RELATIVE ATTRACTIVENESS OF *ANOPHELES GAMBIAE* MOSQUITOES TO SYNTHETIC ODOUR BLENDS AND HUMAN EMANATIONS

By

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DECLARATION

Candidate

I hereby declare that this thesis is my original work and has not been presented for a degree or any other award in any other university.

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We confirm that the candidate under our supervision carried out the work reported in this thesis. We have read and approved this thesis for examination.

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DEDICATION

This work is dedicated to my parents Lordvicus and Eudia Olanga and my siblings Christine, Daniel and Beatrice who gave me moral support during my entire study.

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ABBREVIATIONS AND ACRONYMS

DDT	dichloro-diphenyl-trichloroethane
EAG	Electroantennogram
FNIH	National Institutes of Health
GCGH	Grand Challenges in Global Health
GDP	Gross Domestic Product
HA	Highly Attractive
ICIPE	International Centre of Insect Physiology and Ecology
IGR	Insect Growth Regulators
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
KEMRI	Kenya Medical Research Institute
LA	Least Attractive
LDC	Least Developed Countries
LLINs	Long Lasting Insecticidal-treated Nets
MMX	Mosquito Magnet® X
NPV	Nucleopolyhedrovirus
PVC	Polyvinyl chloride
RH	Relative humidity
SP	Sulfadoxine-pyrimethamine
TOC	Thomas Odhiambo Campus
U.S.A.	United States of America
WHO	World Health Organization

ABSTRACT

Mosquito host seeking behaviour is mediated by chemical, physical and visual cues. Identification of chemical and physical cues utilized by mosquitoes in host location can lead to the development of novel vector management strategies. This research set out to identify synthetic odour blends that simulate human body emanations that can be used to develop mass trapping devices for vector management. The relative attractiveness of human body emanations and synthetic odour blends as attractants of *Anopheles gambiae* mosquitoes was investigated under semi-field conditions at the International Centre for Insect Physiology and Ecology (ICIPE) Mbita Point, western Kenya. The effect of heat and moisture on the response of *An. gambiae* to synthetic odours was also investigated. Binary choice tests were conducted by exposing mosquitoes to natural odours from a human volunteer occupying one tent and synthetic odour blends delivered through a Mosquito Magnet-X® trap positioned in a second tent. Two human male volunteers previously established as person less attractive (Person LA) and person highly attractive (Person HA) to host seeking female *An. gambiae* mosquitoes, were recruited into the study to serve as sources of natural host seeking cues. Mosquito responses to foot odours adsorbed onto the persons' worn socks were also evaluated. This acted as an alternative natural source for host seeking cues. Synthetic stimuli were derived from two odour blends, a standard blend consisting of carbon dioxide, ammonia and distilled water and Blend 1 consisting of seven carboxylic acids, lactic acid, carbon dioxide, ammonia and distilled water. One hundred female mosquitoes aged between 3 – 6 days old and starved for 8 hours were used in each binary assay. Experiments were conducted between 1930 – 2000 hours and 2030 – 2100 hours each night. Mosquitoes attracted to the odours from the two sources, human volunteers and synthetic stimuli or foot odours were counted and recorded for every replicate. Data analysis was carried out in the General Statistical analysis software (Genstat®). Using Generalized Linear Model the number of mosquitoes attracted to odour baits person's emanations, their foot odours or synthetic attractants were modelled as a proportion of the total number of mosquitoes recovered. The data was transformed to assume normal distribution using a logarithm link function. Person LA attracted a higher proportion of female mosquitoes when compared to the standard blend ($P = 0.001$). However, the proportion of mosquitoes attracted to person LA when compared to foot odours and Blend 1 did not differ ($P = 0.417$; $P = 0.163$, respectively). Person HA on the other hand attracted a significantly higher proportion of mosquitoes when compared to his foot odours, standard blend and Blend 1 ($P = 0.001$). Augmentation of synthetic blends with heat (25-27^{o}C) did not increase attractiveness of these odours to mosquitoes. Both persons attracted a significantly higher proportion of mosquitoes when compared to their foot odours, standard blend and Blend 1 when these were augmented with moisture (75 – 85%) ($P = 0.001$). A significantly higher proportion of mosquitoes were attracted to person LA compared to the standard blend ($P < 0.001$) and his foot odours ($P < 0.001$) when each was augmented with both heat (24.9^{o}C) and moisture (75%). There was no difference in the proportion of mosquitoes attracted to person LA when compared to blend 1 ($P = 0.416$) augmented with both heat (24.9^{o}C) and moisture (75%). The findings of this study have demonstrated that heat and moisture had no effect on attraction of mosquitoes to synthetic odours. Synthetic blend 1 was the most potent mosquito attractant. This synthetic blend should be incorporated in traps to mediate mosquito host seeking behaviour that would enhance capture of mosquitoes in field studies

. CHAPTER ONE

INTRODUCTION

1.1 Background

Mosquitoes (Diptera: Culicidae) are important vectors of diseases globally. They transmit parasites that cause malaria, bancroftian filariasis and arboviruses that cause yellow fever, dengue fever and rift valley fever among others. Malaria is a major public health problem in Africa despite the implementation of control strategies in most endemic areas and concerted research efforts to develop new control tools. The disease is a cause of poverty and impedes economic development in Africa through diminished worker productivity, absenteeism (Burton *et al.*, 1999) and the huge medical costs incurred on prevention, diagnosis and treatment (Gallup and Sachs, 2001; Sachs and Malaney, 2002). All age groups are susceptible to malaria but children less than five years of age and pregnant women are most vulnerable due to their lower levels of malaria immunity in both groups (WHO, 2006).

Human malaria is caused by the protozoan parasites *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae*. In Africa, 99% of the malarial parasites are the *P. falciparum* forms (Miller *et al.*, 2002; WHO, 2005). Malarial parasites are transmitted by mosquitoes belonging to the genus *Anopheles*. *Anopheles gambiae* Giles, *Anopheles arabiensis* Patton and *Anopheles funestus* Giles are the main vectors of malaria in sub-Saharan Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee *et al.*, 2000).

Mosquito control aims at reducing biting nuisances and preventing disease transmission. Current strategies that have been adopted to control malaria include chemotherapy (Wistanley, 2000), indoor residual spraying (IRS) (Mabaso *et al.*, 2004), insecticide treated nets (ITNs) (Rozendaal, 1997; Lengeler, 2004; Curtis *et al.*, 2006) and long lasting insecticidal-treated nets (LLINs) (Invest, 2008). These efforts have had success in some parts of Africa (WHO, 2005), but the disease is still rampant. This is due to the emergence and spread of vector resistance to insecticides (Chandre *et al.*, 2000; Nauen, 2007) and widespread resistance of parasites to drugs (Wistanely, 2000; Trape, 2001). In addition, political instabilities and poverty in much of the African continent offer a serious challenge to the management of malaria (Onori, 1988; Greenwood and Mutabingwa, 2002; Kager, 2002). Climatic and environmental changes, both natural and man-made (dams and reservoirs) and changes in the behaviour of the vector further facilitate failure of vector control (Kager, 2002). Besides these, poor organization of control programs and inadequate infrastructure in least developed countries (LDCs) disable implementation of recommended interventions (Kager, 2002).

