in Okyereko ($F_{(3,56)} = 71.07$, $p = 0.0001$) with *An. gambiae* s.l. (12.13 ± 2.19 , 95 % CI [7.43, 16.84]) showing the highest significant density. The mean densities of *An. funestus* (0.13 ± 0.13 , 95 % CI [-0.15, 0.42]) and *Culex sp.* (0.13 ± 0.13 , 95 % CI [-0.15, 0.42]) were not significantly different ($p = 1.00$).

One village (Osorongma) was sampled from the FTEZ. Results indicate almost exclusive presence of *An. gambiae* s.l. (91 specimens) with only one specimen of *An.*

funestus. The mean indoor resting density was 10.00 ± 2.49 *An. gambiae* per room (95 % CI [4.38, 15.62]).

4.2.2 Prevalence of the members of the *An. gambiae* complex

Specimens were identified as *An. gambiae* s.s. or *An. coluzzii* based on amplicon size (Fig. 4.3). From a total of 308 female *An. gambiae* s.l. processed, 176 (57.71 %) were identified as *An. coluzzii* and 132 (42.86 %) as *An. gambiae* s.s. Data showing the percentages of the two species in all the six villages sampled are presented in Table 4.2. *An. gambiae* s.s. was predominant in the FEZ, making up 91 % (n = 98) of the total collections. The 29 specimens processed from Kusa Dinkyae were exclusively *An. gambiae* s.s. and the 47 specimens processed from Atatam had significantly higher ($t = -34.66$ df = 4, $p = 0.0001$) proportion of *An. gambiae* s.s. (98.04 ± 1.96 %) than *An. coluzzii* (1.96 ± 1.96 %). Similarly, in Agyenkwaso, of the 22 specimens processed, there were significantly higher ($t = -26.85$, df = 2, $p = 0.001$) proportion of *An. gambiae* s.s. (76.39 ± 1.39 %) than *An. coluzzii* (23.61 ± 1.39 %).

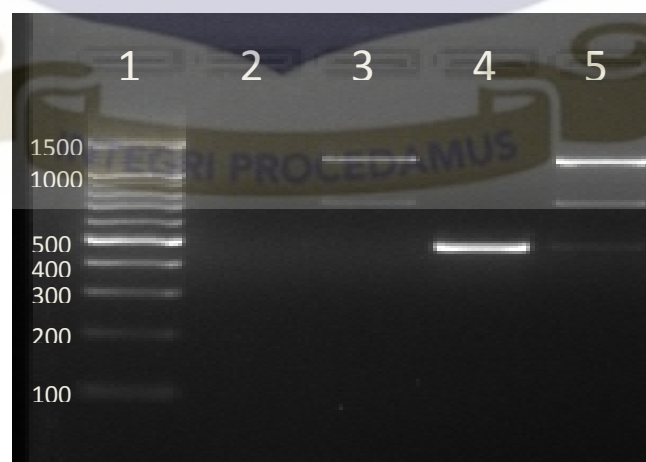


Fig. 4.3: A 2.5 % agarose gel stained with ethidium bromide for visualising species-specific DNA amplicons for *An. gambiae* s.s. and *An. coluzzii*. Lane 1 - 100 base pair molecular weight marker; lane 2 – negative control; lane 3 - M form; lane 4 – S form; lane 5 – MS hybrid.

Collections from Osorongma, the only village sampled in the FTEZ were similar to those from the FEZ, showing significantly higher ($t = -18.45$, $df = 3$, $p = 0.0001$) percentage of *An. gambiae s.s.* (94.00 ± 3.24 %) compared to the *An. coluzzii* (6.00 ± 3.24 %).

A total of 167 female *An. gambiae s.l.* was processed from the CSEZ made up of 79 from Adawukwa and 88 from Okyereko. All the samples from these two villages in the CSEZ were exclusively *An. coluzzii*.

Table 4.2: Relative prevalence of *An. gambiae s.s.* and *An. coluzzii* in six villages from three ecological zones of Ghana

Village	N	Percentages ± S.E.	
		<i>An. coluzzii</i>	<i>An. gambiae s.s</i>
FEZ			
Atatam	47	1.96 ± 1.96 ^b	98.04 ± 1.96 ^a
Agyenkwaso	22	23.61 ± 1.39 ^b	76.39 ± 1.39 ^a
Kusa Dinkyae	29	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a
Sub-Total	98	8.52 ± 1.12 ^b	91.48 ± 1.12 ^a
FTEZ			
Osorongma	43	6.00 ± 3.24 ^b	94.00 ± 3.24 ^a
CSEZ			
Adawukwa	79	100.00 ± 0.00 ^a	0.00 ± 0.00 ^b
Okyereko	88	100.00 ± 0.00 ^a	0.00 ± 0.00 ^b
Sub-Total	210	100.00 ± 0.00 ^a	0.00 ± 0.00 ^b
Total	308	57.71 ± 11.76 ^a	42.86 ± 11.69^a

Means in the same row with different letters as superscripts are significantly different at the 0.05 confidence level by Student's *t*-test.

N = Total number processed.

4.2.3 Prevalence of *kdr* mutations in *An. gambiae* s.l.

Figure 4.4 shows a typical plot of VIC and FAM fluorescence used for determining the presence or absence of *kdr* mutation in specimens collected. A total of 304 specimens were processed for the East and West African *kdr* mutation made up of 45, 21, 29, from Atatam, Agyenkwaso and Kusa Dinkyae respectively in the FEZ, 43 from Osorongma in the FTEZ, 79 from Adawukwa and 87 from Okyereko both in the CSEZ. The West African *kdr* mutation, Leucine to phenylalanine (L1014F) is very high in all the villages sampled (Table 4.3). For the FEZ, it appears to be fixed in the Kusa Dinkyae population ($R = 1$). The L1014F allele frequency was 0.97 in Atatam and 0.98 in Agyenkwaso. The allele frequencies of the same mutation were similarly high in Osorongma (0.98) in the FTEZ but slightly lower, though not statistically significant ($P > 0.05$) in Adawukwa and Okyereko populations from the CSEZ.



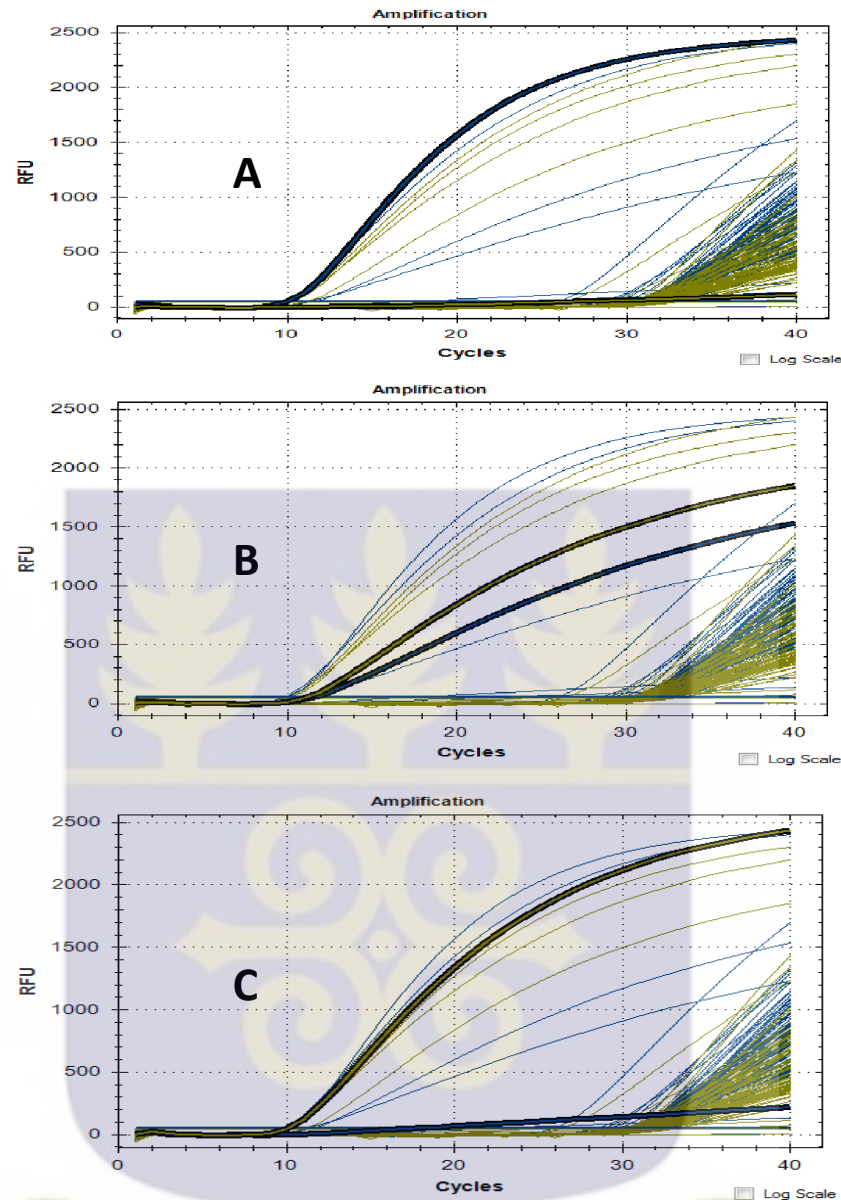


Fig. 4.4: Identification of the *kdr* mutation in *An. gambiae s.l.* using the Taqman real-time PCR assay. A – substantial increase in FAM fluorescence indicating homozygous mutant; B – intermediate increase in both FAM and VIC fluorescence indicating heterozygous mutant; C – substantial increase in VIC fluorescence indicating homozygous wild type.

Contrary to expectations for West Africa, the East African *kdr* mutation, Leucine to Serine (L1014S) was found in few of the samples from two ecological zones, FEZ and CSEZ (Table 4.4). It was not present in populations from Atatam and Kusa Dinkyae in the FEZ or in Osorongma in the FTEZ but was present in Agyenkwaso in

the FEZ, Adawukwa and Okyereko in the CSEZ with allele frequencies of 0.02 in all three locations.

The L1014F mutation was present in both *An. gambiae* s.s. and *An. coluzzii* (Table 4.5). The allele frequencies were slightly lower in *An. coluzzii*, ranging between 0.75 in the Osorongma population to 0.90 in the Agyenkwaso population. In Adawukwa and Okyereko, the allele frequencies were 0.87 and 0.86 respectively. In *An. gambiae* s.s. it was almost fixed in all the populations, being 0.97 in Atatam, 0.99 in Osorongma and 1.00 in Agyenkwaso and Kusa Dinkyae.

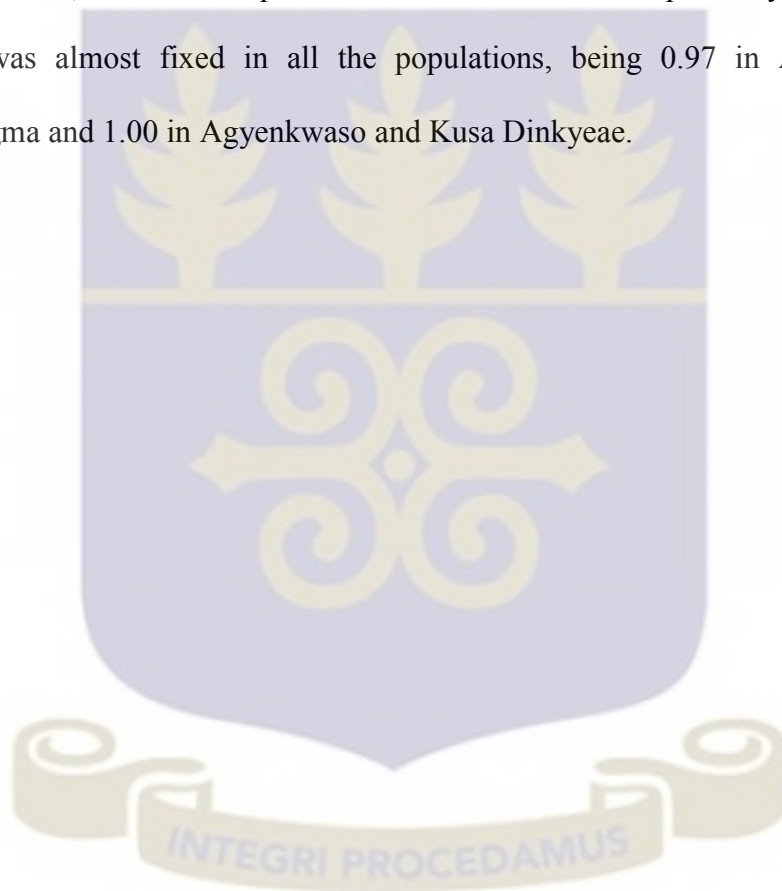


Table 4.3: Allele frequency and distribution of the West African *kdr* mutation in indoor resting *An. gambiae s.l.* populations from six villages in three ecological zones of Ghana.

Village	Genotype Frequencies						Allele Freq.			
	RR		RS		SS		R	S	χ^2	P-value
	Observed	Expected	Observed	Expected	Observed	Expected				
FEZ										
Atatam	42	42.05	3	2.9	0	0.05	0.97 ^a	0.03	0.05	P>0.05
Agyenkwaso	21	21.1	1	0.9	0	0.01	0.98 ^a	0.02	0.01	P>0.05
Kusa Dinkyae	29	29	0	0	0	0	1.00 ^a	0	-	-
FTEZ										
Osorongma	41	41.02	2	1.95	0	0.02	0.98 ^a	0.02	0.02	p>0.05
CSEZ										
Adawukwa	61	59.4	15	18.21	3	1.4	0.87 ^a	0.13	2.45	P>0.05
Okyereko	66	64.78	19	21.45	3	1.78	0.86 ^a	0.14	1.15	P>0.05

Values in the same column with same lower case letter as superscripts are not significantly different at the 0.05 confidence level by one way ANOVA and Tukey's Test.

Table 4.4: Allele frequency and distribution of the East African *kdr* mutation in indoor resting *An. gambiae s.l.* populations from six villages in three ecological zones of Ghana.

Village	Genotype Frequencies						Allele Freq.			
	RR		RS		SS					
	Observed	Expected	Observed	Expected	Observed	Expected	R	S	χ^2	P-value
FEZ										
Atatam	0	0	0	0	45	45	0 ^a	1	-	-
Agyenkwaso	0	0.01	1	0.98	20	20.01	0.02 ^a	0.98	0.01	P>0.05
Kusa Dinkyae	0	0	0	0	29	29	0 ^a	1	-	-
FTEZ										
Osorongma	0	0	0	0	43	43	0 ^a	1	-	-
CSEZ										
Adawukwa	0	0.03	3	2.94	76	76.03	0.02 ^a	0.98	0.03	P>0.05
Okyereko	0	0.05	4	3.91	83	83.05	0.02 ^a	0.98	0.05	P>0.05

Values in the same column with same lower case letter as superscripts are not significantly different at the 0.05 confidence level by one way ANOVA and Tukey's Test.

*D = Dinkyae.

Table 4.5: Allele distribution of the West African *kdr* mutation in the two sibling species of *An. gambiae* s.l. from six villages in three ecological zones of Ghana.

Village	Dominant Species (%)	<i>kdr</i> Allele Frequencies (n)		
		<i>An. coluzzii</i>	<i>An. gambiae</i>	<i>s.s.</i>
FEZ				
Atatam	<i>An. gambiae s.s.</i> (98.04)	-	0.97	(48) ^a
Agyenkwaso	<i>An. gambiae s.s.</i> (76.39)	0.90 (5) ^a	1.00	(17) ^a
Kusa Dinkyee	<i>An. gambiae s.s.</i> (100.00)	-	1.00	(29) ^a
FTEZ				
Osorongma	<i>An. gambiae s.s.</i> (94.00)	0.75 (2) ^a	0.99	(39) ^a
CSEZ				
Adawukwa	<i>An. coluzzii</i> (100.00)	0.87 (79) ^a	-	
Okyereko	<i>An. coluzzii</i> (100.00)	0.86 (88) ^a	-	

- Not enough specimens of that form were recorded from that location for analysis.
Values in the same column with same lower case letter as superscripts are not significantly different at the 0.05 confidence level by one way ANOVA and Tukey's Test.

4.2.4 Vectorial capacity of indoor resting anophelines

Results for blood meal source identification assay are shown in Tables 4.6 and 4.7. The PCR method (Fig. 4.5) could not identify the source of blood meal in some specimens despite physical evidence of blood in the abdomen. A total of 419 specimens (299 *An. gambiae* s.l. and 120 *An. funestus*) were assayed for blood meal source with success rate ranging between 54 % (n = 39) for *An. gambiae* s.l. populations in Osorongma and 100 % for *An. funestus* populations in Osorongma (n = 1) and Okyereko (n = 2). A high level of anthropophagy was recorded for both *An. gambiae* s.l. and *An. funestus* in all the villages sampled. *An. funestus* populations from the six villages sampled were all exclusively anthropophagic (HBI = 1) i.e. no animal feeding was observed for this species. *An. gambiae* s.l. populations were

similarly highly anthropophagic (HBI = 1), however, evidence of animal blood feeding was recorded from some of the villages in all three ecological zones. In FEZ, 3 % (n = 33) and 4 % (n = 25) *An. gambiae* s.l. specimens from Atatam and Kusa Dinkyae respectively showed evidence that they had taken blood meal from an animal. These specimens had also fed on humans explaining the HBI of 1 observed in FEZ. Similar results were observed for *An. gambiae* s.l. from the FTEZ with 38 % (n = 21) having had an animal blood meal in Osorongma. In CSEZ 4 % (n = 56) of *An. gambiae* s.l. assayed had fed on animal blood in Okyereko. All the *An. gambiae* s.l. that fed on animal blood in these ecological zones had also fed on human blood except for one specimen from Okyereko that had exclusively fed on animal blood (cow). HBI for *An. gambiae* s.l. was 1 for all the villages except Okyereko where it was 0.98.



Fig. 4.5: A 2.5 % agarose gel stained with ethidium bromide for visualising species specific DNA fragments for host blood in *An. gambiae* s.l. and *An. funestus*. Lane 1 - 100 base pair molecular weight marker; lane 2 – negative control; lanes 3-7 - positive controls for human, cow, pig, goat and dog blood respectively.

Table 4.6: Blood meal source for *An. funestus* collected from six villages of two ecological zones in Ghana

			Blood Meal Source (%)			
Village	No. Tested	NIS (%)	Human	Animal	Mixed	HBI
FEZ						
Atatam	62	55 (89)	55 (100)	0 (0)	0 (0)	1
Agyenkwaso	35	28 (80)	28 (100)	0 (0)	0 (0)	1
Kusa D*	12	9 (75)	9 (100)	0 (0)	0 (0)	1
FTEZ						
Osorongma	1	1 (100)	1 (100)	0 (0)	0 (0)	1
CSEZ						
Adawukwa	8	6 (75)	6 (100)	0 (0)	0 (0)	1
Okyereko	2	2 (100)	2 (100)	0 (0)	0 (0)	1

NIS: Number identified successfully. Values in parentheses indicate percentages. For values under NIS, they are percentages of number tested and for values under blood meal source; they are percentages of NIS. Percentages under blood meal source may not add up to 100 as some specimen had mixed blood meal

**D = Dinkyeae*



Table 4.7: Blood meal source for *An. gambiae* s.l. from six villages of three ecological zones in Ghana

			Blood Meal Source (%)			
Village	No. Tested	NIS (%)	Human	Animal	Mixed	HBI
FEZ						
Atatam	47	33 (70)	33 (100)	1 (3)	1 (3)	1
Agyenkwaso	22	13 (59)	13 (100)	0 (0)	0 (0)	1
Kusa D*	29	25 (86)	25 (100)	1 (4)	1 (4)	1
FTEZ						
Osorongma	39	21 (54)	21 (100)	8 (38)	8 (38)	1
CSEZ						
Adawukwa	74	52 (70)	52 (100)	0 (0)	0 (0)	1
Okyereko	88	56 (70)	55 (98)	2 (4)	1 (2)	0.98

NIS: Number identified successfully. Values in parentheses indicate percentages. For values under NIS, they are percentages of number tested and for values under blood meal source; they are percentages of NIS. Percentages under blood meal source may not add up to 100 as some specimen had mixed blood meal.

*D = *Dinkyae*.

Plasmodium falciparum infection rate as recorded from ELISA assays (Fig. 4.6) was determined for 430 specimens comprising 306 *An. gambiae* s.l. (131 *An. gambiae* s.s. and 175 *An. coluzzii*) and 123 *An. funestus*, (Table 4.8). There was evidence of infection in all the villages except Okyereko where *P. falciparum* circumsporozoite proteins were not present in any of the 90 specimens processed (2 *An. funestus*, 88 *An. gambiae* s.l.). Sporozoite infection rate was 4.54 % (n = 110) for *An. funestus*, 3.26 % (n = 92) for *An. gambiae* s.s. and 16.67 % (n = 6) for *An. coluzzii* from FEZ. In CSEZ an infection rate of 7.69 % (n = 13) for *An. funestus*, 5.13 %, (n = 39) for *An. gambiae* s.s. and 0.59 % (n = 169) for *An. coluzzii* was recorded. After pooling the data from both ecological zones, infectivity was highest in *An. funestus* (4.88 %, n = 123) followed by *An. gambiae* s.s. (3.80 %, n = 131) and least in *An. coluzzii* (1.14 %, n =

176). It was difficult to make comparisons for sporozoite infection rates between *An. funestus*, *An. gambiae* s.s. and *An. coluzzii* from individual villages or ecological zones as none or too few of some of the species were collected from some villages.

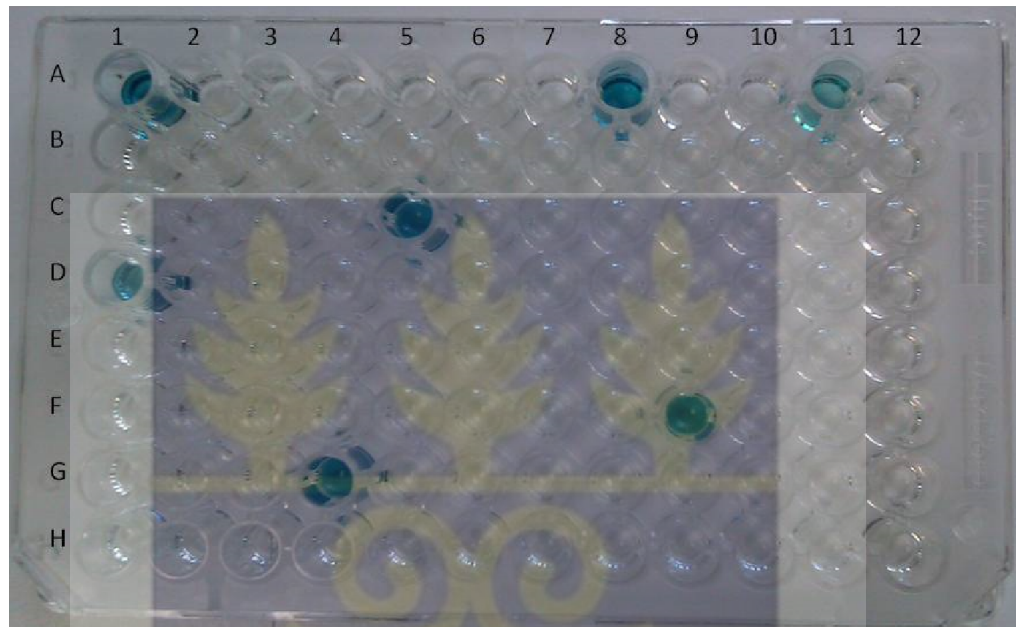


Fig. 4.6: ELISA plate for detection of the presence of *Plasmodium falciparum* circumsporozoite proteins in head and thorax homogenates of mosquito specimens. Well A1 - Positive control; wells A8, A11, C5, D1, F9 and G4 - samples showing positive reaction.



