

**FIELD EVALUATION OF THE BIO-EFFICACY OF THREE BRANDS OF
PYRETHROID-BASED MOSQUITO COILS AGAINST *ANOPHELES GAMBIAE*
SENSU LATO, A MALARIA VECTOR, AT POKUASE IN THE GA WEST DISTRICT
OF GREATER ACCRA REGION, GHANA**

BY

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DECLARATION

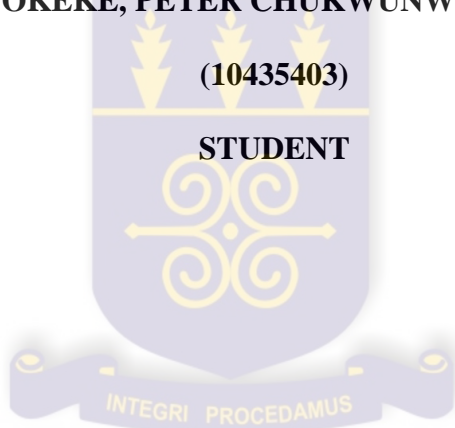
I hereby declare that all experimental works described in this thesis were carried out by me with exception to references made to other people's works published or not but who have all been duly acknowledged. This thesis has never been submitted anywhere else for award of a similar or different degree neither in whole nor in part.

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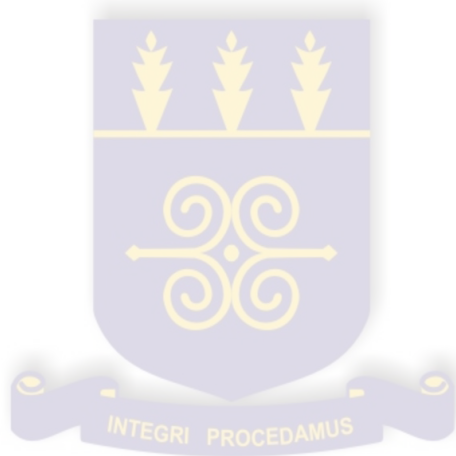
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DEDICATION

This work is dedicated to my lovely parents and siblings for their prayers and encouragement throughout my stay in Ghana.



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I thank God Almighty, who has remained my shield and my strong hold, and by his constant love and mercies has preserved and brought me thus far.

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ABSTRACT

As a result of the increasing demand for the use of mosquito coils in Ghana, as a means of personal protection against *Anopheles gambiae sensu lato*, it was important to evaluate the effectiveness of three selected mosquito coils under field conditions and also determine the resistance status of *Anopheles gambiae sensu stricto* against pyrethroid-based mosquito coils. Three brands of pyrethroid-based mosquito coils; Angel Jumbo coil[®], Lord Insecticide antimosquito coils[®] and Heaven Jumbo coil[®], were therefore evaluated at Pokuase, Ga district, in Greater Accra Region of Ghana. The human landing catch (HLC) technique was used in a Latin square design to collect indoor biting mosquitoes from three different rooms, each treated with the different brands of mosquito coils and a fourth room with no coil as the control room. Collection from each room was replicated four times for each of the different brands of the mosquito coil. Species identification of mosquitoes was done using morphological characteristics, PCR and *HhaI* restriction digest. A total of 935 *Anopheles gambiae sensu lato* were collected from rooms treated with the different coils as follows; 27 % (250), 16 % (150), 22 % (204) and 35 % (331) from rooms treated with Angel Jumbo coil[®], Lord Insecticide antimosquito coils[®], Heaven Jumbo coil[®] and control (no coil) respectively. The population of indoor biting mosquitoes from the treated rooms were significantly ($P = 0.006$) reduced in comparison with those from the control rooms. A percentage repellency of 26.9 %, 33.6 %, 51.4 % and 0 % was provided by Angel Jumbo coil[®], Lord Insecticide antimosquito coils[®], Heaven Jumbo coil[®] and control (no coil) respectively. The one hundred (100) *An. gambiae* s.l. examined were all identified as *An. gambiae* s.s., and consisted of 97 % S molecular forms, 2 % M forms and 1 % M/S hybrid. High level of resistance against the three pyrethroid-based coils was however observed because of the detection of the *kdr* gene in 98 % of the *An. gambiae* s.s. that were examined. The research findings, therefore, suggest that the tested coils cannot provide full protection against the malaria vector when used alone in areas where pyrethroid resistance has been developed by the mosquito vectors.

CONTENTS

TITLE PAGE	i
DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
CONTENTS	vi
LIST OF ILLUSTRATIONS	x
LIST OF TABLES	xi
LIST OF APPENDICES	xii
LIST OF ABBREVIATIONS	xiii
CHAPTER ONE	1
1.0 INTRODUCTION	1
1.1 Rationale	2
1.2 Objectives	3
1.2.1 Main Objective	3
1.2.2 Specific Objectives	3
CHAPTER TWO	4
2.0 LITERATURE REVIEW	4
2.1 Malaria	4
2.2 Malaria Vector	5
2.2.1 Taxonomic Information and Species Identification	5

2.2.2 Dominant Malaria Vector Species in Africa.....	6
2.2.2.1 <i>Anopheles gambiae</i> s.s.....	7
2.2.2.2 <i>Anopheles arabiensis</i>	9
2.2.2.3 <i>Anopheles melas</i>	10
2.2.2.4 <i>Anopheles merus</i>	12
2.2.3 Molecular Forms of <i>An. gambiae</i> s.s.	13
2.3 Malaria Control Strategies	14
2.3.1 Chemotherapy.....	15
2.3.2 Vaccination.....	16
2.3.3 Principles of Vector Control.....	16
2.4 Vector Control Strategies.....	17
2.4.1 Indoor Residual Spraying (IRS).....	18
2.4.2 Larval Control.....	18
2.4.3 Environmental Management and Source Reduction.....	19
2.4.4 Personal Protection	20
2.4.5 Novel Vector Control Strategies.....	20
2.5 Mosquito Coils.....	22
2.5.1 Pyrethroid-based Mosquito Coils	23
2.5.2 Mode of Action of Pyrethroids	25
2.6 Pyrethroid Resistance in <i>Anopheles gambiae</i>	26
2.6.1 Knockdown Resistance (<i>kdr</i>) Allele and the Excito-Repellent Effects of Pyrethroids	29
CHAPTER THREE	30

3.0 MATERIALS AND METHODS	30
3.1 Study Area	30
3.2 Selected Mosquito Coils Evaluated in the Field	31
3.3 Experimental Design	32
3.4 Mosquitoes Collection	32
3.5 Morphological Identification of Adult Mosquitoes	33
3.6 Molecular Studies	34
3.6.1 Extraction of Genomic DNA	34
3.6.2 Species Identification of <i>Anopheles gambiae</i> s.l. Using PCR Method	35
3.6.3 Gel Electrophoresis	36
3.6.4 Identification of Molecular Forms of <i>An. gambiae</i> s.s.	36
3.6.5 Detection of knockdown resistance (<i>kdr</i>) alleles in <i>Anopheles gambiae</i> complex	37
3.7 Data Entry and Analyses	38
3.7.1 Repellency Effect of the Coils	39
3.7.2 Biting Pressure of the Mosquitoes	39
CHAPTER FOUR	40
4.0 RESULTS	40
4.1 Mosquito Catches	40
4.2 Species Composition of the <i>Anopheles gambiae</i> s.l.	41
4.3 Identification of <i>Anopheles gambiae</i> s.s. Molecular Forms	42
4.4 Biting Pressure of <i>Anopheles gambiae</i> s.l. in the Study Area	44
4.5 Repellency Effect of the Mosquito Coils against <i>Anopheles gambiae</i> s.l.	45

4.6 Knockdown Resistance (<i>kdr</i>) Mutation in <i>An. gambiae s.s.</i>	48
CHAPTER FIVE	50
5.0 DISCUSSION	50
CHAPTER SIX	55
6.0 CONCLUSION AND RECOMMENDATIONS	55
6.1 Conclusion	55
6.2 Recommendations.....	56
REFERENCES	57
APPENDICES	82



LIST OF ILLUSTRATIONS

Figure 1: Map of Ghana showing Pokuase community where the sample collection was carried out31

Figure 2: Indoor collection of adult mosquitoes using the human landing catch (HLC) technique33

Figure 3: Distribution of the various species of mosquitoes collected during the 16 days of sampling.....40

Figure 4: Mean number of mosquitoes collected from the treated and control rooms.....41

Figure 5: Agarose gel electrophoregram of PCR amplified rDNA sequence of *An. gambiae* s.s.....42

Figure 6: Percentages of the different molecular forms of *Anopheles gambiae* s.s. detected in the samples tested.43

Figure 7: Agarose gel electrophoregram of *Hh1* restriction of *An. gambiae* s.s. amplified PCR products.43

Figure 8: Percentages of *Anopheles gambiae* s.l. collected from the rooms containing the different anti-mosquito coils.....44

Figure 9: Biting pressure of *Anopheles gambiae* s.l. for the different anti-mosquito coils45

Figure 10: Average percentage protection provided by the three coils against *An. gambiae* s.l.....46

Figure 11: Biting pattern of *Anopheles gambiae* s.l. in the Pokuase community.....47

Figure 12: Hourly percentage repellency of different anti-mosquito coils in Pokuasi area48

Figure 13: Agarose gel electrophoregram of PCR detection of the *kdr* gene49

LIST OF TABLES

Table 1: Primer sequences of *An. gambiae* s.l., expected band sizes and melting temperature (T_m) of the PCR amplified DNA products35

Table 2: Sizes of DNA fragments after PCR-RFLP for the S and M molecular forms of *An. gambiae* s.s.37

Table 3: Sequence details of the *kdr* primers and their melting temperatures (T_m)38

LIST OF APPENDICES

Appendix 1:	Preparation of standard solutions	82
Appendix 2:	Indoor human landing catches for 16 nights in different rooms treated with the Angel Jumbo coil, Lord insecticide anti-mosquito coil, Heaven Jumbo coil and control (no coil)	84
Appendix 3:	Analysis of variance for indoor biting mosquitoes from the different rooms treated with the different brands of anti-mosquito coils	88
Appendix 4:	Analysis of variance for the percentage repellency of the different brands of anti-mosquito coils against <i>An. gambiae</i>	89
Appendix 5:	Analysis of variance for the biting pressure of <i>An. gambiae</i> in the rooms treated with the different brands of anti-mosquito coils	90

LIST OF ABBREVIATIONS

µl	Microliter
b/m/n	Bites/human/night
bp	Base pair
ddH₂O	Double distilled water
dNTPs	Deoxyribonucleotide phosphates
DDT	Dichlorodiphenyltrichloroethane
EtBr	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
HLC	Human Landing Catches
IRS	Indoor residual spraying
<i>kdr</i>	Knockdown resistance
LLIN	Long lasting insecticide-treated net
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism

s.s.	<i>sensu stricto</i>
s.l.	<i>sensu lato</i>
TAE	Tris-Acetate EDTA
WHO	World Health Organisation
CDC	Centre for Disease Control
PMI	President's Malaria Initiative
NIAD	National Institute of Allergy and Diseases
NMCP	National Malaria Control Programme
GHS	Ghana Health Service

CHAPTER ONE

1.0 INTRODUCTION

Mosquitoes are one of the most important insects of medical importance. Apart from the fact that they cause serious nuisance by their biting activity during blood meal, they are also a threat to health as they transmit a number of diseases, including malaria (Avicor *et al.*, 2013). Malaria is a major public health disease in most tropical countries, causing significant mortality and morbidity despite concerted efforts directed at its control. Malaria remains the most important disease of global public health importance. It has an estimated annual death toll of about six hundred and sixty million people, mainly among pregnant women and children (WHO, 2013; Wang *et al.*, 2006; Piet, 2006). Malaria is hyper-endemic in Ghana (PMI, 2012) and is the leading cause of morbidity in adults as well as infant mortality among children below 5 years of age in the country (NMCP/GHS, 2008).

Malaria is caused by the *Plasmodium* parasite which is transmitted from one person to another through the bite of female *Anopheles* mosquito. Of the different types of mosquitoes that exist in nature, only the *Anopheles* species are known to transmit malaria. In Africa, *Anopheles gambiae* and *Anopheles funestus* are the main vectors of malaria (Sinka *et al.*, 2010). Various strategies have been employed to control the malaria disease, however, the reduction of vector-host contact through vector control has been the most effective. Malaria vectors control in Africa rely almost entirely on indoor residual spraying (IRS) and long lasting insecticide-treated nets (LLINs) in reducing malaria prevalence (Mittal *et al.* 1991; Barutwanayo *et al.*, 1991). These vector control tools have successfully reduced mosquito population densities as well as malaria by targeting

indoor-feeding (endophagic) and indoor-resting (endophilic) mosquitoes (Protopopoff *et al.*, 2007; Kim *et al.*, 2012; Skarbinski *et al.*, 2012). Despite the positive results recorded by the use of these tools, limited coverage has remained a major challenge in the successful control of malaria. Hence, in a bid to circumvent this limitation, several measures, either communal or personal, have been employed to reduce the rate of malaria infection by preventing vector-human contact. One of such methods is the use of anti-mosquito coils, which has been widely used for many years all over the world. In Ghana, burning of anti-mosquito coils is one of the most common personal protection measures used by individuals against mosquitoes (Avicor *et al.*, 2013). Mosquito coils are well known and frequently used (Baume and Franca-Koh, 2011) and are inexpensive to make and purchase, and effective in repelling mosquitoes (Ogoma *et al.*, 2012).

1.1 Rationale

The increase in the use of mosquito coils in Ghana, as a personal protection measure against mosquitoes has been greeted with divergent views as regards its efficacy; with opinions divided as to its potency to kill mosquitoes (Atagra, 2008). It was in this light that the efficacies of three selected pyrethroid-based mosquito coils were assessed in the laboratory (Adu-Acheampong *et al.*, 2014). Although the laboratory assessment of these mosquito coils revealed low percentage mortality, they still needed to be tested in the field. Hence, as a follow up to the laboratory assessment, the bioefficacy of the three pyrethroid-based mosquito coils against *Anopheles gambiae* s.l. were evaluated in the field and the mechanism of insecticide resistance in the mosquito species was also determined so that appropriate measures will be recommended for the control and management of these mosquitoes.

1.2 Objectives

1.2.1 Main Objective

To assess the efficacy of three selected pyrethroid-based anti-mosquito coils namely; Angel Jumbo coil[®], Lord Insecticide antimosquito coil[®] and Heaven Jumbo coil[®], against *Anopheles gambiae* s.l. in the field.