There is an urgent need for developing alternative mosquito control technologies as the measures aimed at controlling malaria have achieved limited success, especially in sub-Saharan Africa. Host seeking is a major component of adult mosquito behaviour (Takken and Knols, 1999; Sutcliffe, 1987) that is amenable for manipulation through exploitation of chemicals responsible for mosquito attraction and repulsion to humans (Zwiebel and Takken, 2004; Logan and Birkett, 2007). Female mosquitoes primarily use olfactory cues to locate their blood meal hosts at long range (Sutcliffe, 1987; Takken, 1991; Takken and Knols, 1999). Visual and physical cues play a crucial role in orientation of the mosquitoes within the close vicinity of the host (Sutcliffe, 1987). Physical cues have been identified as heat and moisture. Studies seeking to identify mosquito attractants have evaluated single active compounds (Braks *et al.*, 2001; Healy *et al.*, 2002) as well as blends thereof (Knols *et al.*, 1997; Smallegange *et al.*, 2005; Silva *et al.*, 2005). Whereas some active compounds have been successfully used to attract host seeking mosquitoes (Knols *et al*., 1997; Braks *et al*., 2001; Healy *et al*., 2002), these have not found application in the development of novel trapping tools for vector control in Africa. Ideally, identifying a synthetic odour blend that attracts mosquitoes much the same as a human being (Brady *et al*., 1997) can enhance the usefulness of mass trapping as a tool for vector control (Day and Sjogren, 1994; Kline, 2007).

Anopheles gambiae Giles *sensu stricto* (Diptera: Culicidae) is the most important malaria vector in Africa (White, 1974; Coetzee, 2004). Several behavioural traits predispose this species as a major vector of human malaria (Takken and Knols, 1999). Females of this species have a preference for feeding on human blood (anthropophagic) inside human dwellings (Day, 2005) at night (White, 1974). This mosquito species mainly uses human specific cues to orient towards its blood meal hosts (Costantini *et al*., 1996; Costantini *et al*., 1999; Takken and Knols, 1999). Whereas this mosquito is attracted to certain synthetic attractants (Okumu *et al*., 2009; Okumu *et al*., 2010a; Lwetoijera *et al*., 2010), there is a dearth of information on how these synthetic compounds compare to humans in attracting the mosquitoes. Thus, the identity of a human substitute for use as a mosquito attractant is far from realization (Brady *et al*., 1997). Furthermore, the potential of using odour baited technology in the surveillance and control of vector borne diseases of humans in Africa remains untapped.

The present study was designed to evaluate the relative attractiveness of synthetic odour blends and natural human odours to female *An. gambiae* mosquitoes. The overall influence of heat and/or moisture on the attractiveness of the synthetic odour blends was also determined. The experiments were carried out under semi-field conditions within a screen house at the

Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (ICIPE) located at Mbita Point, Suba District, western Kenya.

1.2 Statement of the problem and justification of the study

Resistance of mosquito vectors to insecticides (Chandre *et al*., 2000) and of malarial parasites to chemotherapeutic agents (Wistanely, 2000; Trape, 2001) are major threats to effective control of malaria. Human volunteers have been previously used as bait in various mosquito trapping systems. One such method is the human landing catch (a mosquito sampling method where humans are used as bait at night to attract and capture adult mosquitoes) that measures the level of human-mosquito contact (Service, 1993). Other methods like the exposure-free mosquito trapping systems (Mathenge *et al*., 2002; 2004) also use human beings to bait traps for adult mosquitoes. The key challenge is to replace these methods with synthetic odour blends that are as attractive as a human being (Brady *et al.*, 1997).

Mosquito host location is mediated by physical and chemical cues (Sutcliffe, 1987; Takken, 1991). Physical cues have been identified as heat and moisture (Takken, 1991) while the identity of chemical cues is not well known. Some compounds have been reported to attract mosquitoes mainly under laboratory conditions (Cork and Park, 1996; Braks *et al.*, 2001; Smallegange *et al.*, 2005) with few field trials (Costantini *et al*. 2001; Murphy *et al*. 2001; Torr *et al*. 2008). Attractive chemical compounds emanating from humans can be synthesized and blended to mimic a human being (Brady *et al.*, 1997). Since physical cues also play a role in the attraction of mosquitoes to humans (Takken *et al.*, 1997), they might enhance attraction of mosquitoes to chemical-based attractants.

If a highly attractive synthetic blend is isolated, it can be used to develop surveillance tools and mass trapping devices for vector control. However, for such tools and traps to be employed, the efficacy of synthetic odour blends as mosquito attractants must be compared to human emanations. The aim of this study was to compare the relative attractiveness of synthetic odour blends with human emanations to *Anopheles gambiae* mosquitoes and the effect of heat and moisture on the attractiveness of these synthetic odour blends.

1.3 Research questions

(a) What is the relative response of *An. gambiae* mosquitoes to synthetic odour blends and human emanations?

(b) What is the effect of heat and moisture on the attraction of *An. gambiae* mosquitoes to synthetic odour blends?

1.4 Hypotheses

(a) There is no significant difference in the response of *An. gambiae* mosquitoes to synthetic odour blends and human emanations.

(b) There is no significant variation in the response of *An. gambiae* mosquitoes to synthetic odour blends when augmented with heat and moisture.

1.5 Objectives of the study

1.5.1 General objective

To compare the relative attractiveness of *An. gambiae* mosquitoes to synthetic odour blends and human emanations.

1.5.2 Specific objectives

(a) To determine the relative response of *An. gambiae* mosquitoes to synthetic odour blends compared to human emanations.

(b) To investigate the effect of heat and moisture on the response of *An. gambiae* mosquitoes to synthetic odour blends.

1.6 Significance and anticipated output

Malaria is a major disease in endemic areas of Africa where it poses an obstacle to socio-economic development. Previous efforts to control the disease have been unsuccessful due to vector resistance to insecticides and parasite resistance to drugs. A potent mosquito attractant will enhance development of powerful mosquito trapping devices. Such devices can be used as surveillance tools and for disease control through massive, well sustained removal trapping efforts. Measuring the potency of an attractant against a human being helps to ensure that the product so developed can be used to reliably calculate key epidemiological indices of disease. Thus, the devices can be harnessed to increase the success of control programmes through forecasting epidemics accurately and for formulating, planning and rolling out control activities. When done well these approaches can reduce morbidity and mortality associated with human malaria, so promoting socio-economic development.

CHAPTER TWO

LITERATURE REVIEW

2.1 Public health importance of malaria

Malaria is a common infectious disease that causes an enormous public health problem in the tropics. Every year, this leads to about 300-500 million new infections and one to three million deaths worldwide (Breman and Holloway, 2007). More than 90% of these deaths occur in sub-Saharan Africa (Boutin *et al*., 2005). It is still responsible for up to 50% of outpatient cases and 20% of hospital admissions. All age groups experience malaria, but the highest mortality occurs in children under five years of age and pregnant women (Philips, 2001) purportedly due to their lower level of immunity (WHO, 2006).