Table 4.8: *Plasmodium falciparum* infection rate of indoor resting anophelines from six villages in three ecological zones of Ghana.

Village	% Infected (n)			
	<i>An. funestus</i>	<i>An. gambiae</i> s.l.	<i>An. coluzzii</i>	<i>An. gambiae</i> s.s.
FEZ				
Atatam	4.76 (63)	2.13 (47)	0 (1)	2.17 (46)
Agyenkwaso	2.86 (35)	13.64 (22)	20.00 (5)	11.76 (17)
Kusa Dinkyae	8.33 (12)	0 (29)	-	0 (29)
Zone Total	4.54 (110)	4.08 (98)	16.67 (6)	3.26 (92)
FTEZ				
Osorongma	0 (1)	4.76 (42)	0 (3)	5.13 (39)
CSEZ				
Adawukwa	10 (10)	1.27 (79)	1.27 (79)	-
Okyereko	0 (2)	0 (88)	0 (87)	-
Zone Total	7.69 (13)	1.44 (209)	0.59 (169)	5.13 (39)
Grand Total	4.88 (123)	2.28 (307)	1.14 (175)	3.8 (131)

4.3 Discussion

4.3.1 Indoor resting mosquito diversity

Anopheles gambiae s.l. and *An. funestus* s.s. were the only anopheline groups collected from the three ecological zones. The indoor resting densities of these species from the FEZ did not differ except in Kusa Dinkyae where the indoor resting density of *An. gambiae* s.l. was higher than that of *An. funestus*. On the contrary, more *An. gambiae* s.l. were collected indoors compared to *An. funestus* in Adawukwa and Okyereko from the CSEZ. Only one *An. funestus* specimen was collected from Osorongma in the FTEZ. Members of the *An. gambiae* complex showed varied distribution across the study area with collections from the FEZ and FTEZ being predominantly *An. gambiae* s.s. with very few *An. coluzzi* except in Kusa Dinkyae where no *An. coluzzii* was collected. *Anopheles gambiae* s.l. from the CSEZ were

however exclusively *An. coluzzii*. These results are similar to those obtained by Hunt *et al.* (2011) and Yawson *et al.* (2004). All collections done by Hunt *et al.* (2011) in Obuasi, a location closest to the three FEZ sampling sites were *An. gambiae* s.s. Similarly, Yawson *et al.* (2004) reported 100 % *An. gambiae* s.s. from Kumasi a locality in the FEZ, and 98 % from Osorongma in the FTEZ. Results from Okyereko and Adawukwa (100 % *An. coluzzii* from each site) also conform to those of Yawson *et al.* (2004). The relative abundance of these two species, which until recently were considered molecular forms of the same species (*An. gambiae* s.s.) has been associated with breeding site characteristics. *An. coluzzii* tends to be associated with flooded or irrigated sites that provide permanent breeding conditions, whereas *An. gambiae* s.s. is associated with rain-dependent temporary sites (Diabate *et al.*, 2003).

The species composition in Osorongma, a locality in the FTEZ, was similar to those from FEZ. Appawu *et al.* (2001) describes the vegetation around Dodowa, of which Osorongma is a suburb as secondary forest with little of the virgin forest left as a result of deforestation. Considering Osorongma as a transition forest zone, then it is likely that the relative abundance of *An. gambiae* s.s. and *An. coluzzii* is influenced by ecological zones. Yawson *et al.* (2007) concluded that ecological barriers appear to be of greater importance in restricting gene flow between the two species.

The results obtained in the current study tally with several reports across Africa, where the three species occur in sympatry over much of their range (Sinka *et al.*, 2010 and Sinka *et al.*, 2012). Prevalence of anophelines in any locality is influenced by ecological factors such as availability of preferred hosts and breeding sites (Minakawa, 2002; Kipyab *et al.*, 2013). In this study, mosquitoes were collected using

indoor collection techniques; therefore availability of host and resting behaviour were the main expected determinants of occurrence. Since all the species collected in the study are highly anthropophilic, endophagic and endophilic (Sinka *et al.*, 2010), it was expected that they would be equally represented in the collections. However, this was not the case. The differences in the distribution observed could be attributed to the availability of breeding sites around the villages where mosquitoes were sampled.

Presence of breeding sites suitable for proliferation of *An. gambiae s.s.* and *An. funestus* i.e. availability of temporary water puddles during the rainy season due to poorly permeable clay soils and streams around the villages respectively in Atatam and Agyenkwaso could be the underlying factors for the presence of both species in equal densities in the two villages. On the contrary, there were no permanent water bodies in Kusa Dinkyae, thus, explaining the predominance of *An. gambiae s.s.* in this village. The breeding sites available around villages in the CSEZ are different from those in the FEZ. Okyereko is an irrigated rice cultivating community and Adawukwa has a vast swamp and a stream close by. The rice fields situated close to these two villages are ideal breeding sites for *An. coluzzii* and to a lesser extent *An. funestus* while the edges of the stream and swamps are ideal sites for *An. funestus* breeding. This probably explains the presence of both species in Okyereko and Adawukwa. The highest density of *An. funestus* was recorded in Adawukwa which is located closer to a swamp ideal for *An. funestus* breeding.

Our findings agree with those of Charlwood and Edoh (1996) who reported that anopheline adult density is negatively correlated with the distance between larval habitats and houses. The complete absence of *An. funestus* in Osorongma could be

explained by absence of suitable breeding sites, as there are no permanent water bodies suitable for *An. funestus* breeding near this village.

The densities reported here might not represent the actual annual anophelines distribution in the study areas. Sampling was only limited to two season; minor rainy season (September to November) and the dry season (December to January) of 2011 and 2012. Species composition and abundance varies with time of year (Hunt *et al.*, 2011). Yawson *et al.* (2004) collected 58 *An. funestus* out of 341 specimen from 10 collections in Osorongma during the major rainy season (June to September), whereas in the present study, only one specimen of *An. funestus* was collected from the same village during the dry season. In this study, more *An. gambiae* was collected during the minor rainy season than the dry season and vice versa for *An. funestus* (data not shown).

4.3.2 Prevalence of the *kdr* mutation

The results from this study shows high variability in the prevalence of the L1014F and L1014S mutations in the three ecological zones. The near fixation of the L1014F mutation in the populations from the areas sampled is understandable. This mutation is widespread and predominant in the West African sub-region (Santolamazza *et al.*, 2008). High frequencies of the L1014F mutation has been reported from Accra (Santolamazza *et al.*, 2008), Abia, Dodowa, Osorongma, Odumase, Ayikuma, Ayenya and Mampong all in the FTEZ of Ghana, and from Okyereko in the CSEZ, (Yawson *et al.*, 2004). It has also been reported from Kumasi (Yawson *et al.*, 2004), Obuasi (Coetzee *et al.*, 2006; Hunt *et al.*, 2011), Ahafo, Akyem and Tarkwa (Hunt *et al.*, 2011) in the FEZ of Ghana. Results from this study show high frequencies of the

same mutation in populations from all the three ecological zones. The only difference between results from the current study and those of previous work is that the L1014F mutation has been predominantly reported in *Anopheles gambiae* s.s. relative to *An. coluzzii*. Results from the current study shows that the mutation is prevalent in both *An. coluzzii* (90 %) and *An. gambiae* s.s. (98 %). The frequency of the mutation in this study is far higher than those reported by Yawson *et al.* (2004) in *An. coluzzii* from Abia (1 %), Okyereko (3%) and Mampong (1 %) all in Southern Ghana and Bonia (6 %) in northern Ghana. This is not the first time that the L1014F mutation has been found at high frequency in *An. coluzzii*. Djegbe *et al.* (2011) reported a near fixation of the allele in this species from Cotonou and a significant temporal increase over a two-year period at Bohicon and Malanville in Benin. Santolamazza *et al.* (2008) concluded that the distribution of the *kdr* mutation is non-uniform on the African continent and that its origin and spread in *An. gambiae* s.l. is an ongoing process.

The L1014S mutation was originally recorded from Kenya (Ranson *et al.*, 2000; Stump *et al.*, 2004) and was subsequently found in Uganda (Verhaeghen *et al.*, 2006), Gabon (Pinto *et al.*, 2006), Cameroon (Etang *et al.*, 2006), and Equatorial Guinea (Moreno *et al.*, 2008). More recently, it has been reported in Congo (Koekemoer *et al.*, 2011) and for the first time in Benin (Djegbe *et al.*, 2011) and Burkina Faso (Namountougou *et al.*, 2013). In the current study, the mutation was recorded in both *An. gambiae* s.s. and *An. coluzzii* though at very low frequencies. The fact that this is the first time the mutation has been reported in Ghana may not mean it is new but could be part of an on-going East-West spread of the mutation as suggested by Santolamazza *et al.* (2008).

The spread of the *kdr* mutation on the African continent has been attributed to introgression (Weill *et al.*, 2000) and to some extent migration (Djebge *et al.*, 2011) driven mainly by widespread use of insecticides in agriculture and possibly for domestic purposes (Pinto *et al.*, 2007). Insecticide usage is widespread in the three ecological zones where this study was carried out (Fianko *et al.*, 2011). In the FEZ, cocoa farming is the major agricultural activity, in which it has been or still reliant on usage of insecticides of all classes including organochlorines for capsid (*Distantiella theobroma* and *Sahlbergella singularis*) control (Fianko *et al.*, 2011). Irrigation rice production around Okyereko and Adawukwa in the CSEZ and mango plantations of the eastern mango enclave of Ghana, around Osorongma rely heavily on insecticide usage. Akogbetor *et al.* (2005) demonstrated that insecticide-resistant *An. gambiae* populations are able to survive and proliferate in insecticide contaminated soils and water from vegetable and cotton farms. Insecticide residues have been detected in water and soil samples from different parts of Ghana (Fianko *et al.*, 2011), suggesting the presence of heavy selection pressure due to insecticides on natural populations of anophelines from both ecological zones. Thus whether the mutation gets into the population by independent mutation events, introgression or by migration, it will easily be sustained and spread in the population.

4.3.3 Vectorial capacity

The success rate of the PCR method in identifying blood meal source was generally high (75 to 100 % for *An. funestus* and 54 to 86 % for *An. gambiae* s.l.). The PCR method used in this study did not include primers for all the possible hosts found in the villages where mosquitoes were sampled such as chicken, sheep, bats and house mice. It could be that specimens for which blood meal source could not be determined

had fed on animals whose primers were not included. It is important to take note of all possible hosts within a locality where samples are being collected and include primers specific for all hosts as much as possible in the PCR reaction. Alternatively, the method of Molaei *et al.* (2006) could be used. This involves screening of mosquito blood meals with avian- and mammalian-specific primer pairs followed by sequencing. Analysis of the sequences can be used to determine the actual species each mosquito specimen fed on.

Anopheles gambiae s.l. and *An. funestus* from the two ecological zones show high levels of anthropophagy with *An. funestus* being exclusively anthropophagic. This is consistent with the feeding behaviour of both species. According to Sinka *et al.* (2010), *An. funestus* shows fairly consistent behaviour (generally anthropophilic and endophilic) throughout its range whereas *An. gambiae* s.l. appears to exhibit greater phenotypic plasticity and opportunism in blood feeding than commonly thought. In the present study, *An. gambiae* s.l. demonstrated some level of animal feeding, but all except a single specimen that fed on animals also fed on humans. This could imply that, in the absence of humans, they will readily feed on animals but given the chance will supplement with human blood.

As vectors of disease, anophelines will only transmit if the parasite is present in the population. With the high HBI recorded for both species in the two ecological zones, the sporozoite rate should reflect the prevalence of the parasite within the populations. This is evident in the variable sporozoite rates recorded for the three species. Thus the sporozoite rate only reflects the prevalence of parasite in the population and not the vectorial capacity of the vectors. The sporozoite rates recorded

in the current study fall within the range of those recorded by other workers (Appawu *et al.*, 2001; Coetzee *et al.*, 2006; Owusu-Agyei *et al.*, 2009; Hunt *et al.*, 2011, Badu *et al.*, 2013) in Ghana, confirming these areas as endemic to malaria. However, a caveat on this data is that the ELISA method is known to often give false positive results, especially for *P. falciparum* and for zoophilic species (Durnez *et al.*, 2011). The highest sporozoite rate of 20 % in this study was recorded from Agyenkwaso where some amount of zoophily was also recorded. Furthermore, the small sample size could also affect the sporozoite rate as all the sites with high rates had small sample sizes. Further work is therefore required before any conclusion can be drawn on sporozoite rates in the study areas.

4.4 Conclusions

Anopheles gambiae s.s., *An. coluzzii* and *An. funestus* are the major indoor resting malaria vectors in all the three ecological zones, where this study was carried out. The three species however do not appear to be sympatric throughout these ecological zones with *An. gambiae* s.s. present in the FEZ and FTEZ, *An. coluzzii* present predominantly in the CSEZ and *An. funestus* present mainly in the FEZ and the CSEZ. Thus, any research and development activity that focuses on developing control tools targeting these major malaria vectors may have to be carried out across all three ecological zones to be relevant for area-wide implementation. Similarly, to carry out any control program across these ecological zones, the tools must target all three species.

The distribution of *An. gambiae* s.s. and *An. coluzzii* appear to be influenced by ecological factors. *An. gambiae* s.s. is more prevalent in the FEZ and the FTEZ whiles

the *An. coluzzii* is more prevalent in the CSEZ. This distribution is highly influenced by breeding site availability.

The L1014F *kdr* mutation is present at very high frequencies in all three ecological zones with its spread in the population reaching a point of fixation in both *An. gambiae* s.s. and *An. coluzzii*. This implies that control programs must include resistance management strategies and incorporate novel tools that are not affected by the *kdr* mutation. An important finding in this study is the first report of the L1014S mutation in Ghana, this calls for extensive resistance monitoring in the country.

All the endophilic populations of *An. funestus* are exclusively anthropophagic throughout all the three ecological zones. *An. gambiae* s.s., though, highly anthropophagic, also exhibits some phenotypic plasticity in host preference. This presents the risk of the spread of zoonotic diseases in the human populations. The level of zoophagy is however too low for the consideration of zooprophyllaxes for malaria control.

The main aim of this study is to understand the indoor resting behaviour of anophelines and exploit it for their control. Since all the populations in the FEZ are similar in terms of species diversity and the *kdr* mutation as well as in their anthropophagy, they can be pooled together and treated as one population. The same applies to the populations from the CSEZ. The population from the FTEZ is similar to the FEZ populations except in the absence of *An. funestus*. Thus for *An. gambiae*, the FTEZ population can be pooled together with the FEZ populations. In subsequent chapters therefore, the populations will be pooled into FEZ made up of collections

from Atatam, Agyenkwaso, Kusa Dinkyae and Osorongma and CSEZ made up of Okyereko and Adawukwa.



CHAPTER FIVE

INDOOR RESTING SITE PREFERENCES OF ANOPHELINE MOSQUITOES

5.1 Background

Malaria control has seen great success in the last decade, resulting in a reduction of the disease burden by as much as 33 % in the hardest-hit region, sub-Saharan Africa (WHO, 2012a). This has been attributed to the key intervention tools among them effective case management using drugs, preventive treatment of pregnant women, ITNs and IRS (Slutsker and Kachur, 2013). This progress appears to have slowed down mainly due to wide-spread development of insecticide resistance in the mosquito vector to majority of insecticides used for ITNs and IRS (WHO, 2012b).

Continued success in the fight against malaria requires persistence, cunning and most of all knowledge (Slutsker and Kachur, 2013). It is important to preserve and improve utility of the available insecticide-based malaria vector control interventions through critical insecticide resistance management (IRM) and also development of novel tools to supplement these interventions.

A number of novel malaria vector control strategies including; insecticide-treated plastic sheeting (ITPS) [Djenotin *et al.*, 2009] and entomopathogenic fungi (Scholte *et al.*, 2004) are under development. For effective incorporation of these strategies into current vector control methods, it is important to understand the bionomics of the populations targeted. The success of IRS was mainly based on the understanding the resting behaviour of malaria vectors (De Meillon, 1934). In his study, De Meillon demonstrated that the malaria vector *An. funestus* entered houses to obtain blood

meals and remained indoors until oviposition. Based on this knowledge, he was able to control this vector by spraying pyrethrum inside houses and this laid the foundation for IRS (De Meillon, 1936). Recently, Mnyone *et al.* (2012) were able to infect wild *An. gambiae* s.l. with fungal biocontrol agents by exploiting the behaviour of adults entering houses. In their experiments, the authors achieved 69 % and 76 % infection of *An. gambiae* s.l. with *Metarhizium anisopliae* treated eave baffles (targeting house entering adults) and bed net strips (targeting host seeking adults) respectively. This demonstrates the importance of understanding behaviour of vector species and its role in effective implementation of control strategies.

The behaviour depicted by malaria vector species appears to vary with species and location. For example, in Columbia, *An. darlingi* and *An. marajoara* were found to have a preference of resting close to the ground whereas *An. oswaldoi* and *An. rangeli* rested higher up (Quinones and Suarez, 1990). On the other hand, in Brazil, *An. darlingi* were collected mostly resting on the ceiling (Roberts *et al.*, 1987). This clearly shows heterogeneity in mosquito population behaviour depending on ecological setting and therefore highlights the importance of understanding the indoor resting behaviour of any vector populations in a given setting before implementation of any novel or conventional malaria vector control tools. To date, there appears to be limited information on the resting behaviour of African malaria vectors especially as it regards where they exactly rest inside houses. This part of the study, therefore, focused on the understanding of indoor resting behaviour of anopheline vectors and factors that influence their choice of resting sites inside houses.

5.3 Results

5.3.1 Indoor resting site preferences

Anopheline mosquitoes belonging to three species were collected from 33 different indoor resting sites across the three ecological zones sampled (Table 5.1). Of these resting sites, 8 were considered important based on a cut-off point of 5 % of at least one species resting on a site. For *An. gambiae* s.s., the preferred resting site in order of prevalence was, roofing beam (28.24 %), roof (19.08 %), clothing (12.21 %), ceiling (8.40 %), wall (8.40 %), mosquito net (8.40 %) and table (5.34 %). The most preferred resting site for *An. coluzzii* was the window (20.11 %) where most specimens were collected trapped in the netting or resting on the frames. Other preferred resting sites for *An. coluzzii* were, roof (18.39 %), mosquito net (11.49 %), wall (9.20 %), table (7.47 %) and roofing beam (6.32 %). For *An. funestus*, the highest proportions were found resting on the roof (24.77 %), wall (22.31 %), roofing beam (18.46 %), ceiling (14.62 %) and table (5.38 %). Chi square analysis shows that the preference for resting site was not species dependent ($\chi^2 = 57.61$, df = 64, P = 0.70).

For mobility of resting site (Fig. 5.1), all three species collected across the two ecological zones preferred to rest on immovable sites ($\chi^2 = 9.12$, df = 2, P = 0.01). The proportions of specimens collected on immovable resting sites were 63.64 %, 63.92 % and 80.79 % for *An. coluzzii*, *An. gambiae* s.s. and *An. funestus* respectively.

Table 5.1: Frequency of anopheline mosquitoes collected from common indoor resting sites across three ecological zones in Ghana.

Resting Site	% Collection per Site		
	<i>An. gambiae</i> s.s.	<i>An. coluzzii</i>	<i>An. funestus</i>
Roof	19.08	18.39	25.77
Roofing beam	28.24	6.32	18.46
Ceiling	8.40	0.57	14.62
Eve	0.00	1.15	0.00
Wall	8.40	9.20	22.31
Door	0.00	0.00	0.38
Window/frame/net	0.76	20.11	0.77
Aluminium sheet	0.00	1.72	0.00
Bed	0.00	0.00	1.15
Shelf	0.76	0.57	0.00
Table	5.34	7.47	5.38
Chair	0.00	0.57	0.38
Cobweb	2.29	0.57	0.77
Cloth/Shoe hanger	0.00	0.57	0.00
Cupboard	0.00	0.00	0.00
Curtain	0.00	1.72	0.38
Mosquito net	8.40	11.49	4.23
Electric cable	0.00	0.00	0.00
Rope	3.05	1.72	0.00
Clothing	12.21	4.02	3.46
Suitcase	3.05	2.30	1.15
Mat	0.00	0.57	0.00
Shoe	0.00	2.30	0.38
Domestic plastic ware	0.00	1.72	0.00
Umbrella	0.00	0.00	0.00
Sac	0.00	0.00	0.00
Loudspeaker	0.00	0.57	0.00
Picture frame	0.00	0.00	0.00
Polythene sheet	0.00	0.57	0.00
Cardboard box	0.00	1.72	0.00
Orange	0.00	1.15	0.00
Belt	0.00	0.57	0.38
Table cover	0.00	2.30	0.00

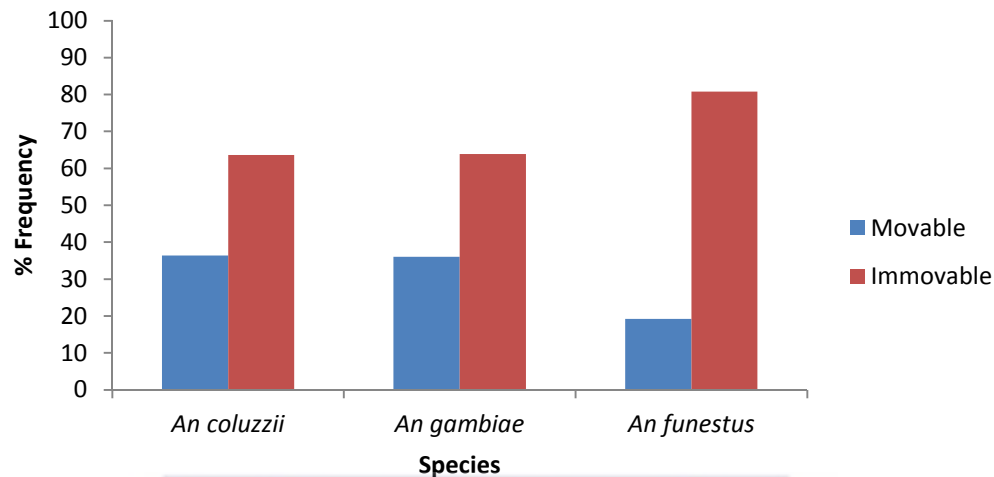


Fig. 5.1: Frequency of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* collected from resting sites located on moveable or immovable materials in village rooms.