1.2.2 Specific Objectives

1. To determine the species composition of the *Anopheles gambiae* s.l. in Pokuase, Ga West District of Greater Accra region, Ghana.
2. To determine the biting pressure of the mosquitoes at Pokuase, Ga West District of Greater Accra Region, Ghana.
3. To determine the repellency effect of three mosquito coils; Angel Jumbo coil[®], Lord Insecticide antimosquito coils[®] and Heaven Jumbo coil[®] on *Anopheles gambiae* s.l.
4. To determine the knockdown resistance (*kdr*) gene frequency in the *Anopheles gambiae* s.s. collected from the study area.

CHAPTER TWO

2.0

LITERATURE REVIEW

2.1 Malaria

Malaria is a mosquito-borne infectious disease caused by a protozoan parasite of the genus *Plasmodium* (WHO, 2014). Even though more than 100 different species of *Plasmodium* exist (National Institute of Allergy and Diseases, 2007), the species known to infect humans include; *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*, *P. knowlesi* (CDC, 2012) and *P. cynomolgi* (Ta *et al.*, 2014). Symptoms of malaria appear about 9-14 days after a bite from an infected mosquito, and they typically include fever, sweating, rigours (shakes), fatigue, vomiting, headaches and other flu-like symptoms (CDC, 2012; Caraballo, 2014). Symptoms may also vary with the different *Plasmodium* species, for example malaria caused by *P. falciparum* is life threatening and can cause multiple organ damage, coma and death (CDC, 2012). Thus, malaria remains the most important vector-borne human disease (Frank and Susan, 1995) and its transmission relies on a dynamic interaction between the *Plasmodium* species, the vector and the vertebrate host (Galinski and Barnwell, 2009).

Despite concerted efforts and increasing international funding directed at the control and elimination of malaria throughout the world (Feachem and Phillips, 2009), the disease is still a major public health challenge, with an estimated population of 3.3 billion people being at risk of malaria, of whom 1.2 billion are at high risk. In high-risk areas, more than one malaria case occurs per 1000 population (WHO, 2014). In 2013, about 198 million cases of malaria and about 584,000 deaths were recorded worldwide, out of which 90 % of all malaria deaths occur in Africa (WHO,

2014). Malaria can be found in both the tropical and sub-tropical regions of the world. Areas of high transmission are found predominantly in rural areas in South America, South-East Asia, Western Pacific and throughout sub-Saharan Africa (CDC, 2012) where it contributes in large parts to the continued impoverishment of the populations in these areas, and is the main cause of infant mortality and adult morbidity (Holstein, 1958; National Institute of Allergy and Diseases, 2007).

Malaria is hyper-endemic and perennial in all parts of Ghana, placing the entire population of 24.2 million at risk of malaria infection (PMI, 2014). In Ghana, malaria is a major cause of low productivity and poverty (NMCP/GHS, 2008) accounting for 33 % of hospital deaths in children under five years, 38 % of all outpatient illness and 36 % of all admissions (PMI, 2014).

2.2 Malaria Vector

The malaria parasite is typically transmitted to people through the bite of an infected female mosquito of the genus *Anopheles* during a blood meal. This intake of blood meal by the female mosquito constitutes the link between the human host and the mosquito vector in the life cycle of the *Plasmodium* parasite (CDC, 2012).

2.2.1 Taxonomic Information and Species Identification

Mosquitoes have been grouped into 41 genera which contain 3,500 species. Out of the 430 *Anopheles* species that have been recognised, only about 30 to 40 species transmit malaria in nature (CDC, 2012). In Africa, the main transmitter of the malaria parasite is the *Anopheles gambiae* (Yeya *et al.*, 2004), which includes the most important vectors of malaria in sub-Saharan Africa, and was recognised as a complex in the 1960s (Coetzee *et al.*, 2000). The *Anopheles gambiae*

Giles complex comprises 7 cryptic species (Scott *et al.*, 1993; Yeya *et al.*, 2004); these include six named species and one unnamed species (Coetzee *et al.*, 2000). The six named species were assigned the following scientific names: *An. gambiae* s.s., *An. arabiensis*, *An. quadriannulatus*, *An. bwambae*, *An. merus* (East African salt-water breeder), and *An. melas* (West African salt-water breeder). The unnamed species from Ethiopia was reported in 1998, and is known as *An. quadriannulatus* species B (Coetzee *et al.*, 2000). Although individual species of the *Anopheles gambiae* complex are morphologically indistinguishable, they however they exhibit different behavioural traits (Fanello *et al.*, 2002).

Owing to the fact that individual species in the *Anopheles gambiae* complex are morphologically indistinguishable, various methods have been employed to accurately identify each of them. These methods include chromosomal banding arrangements (Coluzzi, 1968), isoenzyme electrophoresis (Miles, 1979), cross-mating techniques (Davidson *et al.*, 1967), salinity tolerance tests (Muirhead-Thomson, 1951) and DNA studies using PCR (Scott *et al.*, 1993).

2.2.2 Dominant Malaria Vector Species in Africa

The bulk of the malaria burden is found in Africa due, in part, to the presence of *An. gambiae* complex in the continent (Sinka *et al.*, 2010). The dominant malaria vector species are found in the *An. gambiae* complex, *An. funestus* group, *An. nili* and *An. moucheti*. Within the *An. gambiae* complex, the dominant vector species of human malaria include *An. gambiae* s.s., *An. arabiensis*, *An. merus* and *An. melas*.

2.2.2.1 *Anopheles gambiae* s.s.

The *An. gambiae* s.s. is one of the most well studied malaria vector, probably because of its high vectorial capacity which has given it the status of one of the most efficient vectors of malaria in the world (Coetzee, 2004). Studies have shown that it is undergoing a complicated process of incipient speciation, particularly in West Africa and has been characterized at both chromosomal and molecular levels (Coluzzi *et al.*, 2002). These species include five chromosomal forms: Forest, Bamako, Savannah, Mopti and Bissau, that are separated based on the variable ecological conditions within the large geographical range of the *An. gambiae* (Coluzzi *et al.*, 1985), and two molecular forms: ‘M’ and ‘S’ (della Torre *et al.*, 2001). Both categories are adapted to different larval breeding habitats, for example the Mopti and M forms are typically found in semi-permanent, often man-made, larval habitats such as rice fields or flooded areas, while the Savannah/Bamako and S forms are seen more commonly in temporary, rain-dependent sites such as ground puddles (della Torre *et al.*, 2005; Diabate *et al.*, 2008).

(i) **Breeding site ecology**

The larvae of *Anopheles gambiae* s.s. are typically found in sunlit, shallow, temporary bodies of fresh water such as ground depressions, puddles, pools and hoof prints (Minakawa *et al.*, 2004; Edillo *et al.*, 2002). This choice of larval habitat allows members of the *An. gambiae* complex to avoid most predators, and also the larvae are able to develop very quickly; six days from egg to adult under optimal conditions and temperatures (Gillies and de Meillon, 1968). This rapid development may be a physiological adaptation to the temporal and short-lived nature of their larval habitats (Sinka *et al.*, 2010). Water in these larval sites can appear clear, turbid or polluted (Edillo *et al.*, 2002). Typically *An. gambiae* s.s. larval habitats are described as containing no (or very sparse) vegetation due to their temporary nature.

(ii) Host preference

The *Anopheles gambiae* s.s. is a relatively long-lived species (Olayemi and Ande, 2009) with a short larval development period. These larvae are often found in habitats associated with human activity, where they can easily gain access to their human hosts. Even though *An. gambiae* s.s. is considered to be highly anthropophilic and shows high preference for human hosts, (Dabire *et al.*, 2008), a number of studies indicate that *An. gambiae* s.s. is not very strict in its host specificity, but rather opportunistic in its selection of host. In other words, its choice of host is influenced by location, host availability and the genetic make-up of the mosquito population (Sinka *et al.*, 2010). This holds true for various studies conducted in which *An. gambiae* s.s. showed no statistically significant difference in its host preference when presented with human-baited net trap and calf-baited net trap (Diatta *et al.*, 1998; Duchemin *et al.*, 2001; Bøgh *et al.*, 2001).

(iii) Feeding behaviour

The *An. gambiae* typically feeds late at night, and is often described as endophagic (biting indoors) and endophilic (resting indoors) (Appawu *et al.*, 2004). While this endophagic and endophilic behaviour may hold true for some studies, other studies have shown that there is no difference in the number of females collected from indoor and outdoor human-landing catches when compared (Wanji *et al.*, 2003; Cano *et al.*, 2004; Oyewole and Awolola, 2006). One of such study is by Odiere *et al.* (2007), in which outdoor resting females were sampled in western Kenya using clay pots. The study showed that there was no clear preference for indoor or outdoor resting sites. They therefore suggested that the designation of *An. gambiae* s.s. as a predominantly endophilic species may be as a result of poor sampling comparisons. Thus, *An. gambiae* s.s. appears to be more opportunistic in its blood feeding and resting locations than commonly thought. Considering the fact that, in Sierra Leone the Forest forms of *An. gambiae* s.s. demonstrated stronger exophily

while the Savannah forms were mostly endophilic, the exophilic and endophilic behaviour of *An. gambiae* s.s. may therefore be linked to their chromosomal forms (Bockarie *et al.*, 1993).

2.2.2.2 *Anopheles arabiensis*

Depending on geographical location, *An. arabiensis* is known to have a wide range of feeding and resting patterns (Sharp and Lesueur, 1991), however, when compared to *An. gambiae* s.s. it is said to be zoophilic, exophagic and exophilic (White, 1972). As a result of its wide range of feeding and resting patterns, *An. arabiensis* exhibit some forms of behavioural plasticity which confers adaptive advantage on it. This enables it to evade the effect of indoor residual spray (IRS) used in the control of endophilic mosquitoes (Coluzzi *et al.*, 1979), by avoiding sprayed surfaces (behavioural avoidance) depending on the type of insecticide used (Ameneshewa and Service, 1996).

(i) Breeding site ecology

Anopheles arabiensis is typically found in dry, savannah environments and sparse woodland. It is known to occur in forested areas that have recently been cleared or disturbed by human activity (Coetzee *et al.*, 2000). Its larvae are generally found in small, temporary, sunlit, clear and shallow fresh water pools, similar to those of *An. gambiae* s.s. (Edillo *et al.*, 2002; Himeidan and Rayah Eel, 2008). In addition to these habitats, they are also associated with slow flowing streams that are partially shaded (Shililu *et al.*, 2007) muddy waters (Gimnig *et al.*, 2001) and a variety of large and small natural and man-made habitats.

(ii) Host preference

Anopheles arabiensis has been reported to exhibit either anthropophilic or zoophilic behaviour (Sinka *et al.*, 2010). It shows a great deal of behavioural variability with respect to its feeding preference (Bøgh *et al.*, 2001). Although studies have shown that the populations found in western Africa display higher levels of anthropophily, and preferentially feed and rest indoors, whereas those in the east exhibit greater zoophily and exophilic behaviours. The biting patterns tend to be generally exophagic (Tirados *et al.*, 2006) especially when compared with *An. gambiae* s.s., a highly endophagic species (Sinka *et al.*, 2010).

(iii) Feeding behaviour

They generally feed at night and have peak biting times which begin in the early evening (19:00 hrs.) or early morning (03:00 hrs.) (Tirados *et al.*, 2006; Oyewole and Awolola, 2006). It also has a predisposition to exophilic behaviour regardless of where it has blood fed or the source of its meal (Tirados *et al.*, 2006; Mahande *et al.*, 2007).

2.2.2.3 *Anopheles melas*

Despite the fact that *An. melas* has a comparably lower sporozoite rate than either *An. gambiae* s.s. or *An. arabiensis*, it is still a vector of malaria especially in coastal areas where it can occur in very high densities (Bryan, 1983). In areas where it occurs in sympatry with *An. gambiae* s.s. or *An. arabiensis* however, it is generally considered to be a vector of less importance (Sinka *et al.*, 2010).

(i) Breeding site ecology

Anopheles melas is generally restricted to coastal areas (Muirhead-Thomson, 1946; Muirhead-Thomson, 1948) and is known to utilize saline environments that other species cannot tolerate, and commonly associated with brackish water (Caputo *et al.*, 2008) although it does not appear to require brackish water for the development of its larvae (Muirhead-Thomson, 1946). As an adaptation to surviving some degree of desiccation during low tide, *An. melas* preferentially oviposits on damp ground, rather than in open water (Muirhead-Thomson, 1946). This behaviour ensures that the larvae have ample time to complete the larval development and pupate in the less saline water of the new tide before it begins to recede, and at this point the water either becomes too salty or dries out completely (Giglioli, 1965).

(ii) Host preference

Anopheles melas has been described as a highly anthropophilic species, and at the same time, a zoophilic species as well, however, adult biting behaviour appears to be opportunistic (Tuno *et al.*, 2010), as they have been shown to feed more on the most available host. Experiments have shown that in areas where there are more animal hosts than human hosts, *An. melas* would feed more on the animal hosts, but still feed on the human hosts. Conversely, when the human host is more than the animal host, more *An. melas* would feed on the human host. When sampling for the purpose of determining the host preference of *An. melas*, bias towards anthropophily may be reported when blood fed females tested for host blood type were collected resting indoors (Muirhead-Thomson, 1948).

(iii) Feeding behaviour

Blood feeding activities of *An. melas* appears to be fairly continuous all through the night (Tuno *et al.*, 2010). Generally, *An. melas* appears to rest outdoors after feeding (Muirhead-Thomson, 1948), however, females that bite and rest indoors are more likely to have fed on humans, and those biting or resting outdoors are more likely to have fed on animals (Sinka *et al.*, 2010).

2.2.2.4 *Anopheles merus*

(i) Feeding behaviour

Anopheles merus, hitherto considered as an unimportant vector and ‘potentially unable to sustain malaria transmission alone (White, 1974), has been incriminated in the transmission of malaria along the Tanzanian coast (Temu *et al.*, 1998). Before the advent of molecular techniques, identification of *An. merus* was based on physiological characteristics involving larval salinity tolerance tests. This may be because the morphological differences in the egg and larvae used to distinguish *An. melas* from *An. gambiae* do not occur in *An. merus* (Muirhead-Thomson, 1951).

(ii) Breeding site ecology

Anopheles merus is usually associated with shallow brackish pools and marsh or swamp areas along the coast, where they are found in high numbers. They are also known to occur further inland where they use salt pans and saline pools as larval habitats (Govere *et al.*, 2000; Masendu *et al.*, 2005).

(iii) Host preference

An. merus is generally not strict in its choice of host rather it feeds on any available host. Thus, it is opportunistic in its host selection and has a tendency to bite and rest outdoors (Iyengar, 1962). Whilst a test in which females of *An. merus* were given a choice of human and animal host showed that *An. merus* has a preference for animal host, as they consistently fed on the animal host (Gillies and de Meillon, 1968). Another study indicated that there is no obvious preference, based on the fact that blood meal analysis of mosquitoes collected resting indoors indicated that a higher percentage of the mosquitoes had fed on humans, likewise, those collected resting outdoors indicated that a higher percentage of the mosquito had fed on cattle (Iyengar, 1962). An objective conclusion therefore, on the feeding preference of *An. merus* must be based on the comparative analysis of the test results from indoor and outdoor resting specimens. Examination of the biting times of *An. merus* showed the peak biting period to occur between midnight and 01:00 hrs. (Mutero *et al.*, 1984).