The disease is a major cause of poverty and a hindrance to socio-economic development (Sachs and Malaney, 2002). Malaria impedes economic development not only by causing premature deaths but also through lost productivity, absenteeism, huge medical costs and negative impact on fertility, and a country's savings and investments (Sachs and Malaney, 2002). Malaria and poverty are intimately connected (Gallup and Sachs, 2001). The annual gross domestic product (GDP) of countries where malaria thrives is up to five-fold lower than in countries without intensive malaria (WHO, 2006).

Human malaria, transmitted by *Anopheles* mosquitoes, is caused by single or multiple infections of four *Plasmodium* species. These include *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. Recently, *P. knowlesi* has been reported to cause malaria in Southeast Asia (Cox-Singh *et al*., 2007; Lee *et al*., 2009; Cox-Singh *et al*. 2010). *Plasmodium vivax* is the most widely distributed species while *P. falciparum*, which is the most widespread species in sub-Saharan Africa, causes the most severe complications. Common clinical manifestations of malaria include nausea, shivering, fever, jaundice, arthralgia (joint pains), headaches, anaemia, vomiting and diarrhoea.

2.2 Malaria transmission in Africa

There are approximately 430 species of *Anopheles* mosquitoes about 70 of which are known malaria vectors (White, 1974). Malaria in Africa is mainly transmitted by three mosquito species namely *An.gambiae* Giles, *Anopheles arabiensis* Patton and *Anopheles funestus* Giles. *Anopheles gambiae* and *An. arabiensis* belong to the *Anopheles gambiae* species complex. *Anopheles funestus* belongs to the *Anopheles funestus* group.

2.2.1 The *Anopheles gambiae* complex

The *An. gambiae* complex contains the most important malaria vectors in Africa (Coetzee *et al*., 2000; Coetzee, 2004). The complex consists of seven morphologically indistinguishable sibling species namely *An. gambiae s.s.*, *An. arabiensis*, *Anopheles bwambae*, *Anopheles merus*, *Anopheles melas*, *Anopheles quadriannulatus* species A (White, 1974; Coetzee *et al*., 2000) and *Anopheles quadriannulatus* species B (Hunt *et al.*, 1998). Two species within the complex namely *An. gambiae* and *An. arabiensis* are responsible for most malaria transmission in Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee *et al*., 2000; Coetzee, 2004). Several behavioural traits predispose *An. gambiae* to be a major human malaria vector (Takken and Knols, 1999; Day, 2005). Females of this species have a focused preference for humans over animal blood (anthropophagic) and exhibit distinct nocturnal host-seeking patterns (White, 1974), although variations to these behavioural trait have been reported (Diatta *et al*., 1998; Duchemin *et al*., 2001; Lefevre *et al*., 2009). In addition, these mosquitoes blood feed (endophagy) and rest (endophily) inside human dwellings (Githeko, 1996; Clements, 1999; Costantini *et al*., 1999). Moreover, they oviposit in water bodies created through anthropogenic activities (Day, 2005). *Anopheles gambiae* females are also highly susceptible to infection with *Plasmodium* species (Beier, 1998). The vectorial capacity of *An. arabiensis* is slightly lower than that of *An. gambiae.* This is because of its ability to feed on other animals when humans are unavailable (Gilles and Coetzee, 1987; Githeko, 1996).

2.2.2 The *Anopheles funestus* group

Anopheles funestus consists of a group of nine species including *An. funestus* Giles (at times referred to as *An. funestus sensu stricto*), *Anopheles rivulorum* Leeson, *Anopheles parensis* Gillies, *Anopheles vaneedeni* Gillies and Coetzee, *Anopheles leesoni* Evans, *Anopheles fuscivenosus* Leeson, *Anopheles aruni* Sobti, *Anopheles brucei* Service and *Anopheles confusus* Evans and Lesson (Gillies and Meillon, 1968; Gilles and Coetzee, 1987). Species in the *An. funestus* group have slight morphological differences. *Anopheles funestus*, which is endophilic and anthropophilic, and *An. rivulorum* are the only two species within the *An. funestus* group that have been incriminated as malaria vectors (Gillies and Meillon, 1968; Wilkes *et al*., 1996; Cohuet *et al*., 2004).

2.3 Adult mosquito behaviour

The life of a female mosquito comprises of several major behaviours: mating, foraging and oviposition (Takken and Knols, 1999). These behaviours are regulated by internal and external factors (Figure 1). Internal factors comprise genetic make-up, age, size, gonotrophic status and nutritional state, while external factors include stimuli that mosquitoes perceive from the environment such as pheromones, animal host odours and plant volatiles. Although mosquito behaviours are genetically determined, their responses to external stimuli depend on

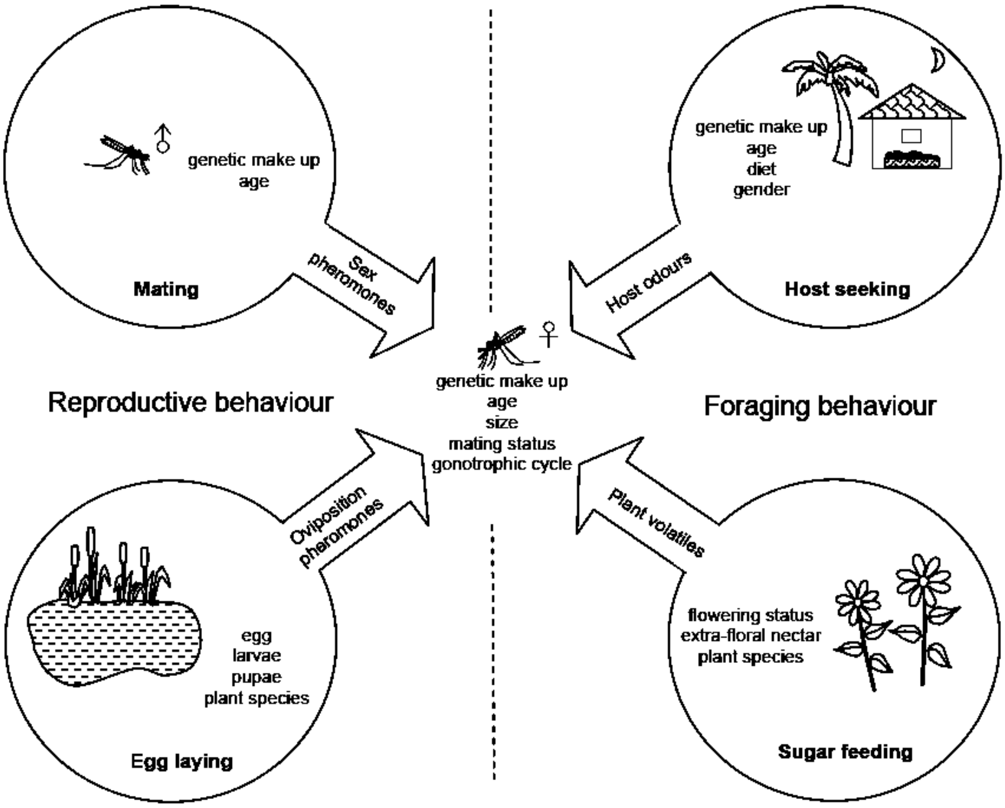

Figure 1: The four life history behaviours of adult female mosquitoes (After Takken and Knols, 1999).

their physiological state (Klowden, 1996). For instance, even though host stimuli may be present at certain times, the insect may not always express host-seeking behaviour. The physiological state, including such factors as age, nutritional state, presence of eggs and circadian rhythmicity may affect this behaviour (Klowden, 1996).

