A STUDY OF BITTNG PATTERNS IN *ANOPHELES GAMBIAE SENSU LATO* (S.L.)

By

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DECLARATION

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

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This thesis is dedicated to God Almighty, my family and friends

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ABSTRACT

The recent evidence indicating a modification in the biting pattern of vector populations could pose a challenge to the increased use of Insecticide treated bed nets as a malaria vector control strategy. This modification includes adaptation to biting humans when they are out of bed that is early evening and dawn together with increased outdoor biting. This modification might not necessarily be as a result of an adaptive change in vector behaviour but due to selective pressure against mosquitoes biting when bed nets are in use. This study therefore seeks to determine if discrete vector populations with specific biting times exist within *Anopheles gambiae* populations. *An. gambiae* sampled from 6pm to 6am in the field were pooled into four categories based on their time of biting; 6pm-9pm, 9pm-12am, 12am-3am and 3am-6am. Generations were raised on which feeding experiment and selection were performed. Non anophelines were separated from anophelines and PCR run on *An. gambiae* for species identification and molecular forms. *An. gambiae* formed the dominant species comprising 90.7% (739/815), 7.6% (62/815) for *Culex* species, 0.6% (5/815) for *Mansonia*, 0.5% (4/815) for *Aedes*, 0.5% (4/815) for *An. funestus* species and the least being *An. pharoensis* with 0.1% (1/815). *Anopheles* sampled comprised of *An.* gambiae (99.3%), *An.* funestus (0.5%) and *An.* pharoensis (0.1%) [n=744]. Different numbers of F_1 and F_2 progeny were obtained and used for feeding experiments for the different time groups. The percentages for the F_1 groups that fed at same time periods as parents in the field (compared with feeding at other time points pooled together) were 90.5% (P=0.013) for 9pm-12am, 66.3% (P=0.029) for 12-3am and 62.3% (P=<0.0001) for 3-6am, whilst F_2 had100% (P=0.228) for 9-12am and 91.2% (P=0.037) for 12-3am. The comparison of the feeding frequencies of F_1 and F_2 progeny for the time groups were [9pm-12am, F_1 = 90.5% (38/42) and F_2 = 100% (12/12)] and [12am-3pm, F_1 = 66.3% (252/380) and F_2 = 91.2% (52/57)]. However, irrespective of the period F_1 and F_2 from different collection times were exposed to feed, a high proportion was found to take a blood meal. PCR run on 138 *Anopheles gambiae* showed 97.1% *An. gambiae s.s.* with no amplification for 2.9%. Furthermore, 134 *Anopheles gambiae s.s.* which were identified by PCR and subjected to restriction fragment length polymorphism (RFLP) analysis for the various molecular forms gave 94.8% S forms and 5.2% M forms. Vector behaviours such as host preference, biting pattern and resting have been linked to genetic influence but results obtained for F_1 and F_2 generations showed otherwise probably due to extrinsic factors.

CHAPTER ONE

INTRODUCTION

1.0 Background

In sub-Saharan Africa, the incidence of malaria still remains a serious problem to the people living in various endemic countries. According to World Health Organisation (WHO), the disease is responsible for about 863,000 deaths worldwide with 89% from the total number of deaths occurring in Africa (WHO, 2011). Malaria can result in anaemia and in severe conditions can cause renal dysfunction, jaundice and cerebral complications leading to coma in infected individuals (WHO, 2006a).The causative parasites are protozoans of the genus *Plasmodium.* Human malaria is caused mainly by four morphologically distinct *Plasmodium* species namely *P. falciparum, P. vivax, P. ovale* and *P. malariae* (Collins, 1995; Lee *et al*., 2009) with occasional human infections with the monkey malaria parasite *P. knowlesi* (Cox-Singh and Singh, 2010; Tang*et al.*, 2010). Anopheline mosquitoes are the principal vectors involved in malaria transmission with *Anopheles gambiae s.l.* and *Anopheles funestus* serving as major vectors in Africa (Appawu *et al.*, 2004; Service, 1984; Takken and Knols, 1999). There are also a few secondary vectors such as *A. nili, A. moucheti* and *A. pharoensis* which may also be responsible for localised transmission (Service 1984).

The Global Malaria Programme (GMP) of the World Health Organisation (WHO) as part of its malaria management strategies saw the need to review and re-design its policy-setting towards the elimination, control and prevention of malaria. This therefore resulted in the setting up of the Malaria Policy Advisory Committee (MPAC) in 2012 to provide strategic advice and technical input to WHO on all areas

pertaining to malaria control and elimination (WHO, 2012). The control of malaria involves early diagnosis and treatment with effective antimalarial drugs, usage of insecticide treated nets (ITNs) preferably long-lasting insecticidal treated nets (LLNs) and indoor residual spraying (IRS) (WHO, 2006b). *Anopheles gambiae* complex, the major vector for malaria infection in Africa exhibits high anthropophilic behaviour (Marquardt *et al.,* 2005) but in the absence of humans, animals can serve as alternative hosts. Studies in Madagascar showed *An. gambiae s.s.* and *An. Arabiensis* exhibiting a high zoophilic tendency with preference for cattle odours (Duchemin *et al.,* 2001) and in São Tomé *An. gambiae s.s.* showed a preference for dogs to humans (Sousa *et al.,* 2001). The *An. gambiae* complex is predominantly endophagic and endophilic (Padonou *et al.*, 2012) but their tendency to exhibit this character can be altered by the effective implementation of intra-domiciliary vector control tools such as long-lasting insecticidal treated nets (LLINs) and indoor residual spraying(IRS). This can therefore result in an increased selection for more exophagic malaria vectors as was observed by Russell *et al.*, (2011) that the usage of ITNs resulted in a change in the vector composition and behaviour of *An. gambiae* complex from the highly anthropophagic and endophagic *An. gambiae s.s.*to the more zoophagic and exophagic *An. arabiensis*. Thus the use of intra-domicilary vector control tools result in exophagic vectors having a higher reproductive advantage over the endophilic ones.

1.1 Rationale

The use of insecticide-treated nets (ITNs) faces several challenges such as the development of insecticide resistance in malaria vectors to the commonly used

insecticides (Pates and Curtis, 2005; Chandre *et al.*, 1999; Reimer *et al.*, 2005) and changes in the behaviour of vectors (Reddy *et al.*, 2011) which could hamper the successful control of malaria. The biting cycle of *An. gambiae* complex begins with low biting rates up to 2100hrs-2200hrs, followed by an increase in biting reaching a peak period from midnight to 0400hrs but with relatively low biting occurring just before dawn (Wanji *et al.*, 2003). This general biting pattern appears to be modified with the introduction of ITNs. This modification in biting patterns in malaria vectors involve changes in the peak biting time such as early evening biting (Appawu *et al.*, 2004; Mbogo *et al.*, 1996) and early morning biting (Reddy *et al.*, 2011). Also, modifications may include shifts in host feeding from humans to animals as was observed in Papua New Guinea (pigs and dogs) and Tanzania (cattle) (Takken, 2002) as well as increased outdoor biting observed in Papua New Guinea and Kenya (Takken, 2002) and Bioko Island, Equatorial Guinea (Reddy *et al.*, 2011).

Understanding the observed changes in biting patterns is critical for any modification of control strategies. The above reports explain that this change in biting pattern might be as a result of an adaptive change in behaviour. However, we propose a second hypothesis that the observed changes could be due to a selection within discrete specific temporal biting populations within the *Anopheles gambiae* complex.

1.2 Objectives

To determine if there are (genetically) discrete biting populations within *An. gambiae* s.s. that bite at specific times during the night (and on which selective pressures imposed by bednet interventions could operate).

1.2.1 Specific objectives

1. To collect biting *An. gambiae* complex within specific time periods in the night.

2. To compare the feeding frequencies of F_1 and F_2 generations for the various time groups.

3. To determine if progeny can be selected in the laboratory with peak biting times observed at time periods when the parental populations were collected from the field.

4. To determine the sibling species of *An. gambiae* complex and identify the molecular forms of *An. gambiae s.s.*.

CHAPTER TWO

LITERATURE REVIEW

2.0 General introduction

Malaria, a vector borne disease is predominant in tropical, sub-tropical zones in Sub-Saharan Africa and even in some temperate regions of the world (Service, 1980). It is responsible for numerous reported clinical cases of infants normally less than five years and adults mostly pregnant women, normally resulting in high morbidity and mortality in affected individuals. Reports indicate that it is still the leading cause of health problems and death in Africa south of the Sahara (WHO, 2006a). This disease receives a lot of monitoring, surveillance, control and global funding but still remains a problem globally. The poor are most affected since it accounts for almost 34 percent of their income, whilst the rich spend only about one percent of their income on the disease hence it being known to be a disease of the poor (Kimani *et al.*, 2006). This disease is the cause of poverty among affected people but not the reverse since countries like Oman and United Arab Emirates with high income per capita are still battling with malaria after several vector control and elimination programmes (Gallup and Sachs, 2001). Malaria also has serious draw backs on the economic growth and social development of endemic areas. Examples can be seen in the economies of Greece, Spain and Italy all in southern Europe where there was a decline in the growth of their economies as a result of malaria but growth accelerated after elimination and complete eradication of the disease (Gallup and Sachs, 2001) Studies in Africa has also revealed that economic growth is slowed down by up to 1.3 percent each year due to the fact that almost forty percent of African health budgets is spent on malaria each year (Kokwaro, 2009). Therefore, the control of malaria will result in increases in economic indices, social and political stability, and

then improve foreign trade and investments (Breman, 2001). Malaria is a vector-borne disease transmitted by the protozoan *Plasmodium* of which there are four main species with humans serving as natural hosts for disease transmission; *P. falciparum* which evolved recently is the most virulent and responsible for most malaria infections in Africa, *P. vivax* which is widespread in Central and South America and Asia is associated with relapse months after infection with high morbidity rates, *P. malariae* involves patients being mainly parasitemic and asymptomatic for years if not diagnosed and treated well and lastly *P. ovale* principally found in Africa is a relapsing parasite and rare (Breman, 2001). More than hundred *Plasmodium* species exist and have been reported to be involved in the infection of hosts like reptiles, birds, rodents, primates and other mammals (Garnham, 1966). Infections with simian malaria parasite *P. knowlesi* have also been reported in some instances with most cases occurring in Asia. One of such cases was observed in Malaysia at the Kapit Hospital where four *P. knowlesi* cases were misdiagnosed as *P. malariae* due to morphological similarities in both *plasmodium* species, whilst transmission of this parasite is limited to *Anopheles leucosphyrus* group of mosquitoes (Cox-Singh and Cox, 2010). The female mosquito of the genus *Anopheles* out of the three genera under the subfamily Anophelinae has been implicated as consisting of several important malaria vectors of medical importance involved in the transmission of malaria. However, not all *Anopheles* mosquitoes are considered as efficient vectors only about forty out of three hundred and eighty different species are involved in malaria transmission (Marquardt *et al.,* 2005), with *Anopheles gambiae* out of the *Anopheles gambiae* complex probably being the world's most efficient vector in the transmission of malaria (Service, 1986). Infection of the disease is characterised by symptoms such as chills, headaches, fever, anaemia and general weakness. In severe

cases, the disease can result in complications such as cerebral malaria (which can cause hypoglycemia, decreased cerebral perfusion/hypoxia and cytokine induction resulting in paralysis, tissue damage, blindness, epilepsy, hearing impairment and behaviour disorders), respiratory problems, neurological damage, impaired growth and development in affected individuals, low birth weight in babies born by infected mothers, miscarriages in pregnant women and above all mortality (WHO, 2006a). Studies by (Breman *et al.,* 2001) indicated that more than seven percent of pregnant mothers with severe anaemia is due to malaria infection. However, expectant mothers as a preventive measure to remain malaria-free during pregnancy can be given intermittent preventive treatment (IPT_P) doses of sulphadoxine-pyrimethamine (SP) as therapy.

2.1 Global distribution of malaria

The burden of malaria continues to receive inadequate monitoring, surveillance, case detections and reporting hence only few cases are made known to various health institutions therefore posing a lot of problems to endemic countries. This phenomenon can be likened to a hippopotamus floating on water surface with only ears showing and the largest part of the body submerged in water (Breman, 2001). Approximately over 243 million malaria cases worldwide was reported in 2008 by WHO with about eighty five percent cases coming from Africa, ten percent from South-East Asia and four percent from the Eastern Mediterranean regions with total mortality that same year estimated as 863,000 with eighty nine percent from Africa, six percent from the Eastern Mediterranean and five percent from the South-East Asian Regions (WHO, 2011).Malaria currently is still endemic in 109 countries with either tropical or sub-tropical zones despite the fact that for the past five years global funds amounting to US$ 250 million is contributed yearly towards the control of

malaria by the Global Fund to fight malaria, AIDS and Tuberculosis, the World Bank's Booster programme and President's Malaria Initiative (PMI) (WHO, 2008)