2.2.3 Molecular Forms of *An. gambiae* s.s.

Studies of the polytene chromosome of populations of *An. gambiae* in West Africa have revealed the existence of five chromosomal forms based on paracentric inversion polymorphism on the second chromosome (Coluzzi *et al.*, 1979; Toure *et al.*, 1994). These chromosomal forms are associated with specific ecological zones and have been designated with a non-Linnaean nomenclature: Bamako, Mopti, Savanna, Forest and Bissau (Toure *et al.*, 1998). Further research on the pattern of variation observed with molecular markers that are not linked to inversion polymorphism revealed the existence of two genetic variants referred to as ‘M and S’ forms (Favia *et al.*, 1994; della Torre *et al.*, 2001; Wondji *et al.*, 2002). The M form of *An. gambiae* s.s. is known to be associated with permanent breeding conditions such as those found in flooded or irrigated

sites, whereas the S form is typically found in temporary, rain-dependent breeding sites (Diabate *et al.*, 2005; Kabula *et al.*, 2011).

The two molecular forms have been observed to exist in sympatry, as revealed by various studies in Ghana (Kabula *et al.*, 2011), Mali (Edillo *et al.*, 2002.) and Kenya (Gimnig *et al.*, 2001). However, the ability of one form to predominate a particular site is an indication that the two forms do not have the same ability to exploit the shared habitat (Kabula *et al.*, 2011). Despite the fact that these molecular forms exist in sympatry, low level of hybridisation has been observed between them (della Torre *et al.*, 2001; Kabula *et al.*, 2011). This has been hypothesized to be due to partial reproductive isolation among the molecular forms, with gene flow occurring only in certain geographical locations or at certain seasons (Black and Lanzaro, 2001). Determination of the intra- and inter-form variation in M and S forms in Ghana and southern Burkina Faso, using microsatellite loci, suggested that inter-form hybridisation occurs at significant levels (Yawson *et al.*, 2007).

2.3 Malaria Control Strategies

As a result of the importance of the malaria in terms of economic burden and lives lost, various control measures have been employed to effectively control the disease. One of the initial attempts by the World Health Organization (WHO) to eliminate malaria was a massive worldwide campaign launched in the mid-1950s. The program which combined the use of insecticide spray and drug treatment recorded many successes at the beginning, completely eliminating malaria in some areas of the world (National Institute of Allergy and Diseases, 2007). It was however not long before a combination of factors soon negated the initial success, resulting in the resurgence of malaria in some areas where the disease had been eliminated. While some of the factors that

impinged on the successful control of malaria were administrative, and others financial, the most disconcerting challenge was the development of resistance by the *Plasmodium* parasite to antimalarial drugs and the vectors to insecticides (National Institute of Allergy and Diseases, 2007; Corbel *et al.*, 2012). Some of the strategies employed in the control of malaria include the use of chemotherapy and vector control.

2.3.1 Chemotherapy

The use of anti-malarial drugs have historically played a key role in reducing the prevalence of malaria infection and the incidence of clinical cases, thereby resulting in the control of malaria in endemic areas (National Institute of Allergy and Diseases, 2011). Mortality and morbidity from malaria among young children and pregnant women living in endemic areas have also been effectively reduced using chemoprophylaxis. This is however, difficult to sustain and has been shown to impair the development of natural acquired immunity (Greenwood, 2004).

One of the major challenges in the control of malaria using antimalarial chemotherapy is the development of resistance to anti-malarial drugs by the *Plasmodium* parasite (Caraballo and Rodriguez-Acosta, 1999). The spread of this resistance over the years has contributed to the re-emergence of malaria in endemic areas, especially sub-Saharan Africa (National Institute of Allergy and Diseases, 2011). Hence, in order to prevent or minimise the development and spread of drug resistant malaria, there is the need for the optimization of malaria treatment policies, with special emphasis on drug quality, rational prescription and the determination of the most appropriate and most effective pattern of drug use under different circumstances (Geoffrey, 1984).

2.3.2 Vaccination

The development of an effective vaccine against malaria will be a huge step towards the successful control of malaria, especially in endemic regions. Unfortunately, there is, at the moment, no licensed vaccine against malaria in existence. That notwithstanding, several candidate vaccines that target various life cycle stages of the malaria parasite are in development including the exploration of novel vaccine strategies, such as transmission-blocking vaccines, which work by blocking transmission of the malaria parasite to the mosquito vectors (National Institute of Allergy and Diseases, 2011).

The promising results of the RTS,S malaria vaccine, which is currently in its third phase of clinical trials, is an indication that the time and resources invested in the development of a malaria vaccine that can be administered by a safe and simple schedule seem to be paying off. It is therefore expected that an effective, long lasting malaria vaccine will be available for general use in the nearest future (MVI, 2015).

In the face of these challenges, eliminating malaria from most endemic areas remains a distant, huge, but surmountable task. In furtherance of this goal of eliminating malaria, the use of artemisinin-based combination therapy, long-lasting insecticidal nets (LLIN), indoor residual spraying of insecticides (IRS) and intermittent preventive treatment during pregnancy are the recommendations of the World Health Organisation to combat malaria (Corbel *et al.*, 2012).

2.3.3 Principles of Vector Control

As a strategy in the control of malaria, the main goal of vector control is to decrease the vectorial capacity of the mosquitoes below the critical threshold needed to achieve a malaria reproduction

number (R_0 , expected number of hosts who would be infected after one generation of the parasite by a single infectious person in a population) of less than one (Corbel *et al.*, 2012).

Targeting the adult mosquito vector as a strategy for the control of malaria is generally favoured because of the relative ease with which houses can be identified and mapped, as well as the correlation that exist between transmission intensity and the mortality rate of adult female mosquito vectors (Corbel *et al.*, 2012). In order for a mosquito vector to transmit malaria, it must ingest infectious gametocyte forms of the malaria parasite from an infected person during a blood meal, survive long enough for the gametocytes to transform into infectious sporozoites, and then transfer the sporozoites to another susceptible human host during a blood meal. Therefore, control measures that increase the mortality rate of adult female mosquitoes entering houses breaks the transmission cycle, thereby suppressing malaria transmission (Kouznetsov, 1977).

2.4 Vector Control Strategies

Current interventions in the control of malaria are geared towards domestic protection against adult mosquitoes (Guyatt *et al.*, 2002). This is aimed at reducing human-vector contact (WHO, 2009; Enayati and Hemingway, 2010), thereby reducing malaria transmission, thus, vector control is a fundamental element of the existing global strategy to fight malaria. Disease transmission in endemic areas has been successfully reduced using vector control measures. Some of the strategies used against the malaria vector include the use of indoor residual spraying (IRS) (Pluess *et al.*, 2010), larval control, environmental management and source reduction (National Institute of Allergy and Diseases, 2011), and the use of personal protection such as long lasting insecticide treated bed nets (Noor *et al.*, 2009) and anti-mosquito coils.

2.4.1 Indoor Residual Spraying (IRS)

Indoor residual spraying (IRS) involves careful, controlled spraying of insecticides along the inside walls of a building (Kolaczinski *et al.*, 2007). Its impact is dependent on a number of factors such as; the use of an effective insecticide, adequate coverage, and a sufficient number of spray rounds per year to adequately reduce the population of the vector (Skarbinski *et al.*, 2012). Chemicals recommended by WHO for use in IRS, include dichlorodiphenyltrichloroethane (DDT), bendiocarb, malathion, lambda-cyhalothrin and alphacypermethrin (WHO, 2006).

Indoor residual spraying (IRS) is considered to be one of the most effective methods for reducing malaria burden worldwide (Kolaczinski *et al.*, 2007) as it has become an increasingly popular method of malaria control using insecticides, and many recent studies have reported on its efficacy in reducing or interrupting malaria transmission in a single community or region (Kim *et al.*, 2012). Evidence of the efficacy of IRS has been available since the 1940s, and many historical examples of successful IRS programs exist from the Global Malaria Eradication Program era in the 1950s to 1970s (Najera *et al.*, 2011).

As a result of its efficacy, IRS is increasingly being adopted as a vector control strategy in areas of intense transmission in sub-Saharan Africa (Skarbinski *et al.*, 2012). This accounts for the recent increase in the use of IRS in sub-Saharan Africa, which has had a huge impact in malaria control (Protopopoff *et al.*, 2007).

2.4.2 Larval Control

Larval control involves the use of measures that eliminate mosquito larvae before they reach the adult stage which can transmit malaria. While this may seem to be an ideal approach to mosquito control, theoretically, it has however not been an effective approach. These shortcomings in the

designation of the larval stage as a target for vector control could be attributed to, but not limited to, the fact that larval habitats may be small, widely dispersed, and transient (CDC, 2012).

Larval mosquito control for prevention of malaria in Africa has not been attempted on a large scale, because of the difficulty in predicting when and where the larval breeding sites of the *An. gambiae* vector will form so as to treat them before the adult mosquitoes emerge (CDC, 2012).

2.4.3 Environmental Management and Source Reduction

The fundamentals of the concept of environmental management are the environmental modification and manipulation of the vector breeding sites and the reduction of human/vector contact (Rojanapremsuk *et al.*, 1988). The control of mosquito vectors by this means has been known to be effective and simple (WHO, 1982; Thevasagayam, 1985), and a significant decrease in both the malaria vector and the incidence of malaria has been recorded in endemic areas where environmental management had been implemented (Rojanapremsuk *et al.*, 1986).

A caveat in the implementation of environmental management is the fact that naturally occurring predator-prey relationships may be destroyed, plant refuges may be eliminated, or the overall community structure may be altered. Disruption of any or all of these elements may inadvertently promote vector populations and/ or create niches for other disease-transmitting mosquito species. There is therefore the need for the documentation of changes to the overall community structure so as to ensure accurate assessment of the long term impact of the strategy, in terms of practicality and effectiveness (Grieco *et al.*, 2005).

2.4.4 Personal Protection

Personal protection measures include the use of window and door screens, long lasting insecticide-treated nets (LLINs), repellents such as mosquito coils and wearing light-colored clothes such as trousers, and long-sleeved shirts, especially when outdoors in areas where mosquitoes bite outdoors (CDC, 2012).

Bednets impregnated with pyrethroids can significantly reduce vector/host contact, thereby reducing feeding on humans. By so doing, they effectively reduce the annual infective biting rate and markedly reduce transmission potentials (Pedersen and Mukoko, 2002). Well-constructed houses with window screens, as a personal protection measure, are effective for protection against indoor biting mosquitoes. Recent studies suggest that repellents may also be effective in reducing malaria transmission (CDC, 2012).

2.4.5 Novel Vector Control Strategies

(i) Sterile male release

This involves the release of sterile male mosquitoes into the area so that they can mate with the female mosquitoes, which are the vectors. This results in the production of non-viable offspring by the female mosquito. Thus reducing the population of mosquito vectors in the area; and consequently, reducing malaria transmission in the area. This has been successfully applied in several small-scale areas. However, the need for large numbers of mosquitoes for releases makes this approach too expensive and impractical for most areas (CDC, 2012).

(ii) Genetic modification of malaria vectors

Genetic modification aims to develop mosquitoes that are refractory to the parasite. This approach is, however, still many years from application in field settings (CDC, 2012).

Of the different strategies available for vector control, the most successful are indoor residual spraying (IRS) and insecticide-treated nets (ITNs), including long-lasting insecticide treated nets (Raghavendra *et al.*, 2011), which have had very huge impact in endemic countries where outstanding successes have been recorded (WHO, 2009; Feachem and Sabot, 2008). Despite the considerable success of malaria control programs in the past, using IRS and LLINs, malaria still remains a major public health problem in several countries due to limited coverage of control interventions. This limitation in malaria vector control was corroborated by WHO (2013) which reported a slowdown in the expansion of interventions to control mosquitoes for the second successive year, particularly in providing access to insecticide-treated bed nets. This has been primarily due to lack of funds to procure bed nets in countries that have ongoing malaria transmission.

In order to further reduce the disease burden, there is thus the need to explore other synergistic approaches and to ensure integration of vector control activities. The need for this synergistic approach cannot be over emphasized as past events have shown that parasites and mosquitoes will, over time, continue to evolve ways of circumventing malaria control tools, no matter how effective they may be, if used in isolation or used ineffectively. One of such tools that have been recommended for use as a supplement to other control measures is the use of anti-mosquito coils (Avicor *et al.*, 2013).

2.5 Mosquito Coils

The use of chemical repellants in mosquito coils have been in existence since 1980 when stick-type mosquito coils were first commercialized in Japan. In 1985 the shape was improved to the spiral type which is widely used presently, and still tops the list of annual worldwide use of the four major types of household insecticide formulations of mosquito coils, aerosols, vapourizing mats and liquid vapourizers (Katsuda *et al.*, 2008). Mosquito coils, which are inexpensive to make and purchase (Ogoma *et al.*, 2012), are made from a paste of powdered insecticide and a filler such as sawdust, which is moulded into a spiral shape. When using the coil, it is mounted on a metal stand, and the free end of the spiral is lit. Once alight, the coil will smoulder at a steady rate, releasing smoke that acts as a carrier to distribute the insecticide throughout the room. Coils thus function as fumigants (Lawrance and Croft, 2004), and are effective in repelling mosquitoes (Ogoma *et al.*, 2012).

The mosquito coil is one of the most common household insecticide formulations used in the control of adult mosquitoes. Official guidelines on malaria prevention often recommend the use of mosquito coils as an effective means to prevent malaria (Bradley and Bannister, 2001). They are readily portable, and may appeal to some travelers who do not wish to take anti-malaria drugs. Compared to malaria chemoprophylaxis, mosquito coils are affordable (Lawrance and Croft, 2004). They are commonly used in numerous households in Asia, South America and Africa (Liu *et al.*, 2003), including Ghana where about 43 % of mosquito coil users use them on a daily basis (Baume and Franca-Koh, 2011; Adu-Acheampong *et al.*, 2014).

2.5.1 Pyrethroid-based Mosquito Coils

Coils may differ markedly in their active components, whereas some contain allethrin or another pyrethroid, others may contain DDT or lindane (Stürchler, 2001). The main active ingredients recommended by the World Health Organization (WHO) for use in the vapour phase, however, all belong to the pyrethroid chemical class, and the most commonly used format for the vapor phase is mosquito coil (Liu *et al.*, 1987). In pyrethroid-based mosquito coils, pyrethroids and natural pyrethrins are used as active ingredients, due to their rapid effects on insects at minimal dosages (Katsuda *et al.*, 2008), hence, conferring protection against mosquito bites by the spatial action of the emanated airborne pyrethroid particles (Ogoma *et al.*, 2012); the airborne insecticide particles present inside and around houses create a chemical barrier that prevents mosquitoes from entering the homes (Pal, 1964).