Female mosquitoes utilize semiochemicals to find sugar sources, mates, blood meal hosts and oviposition sites (Takken and Knols, 1999; Zweibel and Takken, 2004). Semiochemicals are signalling chemicals produced by one organism that induce changes in the behaviour or physiology of another organism. The semiochemicals exploited by mosquitoes while searching for sugar sources and mating partners include plant volatiles and sex pheromones, respectively. Host odours and oviposition pheromones are utilized by mosquitoes to locate hosts and oviposition sites, respectively.

2.3.1 Feeding behaviour

Nectar produced by plants is the only food source of male mosquitoes, while the females of many mosquito species take a sugar meal before blood feeding. The sugar meal is the source of energy used to sustain the host-seeking flight. Mosquitoes locate sugar sources mainly by using odours (plant volatiles) emitted by flowering plants (Foster and Takken, 2004). A considerable proportion of *Anopheles* females take a blood meal prior to mating (Takken and Knols, 1999). Only female mosquitoes feed on blood which is required for egg maturation.

2.3.2 Mating behaviour

Mosquitoes mate within the first 24 hours to 5 days of adult life (Verhoek and Takken, 1994). Mating in *Anopheles* mosquitoes occurs during twilight above specific features in the environment (called swarm “markers”) over which male swarms are formed. Females that approach the swarm are seized (Charlwood *et al.*, 2002) and, upon entering the swarm, are recognized by their flight tone (wing beat) which is slightly lower than that of the male counterparts (Brogdon, 1998). A male then couples with a female and the two leave the swarm *in copula*. Swarms formed by *An. gambiae* mosquitoes are rarely observed and it is possible that this species employs other cues for mate finding and recognition (Takken and Knols, 1999). Species-specific contact pheromones, a mechanism for the males to recognize their conspecific females upon contact, were found for several mosquito species (Kliewer *et al.*, 1967; Lang, 1977). *Aedes* and *Mansonia* males usually respond to host odours and they intercept and mate with females within the vicinity of the host (Jaenson, 1985).

2.3.3 Oviposition behaviour

Mosquitoes select oviposition sites by using visual and chemical cues. Chemical cues originate from natural water bodies as breakdown products of bacterial origin or from mosquitoes as oviposition pheromones (Bentle and Day, 1989). The stimuli are responsible for the aggregation of eggs in sites suitable for larval development (McCall and Cameron, 1995). *Anopheles gambiae s.l.* mosquitoes are nocturnal in their oviposition activities and the time of oviposition is determined by many factors including ambient temperature and light conditions and the time the mosquito obtains its blood meal (Clements, 1999). Studies carried out on *Anopheles gambiae s.l.* showed that its peak oviposition time is regulated by the light-dark cycle rather than oviposition habitat characteristics or feeding times (Sumba *et al.*, 2004).

2.3.4 Mosquito host seeking behaviour

Mosquitoes locate their preferred hosts using visual, physical and chemical cues (Sutcliffe, 1987; Takken, 1991; Takken and Knols, 1999). However, the identity of kairomones utilised by female mosquitoes to locate their hosts are largely unknown. Kairomones are chemical substances emitted by one species, in this case humans, but which evoke behavioural or physiological responses in another species, the mosquito in this case, where the responses elicited are favourable to mosquitoes but not humans (Clements, 1999). Visual and physical cues comprising of heat and moisture play a crucial role in orientation of the mosquito in the close vicinity of the host (Sutcliffe, 1987; Costantini *et al*., 1999).

The process by which mosquitoes locate a host is composed of a series of responses to internal and external stimuli. This follows a chain of events whose outcome is an increased probability of encountering and contacting the host. The events include appetitive search, activation, attraction, landing, probing, biting and engorgement (Sutcliffe, 1987; Dodd and Burgess, 1995). The mosquito, driven by endogenous rhythms and hunger, engages in appetitive search which is a behaviour that increases the probability of encountering stimuli signifying the presence of a host (Sutcliffe, 1987). Mosquitoes fly upwind when searching for hosts. The second behaviour designated as activation is a switchover from search behaviours to active host location brought about by host-distinctive stimuli. The process by which the mosquito comes in the immediate vicinity of the host is termed attraction. This relies on host-derived stimuli encompassing chemical, physical (heat and moisture) and visual cues (Sutcliffe, 1987). Thereafter, the mosquito will land on the host, probe and commence blood feeding.

2.3.4.1 The process of mosquito host location

The distances over which various stimuli act are divided into three ranges including long, medium and short-range phases (Figure 2) (Gillies and Wilkes, 1969; Sutcliffe, 1987; Gibson and Torr, 1999). Long distance orientation (between 20 - 70 metres) is governed entirely by body odour (chemical cues) other than carbon dioxide (Gillies and Wilkes, 1969). Medium-distance orientation (between two and 20 metres) starts when the mosquito not only perceives chemical cues, but in addition a distinctive carbon dioxide concentration above the background threshold levels (Gillies and Wilkes, 1969). It ends when the mosquito approaches the host (in this case upon entering the house). Short-distance orientation commences in the vicinity of the host (less than two metres) when the mosquito perceives chemical (including carbon dioxide), physical (heat and moisture) and visual cues (Gillies and Wilkes, 1969; Sutcliffe, 1987).

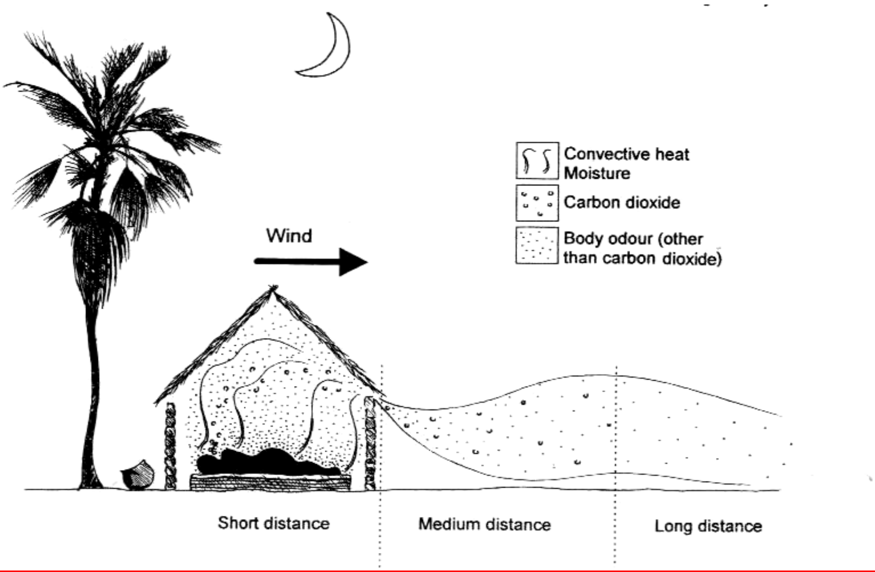

Figure 2: Stimuli perceived downwind by host-seeking *Anopheles gambiae* (After Gillies, 1988).