5.3.2 Resting material and background colour preferences

The 33 resting sites across the two ecological zones were made up of 26 different materials (Table 5.2). Out of these eight were regarded as important based on the 5 % cut-off i.e. at least 5 % of each species resting on such material. Materials can be categorised into roof and ceiling materials (i.e. brick tiles, aluminium sheets, and straw, wood, bamboo and palm fronds), wall material (i.e. clay and cement) and fabrics (i.e. cotton, polyester and nylon). *An. gambiae* s.s. were found resting in order of preference on, wood (29.91 %), polyester (11.21 %), aluminium sheets (10.28 %), cotton (10.28 %) bamboo (7.48 %), clay (7.48 %) and palm frond (5.61 %). The most preferred resting material for *An. coluzzii* was nylon (18.29 %), wood (14.86 %), brick tile (13.14 %), polyester (10.86 %), cement (9.14 %), cotton (7.43 %) and straw (5.71 %). Wood (30.47 %) was the most preferred resting material for *An. funestus* followed by bamboo (19.74 %), clay (18.03 %), palm frond (6.87 %) and aluminium sheet (5.15 %). No *An. gambiae* s.s. specimens were collected on straw and brick tiles and

no *An. coluzzii* was collected on bamboo and clay. Preference for resting material was species dependent ($\chi^2 = 74.94$, df = 54, P = 0.03).

Table 5.2: Frequency of anopheline mosquitoes collected from common indoor resting materials across three ecological zones in Ghana.

Material	% Resting on Material		
	<i>An. gambiae</i> s.s.	<i>An. coluzzii</i>	<i>An. funestus</i>
Brick tile	0.00	13.14	0.43
Aluminium sheet	10.28	2.29	5.15
Straw	0.00	5.71	1.29
Wood	29.91	14.86	30.47
Bamboo	7.48	0.00	19.74
Palm frond	5.61	0.57	6.87
Coconut frond	3.74	1.14	0.86
Cardboard	0.00	2.29	0.00
Cement	4.67	9.14	2.58
Clay	7.48	0.00	18.03
Glass	0.00	0.00	0.00
Metal	0.00	0.57	0.00
Cotton	10.28	7.43	3.87
Polyester	11.21	10.86	4.72
Nylon	1.87	18.29	2.58
Linen	0.00	0.57	0.43
Satin	0.94	0.57	0.00
Velvet	1.87	0.57	0.00
Silk	0.00	0.00	0.00
Jute	0.00	0.00	0.00
Polythene sheet	0.93	1.14	0.00
Cobweb	2.80	0.57	2.58
Plastic	0.00	2.86	0.00
Leather	0.93	4.00	0.00
Orange	0.00	1.14	0.00
PVC	0.00	2.29	0.00

With regards to background colour of resting surface (Fig. 5.2), there was no variation in the preference recorded for all the three anopheline species collected across the two ecological zones ($\chi^2 = 41.80$, df = 30, P = 0.74). All species preferred resting on normal coloured (original colour of material without any artificial painting or

colouring) background, with 77.43 % of *An. funestus*, 58.02 % of *An. gambiae* s.s. and 33.72 % of *An. coluzzii* collected resting on such background. For *An. coluzzii*, other resting background colours which were significant in proportions were blue (14.69 %) white (12.43 %), yellow (10.19 %), green (9.04 %), black (5.65 %) and brown (5.08 %). For *An. gambiae* s.s. smoke-tanned (12.21 %), green (9.92 %) and black (5.34 %) were the other preferred background colours. whereas for *An. funestus*, white was the only other preferred resting background colour accounting for 8.41 %.

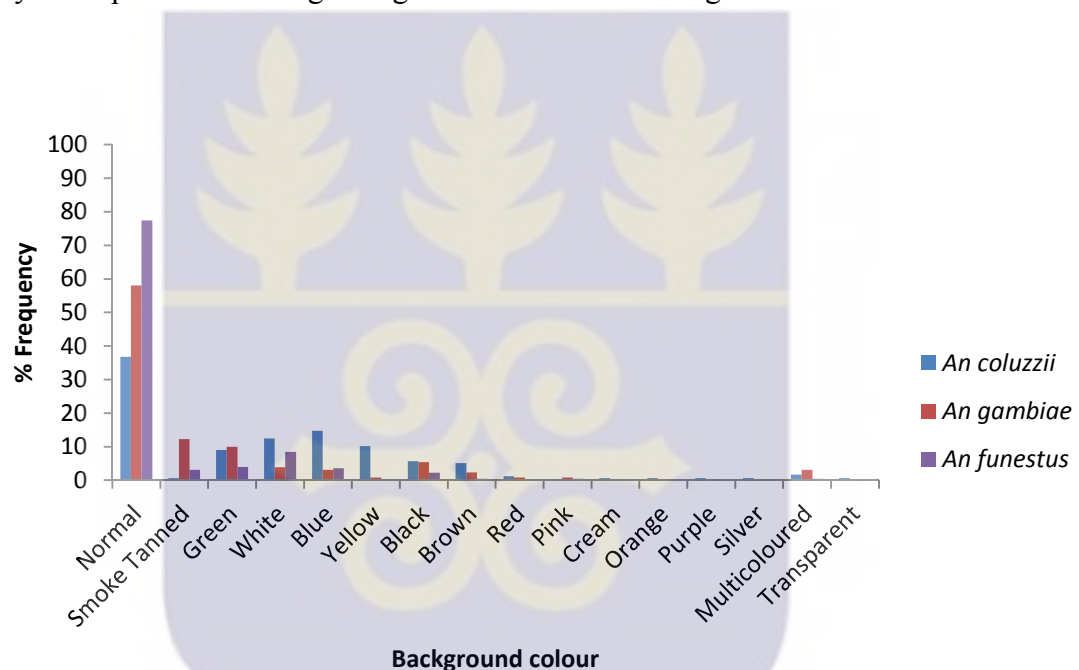


Fig. 5.2: Background colour of indoor resting sites of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* across three ecological zones of southern Ghana.

5.3.3 Three dimensional location of indoor resting sites

Results for preference of resting with reference to the main entrance of rooms are presented in Fig. 5.3. The preference of the three species for resting on front, back, left or right wall was statistically significant ($\chi^2 = 18.81$, $df = 6$, $P = 0.01$). More *An. coluzzii* were collected on the back wall (46.48 %) compared to the front (24.65 %), left (19.01 %) and right (9.86 %) walls. The front wall (37.01 %) was the most

preferred resting location for *An. gambiae* s.s. followed by back (24.42 %), right wall (22.05 %) and left wall (16.54 %). Similar proportions of *An. funestus* were recorded on front, back and left (26.09 %, 28.50 % and 28.02 % respectively) with the right wall recording the least (18.39 %).

Across ecological zones more specimens were collected higher up the roof than closer to the floor (Fig 5.4). Statistical analysis showed a positive correlation between height from the floor and number of specimens collected for *An. coluzzii* ($r = 0.95$, $P = 0.01$) and *An. gambiae* s.s. ($r = 0.97$, $P = 0.01$). Though there was a similar positive correlation for *An. funestus*, this was not statistically significant ($r = 0.78$, $P = 0.12$). Large proportion of *An. gambiae* s.s. (75.94 %), *An. funestus* (73.60 %) and *An. coluzzii* (58.19 %) were found resting at heights above 200 cm from the floor. On the contrary, using the closest wall as a reference point, the further one moved from the walls, the less were the chances of collecting mosquitoes (Fig 5.5). Statistical analysis showed a negative correlation between mosquito frequencies and distances from the walls for all the three species *An. coluzzii* ($r = -0.67$, $P = 0.004$), *An. gambiae* s.s. ($r = -0.85$, $P = 0.000$) and *An. funestus* ($r = -0.78$, $P = 0.000$). The highest proportions of *An. gambiae* s.s. (68.38 %), *An. coluzzii* (80.98 %) and *An. funestus* (68.29 %) were collected within 50 cm from the closest wall.

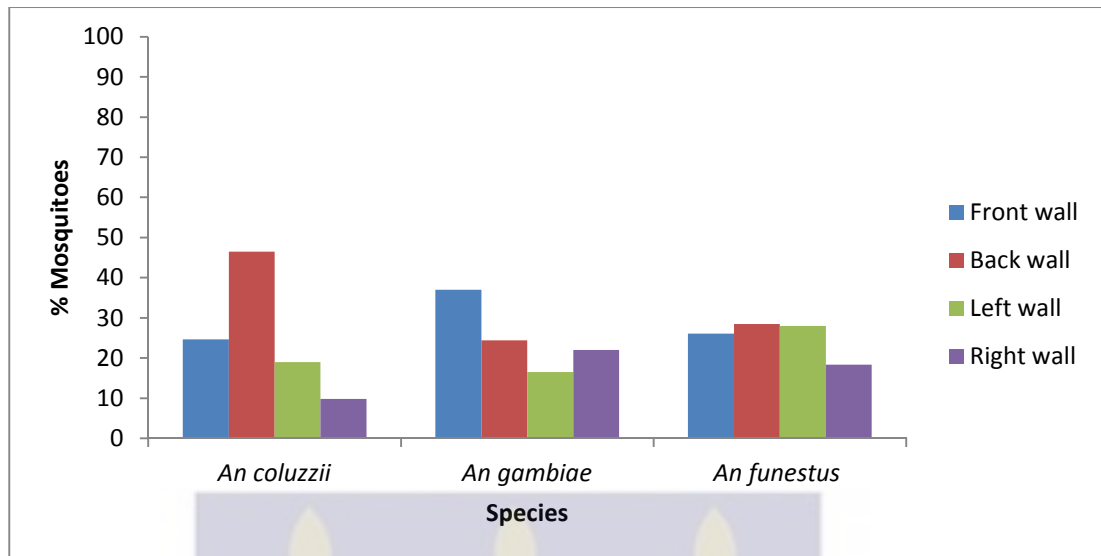


Fig. 5.3: Frequency of anopheline mosquitoes resting on all four walls in a room with reference to the main entrance.

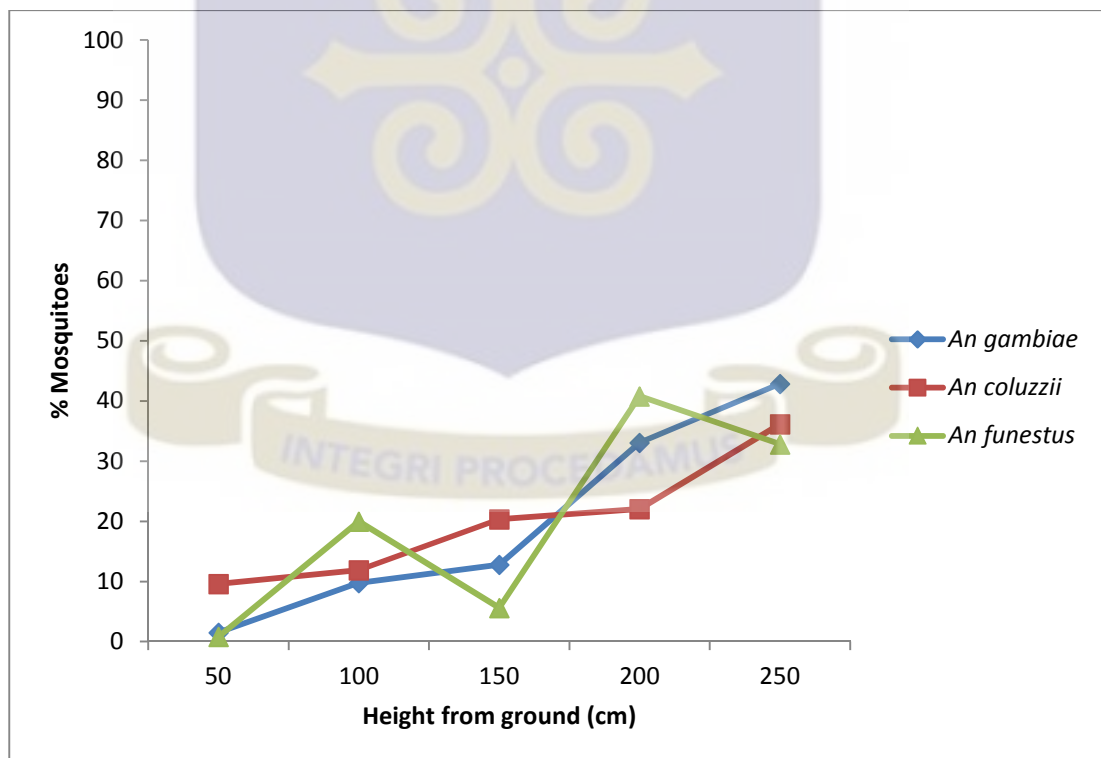


Fig. 5.4: Distribution of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* resting at different heights in village rooms across three ecological zones of Ghana.

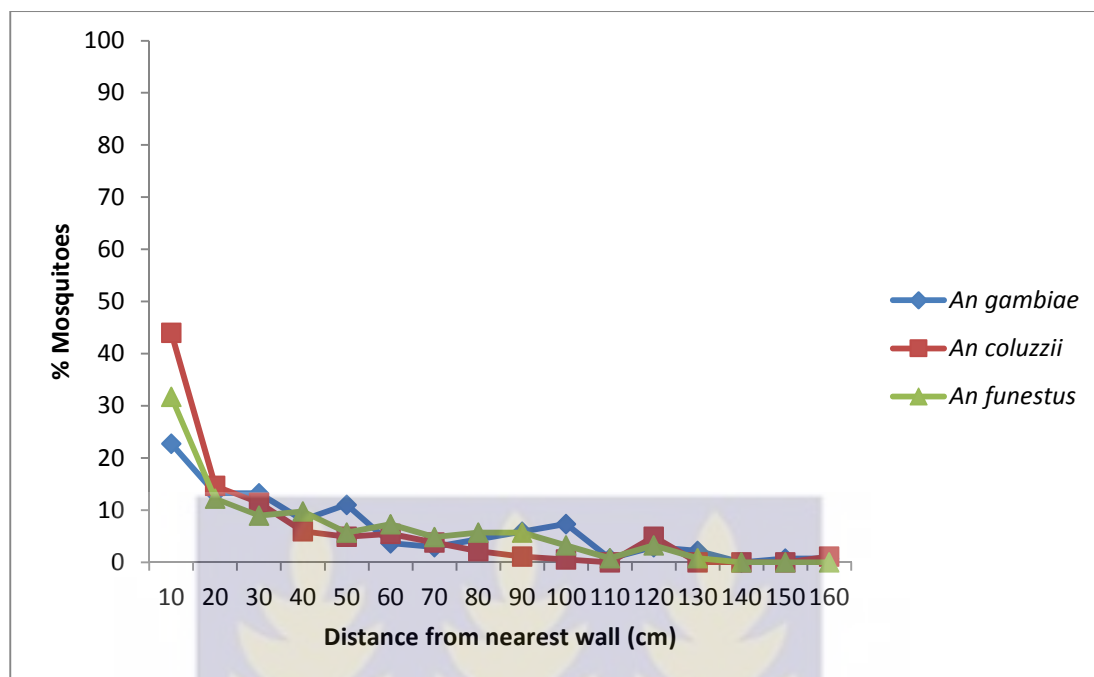


Fig. 5.5: Distribution of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* resting at different distances from nearest wall in village rooms across three ecological zones of Ghana.

5.4 Discussion

The three major malaria vectors showed varying indoor resting behaviour in terms of resting site preference, preference of resting material, background colour of resting material, distance from the nearest wall and height from ground across the two ecological zones. The preferred indoor resting sites appeared to differ between species and locations. In Brazil, Roberts *et al.* (1987) collected 59 %, 37 % and 4 % of *An. darlingi* on the ceiling; walls and floor respectively and in Guatemala, the greatest number (53 %) of *An. albimanus* were found resting on the under-surface of roofs (Ogata *et al.*, 1992). Nagpal *et al.* (2012) working on *An. stephensi* in India reported that this species preferred resting on hanging objects and household objects as a behavioural adaptation to avoid insecticide treated surfaces. In Tanzania, *An. gambiae* was found to prefer resting on the under-surface of thatched roofs, on mud walls and

on household objects in village huts (Smith, 1955, 1962a, b). Mathis *et al.* (1963) collected 94.6 % of *An. gambiae* and *An. funestus* on ceilings in a village room in Burkina Faso. Data from the present study agrees with what was reported for these vector species in Tanzania and Burkina Faso. In this study, *An. gambiae* s.s., *An. coluzzii* and *An. funestus* were found to prefer resting on the under-surface of roofs, roofing beams, ceilings, walls, windows, tables, mosquito nets and clothing. The distribution of indoor resting adults observed may be affected by factors such as type of house, building materials used, availability of alternative resting places, such as clothing and furniture as well as the presence of irritants such as insecticides on the walls (Nagpal *et al.*, 2012), smoke from fire (Service, 1976) and increasing temperature of the resting surface as the sun heats walls or roofs (Smith *et al.*, 1966).

The highly preferred resting sites for the three indoor resting vector species were made of different materials. The roofs were made either of brick tiles, aluminium sheets, straw or bamboo, resulting in higher frequencies of indoor resting mosquitoes of all three species on these materials. Wood had especially highest frequency of all three vector species because the roofing beams and ceilings as well as the other preferred resting sites such as tables, and window frames are usually made of wood. Other important resting sites for the three vector species were walls, tables and mosquito nets. The walls were usually made of cement or clay. No *An. coluzzii* was collected on clay walls, this does not mean that they prefer to rest on cement walls, rather, it was the only species collected from the coastal savannah zone, where the villages sampled, being peri-urban villages where almost all the houses were made of cement. Similarly, no *An. coluzzii* was collected on bamboo because this material is not commonly used in the peri-urban village buildings. *Anopheles gambiae* s.s. was

not collected from brick tile and straw since these materials are used in buildings only from the peri-urban villages of the coastal savannah ecological zone where no *An gambiae* s.s. was collected. This implies that though these vector species show some preference for resting on certain materials, this preference is influenced by the availability of those materials in the indoor environment. Further, some materials that appear to be preferred resting materials have consequential high numbers rather than preferential high numbers. For example, high numbers of *An. stephensi* were found resting on household objects as a result of avoiding insecticides on sprayed walls and ceilings (Nagpal *et al.*, 2012). The high numbers of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* found resting on ITNs was as a result of improper fixing or holes in the nets, causing mosquitoes that enter for a blood meal to become trapped. The choice of resting material may thus be responsible for the hopping behaviour (Nagpal *et al.*, 2012) exhibited by blood-fed females as they must land on the material first to be able to determine its suitability. If the material is not suitable they may fly off to another site till they find a suitable material.

Though the data shows preference for resting sites and for different materials, it is not clear if the choice of resting sites is influenced by extrinsic factors such as the type or colour of the material or by intrinsic factors such as species and/or the physiological state of the individual mosquito. In the present study, the three major anopheline vectors appear to show some variations in their resting behaviour. However, the study did not consider the physiological state of specimens collected, which is required before any conclusion can be drawn on the intrinsic factors influencing the choice of resting site. By general observation, mosquitoes prefer to rest on darker coloured materials. Data from this study show that higher proportions of all the three vector

species were collected on background colours classified as normal. These normal colours are usually woody brown colours of wood, bamboo, palm or coconut frond and straw used mainly for the roofing of village houses. This again raises the question of which extrinsic factors are more important in determining where endophilic anopheline vectors rest. The darker colours viz, normal, smoke-tanned, black and brown, together have more of all the three species resting on them than the brighter colours (white, green yellow and blue) put together. Background colour may therefore be one of the extrinsic factors influencing the choice of resting site.

All three vector species appear to show some trends in the three dimensional location of their resting sites. *An. gambiae* s.s. and *An. coluzzii* prefer to rest on or close to the front and back walls respectively whiles *An. funestus* appears to rest randomly on any wall. The choice of which wall these vector species rest on could be dependent on how much interference they experience from indoor human activity. Thus depending on the position of the bed, other furniture and materials in the rooms, some areas will have less human interference than others and logically, mosquitoes will prefer to rest on areas with less interference. This is supported by the fact that greater proportions of all the three vector species were collected on immovable resting sites. Furthermore, significant proportions of all the vector species were resting at heights from 200 cm upwards. Thus, they preferentially rest above the height of an average human being, supporting the idea that they will rather rest where they will experience less human interference. Other workers have reported higher proportions of *An. gambiae* s.s. (Gibbins, 1933; Haddow, 1942) *An. funestus* (Garnham, 1929; Haddow, 1942) and *An. culicifacies* (Bhatia *et al.*, 1957) resting higher up in village rooms, on the roof, ceiling and upper walls. This implies that targeting those areas for deployment of

vector control agents such as insecticides and entomopathogenic fungi will yield good results. Djenontin *et al.* (2009) achieved 80.3 % mortality in recaptured *An. gambiae* released in experimental huts treated with ITN combined with woven polypropylene treated with bendiocarb on the upper one-third of walls and 100 % for similar treatment but covering all the walls with the bendiocarb-treated woven polypropylene. Similar results were obtained by Corbel *et al.* (2012). By suspending 3 m² *M. anisopliae* impregnated black cotton sheets (“targets”) on ceilings in traditional houses in a rural village in Tanzania, Scholte *et al.* (2005) were able to achieve 23 % infection of *An. gambiae* and 75 % reduction in malaria transmission. Extending such treatment to cover the whole ceiling and upper one-third of the walls is likely to increase fungal infection rate and further reduction in transmission.

5.5 Conclusions

The preferred indoor resting sites of anopheline vectors appear to differ between species and locations. The populations of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* in the current study appear to follow similar trends across southern Ghana and agrees with those reported for these species in other parts of Africa. All the species preferred to rest on darker coloured wooden and wall materials as well as clothing materials. Based on these data, it should be possible to use darker wooden or cloth materials to construct point source dissemination devices for entomopathogenic fungi or even other synthetic insecticides. These devices could be placed higher up and closer to walls in village rooms to target anopheline vectors of disease in Ghana.

Since the behaviour of organisms is influenced by both intrinsic and extrinsic factors and the current study focused on extrinsic factors, it is recommended that future studies take cognisance of intrinsic factors such as age and physiological status.



CHAPTER SIX

EFFECT OF MICROCLIMATIC CONDITIONS ON THE CHOICE OF RESTING SITES BY ENDOPHILIC ANOPHELINE MOSQUITOES

6.1 Background

The physiology of insects is highly influenced by the surrounding environment (Bayoh and Lindsay, 2004; Gray and Bradley, 2005). Various studies have shown that variations between indoor and outdoor climate (Afrane *et al.*, 2006; Paaijmans and Thomas, 2011; Cator *et al.*, 2013) as well as internal variations of microclimatic factors within the indoor environment (Okech *et al.*, 2003) create mosaics of microhabitats which can be exploited by mosquitoes at different physiological stages. Microclimatic factors that have been identified to influence mosquitoes include: temperature, relative humidity, light intensity and wind speed (Shannon, 1934; Muirhead-Thomson, 1938; Afrane *et al.*, 2006; Paaijmans and Thomas, 2011; Cator *et al.*, 2013), of which temperature and relative humidity are the most studied.