The spatial activity of airborne insecticide is dependent on environmental factors such as airflow, wind speed, temperature and humidity within the treated area (Kawada *et al.*, 2006). Increased air current increases the rate at which the insecticide particles are dispersed over a specified area, thereby diluting the chemical attractants from the humans and consequently reducing host attack by mosquitoes (Hoffmann and Miller, 2002).

Generally, mosquito responses to airborne insecticide particles of mosquito coils are classified into five known antimosquito properties namely: feeding inhibition, repellency, deterrence, knock-down and mortality (Ogoma *et al.*, 2012). Feeding inhibition is where mosquitoes are prevented from taking blood meal from humans. The insecticidal vapor released in the smoke of a burning mosquito coil is effective in reducing the number of biting mosquitoes, or at least the number of mosquito bites received by a host in the immediate vicinity of a burning coil (Lawrance and Croft,

2004). In the field, deterrence has been measured by comparing the number of mosquitoes entering houses treated with insecticides and those without (Ogoma *et al.*, 2012). Studies have also shown that coils containing pyrethrins deter between 45 % and 80 % mosquitoes. They also induce up to 95 % mortality in laboratory assays compared to very low levels observed in field assays (3 % - 16 %). This is attributed to volume and/or ventilation limitations that may occur in some laboratory assay spaces, which reduce insecticide dispersion, consequently increasing relative insecticide concentration (Ogoma *et al.*, 2012).

Although most studies report a reduction in the feeding activities of mosquitoes in rooms treated with coils, it is however possible that the bite reduction reported in the majority of trials could have been caused by more than one of the five antimosquito properties of coil smoke. For example, a combination of direct bite inhibition, repellence and deterrence may work additively or synergistically to interfere with a mosquito's ability to locate a suitable host, thus, it is difficult to quantify precisely how effective coils are in terms of these modes of action (Lawrance and Croft, 2004).

Msangi *et al.* (2010) had earlier stated that mosquito coils are effective personal protection tools against mosquitoes, Avicor *et al.* (2013), on the other hand, opined that mosquito coils cannot offer an overwhelming degree of protection against mosquitoes when used as the only anti-mosquito method. Ordinarily, one would assume that a reduction in the number of mosquito bites would seem likely to lower the potential for vector-borne disease. This may not be the case for malaria transmission as it cannot be inferred that mosquito coils reduce transmission (Lawrance and Croft, 2004) considering the fact that just one infective anopheline bite is needed to transmit malaria

(Winstanley 1998). Thus a modest reduction in bite numbers could have no clinical impact on malaria transmission (Lawrance and Croft, 2004).

The use of mosquito coils as a personal protection measure exposes users to the smoke from burning mosquito coils. Such exposure may pose some potentially harmful effects on the health of human users (Krieger *et al.*, 2003; Liu *et al.*, 2003). There is some awareness among travel medicine advisors that the particles and gases generated through burning mosquito coils may affect persons with breathing problems (Cupp, 2001) or may cause possible airway irritation (Stürchler, 2001). It could also be a health risk potentially for lung cancer (Krieger *et al.*, 2003).

2.5.2 Mode of Action of Pyrethroids

Chemical control of malaria vector is currently dependent on pyrethroid-based insecticides (Zaim *et al.*, 2000). This may be due to the fact that pyrethroids have a rapid effect on mosquitoes, even at minimal dosages (Katsuda *et al.*, 2008). Pyrethroids are acute neurotoxicants (Soderlund *et al.*, 2002) that act on the voltage-gated sodium channel protein in the insect nerve membrane, disrupting the normal function of nerve impulses, and eventually leading to the death of the insect (Adams and Miller, 1980). In other words, when a pyrethroid binds to the voltage-gated sodium channel protein, it delays the closing of the sodium channel, prolonging the action potential and causing repetitive neuron firing, paralysis and eventual death of the insect (Ranson *et al.*, 2011). This effect of pyrethroids on the voltage-gated channel protein of insects is outwardly expressed as hyper-excitation, tremors and paralysis developed by the insects when intoxicated with pyrethroids (Narahashi, 1971).

There may also be situations whereby an insect is exposed to a dosage of pyrethroids that is not sufficient to bring about its death (sub-lethal dose), as in the case of repellents in mosquito coils. In such instances, the insect's ability to perceive and respond to host's attractants is rather inhibited (McMahon *et al.*, 2003). Repellents either activate or inhibit action of olfactory receptors thereby interfering with the host-seeking behaviour of mosquitoes, resulting in repellency or anti-feeding. Repellent pyrethroids thus disrupt insect behaviour by inhibiting the response of odorant receptors to attractants and not through their action on the voltage-gated sodium channel (Bohbot *et al.*, 2011).

2.6 Pyrethroid Resistance in *Anopheles gambiae*

Pyrethroids are the main class of insecticides used in the control of the malaria vector, and are being used in various formulations (Zaim *et al.*, 2000). They are preferably used because of their high effectiveness in terms of fast knockdown and strong excito-repellent effect, yet have lower mammalian toxicity than organochlorine, carbamate and organophosphate compounds (Mittal *et al.*, 1991; Zaim *et al.*, 2000). As a result of years of intensive usage, the efficacy of insecticides in the control of mosquitoes is seriously being threatened by the rapid development and spread of insecticide resistance by the mosquitoes (Ranson, *et al.*, 2009). Of particular concern, is the fact that pyrethroid resistance in *An. gambiae*, which was first reported in Cote d'Ivoire in 1993 (Chandre *et al.*, 1999; Corbel *et al.*, 2007), has become widespread in different regions of Africa (Etang *et al.*, 2003) including Ghana (Okoye *et al.*, 2008). This development may, therefore, represent a threat for the successful and sustainable implementation of vector control strategies using pyrethroid-based insecticides.

Target-site mutation and metabolic resistance are the two major mechanisms by which pyrethroid resistance develop in the African malaria vectors (Ridl *et al.*, 2008; Kawada *et al.*, 2014). Other mechanisms such as cuticle alteration have also been elicited (Nkya *et al.*, 2013).

(i) Target-site mutation

The target-site mutation is characterized by a marked reduction in the sensitivity of the insect nervous system to pyrethroids. This is caused by mutations in the target protein, thereby reducing the insecticide binding (Ridl *et al.*, 2008; Nkya, *et al.*, 2014). It also confers resistance to DDT, which shares a similar mode of action as pyrethroids (Martinez-Torres *et al.*, 1998). This mechanism was first identified in DDT-resistant houseflies, *Musca domestica*, and was termed knock-down resistance (*kdr*) (Milani, 1954). Knock-down resistance is due to an alteration of the insecticide affinity for its binding site on the sodium channel (Pauron *et al.*, 1989). Several alterations in the sodium channel have been associated with pyrethroid resistance in a variety of insects (Davies *et al.*, 2007). However, one of the most common amino acid replacements, and so far the only residue associated with pyrethroid knock-down resistance (*kdr*) in malaria vectors, is the point mutation in which the leucine residue found at codon 1014 is replaced by either phenylalanine (1014F) or serine (1014S).

This mutation was demonstrated in a study conducted by Martinez-Torres *et al.* (1998), in which the S6 transmembrane segment of the domain II region of the para-type sodium channel from pyrethroid susceptible and resistant strain of *An. gambiae* were observed and a single point mutation from leucine to phenylalanine was discovered in the resistant strain. This confirms that a point mutation of leucine to phenylalanine in this region is associated with the *kdr* resistance in the malaria vector (Pauron *et al.*, 1989).

The *kdr* resistance has been observed to occur at very high levels in S-form populations but at very low levels in the M form, even in areas where both molecular forms occur in sympatry (della Torre *et al.*, 2001; Yawson *et al.*, 2004). Thus, Awolola *et al.* (2003) suggested that this occurrence of high levels of *kdr* in S forms only may be as a result of the existence of an additional resistance mechanism in *An. gambiae* s.s.

It has also been hypothesized that the *kdr* mutation may have introgressed from the S form into the M form (Weill *et al.*, 2000), raising the possibility that there could be numerous adaptively and epidemiologically important genetic exchanges between forms.

(ii) Metabolic resistance

Metabolic resistance is characterized by elevated activities of one or more detoxification enzymes which results in a sufficient proportion of the insecticide being sequestered or detoxified before it reaches the target site to impair the toxicity of the insecticide (Ridl *et al.*, 2008; Nkya, *et al.*, 2014). In insects, the cytochrome P450s are the primary enzyme family responsible for pyrethroid metabolism (Feyereisen, 2005), of which CYP6M2, CYP6P3 and CYP6Z2 are the three candidate P450 genes that have been identified to be repeatedly over expressed in pyrethroid resistant populations of *An. gambiae* (Djouaka *et al.*, 2008). Although all of these genes encode for enzymes that are able to bind to pyrethroid insecticides, only CYP6P3 and CYP6M2 can metabolise the insecticide (Muller *et al.*, 2008). Other enzyme families might also play a secondary role in pyrethroid resistance (Ranson *et al.*, 2011).

2.6.1 Knockdown Resistance (*kdr*) Allele and the Excito-Repellent Effects of Pyrethroids

One advantage of pyrethroids is their excito-repellent effect on malaria vectors (Kawada *et al.*, 2014). This effect may however, be influenced by the rapid development of pyrethroid resistance which is clearly widespread in malaria vectors across Africa. With the dramatic increase in the frequency of the insecticide resistance allele in *An. gambiae* in recent times (Ranson *et al.*, 2011), there may be a relationship between the mode of insecticide resistance and excito-repellency in pyrethroid-resistant mosquitoes. Studies to determine resistance mechanisms are necessary to complement bioassay results and to evaluate their impact on control programme (Awolola *et al.*, 2003).

It has been observed that behavioural changes in pyrethroid resistant mosquitoes makes them less repelled by the insecticide thereby enabling them to remain on pyrethroid-treated materials for longer periods (Darriet, *et al.*, 2000). This may be attributed to the fact that *kdr* mosquitoes loose repellency to pyrethroids, whereas those lacking *kdr* maintain high repellency irrespective of whether they possess metabolic resistance factors to pyrethroids or not (Kawada *et al.*, 2014). It might, therefore, be reasonable to consider that the point mutation in the voltage-gated sodium channel interferes with the sensitivity of the sensory nervous system to pyrethroid as well as with the central nervous system, causing less irritancy to mosquitoes, resulting in slower avoidance or reduced repellency (Kawada, 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out at Pokuase, in Ga West District of the Greater Accra Region of Ghana (Figure 1), where an initial survey was carried out by Adu-Acheampong *et al.* (2014) to evaluate community perception and choice of mosquito coil usage. Greater Accra lies in the Coastal Savannah ecological zone, which is characterized by low rainfall and dry climate with geographic coordinates 5°33'21"N, 0°11'48"W. The peak rainy seasons in Pokuase are April to July and August to October, with annual rainfall ranging from 800 mm to 1270 mm. The area has a relative humidity which ranges from 69 to 94 % with an average of 75 %, while the temperature ranges from 25.1 °C in August and 28.4 °C in February and March (Ministry of Food and Agriculture, Ghana, 2010).

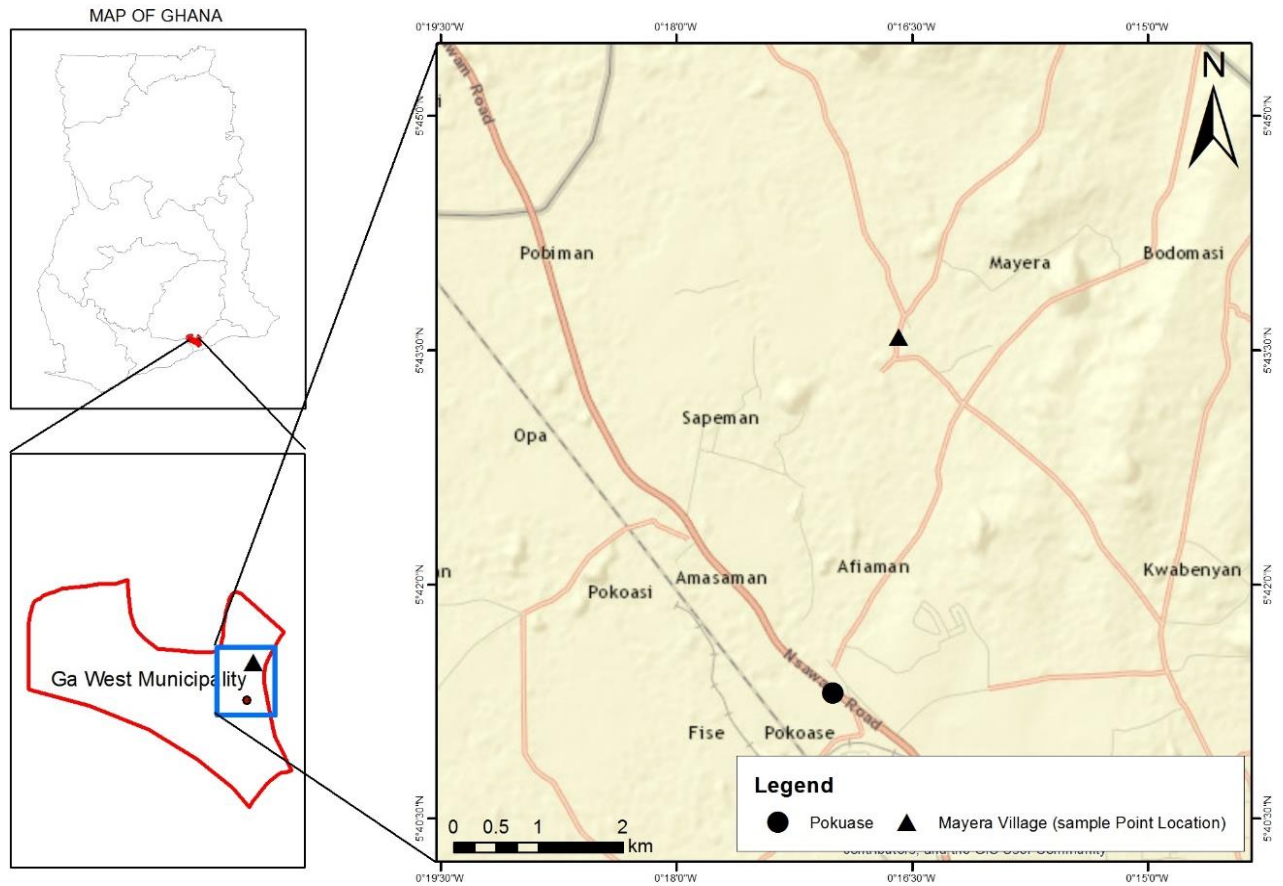


Figure 1: Map of Ghana showing Pokuase community where the sample collection was carried out.