2.3.4.2 Differential attractiveness of human individuals to mosquitoes

Human beings exhibit interpersonal variations in attractiveness to host seeking mosquitoes (Lindsay *et al.*, 1993; Mukabana *et al.*, 2002; Qiu *et al.*, 2006). It has been reported that

Anopheles species prefer to feed on pregnant women rather than their non-pregnant counterparts (Lindsay *et al.*, 2000; Ansell *et al.*, 2002), adults rather than children (Muirhead-Thomson, 1951) and men rather than women (Muirhead-Thomson, 1951). This differential attractiveness of humans to mosquitoes has been attributed to compounds in human body emanations (Lindsay *et al.*, 1993; Mukabana *et al.*, 2002; Qiu *et al.*, 2006). Qiu *et al* (2006) reported that emanations from 27 human individuals collected on glass marbles differed in attractiveness to *An. gambiae* mosquitoes. Mukabana *et al* (2002) ranked nine Kenyan men and concluded that variability in attractiveness of humans to *An. gambiae* is attributed to differences in chemical composition of their total body emanations.

2.4 Malaria control

Malaria control efforts are either targeted at *Plasmodium* parasites or mosquito vectors that transmit them. Parasite control currently entails the use of anti-malaria drugs (Wistanley, 2000; Wistanley and Ward, 2006) with a promise of using vaccines in the future (Ballou *et al.*, 2004). Control strategies against the vectors target the adult or aquatic stages. Control measures aimed at adult mosquitoes include indoor residual spraying, personal protection that involves use of insecticide treated nets (Lengeler, 2004; Curtis *et al.*, 2006), and repellents (Rozendaal, 1997). Measures used against immature stages include biological control (Walker and Lynch, 2007), larviciding with microbial insecticides (Fillinger *et al.*, 2003) and environmental management (Keiser *et al.*, 2005; Lindsay *et al.*, 2004).

2.4.1 Control of malaria parasites

Over the centuries, a number of drugs have been used to cure patients suffering from malaria. Quinine, extracted from the bark of the cinchona tree, was used as an anti-malarial agent as early as 1632 (Baird *et al.*, 1996) and by the 19th century it was the only known anti-malarial agent. Chloroquine followed shortly after in 1934 (Thompson *et al*., 1972). Around 1946 it was designated the drug of choice for treatment of malaria (Coatney, 1963). Chloroquine and sulfadoxine-pyrimethamine (SP)-based drugs such as Fansidar have for a long time been used as anti-malarial drugs in Africa (Wistanely, 2000; Trape, 2001).

In the 1970s, *P. falciparum* resistance to chloroquine was reported from Eastern Africa and spread progressively to the west (Campbell *et al*., 1979). Over the past decade, new groups of antimalarials, -the artemisinin-based compounds, have been deployed on a large scale. These drugs are now recommended by the World Health Organization (WHO) as first-line treatment of uncomplicated falciparum malaria in all malaria is endemic areas (WHO, 2006). Artemisinin-based combination therapies are tolerated by patients, and have good parasite clearance rates (White, 2008).

Recently, there have been signs that the efficacy of artemisinin-based combination therapy have declined in western Cambodia (Dandorp *et al*., 2009). Artemisinin resistance would be disastrous for global malaria control and increased efforts are required to prevent this occurrence.

2.4.2 Adult mosquito control

Efforts aimed at controlling adult mosquitoes mainly rely on indoor residual spraying and personal protection measures which include insecticide treated nets, insecticide vaporizers and repellents.

2.4.2.1 Indoor residual spraying

Indoor residual spraying is the application of long-acting chemical insecticides on walls and eaves of houses in order to kill the adult mosquitoes that land and rest on these surfaces. House spraying is expected to i) reduce the life span of mosquitoes so they can no longer transmit malaria parasites from one person to another ii) to reduce number of mosquitoes that enter the house and iii) to reduce the number that having bitten infected people, leave the sprayed house (WHO, 2006). However, indoor spraying will only kill the endophilic fraction of the vector population, while the exophilic fraction will not be affected. House-spraying remains a valuable tool in malaria control (WHO, 1995; 2006), but insecticide resistance by mosquitoes hampers its effectiveness (Chandre *et al*., 2000; Nauen, 2007). Members of the *An. gambiae* complex in Africa have been reported to show resistance to DDT, organophosphates and carbamates (WHO, 2006).

2.4.2.2 Personal protection

Personal protection measures include i) house screening of windows and doors, ii) electrically heated vaporising mats, iii) mosquito coils containing volatile pyrethroids (Curtis and Townsend, 1998) and iv) repellents used on the skin or clothing namely DEET® (N, N-Diethyl-meta-toluamide). Although repellents applied to skin or clothing or volatile pyrethroids in vaporising mats are of proven efficacy in reducing the number of mosquito bites, their impact on malaria has not been quantified.

Bednets, another personal protection measure, have been used since historical times as a protective barrier against mosquito bites (Lindsay and Gibson, 1988). Insecticide treated bed nets (ITNs) have become an important defence against malaria transmission (Lengeler, 2004; WHO, 2006). The initial pyrethroid-treated bednets had the main drawback that they had to be re-treated every 6 to 12 months. Long lasting insecticidal nets (LLINs) which could last for 3 to 5 years were then introduced. The use of ITNs alone has been shown to significantly reduce morbidity and mortality due to malaria (Alonso *et al*., 1991; 1993; Nahlen *et al*., 2003; Philips-Howard *et. al*., 2003). However, the existing widespread use of bednets and indoor residual spraying is expected to enhance insecticide resistance (Hemingway and Ranson, 2000; Hemingway *et al*., 2002; Nauen, 2007).

2.4.3 Larval mosquito control

Larviciding is the use of chemicals and living agents to kill mosquito larvae in aquatic habitats. Larviciding is feasible and effective when breeding sites are relatively few in number and easily identified and treated. Larviciding involves the use of two methods namely chemical larviciding and biological control.