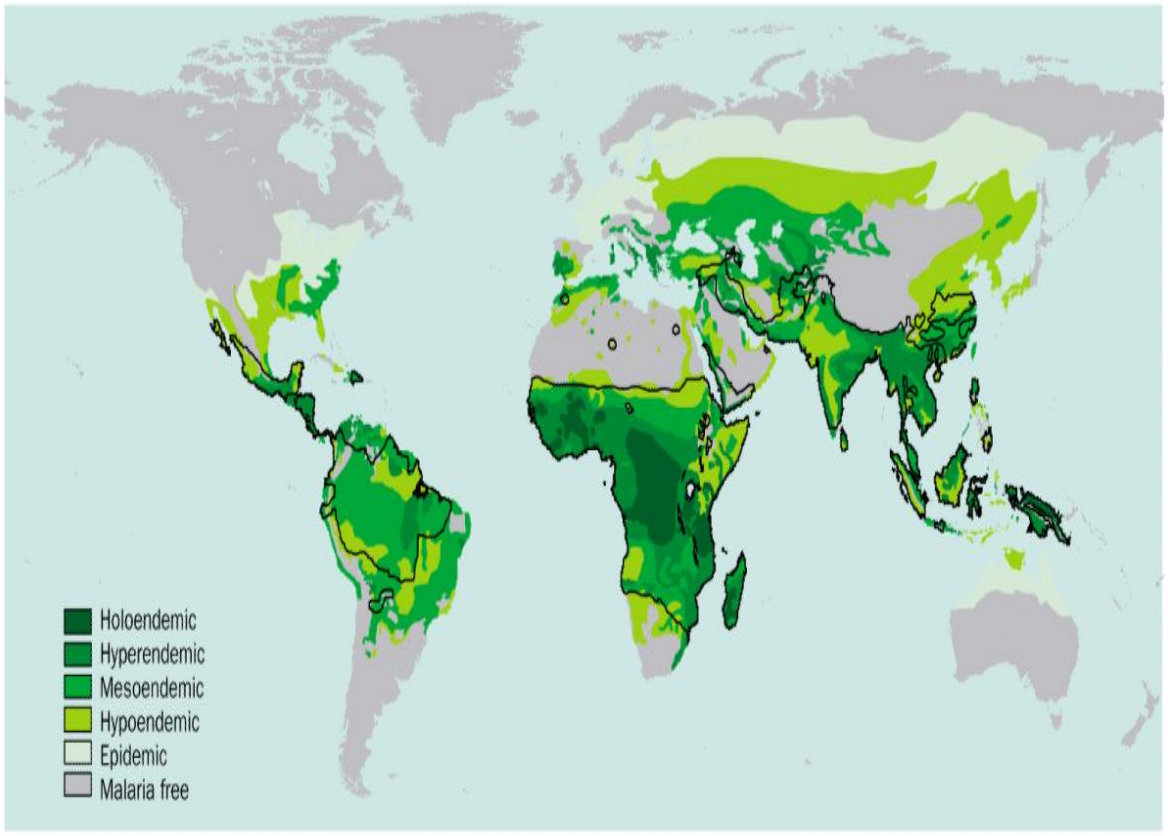


Figure 1 Map showing the global distribution of malaria

2.2 Malaria in Ghana

Asenso-Okyere and Dzator, (1997) in their research indicated malaria as a serious problem in Ghana. However, there is very little evidence showing a decline in the prevalence of the disease despite the implementation of various control interventions, and also since it contributes to a greater number of outpatient morbidity and mortality particularly among children and pregnant women. Reports showed that the burden continues to be high with about 323 per every 1,000 infections among children lesser than 5 years in the year 2008 (Asante *et al.*, 2011). Information from the ministry of health (MOH) in Ghana also indicate the rate of infection to be high in children from the ages of five to nine years, with infection rates as high as eighty

percent but low in adults (Ministry of Health, 2009). Malaria is responsible for about forty percent of outpatient clinic visits and resulting in twenty percent of deaths in children normally below the age of five (Ahorlu *et al.,* 2009). In the year 2006, it accounted for almost thirty seven percent admissions in general with approximately fourteen and nine percent representing admission and mortality in pregnant women respectively. Studies by (Koram *et al.,* 2003) reported that malaria and anaemia accounted for about fifty nine percent of deaths in 1996 in the Kassena-Nankana (KND) District in Northern Ghana. In the year 2006, seven million cases of malaria were reported therefore placing Ghana 8th out of 19 countries estimated to have ninety percent of cases in Africa (Crookston *et al.,* 2010).

2.3 Life cycle of *Plasmodium* parasite

An infective female anopheline mosquito in an attempt to take a blood meal from its human host for reproduction in the process injects saliva containing sporozoites into its host. The sporozoites then move to the hepatic cells of the liver where they undergo an asexual cycle termed exoerythrocytic schizogony. This process leads to the production of merozoites which are released from the hepatic cells into the blood upon rupture. Some merozoites termed as hypnozoites hide within the tissues of the liver as can be observed in *P. ovale* and *P. vivax* causing relapse of the disease in infected individuals. The merozoites invade the erythrocytes where they undergo a trophic stage and in the process metabolise the cytoplasm and other cell contents. At this stage, the parasite is termed as a trophozoite and its shape during this period can be likened to a ring hence the name ring form. The trophozoites enlarge and transform into schizont after growing to a certain stage causes the erythrocytes to rupture releasing merozoites which reinfect the erythrocytes; this cycle is termed erythrocytic schizogony. However, not all the merozoites are released into the blood

some differentiate into sexual forms namely macrogametocytes (female) and microgametocytes (male). The mosquito vector is considered as the definite host since the sexual cycle takes place within it while human is the intermediate host. A female anopheline mosquito in an attempt to take a bloodmeal can pick up these sexual forms of the parasite which upon entering the midgut of the mosquito results in the shedding of flagella by the macrogametocytes by a process known as exflagellation and eventually the fusion of the micro and macro gametes to form a zygote (Mckenzie and Collins, 2008) and the cycle repeating itself in that order (Kuehn and Pradel, 2010). This transforms into an ookinete which further differentiates into an oocyst which then penetrates the epithelial cells of the walls of the midgut into the peritoneal matrix. The oocyst eventually ruptures and releases sporozoites by a process known as sporogony into the haemocoel and then transported by the haemolymph to all parts of the insect's body but a greater proportion of the sporozoites infecting the salivary glands. At this level, the mosquito is termed as infective and can transmit the sporozoites upon taking a bloodmeal.

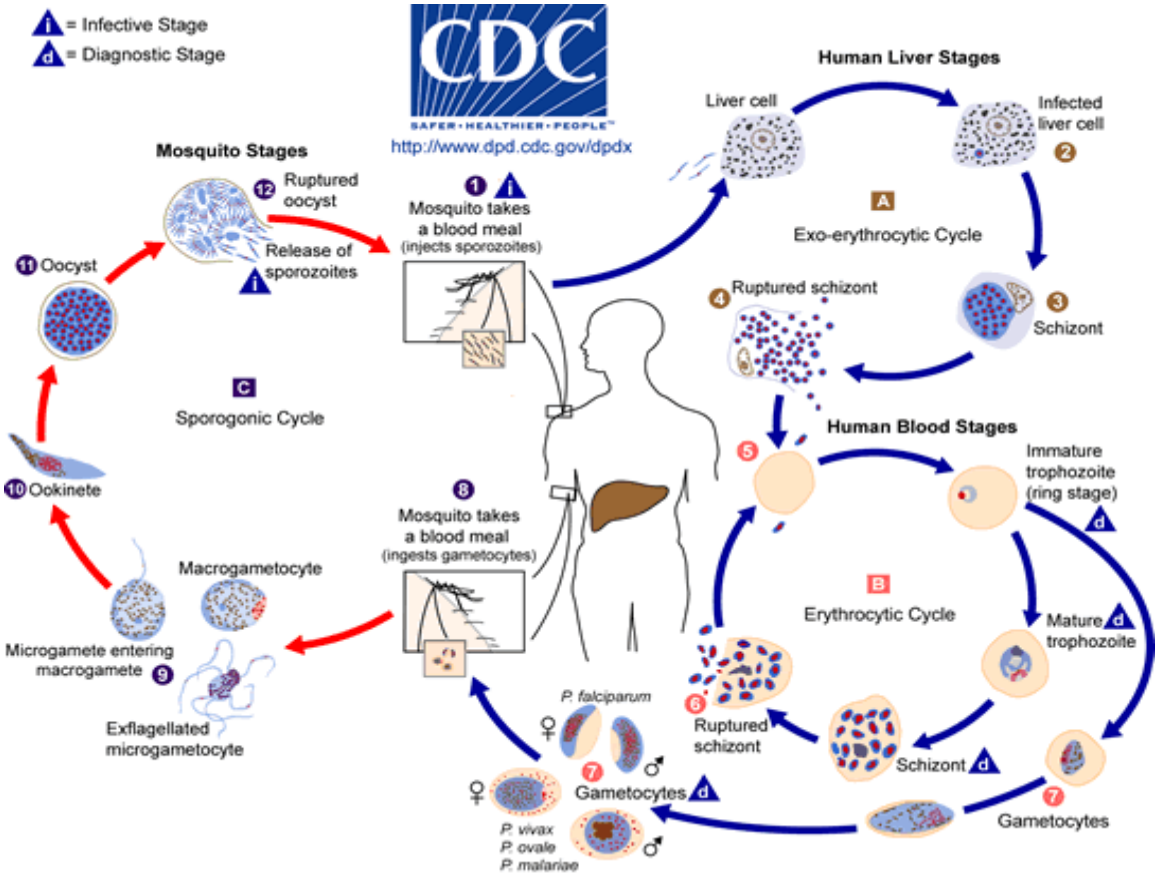


Figure 2 The life cycle of the malaria parasite
www.cdc.gov/malaria/about/biology/index.html

2.4 Mosquito biology

There are about 4,000 mosquito species distributed worldwide, belonging to the family Culicidae and grouped into 41 genera (CDC, 2004). They belong to the order Diptera which implies that they are two-winged or true flies and in the suborder Nematocera. Mosquitoes fall into three subfamilies namely Toxorhynchitinae, Anophelinae and Culicinae (Marquardt *et al.*, 2005). They are mostly found breeding in pools of water, tracks of car tyres, at the edges of streams, empty cans, containers serving as water reservoirs, swampy areas, rice fields, irrigated fields et cetera. It should however be noted that the breeding places for mosquitoes is species dependant on several factors. An example is the *Anopheles* which mostly prefer

clean, sunlit and shallow standing water (Minakawa *et al.*, 2002). *Culex* however thrives well in slightly polluted water (Curtis and Feachem, 1981) whilst *Mansonia* and *Aedes* prefer the edges of streams and ponds with vegetation covering the water surface. In the case of *Mansonia*, the larvae using its siphon pierce the roots of vegetation in order to derive oxygen. Mosquitoes undergo complete metamorphosis involving the egg, larvae (1^{st}, 2^{nd}, 3^{rd} and 4^{th} instars) also known as wigglers and pupal (tumbler) stages which are the immature aquatic forms. The fourth stage which is the adult is terrestrial and the matured form of the mosquito and this can be either male or female. Larvae mostly feed on bacteria, yeast, protozoans, diatoms, micro crustaceans and in the case of toxorhynchites larvae, they sometimes prey on the larvae of other mosquitoes hence it being considered in the biological control of mosquitoes (Linley, 1995). Adults, both males and females feed on nectar from flowers, fruits and honey dew. Females on the other hand require blood meal for ovary and egg development termed as anautogeny (Straif and Beier, 1996) whilst some few females use nutrients obtained during larval growth to nourish their first batch of eggs to maturity but subsequent eggs however need a blood meal in order to develop to maturity. This phenomenon is known as autogeny and can be seen in *Culex pipens, Culextarsalis* and *Wyeomyiasmithi* (Service, 1993). A female mosquito can lay between 30 to 300 eggs during oviposition and the eggs laid come in different shapes. Eggs laid by *Anopheles* are boat-shaped, have floats attached to them and are laid singly whilst those laid by *Culex* are grouped and form a raft (Gillies and Demeillon, 1968). Male and female mosquitoes form swarms mostly in the evening and also from dawn to dusk to mate. A female mosquito mated once has enough sperms stored in the spermatheca to fertilize eggs during every oviposition.

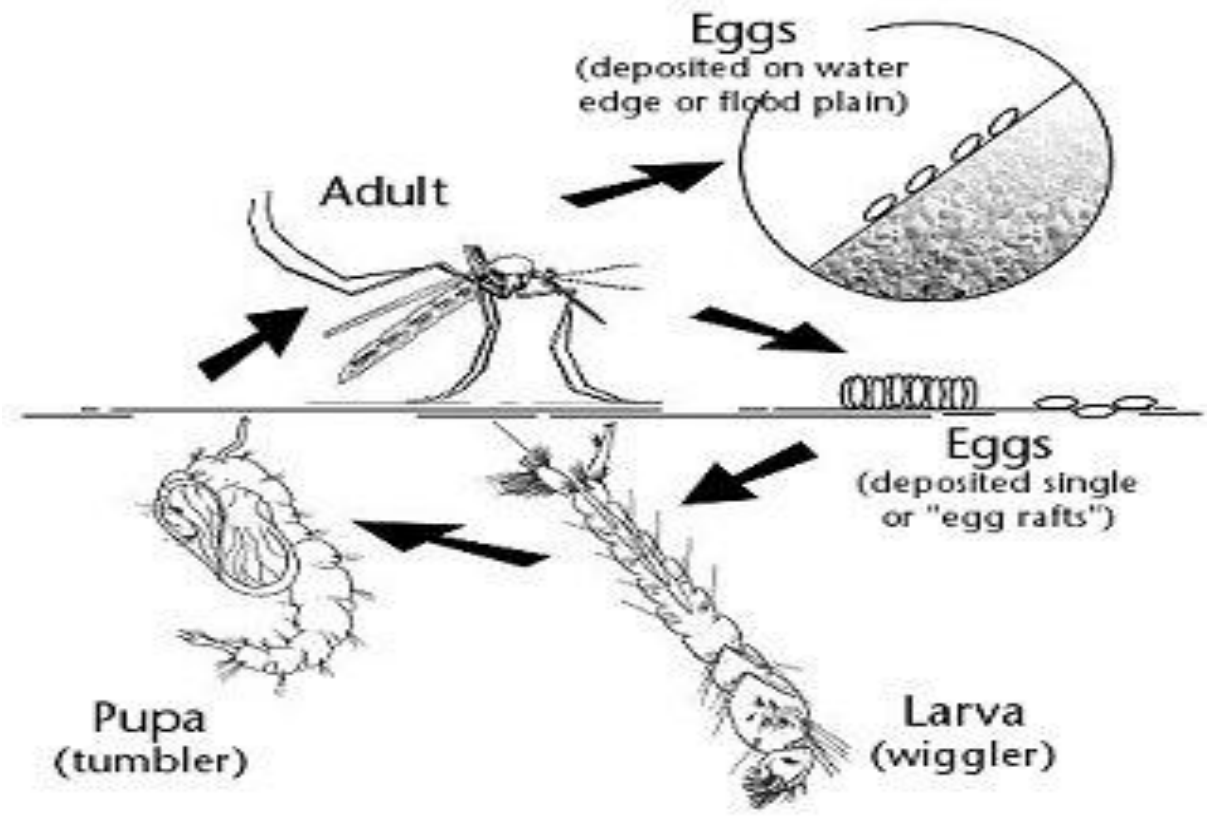


Figure 3 The life cycle of the mosquito
Source: http://whatcom.wsu.edu/commun/wnvhomeowners.htm

2.4.1 Diseases transmitted by mosquitoes

Various important diseases of medical, veterinary and public health concern have mosquitoes serving as vectors. Some examples of these diseases are Eastern equine encephalitis (*Coquilletidia pertubans*), Venezuelan encephalomyelitis (*Culex pipiens*), Dengue (*Aedes aegypti* and *Aedes albopictus*), Japanese encephalitis (*Culex tritaeniorhynchus*), Yellow fever (*Aedes aegypti* and *Aedes africanus*), Malaria (*Anopheles* species), Lymphatic filariasis (*Culex*, *Anopheles* and *Mansonia* species) (Marquardt *et al.,* 2005).