Effects of temperature and relative humidity on the population dynamics (Afrane *et al.*, 2006; Paaijmans *et al.*, 2010; Parham and Michael, 2010) distribution (Craig *et al.*, 1999) and behaviour (Blanford *et al.*, 2009; Paaijmans and Thomas, 2011; Cator *et al.*, 2013) of mosquitoes are relatively well characterised. Other studies on the reactions of mosquitoes to temperature and relative humidity (Shannon, 1934; Muirhead-Thomson, 1938; Kirby and Lindsay, 2004; Blanford *et al.*, 2009) have clearly demonstrated that mosquitoes exhibit preferences for certain temperature and humidity ranges. Indeed, Blanford *et al.* (2009) indicated that, *An. stephensi* shows some capacity to behaviourally thermoregulate by resting within a narrow range of ambient temperature where body temperature is optimised for other physiological

processes such as digestion and oogenesis. However, little exists on the influence of these microclimatic parameters on the choice of resting site. It is also important to note that studies on the effects of microclimatic factors on mosquitoes are limited to measuring general indoor climates (Okech *et al.*, 2003; Okech *et al.*, 2004; Afrane *et al.*, 2006; Paaajmans *et al.*, 2010; Paaajmans and Thomas, 2011) and not microclimatic conditions at the actual resting sites of mosquitoes. This, despite clear evidence that microclimatic conditions can be significantly different from surrounding environmental conditions (Okech *et al.*, 2004; Afrane *et al.*, 2006; Atieli *et al.*, 2009).

Apart from temperature and relative humidity, the effects of other microclimatic factors such as light intensity and especially, wind speed have received very little attention. Light intensity is a key factor in *Anopheles* resting site selection (Clements, 1999). Anophelines avoid light situations during the day and the one important requirement for a resting place appears to be relative darkness (Christophers and Missiroli, 1933). However, very few quantitative records exist on the preferred range of light intensities within which mosquitoes rest. Similarly, most records on the effect of wind speed on mosquitoes focus on flight, dispersion, swarming and host location (Hoffmann and Miller, 2001; Ritchie and Rochester 2001; Hagemoen and Reimers, 2002; Omrani *et al.*, 2010). It is known that mosquitoes appear to avoid places with high wind speeds (Hoffmann and Miller, 2001), but exactly what range of wind speed is preferred for resting by anophelines is not known.

In nature, temperature, relative humidity, light and wind speed often interact with each other and with other biotic and abiotic factors to create mosaics of microhabitats. To understand how microclimate influences resting site selection, it is important to

take a holistic look at all the factors and determine how these interact to create suitable resting sites for the target mosquitoes. This chapter therefore focuses on the determination of prevailing microclimatic factors and how they interact to influence the selection of resting sites of endophilic anophelines.

6.2 Results

6.2.1 Indoor and outdoor climate

Variations in mean indoor and outdoor climate parameters were analysed separately for all six villages using ANOVA and Tukey's test as summarised in Table 6.1. Mean indoor temperature was significantly different ($F_{5,66} = 7.825$, $p = 0.0001$) across the six villages, being lowest in Kusa Dinkyae (27.46 ± 0.65 °C) but not significantly different from those of Atatam (28.30 ± 0.31 °C) and Adawukwa (29.24 ± 0.37 °C) but significantly different from mean indoor temperatures for Osorongma, Agyenkwaso and Okyereko (29.94 ± 0.43 °C, 30.30 ± 0.64 °C and 30.59 ± 0.32 °C respectively). Mean outdoor temperature was lowest in Agyenkwaso (28.40 ± 0.52 °C) and highest in Okyereko (32.27 ± 2.47 °C), however, there was no significant difference ($F_{5,12} = 0.806$, $p = 0.567$) between mean outdoor temperatures for all six villages. Pair-wise comparison of indoor and outdoor mean temperatures for each village did not show any significant difference ($p > 0.05$).

Mean indoor relative humidity did not differ significantly ($F_{5,66} = 0.725$, $p = 0.607$) between the villages sampled, ranging between 66.50 ± 2.36 % and 72.58 ± 1.25 %. The same was true for outdoor relative humidity ($F_{5,12} = 0.462$, $p = 0.0797$) with a minimum of 64.93 ± 5.87 % and maximum of 71.50 ± 0.93 %. Pair-wise comparisons of indoor and outdoor relative humidity for each village showed no significant

difference ($p > 0.05$) except for Okyereko where mean indoor relative humidity (71.31 ± 0.53 %) was significantly higher ($t_{1,6} = 2.472$, $p = 0.025$) than the mean outdoor value (64.93 ± 5.87 %).

Mean light intensity ranged between 6.88 ± 3.40 Lux and 29.09 ± 8.45 Lux indoors and between $4,079.00 \pm 1,706.00$ Lux and $10,530.00 \pm 3,150.00$ Lux outdoors. Comparing the mean indoors light intensities between the six villages shows no significant differences ($p > 0.05$). The same was true for outdoor light intensity ($P > 0.05$), however, pair-wise comparisons of indoor and outdoor light intensities for each village showed that outdoor light intensity was significantly higher ($p < 0.05$) than indoor values in all villages.

Wind speed followed a similar trend as light intensity, being significantly lower indoors than outdoors in all the villages ($p < 0.05$) but not significantly different between indoor ($F_{5,66} = 1.503$, $p = 0.201$) or outdoor ($F_{5,12} = 1.605$, $p = 0.232$) sites across villages. Indoor wind speed was 0.00 ms^{-1} in almost all the villages except Atatam ($0.01 \pm 0.01 \text{ ms}^{-1}$) and Adawukwa ($0.10 \pm 0.10 \text{ ms}^{-1}$), whereas outdoor values ranged between $0.40 \pm 0.05 \text{ ms}^{-1}$ and $1.83 \pm 0.77 \text{ ms}^{-1}$.

Results from the Pearson's correlation (Table 6.2) showed that indoor temperature decreased significantly ($r = -0.24$, $p = 0.0001$) as you move from the floor towards the roof and increased significantly ($r = 0.10$, $p = 0.003$) as you move further from the walls. Wind speed was also found to significantly ($r = -0.17$, $p = 0.0001$) affect temperature variations indoors with temperature decreasing as wind speed increased. Light intensity and wind speed were higher towards the roof but did not differ on the

horizontal scale (light intensity: $r = 0.10$, $p = 0.002$; wind speed: $r = 0.07$, $p = 0.024$). Relative humidity was uniform indoors with no vertical ($r = -0.03$, $p = 0.36$) or horizontal ($r = -0.03$, $p = 0.364$) variations (Table 6.2).

6.3.2 Temperature variations at resting sites

Mean temperatures at the resting sites of all three endophilic anophelines collected from all the villages were compared alongside the mean general indoor temperature as summarised in Table 6.3. Results from a one way ANOVA indicated no significant difference between mean resting site temperatures of the three vectors investigated and the indoor temperatures recorded ($F_{3,499} = 1.832$, $p = 0.140$). However, number of mosquitoes collected was highest for all three species between 27 and 30 °C (Fig. 6.1). General indoor temperatures ranged between 24.8 °C and 33.0 °C (Table 6.1) and resting site temperatures between 21.7 °C and 36.0 °C (Table 6.3).

6.3.3 Variations in relative humidity at resting sites

General indoor relative humidity ranged between 49.70 % and 80.60 % with a mean of 71.06 % (± 0.58 %), (Table 6.1) which was not significantly different ($p > 0.05$) from the mean resting site relative humidity of *An. gambiae* s.s. and *An. coluzzii* but significantly higher ($p < 0.05$) than that of *An. funestus* (Table 6.3). The distribution of all three species along a resting site relative humidity gradient (Fig. 6.2) showed high proportions of *An. gambiae* s.s. (92 %), *An. coluzzii* (94 %) and *An. funestus* (74 %) resting at locations with relative humidity between 65 % and 75 %. The lowest resting site relative humidity was recorded for *An. funestus* was 49.50 % and the highest was 84.20 % for *An. coluzzii* (Table 6.3).

Table 6.1: Indoor and outdoor climate parameters for six villages across southern Ghana.

Village	N	Indoor				Outdoor					
		Mean ± SE	Min	Max	95 % CL		Mean ± SE	Min	Max	95 % CL	
					Lower	Upper				Lower	Upper
Temperature (°C)											
Osorongma	10	29.9 ± 0.4 ^{ab*}	28.1	32.6	29.0	30.9	28.9 ± 1.0 ^{a*}	27.1	30.4	24.8	33.1
Atatam	20	28.3 ± 0.3 ^{bc*}	25.8	30.4	27.7	28.9	29.6 ± 1.8 ^{a*}	26.3	32.6	21.7	37.4
Kusa Dinkyae	8	27.5 ± 0.7 ^{c*}	24.8	29.3	25.9	29.0	29.2 ± 1.7 ^{a*}	26.8	32.5	21.9	36.5
Agyenkwaso	11	30.3 ± 0.6 ^{a*}	27.0	32.8	28.9	31.7	28.4 ± 0.5 ^{a*}	28.5	29.3	26.2	30.6
Adawukwa	8	29.2 ± 0.4 ^{abc*}	28.1	31.4	28.7	30.1	29.4 ± 0.5 ^{a*}	28.6	30.2	27.3	31.4
Okyereko	15	30.6 ± 0.3 ^{a*}	27.8	33.0	29.9	31.3	32.3 ± 2.5 ^{a*}	29.5	37.2	21.6	42.9
Relative Humidity (%)											
Osorongma	10	70.37±1.30 ^{a*}	63.00	77.00	67.42	73.32	68.33±3.55 ^{a*}	64.20	75.40	53.06	83.61
Atatam	20	72.16±1.06 ^{a*}	61.30	80.60	69.93	74.38	67.97±4.39 ^{a*}	60.90	76.00	49.10	86.84
Kusa Dinkyae	8	73.46±1.14 ^{a*}	70.20	79.20	70.78	76.15	71.37±3.06 ^{a*}	65.40	75.50	58.22	84.52
Agyenkwaso	11	66.50±2.36 ^{a*}	49.70	76.30	61.25	71.75	71.50±0.93 ^{a*}	70.00	73.20	67.50	75.50
Adawukwa	8	72.58±1.25 ^{a*}	67.90	78.30	69.62	75.53	68.73±0.83 ^{a*}	67.10	69.80	65.17	72.30
Okyereko	15	71.31±0.53 ^{a*}	68.60	76.20	70.17	72.44	64.93±5.87 ^{a**}	53.20	71.20	39.67	90.20

Means in the same column for each parameter with different lower case letters as superscripts are significantly different at the 0.05 confidence level by one way ANOVA and Tukey's test.

Indoor and outdoor mean values for the same village with same number of asterisks (*) are not significantly different at the 0.05 confidence level by Student's t-test.

Table 6.1: Indoor and outdoor climate parameters for six villages across southern Ghana (continued).

Village	N	Indoor					Outdoor				
		Mean ± SE	Min	Max	95 % CL		Mean ± SE	Min	Max	95 % CL	
					Lower	Upper				Lower	Upper
Light Intensity (Lux)											
Osorongma	10	10.80±6.81 ^{a*}	0.00	68.00	-4.61	26.21	9170±1303 ^{a**}	2257	6680	3564	14776
Atatam	20	13.00±8.41 ^{a*}	0.00	167.00	-4.60	30.60	8587±2110 ^{a**}	6160	12790	-492	17665
Kusa Dinkyae	8	6.88±3.40 ^{a*}	0.00	22.00	-1.17	14.92	10520±3150 ^{a**}	6540	16740	-3034	24075
Agyenkwaso	11	29.09±8.45 ^{a*}	0.00	99.00	10.26	47.92	9617±1133 ^{a**}	7540	11440	4742	14491
Adawukwa	8	9.88±5.05 ^{a*}	0.00	41.00	-2.06	21.81	5217±1993 ^{a**}	1650	8540	-3357	13791
Okyereko	15	15.40±8.74 ^{a*}	0.00	136.00	-3.34	34.14	4079±1706 ^{a**}	1997	7460	-3259	11417
Wind Speed (ms ⁻¹)											
Osorongma	10	0.00 ^{a*}	0.00	0.00	-	-	1.33±0.32 ^{a**}	0.80	1.90	-0.03	2.70
Atatam	20	0.01±0.01 ^{a*}	0.00	0.20	-0.01	0.03	0.40±0.05 ^{a**}	0.30	0.50	0.15	0.65
Kusa Dinkyae	8	0.00 ^{a*}	0.00	0.00	-	-	1.83±0.77 ^{a**}	0.70	3.30	-1.47	5.14
Agyenkwaso	11	0.00 ^{a*}	0.00	0.00	-	-	0.57±0.12 ^{a**}	0.40	0.80	0.05	1.08
Adawukwa	8	0.10±0.10 ^{a*}	0.00	0.80	-0.14	0.34	0.83±0.35 ^{a**}	0.30	1.50	-0.68	2.35
Okyereko	15	0.00 ^{a*}	0.00	0.00	-	-	0.90±0.45 ^{a**}	0.40	1.80	-1.04	2.84

Means in the same column for each parameter different with lower case letters as superscripts are significantly different at the 0.05 confidence level by one way ANOVA and Tukey's test.

Indoor and outdoor mean values for the same village with same number of asterisks (*) are not significantly different at the 0.05 confidence level by Student's t-test.

Table 6.2: Correlation between resting site location and microclimatic conditions across all villages.

Parameters	N	r	p-value	Significance
HFG x Temperature	918	-0.24	0.0001	**
HFG x RH	918	-0.03	0.360	n.s.
HFG x LI	918	0.10	0.002	**
HFG x WS	918	0.07	0.024	*
DFNW x Temperature	918	0.10	0.003	**
DFNW x RH	918	-0.03	0.364	n.s.
DFNW x LI	918	-0.06	0.069	n.s.
DFNW x WS	918	-0.13	0.387	n.s.
Temperature x WS	918	-0.17	0.0001	**

****Significant at 0.01 confidence level, *significant at 0.05 confidence level, HFG = Height from ground, DFNW = distance from nearest wall, RH = relative humidity, LI = light intensity, WS = wind speed, N = total number of samples, r = correlation coefficient.**

6.3.4 Variations of light intensity at resting sites

Light intensity varied widely (Table 6.1) with general indoor values ranging between 0.00 Lux and 167.00 Lux with a mean of 14.63 ± 3.34 Lux. Mean light intensity was highest for *An. coluzzii* (17.52 ± 4.08 Lux), which was not significantly different ($p > 0.05$) from the mean general indoor light intensity (Table 6.3). The mean resting site light intensities for *An. gambiae* s.s. (4.64 ± 1.04 Lux) and *An. funestus* (6.99 ± 1.49 Lux) were significantly ($p < 0.05$) lower than those recorded for *An. coluzzii* (17.52 ± 4.08 Lux) and the general indoor value (Table 6.3). The minimum resting site light intensity was 0.00 Lux for all the three species and the highest being 421.00 Lux, recorded at a resting site for *An. coluzzii*. High proportions [96 % (N = 132), 86 % (N = 176) and 89 % (N = 123)] of all three species (*An. gambiae* s.s., *An. coluzzii* and *An. funestus* respectively) were collected at resting sites with light intensities between 0.00 Lux and 20.00 Lux (Fig. 6.3).

6.3.5 Variation of wind speed at resting sites

Mean indoor wind speed was generally low (Table 6.1) being 0.00 ms^{-1} in Osorongma, Kusa Dinkyae, Agyenkwaso and Okyereko and $0.10 \pm 0.10 \text{ ms}^{-1}$ in Adawukwa, compared to outdoor wind speed ranging from a minimum of $0.40 \pm 0.05 \text{ ms}^{-1}$ in Atatam to a maximum of $1.83 \pm 0.77 \text{ ms}^{-1}$ in Kusa Dinkyae (Table 6.1). There was no significant difference ($F_{3,499} = 2.123$, $p = 0.196$) between the mean general indoor wind speed ($0.01 \pm 0.01 \text{ ms}^{-1}$) and the mean wind speed at resting sites of *An. gambiae* s.s., which was zero, *An. coluzzii* ($0.03 \pm 0.01 \text{ ms}^{-1}$) and *An. funestus* ($0.02 \pm 0.01 \text{ ms}^{-1}$) (Table 6.3). However, all *An. gambiae* s.s. specimens (100 %) and high proportions of *An. coluzzii* (96 %) and *An. funestus* (90 %) collected from all six villages throughout the study period were resting at locations with zero wind speed (Fig. 6.4).

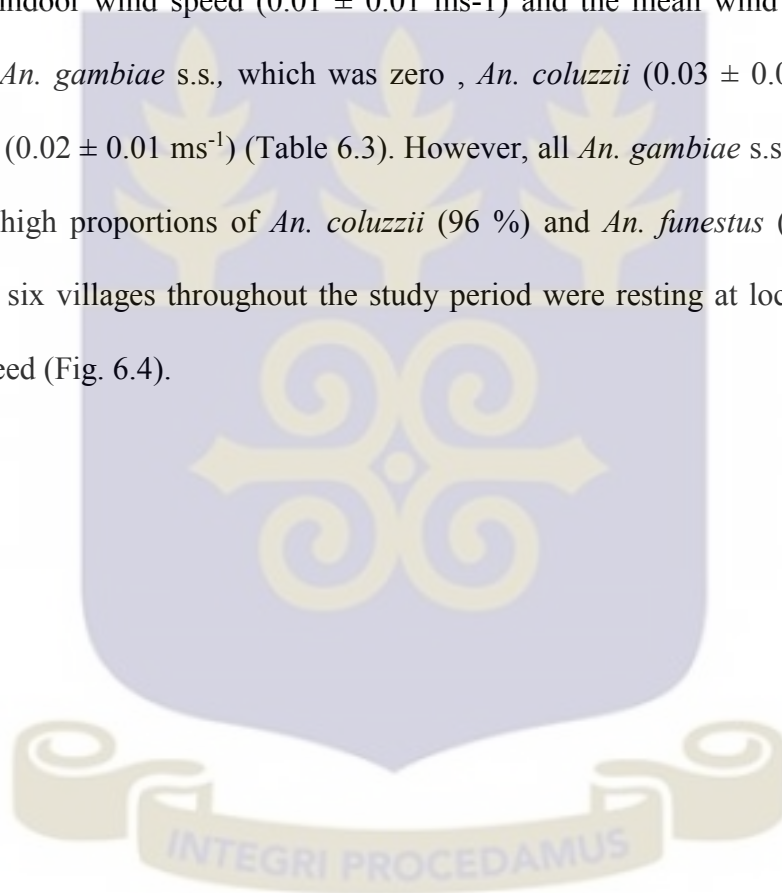


Table 6.3: Indoor resting site microclimate parameters for the three endophilic anopheline mosquito species, *An. gambiae* s.s., *An. coluzzii* and *An. funestus* in the study communities in southern Ghana.

Species	N	Mean±SE	Min	Max	95 % CL	
					Lower	Upper
Temperature (°C)						
General Indoor	72	29.33±0.22 ^a	24.80	33.00	28.90	29.77
<i>An. gambiae</i> s.s.	132	29.11±0.14 ^a	25.20	32.80	28.82	29.39
<i>An. coluzzii</i>	176	29.62±0.15 ^a	21.70	36.00	29.32	29.91
<i>An. funestus</i>	123	29.34±0.19 ^a	24.50	33.20	28.95	29.72
Relative Humidity (%)						
General indoor	72	71.06±0.58 ^a	49.70	80.60	69.90	72.22
<i>An. gambiae</i> s.s.	132	72.20±0.37 ^a	61.90	83.20	71.52	72.99
<i>An. coluzzii</i>	176	72.70±0.32 ^a	58.60	84.20	72.06	73.33
<i>An. funestus</i>	123	68.74±0.54 ^b	49.50	80.50	67.68	69.80
Light Intensity (Lux)						
General indoor	72	14.63±3.43 ^a	0.00	167.00	7.78	21.47
<i>An. gambiae</i> s.s.	132	4.64±1.04 ^c	0.00	90.00	2.64	6.65
<i>An. coluzzii</i>	176	17.52±4.08 ^{ab}	0.00	421.00	9.48	25.57
<i>An. funestus</i>	123	6.99±1.49 ^{bc}	0.00	128.00	4.04	9.94
Wind Speed (ms⁻¹)						
General indoor	72	0.01±0.01 ^a	0.00	0.80	-0.009	0.037
<i>An. gambiae</i> s.s.	132	0.00 ^a	0.00	0.00	-	-
<i>An. coluzzii</i>	176	0.03±0.01 ^a	0.00	1.10	0.006	0.047
<i>An. funestus</i>	123	0.02±0.01 ^a	0.00	0.20	0.009	0.030

Differences in lower case letters indicate significant differences between means for each parameters at the 0.05 confidence level.

N = Number of sites for which microclimatic data were recorded.

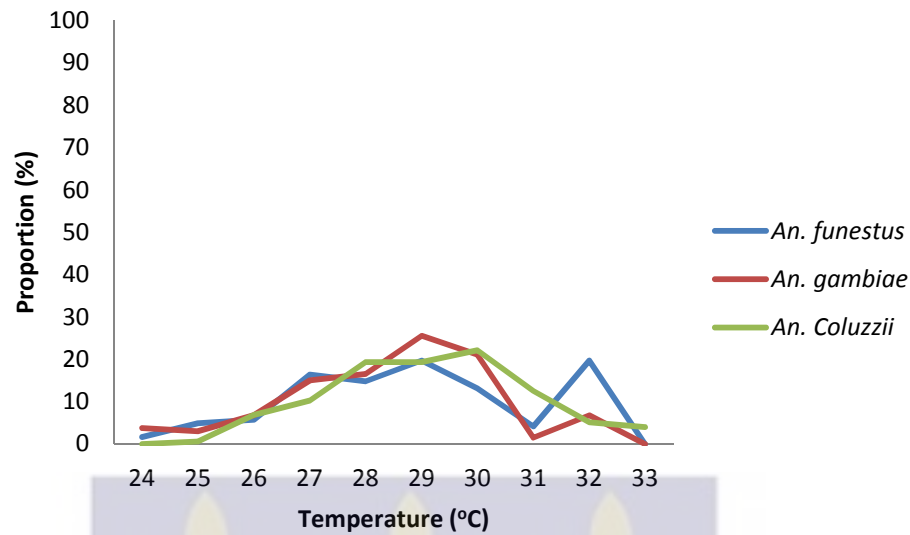


Fig. 6.1: Distribution of *An. funestus*, *An. gambiae* s.s. and *An. coluzzii* resting at varying indoor resting site temperatures at the study communities in southern Ghana.