2.4.3.1 Chemical larvicides

The aim of chemical larviciding is to eliminate or reduce vector populations by killing the immature stages of mosquitoes. A range of chemicals has been successfully used as larvicides against malaria vectors. Petroleum oils were applied over 100 years ago to asphyxiate larvae of malaria vectors and other mosquitoes (Gratz and Pal, 1988). The larvicidal stomach poison Paris Green (copper acetoarsenite) was employed against anophelines extensively until the 1940s, by application as a fine powder that floated on the water surface and where it was eaten by *Anopheles* larvae (Rozendaal, 1997). Systematic use of Paris Green during the 1930s contributed to the elimination of *An. gambiae s.l* in north-east Brazil. (Soper and Wilson, 1943). The use of Paris Green is no longer recommended due to its high toxicity (Coosemans and Carnevale, 1995). Heavy petroleum oils have also been replaced with lighter products such as monolayer films which may show good efficacy against anophelines under certain conditions (Karanja *et al.*, 1994; Bashir *et al.*, 2008). In the 1960s several organophosphate-based larvicidal formulations were developed. In particular, Temephos exhibited very low mammalian toxicity (Gratz and Pal, 1988) and has been used for malaria control in several countries including India (Kumar *et al.*, 1994), Mauritius (Gopaul, 1995) and Oman (Parvez and Al-Wahaibi, 2003).

2.4.3.2 Insect Growth Regulators

Insect growth regulators (IGRs) are especially recommended due to their great persistence and the apparent lack of non-target effects (Graf, 1993). Pyriproxyfen, which interferes with larval/pupal/adult metamorphosis, is more durable and cost effective than temephos and other larvicides. Several pilot projects involving larviciding with IGRs show promising results. In Thailand applications of Pyriproxyfen at a rate of 0.005mg/l to slow moving rivers significantly reduced adult populations of several vector species (Kanda *et al.*, 1995). In Dar es salaam, Tanzania, larviciding with IGRs in concert with drain maintenance was identified as the most cost-effective and suitable vector control method (Castro *et al.*, 2004). Larval resistance to some of the more widely applied larvicides such as Temephos is a growing problem (Coosemas and Carnevale, 1995).

2.4.3.3. Biological control

Many organisms help to regulate *Anopheles* populations naturally through predation, parasitism and competition. Biological control is the introduction or manipulation of these organisms to suppress vector populations. At present, fish and bacterial pathogens (*Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus*) that attack larval mosquito stages are employed (Das and Amalraj, 1997). Several pathogens in the fungal genera *Metarhizium* and *Beauveria* show promise as larvicides (Scholte *et al.*, 2003). Another biological control agent includes the nematode *Romanomermis culicivorax* and the aquatic plant *Azolla* (Lacey and Lacey, 1990). The advantages of biological larval control agents in comparison to chemical control includes their effectiveness at relatively low doses, safety to humans and non-target wildlife, low cost of production in some cases, and lower risk of resistance development (Yap, 1985).

Two bacterial species, *Bacillus thuringiensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) have been widely demonstrated to be effective larvicides against mosquitoes. Both *Bti* and *Bs* function as stomach poisons in the mosquito larval midgut. The former acts as a broad-spectrum biological insecticide (Fillinger and Lindsay, 2006). Several formulations of *Bti* have been developed for use against larvae of mosquitoes such as *Anoheles albimanus*, *Anopheles sinensis* and *Anopheles stephensi* as well as members of the *An. gambiae*, *Anopheles maculatus*, *Anopheles maculipennis* and *Anopheles sundaicus* complexes (Lacey and Lacey, 1990; Becker and Margalit, 1993; Mittal, 2003). The lethal effect of *Bti* is largely due to protoxins in parasporal crystals and the spore coat, rather than the actual infection. Field trials of *Bti* to control *An. gambiae s.l.* larvae in Africa generally have shown good control *albeit* with a short duration of residual activity (Karch *et al*., 1991; Romi *et al.*, 1993; Fillinger *et al.*, 2003).

Research on mosquito pathogenic viruses has been limited, in part, due to the inability to effectively transmit them to the larval mosquito host. Recently, there have been tremendous advancements in the ability to transmit some mosquito pathogenic viruses (Becnel *et al*., 2001). The viral microbial agents that infest the mosquito larval stages belong to the Nucleopolyhedrovirus group (NPV). In mosquito larvae, these viruses are specific to the midgut tissues (Becnel *et al*., 2001). Viral-induced mortality is usually caused by toxic proteins that accumulate during the reproductive cycle of the virus.

Predatory fish (particularly in the family Cyprinodontidae) that eat mosquito larvae, have been used for mosquito control for at least 100 years (Meisch, 1985). Prior to the 1970s, the most commonly used species was the mosquito fish *Gambusia affinis affinis* (Cyprinodontiformes: Peociliidae), a fresh water species native to the south-eastern U.S.A. The practice has since been discouraged as the efficacy is highly variable and negative impacts of this voracious and aggressive fish on native fauna have been quite significant (WHO, 1982).

Fish may be useful in controlling malaria vectors associated with rice fields (Lacey and Lacey, 1990). In Asia, introduction of larvivorous fish has been effective where pisciculture provides additional economic, agricultural and nutritional benefits (Wu *et al.*, 1991; Victor *et al.*, 1994). Larvivorous fish also show promise in controlling malaria vectors in human-made containers, particularly in urban areas. In an urban area in Ethiopia, Fletcher *et al.* (1992) found that the indigenous fish *Aphanius dispar* effectively suppressed *Anopheles culicifacies adenensis* larvae breeding in wells and containers.

2.4.4 Environmental management for malaria control

Since the discovery of the role of *Anopheles* mosquitoes in malaria transmission, malaria control programmes have targeted potential mosquito larval breeding sites and have helped reduce or eliminate malaria transmission in many parts of the world (Baer *et al.*, 1999; Lindsay *et al.*, 2004). Environmental management for malaria control involves the performance of activities that lead to the modification or elimination of aquatic habitats so as to reduce mosquito breeding. Environmental management is a re-emerging vector control strategy (Utzinger *et al.*, 2001). Techniques of environmental management are grouped into three main categories namely environmental modification, environmental manipulation and modification of human habitations (WHO, 1982; Rafatjah, 1988; Ault, 1994). The first two categories generally target the larval stages whereas the third targets adult vectors.

Environmental modification involves a physical change, often long-term, of potential breeding sites. The physical changes include drainage, land levelling and filling (WHO, 1982). In Africa, several environmental modification projects in Zambia (Watson, 1953; Baer *et al*., 1999; Utzinger *et al*., 2001), Uganda (Lindsay *et al*., 2004) and Tanzania (Castro *et al*., 2004) were associated with reduced vector populations. However, poorly maintained projects may increase larval breeding habitats (Takken *et al*., 1990; Castro *et al*., 2004).

Environmental manipulation refers to activities that reduce larval breeding sites through temporary changes in the aquatic environment in which larvae develop. Techniques include changing water levels in reservoirs, flushing streams or canals, providing intermittent irrigation to agricultural fields (particularly rice), flooding or temporarily draining man-made or natural wetlands and changing water salinity (Rafatjah, 1988). Intermittent irrigation in rice fields which involves periodic draining of fields timed at a frequency to prevent mosquito larvae from completing development has been successful in India, China and other parts of Asia (Lacey and Lacey, 1990).