2.4.2 Mosquito biting behaviour

Knowledge of the host preference, biting and resting behaviours especially of female mosquitoes is important in determining and establishing appropriate vector control measures for implementation and also understanding the epidemiology of any disease transmitted by the vector mosquito (Hassan *et al.,* 2001). Some female mosquitoes prefer biting humans (anthropophagic) and resting indoors (endophilc) whilst others prefer animals (zoophagic) and rest outdoors (exophilic) (Pates and Curtis, 2005). Studies on the biology of *Anopheles gambiae* in South East Benin (Padonou *et al.,* 2011), Ghana (Tuno *et al.*, 2010)and Mount Cameroon (Wanji *et al.*, 2003) showed this species to be highly endophagic and endophilic. Anthropophagous behaviour is also shown by *Aedes aegypti* the vector for yellow fever (Harrington *et al.,*2001) whilst studies in Macao, China revealed the vector for dengue *Aedes albopictus* to be mainly exophagic and exophilic (Almeida *et al.,* 2005). *Anopheles arabiensis* a cryptic species of the *Anopheles gambiae complex* exhibited partial zoophily and exophily in studies conducted in Eritrea to assess its host preference (Shililu *et al.,* 2004). Similarly, this behaviour has also been observed in this same species which occurs all over Madagascar (Duchemin *et al.,* 2001). *Anopheles quadriannulatus* also a sibling species of the *Anopheles gambiae complex* is mainly zoophilic (Dekker and Takken, 1998). Studies by (Reddy *et al.*, 2011) observed a shift in the biting behaviour of *An. gambiae s.s.* from an indoor to an outdoor biting species on the Bioko Island in Equatorial Guinea due to the implementation of ITNs as a vector control tool.

2.4.3 Phenotypic plasticity

Most mosquitoes however exhibit phenotypic plasticity or are catholic in their blood feeding behaviour since majority of them are not entirely anthropophilic or zoophilic in their biting. This behaviour is expressed especially in the absence of a particular host (Lefèvre *et al.*, 2009) when there is the implementation of any vector control measure such as Indoor Residual Spraying (IRS) or the usage of Insecticide Treated Nets (ITNs) as well as change in season or place of habitation of mosquitoes (Service, 1993). *Anopheles gambiae s.s.* predominantly anthropophilic exhibited zoophily in studies by (Sousa *et al.*, 2001) probably due to the predominance of dogs compared to the human and pig population in Sao Tome. Dichlorodiphenyltrichloroethane (DDT) being used as a vector control tool in malaria transmission resulted in the shift of biting of the mostly endophilic and endophagic *Anopheles minimus s.l.* species in Thailand (Potikasikorn *et al.*, 2005), *An. Sundaicus* in Southern Java in Indonesia (Sundavaraman, 1958) and *An. sacharovi* in Greece (Pates and Curtis, 2005) to a more zoophilic and exophilic species.

2.4.4 Biting cycles of mosquitoes

Mosquitoes have biting patterns or rhythms with which they forage for their hosts. These are cycles to which they have adapted to over time in order to have greater accessibility to available host, likewise having much information on such activities gives much insight into the appropriate vector control measure to implement at a particular location. Studies show *Anopheles gambiae* the major malaria vector in Sub-Saharan Africa (Appawu *et al.*, 2003) together with its sibling species having an initial period of low biting activity up to 2100-2200 hours followed by a gradual increase in biting with peak normally occurring from midnight to about 0400 hours

then with considerable biting continuing just before dawn (Wanji *et al.*, 2003; Gillies and Demeillon, 1968; Mutero *et al.,* 1984). Service (1963a) reported his findings on studies carried out in Northern Nigeria indicating that biting in *An. nili* begins steadily in the early evening with biting rising to a peak between 2200 and 0100 hrs and gradually declining till dawn. *An. funestus* another important vector incriminated in the transmission of malaria in Africa (Fornadel *et al.,* 2010) starts biting at very low rates during the early hours of the night, increasing steadily after 2200 hrs, then with biting reaching a steady plateau around 0100 till dawn (Gillies and Furlong, 1964). A study (Rubio-Palis and Curtis, 1994) investigating the biting and resting behaviour of Anophelines in Western Venezuela and their implications for the control of malaria transmission observed steady biting in the early hours of the evening with peak biting occurring just after midnight to 0200 hours followed with considerable biting till dawn for *An. nuneztovari* whilst *An. Triannulatus* showed early biting peaks from about 1900 to 2200 hours in the early hours of the night continuing with relative biting till dawn.

2.5 Malaria vectors of the world

Human malaria is exclusively caused by female Anopheline mosquitoes. *Anopheles* is distributed world-wide, occurring in both tropical and temperate regions (Service, 1993). They belong to the Order Diptera, Sub-order Nematocera, Family Culicidae and Sub-family Anophelinae. However, not all Anophelines transmit malaria as it was formerly thought that many species of African *Anopheles* played local or a minor role in the transmission of malaria (Gillies and Demeillon, 1968). There are approximately 430 known species of which 40 have been implicated as malaria vectors (Catteruccia, 2007) implying that the ability of a vector to support parasite development depends largely on differences in genetics, ecology and behaviour

between mosquito species (Costantini *et al.,* 1999). Anopheline malaria mosquitoes can be classified as being primary or secondary (incidental) vectors with the secondary showing their importance in their localised area (Service, 1984). However, this classification is rather unsatisfactory because a species purported as being primary in an area can be a non-vector or secondary in a different location.

2.5.1 Factor affecting malaria transmission

For a mosquito to be considered as a vector in the transmission of malaria certain entomological indices must satisfy the process. The said vector involved must be abundant, susceptible to infection, exhibit anthropophily, have a proportion of anophelines carrying infective sporozoites and live longer (Service, 1984; Catteruccia, 2007; Hay *et al.*, 2011; Onori and Grab, 1980). Likewise, meteorological factors like rainfall as was observed in India (Young and Majid, 1930) together with relative humidity and temperature which was observed in Argentina (Alvarado, 1948) also have direct impact on the entomological indices involved in malaria transmission. Environmental factors such as irrigation sites, drainage canals, destruction of forests and the construction of dams and urbanisation can enhance the proliferation and colonisation of new areas by efficient vectors increasing the risk of malaria transmission (Guerra *et al.*, 2008)

Table 1: A list of Anopheline vectors involved in malaria transmission world-wide (Service, 1993).

ZONES	VECTORS
Mexico and Central America	*An. albimanus, An. albitarsis, An. aquasalis, An. aztecus, An. darlingi, An. punctimaculata*
South America	*An. albimanus, An. punctimaculata, An. cruzii, An. albitarsis, An. aqualis, An. bellator, An. darlingi, An.nuneztovari, An.pseudopunctipennis*
Australasian Area	*An. bancrofti, An. punctulatus complex*
Africa South of the Sahara	*An. Gambiae s.s., An. arabiensis, An. melas, An. merus, An. bwambae, An. quadriannulatus, An. funestus, An. pharoensis, An. hargreavesi, An. nili, An. mouchetti*
China, Taiwan and Korea	*An. balabaciensis, An. lesteri, An. pattoni, An. sinensis*
South East Asia	*An. aconitus, An. campestris, An. culicifascies, An. hycranus, An. lesteri, An. sinensis, An. letifer, An. umbrosus, An. leucosphyrus, An. maculatus, An. minimus, An. nigerrimus, An. subpictus, An. sundaicus*
Indian Subcontinent	*An. superpictus, An. culicifacies, An. fluvialitis, An. minimus, An. stephensi*
North Africa and Middle East	*An. atroparvus, An. claviger, An. labranchiae, An. pharoensis, An. sacharovi, An. sergentii, An. stephensi, An. superpictus*

2.6 African malaria vectors

Generally, in Sub-Saharan Africa, *An. gambiae s.l.* and *An. funestus* are the most efficient vectors involved in malaria transmission (Service, 1984; Fornadel *et al.*, 2010) However, localised transmission might involve vectors such as *nili, mouchetti, merus* and *melas* (Gillies and Demeillon, 1968). Secondary or incidental vectors like *pharoensis, coustani, hargreavesi, hancocki, squamosus* et cetera might also play minor roles in the spread of the disease (Gillies and Demeillon, 1968). *An. gambiae* complex consist of West Africa's three most efficient malaria vectors namely *An. gambiae sensu stricto* (*s.s.*), *An. arabiensis*and *An. melas* (De Souza *et al.*, 2010). *An.gambiae* can be found in West, East, South, Central, North Africa together with surrounding islands including Madagascar, Saudi Arabia and Yemen since it is

widely distributed (Coetzee *et al.*,2000). *An. funestus* which is second only to *gambiae* as a vector of malaria in some areas may be responsible for majority of transmission. This malaria vector has a wide distribution covering tropical, subtropical regions of Africa and Madagascar (Garros *et al.*, 2004).

2.6.1 *Anopheles gambiae* complex

Before 1956, *Anopheles gambiae* which was initially thought of as being a single species was discovered to be a complex through hybridisation experiments being conducted to investigate the inheritance of insecticide in *An. gambiae* (Davidson 1956, 1962). This was further clarified from many cross mating experiments (Service, 1984) revealing seven closely related, morphologically indistinguishable sibling species but varying in their ecological, behavioural and vector competence (Djogbénou *et al.*, 2010). They include *An. gambiae s.s., An. arabiensis, An. melas, An. merus, An. bwambae, An. quadriannulatus* A and B (Coetzee *et al.*, 2000)

2.6.2 *Anopheles gambiae sensu stricto* (*s.s.*)

This is the most efficient vector in the *gambiae* complex, widely distributed in Africa and which normally occurs in sympatry with *An. arabiensis* since both are good malaria vectors (Fanello *et al.*, 2002). This sibling species is highly anthropophilic and endophilc with larvae found breeding in sunlit pools having bare soil at the edges (Service, 1984). The adult is found mostly in humid savannah and forest areas especially during the wet season (Wondji *et al.*, 2002). It is however better adapted to wetter regions than the savannah (Lindsay, 1998).

2.6.3 *Anopheles arabiensis*

This sibling species is also widely distributed in Africa just like *An. gambiae s.s.* It is the next efficient malaria vector in the complex exhibiting some degree of anthropophily and endophily (Coluzzi, 1984). However, *An. arabiensis*is known to

have a greater behavioural plasticity with respect to feeding and resting (Gillies and Coetzee, 1987), hence, its ability to be readily directed to cattle (zoophily) and also displaying higher exophilic tendencies (Service, 1984). This exophilic behaviour will therefore make it more difficult to be controlled with residual insecticides (White, 1974). In West Africa, this species is normally found in the arid savannah and steppes including those of South-Western part of Arabian Peninsula due to its high tolerance for dry environment (Coetzee *et al.*, 2000). However, notwithstanding this environmental preference, studies in Benin (Djogbénou *et al.*, 2010) has hypothesised that this species had extended its range from the drier savannah to the more humid forest areas corroborating similar findings from Nigeria (Kristan *et al.*, 2003).

2.6.4 *Anopheles melas*/*merus*

This halophytic species has larvae normally breeding in brackish water mainly in coastal saline habitats such as swamps and mangroves (Coetzee *et al.*, 2000). Their role in malaria transmission is secondary due to their strong zoophilic and exophilic behaviours (Djogbénou *et al.*, 2010). *An. merus* is a West African species whilst *An. melas* is found in East Africa with their ecological and morphological similarities seemingly reflecting evolutionary convergence (Coluzzi *et al.*, 2002).

2.6.5 *Anopheles bwambae*

Anopheles bwambae species is known to be restricted to the mineral hot springs in Bwamba County found in the Semiliki forest of Uganda, where it breeds in brackish water together with other halophilic mosquitoes with the pH of water ranging between 8.2-8.3 (Sueur and Sharp, 1988). The natural host of this species is unknown but they do sometimes bite humans complementing studies by White (White, 1985) who found sporozoites and developing larvae of *Wuchereria bancrofti* in females therefore inferring it as being a vector involved in local transmission of malaria and filariasis. This species is said to display anthropophilic and endophilic tendencies but in the absence of man, animals can serve as alternate hosts as was the case in the Semiliki forest (White, 1985).

2.6.6 *Anopheles quadriannulatus*A/B

Anopheles quadriannulatus A is mainly zoophilic since it is mostly attracted to cattle, hence it being of less direct medical interest (Coetzee *et al.*, 2000). This species occurs sympatrically with *An. arabiensis* but less frequent with *An. gambiae* (White, 1974), as well as have their larvae to be fresh water breeders (Gillies and Demeillon, 1968). It occurs mainly in South-Eastern Africa, Southern Africa, Ethiopia and Zanzibar (Coluzzi *et al.*, 1978). However, high levels of exophily occur

in species from Zanzibar and Southern Africa, whilst species at higher altitudes in Ethiopia tend to be endophilic (Coluzzi, 1978). *Anopheles quadriannulatus* A is only found in Ethiopia and has been likened to *Anopheles quadriannulatus* B therefore considering it to have the same vector capacity and behaviour (Coetzee *et al.,* 2000). However, this might not be the case, therefore much studies is needed for this species.