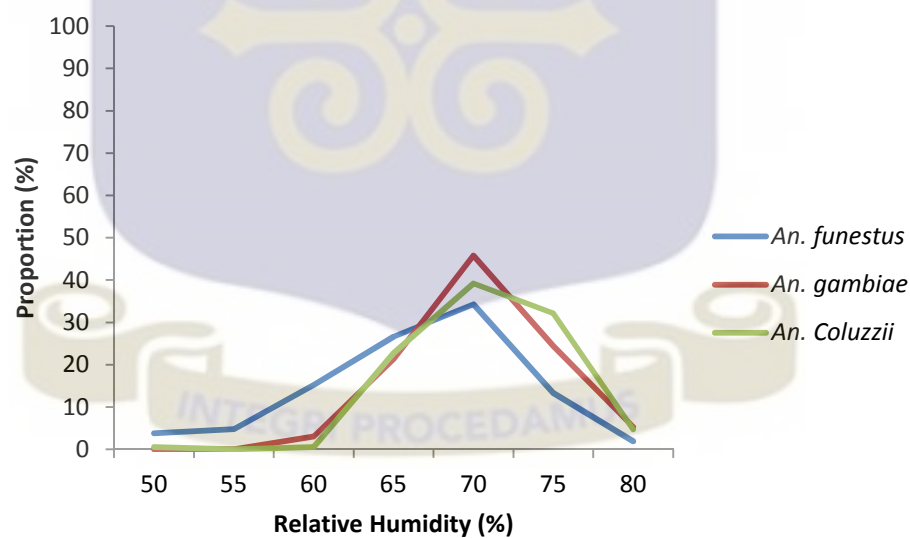


Fig. 6.2: Distribution of *An. gambiae* s.s., *An. coluzzii* and *An. funestus* resting at varying indoor resting site relative humidity at the study communities in southern Ghana.

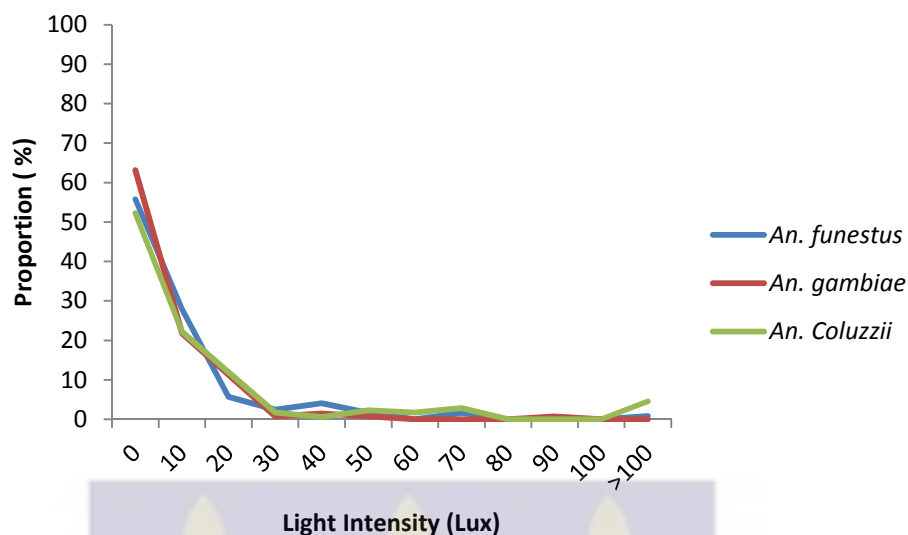


Fig. 6.3: Distribution of *An. funestus*, *An. gambiae* s.s. and *An. coluzzii* resting at varying indoor resting site light intensities at the study communities in southern Ghana.

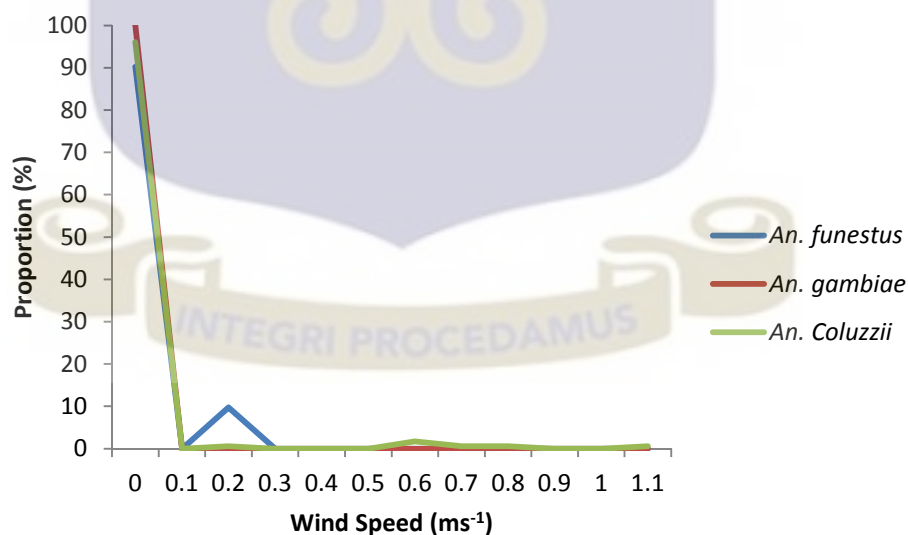


Fig. 6.4: Distribution of *An. funestus*, *An. gambiae* s.s. and *An. coluzzii* resting at varying indoor resting site wind speed in southern Ghana.

6.3 Discussion

The choice of resting site by different anophelines depends largely upon presence of favourable microclimatic conditions (Shannon, 1934) and different species of *Anopheles* differ to some degree in their choice of microclimate (Martini and Taubner, 1933). It has however been established that indoor microclimate varies with season and location (Afrane *et al.*, 2006, 2007, 2008) as well as the nature and materials of the building structure (Okech *et al.*, 2004; Atieli *et al.*, 2009). Additionally, even within a single room, there is likely to exist a gradient of microclimatic factors (Okech *et al.*, 2003) creating a mosaic of microhabitats. The current study aimed at determining the microclimates prevailing at the indoor resting sites of anophelines and to establish any effect of these microclimatic factors on the choice of resting sites by endophilic anopheline vectors. The main species encountered in this study were *An. gambiae* s.s., *An. coluzzii* and *An. funestus*.

The results obtained indicate that outdoor climate and indoor microclimatic factors including relative humidity, light intensity and wind speed did not vary between the different villages (Table 6.1). However, indoor temperatures varied between villages, despite the lack of significant variation in outdoor parameters. This variation is likely due to the nature and materials of the buildings (Okech *et al.*, 2004; Atieli *et al.*, 2009). Despite the variations in indoor temperature between the villages and even within rooms as indicated by the significant correlations of temperature with height of resting site from the floor of the room and the distance from the nearest wall, there was no difference in the mean temperatures at resting sites of all three anopheline species. The resting site temperatures of the three anophelines did not differ significantly from each other and from the mean general indoor temperature. However, about two thirds of all species were found resting within the narrow

temperature range of 27 – 30 °C. Blanford *et al.* (2009) reported that less than half of *An. stephensi* exposed to temperature gradients preferred to rest between 24 – 27 °C. The authors attributed this to acclimation of the laboratory colony, which was reared at 26 – 27 °C. Cator *et al.* (2013) recorded differences of the order 1-3 °C for *An. stephensi* resting sites in India. Although this study was not a preference study *per se*, data do suggest that all three vectors prefer to rest in cool, humid areas. Kessler and Guerin (2008) showed that newly emerged *An. gambiae* s.s. use their themohygroreceptor cells to guide them towards cooler and more humid locations that facilitate survival. Anophelines are thus doubtlessly sensitive to temperature, and the choice to rest within a narrow range of the available temperature gradient reflects a preference for resting sites where body temperature is optimised for physiological processes (Blanford *et al.*, 2009).

The range of indoor relative humidity available in the study area was between 50 – 80 %, however, significant proportions of the three species of *Anopheles* were found resting at locations with relative humidity between 65 and 75 %. This indicates some preference for resting at sites within a narrower range of available relative humidity. Although Shannon (1934) concluded that temperature was a more important environmental determinant of resting site choice than humidity, the two are interlinked (Blanford *et al.*, 2009) and the three species investigated here, clearly showed some kind of preference for humidity when choosing resting sites (Fig. 6.2). For many species, there exists a range of temperature and humidity within which biological function and survival is optimal (Muirhead-Thomson 1938; Kirby and Lindsay, 2004; Blanford *et al.*, 2009).

It is worth noting that the mean resting site relative humidity, varied between species with *An. funestus* being collected at sites with lower relative humidity than the other species. Lyons (2013) reported that *An. funestus* could tolerate lower humidity for longer periods than *An. arabiensis*, a sibling species of both *An. gambiae* s.s. and *An. coluzzii*. Charlwood *et al.* (2000) also indicated that *An. gambiae* s.s. has a greater dependency on humid conditions than *An. funestus* and *An. arabiensis*. In this study, greater numbers of *An. funestus* were collected during the dry season (data not shown), during which relative humidity was generally lower. The tolerance of *An. funestus* for lower humidity may be attributed to several factors. Survival during the dry season requires adaptation to lower relative humidity and this species-specific variation in humidity tolerance could be due to variations in cuticular properties as well as physiological activity (Charlwood *et al.*, 2000; Gray and Bradley, 2005; Wood *et al.*, 2010). It can therefore be concluded at this stage that relative humidity might be another important microclimatic factor influencing the choice of resting site by endophilic anophelines in southern Ghana.

Light intensity is also an important factor determining where mosquitoes rest (Christophers and Missiroli, 1933; Shannon, 1934; Clements, 1999). In addition, it has been shown to function as cue for guiding mosquitoes in the early morning hours to their daytime resting sites (Shannon 1934). Here, light intensity was shown to significantly influence resting site behaviour of the three investigated vector species. Generally, light intensity is higher outdoors than indoors and even within the indoor environment, it varies vertically but not horizontally. The increase in light intensity as you move up from the floor of rooms is likely due to light entering the rooms from open eaves and diffusing downwards. In effect, there is higher light intensity on the roof ceilings and upper walls than other parts of village rooms, and these same areas

harbour greater proportions of indoor resting mosquitoes (see Chapter 5). The mean resting site light intensity ranged between 5 – 18 Lux with higher proportions of all mosquitoes collected resting at sites with light intensity below 20 Lux (Fig. 6.3). These, coupled with the fact that the mean resting site light intensity varied between species shows that light should be regarded as a critical microclimatic factor influencing choice of resting site. According to Shannon (1934) light intensities of 1 – 5 candle-foot (10.76 – 53.82 Lux) is preferred by anophelines and anything higher or lower is avoided. Usually, dark places are cooler and more humid resulting in a greater proportion of resting mosquitoes (Shannon, 1934).

Indoor wind speed ranges between less than 0.05 ms^{-1} in rooms without mechanical ventilation (Melikov *et al.*, 1988) to around 0.1 ms^{-1} in mechanically ventilated rooms (Kovanen *et al.*, 1989). Data from village rooms in the current study fall within this range and are lower than the maximum tolerable wind speed range of mosquitoes. Mosquitoes appear to avoid places with wind speeds that approach their own flying speed ($0.4 - 1.6 \text{ ms}^{-1}$) (Hoffmann and Miller, 2001). This means that minimum tolerable wind speed for mosquitoes is about four-folds greater than the maximum indoor wind speed and thirteen-folds greater than the maximum resting site wind speed recorded in the current study. This coupled with the fact that close to one hundred percent of all anophelines collected in the current study rest at locations with wind speed less than 0.1 ms^{-1} implies that indoor wind speed and the wind speed at resting sites are way below levels that can influence choice of resting site by endophilic anophelines in southern Ghana.

Other factors influencing resting site behaviour are material type, background colour and physical location of suitable resting sites which all ultimately affect the climatic

conditions and can create mosaics of microhabitats within the indoor environment. Of particular interest to this project are the implications of these resting site microhabitats for the use of entomopathogenic fungi. Fungal activity is strongly influenced by temperature, relative humidity and solar (UV) radiation (Moore *et al.*, 1996 a, b; Milner *et al.*, 2003; Vanninen *et al.*, 2000; Yeo *et al.*, 2003; Shipp *et al.*, 2003; Hsiao *et al.*, 2009). For example, the pathogenic nature of any isolate of entomopathogenic fungi is dependent not only on the target insect but also the temperature conditions (Yeo *et al.*, 2003) and between 21 and 27 °C, 100 % mortality of aphids infected with *Verticillium lecanii* was observed. This implies that the preferred range of temperatures for the endophilic anophelines recorded in the current study would be ideal for fungal activity so long as humidity remains ideal.

Humidity affects the infection rate of entomopathogenic fungi. According to Shipp *et al.* (2003), at humidity of 97.5 %, infection of 60-88 % was observed in greenhouse pests (insects and mites) under controlled conditions in petri dish. However, at 75-80 %, only 15.3-43.9 % infection was achieved. This notwithstanding, on leaf surfaces, the difference in humidity did not cause as great variation in infection as in the petri dish trials. According to Hsiao *et al.* (2009), fungus-induced mortality in aphids does not appear to be dependent on relative humidity but relative humidity does affect sporulation. At higher relative humidity of 75% and above, spores were produced from dead cadavers (Hsiao *et al.*, 2009), a condition that is likely to enhance horizontal transmission of fungal spores if dead mosquitoes remain at the resting sites. Though the preferred range of resting site relative humidity recorded in the current study are lower than those ideal for the performance of entomopathogenic fungi, enhanced formulations such as oil formulations are likely to compensate for this drawback.

With reference to light, fungal spores are very quickly deactivated by solar (UV) radiation (Moore *et al.*, 1996). However, when spores are applied in more protected environments, persistence can be extended to months or even years (Milner *et al.*, 2003; Vanninen *et al.*, 2000). Since the application of fungal biopesticides for mosquito control will most likely be in indoor or at least shaded environments with low light intensities as recorded in the current study, UV radiation is unlikely to be a constraint, suggesting that persistence might be good.

6.4 Conclusion

Resting site behaviour of the three endophilic vectors investigated here, *An. gambiae* s.s., *An. coluzzii*, and *An. funestus*, is determined by temperature, humidity, light intensity and a combination of these factors. Although wind speed is a known deterrent above a certain speed for some mosquito species (Hoffmann and Miller, 2001), in this study, windspeed did not significantly differ within indoor locations or between villages, and therefore, had little influence on resting site behaviour of mosquitoes collected. It appears that these indoor microclimatic factors interact in a complex manner to create mosaics of microhabitats. It is worth noting that resting site behaviour of sympatric populations of the different anophelines encountered in the current study does not show much variation. This implies that any vector control strategy developed on the basis of resting site behaviour should be effective against all the endophilic anopheline species in southern Ghana. In general, the resting site microhabitats are ideal for the survival, infection and persistence of entomopathogenic fungi.

CHAPTER SEVEN

ATTRACTIVENESS OF RESTING DEVICES MADE FROM DIFFERENT MATERIALS FOR ENDOPHILIC ANOPHELINE MOSQUITOES

7.1 Background

Essential to the use of entomopathogenic fungi as a vector control tool is the need to have effective application methods. Central to the development of application methods is a need to understand pre- and post-prandial behaviour of adult female mosquitoes. Though information on this subject exists (Forattini *et al.*, 1993; Bøgh *et al.*, 1998; Tuno *et al.*, 2003; Perich *et al.*, 2004; Sumodan *et al.*, 2004; Kent *et al.*, 2006; Mahande *et al.*, 2007), the implications resting behaviour for the development of fungi as biopesticides is yet to be established. It is known that teneral, blood-fed and gravid females in the family Culicidae (Diptera) prefer dark, cold and humid sites for resting (Shannon, 1934). In Chapter Four, *Anopheles gambiae* s.s., *An. coluzzii* and *An. funestus* were identified as the major endophilic vectors in the study villages in southern Ghana. These vectors show little variations in their resting behavior. However general trends were observed as highlighted in Chapters Five and Six. All three species preferred to rest at cool humid and darker shaded areas, similar to the findings from other parts of Africa (Smith, 1955; Smith, 1962a; Smith, 1962b). Wood, wall materials such as clay and cement and cloth materials appeared to be the preferred resting materials. Based on these findings, it should be possible to develop resting devices that are attractive to indoor resting *Anopheles* species.

Different materials have been used to collect endophilic and exophilic mosquitoes. The common materials used include; black cotton cloth, wicker baskets, earthenware pots, and wooden boxes (Crans, 1989; Scholte, *et al.*, 2005; Harbison *et al.*, 2006;

Odiere *et al.*, 2007). Black felt is routinely used in most insectaries to construct resting sites for adult mosquitoes. Each of these materials has been shown to effectively attract mosquitoes seeking resting sites. However, the relative efficiency of these materials in attracting mosquitoes is not known.

This chapter covers investigations on the evaluation of resting devices made from six different materials for their attractiveness to anopheline mosquitoes. The hypothesis of this study is that resting devices made from different materials will create specific microclimates that will show varying attractiveness to endophilic *Anopheles* mosquitoes. The objective was therefore to find the most attractive materials for development of point source dissemination devices for entomopathogenic fungi.

7.2 Results

7.2.1 Screen house trials

Results for non-choice experiments presented in Table 7.1 show that a higher proportion of blood fed mosquitoes rested on the black felt box ($61.67 \pm 2.19\%$). This part of the study was carried out with colonised laboratory mosquitoes. Generally, blood fed females were more prone to finding resting sites than newly emerged females and second time feeders as shown in the choice and non-choice tests (Tables 7.1 and 7.2). There was no significant difference in the preference for all the resting devices for newly emerged females and second time feeders in all the screen house trials (Tables 7.1 and 7.2) except for the felt tested with newly emerged females in the choice test (Table 7.1). In both the choice and non-choice screen house trials, blood fed females had the highest significant preference for the felt box ($56.67 \pm 5.24\%$ and $61.67 \pm 4.48\%$ respectively). The next preferred in both cases is the black cotton box

(14.33 ± 2.24 and 28.67 ± 4.48 for choice and non-choice tests respectively). In the choice test for blood fed *An. gambiae* s.s., jute box, wicker box, wooden box and clay pot had 7.00 ± 4.00 , 5.17 ± 0.17 , 3.33 ± 0.88 and 2.67 ± 1.20 percent respectively of all released mosquitoes resting in them. Similarly, in the non-choice test, 11.33 ± 0.67 , 9.00 ± 1.15 , 7.33 ± 1.20 and 5.33 ± 2.40 percent *An. gambiae* s.s. were recorded in jute box, wicker box, wooden box and clay pot respectively

Table 7.1: Preference of adult female mosquitoes for resting devices made from different materials in a screen house (choice test)

Resting Device	Percentage of Released Females Resting in Devices ($\pm$ S.E.)		
	Newly Emerged	Blood Fed	After One Gonotrophic Cycle
Wooden Box	0.50 ± 0.28^{aB}	3.33 ± 0.88^{bB}	0.33 ± 0.33^{aA}
Wicker Box	0.00 ± 0.00^{aB}	5.17 ± 0.17^{bB}	0.67 ± 0.33^{aA}
Clay Pot	0.67 ± 0.67^{aB}	2.67 ± 1.20^{aB}	1.17 ± 0.44^{aA}
Jute Box	0.17 ± 0.17^{aB}	7.00 ± 4.00^{aB}	1.33 ± 0.88^{aA}
Black Cotton Box	0.67 ± 0.44^{aB}	14.33 ± 2.24^{bB}	1.83 ± 0.33^{aA}
Black Felt Box	2.00 ± 0.50^{aA}	59.67 ± 5.24^{bA}	2.00 ± 1.04^{aA}

Means in the same row with different lower case letters as superscript are significantly different at the 0.05 confidence level. Tukey's Test. Means in the same column with different upper case letters as superscripts are significantly different at the 0.05 confidence level. Tukey's Test.

Table 7.2: Preference of adult female mosquitoes for resting devices made from different materials in a screen house (non-choice test)

Percentage of Released Females Resting in Device ($\pm$ S.E)		
Resting Device	Newly Emerged	Blood Fed
Wooden Box	7.00 $\pm$ 1.53 ^{aA}	7.33 $\pm$ 1.20 ^{aB}
Wicker Box	3.33 $\pm$ 0.88 ^{aA}	9.00 $\pm$ 1.15 ^{bB}
Clay Pot	6.33 $\pm$ 2.33 ^{aA}	5.33 $\pm$ 2.40 ^{aB}
Jute Box	5.00 $\pm$ 2.08 ^{aA}	11.33 $\pm$ 0.67 ^{bB}
Black Cotton Box	11.67 $\pm$ 0.88 ^{aA}	28.67 $\pm$ 4.48 ^{bB}
Black Felt Box	3.67 $\pm$ 0.88 ^{bA}	61.67 $\pm$ 2.19 ^{aA}

Means in the same row with different lower case letters as superscript are significantly different at the 0.05 confidence level. Tukey's Test. Means in the same column with different upper case letters as superscripts are significantly different at the 0.05 confidence level. Tukey's Test.

7.2.2 Field trials

Field trials were conducted to evaluate the attractiveness of the different resting devices to wild mosquitoes. A total of 3015 mosquitoes were collected from Adawukwa and Osorongma (1943 and 1072 respectively). All samples from Osorongma were identified morphologically as *An. gambiae* s.l. and those from Adawukwa were made up of *An. gambiae* s.l. (1411) and *An. funestus* (532). Based on physical observation of the abdomen, only blood fed females were found resting in the devices regardless of the material. There was no significant difference in the mean number of mosquitoes collected from rooms in which the different devices were placed in both villages (Table 7.3). In Osorongma, the mean number of mosquitoes collected per room ranged between 13 ± 05 and 36 ± 21 and in Adawukwa it ranged between 26 ± 06 and 117 ± 60 . Pearson's correlation did not show any significant correlation ($r = 0.27$, $P = 0.265$ for Osorongma and $r = -0.16$, $P = 0.380$ for

Adawukwa) between the percentage of mosquitoes resting in the devices and total number of mosquitoes collected from the rooms.

The trend observed in the screen house trials were repeated in the field trials. Black felt box and black cotton box were preferred for resting in village rooms in Osorongma (13.00 ± 6.00 and 12.00 ± 4.00 percent respectively) and Adawukwa (8.00 ± 2.00 and 12.00 ± 3.00 percent respectively) (Table 7.3). In Osorongma, wooden box and wicker box had means of 1.00 ± 1.00 % mosquitoes resting in each of them where as jute box and clay pot did not attract any mosquitoes at all. In Adawukwa, wooden box, clay pot, jute box and wicker box attracted 4.00 ± 3.00 , 3.00 ± 3.00 , 2.00 ± 1.00 and 1.00 ± 1.00 percent mosquitoes respectively.

Table 7.3: Preference of adult female mosquitoes for resting devices made from different materials in houses at Osorongma and Adawukwa.

Resting Device	% of Total No. of Mosquitoes Collected Resting in Device ($\pm$ S.E.)			
	Osorongma		Adawukwa	
	Total Collected	% In Device	Total Collected	% in Device
Wooden Box	29 ± 21^a	1.00 ± 1.00^a	117 ± 60^a	4.00 ± 3.00^a
Wicker Box	27 ± 21^a	1.00 ± 1.00^a	47 ± 31^a	1.00 ± 1.00^a
Clay Pot	26 ± 14^a	0.00 ± 0.00^a	73 ± 33^a	3.00 ± 3.00^a
Jute Box	25 ± 18^a	0.00 ± 0.00^a	62 ± 06^a	2.00 ± 1.00^a
Black Cotton Box	13 ± 05^a	12.00 ± 4.00^b	26 ± 06^a	8.00 ± 2.00^b
Black Felt Box	36 ± 21^a	13.00 ± 6.00^b	61 ± 32^a	12.00 ± 3.00^b

Means in the same column with different lower case letters as superscripts are significantly different at the 0.05 confidence level. Tukeys's Test. Mean temperature at time of collection: Osorongma = 27.62 ± 0.19 (Range: 26.16 – 29.11), Adawukwa = 26.73 ± 0.17 (23.39 – 30.91). Mean RH at time of collection: Osorongma = 82.15 ± 0.56 (Range: 77.9 – 86.5), Adawukwa = 85.60 ± 0.59 (Range: 73.5 – 90.1).