3.2 Selected Mosquito Coils Evaluated in the Field

The three anti-mosquito coils tested were all pyrethroid-based mosquito coils namely; Angel Jumbo coil[®], Lord Insecticide antimosquito coils[®] and Heaven Jumbo coil[®]. The Angel Jumbo coil[®] is manufactured in Malaysia and distributed in Ghana by M. Hiranand (Ghana) Ltd. Its active ingredient is 0.32 % (w/w) D-allethrin. Lord Insecticide antimosquito coils[®] is produced by Beatex Ltd., Tema, Ghana, and its active ingredient is also 0.32 % (w/w) D-allethrin, while Heaven Jumbo coil[®] manufactured in China for Menkish Impex Ltd., has 0.32 % (w/w) Dimefluthrin as its active

ingredient. The choice of the selected coils was based on the fact that these three brands of mosquito coils are the most used in the study area at Pokuase; (Adu-Acheampong *et al.*, 2014).

3.3 Experimental Design

Four test rooms were used in the study in a 4×4 Latin square design. Three of the rooms were treated with the mosquito coils, while the fourth was the control room, which had no treatment. Mosquitoes were collected by four trained collectors (three treatments and one control) every night. The experiment was repeated for 16 nights by systematic rotation of coils in the test rooms to avoid household positional and individual attractiveness bias.

3.4 Mosquitoes Collection

The mosquitoes were collected from each of the four test rooms by trained collectors using the human landing catches (HLC) technique (Figure 2). Landing catches were performed 1.5 m from the position of the coils from 21:00 to 06:00 hours, with 10 minutes breaks every hour. Mosquito collectors wore long sleeve shirts to ensure that blood-seeking mosquitoes had access to only their lower legs which were exposed (WHO, 2009). Using a test tube, mosquitoes were collected once they landed on the exposed lower legs of the collectors, but before biting commenced, and transferred to pre-labelled holding cups which were changed after each hour to provide hourly measure of repellence (Dadzie *et al.*, 2013). They were later taken to the laboratory for species identification and counting. Indoor temperature and relative humidity were recorded during the collection period in one of the test rooms selected at random (WHO, 2009). After each night, a period of 48 hours elapsed before the rooms were used again for further testing (WHO, 2009). This was to avoid contamination of the test rooms due to coil effects.



Figure 2: Indoor collection of adult mosquitoes using the human landing catch (HLC) technique

3.5 Morphological Identification of Adult Mosquitoes

In the laboratory, adult mosquitoes were identified morphologically using identification keys as outlined by Gilles and de Meillon (1968). *Anopheles* mosquitoes were differentiated from other mosquitoes by their characteristic five distinctly pale spots on the costal margin of the wings. The

palps of the females, which are almost as long as the proboscis, and the white tibia ornamentation (spots in the medium part) on the legs (Gilles and de Mellion, 1968).

Female *Anopheles* mosquitoes were kept in dry silica gel and stored at 4 °C until they were required for use.

3.6 Molecular Studies

3.6.1 Extraction of Genomic DNA

The genomic DNA of the *Anopheles* mosquito was extracted from a whole mosquito using the Bender buffer method (Collins *et al.*, 1987) with a slight modification. The extract was then used as a DNA template for the species identification and the determination of the *kdr* frequency.

The Bender buffer was prepared using a mixture of 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl (pH 7.5), 0.05 M EDTA (pH 9.1) and 0.5 % SDS all mixed well by vortexing and then autoclaved. 100 µl of the Bender buffer solution, pre-heated at 65 °C, was added to each of the 1.5 ml microcentrifuge tubes containing a whole mosquito, and homogenized using a sterile hand-held plastic pestle. The homogenate was incubated at 65 °C for 30 minutes, after which 15 µl of pre-chilled 8 M potassium acetate was added to each tube and vortexed for 5 seconds to mix well. The tubes were then incubated in ice for 30 minutes, centrifuged at 14,000 rpm for 10 minutes, and the supernatants transferred into a fresh 1.5 ml microcentrifuge tubes to which 250 µl of pre-chilled 100 % ethanol was added and inverted several times to mix well and precipitate the DNA. The tubes were incubated at -40 °C for two hours and centrifuged at 14,000 rpm for 5 minutes to pellet the DNA. The supernatants were discarded and the DNA pellets were washed with 200 µl of 70 % ethanol, by centrifugation at 10,000 rpm for 5 minutes. Again, the supernatants were discarded

and the tubes were left to dry by evaporation. The DNA pellets were then re-dissolved in 200 μ l of sterile double distilled water and stored in -20 °C freezer until time of use.

3.6.2 Species Identification of *Anopheles gambiae* s.l. Using PCR Method

The polymerase chain reaction (PCR) method described by Scott *et al.*, (1993) with slight modification was used to identify the *An. gambiae* s.l. sibling species. Species specific DNA sequences were amplified using four sets of primers abbreviated as UN, GA, ME, and AR (Table 1). These primers were designed from the DNA sequences of the intergenic spacer region of the ribosomal DNA (rDNA) of *An. gambiae* complex. UN is a universal primer and anneals to the same binding position on the rDNA sequences of all species in *An. gambiae* complex. GA is specific to *An. gambiae* s.s, ME is specific to *An. melas* and *An. merus*, and AR is specific to *An. arabiensis*.

Table 1: Primer sequences of *An. gambiae* s.l., expected band sizes and melting temperature (T_m) of the PCR amplified DNA products (Scott *et al.*, 1993).

Primers	Sequences (5'-3')	Band size (bp)	T _m (°C)
UN	GTG TGC CCC TTC CTC GAT GT	468	56
GA	CTC GTT TGG TCG GCA CGT TT	390	62
ME	TGA CCA ACC CAC TCC CTT GA	464	90
AR	AAG TGT CCT TCT CCA TCC TA	315	78

A master mix solution of 20 μ l was prepared for each sample to be run for PCR. This mixture contained 1 \times PCR buffer (Invitrogen, U.S.A), 0.6 μ l of 1.5 mM MgCl₂ (Invitrogen, U.S.A), 0.8 μ L of 0.4 mM deoxyribonucleotide triphosphates (dNTPs) mix (Invitrogen, U.S.A), 0.6 μ l of 10 μ M of the oligonucleotide primers (Invitrogen, U.S.A), 0.2 μ l of DNA Taq polymerase (Invitrogen,

U.S.A) and 1 μ l of the DNA. The reaction mixture was topped up to 20 μ l with sterile double distilled water, after which it was placed in a thermocycler for amplification, using the following thermocycling profile; an initial denaturing step of 3 minutes at 94 °C to activate the DNA polymerase followed by 35 cycles each consisting of 30 seconds denaturation at 94 °C, 30 seconds annealing at 50 °C and 1 minute extension at 72 °C and an extension of the final cycle products for 7 minutes at 72 °C.

3.6.3 Gel Electrophoresis

The PCR products were electrophoresed on 2 % agarose gel stained with 0.8 μ g/ml of Ethidium bromide (EtBr) to detect the presence of DNA fragments; 8 μ l of the PCR product mixed with 1 μ l of Orange G (a loading dye) were loaded into the wells of the gel and electrophoresed. Electrophoresis was done in 1 \times Tris Acetate-EDTA (TAE) buffer at 100V for 1 hour. The gel was then viewed under a UVP dual transilluminator and photographs taken with the aid of a Polaroid direct screen instant camera with a Polaroid type 667 film. With the aid of a molecular weight ladder (Invitrogen, U.S.A) the sibling species were identified by comparing the band sizes of the PCR product (Table 1) to the mobility of the 100 bp DNA molecular weight ladder.

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

The method used in the identification of the M and S forms of *An. gambiae* s.s. was that described by Fanello *et al.* (2002) with slight modifications. This method involves the use of restriction fragment length polymorphism (RFLP). This PCR-RFLP uses restriction enzyme *Hha* 1 to cut the S forms of *An. gambiae* s.s. at its restriction sites thus differentiating it from the M form which has no restriction site for this enzyme. The S form was therefore characterized by two fragments 110 base pairs (bp) and 257 bp, while the M form is characterized by a single 367 bp (Table 2). The

procedure involved the preparation of a 15 µl reaction mixture which was made up of 3.3 µl of ddH₂O, 1.5 µl of enzyme buffer M (Invitrogen, U.S.A), 0.2 µl of *Hha I* enzyme (Invitrogen, U.S.A) and 12 µl of the PCR product. The reaction mixture was centrifuged briefly and placed in a thermocycler at 37 °C for 5 hours. The samples were then run on EtBr stained 2 % Agarose gel as described in Section 3.8.3.

The M and S molecular forms were then identified by comparing the band sizes of the restriction products to the mobility of the 100 bp DNA molecular weight ladder (Invitrogen, USA).

Table 2: Sizes of DNA fragments after PCR-RFLP for the S and M molecular forms of *An. gambiae* s.s. (Fanello *et al.*, 2002)

Species	Fragment lengths (bp) after digestion with <i>Hha I</i>		
<i>An. gambiae</i> S-form	257	110	23
<i>An. gambiae</i> M-form	367	23	

3.6.5 Detection of knockdown resistance (*kdr*) alleles in *Anopheles gambiae* complex

The *kdr* gene mutation in mosquitoes was detected using the method described by Martinez-Torres *et al.* (1998). The DNA extraction was performed as described in Section 3.8.1. The primers used were Agd1, Agd2, Agd3 and Agd4 (Table 3).

Table 3: Sequence details of the *kdr* primers and their melting temperatures (T_m) (Martinez-Torres *et al.*, 1998).

Primer	Sequence (5'-3')	T _m (°C)
Agd1	ATA GAT TCC CCG ACC ATG	54
Agd2	AGA CAA GGA TGA TGA ACC	64
Agd3	AAT TTG CAT TAC TTA CGA CA	40
Agd4	CTG TAG TGA TAG GAA ATT TA	52

A 20 µl reaction mixture containing 4.1 µl of ddH₂O, 12 µl of Gotaq, 1 µl of DNA, 0.6 µl of 10 µM each of Agd1, Agd2, Agd3 and Agd4 was prepared. The reaction mixture was amplified using the following PCR cycling condition; an initial denaturing at 94 °C for 3 minutes followed by 40 cycles at 94 °C for 30 seconds, at 48 °C for 1 minute 50 seconds and at 72 °C for 2 minutes. A final extension cycle at 72 °C for 5 minutes was performed followed by cooling at 4 °C. The product was electrophoresed through ethidium bromide-stained 2 % agarose gel and visualized under UV light. The *kdr* genotypes of both the resistant and susceptible individuals were then recorded.

The expected band sizes for the reaction are 293 bp for the control band, 195 bp for the resistant (RR) mosquito and 137 for the susceptible (SS) mosquito.

3.7 Data Entry and Analyses

The data collected were entered into the Excel sheet and log transformed to annul the effect of some violations of the assumptions of Analysis of variance (ANOVA) such as the multiplicative effect of the main treatment effect, and the correlation between the variances and the means of the treatments. The data on biting pressure and the percentage repellency from the treatment were compared with those in the control rooms using ANOVA, at 5 % significance level (WHO, 2009).

3.7.1 Repellency Effect of the Coils

The numbers of mosquitoes collected by human catches in the treated rooms was compared with the mosquitoes collected by human catches in the control room. The percentage repellency or percentage protection time was calculated using Abbott (1987) formula;

$$\text{Percentage repellency (\%)} = \frac{N_c - N_t}{N_c} \times 100$$

Where:

N_t = Number of mosquitoes collected by human catchers in rooms with coils

N_c = Number of mosquitoes collected by human catchers in rooms without coils (control).

3.7.2 Biting Pressure of the Mosquitoes

This was determined by the number of mosquitoes collected by each of the trained collectors each of the sixteen nights. This was calculated using a formula by Lines *et al.* (1991):

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

Where:

Man nights = Number of collectors \times Number of nights

MBR = Man biting rate

CHAPTER FOUR

4.0

RESULTS

4.1 Mosquito Catches

During the 16 days of sampling of adult mosquitoes, a total of 978 mosquitoes were collected. *Anopheles gambiae* s.l. constituted 95 % (935) of the total number of mosquitoes collected while *Culex*, *Aedes* and *Mansonia* species made up the remaining 5 % (43) (Figure 3).

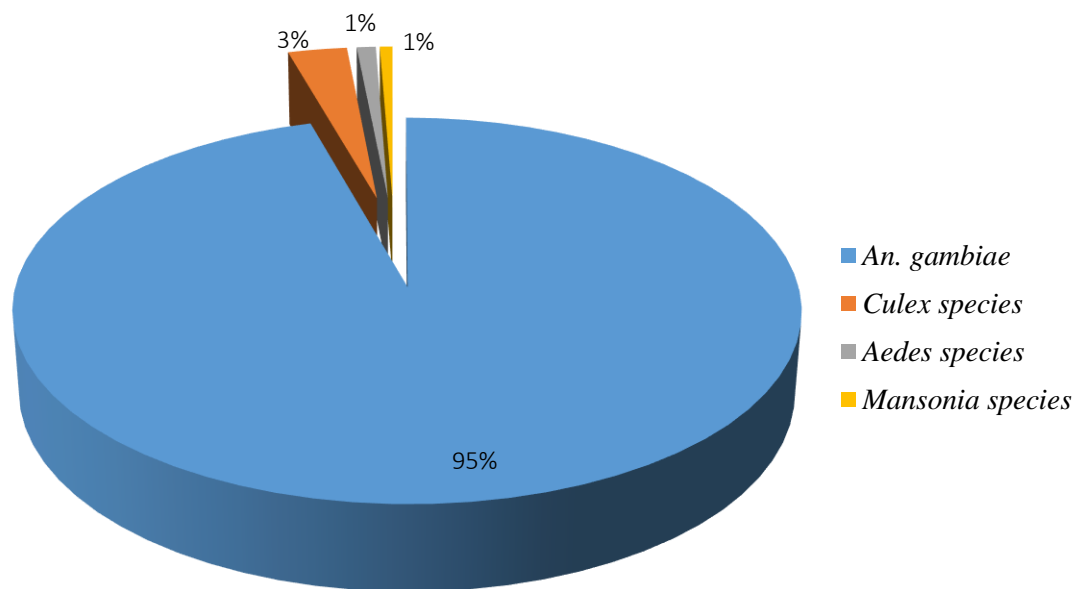


Figure 3: Distribution of the various species of mosquitoes collected during the 16 days of sampling.

Other factors such as day, rooms, and collectors had no effect on the experiment except mosquito populations caught indoors in each treatment. The total number of *Anopheles* mosquitoes collected from the control rooms were significantly higher ($df = 3$, $F = 12.39$, $P = 0.006$) than those collected

from the treated rooms. There was however no significant difference in the number of mosquitoes collected from the rooms containing the different brands of coils (Figure 4).