2.7 Genetic variation within the *An. gambiae* Complex

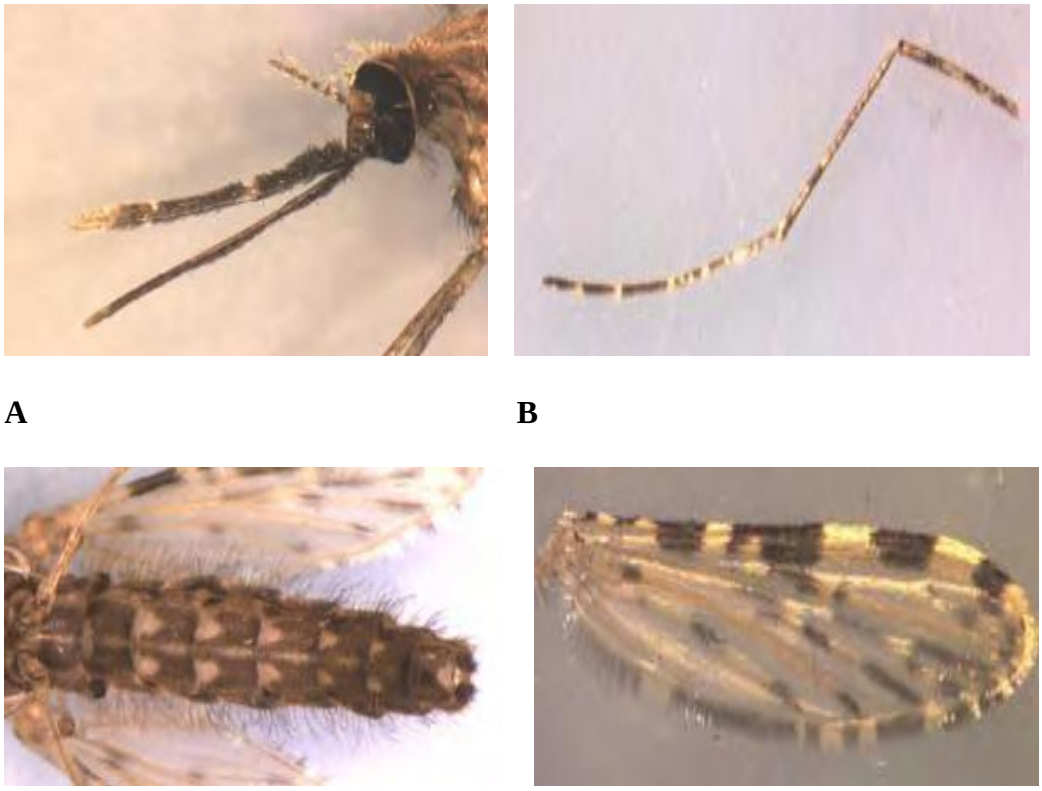
Non-stochastic associations and frequencies of some polymorphic chromosomal inversion arrangements within populations of the major malaria vector *An. gambiae s.s.* indicate the existence in West Africa of five chromosomal forms namely Bissau, Mopti, Savannah, Forest and Bamako (Coluzzi *et al.,* 1985). The Forest and Bissau chromosomal forms are found in humid and coastal areas of West Africa, whilst Bamako, Mopti and Savannah are more adapted to dry environments (Favia *et al.*, 1997). The Forest chromosomal form is characterised by inversion systems 2Rb, 2Rd and 2La; the Savannah form having the inversion arrangements 2Rbc and 2Rbcd; Mopti chromosomal form also characterised by inversions 2Rbc/2Ru and sympatric with Savannah and/or Bamako; Bissau form is also characterised by the arrangement 2Rd and lastly Bamako which is characterised by the inversion j and mostly sympatric with Mopti and Savannah (Yawson *et al.*, 2007; Coluzzi *et al.,* 1985). Further characterisation of the chromosomal forms within *Anopheles gambiae s.s.* resulting in the analyses of the intergenic spacer (IGS) and internal transcribed spacer regions (ITS) of the rDNA, revealed nucleotide substitutions which differentiate into two forms namely M and S forms in the case of IGS (della Torre *et al.*, 2001) and Types I and II in the case of ITS (Gentile *et al.*, 2002). M/S hybrids are absent or very rare in sympatry (Wondji *et al.,* 2002) as very low frequency (less

than 2%) was observed in Nigeria, Burkina Faso and Côte d'Ivoire (Djogbénou *et al.,* 2010) but with relatively high levels (up to 24%) being recorded in Gambia and Guinea Bissau (Oliveira *et al.,* 2008). Notwithstanding this, interbreeding sometimes does occur under laboratory conditions yielding fertile progeny (Diabaté *et al.,* 2007). The knockdown resistance gene (kdr) was previously known to be restricted to the S form (Chandre *et al.,* 1999) but recent studies have shown its occurrence in the M form but at low frequencies (Yawson *et al.,* 2007; Fanello *et al.,* 2002; Awolola *et al.,* 2003; Diabate *et al.,* 2003) possibly through introgression from the S form (Weill *et al.,* 2000). Recently, the observation of the ace-1^Rallele (responsible for cross-resistance to carbamates and organophosphates insecticides) in both M and S forms is also an indication of introgression between them (Djogbénou *et al.,* 2008). The linkage of M and S molecular forms with Mopti and Savannah/Bamako respectively in Mali and Burkina Faso breaks down in other parts of Arica (Gentile *et al.,* 2002). The S form is mostly dependent on temporary breeding sites and rainfall, whilst M form is better adapted to permanent breeding sites such as rice fields and irrigation plains (Diabaté *et al.,* 2005; de Souza *et al.,* 2010).

2.8 Morphological identification of *Anopheles gambiae* complex

Several techniques have been employed in the identification of various species of mosquitoes; this could either be morphological, molecular or cytotaxonomical (Collins *et al.,* 2000). Gillies and Demeillon, (1968) and Gillies and Coetzee, (1987)provide detailed information on the morphological identification of Anopheline mosquitoes. *Anopheles gambiae s.l.* female adults are identified by three smooth pale bands on their palps, having the apical band being broad; median band less than half the width of the apical band; and a basal band. The femora, tibiae and first tarsal segment are speckled and sometimes have these speckles fused to form

short lines and rarely forming complete rings. The abdomen is mainly light brown in colour with hairs, but the eighth tergite usually has scales which may extend to the seventh. Wings also have pale markings which can either be yellowish or cream in colour.



A **B**

C **D**

Figure 4: Diagrams showing the A: Palp. B: Protarsi. C: Abdomen. D: Wing of *An. gambiae* (Benedict, 2007).

2.9 *Anopheles funestus* group

Within the *An. funestus* group, *An. funestus* serves as a highly efficient malaria vector second to *An. gambiae*, and sometimes playing a major role in malaria transmission at a particular location (Garros *et al.*, 2004). However, other species of the group are mainly zoophilic, biting human outdoors and playing little or no role in malaria transmission (Garros *et al.*, 2004). The funestus group consists of at least

eleven species namely *An. funestus, An. vaneedeni, An. rivulorum, An. rivulorum*-like, *An. confusus, An. parensis, An. brucei, An. aruni, An. fuscivenosus, An. Leesoni* and the Asian member *An. fluviatilis* (Benedict, 2007) *An. funestus* has a wide geographical distribution including subtropical Africa and Madagascar, while the others are restricted in their distribution in Africa (Gillies and Coetzee, 1987).

2.9.1 Morphological identification of *Anopheles funestus group*

Gillies and Demeillon, (1968) described this group as consisting of small dark species exhibiting uniformity in adult characters; the main dark and light areas of costa and first vein always well developed and pale spots on other veins reduced. Wing markings of *An. funestus* species showing the characteristic 4 pale spots (numbered) on the costa (Hervey *et al*., 1998).

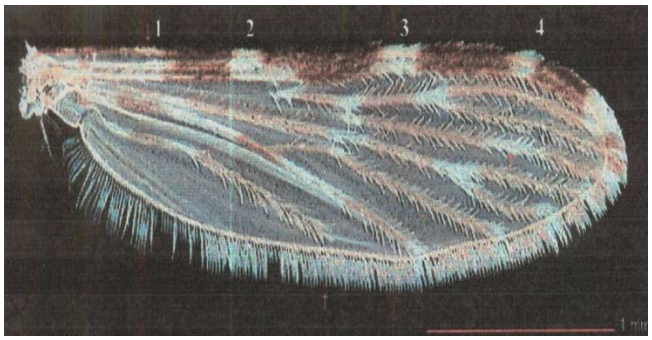


Figure 5: Wing of *An. funestus* species (Hervey et al., 1998)

2.10 Malaria control

The global malaria incidence in 2010 was between (154–289 million) cases with (490,000–836,000) estimated deaths (WHO, 2013). Despite the frantic efforts to control malaria through vector control and chemotherapy, an increase in the incidence of the disease has been realised for the past thirty years mainly due to socio-economic underdevelopment together with insecticide and drug resistance (Phillips, 2001). Funds for malaria control also increased appreciably from

approximately US$100 million in 2003 to US$1.5 billion in 2010 with more than three-forth going to sub-Saharan Africa (WHO, 2010a) but this disease still continues to be a threat particularly in Africa. Between 1955 and 1969, the World Health Organisation's Global Malaria Eradication Programme in an attempt to globally eradicate malaria, advocated for the spraying of houses with residual deposits of organochlorines; dieldrin and Dichlorodiphenyltrichloroethane (DDT), which apparently led to significant reductions of the disease in endemic regions including countries on the Indian sub-continent and South America (Cueto *et al.,* 2007). However, this action unfortunately resulted in the development of resistance in malaria vectors upon exposure to the insecticide therefore eventually leading to the abandonment of insecticidal spraying in several countries (Takken, 2002). Hence the plausibility of completely eradicating malaria especially in Africa might not be feasible due to the existence of conducive climatic conditions for the resilient effective vector *An. gambiae* and also the possibility of disease resurgence. Therefore global eradication might not be possible but the maintenance of low transmission levels by effective vector control is recommendable. Hence a renewed global campaign towards the eradication must be put in place due to the fact that abandonment of malaria control has led to resurgence of the disease, the possibility of reaching the very poor with existing control methods, success in the complete sequencing of the genome of the malaria vector and parasite paving the way for new targets for drug and vaccine development (Hoffman *et al.,* 2002); as well as the establishment of new programmes to support global control efforts (Sachs, 2002). Several global commitments such as the African heads of state summit held in Abuja in 2000; the establishment of the Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM) in 2001; new research collaborations such as the multilateral

initiative on malaria in 1997; the Roll Back Malaria by the United Nations in 2000 and the establishment of various National Malaria Control programmes at the various health ministries of endemic countries (WHO, 2003). Elimination of malaria which involves the cessation of local malaria transmission but with disease occurrence due to the introduction of cases attained elsewhere (Greenwood, 2008) must target countries bordering areas with high malaria transmission and when success has been attained, malaria could be rolled back (Feachem and Sabot, 2008). The core strategies for the implementation of effective malaria control in Africa include; access to proper treatment, vector control (usage of LLNs and IRS) and any appropriate control measure locally, early detection and response to epidemics and the prevention and treatment of the disease in pregnant women (WHO, 2006a). Malaria control involves an integrated approach consisting of effective vector control and treatment with effective antimalarials. The current approach to malaria control is asynergistic action consisting of proper environmental management, usage of insecticides, bednets, chemotherapy and proper case management. However, this has unfortunately been hampered by the development of resistance to antimalarials and insecticides directed to malaria parasites and vectors respectively and resurgence of the disease in places where it has already been eradicated.

2.10.1 Chemotherapy in malaria control

The main aim of this line of treatment is to reduce morbidity and mortality in infected individuals by; preventing human malaria parasite reservoir of infection and infectivity, avoiding the progression of uncomplicated malaria to severe cases and the prevention of malaria during pregnancy (WHO, 2010b). It is recommended that uncomplicated malaria be treated with an artemisinin-based combination therapy (ACT), but due to the fact that the anti-gametocidal effect of artemisinin is

incomplete the addition of primaquine to artemisinin-based therapies is recommended to interrupt transmission more effectively (WHO,2010). The five recommended ACTs by WHO are artemether-lumefantrine (Coartem), artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulphadoxine and artesunate-pyrimethamine (WHO, 2006a). This approach is mostly effective where malaria transmission is very low and infected individuals seek early treatment. A similar situation which was observed at the Thai-Burmese border (Nosten *et al.*, 2000) and South Africa (Barnes *et al.*, 2005) where malaria transmission was low, observed significant reductions in the incidence of *P. falciparum* after the introduction of ACTs but the reverse was seen in higher transmission zones. However, this mode of control is faced with the challenge of malaria parasite resistance against antimalarials as was observed in East Africa in the 1970s, where the first case of the emergence of *P. falciparum* resistance to chloroquine was initially recorded, and has now spread to the rest of Africa (Collins *et al.*, 2000)

2.10.2 Intermittent Preventive Treatment (IPT_P) in pregnant women

Pregnant women and infants include the most vulnerable in terms of malaria in sub-Saharan Africa (Okeibunor *et al.*, 2011). Malaria in expectant mothers most often increases the risk of still births, miscarriages and low birth weight (Shane, 2001). Normally, antenatal care recommends the usage of insecticide-treated nets (ITNs) and two treatment doses of sulphadoxine-pyrimethamine (SP) (15000/75 mg) as an intermittent preventive treatment (IPT_P) in pregnancy (Okeibunor *et al.*, 2011). IPT_p involves the administration of an antimalarial drug or combination at specified times most often in corresponding to the time when a subject has access to health services and individuals at risk (whether or not they are infected) (Greenwood, 2008). The implementation and adoption of WHO recommendation for the control of malaria in

pregnant women in Sub-Saharan Africa as was implemented in studies conducted in Côte d'Ivoire (Vanga-Bosson *et al.,* 2011) and Ghana (Tutu *et al.,* 2011) accounted for significant reductions in malaria parasitaemia. Studies by (Okeibunor *et al.,* 2011) also recommend the implementation of a community-directed programmes approach to help enhance health delivery system for pregnant women.

2.10.3 Biological vector control agents

Due to the avoidance of possible insecticide resistance upon exposure to chemicals and the impact they have on the environment, the usage of biological agents could also enhance malaria control. The larvivorous fish, *Gambussia affinis* which preys on the larvae of mosquitoes has successfully been used in malaria control programmes to reduce malaria incidence in Greece and Italy where malaria transmission was unstable (Wickramasinghe and Costa, 1986). The bacteria, *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* produce toxic endospores which have been used as larviciding agent (Collins, 1995). Unfortunately, the toxin is expensive to produce and has no residual effect therefore requiring several applications culminating into extra expenses. The usage of entomopathogenic fungi directed against adult mosquitoes has proven effective under laboratory (Howard *et al.,* 2010) and field (Lwetoijera *et al.,* 2010) conditions. Field studies in Benin, (Howard *et al.,* 2011) following the exposure of insecticide-resistant malaria vector *An.gambiae s.s.* VKPER laboratory colony strain with fungi (*Beauveria basiana*) infested polyester netting showed promising results due increased mortality in mosquitoes. Similarly, insecticide-susceptible malaria vectors in Tanzania infected with the fungi *Metarhizium anisopliae* resulted in a shorter life span in mosquitoes infected (Lwetoijera *et al.,* 2010). Notwithstanding the success achieved using this method of control, this approach still needs to be further researched into on the field in order to

examine the effects of environmental conditions on the virulence and viability of the fungi (Farenhorst *et al.*, 2010).