7.3 Discussion

Black felt box and black cotton cloth box were the most attractive indoor resting devices for endophilic *Anopheles* in this study. In both screen house and field experiments a significant number of mosquitoes were found resting on black felt and black cotton boxes compared to clay pot, wicker basket, wooden box and jute box. Clay pots, wicker basket and wooden boxes have all been used successfully to sample mosquitoes, albeit as outdoor collection methods (Harbison *et al.*, 2006; Odiere *et al.*, 2007; Crans, 1989). The resting site preferences of endophilic and exophilic mosquitoes vary (Paaijmans and Thomas, 2011). *Anopheles gambiae* s.l. and *An. funestus*, which were considered in this study, are highly endophilic (Sinka *et al.*, 2010), therefore, expected to have different resting site preferences compared to exophilic species. Contrary to this, a significant proportion of mosquitoes were caught on black cotton box which is traditionally used to collect exophilic mosquitoes (Kweka *et al.*, 2009). In their experiment, Kweka *et al.* (2009) recorded 15.1 % (N = 9558) of *An. arabiensis* collected on black cotton box placed outdoors. Similarly, Scholte *et al.* (2005) collected 53.6 % of indoor resting *An. gambiae* on black cotton sheets suspended from ceilings in traditional houses.

From the present study, it appears that anophelines prefer to rest on darker materials, these result tallies with the findings in Chapters Five and Six of this thesis as well as those of Kweka *et al.* (2009) and Scholte *et al.* (2005). This is further supported by Shannon (1934) who established that mosquitoes prefer to rest in darker areas. The black felt and black cotton cloth boxes create darker shaded conditions inside them compared to the other resting devices used in this study. Although the earthenware pots used were painted black outside, the brick red interior did not seem to create the

kind of dark conditions required by anophelines seeking resting sites, probably explaining its poor performance.

Blood-fed females are more prone to resting than blood seeking females. In this study, all mosquitoes collected from the resting devices during the field trials were blood-fed females. Added to this, more blood-fed females rested in the devices in the choice and non-choice screen house trials. This is expected as blood fed females require favourable locations to digest the blood meal and develop their eggs. Implications are that any success with these resting devices as point sources for dissemination of entomopathogenic fungi may be targeting blood-fed females seeking suitable resting sites to digest the blood meal and develop their eggs. Since both *Metarhizium anisopliae* and *Beauveria bassiana* impair parasite development in infected female anophelines (Blanford *et al.*, 2005) targeting blood fed females will reduce malaria transmission even if the fungus does not kill the mosquito before it is ready for the next blood meal.

In conclusion, the black felt box and black cotton box are the most preferred resting devices and are most attractive to blood fed females. Further research on the design as well as efficacy and persistence of fungal spores on these surfaces are however required before these materials can be deployed as point source fungus dissemination devices against endophilic *Anopheles* vectors.

CHAPTER EIGHT

GENERAL DISCUSSIONS AND CONCLUSIONS

8.1 General Discussion

Vector control is considered an important strategy for insect-borne disease management as it breaks the transmission cycle, thereby reducing morbidity and mortality (WHO, 2013). Currently, the most effective and widely adopted vector control strategies include: indoor residual spraying (IRS), insecticide treated nets (ITNs) and to some extent larviciding of breeding habitats (Mabaso *et al.*, 2004; Fillinger and Lindsay, 2006; Kleinschmidt *et al.*, 2006). These strategies together with other interventions have resulted in significant progress in the fight against major vector-borne diseases such as malaria and lymphatic filariasis (Njenga *et al.*, 2011; WHO, 2012a).

Despite these interventions, vector-borne diseases still remain a major public health problem. For example, regardless of the adoption of ITNs and IRS in 2004 and 2005 respectively by the NMCP as major vector control tools, malaria incidence in Ghana still remains high (WHO, 2012a). This is likely due in part to the widespread insecticide resistance and changes in the behaviour of the vectors (Yawson *et al.*, 2004; Coetzee *et al.*, 2006; Owusu-Agyei *et al.*, 2009; Hunt *et al.*, 2011). To overcome these challenges and win the war against vector-borne diseases, several novel vector control strategies including; the sterile insect technique (SIT) and entomopathogenic fungi for the control of wild vector mosquito populations irrespective of their insecticide resistance status are being developed (Benedict and Robinson, 2003; Scholte *et al.*, 2005; Farenhorst *et al.*, 2009; Munhenga *et al.*, 2011).

Critical to the implementation of any novel strategy is the understanding of the ecology, bionomics and behaviour of the target population (De Meillon, 1934; De Meillon, 1936; Kuzoe and Schofield, 2004; Paaijmans and Thomas, 2011; Mnyone *et al.*, 2012). Therefore, to implement entomopathogenic fungi for vector control in Ghana, this project aimed at studying the diversity, bionomics and resting behaviour of endophilic anopheline vectors at selected communities in southern Ghana.

8.2 Major findings of the study

8.2.1 Indoor *Anopheles* diversity and population characteristics

The first specific objective of the study was to determine the diversity and population characteristics of endophilic *Anopheles* populations in six selected villages across three major ecological zones of southern Ghana. Indoor resting collections from six villages in three ecological zones were carried out, all specimens were morphologically identified and a sub-sample confirmed by PCR. Further discrimination between sibling species using PCR, the prevalence of the L1014F and L1014S *kdr* mutation, blood meal source by PCR and sporozoite rates by ELISA were also determined.

***Anopheles* diversity and distribution**

The results obtained indicate that *Anopheles gambiae* s.s. and *An. coluzzii* (M form of *An. gambiae* s.s. in previous literature) were the only members of the *An. gambiae* complex resting indoors alongside *An. funestus* (the only member of the *An. funestus* group) in southern Ghana. The three species appeared to vary in their distribution with *An. gambiae* s.s. collected mainly from the FEZ and FTEZ whiles *An. coluzzii* dominated collections from the CSEZ. *Anopheles funestus* was collected from all the

ecological zones except the FTEZ where the population was similar to those from the FEZ save the near absence of *An. funestus*. The distribution of these species appeared to depend on the availability and proximity of preferred breeding sites around the sampled villages (Charlwood and Edoh, 1996; Minakawa *et al.*, 2002) since all the three species are highly anthropophilic, endophagic and endophilic (Sinka *et al.*, 2010).

Prevalence of the *kdr* mutation

Both the L1014F and L1014S *kdr* mutations were present in the members of the *An. gambiae* complex sampled, albeit at varying frequencies. The L1014F mutation appeared almost fixed in *An. gambiae* s.s. (0.97 - 1.00) and *An. coluzzii* (0.75 - 0.90) from southern Ghana. The fixation of this mutation in *An. gambiae* s.s. from southern Ghana is not surprising as the mutation was originally identified in this species and is widespread in the West African sub-region (Martinez-Torres *et al.*, 1998; Santolamazza *et al.*, 2008). However in Ghana, it has been reported mainly from *An. gambiae* s.s. (Yawson *et al.*, 2004; Coetzee *et al.*, 2006; Santolamazza *et al.*, 2008; Hunt *et al.*, 2011) and to some extent in *An. coluzzii*, though at very low frequencies between 1 and 3 % (Yawson *et al.*, 2004). This is the first time that the L1014F mutation is being reported at such high frequencies in any *An. coluzzii* population from Ghana, but not the first for the species as a near fixation has been reported previously for populations from Benin (Djegbe *et al.*, 2011).

Another important finding from this population study is the presence of the L1014S mutation in Ghanaian *An. gambiae* s.s. and *An. coluzzii* populations. This mutation was originally recorded from Kenya (Ranson *et al.*, 2000; Stump *et al.*, 2004) and has

since been reported in populations from several other African countries (Etang *et al.*, 2006; Pinto *et al.*, 2006; Verhaeghen *et al.*, 2006; Moreno *et al.*, 2008; Santolamazza *et al.*, 2008; Djegbe *et al.*, 2011; Koekemoer *et al.*, 2011; Namountougou *et al.*, 2013). This first record of the mutation in Ghana is further evidence to the East-West spread hypothesis suggested by Santolamazza *et al.* (2008).

Vectorial capacity

All the endophilic *Anopheles* collected in this study were highly anthropophagic, consistent with their feeding behaviour (Sinka *et al.*, 2010). Though some level of zoophagy was recorded, in *An. gambiae s.l.* populations, almost all the specimens that fed on animals also demonstrate the tendency to supplement with human blood. With the high level of anthropophagy recorded in the study, sporozoite rate appeared to mirror the prevalence of parasites within the human populations. These data are consistent with previous records (Appawu *et al.*, 2001; Coetzee *et al.*, 2006; Owusu-Agyei *et al.*, 2009; Hunt *et al.*, 2011) from Ghana confirming these areas as endemic to malaria.

8.2.2 Indoor resting behavior

Chapter Five reports the indoor resting site and material preferences of the endophilic *Anopheles* populations from southern Ghana and Chapter Six covers the effects of microclimatic factors on the choice of resting site. All the three species, viz *An. gambiae s.s.*, *An. coluzzii* and *An. funestus* preferred to rest high up in rooms on the underside of roofs, roofing beams, ceilings and on upper wall surfaces as well as on windows, tables, mosquito nets and clothing. These findings are consistent with previous reports for these species in other parts of Africa (Garnham, 1929; Gibbins,

1933; Haddow, 1942; Smith, 1955; Smith, 1962a; Smith, 1962b; Mathis *et al.*, 1963).

Resting higher up in rooms could be an adaptation to avoid human interference.

The general trend in resting site location is not reflected in the material preference as the different species and populations appeared to exhibit varying resting material preferences across the study area. This could be attributed to the variations in building style and materials used (Smith, 1955; Smith, 1962a; Smith, 1962b; Mathis *et al.*, 1963). Woody materials used in roofing, ceilings, windows and furniture appear to be the most preferred resting materials. These woody materials are usually not painted and so were classified under normal coloured materials resulting in high preference for normal coloured materials for resting by all three species. It is known that mosquitoes avoid brighter areas but will also not rest in absolute darkness (Christophers and Missiroli, 1933; Shannon, 1934) and since background light intensity depends on the colour of the material, woody brown colour may be providing somewhat ideal background light intensity that is suitable for mosquito resting.

Indoor microclimatic factors including temperature, relative humidity, light intensity and wind speed, all appear to influence the choice of resting site to varying extents (see Chapter 6). Data presented in Chapter Six show that indoor microclimate depends on the nature and material of buildings and even within the same room, varies vertically and to some extent horizontally, creating mosaics of microhabitats as reported previously by Okech *et al.* (2004) and Atieli *et al.* (2009). These microhabitats are influenced by a complex interaction between physical factors (such as material type and colour), microclimatic factors (especially temperature, relative

humidity and light intensity) and human factors. Exactly how these interactions work together to influence resting site selection however is not clear from this study.

8.2.3 Attracting endophilic *Anopheles* mosquitoes to preferred resting sites

Based on the resting behaviour studies reported in Chapters Five and Six, and reports in the literature on materials that have been used to collect mosquitoes in the field, the possibility of attracting endophilic *Anopheles* mosquitoes to resting devices made from different materials were explored. The hypothesis for this study reported in Chapter Seven was that, resting devices made from different materials will show varying attractiveness to endophilic *Anopheles* mosquitoes.

The data show that boxes made from black felt and black cotton cloth were the most attractive to endophilic *Anopheles* mosquitoes. These results *vis-a-vis* those in Chapters Five and Six showed that endophilic *Anopheles* mosquitoes prefer to rest on darker backgrounds, consistent with the findings of other workers (Shannon, 1934; Scholte *et al.*, 2005; Kweka *et al.*, 2009).

Another important finding from this study is that blood-fed female *Anopheles* mosquitoes are more prone to finding resting sites than non-blood fed host-seeking females. Finding resting sites with ideal microclimatic conditions and fewer disturbances is a key requirement for blood fed females to digest blood meal and develop their eggs (Paaijmans and Thomas, 2011).

8.3 Future Perspectives and Policy Implications

The presence of the three most efficient vectors of malaria from indoor collections in southern Ghana and the fact that they were highly anthropophilic and effectively transmitting malaria is a high call for intensifying vector control efforts. Current control strategies are highly dependent on chemical insecticides and cannot be solely relied on to win the fight against vector-borne diseases including malaria and filariasis as the populations show high prevalence of the *kdr* resistance mutations.

The high levels of the L1014F *kdr* mutation in *An. coluzzii* and the first report of the L1014S in both *An. gambiae* and *An. coluzzii* from southern Ghana confirms the spread of these resistance mechanisms in West Africa where they coexist with other resistance mechanisms (Namountougou *et al.*, 2013). To curb this and make any significant impact on the malaria situation in Ghana, it is important for the NMCP and the malaria research community to put in place proper policies and programs to establish the distribution of these mutations nationwide and implement insecticide resistance management (IRM) plans.

Since the *kdr* is a mutation on the sodium-gated-channel of the insect nervous system, insecticides that act on this channel such as Pyrethroids and DDT may no longer be effective vector control agents in southern Ghana. This is evident in the high numbers of *Anopheles* mosquitoes collected on ITNs in this study. Unfortunately only pyrethroids are recommended for use on ITNs, a flagship vector control tool. To win the fight against malaria, there is the need to introduce other insecticides into the major vector control strategies of IRS and ITNs. For example, ITNs and IRS using carbamates and organophosphates that are not affected by the *kdr* mutation should be

integrated in IVM strategies. In addition, efforts must be made to speed up the implementation of promising novel vector control strategies such as entomopathogenic fungi, insecticide treated wall linings and SIT. These novel tools, in addition to effectively reducing vector populations and disease transmission also have the potential for insecticide resistance management as they are effective even against insecticide resistant populations.

Key to any effective IRM is the development and implementation of integrated vector management (IVM) which incorporates several control strategies. One of the most important strategies that can make great impact as part of an IVM is the use of entomopathogenic fungi for vector control. Biological insect control with fungal entomopathogens is a well founded technology, which is so far only being deployed on a wide scale against agricultural pest insects and not yet against disease vectors such as mosquitoes. In Brazil and China, more than one million hectares is treated with fungal biopesticides annually (Li *et al.*, 2010), and a locust control product based on *Metarhizium* spores is now registered throughout Africa and commercially produced in South Africa and Senegal (Douthwaite *et al.*, 2001; Lomer *et al.*, 2001). There is thus ample knowledge available on the implementation and production of fungal biopesticides, which can be used for the future deployment of fungi as malaria vector control agents. Indeed two species of hyphomycetes fungi; *Metarhizium anisopliae* and *Beauveria bassiana* have been demonstrated to hold great potential as vector control agents. A range of *M. anisopliae* and *B. bassiana* isolates have been shown to be successful in infecting and killing *Anopheles*, *Aedes* and *Culex* mosquitoes when applied on several different substrates, irrespective of their resistance status (Scholte *et al.*, 2003a; Blanford *et al.*, 2005; Scholte *et al.*, 2005,

2007). In addition to killing malaria vectors, fungi have also been shown to prevent the development of *Plasmodium* sporozoites, reduce blood feeding frequency and reproductive fitness prior to death, which could further impact on the mosquito's capacity to transmit disease (Blanford *et al.*, 2005; Scholte *et al.*, 2006; Hancock *et al.*, 2009; Read *et al.*, 2009). What is remaining is their introduction into our vector control mix.

Key to the implementation of these entomopathogenic fungi is understanding the behaviour of the target vectors so as to increase the chances of infecting them with fungal spores (Mnyone *et al.*, 2012). Our understanding of the indoor resting behaviour of the three major endophilic *Anopheles* vectors of southern Ghana paves the way for developing fungal spore deployment strategies for field implementation. This study, has established that all the three endophilic *Anopheles* species in southern Ghana rest higher up in village rooms and prefer to rest on darker coloured materials in cool humid areas. It has also further demonstrated that black felt and black cotton cloth are attractive to blood-fed females seeking resting sites to digest blood meal and develop their eggs. Guided by this knowledge, future research should focus on designing these preferred materials into resting devices that create suitable microhabitats to which endophilic anopheline mosquitoes can be attracted and contaminated with fungal spores.

Further research is also needed to determine the efficacy and persistence of fungal spores on these preferred resting materials so as to determine application rates and frequency as has been done for other materials for conventional insecticide-treated targets and screens (Blanford *et al.*, 2012).

8.4 Conclusion

The indoor resting populations of *Anopheles* mosquito vectors and their population characteristics have been determined for the three major ecological zones of southern Ghana with the highest frequency of the L1014F *kdr* mutation in *An. coluzzii* so far and the first report of the L1014S mutation in both *An. gambiae* and *An. coluzzii* from Ghana. It has also been established that the populations are highly anthropophagic and are effectively transmitting malaria, hence requiring effective action to control them.

The indoor resting behaviour of the three major vector species in southern Ghana has been determined. The data showed that all three species prefer to rest on darker materials in a cool humid area, higher up in village rooms. Black felt and black cotton materials have been demonstrated to be attractive to blood fed females seeking a resting site to digest blood meal and mature their eggs.

With the current knowledge on the indoor resting behaviour of the vector species and availability of commercial formulations of fungal spores, future efforts should focus on development of fungal spore dissemination devices and their field evaluation. Future developments of integrated vector management strategies incorporating entomopathogenic fungi could provide a new malaria vector control approach with potential to be successful in Ghana where insecticide resistance is widespread and hampering the effectiveness of existing malaria interventions.

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Resting Behaviour of Anopheline mosquitoes in Two Ecological Zones in Ghana Village Datasheet

M.O. / Datasheet / June 2011

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APPENDIX IV: Collins DNA Extraction Protocol**Equipment and reagents required**

The materials and equipment listed here are recommendations and alternative reagents may be substituted depending on the specific need of your DNA

Equipment:

- Micro-centrifuge
- Vortex/mixer
- Heating block
- 20 – 200 μ l Micropipette
- 2 – 20 μ l Micropipette
- 0.2 – 2 μ l Micropipette

Reagents:

- Ice cold 100% ethanol
- Ice cold 70% ethanol
- TE buffer (10 mM Tris HCl, 0.1 mM EDTA pH 8)
- 8M potassium acetate
- Distilled H₂O
- Grinding buffer (see recipe below)

Plasticware:

- Autoclaved 1.5 ml eppendorf tubes
- Clean, autoclaved pestles
- Autoclaved tips (20 – 200 μ l), (2 – 20 μ l), (0.2 – 2 μ l)

The preparation and storage of reagents**70% Ethanol:**

- Add 30 ml distilled water to 70ml absolute ethanol
- Store at -20°C in a freezer (indefinitely)

Grinding Buffer:

In final volume of 20 ml:

- 1 600 μ l 1M NaCl
- 1.095 g Sucrose
- 2 400 μ l 0.5M EDTA
- 1 000 μ l 10% SDS (if it has precipitated then microwave for $\pm$ 30 seconds)

- 2ml 1M Tris-Cl (pH 8.6)

Make up final volume to 20 ml ($\pm$ 13 ml), using distilled water.

Grinding buffer lasts about 3 weeks if kept at 4°C.

8M KAc (*Potassium Acetate*):

Molecular weight of KAc = 98.1417

$8M = M_w \times 8/L = 785.1336g/L$

10 ml 8M KAc = $785.1336/100 = 7.851336g$ in 10 ml distilled water

Detailed step-by-step instructions on how to perform the test

- Step 1:** Ensure that you have autoclaved enough tips, 1.5ml eppendorf tubes and pestles. UV treat the pestles for 15 minutes prior to use.
- Step 2:** Place the whole mosquito or part thereof into a 1.5ml eppendorf tube. Using a permanent marker, label the tube.
- Step 3:** Using a clean pestle for each specimen, grind the specimen until very fine. Add 200 μ l of grinding buffer and continue to grind using the pestle. Grind the specimen as fine as possible.
- Step 4:** Remove the pestle, ensuring that no mosquito parts remain on it.
- Step 5:** Incubate at 70°C in a heating block for 30 minutes. Never incubate for more than 1 hour.
- Step 6:** Remove the tube from the heating block and add 28 μ l 8M KAc. Mix gently by tapping gently with a finger.
- Step 7:** Incubate **on ice** for 30 minutes, max. 1 hour.
- Step 8:** Centrifuge for 20 minutes at 13 000 rpm, with the eppendorf tube hinge facing.
- Step 9:** Pipette off all the liquid into a **NEW** eppendorf tube, **without** disturbing the pellet. Using a permanent marker, label the new tube. Discard the old eppendorf tube containing the pellet.
- Step 10:** Add 400 μ l of ice cold 100% ethanol (from -20°C freezer). Mix by inverting tube.

- Step 11:** **EITHER** incubate at room temperature for 5 minutes; **OR** preferably incubate in a freezer overnight at -20°C.
- Step 12:** Pipette off and discard the 100% ethanol, without disturbing the pellet.
- Step 13:** Without disturbing the pellet, add 200 µl ice cold 70% Ethanol (from -20°C freezer) to the eppendorf tube.
- Step 14:** Centrifuge the tube at 13 000 rpm for 30 minutes.
- Step 15:** Without disturbing the pellet, pipette off and discard the 70% ethanol. Leave the eppendorf tube open on the lab bench to air dry. If necessary use the Eppendorf Concentrator (Speedy vac) to dry the pellet.
- Step 16:** Re-suspend the dry pellet in 200 µl 1x TE (pH 8). The pellet can be resuspended by leaving the tube overnight in the fridge. If however, the DNA is required immediately resuspend by pipetting the TE buffer up and down until the pellet is dissolved.
- Step 17:** The DNA quantity and quality can be determined using a spectrophotometer by measuring the optical density at A260nm wavelength. The A260/A280 nm wavelength ratio should be between 1.8 – 2.0 for high quality DNA preparations. DNA concentration can be adjusted to end user specification using TE buffer.
- Step 18:** DNA can be used immediately in downstream applications or frozen at -20°C.

Any necessary calculations:

None.

Test acceptance criteria

A positive and negative control must be run in parallel with every extraction batch. The negative control is a tube which is treated exactly as a standard extraction but with no mosquito added.

The positive control is normally a mosquito specimen obtained from the Both De Meillon insectary. This positive control should match the species of the PCR identification you intend to perform.