The average maximum and minimum temperature in the room during the period of collection was 27.8 °C and 25.3 °C respectively, while the relative humidity of the room was about 75%.

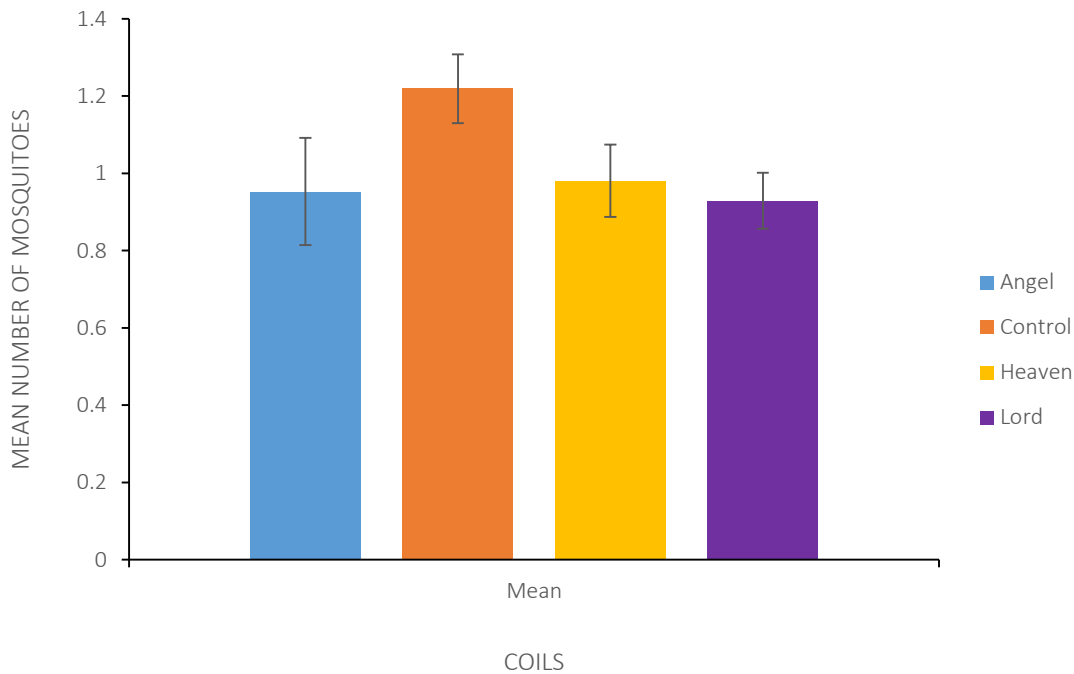


Figure 4: Mean number of mosquitoes collected from the treated and control rooms

4.2 Species Composition of the *Anopheles gambiae* s.l.

One hundred (100) *Anopheles* mosquitoes were identified by PCR after the extraction of genomic DNA from whole mosquito samples to determine their sibling species. All were identified as *An. gambiae* s.s. based on their band size which is a fragment of 390 bp (Figure 5).

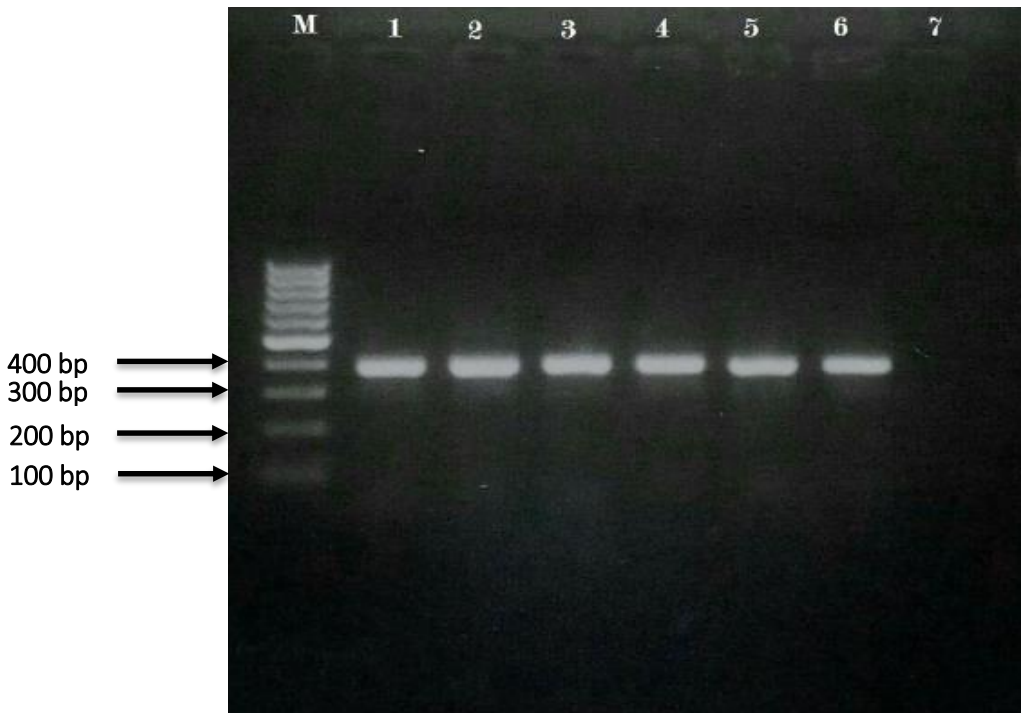


Figure 5: Agarose gel electrophoregram of PCR amplified rDNA sequence of *An. gambiae* s.s. mosquitoes. Lane M = 100 bp molecular weight ladder; Lanes 1-5 = *An. gambiae* s.s.; Lane 6; positive control; Lane 7 = negative control.

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

All 100 samples of *An. gambiae* s.s. were further identified by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) to determine the M and S molecular forms. In all, 2 % (2) were identified as M forms, 97 % (97) were identified as S forms, while 1 % (1) was identified as M/S hybrid (Figure 6). The identification was done by comparing their band sizes with that of the 100 bp molecular weight ladder. The M forms were characterised by a single fragment of 367 bp, S forms were characterised by two fragments of 110 bp and 257 bp, while the M/S hybrid was characterised by three fragments of 367 bp, 257 bp and 110 bp (Figure 7)

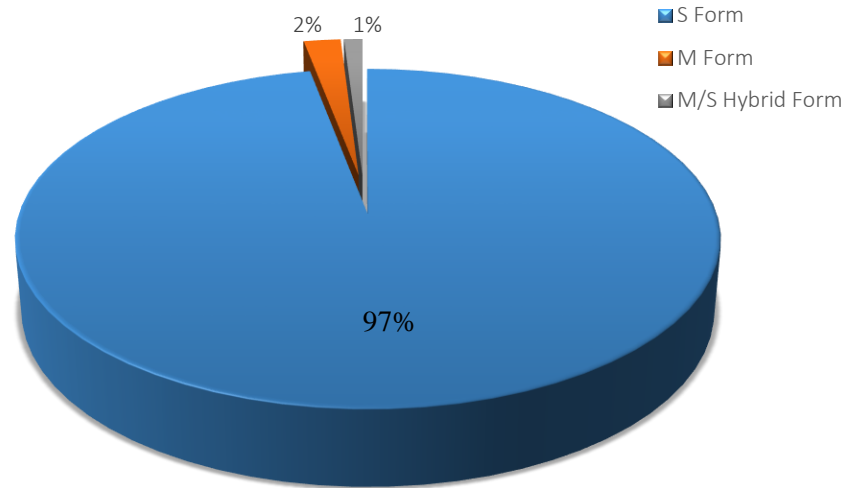


Figure 6: Percentages of the different molecular forms of *Anopheles gambiae* s.s. detected in the samples tested.

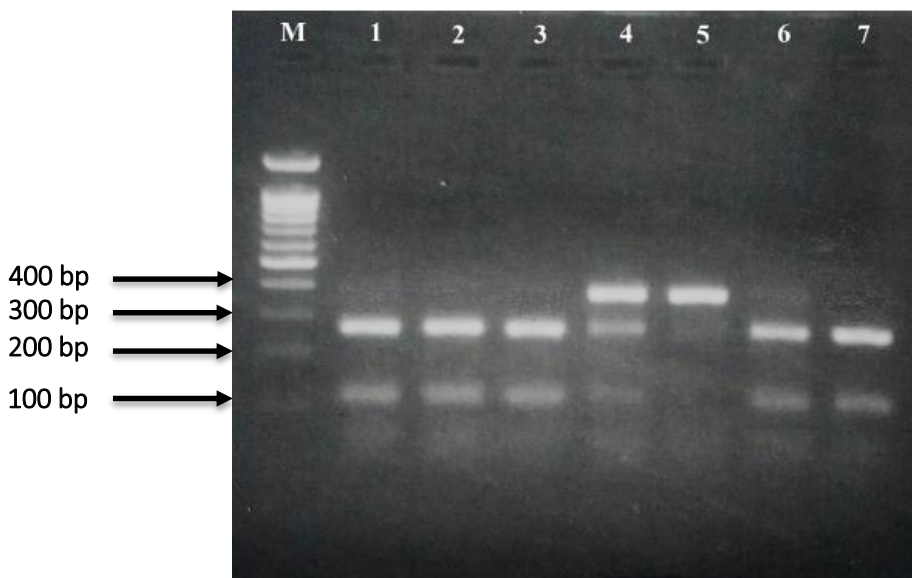


Figure 7: Agarose gel electrophoregram of *Hhl* restriction of *An. gambiae* s.s. amplified PCR products. Lane M = 100 bp molecular weight marker; Lanes 1-3, 6-7 = S forms; Lane 4 = M/S hybrid form; Lane 5 = M form (undigested).

4.4 Biting Pressure of *Anopheles gambiae* s.l. in the Study Area

A total of 64 man-nights captured 978 mosquitoes. *Anopheles* mosquitoes constituted 95 % (935) of which 64.6 % (604) were collected in rooms treated with the different coils (Figure 8); Lord Insecticide antimosquito coils[®] – 16 % (150), Heaven Jumbo coil[®] – 22 % (204) and Angel Jumbo coil[®] – 27 % (250), and the remaining 35 % (331) were from the control rooms (no coil).

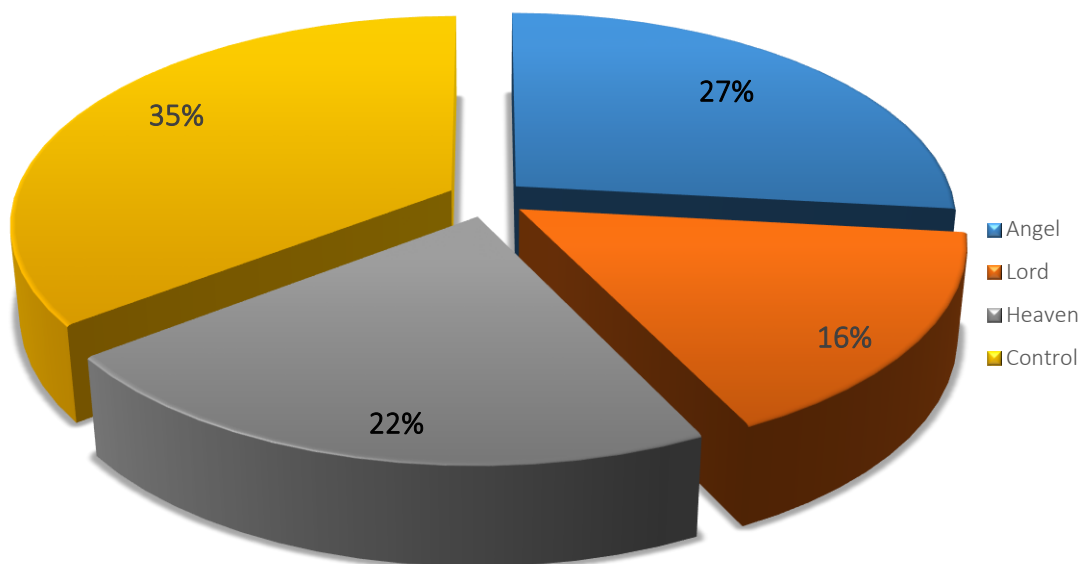


Figure 8: Percentages of *Anopheles gambiae* s.l. collected from the rooms containing the different anti-mosquito coils.

The biting pressure of *Anopheles gambiae* s.l. on unprotected individuals averaged 21 bites/man/night. Rooms treated with Angel Jumbo coil[®], Heaven Jumbo coil[®] and Lord Insecticide antimosquito coil[®] had a biting pressure of 16 bites/person/night, 13 bites/person/night and 9 bites/person/night respectively, a reduction of 24%, 38% and 57% in biting pressure respectively when compared to the control (untreated room). The biting pressure of *An. gambiae* s.l. for the different treatments is however not significantly ($P = 0.05$) different (Figure 9).

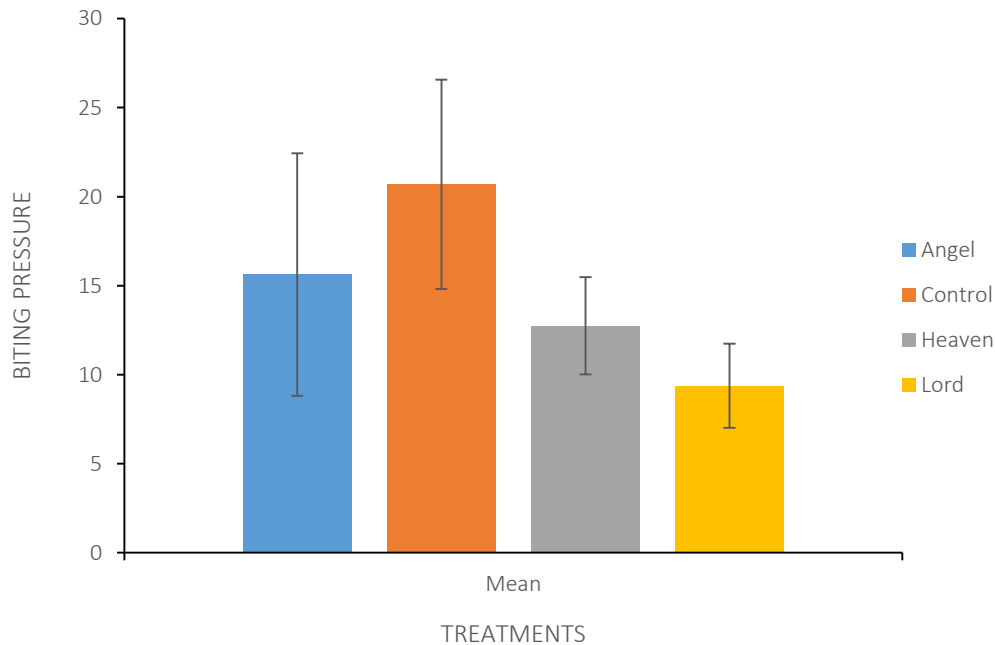


Figure 9: Biting pressure of *Anopheles gambiae* s.l. for the different anti-mosquito coils

4.5 Repellency Effect of the Mosquito Coils against *Anopheles gambiae* s.l.

The total numbers of *Anopheles* mosquitoes collected from the control rooms (no coil) and the treated rooms were used to estimate the percentage repellency or percentage protection of the three brands of anti-mosquito coils. The result showed that Lord Insecticide antimosquito coils[®], Heaven Jumbo coil[®] and Angel Jumbo coil[®] provided average percentage repellency of 51.4 %, 33.6 % and 26.9 %, respectively, with Lord Insecticide antimosquito coils[®] providing a significantly ($P = 0.007$) higher percentage repellency against *Anopheles gambiae* s.l. (Figure 10).