2.11 Malaria control for adult vectors

The two major intradomicilliary vector control interventions which are directed at adults are indoor residual spraying (IRS) and the usage of insecticide treated nets (ITNs). These are used either individually or in combination to prevent malaria transmission in both holoendemic and hyperendemic communities and their usage dependent on both the epidemiological conditions and operational requirements in an area. The joint usage of both control tools has been lauded by the WHO (WHO, 2010b) and various malaria control programmes in the sense that IRS provides a toxic effect and repels mosquitoes upon contact, together with ITNs inhibiting feeding and resulting in mortalities. These when used in conjunction could result in the disruption of malaria transmission. The combination of both interventions in houses in the Zambezi province, Mozambique and Bioko, Equatorial Guinea (Kleinschmidt *et al.*, 2009) accounted for a lower probability of contracting malaria among children. However, IRS and ITNs used concurrently would not efficiently disrupt transmission in areas where malaria vectors are mainly exophilic or where transmission (Entomological Inoculation Rate (EIR) is in the range of hundreds) (Kleinschmidt *et al.*, 2009). Therefore, complementary interventions that target outdoor resting and feeding mosquitoes could be implemented (Ferguson *et al.*, 2010)

2.11.1 Insecticides for malaria vector control

Insecticides elicit different range of behavioural and physiological effects on mosquitoes upon exposure. Chemicals for malaria control belong to four major classes (organophosphates, organochlorines, carbamates and pyrethroids), together with insect growth regulators such as diflubenzuron, methoprene, novaluron and pyriproxyfen having different modes of action (WHO, 2011). Insecticides can act as deterrents or repellents (Roberts *et al.*, 2000) by preventing mosquitoes from entering houses, irritants (Smith, 1965) due to its exiting effects, toxicants (Grieco *et al.*, 2007) by resulting in mortalities, cause feeding inhibition (Hossain *et al.*, 1989) and having sub-lethal effects on mosquitoes (Kennedy *et al.*,1947). The implementation of both IRS and ITNs in an area would therefore require in both instances insecticides with overlapping modes of action for longer protection as was the case in Burundi(Protopopoff *et al.*, 2007) and the avoidance of resistance due to single insecticide usage. The problem encountered most of the time is the development of resistance which normally hampers vector control.

2.11.1.1 Indoor residual spraying (IRS)

This is one of the major malaria intervention tools approved by WHO for adult vector control. Twelve different insecticides have been recommended by the WHO for IRS: oneorganochlorine, six pyrethroids, three organophosphates and two carbamates (WHO, 2006b). It involves the spraying of walls and roofs of houses alongside domestic animal shelters with residual insecticides, mostly targeting indoor resting vectors by repelling and exerting toxic effect on them (Okumu and Moore, 2011). Proper implementation and sustainability of IRS in the Southern part of Africa is known to have effectively reduced malaria transmission and in controlling the disease (Mabaso *et al.*, 2004). However, some concerns have arisen regarding the

usage of IRS for malaria vector control and this includes: the issue with rapid decay of insecticides after sometime, logistics and resources involved (training sprayers and supervisors, equipment for spraying, financial resources and community sensitisation) (WHO, 2006b).

2.11.1.2 Insecticide treated nets (ITNs)

The major strategy of the National Malaria Control Programme is focussed on effective case management and the use of insecticide treated bed nets among vulnerable groups such as children less than five years and pregnant women (Thwing *et al.*, 2011). The wide distribution of ITNs to reduce morbidity and mortality is one of the four main strategies to curb malaria(WHO, 2003),with a target set by African Heads of State to protect 60% of all pregnant women and children by 2005 resulting in large-scale programmes taking off during the last few years. The distribution of ITNs have received wide coverage in many African countries since 2002 as part of Global Fund, United Nation International Children Emergency Fund (UNICEF),International Federation of Red Cross (IFRC), NGOs and the President's Malaria Initiative (PMI) programmes. The procedure used for the distribution of ITNs during these campaigns varied, with few offering assistance to hang the nets. Programmes to monitor interventions are very important as this will help assess the impact of control measures on target vectors over time (Boakye *et al.*, 2007). ITNs offer an additional chemical barrier to just the physical barrier presented by the untreated nets. They serve as a human-baited trap when an individual is inside by intercepting mosquitoes in their search for a blood meal (Eisele and Steketee, 2011). One of the major setbacks to the large-scale use of ITNs is the need for frequent re-treatment with insecticide almost every six months as a result of the loss of efficacy after three washes (N'Guessan*et al*., 2001). ITNs that are not re-treated eventually

become untreated net and in the presence of holes, become less effective as both the chemical and physical barrier become compromised. This factor has therefore resulted in the development of Long-lasting Insecticide Treated Nets (LLINs) which have insecticides incorporated into the fibre of the netting material during their manufacture (N'Guessan *et al*., 2001).They also have the ability to last and retain insecticidal activity for four to five years, and can have up to twenty washes without the need for insecticide re-treatment (Guillet *et al.,* 2001).

Insecticides mostly used for the impregnation of bed nets belong to the class of synthetic pyrethroids with relatively long residual activity and include etofenprox and permethrin (non-alpha-cyanopyrethroid) and cyfluthrin, deltamethrin, alpha–cypermethrin (Alpha–cyanopyrethroids) (Okumu and Moore, 2011). Pyrethroids are preferred to other classes of insecticides for the impregnation of bed nets because of their relatively low mammalian toxicity and lesser environmental effect (Zaim *et al*., 2000).

2.12 Entomological impact of ITNs

The consistent usage of ITNs in an area against anthropophilic malaria vectors can cause mass killing effect against them resulting in a drop in density of the local vector population and decrease in parous and sporozoite rate as recorded in village-scale trials in Tanzania (Magesa *et al.,* 1991).Insecticides used in the impregnation of bed nets upon exposure to insects can exert toxic effect, deterrence, excito-repellency or mortality (Okumu and Moore, 2011). Other plausible effects of using ITNs include: Change in the biting behaviour of mosquitoes which consist of the change in the place of biting (Reddy *et al.*, 2011) change in time of biting (Appawu *et al.*, 2004) and shifts in hosts (Takken, 2002) by mosquitoes.

2.13 Research into malaria control

There is the need for efficient and effective interventions, which combined with existing measures could effectively reduce the malaria burden in endemic areas especially in Africa (Greenwood *et al.,* 2005) therefore prompting the need for more research work. There has been vigorous research works associated with the usage of molecular techniques in malaria vector control, these include; molecular assays for the specific identification of malaria vector species, studies on the molecular basis of insecticide resistance, the characterization of genes expressed in the (midgut, fat bodies and salivary gland) with the sole aim of developing *plasmodium* inhibiting constructs that can be expressed in these tissues (Proux-Gillardeaux *et al.*, 2007) and interaction between the malaria parasite and the mosquito vector during the midgut and salivary gland phases of sporogony (Zieler *et al*., 2000). Research into new diagnostic techniques involving the detection of low numbers of both asexual and sexual stage parasites by molecular methods, and novel approaches to serology is also underway (Mlambo *et al.*, 2008).

2.13.1 Vaccine development

The development of new vaccines is still ongoing since currently there is no accepted vaccine for usage. The complex nature of the life cycle of the malaria parasite presents different stages of the parasite that can be targeted (Tediosi *et al.*, 2009). There are basically three vaccine types being researched into: pre-erythrocytic vaccines (PEV) which has received much clinical advancement (Alonso *et al.*, 2004) reduces the proportion of inoculations from infected bites, blood stage vaccine (BSV) also reduces parasite densities at each time step by a proportion equal to the vaccine efficacy and mosquito stage transmission blocking vaccines (TBV) which

involves the proportional reduction of the probability that a mosquito becomes infected from any one feed on an infectious vaccinated human (Tediosi *et al.*, 2009).

2.13.2 Integrated vector management (IVM)

This is a rational decision-making process involving the optimal use of available resources in vector control and therefore comprising of proper management that will see to the improvement of the efficacy, cost effectiveness, ecological soundness and the sustainability of vector control interventions with available tools and resources (WHO, 2012). In 2004, the WHO globally adopted IVM as a vector control approach (WHO, 2004) consisting of five key elements: evidence-based decision-making; integrated approach; collaboration with health and other sectors; advocacy, social mobilisation, legislation and capacity building (Beier *et al.*, 2008). However, for promising results during the implementation of IVM, it requires a synergy of constant monitoring, evaluation, commitment from governments and international partners (WHO, 2004). The success story achieved in the implementation of IVM for malaria control was observed in Zambia (Chanda *et al.*, 2008) and Dar es Salaam Tanzania (Caldas de Castro *et al.*, 2004) respectively.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Study site

The study was undertaken at Odumasi a suburb of Dodowa in the Dangme West District of the Greater Accra region (Figure 1). Sampling was done in two houses with coordinates and elevation being (N 05.89731°, W 000.07889°, 65m) and (N 05.89776°, W 000.07968°, 66m) respectively. This area is humid, has persistent rainfall and falls within the coastal savannah zone. The inhabitants here are mostly small scale-farmers involved in cassava, mango and vegetable farming. Several ponds and marshes surrounded by shrubs were spotted in the town whilst the major malaria vectors found here were *Anopheles gambiae s.l.* and *An. funestus*. The estimated population size for this area is about 122,836 and houses found here are either mud with thatch roof or concrete with windows.

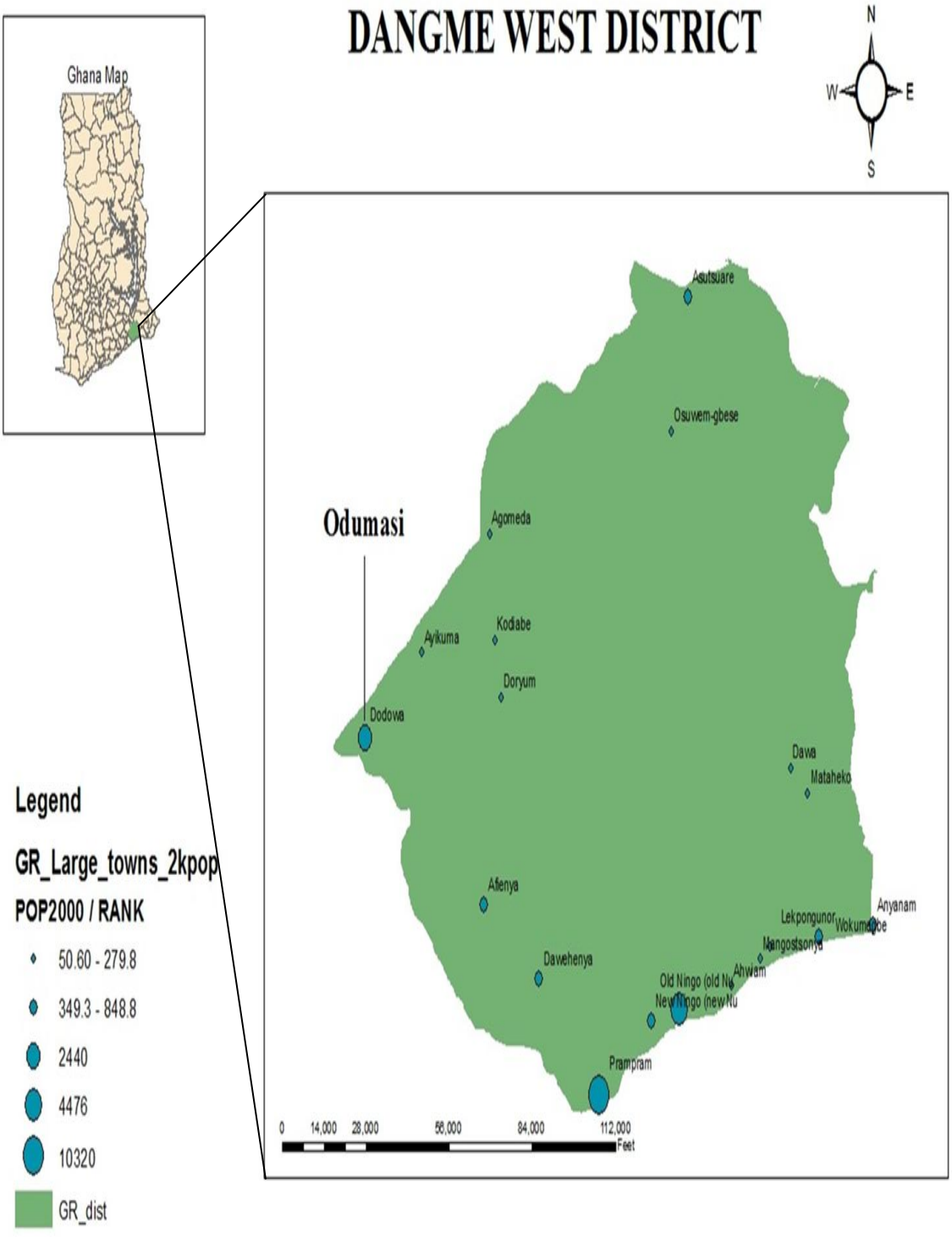


Figure 6: Map of the Dangme West District of Ghana showing the location of the study site Odumasi a suburb of Dodowa

3.1 Field sampling of adult mosquitoes

Indoor and outdoor human landing catches (HLC) of mosquitoes was performed once in late October 2012 at sampling site. The town for sampling was divided into two sections and a house selected from each side for collection. On the night of collection, each house had two individuals collecting mosquitoes both indoors and outdoors from 6pm to 6am. Collection was done by exposing the bare limbs of collectors seated in the dark to mosquitoes attempting to take a blood meal. A flash–light was used to locate host-seeking mosquitoes and a glass tube used to trap them (Figure 7A). Trapped mosquitoes were then released into a holding cup and placed in boxes which were labelled outdoor and indoor. Each box contained twelve cups (one for each hour reflecting the time for sampling) covered with nets which were held in place with elastic bands and on each side of the paper cup was a hole which was covered with foam with a slit in the middle. A small ball of cotton wool soaked with 10% sugar solution was placed on each cup to sustain mosquitoes until transportation to the laboratory. In the laboratory, the captured mosquitoes originally grouped on hourly basis were pooled into four time groups namely 6pm–9pm, 9pm–12am, 12am –3am and 3am-6am hours for rearing.

3.2 Morphological identification of mosquitoes

Samples were sorted out using morphological identification based on the keys of Gillies and Demeillon, (1968) and Gillies and Coetzee, (1987). *Aedes* mosquitoes were identified by clearly defined black and white rings on the legs, with abdomen having black and white scales forming distinctive patterns. *Mansonia* spp. were brown, thick and hairy with pale spots/patches on the legs. *Culex* mosquitoes were dark brown without patches/spots on the legs and wings were entirely dark without

pale spot patterns. *An. pharoensis* was identified by pale patches or spots on the legs which were visible and relatively larger in terms of size compared to *An. funestus* and *An. gambiae s.l. Anopheles funestus* was distinguished morphologically by absence of pale patches on the legs whilst *An. gambiae s.l.* had pale patches/spots on the legs. The number of pale patches or spots on the outer costal margin of the wing was also used to distinguish the *Anopheles* species. *An. gambiae* had five patches whilst *An. funestus* and *An. pharoensis* had four and six respectively.