2.5 Mosquito attractants derived from human hosts

Mosquitoes locate their preferred hosts through chemical, physical and visual cues (Takken and Knols, 1999). Activation beyond the visual range is effected by chemical cues. Insect attractants achieve their purpose by enhancing host-vector contact or facilitating the feeding process. Human skin odour has been shown to account for most of the attractiveness of humans to *An. gambiae* (Costantini *et al*., 1996; Mboera *et al*., 1997; Costantini *et al*., 1998).

Approximately 350 chemical compounds have been identified from human volatiles (Bernier *et al.*, 2000) but few have been singled out as candidates involved in attraction of female mosquitoes to humans (Costantini *et al*, 2001; Braks *et al.*, 2001; Healy *et al.*, 2002).

So far efforts to fractionate and analyse human emanations have been unable to identify chemicals that are solely responsible for human attractiveness to host seeking mosquitoes. However, human sweat, and skin washings have been shown to elicit behavioural responses in mosquitoes under laboratory (Knols *et al.*, 1997; Braks *et al.*, 2001; Smallegange *et al.*, 2005) and semi-field conditions (Njiru *et al.*, 2006). However, none of these by products of human metabolic processes or skin microbial action elicits stronger mosquito responses than a human being.

Carbon dioxide, a major constituent of exhaled breath, has been identified as an attractant for many mosquito species (Gilles, 1980; Takken, 1991; Mboera, 1997). Odour bursts of breath from an adult human are excreted at 300 ml/min^{-1} and contain approximately 4.5% carbon dioxide, while normal atmospheric levels of carbon dioxide are between 0.3 – 0.4% (Gillies 1980). Gillies (1980) suggested that carbon dioxide acts as an activator, possibly initiating flight response, and as a kairomone. However, in *An. gambiae s.s.* it appears to be of minor importance (Mboera *et al.*, 1997). This is based on the fact that since all vertebrates exhale carbon dioxide, and *An. gambiae s.s.* being anthropophilic, must be guided by human-specific compounds when host seeking (Mboera *et al.*, 1997).

Studies undertaken by Takken and Kline (1989) were the first to report that octenol is a mosquito attractant. Octenol and carbon dioxide acted synergistically in attracting significantly great numbers of *Anopheles* mosquitoes and have been used as attractants in traps to capture nuisance mosquitoes (Takken and Kline, 1989; Kline *et al*., 1990). Acetone is a component of human breath and was found in fresh but not in incubated human sweat (Meijerink *et al.*, 2000). The combination of CO_2 and acetone at a low concentration elicited attraction of *An. gambiae* in a wind tunnel bioassay (Takken *et al.*, 1997a).

Human sweat was also found to be attractive to female *An. gambiae* mosquitoes (Braks *et al*., 1997; Braks and Takken, 1999; Healy and Copland, 2000). However, incubated sweat was found to be more attractive than fresh sweat, an observation which was attributed to the emission of ammonia produced by microbial activity in sweat (Braks *et al*., 2000). Differences in the abundance of components, such as indole, 1-dodecanol, 6-methyl-5-hepten-2-one and geranyl acetone, were found in the chemical composition of the highly attractive incubated and lowly attractive fresh sweat (Meijerink *et al.*, 2000). Incubated sweat becomes alkaline due to increasing ammonia formation (Braks and Takken, 1999). Ammonia has been found to act as an attractant to *An. gambiae* in olfactometer bioassays (Braks *et al*., 1999, 2001; Smallegange *et al.*, 2005) and under semi-field conditions (Njiru *et al*., 2006).

Human sweat also contains lactic acid (Eiras and Jepson, 1991; Cork and Park, 1996; Healy and Copland, 2000). Human skin emanations have uniquely high levels of this compound compared to skin emanations from other animals (Dekker *et al*., 2002). Although Healy and Copland (2000) reported that *An. gambiae* females land on filter papers impregnated with human sweat, the concentration of lactic acid that was present in sweat did not elicit landing responses. This compound was reported to be slightly attractive by itself (Braks *et al*., 2001) as well as act synergistically with carbon dioxide (Dekker *et al*., 2002; Murphy *et al*., 2001).

Carboxylic acids make up an important part of human sweat (Cork and Park, 1996). Electroantennogram (EAG) studies have shown that *An. gambiae* females can detect saturated carboxylic acids. The short-chain carboxylic acids methanoic, ethanoic, propanoic, butanoic and hexanoic acids elicited higher EAG responses than less volatile, long-chain carboxylic acids (Cork and Park, 1996). Oxocarboxylic acids were found in human blood and urine (Chalmers and Lawson, 1982). Six oxocarboxylic acids composed of 2-oxobutanoic, 2-oxo-3-methylbutanoic, 2-oxopentanoic, 2-oxo-3-methylpentanoic, 2-oxo-4-methylpentanoic and 2-oxohexanoic acids were reported to stimulate landing of *An. gambiae* (Healy *et al.*, 2002). Experiments done in Y-tube olfactometers demonstrated a synergistic effect of ammonia, lactic acid and hexanoic acid on behavioural responses of *An. gambiae* (Smallegange *et al*., 2002).

The compound 7-octenoic acid has been reported to be abundantly produced by and specific to human beings (Zeng *et al*., 1991). A field study in Burkina Faso reported that addition of 7-octenoic acid to carbon dioxide increased the number of *An. gambiae* attracted to odour-baited entry traps (Costantini *et al.*, 2001).

Experiments aimed at describing sites preferred for biting by host seeking mosquitoes demonstrated that female *An. gambiae s.s.* preferred biting the feet and ankles of human volunteers (De Jong and Knols, 1995). It was subsequently shown that Limburger cheese, whose smell is reminiscent to that of human foot odour, attracted *An. gambiae* (Knols and De Jong, 1996). The ripening of Limburger cheese is effected by coryneform bacteria, specifically *Brevibacterium linens* which is closely related to *Brevibacterium epidermidis*, a common constituent of the microflora that abounds on human feet. Limburger cheese contains significant quantities of short-chain fatty acids, compounds that are also present in human sweat (Knols and De Jong, 1996) and, which are known to elicit behavioural responses in *An. gambiae s.s.* (Knols *et al*., 1997). Njiru *et al*., (2006) reported that *An. gambiae* females responded to foot odour collected on worn nylon socks in a semi field system. Laboratory studies had earlier demonstrated strong attraction by female *Anopheles* mosquitoes to nylon stockings worn by a human volunteer (Pates *et al*., 2001).

2.5.1 Synthetic odour blends attractive to host seeking *An. gambiae* mosquitoes

Based on the principle that mosquitoes rely on a combination of chemicals from human skin to locate suitable hosts (Takken, 1991), attempts have been made to synthesize and blend candidate chemical compounds displaying the ability to attract mosquitoes. Knols *et al*., (1997) demonstrated that a synthetic mixture of 12 aliphatic carboxylic acids (C_2-C_{14}) elicited electrophysiological and behavioural responses in *An. gambiae* females in a wind-tunnel.