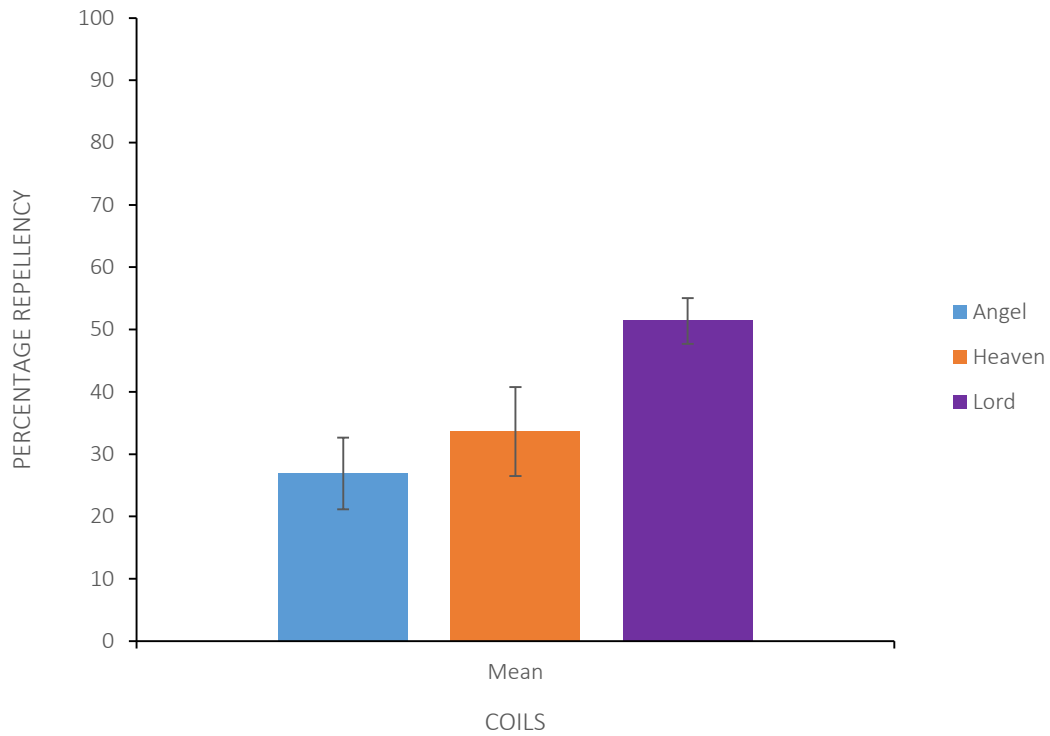


Figure 10: Average percentage protection provided by the three coils against *An. gambiae* s.l.

The hourly collection of mosquitoes from the different rooms showed a peak biting hour of *Anopheles gambiae* to be between 0:00 hours - 01:00 hours (Figure 11), during which period Lord Insecticide antimosquito coils[®] provides the highest percentage repellency (Figure 12).

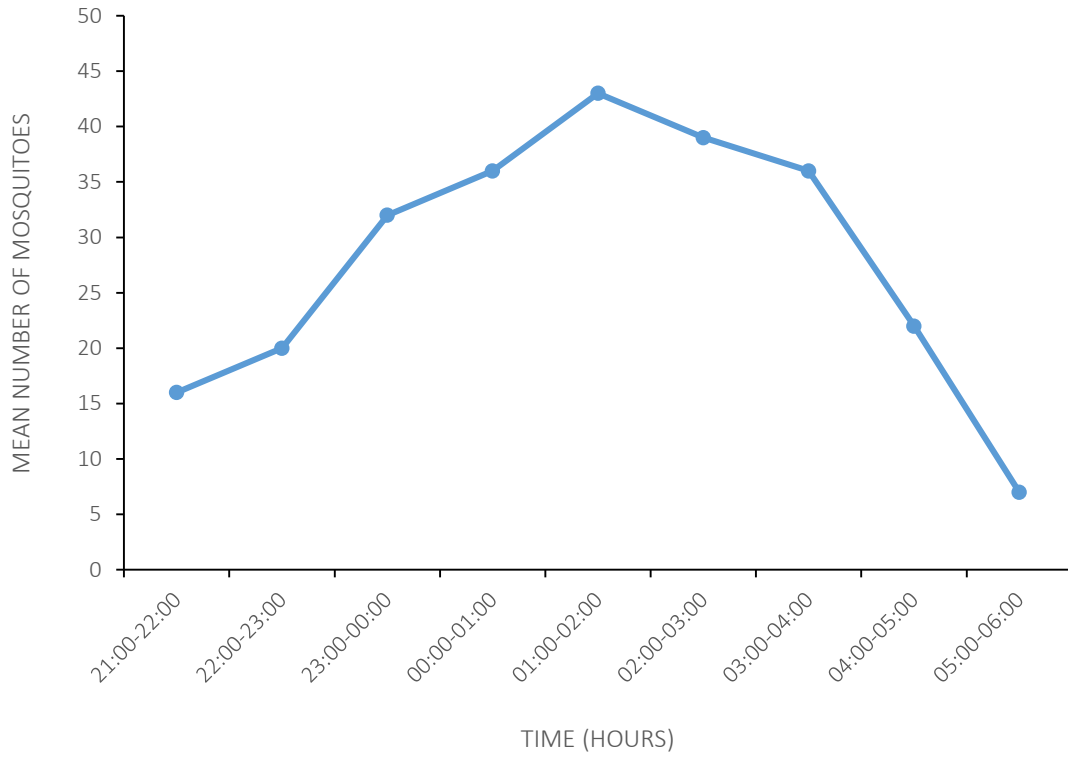


Figure 11: Biting pattern of *Anopheles gambiae* s.l. in the Pokuase community

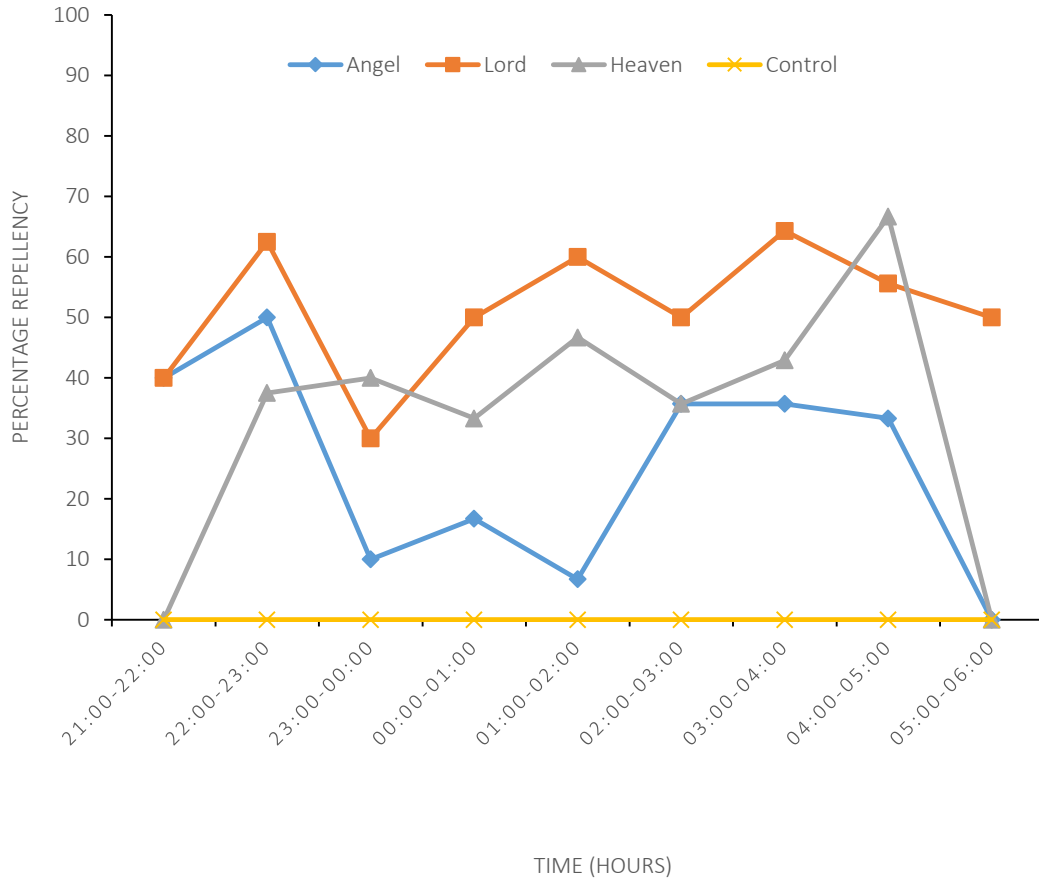


Figure 12: Hourly percentage repellency of different anti-mosquito coils in Pokuasi area

4.6 Knockdown Resistance (*kdr*) Mutation in *An. gambiae s.s.*

A total of 100 *An. gambiae s.s.* samples were analysed for the presence of the *kdr* gene. Molecular analysis revealed the presence of the resistant gene in 98 % (98) of the *An. gambiae s.s.*, while 2 % (2) were shown to be susceptible. The identification of the genes was based on the presence of a control band of 285 bp for all the samples, in addition to a band of 188 bp and 210 bp for the resistant and susceptible samples respectively (Figure 13).

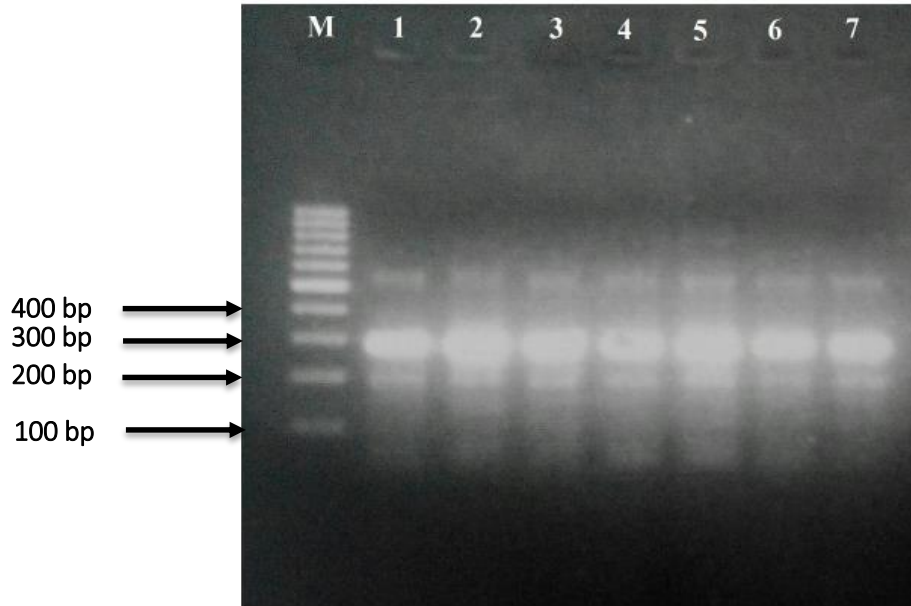


Figure 13: Agarose gel electrophoregram of PCR detection of the *kdr* gene. Lane M = Molecular weight ladder; Lanes 1 – 7 = Resistant *An. gambiae* s.s.

CHAPTER FIVE

5.0

DISCUSSION

Mosquito coils remain one of the most common means of personal protection against mosquitoes in both the rural and urban areas of Ghana (Adu-Acheampong *et al.*, 2014). This may be attributed to the fact that they are very affordable and easy to use (Lawrance and Croft, 2004). Their efficacy, however, still remains in doubt. A laboratory study carried out to evaluate the efficacy of the same type of mosquito coils in the Pokuase community of Greater Accra Region showed that they provided low knockdown effect (Adu-Acheampong *et al.*, 2014). The present study, therefore tested the repellency effect of the coils in the study area in order to evaluate the bio-efficacy of mosquito coils under natural settings in the field. The *kdr* status of *Anopheles gambiae* s.s., the main malaria vector in the study area, was also determined.

In the adult mosquito sampling carried out in the Pokuase community, *An. gambiae* s.l. was the major vector collected from the study area. It made up 95 % of the total collections, thus, indicating that *An. gambiae* s.l. is the main mosquito species in the area. This is consistent with other studies that detected *An. gambiae* s.l. to be the major mosquito feeding on humans in the Greater Accra Region (Kabula *et al.*, 2011), thus confirming this species to be highly anthropophilic (Dabire *et al.*, 2008). Since collections were done indoors, the results also corroborate the fact that *An. gambiae* is predominantly endophagic as observed in previous studies by Appawu *et al.* (2004).

All the *An. gambiae* complex examined by PCR showed that all the species were *An. gambiae* s.s. Considering the fact that the other members of the complex were not identified, it is possible that *An. gambiae* s.s. could be the only member of the *An. gambiae* complex present in the study area.

An. arabiensis was not identified, probably because the study area does not support the breeding of *An. arabiensis* which is typically found in dry savannah environments and sparse woodland. It is also known to occur in forested areas that have recently been cleared (Coetzee *et al.*, 2000). The absence of *An. melas* on the other hand may be attributed to the fact that they are only found in coastal areas (Bryan, 1983).

Out of the 100 samples of *An. gambiae* s.s that were examined, 97 % were S forms, 2 % were M forms and the remaining 1 % was an M/S hybrid form. The result showed that the S form was the dominant molecular form of *An. gambiae* s.s. in the study area. This may be attributed to the fact that the Pokuase community is characterised by mostly temporary breeding sites, such as untarred pot-holed roads, that can support the breeding of more S forms. The distribution of the M and S forms is dependent on ecological and geographical factors since S forms breed preferentially in temporary sites that depend on rainfall with low temperatures (Diabate *et al.*, 2003), while the M forms prefer to breed in permanent environments and habitats created by human activities such as irrigation fields with high temperatures (Wondji *et al.*, 2002). The existence of the two molecular forms in sympatry in the study area is consistent with the findings of Kabula *et al.* (2011) in a study carried out in selected areas of the Accra metropolis.