3.3 Raising of wild mosquitoes in the laboratory

The pooled mosquitoes for each of the time groups were introduced into four well labelled cages using an aspirator and sustained with 10% sugar solution. Mosquitoes were blood fed with immobilised rabbits (Figure 7B) each day till egg laying was observed on labelled oviposition trays placed in each of the cages. The eggs laid were sent to the larval room and washed into labelled larval bowls containing dechlorinated water with a little amount of yeast added to facilitate the hatching of eggs. The first instars hatched from eggs were divided into several larval bowls after two days in order to avoid overcrowding and competition amongst them (Figure 7C). Larvae were fed with finely ground fish meal (TetraFin goldfish flakes). However, the amount of feed added to each larval bowl was dependent on the number and stage of the larvae but it was appropriate feeding them with small amount twice a day than just once so as to avoid contamination of the rearing medium with excess feed. Pupae belonging to the various time groups were pipetted out of the rearing bowls each morning with labelled Pasteur pipettes and transferred into labelled plastic cups which were placed in adult cages in the adult room and left for emergence (Figure 7D).The ambient conditions for rearing adults were $27^{o}C–30^{o}C$ for temperature and 60% –85% relative humidity. A 12h: 12h light and dark cycle was maintained by

switching the lights off at 6pm in the evening to 6am in the morning. The rearing water had temperatures ranging between 26^{o}C–29^{o}C. Regular cleaning of larval bowls was carried out by changing water due to excess feed and microbial growth in order to prevent larval mortality. Shelves on which adult cages were located had their stands mounted in oil to prevent ants and other crawling insects from getting in and also cages were cleaned daily of dead mosquitoes to avoid attracting scavengers as ants.

A

B

C

D

Figure 7: A: Human Landing Catches (HLC). B: Blood feeding with a rabbit. C: Cross-section of larval bowls. D: Cage with pupae and adults.

3.4 Biting behaviours of progeny

F_1 and F_2 progeny obtained from parents were kept for three to five days after emergence to ensure maximum mating before using for feeding experiment. The test was carried out from 6pm to 6am for each of the time groups when enough adults were obtained. For each of the time groups, female mosquitoes were sorted out from males and depending on the number obtained distributed amongst four separate cages labelled as 6pm-9pm, 9pm-12am, 12am–3am and 3am-6am hours respectively. Mosquitoes were starved off sugar for six hours to ensure that they were not satiated before the feeding experiments. They were allowed to feed on an immobilized rabbit placed on their cages for three hours. At the end of every three hour, a new set of immobilized rabbits were then placed on the next set of cages for the mosquitoes to feed. The process was repeated till all the mosquitoes at the different three-hour interval had had their turn in feeding. The number of fed and unfed mosquitoes in each experiment was counted and recorded. The fed mosquitoes in the time group corresponding to the time parents were sampled from the field were selected to obtain the F_2 generation on which feeding experiment was performed. The room where the experiment was carried out was kept completely dark to simulate natural conditions for mosquito biting. Replicates for the feeding experiment were dependent on the availability of mosquitoes. The experimental setup is illustrated in Figure 8.

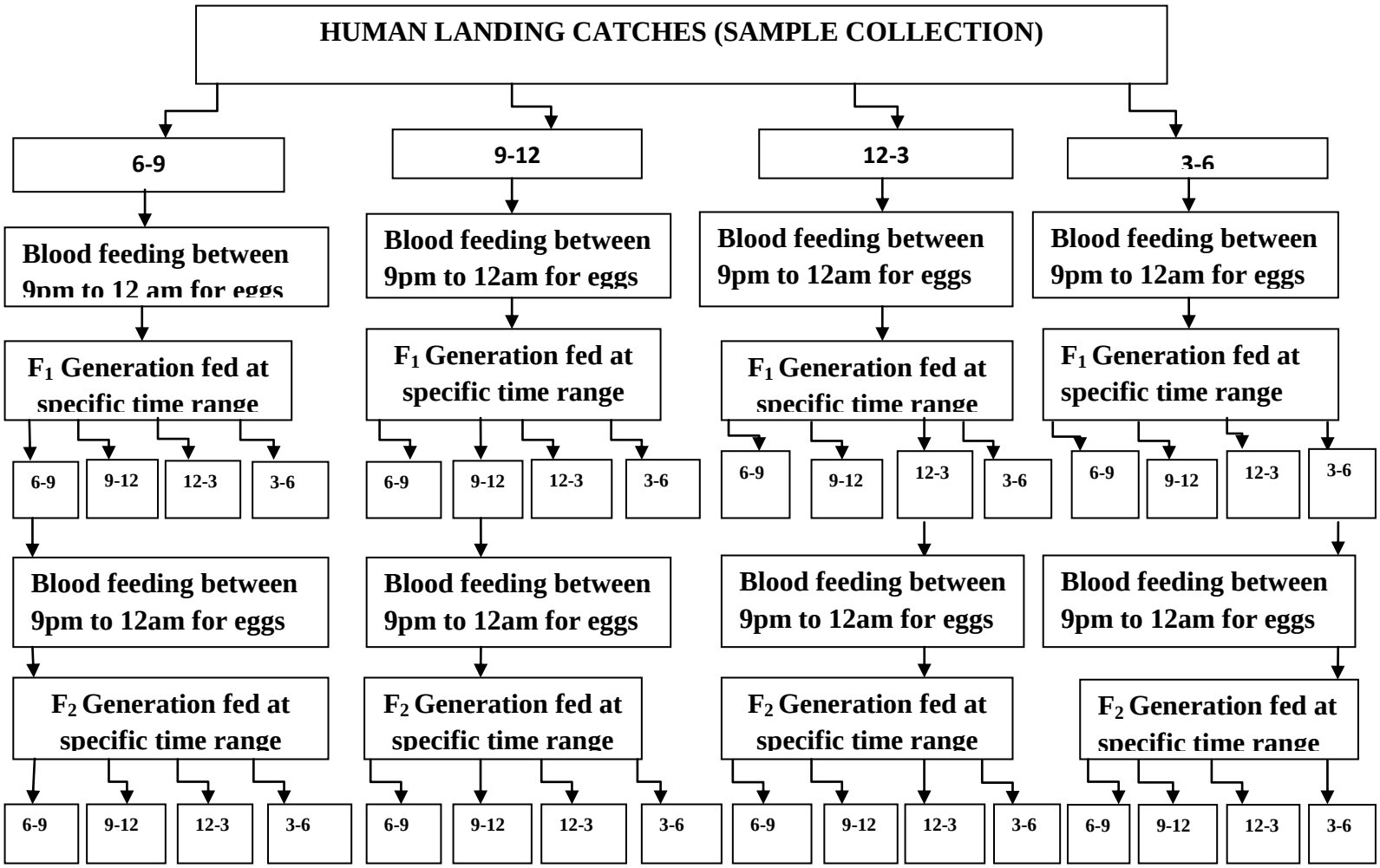


Figure 8: Illustration of experimental procedure

3.5 Molecular identification of *Anopheles gambiae* species complex

Genomic DNA extracted from the legs of parent *Anopheles gambiae* mosquitoes collected from the field was used as template for PCR to identify the sibling species. A restriction digest was carried out on identified *An. gambiae s.s.*

3.5.1 Genomic DNA extraction

Genomic DNA extraction from adult females was carried out using the ground leg extract protocol of (Xu *et al.*, 1998). It involved the grinding of two mosquito legs with a sterile Konte's plastic pestle in 30 µl of sterile double distilled water (sddH_20) in 1.5 ml Eppendorf tube. The homogenate was boiled at 90 ºC for 15 minutes and used directly as DNA template for PCR. The DNA extract was stored at -40 when not in use.

3.5.2 Amplification of ribosomal DNA of *Anopheles gambiae* complex

The protocol of (Scott *et al.*, 1993) polymerase chain reaction (PCR) was used for the identification of *An. gambiae* sibling species. Four oligonucleotide primers, abbreviated GA, ME, AR and UN denoting '*gambiae'*, '*merus/melas'*,*'arabiensis',* and 'universal' respectively designed from the DNA sequences of the intergenic spacer (IGS) region of *Anopheles gambiae* complex ribosomal DNA (rDNA) were used.

Table 2: Sequence details, melting temperatures (Tm) and diagnostic band sizes of oligonucleotide primers used for the PCR identification of the *An. gambiae* species complex (Scott *et al*., 1993).

Primer	Sequence (5'-3')	Melting Temp (Tm^oC)	Expected amplified DNA size (bp)
UN (F)	GTG TGC CCC TTC CTC GAT	58.3	468
GA (R)	CTG GTT TGG TCG GCA CGT TT	59.3	390
ME (R)	TGA CCA ACC CAC TCC CTT GA	57.2	464
AR (R)	AAG TGT CCT TCT CCA TCC TA	47.4	315

The PCR reaction mix of 20 µl contained 1X PCR buffer (Invitrogen, USA), 0.25 mM $MgCl_2$ (Promega, USA), 0.25 mM each of the 4 deoxyribonucleotide triphosphates (dNTPs), 0.15 µM of oligonucleotide primers, 0.5U of *Taq* polymerase enzyme (Promega, USA) and 3µl of the extracted genomic DNA. Sterile double distilled water (sddH_2O) was added to make up the volume to 20 µl. The reaction mix was overlaid with a drop of mineral oil to avoid evaporation and refluxing during thermocycling.

The amplification reaction was carried out using PTC 100 thermo-cycler (MJ Research Inc., USA). The cycling parameters for the reactions were as follows: 94 ºC for 3 minutes (initial denaturation), followed by 35 cycles of 94 ºC for 30 sec, 50 ºC at 1 min (annealing), 72 ºC for 1 min (extension) and ended with a cycle of 94º C for 30 sec, 50ºC for 30 sec and 72ºC for 7 min. The PCR machine was set to hold and keep products at 4°C indefinitely until removed from thermo cycler for storage. For each reaction, a positive control containing extracted DNA of previously identified *An. gambiae s.s.* and a negative control which contained no DNA but sdd H_20 were also included in each amplification.

3.5.3 Identification of *Anopheles gambiae s.s.* M and S molecular forms

This method is based on the fact that GCG^C restriction site for *Hha*I enzyme (Favia *et al.,* 1997) lies within the *An. gambiae* specific fragment (Scott *et al.*,1993)which makes it possible to digest this fragment directly in order to differentiate M and S molecular forms. Enzyme restriction of PCR products of identified *An. gambiae* s.s was done using restriction enzyme *Hha*I to determine the M and S molecular forms (Fanello *et al.,* 2002). This digest contained 10µl of amplified DNA product, 0.067U *Hha*I, 0.5µl of reaction buffer, 0.5µl of bovine serum albumin (BSA) and sdd water

was added to make up the final volume of 15µl. This was mixed, centrifuged briefly and incubated at 37ºC for three hours.

Table 3: Fragment of the IGS region in molecular forms of the An. gambiae s.s, bolded and underlined regions indicate the restriction site for *Hha*I enzyme for M and S molecular forms (Scott *et al.*, 1993).

Molecular forms	Sequence
M form	3' GTGCCCCTTCCTCGATGGC**GC**AACGAACCATCTTGGTCTG 5'
S form	3' GTGCCCCTTCCTCGATGGC**AT**AACGAACCATCTTGGTCTG 5'

3.5.4 Analyses of PCR and restriction products

PCR and restriction digest products were electrophoresed on 2% agarose gels stained with 0.5µg/ml ethidium bromide. 8µl of each sample was added to 1µl of orange G (5X) gel loading dye for the electrophoresis. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system (BIORAD USA) at 100 volts for 50 minutes and the bands observed using a UV transilluminator Sony Toyobo FASIII. The sizes of the PCR products were estimated by comparison with the mobility of a 100 base pair molecular marker (Promega, USA).

3.6 Data Analyses

Data were entered into SPSS (Version 16) and the comparison of the feeding rates of progeny for both the F_1 and F_2 generations across and along generations were analysed using Chi-Square Test.

RESULTS

4.0 Mosquito populations in the study area

A total of 815 mosquitoes were collected from Odumasi, the study area, using human landing catches (HLCs).

4.1 Species composition

The details of species composition of the total mosquitoes sampled during the study period are given in (**Appendix I**). *An. gambiae* s.l. formed the dominant species comprising 90.7% (739/815), 7.6% (62/815) for *Culex* species, 0.6% (5/815) for *Mansonia*, both *Aedes* and *An. funestus* species had 0.5% (4/815) and the least being *An. pharoensis* with 0.1% (1/815). *An. gambiae* s.l. and *An. funestus* group were the only malaria vectors encountered in the study area.

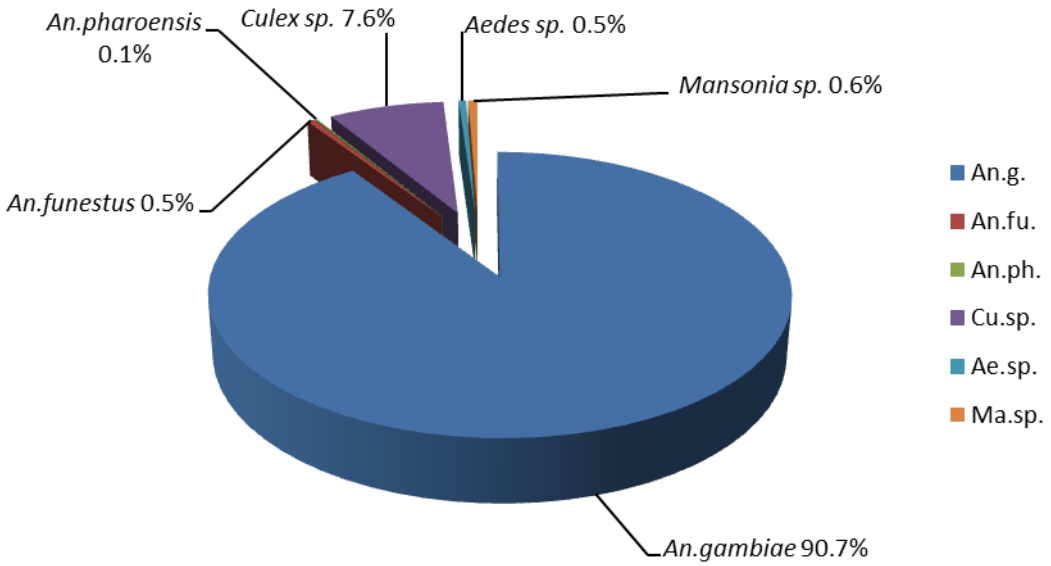


Figure 9: Species composition of biting mosquitoes collected during the study period.

4.2 Hourly distribution of mosquito species collected during the study period

The hourly distribution of mosquitoes collected for the various time groups for both outdoor and indoor collections pooled together is indicated in (**Appendix II**). The biting of mosquitoes sampled from the field started from 6pm, with a drop in biting activity between the hours of 7pm-8pm. This was followed with a steady increase in biting, with peaks between the hours of 1am–2am and 4am-5am and eventually declining till 6am.