Interpretation of test results

Not applicable

Where applicable, biological reference intervals and units

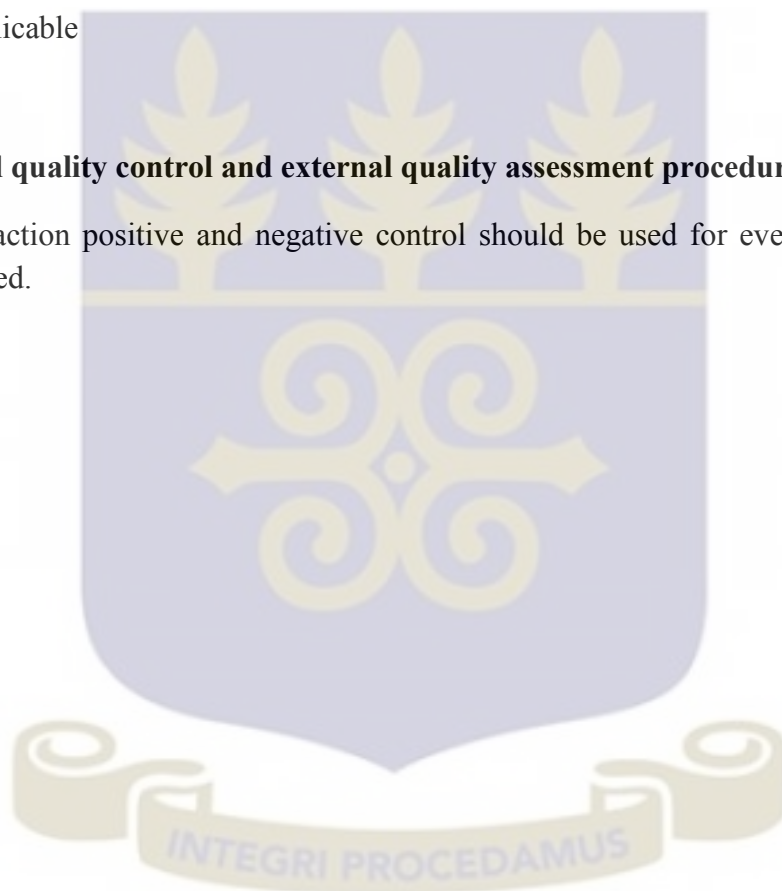
Not applicable

Potential sources of variability

Not applicable

Internal quality control and external quality assessment procedures

An extraction positive and negative control should be used for every extraction set performed.



APPENDIX V: *Anopheles gambiae* complex PCR identification protocol (Scott *et al.*, 1993)

Equipment and reagents required

Forceps:	Sterile forceps, cleaned with rectified spirits
Pipettes:	20 – 200 µl Micropipette 2 – 20 µl Micropipette 0.2 – 2 µl Micropipette
Centrifuge:	A desktop centrifuge or microfuge capable of holding 1.5 ml microcentrifuge tubes
Reagents:	dNTP mixture (each 2.5mM), 25mM MgCl ₂ , 10x PCR Buffer, Primers (GA, AR, ME, QD, UN), taq (5 U/µl), PCR quality water, 6x loading dye
Plasticware:	Autoclaved tips (20 – 200 µl), (2 – 20 µl), (0.2 – 2 µl) Autoclaved 1.5 ml Microcentrifuge tubes Autoclaved 0.2 ml PCR tubes Sterile pestles for grinding specimen Fine tipped permanent marker pen
2.5% TAE gel:	10 g agarose, 400 ml 1 x TAE solution, ethidium bromide dye (10 mg/ml), measuring cylinder, beaker, microwave, casting trays and combs

Detailed step-by-step instructions on how to perform the test

Step 1: Place all frozen reagents on ice and allow to thaw completely.
Prior to using the reagents, vortex the tubes and then briefly centrifuge in a desktop microfuge.

Step 2: Prepare the PCR master mix.

The recipe below is per specimen. Multiply the “1x” volumes by the number of PCR tubes required.

(The number of PCR tubes required = the number of specimens identifying + 4 positive controls + 1 PCR negative control.)

It is recommended that for larger PCR master mix volumes, that you round up the total master mix value to the nearest five. For example: if you calculate that 31 PCR tubes are required, round up the master mix to prepare a 35x master mix. See the table below for an example.

Reagent	1x	35x
10x PCR Buffer	1.25µl	43.75 µl
10x dNTPs	1.25µl	43.75 µl
MgCl ₂ solution	0.5 µl	17.5 µl
Quad Primer	0.5 µl	17.5 µl
Other primers	1.0 µl each	35.0 µl
Deionised water	4.9 µl	171.5 µl
Taq	0.1 µl	3.5 µl

Step 3: Pipette each of the required volumes of the PCR master mix components into a 1.5 ml microcentrifuge tube. Remember to use a clean tip for each reagent. Vortex the master mix and then centrifuge briefly in a desk top microfuge.

Step 4: For each specimen or control: use the sterile forceps to add a single leg or wing from the specimen into a 0.2 ml PCR tube. Label the PCR tube using the permanent marker pen.

Repeat for each of the specimens and controls. Remember to wipe the forceps clean with rectified spirits between each sample.

Step 5: Aliquot 12.5 μ l of the PCR master mix onto the leg or wing in the 0.2 ml PCR tubes.

When pipetting the PCR master mix, ensure that the leg or the wing remains inside the PCR master mix solution. If the leg or wing moves out of the PCR master mix solution, use a tip to push it back in.

Remember to use a clean pipette tip for each aliquot of master mix.

Step 6: Place the PCR tubes into the PCR machine. Close the lid. Select and run the *An. gambiae* PCR program.

Step 7: Prepare a 2.5% TAE agarose gel according to the method below. Pour 400 ml of 1x TAE Buffer into a larger beaker. To this, add 10g agarose. Agarose can be found on the chemical shelf in the PCR room. Mix well and boil for 10 minutes in the microwave. Do not walk away from the microwave. Stand and watch the gel whilst it is boiling as it can boil over. Ensure that the agarose solution is clear. If the solution is not completely clear, continue to boil the agarose mix until it becomes clear. Leave the hot solution on the bench top to cool for 5 minutes. Whilst the solution is cooling, set up the casting tray and combs.

After 5 minutes, remove the skin from the surface of the liquid.

Add 12 μ l ethidium bromide (biohazard) and mix well.

Pour the molten gel into the casting tray and allow to set.

Used agarose gels can be re-melted to minimize costs and wastage. The method is described below:

- Weigh the gel. Make up any lost weight with fresh 1x TAE buffer.
- Place the used gels into a large beaker and boil for 5 to 10 minutes in the microwave. Ensure that the gel has dissolved completely. If the solution is not completely clear continue to boil until it becomes clear.
- Make up lost volume with 1 x TAE and boil for 5 minutes.
- Cool and add ethidium Bromide (4 μ l per 400 ml of gel).
- Pour the gel into a casting tray and allow to set.

Step 8: Load the PCR products into a gel that has been placed in an electrophoresis tank containing 1 x TAE buffer. For every 5 μ l of PCR product, add 1 μ l of loading dye. Onto each gel you should load the following:

2 x Mw markers (one at each side of the gel)

4 x positive controls

1 x PCR negative control

Use the remaining lanes to load your unknown specimens.

Electrophorese at 100V and not more than 120-150 mA until the smallest fragment of the Mw marker has reached the end of the gel.

Test acceptance criteria

- The negative control should not amplify. If amplification occurs in your negative control, the PCR has failed.
- The positive controls should yield amplicon for the *An. gambiae* species listed below. Failure of more than one positive control will result in the run/batch being rejected.

Interpretation of test results

The amplicons are analyzed by gel electrophoresis on 2.5% TAE agarose gel. The settings on the power pack should be 100V and not more than 120-150 mA. The gel must be electrophoresed until the smallest fragment in the DNA marker has reached the end on the gel.

The expected sizes of the amplicons are:

464 bp *An. merus*

390 bp *An. gambiae*

315 bp *An. arab*

153 bp *An. quad*

For an example of an acceptable diagnostic gel, refer to Figure 1 below.

Where applicable, biological reference intervals and units

Not Applicable

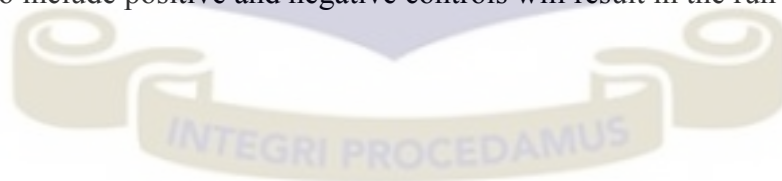
Potential sources of variability

Not Applicable

Internal quality control and external quality assessment procedures

Every set of PCR reactions must have positive and negative controls.

Failure to include positive and negative controls will result in the run being rejected.



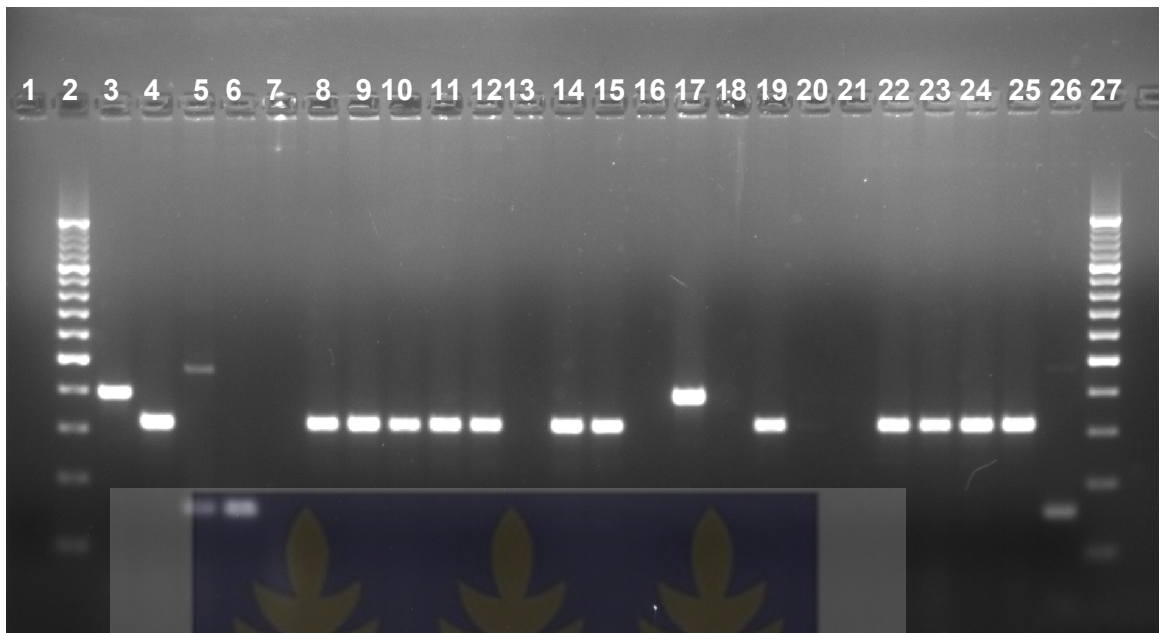


Figure 1. Example of an acceptable *An. gambiae* diagnostic gel

Lane 1: PCR –ve Control (Note: There is no amplification present)

Lanes 2 and 17: Mw Marker (Note: the Mw Marker has separated and there are two present on the gel)

Lanes 3 – 6: Positive PCR Controls. A minimum of 3 out of four have amplified



APPENDIX VI: Protocol for distinguishing between *An. gambiae* s.s. and *An. coluzzii* (Favia *et al.*, 2001)

Equipment and reagents required

Pipettes:	20 – 200 µl Micropipette; 2 – 20 µl Micropipette
Centrifuge:	A desktop centrifuge or microfuge capable of holding 1.5ml microcentrifuge tubes.
Reagents:	Primers: R3, R5, B/S and Mopint; 10x PCR buffer containing MgCl ₂ , Supertherm taq; dNTP mix; Deionised water
Plasticware:	Autoclaved tips (2 - 20 µl and 20 – 200 µl) 1.5 ml Microcentrifuge tubes, autoclaved 0.2 ml PCR tubes, autoclaved
Electrophoresis:	1 x TAE running buffer, electrophoresis tank and power pack, 2.5% TAE agarose gel containing ethidium bromide

Detailed step-by-step instructions on how to perform the test

Step 1:

- Remove all the reagents from the -20°C freezer and place them on ice to thaw completely.

Step 2:

- Vortex and centrifuge the thawed reagents. Do not vortex the Taq. Centrifugation might be necessary to ensure the contents are at the bottom of the tube.
- Ensure all the reagents are kept on ice.

Step 3:

- Prepare the master mix as follows: (This is for 1 specimen. Multiply for the number of specimens including two positive and 1 negative control.)

Reagents	1x
10x PCR Buffer with MgCl ₂	2.5 µl
dNTPS	2.5 µl
Primers:	
R5	2 µl
B3	2 µl
Mopint	2 µl
B/S	2 µl
Deionised water	11.5 µl
Supertherm gold Taq	0.2 µl

Step 4:

- Pipette the required volume of each reagent into a 1.5ml microcentrifuge tube. Mix well by briefly vortexing. Centrifuge in a desktop microfuge to collect the contents of the tube at the bottom.

Step 5:

- Aliquot 24.7µl of master mix into each 0.2 ml PCR tube.
- Where necessary, add 1.0 µl of DNA. Remember that the negative control should not contain any DNA, but 1µl of water instead.

Step 6:

- Place the tube in PCR machine and run at the recommended cycling conditions listed below:

94°C for 10minutes	} cycle 25 times
94°C for 30 seconds	
62°C for 30 seconds	
72°C for 30 seconds	
72°C for 7mintues	

Step 7:

- Electrophorese the amplicons on a 2.5% TAE agarose gel containing ethidium bromide.

Test acceptance criteria

- The negative control should not amplify when used in PCR.
- The positive control should yield amplicon for the M and S forms when used in PCR.

Interpretation of test results

- Expected size of amplicons size :

1,3 kb	<i>An. gambiae</i> band
700bp	Mopti band
500bp	Savannah band

Where applicable, biological reference intervals and units

- Not Applicable

Potential sources of variability

- Although it is rare, hybrid mosquitoes will produce both the M and S amplicons.

Internal quality control and external quality assessment procedures

- Every set of PCR must have both positive and negative controls.
- Failure to include positive and negative controls will result in the run being rejected.

APPENDIX VII: *An. funestus* group PCR identification protocol (Koekemoer et al., 2002)**Equipment and reagents required**

Reagents: 10 X PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl)

25 mM MgCl₂

dNTP mixture (mixture containing 2.5 mM of each dNTP)

3.3 pmol Primers (UV, FUN, LEES, PAR, RIV, VAN)

Taq (5 units/μl)

Sterilized distilled H₂O

Template DNA

Equipment:

Thermal Cycler: A thermal cycler with a hot lid

Pipettes: 0.1 -10 μl, 20 – 200 μl and 100 - 1000 μl pipettes

Centrifuge: A desktop centrifuge or microfuge capable of holding 1.5 ml microcentrifuge tubes as well as the PCR tubes

Vortex: Desktop vortex

Consumables: Autoclaved tips (0.2-10 μL; 20-200 μL; 100-1000 μL)

0.2 ml and 1.5 ml Microcentrifuge tubes

Thin walled, flat cap PCR tubes

Electrophoresis: Electrophoresis tank

Power supply unit

1 X TAE buffer

2.5% TAE agarose gel containing ethidium bromide

Loading buffer (Fermentas O'Range 6 X Loading Dye)

Molecular weight marker (Fermentas O'Range Ruler 100bp)

The preparation and storage of reagents

- Stock primers are stored in a stock box at -20 °C freezer. To each 10 µl stock primer (already aliquoted), add 290 µl sterilized distilled H₂O to obtain a 3.3 pmol solution.
- Mw Marker is best stored at -20°C and loading buffer should be stored at 4°C.
- TAE buffer and ethidium bromide may be stored at room temperature
- Always wear a lab coat and gloves.
- Before starting, wipe down your lab bench and pipettes with 70% ethanol.
- Fill an ice- box with ice and place it on the lab bench.
- Remove all the PCR components from the -20°C freezer and place them on ice to thaw completely.
- Once all the reagents are thawed, mix (either by finger tapping or inverting the tube or using the vortex). Use the centrifuge to spin the tubes so that all the liquid collects at the bottom of the tube. Place all tubes back onto the ice.

From this point forward, all the preparations are carried out on ice.

- * Prior to use, all frozen reagents should be placed on ice to thaw. Once thawed, briefly vortex the reagents and spin them down in a desktop centrifuge.

Detailed step-by-step instructions on how to perform the test

- Step 1:** Count how many samples you will need to do PCR on. Remember to include the positive control for the DNA extraction, the negative control for the DNA extraction and a no DNA control for the PCR. This number will be the value that you use when calculating your master mix volumes.
- Step 2:** Label the clean, autoclaved PCR tubes. There should be a tube for each specimen, including one for each of the controls.
- Step 3:** Add 0.5 µL of the DNA sample into the corresponding labeled PCR tube. Remember to include PCR tubes for the controls.

Step 4: Make up the master mix using the recipe below:

Reagent	x1	x10	x100
Buffer	1.25 μ l	12.5 μ l	125 μ l
dNTP	1 μ l	10 μ l	100 μ l
MgCl ₂	0.75 μ l	7.5 μ l	75 μ l
Primer UN	1 μ l	10 μ l	100 μ l
FUN	1 μ l	10 μ l	100 μ l
LEES	1 μ l	10 μ l	100 μ l
PAR	1 μ l	10 μ l	100 μ l
RIV	1 μ l	10 μ l	100 μ l
VAN	1 μ l	10 μ l	100 μ l
<i>Taq</i>	0.1 μ l	1 μ l	10 μ l
H ₂ O	2.9 μ l	29 μ l	290 μ l
Template	0.5 μ l	0.5 μ l each	0.5 μ l each
Total	12.5 μ l		

When making up the master mix, remember to multiply each volume by the number of samples that you have. Pipette the required volume of PCR reagent into a 1.5 ml microcentrifuge tube.

Mix this tube well by briefly vortexing. Centrifuge the master mix tube briefly in a desktop centrifuge.

Step 5: Aliquot 12 μ l from this larger master mix tube into all PCR tubes.

Step 6: Close the lids of the PCR tubes. Mix the DNA and master mix by tapping the tube with your finger.

- Step 7:** Centrifuge the tubes briefly in a desktop centrifuge. When removing the tubes from the centrifuge ensure that the contents have collected at the bottom of the tube.
- Step 8:** Load all the PCR tubes into the PCR machine and set up the cycling conditions as follows:

94 °C – 2 mins

94 °C – 30 secs

45 °C – 30 secs

72 °C – 40 secs

72 °C – 5 mins

} X 30

- Step 9:** After the PCR cycle has completed, remove all the PCR tubes from the PCR machine. Load the samples into a 2.5% to 3% TAE gel containing ethidium bromide. Use 2.5 µl loading buffer for each 12.5 µl PCR product. Remember to include at least one lane of Mw Marker and include all the controls on the same gel.

- Step 10:** Electrophorese the gel at 100 V (<400 mAmps), until the loading dye has separated and the leading dye front has just about run off the bottom of the gel.

- Step 11:** Visualise the amplicons on the gel using a gel doc system. Ensure that the PCR has worked correctly by sizing the products against the molecular weight marker.

Any necessary calculations

Size the resulting amplicons against the Mw Marker. See Figure 2 below.

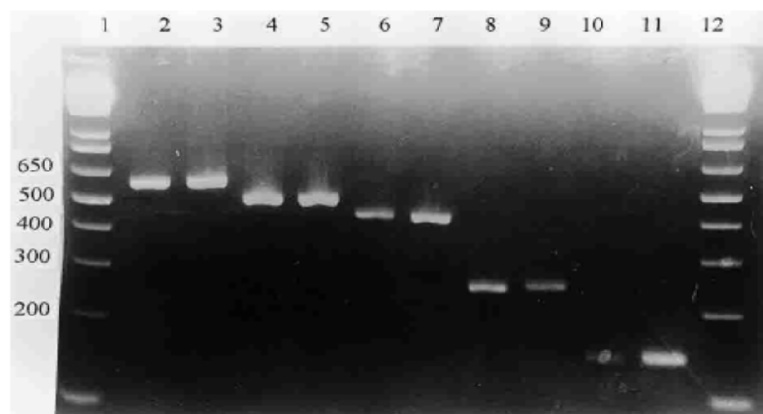


FIGURE 2. Amplified fragments using the species-specific polymerase chain reaction assay to identify members of the *A. funestus* group. Lanes 1 and 12: 1-Kb-plus DNA ladder; Lanes 2 and 3: *A. vaneedeni*; Lanes 4 and 5: *A. funestus*; Lanes 6 and 7: *A. rivulorum*; Lanes 8 and 9: *A. parensis*; Lanes 10 and 11: *A. lesoni*. The fragment sizes of the 1-Kb-plus DNA ladder is indicated on the side in base pairs.

(Courtesy of Koekemoer *et al.*, 2002)

Test acceptance criteria

The no DNA PCR control must not have any amplification in the product as visualised by no bands in this lane. At least three of the five positive controls must have successfully amplified.

Interpretation of test results

Successful PCR amplification of the unidentified specimens with the following molecular weights: *An. funestus* (505 bp), *An. lesoni* (146 bp), *An. parensis* (252 bp), *An. vaneedeni* (587 bp), and *An. rivulorum* (411 bp).

Where applicable, biological reference intervals and units

Not Applicable

Potential sources of variability

Not Applicable

Internal quality control and external quality assessment procedures

- Every set of PCRs run, must have positive and negative controls.
- Failure to include positive and negative controls will result in the run being rejected.

- A minimum of three of the five positive controls must amplify for the PCR run to be accepted/
- Should external quality assessment be required, this can be performed by an independent repeat of the protocol or sequencing of the PCR products to confirm the species identification.

Anopheles funestus group species-specific primer sequences with respective T_m temperatures

Primer name	Sequence (5' to 3')	T _m (°C)
UV	TGT GAA CTG CAG GAC ACA T	55.34
FUN	GCA TCG ATG GGT TAA TCA TG	52.4
VAN	TGT CGA CTT GGT AGC CGA AC	58
RIV	CAA GCC GTT CGA CCC TGA TT	58.8
PAR	TGC GGT CCC AAG CTA GGT TC	60.5
LEES	TAC ACG GGC GCC ATG TAG TT	60.2



APPENDIX VIII: Protocol for detection of the L1014F and L1014S resistance mutations (Bass et al., 2007)**Equipment and reagents required**

Reagents:	IQ Supermix
	10 μ M Forward primer
	10 μ M Reverse primer
	10 μ M WT probe
	10 μ M <i>kdr</i> E or W probe
	PCR grade dH ₂ O
	Template DNA
Thermal Cycler:	A real-time PCR cycler
Pipettes:	0.1 -10 μ l, 10-200 μ l and 100-1000 μ l pipettes
Centrifuge:	One that can accommodate 96 well plates.
Consumables:	96 well plates for conducting qPCR, 1.5 ml tubes

The preparation and storage of reagents

- Stock primers are stored in a stock box at -20 °C. To each 10 μ l stock primer (already aliquoted), add 90 μ l sterilized distilled H₂O to obtain a 10 μ M solution.

Detailed step-by-step instructions on how to perform the test

- Step 1:** Count how many samples you will need to do KDR identification on. Remember to include the positive controls and the negative control for the hydrolysis probe reaction. This number will be the value that you use when calculating your master mix volumes
- Step 2:** Prepare a master mix. Use the table below to assist in your calculations.