Hybridisation among the two molecular forms is a rare occurrence even though they exist in sympatry. Some previous studies on the molecular forms of *An. gambiae* s.s. carried out in Ghana had not reported on the presence of any hybrid forms (Yawson *et al.*, 2004; Kabula *et al.*, 2011), however, the present study observed 1 % M/S hybrid form. This finding is consistent with previous finding that inter-form hybridisation does occur at low levels (Yawson *et al.*, 2007; della Torre *et al.*, 2001; Tripét *et al.*, 2001; Taylor *et al.*, 2001), and has been hypothesized by Black and Lanzaro

(2001) to be due to partial reproductive isolation among the molecular forms, with gene flow occurring only in certain geographical locations or at certain seasons.

The observation that biting pressure of the mosquitoes in the rooms treated with the different coils was not significantly different from those of the control rooms (no coil), as well as the fact that an average percentage repellency of only 51.4 % was provided by Lord Insecticide antimosquito coil[®] during the peak biting period (01:00 - 02:00 hrs.), is an indication that the anti-mosquito coils do not provide sufficient protection against the malaria vector. This therefore points to the fact that the tested anti-mosquito coils are not very effective personal protection tools against the malaria vectors.

Some laboratory studies indicated that the low mortality induced by pyrethroid-based anti-mosquito coils can be attributed to the development of pyrethroid resistance by the malaria vectors (Avicor *et al.*, 2013; Adu-Acheampong *et al.*, 2014). Similarly, the high level of pyrethroid resistance observed in the *An. gambiae* s.s. population in the study area probably accounted for the low repellency effect offered by the tested anti-mosquito coils under field conditions. Conversely, in a similar study carried out in Tanzania by Msangi *et al.* (2010), the evaluated mosquito coils had more than 75 % feeding inhibition for *An. gambiae* s.l. Such high level of protection by the anti-mosquito coils was attributed to the fact that the *An. gambiae* s.l. population in the study area lacked the *kdr* gene, thus maintained a high repellency to the tested pyrethroid-based mosquito coils. These findings support the observation that knockdown resistant (*kdr*) mosquitoes lose repellency to pyrethroids, whereas those lacking *kdr* maintain high repellency (Kawada *et al.*, 2014). It might, therefore, be reasonable to infer that the point mutation in the voltage-gated sodium channel interferes with the sensitivity of the sensory nervous system to pyrethroid as well

as with the central nervous system, causing less irritancy to mosquitoes, and resulting in slower avoidance or reduced repellency (Kawada, 2000).

The presence of the *kdr* gene, which is associated with pyrethroid resistance and cross resistance with DDT in malaria vectors, was also tested for. Ninety-eight percent (98 %) of the total samples (100) examined tested positive for the *kdr* gene. This is an indication that the study area has high pyrethroid resistance, hence, the study also agrees with other studies in the Greater Accra Region which reported resistance to pyrethroid based vector control methods (Adeniran, 2002; Achonduh *et al.*, 2008; Boakye *et al.*, 2009). The high pyrethroid resistance recorded in the study area may be attributed to the fact that this same group of insecticides is continuously being used by the inhabitants in the form of aerosols or coils to control mosquitoes (Adu-Acheampong *et al.*, 2014), thereby, exerting a selection pressure which allows only *An. gambiae* s.s. with the *kdr* gene to survive and perpetuate in the population.

The fact that *kdr* mutation is mostly associated with the S forms (Chandre *et al.*, 1999) was corroborated by the findings of the present study, as 100 % of the S forms identified in the study area were all resistant to pyrethroids. Pyrethroid resistance has been reported to occur in the M forms as well (Weill *et al.*, 2000; Yawson *et al.*, 2004), however, the present study observed no *kdr* gene in all the M forms collected from the study area. This result is consistent with the findings of Awolola *et al.* (2003) in a study carried out in Nigeria, where the *kdr* mutation was also found in only the molecular S forms, even in areas where both molecular forms of *An. gambiae* s.s. occurred in sympatry, and therefore agrees with the suggestion that an additional pyrethroid resistance mechanism may be present in *An. gambiae* s.s. (Awolola *et al.*, 2003).

This reduction in the efficacy of anti-mosquito coils may be responsible for the decline in the use of anti-mosquito coils in the Greater Accra Region, as observed by Adu-Acheampong *et al.* (2014). Though the high level of *kdr* mutation may play a role in the reduced efficacy of mosquito coils, other factors such as the manufacturing of sub-standard coils could also be involved.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The study showed that the main malaria vector in the Pokuase community of Greater Accra Region is *An. gambiae* s.s. The M and S molecular forms were seen to occur in sympatry, with the S molecular form being predominant in the area. The observation of one M/S hybrid form indicated the existence of some level of hybridisation between the M and S molecular form in the area. A high *kdr* frequency which was predominant in the S molecular forms was also recorded.

The average percentage repellency of 51 % provided by Lord Insecticide antimosquito coils[®] was significantly higher than those of the Angel Jumbo coil[®] and the Heaven Jumbo coil[®]. That notwithstanding, it was still too low to confer the needed protection against the malaria vectors. On the other hand, the biting pressure of mosquitoes in all the rooms treated with the tested anti-mosquito coils was not significantly different from that of the control room, where there was no coil. This implies that the tested coils do not provide any significant reduction in the number of mosquito bites users are exposed to, hence, the tested coils cannot provide full protection against the malaria vector when used alone in areas where pyrethroid resistance has been developed by the mosquitoes.

6.2 Recommendations

The high level of resistance against all three brands of pyrethroid-based mosquito coils by the *An. gambiae* s.l. in the study area is an indication that mosquito coils cannot be relied upon as the sole means of protection against the malaria vector in the area. It should thus be used in combination with other vector control tools.

The implication of the high pyrethroid resistance in the study area on malaria control using pyrethroid-based vector control measures is of great concern. It is therefore recommended that other non-pyrethroid vector control tools such the use of endosymbiotic bacteria that are able to reduce the vectorial capacity of *An. gambiae* be explored in the study area.

The knockdown resistance is as a result of a point mutation in the voltage-gated sodium channel which reduces sensitivity to pyrethroid insecticides, thereby resulting in loss of their rapid knockdown effect on the insect. The reduced repellency observed in *kdr* resistant mosquitoes, however, suggests the existence of a possible link between the effects of the *kdr* mutation on the rapid knockdown of insects and the effect on excito-repellency. It is therefore recommended that further studies be conducted to better understand the mechanism behind the effect of the *kdr* mutation on the olfactory nerves of *An. gambiae* s.l. which results in reduced sensitivity to excito-repellency of pyrethroid-based repellents.

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APPENDICES

Appendix 1

Preparation of standard solutions

Solutions Used for Molecular Studies

PCR Primers

Primers used were diluted as specified by the manufacturers

Invitrogen Buffer for PCR

300 mM Tris HCl, 75 mM (NH₄)₂ SO₄

Solutions for Electrophoresis

10× TAE Buffer

50× TAE was diluted with ddH₂O to bring the concentration to 1×. This was done by measuring 200 ml of 50× TAE buffer and topping the volume up to 1000 ml with ddH₂O.

2 % Agarose Gel

This was prepared by dissolving 0.4 g of the Agarose powder in 40 ml 1× TAE buffer and microwaved to dissolve the powder and then cooled under running tap water. Then 0.5 ml (10 mg/ml) EtBr was added and the beaker stirred to mix thoroughly. The solution was then poured in

a gel mould (Mini gel system, BIORAD, USA) with combs of <1 mm and left for about 15 minutes to solidify. The combs were removed and wells created in which samples were loaded.

Gel Loading Dye

The loading dye, 5× orange G was prepared and stored at room temperature (27 °C). It is 20 % (w/v) Ficoll, 25 % (w/v) Orange G, and 25 mM EDTA.

DNA Molecular Weight Ladder

All dilutions on the product were carried out as directed by the manufacturer. When loaded the size of the ladder increases in an ascending order. From the base, the first ladder is 100 bp, then 200 bp and up to 1000 bp.

Ethidium Bromide, EtBr (100 mg/ml)

1 g of EtBr was completely dissolved in sterile double distil water and stored at room temperature in a dark room.

Appendix 2

Indoor human landing catches for 16 nights in different rooms treated with the Angel

Jumbo coil

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Time (Hours)																
21:00- 22:00	0	0	0	0	1	0	0	0	1	2	2	2	0	0	1	0
22:00- 23:00	0	2	0	1	2	1	0	0	0	4	1	7	0	0	0	0
23:00- 00:00	0	1	0	3	9	0	0	1	3	4	1	9	1	1	1	0
00:00- 01:00	0	1	0	1	7	0	1	0	4	6	5	9	0	1	3	0
01:00- 02:00	0	1	0	2	8	7	6	1	6	2	4	11	0	1	3	0
02:00- 03:00	0	1	0	0	8	4	1	0	3	6	3	5	0	0	1	1
03:00- 04:00	0	0	0	1	4	8	2	2	1	5	2	8	0	0	4	0
04:00- 05:00	0	0	0	0	8	0	0	2	1	1	2	6	1	0	2	0
05:00- 06:00	0	0	0	0	1	0	1	0	0	1	0	2	0	0	1	0
SUM	0	6	0	8	48	20	11	6	19	31	20	59	2	3	16	1

Appendix 3

**Indoor human landing catches for 16 nights in different rooms treated with the Lord
insecticide anti-mosquito coil.**

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Time (Hours)																
21:00- 22:00	1	0	0	1	0	1	0	1	2	0	0	0	1	0	0	0
22:00- 23:00	0	0	1	0	0	2	0	1	3	0	2	0	0	1	0	3
23:00- 00:00	0	1	0	1	3	1	0	0	4	1	1	4	2	1	2	2
00:00- 01:00	0	5	1	1	1	1	0	0	5	0	1	1	1	1	1	2
01:00- 02:00	0	0	0	2	0	1	1	0	4	3	1	0	1	3	1	5
02:00- 03:00	0	0	0	6	1	1	1	1	2	2	3	0	6	1	3	2
03:00- 04:00	0	0	0	2	0	0	0	0	2	5	2	1	3	0	0	3
04:00- 05:00	3	0	0	1	0	0	0	0	1	6	0	0	2	0	0	2
05:00- 06:00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
SUM	4	6	2	14	5	7	2	3	23	17	10	6	16	7	7	21

Appendix 4

Indoor human landing catches for 16 nights in different rooms treated with the Heaven

Jumbo coil

Heaven Jumbo coil																
Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Time (Hours)																
21:00-22:00	0	2	0	2	5	1	2	2	0	3	0	0	1	0	0	0
22:00-23:00	1	1	0	8	3	0	2	1	0	0	0	1	1	0	0	0
23:00-00:00	0	1	2	4	2	0	3	1	0	3	0	1	5	0	1	0
00:00-01:00	1	2	2	7	4	0	5	0	0	5	1	0	0	1	2	0
01:00-02:00	1	1	0	7	2	1	7	0	0	3	0	1	3	2	2	0
02:00-03:00	1	1	0	8	0	3	6	0	1	2	0	1	4	0	4	2
03:00-04:00	0	1	1	10	3	2	4	0	0	1	2	2	1	2	0	1
04:00-05:00	0	0	0	3	3	1	2	1	0	0	0	0	3	0	1	0
05:00-06:00	0	0	0	3	1	0	1	1	0	0	0	0	1	1	0	0
SUM	4	9	5	52	23	8	32	6	1	17	3	6	19	6	10	3

Appendix 5

Indoor human landing catches for 16 nights in different rooms with no coils

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Time (Hours)																
21:00- 22:00	3	0	0	0	1	0	1	3	2	0	2	0	1	0	1	0
22:00- 23:00	2	1	0	1	0	1	3	9	2	2	3	0	0	0	1	0
23:00- 00:00	1	3	2	4	6	0	2	6	3	0	6	2	1	0	0	1
00:00- 01:00	6	2	5	2	5	0	4	5	2	0	6	2	2	1	4	1
01:00- 02:00	7	3	3	4	7	9	4	11	0	1	7	1	0	0	3	0
02:00- 03:00	2	7	2	4	5	12	4	6	3	0	6	3	0	0	1	0
03:00- 04:00	8	5	3	0	2	7	4	9	3	1	5	1	0	1	3	1
04:00- 05:00	5	3	0	4	5	1	0	5	2	1	2	1	0	2	3	0
05:00- 06:00	0	0	0	0	1	0	0	3	0	0	1	0	0	0	1	0
SUM	34	24	15	19	32	30	22	57	17	5	38	10	4	4	17	3

Appendix 6**Analysis of variance for indoor biting mosquitoes from the different rooms treated with the different brands of anti-mosquito coils**

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rooms stratum	3	2.1913	0.7304	31.47	
Days stratum	3	0.9267	0.3089	13.31	
Rooms.Days stratum					
Coils	3	0.8625	0.2875	12.39	0.006
Residual	6	0.1393	0.0232	0.17	
Rooms.Days.*Units* stratum					
	48	6.6481	0.1385		
Total	63	10.7679			

Tables of means

Grand mean 1.020

Coils	Angel	Control	Heaven	Lord
	0.953	1.219	0.981	0.929

Least significant differences of means (5 % level)

Multiple comparison (Bonferroni test)

Comparison-wise error rate = 0.0083

	Mean	
Lord	0.929	a
Angel	0.953	a
Heaven	0.981	a
Control	1.219	b

Appendix 7

Analysis of variance for the percentage repellency of the different brands of anti-mosquito coils against *An. gambiae*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time stratum	8	3661.6	457.7	2.18	
Time.*Units* stratum					
Coil	2	2881.5	1440.8	6.85	0.007
Residual	16	3363.5	210.2		
Total	26	9906.7			

Tables of means

Grand mean 37.3

Coil	Angel	Heaven	Lord
	26.9	33.6	51.4

Least significant differences of means (5 % level)

Multiple comparison (Bonferroni test)

Comparison-wise error rate = 0.0167

	Mean	
Angel	26.88	a
Heaven	33.64	ab
Lord	51.38	b

Appendix 8**Analysis of variance for the biting pressure of *An. gambiae* in the rooms treated with the different brands of anti-mosquito coils**

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rooms stratum	3	894.86	298.29	12.48	
Rooms.*Units* stratum					
Coils	3	275.32	91.77	3.84	0.051
Residual	9	215.19	23.91		
Total	15	1385.37			

Tables of means

Grand mean 14.6

Coils	Angel	Control	Heaven	Lord
	15.6	20.7	12.8	9.4

Least significant differences of means (5 % level)

Multiple comparison (Bonferroni test)

Comparison-wise error rate = 0.0083

	Mean	
Lord	9.38	a
Heaven	12.75	a
Angel	15.62	a
Control	20.69	a