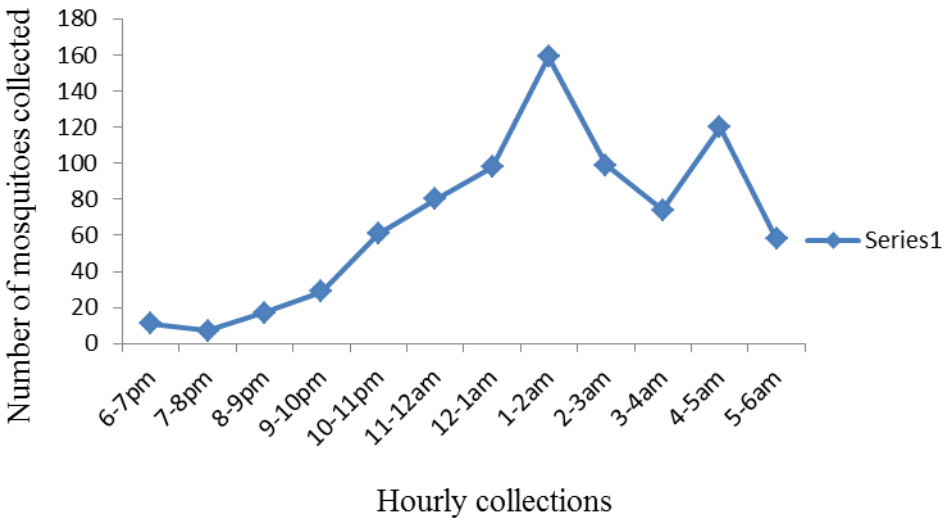


Figure 10: Hourly distribution and the biting pattern of mosquitoes sampled from Odumasy during the study period.

4.3 Comparison of parent feeding time to progeny of first and second generation for the various time groups

A total of two thousand five hundred and sixty eight mosquitoes used in blood feeding experiment for the F_1 generation had 73% (1874/2568) fully fed and 27% (694/2568) unfed for all the time groups pooled together. F_1Progeny from the various parental time groups that fed upon exposure to feed at the different time periods is indicated in Table 4. P-values obtained for progeny feeding at the same

time as parents were sampled from the field (parent feeding time) compared with other time periods within the same time group (non-parent feeding time) were: 9-12am [6-9pm (68%), 9-12am (90.5%, P=0.013), 12-3am (69.0%) and 3-6am (69%)]; 12-3am [6-9pm (95.8%), 9-12am (66.3%), 12-3am (66.3%, P=0.029) and 3-6am (54.5%)] and 3-6am [6-9pm (78.6%), 9-12am (78.6%), 12-3am (85%) and 3-6am (62.3%, P=<0.0001).

A total of two hundred and seventy six mosquitoes which was also used in blood feeding experiment for the F_2 generation resulted in 84% (231/276) fed and 16% (45/276) unfed. F_2 Progeny from the various parental time groups that fed upon exposure to feed at the different time periods is indicated in Table 5. P-values obtained for progeny feeding at the same time as parents were sampled from the field (parent feeding time) compared with other time periods within the same time group (non-parent feeding time) were: 9-12am [6-9pm (83.3%), 9-12am (100%,P=0.228), 12-3am (100%) and 3-6am (83.3%)]; 12-3am [6-9pm (87.7%), 9-12am (77.2%),12-3am (91.2%, P=0.037) and 3-6am (71.9%)]. Values were not obtained for the time group 3pm-6am due to lesser egg production from the F_1 progeny hence much adults were not obtained.

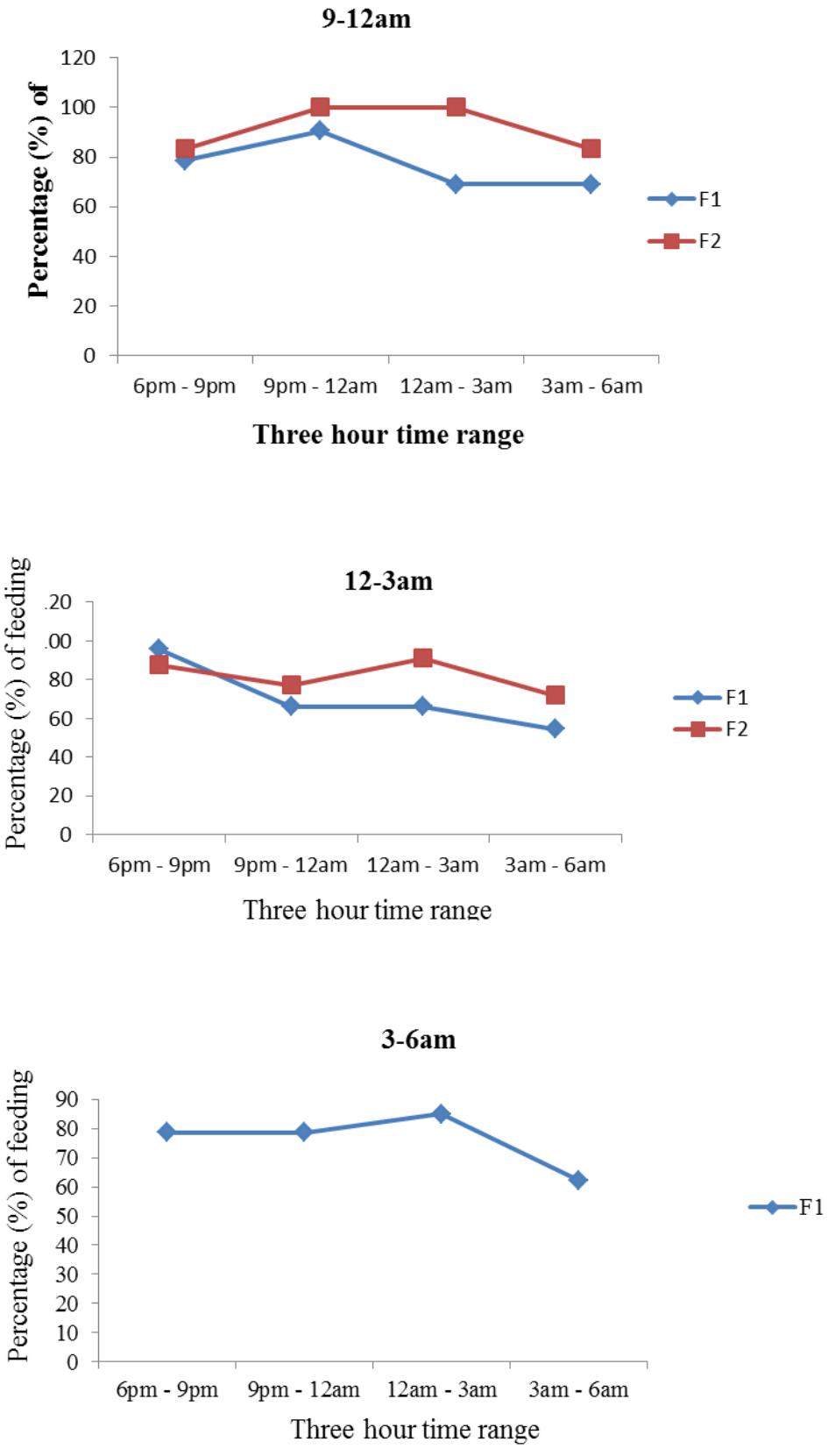


Figure 11: Graphs showing the feeding frequencies of F_1 and F_2 progeny in the various time groups.

4.4 Comparison of the feeding frequencies of F_1 and F_2 progenyfor the various time groups

The F_1 generation for time group 9-12am had total number of fed being 90.5% (38/42) as against the unfed 9.5% (4/42), whilst the F_2 generation for the same time group had 100% (12/12) for fed and 0% (0/12) for the unfed. Results for the F_1 generation of the time group 12-3am also had 66.3% (252/380) fed and 33.7% (128/380) unfed, as compared to the F_2 generation which had 91.2% (52/57) fed and 8.8% (5/57) unfed. These are represented in Tables 6 and 7 respectively.

Table 4: Feeding times of parents compared to their F_1 generation

	Time period F1s were exposed to feed.				
Time period parents of F_1 fed in the field	**6-9pm N (%)**	**9-12am N (%)**	**12–3am N (%)**	**3–6am N (%)**	**Total number of mosquitoes**
9–12am	42 (78.6)	42 (90.5)	42 (69.0)	42 (69.0)	168
12–3am	380 (95.8)	380 (66.3)	380 (66.3)	380 (54.5)	1520
3–6am	220 (78.6)	220 (78.6)	220 (85.0)	220 (62.3)	880
Total	642	642	642	642	2568

N = Total number of F_1s exposed to feed within the various time periods.
% = Percentage of F_1s that fed

Table 5 : Feeding times of parents compared to their F_2 generation

	Time period F_2s were exposed to feed.				
Time period parents of F_2s fed in the field	**6–9pm N (%)**	**9–12am N (%)**	**12–3am N (%)**	**3–6am N (%)**	**Total number of mosquitoes**
9–12am	12 (83.3)	12 (100)	12 (100)	12 (83.3)	48
12–3am	57 (87.7)	57 (77.2)	57 (91.2)	57 (71.9)	228
Total	69	69	69	69	276

N = Total number of F_2s exposed to feed within the various time periods.
% = Percentage of F_2s that fed

Table 6: The feeding frequencies of F_1 and F_2 generations in the time group 9-12am.

			Time fed		
			9-12am fed	9-12am unfed	Total
Generation	F1	Count	38	4	42
		% within Generation	90.5%	9.5%	100.0%
	F2	Count	12	0	12
		% within Generation	100.0%	.0%	100.0%
Total		Count	50	4	54
		% within Generation	92.6%	7.4%	100.0%

Table 7: The feeding frequencies of F_1 and F_2 generations in the time group 12-3am.

			Time fed		
			12-3am-fed	12-3am-unfed	Total
Generation	F1	Count	252	128	380
		% within Generation	66.3%	33.7%	100.0%
	F2	Count	52	5	57
		% within Generation	91.2%	8.8%	100.0%
Total		Count	304	133	437
		% within Generation	69.6%	30.4%	100.0%

4.5 Molecular identification of *Anopheles gambiae* s.l.

One hundred and thirty eighty (n=138) morphologically identified *Anopheles gambiae s.l.* were selected using Cochran's (Bartlett *et al.*, 2001) formula of sample size determination from pooled parents of 6-9pm, 9-12am, 12-3am and 3-6am and were subjected for identification by PCR. PCR results showed 97.1% (134/138) of the samples amplified as *An. gambiae s.s.* and 2.9% (4/138) resulting in no amplification.

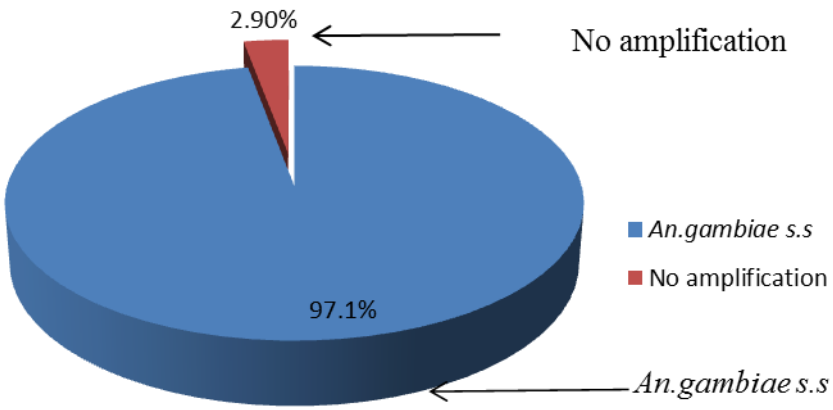


Figure 12: Distribution of sibling species of the *An. gambiae* complex at Odumasi.

1 2 3 4 5 6 7 M

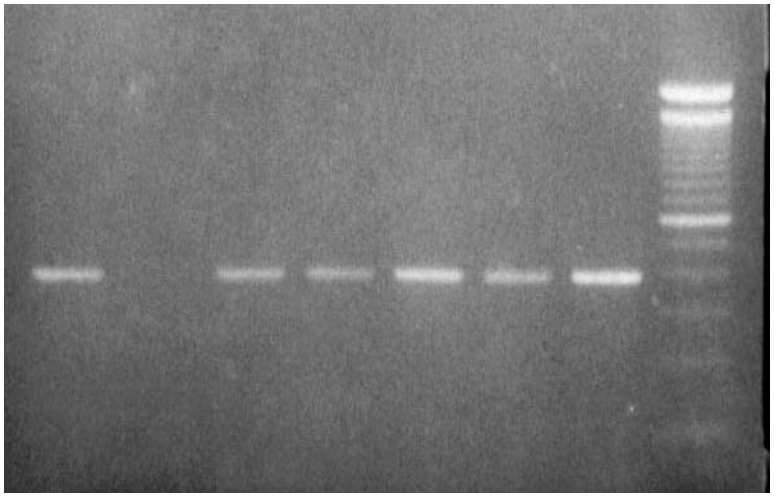


Figure 13: A 2% agarose gel electrophoresis of PCR amplified rDNA sequences of *An. gambiae s.s* mosquitoes. Lane 1 = positive control; Lane 2 = negative control; Lanes 3-7= *An. gambiae s.s*; Lane M= 100bp molecular weight marker.