Reagent	1 x (VCRL modified)***	1 x (Bass et al. 2007)
IQ Supermix	10 μ l	12.5 μ l
Forward primer (10 μ M)	1.6 μ l	2.25 μ l
Reverse primer (10 μ M)	1.6 μ l	2.25 μ l
WT probe (10 μ M)	0.4 μ l	0.5 μ l
kdrE/kdrW probe (10 μ M)	0.4 μ l	0.5 μ l
dH ₂ O	5.0 μ l	6 μ l
DNA	1.0 μ l	1 μ l
Total	20 μl	25 μl

***Add 19 μ l to each tube

Step 3: Aliquot 19ul of master mix to each tube/well. Pipette in your DNA.

Step 4: Program the real time machine. Use the recommended cycling conditions listed below.

Recommended Cycling Conditions:

95 °C – 10 mins

95 °C – 10 secs } **X 40**

60 °C – 45 secs



Plate read

VIC- yellow (530nm excitation & 555nm emission)

FAM – green (470nm excitation & 510 emission)

Any necessary calculations

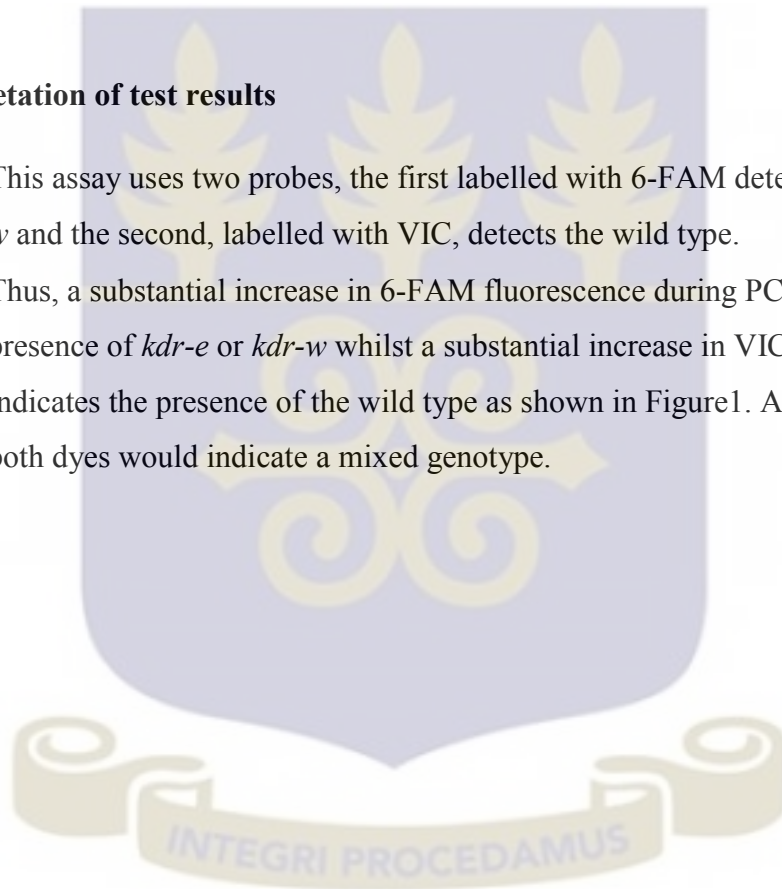
None.

Test acceptance criteria

- The samples are considered acceptable if the amplification curve is substantially higher than the baseline curve.
- All positive controls have to work and the negative control should not amplify any product.

Interpretation of test results

- This assay uses two probes, the first labelled with 6-FAM detects *kdr-e* or *kdr-w* and the second, labelled with VIC, detects the wild type.
- Thus, a substantial increase in 6-FAM fluorescence during PCR indicates the presence of *kdr-e* or *kdr-w* whilst a substantial increase in VIC fluorescence indicates the presence of the wild type as shown in Figure1. An increase in both dyes would indicate a mixed genotype.



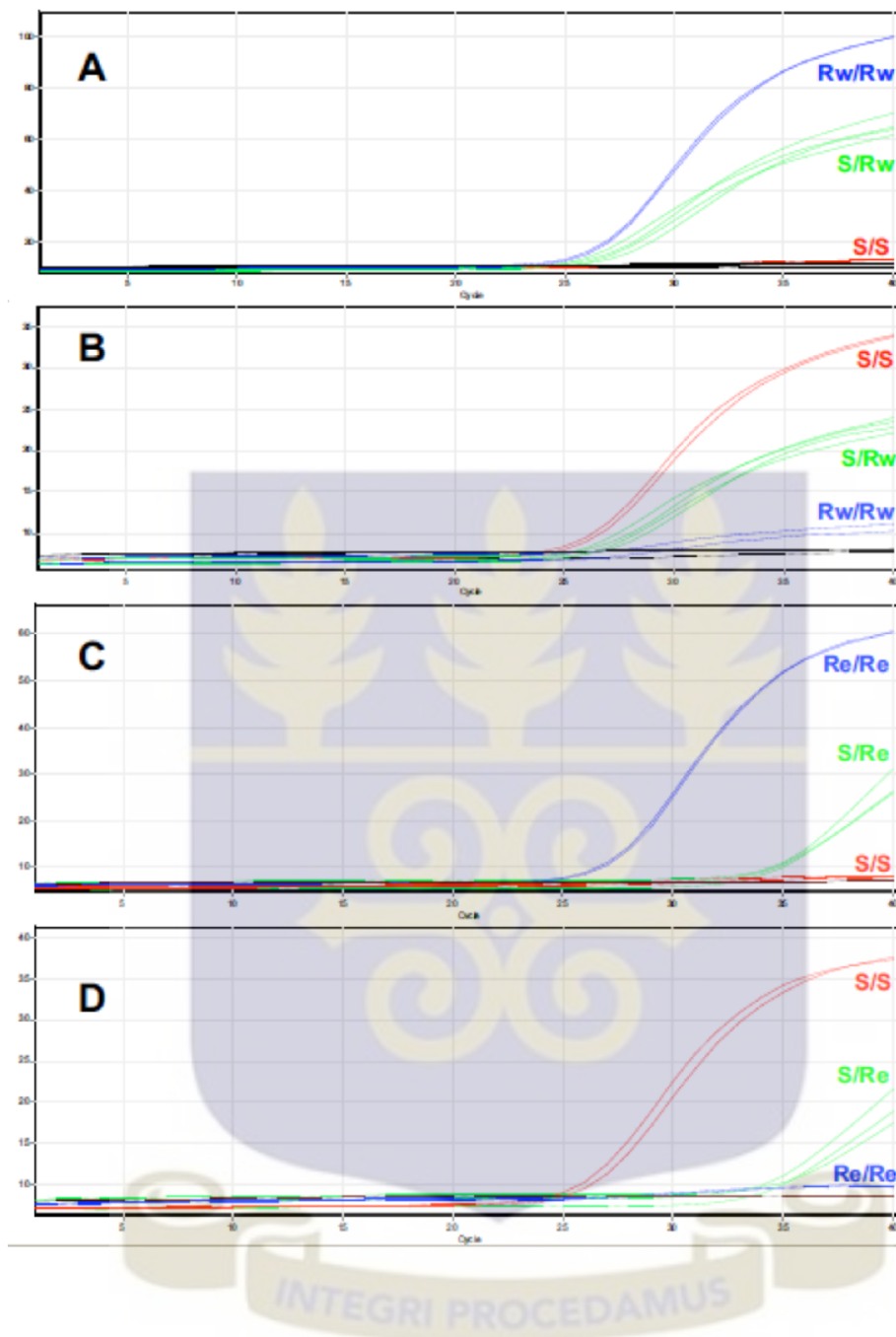


Figure 1. A) and B) Detection of the *kdr-w* mutation. C) and D) Detection of the *kdr-e* mutation. A) Cycling of FAM-labelled probe specific for the *kdr-w* allele. C) Cycling of the FAM-labelled probe specific for the *kdr-e* allele. B) and D) cycling of the VIC labelled probe specific for the wild type allele. S: Wild type allele (L1014), Rw: Resistant allele, West African mutation (L1014F), Re: Resistant allele, East African mutation (L1014S).

Where applicable, biological reference intervals and units

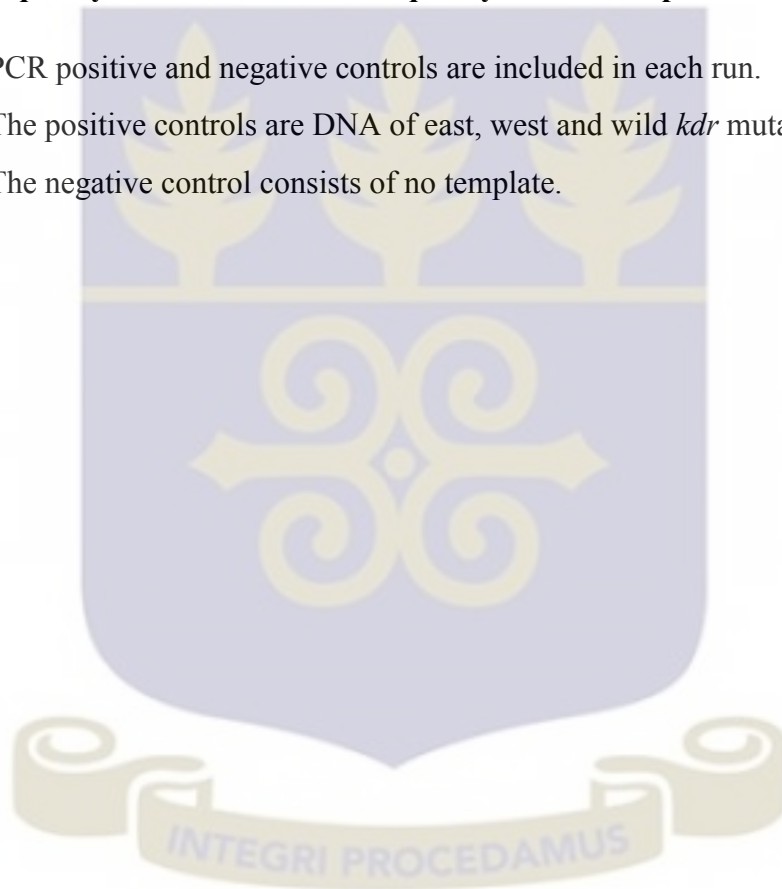
- None

Potential sources of variability

- None

Internal quality control and external quality assessment procedures

- PCR positive and negative controls are included in each run.
- The positive controls are DNA of east, west and wild *kdr* mutations.
- The negative control consists of no template.



APPENDIX IX: PCR protocol for identification of mammalian blood meal in mosquito samples (Kent and Norris, 2005)

Equipment and reagents required

PCR reagents:	10x PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl) 25mM MgCl₂ dNTP mixture (mixture containing 2.5mM of each dNTP) 50 uM of each primer: UnvRev1025, Pig573F, Human741F, Goat894F, Dog368F and Cow121F PCR-grade H₂O <i>Taq</i> DNA Polymerase (5U/μl)
PCR Machine:	A thermal cycler with a hot lid.
Pipettes:	0.2 -10 μl Micropipette; 20 – 200 μl Micropipette; 200 – 1000 μl Micropipette
Centrifuge:	A desktop centrifuge or microfuge capable of holding 1.5ml microcentrifuge tubes as well as the PCR tubes.
Vortex:	Desktop vortex.
Plasticware:	Autoclaved tips (0.2 -10 μl; 20 – 200μl; 200 – 1000 μl) 1.5ml Microcentrifuge tubes Thin walled, flat cap PCR tubes
Electrophoresis:	Electrophoresis tank Power supply unit 1 x TAE buffer 2.5% TAE agarose gel containing ethidium bromide Loading buffer (Fermentas O'Range 6 x Loading Dye) Molecular weight marker (Fermentas O'Range Ruler 100bp)

The preparation and storage of reagents

- When PCR reagents are not in use they can be stored at -20°C .
- Mw Marker is best stored at -20°C .
- Loading buffer should be stored at 4°C .
- TAE Buffer and ethidium bromide may be stored at room temperature.
- Dilute all primers to $50\text{pmol}/\mu\text{l}$ working concentration.

(Note: $1\text{pmol}/\mu\text{l} = 1\text{ }\mu\text{M}$. So for a $100\text{ }\mu\text{l}$ working solution, add $50\text{ }\mu\text{l}$ of a $100\text{ }\mu\text{M}$ stock primer to $50\text{ }\mu\text{l}$ dH_2O).

- Prior to use, all frozen reagents should be placed on ice to thaw.
- Once thawed, briefly vortex the reagents and spin them down in a desk top centrifuge.

Detailed step-by-step instructions on how to perform the test

Step 1:

- Count how many samples you will need to do PCR on.
- Remember to include the $-ve$ and $+ve$ controls for the DNA extraction and a no DNA control for the PCR.
- You should also include PCR positive controls which will be DNA extractions from blood of known origin (pig, goat, dog, cow, human).
- The number of samples above (including all the relevant controls) will be the value that you use when calculating your master mix volumes.
- If your number of tubes is just short of fifty or one hundred, round up to these values and use the tables below.
- Try to round up your number to the nearest five (For example: if you need to prepare for 33 tubes, prepare a master mix for 35).

Step 2:

- Make up the master mix using the recipe below:

Reagent	Per Specimen	Master Mix for 50 tubes	Master Mix for 100 tubes	Master Mix for X tubes
10x PCR Buffer	1.25 ul	62.5 ul	125ul	
10x dNTPs	1.25 ul	62.5 ul	125ul	
25 mM MgCl ₂	0.75 ul	37.5 ul	75 ul	
50 µM UnvRev1025 Primer	0.5 ul	25 ul	50 ul	
50 µM Pig573F Primer	0.5 ul	25 ul	50 ul	
50 µM Human741F Primer	0.5 ul	25 ul	50 ul	
50 µM Goat894F Primer	0.5 ul	25 ul	50 ul	
50 µM Dog368F Primer	0.5 ul	25 ul	50 ul	
50 µM Cow121F Primer	0.1 ul	5.0 ul	10 ul	
rTaq	5.65 ul	282.5 ul	565ul	
dH ₂ O				
Total	12 ul	600ul	1200ul	

Step 3:

- Pipette 12 µl of master mix into each labeled 0.2 ml PCR tube and add 0.5 µl of extracted DNA to make up a 12.5 µl reaction mixture.

Note: Set up positive controls using DNA extracted from Human, Cow, Pig, Dog and Goat blood as well as a non-template negative control containing master mix without DNA.

Step 4:

- Vortex lightly to mix DNA and master mix. Centrifuge briefly.

Step 5:

- Place the tubes in a PCR machine. Set the PCR to run the following program:
95°C for 5min

95°C for 1min }
58°C for 1min } 35 Cycles

72°C for 1min

72°C for 7min

Step 6:

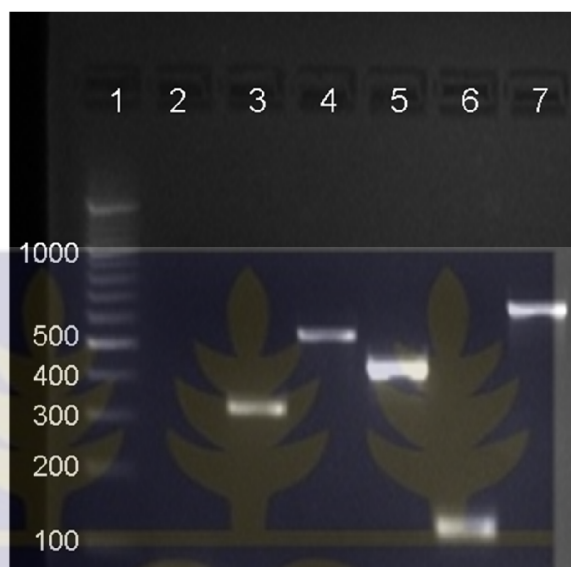
- After the PCR cycle has completed, remove all the PCR tubes from the PCR machine.
- Load the samples into a 2.5% TAE gel containing ethidium bromide.
- Use 2.5ul loading buffer for each 12.5ul PCR product.
- Remember to include at least one lane of Mw Marker and include all the controls on the same gel.
- Electrophorese the gel at 100V (<400mAmps), until the loading dye has separated and the leading dye front has just about run off the bottom of the gel.

Step 7:

- Visualize the amplicons on the gel using a gel doc system.
- Ensure that the PCR has worked correctly by sizing the products against the molecular weight marker.
- The human blood specific product is ~334 bp in length, pig blood is ~453 bp, goat blood is ~132 bp, dog blood is ~680 bp and cow blood is ~561bp.

Any necessary calculations

Size the resulting amplicons against the Mw Marker.



Lane 1: 100 basepair DNA Ladder (Fermentas O'range Ruler), Lane 2: no template control, Lane 3: Human blood, Lane 4: Cow blood, Lane 5: Pig blood, Lane 6: Goat blood, Lane 7: Dog blood.

Test acceptance criteria

The no DNA PCR control must not have any amplification in the product as visualized by no bands in this lane.

Interpretation of Test Results

Amplicon length is indicative of DNA belonging to one of the blood meal sources included in the assay.

Where applicable, biological reference intervals and units

Not Applicable

Potential Sources of Variability

Not Applicable

Internal quality control and external quality assessment procedures

Every set of PCRs run, must have positive and negative controls.

Failure to include positive and negative controls will result in the run being rejected.

Should external quality assessment be required, this can be performed by an independent repeat of the protocol or sequencing of the PCR products to confirm the species identification.



APPENDIX X: ELISA protocol for detecting Plasmodium falciparum in mosquito samples (Wirtz et al., 1992)**Equipment and reagents required****Plasticware:**

- Sterile pestles
- 1.5 ml reaction tubes
- Transparent 96 Well microtitre plate

Equipment:

- Multichannel micropipettes capable of aliquotting 50 and 100 μ l
- Measuring cylinders
- Microplate reader

Reagents:

- Casein
- Sodium hydroxide
- Phenol red
- Phosphate buffered saline
- Phenol Red solution
- Tween-20
- Igepal/CA-20
- Primary antibody (Pf-2A10-28, cat number: CDC-37-01-24-2: KPL)
- Secondary antibody (Pf-2A10 CDC15, cat number: 37-00-24-4: KPL)
- Substrate (50-66-01: KPL)

The preparation and storage of reagents

Buffer preparation:***Phosphate buffered saline (PBS)***

- Dissolve 1 PBS tablet in 1 litre distilled water to obtain a working 1X solution.
- If there are no PBS tablets available the buffer can be prepared as follows

For 1 litre of **10X** PBS:

80g NaCl

19g Na₂HPO₄·7H₂O

2g KCl

2g KH₂PO₄

Make up to 1 litre with distilled water.

PBS-Tween (PBS-T)

- Add 500 µl Tween-20 for every 1 litre of 1X solution

Blocking Buffer (BB)

	500 ml	1000 ml
Casein	2.5 g	5 g
0.1N NaOH	50 ml	100 ml
PBS	450 ml	900 ml
Phenol Red	0.1 ml	0.2 ml

- Suspend casein in NaOH. Use the microwave to bring the solution to the boil.
- Once casein has dissolved, add PBS to solution.
- Adjust pH to 7.0-7.4 using concentrated HCl.
- Add phenol red.

Grinding buffer

- 50 ml blocking buffer
- 250 µl Igepal/NP-40.

Store all buffers at 4°C for a maximum of 2 weeks.

Detailed step-by-step instructions on how to perform the test**Specimen and control preparation:*****Specimens***

- Bisect head and thorax from the abdomen of the female mosquito.
- Place head and thorax in 1.5 ml reaction tube.
- Add 50 µl of Grinding buffer to tube
- Homogenise sample in grinding buffer, using a sterilised pestle
- Wash off biological material from pestle with 150 µl of blocking buffer to result in a total specimen volume of 200 µl.

Positive control

- Mix 1 µl of dilute circumsporozoite protein (labeled vial II) with 50 µl blocking buffer.

Negative control

- Kill 7 samples of insectary reared females of the same species as your test specimens.
- Grind the heads and thoraces as described for specimens above.

Procedure

Step 1: Coating With Primary Antibody

- Make the primary antibody (Pf2A10, **clear** bottle) mixture by mixing 40µl in 5ml PBS/96 well plate
- Add 50 µl antibody mix to each well
- Cover plate with foil to prevent drying and store at 4°C.

Step 2: Blocking the Plate

- Pour Blocking buffer over plate ensuring wells are filled to capacity
- Incubate at room temperature for a minimum of 1 hour.

Step 3: Sample Hybridisation

- Pour off the blocking buffer. If necessary, the plate can be blotted off using paper towel.
- Add 50 µl of sample into the wells. Add one sample per well.
- Add the controls to the plate:
 - a. Add 50 µl of the positive control to well A1
 - b. Add 50 µl of each negative control to the final 7 wells of the plate.
- Incubate samples and control at room temperature for 2 hours.

Step 5: Initial Wash to Remove Unbound Samples.

- Shake samples vigorously out of wells. At this stage, the reagents are non-toxic, therefore they can be poured off down drain.
- Pour a generous (filling all wells) amount of PBS-Tween over plate, swirl and shake out.
- Repeat above step.
- Ensure the plate is dry by tapping out excess PBS on paper towel.

Step 6: Secondary Labeled Antibody

- Prepare the peroxidase antibody mix: add 10 µl peroxidase labeled antibody into 5.6 ml blocking buffer. The antibody is in a BROWN BOTTLE.
- Add 50µl of antibody to each well.
- Incubate the plate for 1 hour at room temperature.

Step 7: Secondary Wash To Remove Unbound Labeled Antibody

- As with step 5, wash wells out vigorously with PBS-Tween.
- Repeat the step until the plate is washed four times.
- Pat the plate dry as before.
- Remember that poor washing at this step will lead to false positives.

Step 8: Addition of Peroxidase Substrate

- Add 100 µl of ABTS 1 step peroxidase substrate aliquot per well.
- Stand at room temperature for 30 - 60 minutes
- Determine optical density at 405 nm on the 96-well plate reader

Any necessary calculations (i.e. Cut-off, etc)

- Calculate the critical cut of value as follows
$$[\Sigma(-ve\ controls)/7] \times 2$$
- All values above this critical value are classed as positive and must be repeated.

Test acceptance criteria (e.g. Negative control must be less than x)

- If the positive control does not change colour, the entire reaction has failed and must be repeated.

Interpretation of test results

- All samples with an optical density above the critical cut-off value are considered to be positive for *Plasmodium falciparum* protein.

Where applicable, biological reference intervals and units

- Not applicable.

Potential sources of variability

- The reaction is inhibited if the pH of the reaction varies from the neutral pH of 7.0 - 7.4.
- Poor washing techniques can lead to false positives.
- Omission of the non-ionic detergent Tween-20 from the washing solutions will lead to high background.

Internal Quality Control and External Quality Assessment procedures

- Positive samples are repeated to confirm positive status.
- When repeating, the positive sample must be heated to 100°C for 5 minutes prior to hybridisation.