4.6 Molecular forms of *Anopheles gambiae s.s.*.

A total of 134 *Anopheles gambiae s.s.* were identified by PCR and subjected to restriction fragment length polymorphism (RFLP) analysis, 5.2% (7/134) were

digested as M form and 94.8% (127/134) as S form (Figure 14). Figure 15 shows an electrophoregram of digested *Anopheles gambiae s.s.*

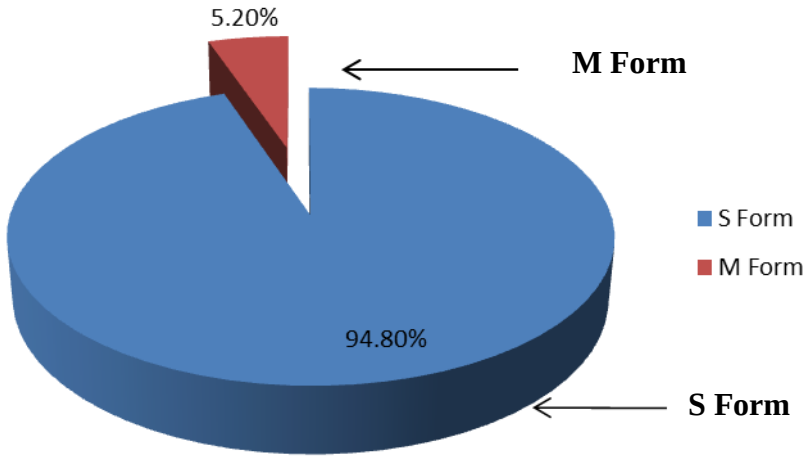


Figure 14: Proportions of M and S forms of *Anopheles gambiae s.s.* at Odumasi

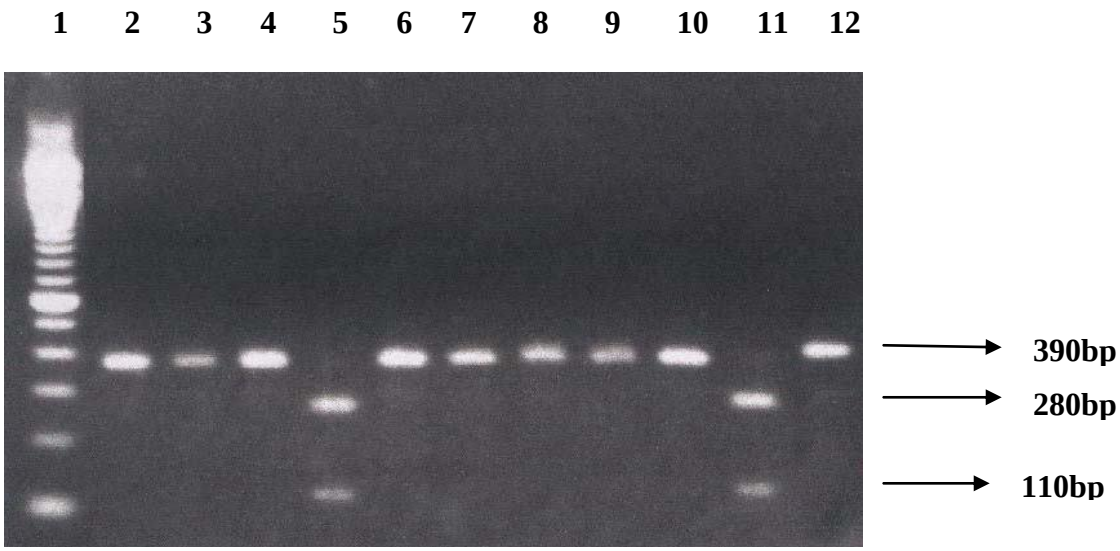


Figure 15: Ethidium bromide-stained 2% agarose gel electrophoregram of Hha1restriction of *An. gambiae s.s* amplified PCR products. Lane 1 is a 100 base pair molecular weight marker; Lanes 2-4, 6-10 and 12 are M forms (undigested). Lanes 5 and 11 are S form.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.0 Discussion

Malaria poses a serious threat to the socio-economic development especially of Sub-Saharan Africa where at least 90% of malaria deaths occur (WHO, 2010a). It is normally responsible for majority of the reported cases in children under five years, and with the more susceptible adult group being mostly pregnant women (WHO, 2006a). This situation therefore presents the disease malaria as a global challenge requiring prompt and sustainable solution. The major control strategies mostly directed against malaria vectors and being employed by various control programmes in preventing transmission includes insecticide treated bed net usage and indoor residual spraying (IRS). However, the continuous usage of bed nets as a malaria vector control strategy can result in the modification of the biting pattern of mosquito populations as recent studies have indicated this phenomenon. These modifications might have therefore led to the adaptive change in the biting behaviour of mosquitoes probably due to selective pressure against those biting when bed nets are in use. These modifications in the biting pattern of mosquitoes (*Anopheles gambiae*) include change in the time of biting (Appawu *et al*., 2004), change in biting behaviour expressed by outdoor biting (Reddy *et al.,* 2011) and shifts in hosts (Takken, 2002).Undoubtedly, these changes in biting pattern of malaria vectors may influence the success of ITNs as a vector control strategy. This present study therefore sought to determine if there are (genetically) discrete biting populations within *An. gambiae* s.s. that bite at specific times during the night (and on which selective pressures imposed by bednet interventions could operate).

The malaria vector *An. gambiae* s.l. which is predominantly involved in malaria transmission mostly in West Africa and Africa as a whole was the dominant *Anopheles* vector caught at Odumasi. However, *An. funestus* also a vector was found in appreciable numbers during the study. Further PCR identification of the *An. gambiae* complex revealed *An. gambiae* s.s. as the only sibling species in the study area (Yawson *et al.,* 2004), which is also the major malaria vector within the *gambiae* complex. The two molecular forms of *An. gambiae* M and S were also found in sympatry, with no detection of hybrid forms M/S (Yawson *et al.,* 2004). S form larvae are mainly associated with or found to breed more frequently in rain-dependent breeding sites occurring during rainy seasons, whilst M form larvae are mainly associated with the presence of permanent breeding conditions such as irrigated farms and ponds resulting from river run-offs (de Souza *et al*., 2010). Therefore, the large number of S forms obtained from the sample site might have been as a result of a series of rainfall that had fallen some few days before sampling.

Studies have shown that the general biting pattern of *Anopheles gambiae* the major malaria vector in sub-Saharan Africa (Appawu *et al.,* 2003) together with its sibling species have an initial period of low biting activity up to 9-10pm followed by a gradual increase in biting with peak biting normally occurring from midnight to about 4am then with considerable biting continuing just before dawn (Gillies and Demeillon, 1968; Mutero *et al.,* 1984; Wanji *et al.,* 2003). The biting pattern observed for both outdoor and indoor collections pooled together for the various hours showed a similar pattern as compared to those observed in other studies. The biting of mosquitoes sampled in the study area had an initial low biting rate during the early hours of the night, with peak biting being realised after midnight between

the hours of 1-2am and another rise in rate of biting mosquitoes between the hours of 4-5am with biting eventually declining till 6am.

Mosquito behaviours such as place of resting and biting (outdoor/indoor), host preference and availability, as well as their biting pattern may be genetically linked (WHO, 1975). In this line of thought, it is possible that mosquito feeding behaviour with respect to the time they prefer to feed may be linked genetically but this is not well understood. To better understand this phenomenon, this study therefore sought to find out whether mosquito behaviour with respect to their time of biting could be genetically linked. This was done by selecting both F_1 and F_2 generations at specific time periods of the night and analysing both generations to assess whether they preferred to feed most, at the same time periods their parents were caught from the field.

Though mosquitoes were not followed to a number of generations the results obtained showed that the time of biting did not have any genetic influence affecting it since P-values obtained for progeny from both $F_{1:}$(9pm-12am, P = 0.013), 12-3am, P = 0.029), (3-6am, P=<0.0001) and $F_{2:}$(9-12am, P=0.228), (12-3am, P=0.037) generations feeding at the same time as parents were sampled from the field, compared with the other time periods from within the same time group were significant. The results for the comparison of the feeding frequencies of F_1 and F_2 progeny for the time groups were [9pm-12am, F_1 = 90.5% (38/42) and F_2 = 100% (12/12)] and [12am-3pm, F_1 = 66.3% (252/380) and F_2 = 91.2% (52/57)]. Results obtained from both analyses indicated that there was no evidence of parent mosquitoes sampled within specific times having their progeny preferring to bite most within those times.

5.1 Conclusions and Recommendations

This study therefore revealed that there was no genetic influence affecting both F_1 and F_2progeny in their time of biting since this vector behaviour might be due to an expression of phenotypically plastic behavioural response to some extrinsic factors such as host availability (Lefèvre *et al*., 2009) and other factors. This therefore allows mosquitoes to adapt their behaviour according to the environmental conditions they encounter. There was also no evidence of parent mosquitoes sampled within specific times having progeny preferring to bite most within those times. In this study progeny were raised only up to the second generation and therefore results obtained did not give much information regarding the biting behaviour of *Anopheles gambiae s.s.*. This observed behaviour in the mosquitoes might therefore be due to extrinsic factors. Hence, the raising of several generations might give us a much better insight into the feeding dynamics of the progeny of parents sampled from the field.

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APPENDICES

APPENDIX I: Raw data of samples collected

HOUSE ONE (OUTDOOR)

TIME (H GMT)	Number Collected	Species						
		An.g.	*An.fu.*	*An.ph.*	*Cu.sp.*	*Ae.sp.*	*Ma.sp.*	Others
1800-1900	5	0	0	0	4	1	0	0
1900-2000	2	1	0	0	1	0	0	0
2000-2100	2	1	0	0	0	1	0	0
2100-2200	11	7	0	0	4	0	0	0
2200-2300	18	14	0	1	1	2	0	0
2300-2400	24	21	0	0	2	0	1	0
2400-0100	30	26	2	0	1	0	1	0
0100-0200	51	49	0	0	2	0	0	0
0200-0300	33	32	0	0	1	0	0	0
0300-0400	20	20	0	0	0	0	0	0
0400-0500	24	22	0	0	0	0	2	0
0500-0600	19	18	0	0	1	0	0	0
Total	239	211	2	1	17	4	4	0

HOUSE ONE (INDOOR)

TIME (H GMT)	Number Collected	Species						
		An.g.	*An.fu.*	*An.ph.*	*Cu.sp.*	*Ae.sp.*	*Ma.sp.*	Others
1800-1900	1	0	0	0	1	0	0	0
1900-2000	0	0	0	0	0	0	0	0
2000-2100	9	8	0	0	0	0	1	0
2100-2200	8	8	0	0	0	0	0	0
2200-2300	24	22	0	0	2	0	0	0
2300-2400	12	12	0	0	0	0	0	0
2400-0100	22	21	0	0	1	0	0	0
0100-0200	25	23	0	0	2	0	0	0
0200-0300	26	24	0	0	2	0	0	0
0300-0400	13	13	0	0	0	0	0	0
0400-0500	13	13	0	0	0	0	0	0
0500-0600	10	10	0	0	0	0	0	0
Total	163	154	0	0	8	0	1	0

HOUSE TWO (OUTDOOR)

TIME (H GMT)	Number Collected	Species						
		An.g.	*An.fu.*	*An.ph.*	*Cu.sp.*	*Ae.sp.*	*Ma.sp.*	Others
1800-1900	4	3	0	0	1	0	0	0
1900-2000	2	1	0	0	1	0	0	0
2000-2100	4	1	0	0	3	0	0	0
2100-2200	6	2	0	0	4	0	0	0
2200-2300	11	8	0	0	3	0	0	0
2300-2400	33	33	0	0	0	0	0	0
2400-0100	22	20	0	0	2	0	0	0
0100-0200	52	46	2	0	4	0	0	0
0200-0300	24	22	0	0	2	0	0	0
0300-0400	29	27	0	0	2	0	0	0
0400-0500	53	53	0	0	0	0	0	0
0500-0600	13	12	0	0	1	0	0	0
Total	253	228	2	0	23	0	0	0

HOUSE TWO (INDOOR)

TIME (H GMT)	Number Collected	Species						
		An.g.	*An.fu.*	*An.ph.*	*Cu.sp.*	*Ae.sp.*	*Ma.sp.*	Others
1800-1900	1	0	0	0	1	0	0	0
1900-2000	3	2	0	0	1	0	0	0
2000-2100	2	2	0	0	0	0	0	0
2100-2200	4	1	0	0	3	0	0	0
2200-2300	8	7	0	0	1	0	0	0
2300-2400	11	9	0	0	2	0	0	0
2400-0100	24	24	0	0	0	0	0	0
0100-0200	31	28	0	0	3	0	0	0
0200-0300	16	14	0	0	2	0	0	0
0300-0400	12	12	0	0	0	0	0	0
0400-0500	30	29	0	0	1	0	0	0
0500-0600	18	18	0	0	0	0	0	0
Total	160	146	0	0	14	0	0	0

APPENDIX II: Total number of mosquitoes sampled hourly for all time groups

Time Range	Total number of mosquitoes sampled
18:00-19:00	11
19:00-20:00	7
20:00-21:00	17
21:00-22:00	29
22:00-23:00	61
23:00-24:00	80
24:00-01:00	98
01:00-02:00	159
02:00-03:00	99
03:00-04:00	74
04:00-05:00	120
05:00-06:00	58

APPENDIX III.a: Replicates for the various time groups for F_1 generation

1. Replicate One: 9-12am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
9-12pm	n	22	22	22	22
	n fed	17	22	14	19
	%fed	77.3	100.0	63.6	86.4

2. Replicate Two: 9-12am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
9-12pm	n	20	20	20	20
	n fed	16	16	15	10
	%fed	80.0	80.0	75.0	50.0

3. Replicate One: 12-3am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	80	80	80	80
	n fed	78	68	56	31
	%fed	97.5	85.0	70.0	38.8

4. Replicate Two: 12-3am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	80	80	80	80
	n fed	75	75	61	49
	%fed	93.8	93.8	76.3	61.3

5. Replicate Three: 12-3am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	220	220	220	220
	n fed	211	183	135	127
	%fed	95.9	83.2	61.4	57.7

6. Replicate One: 3-6am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
3-6am	n	60	60	60	60
	n fed	54	49	50	32
	%fed	90.0	81.7	83.3	53.3

7. Replicate Two: 3-6am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
3-6am	n	60	60	60	60
	n fed	47	50	49	23
	%fed	78.3	83.3	81.7	38.3

8. Replicate Three: 3-6am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
3-6am	n	100	100	100	100
	n fed	72	74	88	82
	%fed	72.0	74.0	88.0	82.0

APPENDIX III.b: Total percentages for the replicates of progeny from F_1 generationthat fed in the various time groups

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
9-12am	n	42	42	42	42
	n fed	33	38	29	29
	%fed	78.6	90.5	69.0	69.0

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	380	380	380	380
	n fed	364	252	252	207
	%fed	95.8	66.3	66.3	54.5

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
3-6am	n	220	220	220	220
	n fed	173	173	187	137
	%fed	78.6	78.6	85.0	62.3

APPENDIX IV.a: Replicates for the various time groups for F_2 generation

1. Replicate One: 9-12am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
9-12am	n	12	12	12	12
	n fed	10	12	12	10
	%fed	83.3	100	100	83.3

2. Replicate One: 12-3am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	35	35	35	35
	n fed	28	23	30	22
	%fed	80	65.7	85.7	62.9

3. Replicate Two: 12-3am

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	22	22	22	22
	n fed	22	21	22	19
	%fed	100	95.5	100	86.4

APPENDIX IV.b: Total percentages for the replicates of progeny from F_2 generationthat fed in the various time groups

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
9-12am	n	12	12	12	12
	n fed	10	12	12	10
	%fed	83.3	100	100	83.3

Time of collection from the field	Sample size	Time of feeding in the laboratory			
		6-9pm	9-12am	12-3am	3-6am
12-3am	n	57	57	57	57
	n fed	50	44	52	41
	%fed	87.7	77.2	91.2	71.9