Experiments with *An. gambiae* females that were conducted in Y-tube olfactometers demonstrated the synergistic effect of the combination of ammonia, lactic acid and hexanoic acid (Smallegange *et al*., 2002). According to Smallegange *et al*. (2005) the combination of ammonia, lactic acid and carboxylic acids was significantly more attractive to *An. gambiae* than these compounds individually in a dual-port olfactometer. This alluded to the earlier thoughts that an odour blend rather than a single compound represented the attractive principle in the host seeking behaviour of mosquitoes (Takken, 1991).

A field study in The Gambia, West Africa, revealed that counter flow geometry traps (MMX® traps) baited with a synthetic odour blend consisting of ammonia, lactic acid, carbon dioxide and 3-methylbutanoic acid was a very attractive odour for most mosquito species (Qiu *et al*., 2007). This candidate odour blend has proven the potential of synthetic blends in trapping mosquitoes, and offers a possible candidate for use in mass trapping devices for the control of vectors (Day and Sjogren, 1994; Kline, 2007).

In a recent field study in Tanzania, an odour blend consisting of known mosquito attractants namely carbon dioxide, ammonia and carboxylic acids were evaluated. Field experiments conducted inside experimental huts found the blend to attract three to five times more *An. arabiensis* than humans when the two baits were in different experimental huts (10 - 100 metres apart) (Okumu *et al.*, 2010b). However, the blend was equally or less attractive than humans when compared side by side within the same huts (Okumu *et al.*, 2010b). The synthetic odour blends tested so far have not equalled or exceeded the attractiveness of human body odour. Therefore the identity and ratios of key chemical compounds that *An. gambiae* relies upon to locate its human hosts remain unknown.

CHAPTER THREE

MATERIALS AND METHODS

The studies reported in this thesis were conducted under semi-field conditions within a screen house located at the ICIPE Thomas Odhiambo Campus (TOC) situated near Mbita Point township, western Kenya. The gable-roof screen house measuring 11.4m long, 7.1m wide and 3m at the apex was fitted with a glass-panelled roof and gauze-covered walls. A layer of sand was spread on the floor and a large mosquito netting cage fitted inside the screen house.

3.1. Rearing of mosquitoes

Experiments were conducted using the Mbita strain of *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *Anopheles gambiae*). The mosquitoes were reared under ambient conditions in an insectary where the natural 12L: 12D photoperiod was maintained. Mosquito eggs were dispensed into plastic basins filled with filtered Lake Victoria water. The eggs hatched into larvae and were fed on Tetramin® baby fish food three times per day. The basins each contained 200 – 250 larvae. The water in which the larvae were reared was replaced every two days by sieving larvae using a metallic sieve and transferring them into fresh water in a clean plastic basin. Pupae were collected daily using pipettes and transferred into clean cups filled with filtered lake water. The water-filled cups containing pupae were then placed in mesh-covered cages (30 × 30 × 30cm) pending adult emergence. Emerged adult mosquitoes were provided with 6% glucose solution on wicks made from tissue paper. Adult female mosquitoes were provided with a cup of filtered lake water on which they laid eggs. Laid eggs were dispensed into plastic basins filled with filtered Lake Victoria water to complete the routine.

Adult female *An. gambiae* mosquitoes aged between three and six days old with no prior access to a blood meal were used in all experiments. The mosquitoes were selected from adult holding colony cages at 1130 and 1230 hours and put into cylindrical mosquito release cups (diameter 10cm, height 12cm). The release cups were covered with opaque polythene paper to exclude visual cues. A piece of mosquito netting with a central hole cut in it was used to cover the mouth of the release cups (Plate 1). Dry cotton wool was placed on the central hole to prevent mosquitoes from escaping. After mosquitoes were placed in the cup, water-wet cotton wool was then placed on the net so as to hydrate the mosquitoes. The release cup (Plate 1) containing the mosquitoes was then placed on a metallic rack at a corner of the insectary for eight hours prior for use in the behavioural assays.

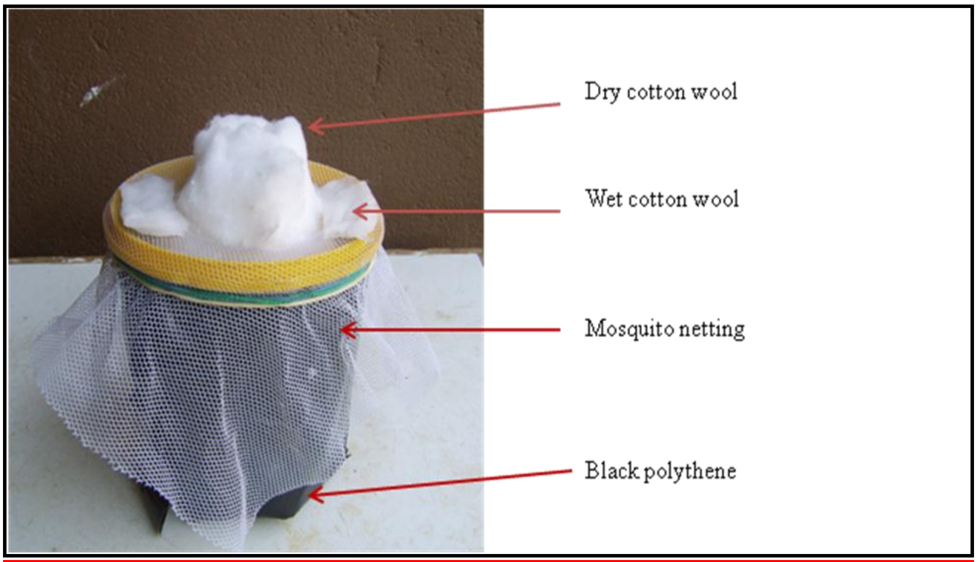

Plate 1: The mosquito release cup.

3.2 The dual-port olfactometer

A dual-port olfactometer made from Perspex measuring 50 × 30 × 20cm connected to two tents by polyvinyl chloride (PVC) pipes (10cm diameter × 75cm) was used for experiments.

The olfactometer consisted of one central choice chamber and two trap chambers (Figure 3). Each trap chamber contained a mesh-covered cage measuring 13 × 17 × 28 cm. The tents (2 × 1 × 1.5m at the apex) were made of wooden framework and covered with thick white cotton cloth to retain odours in the tents (Plate2). Opaque polythene sheets were used to cover the tents, olfactometer and connecting pipes to exclude visual cues. The tents designated A and B had entry slits on the distal ends, cotton sleeves, in which PVC pipes were fitted, were proximal to the olfactometer . Each tent had a white clean cotton bed sheet spread atop a mattress for the human subjects to sleep on. A CDC light trap fan was placed in the pipe on top of the olfactometer lid to draw air out of the tents (approximately 130 L/min/tent). A cylindrical release cup was connected to the dual-port olfactometer from the bottom of the choice chamber. Mosquitoes placed in the release cup flew into the choice chamber in response to odours and were subjected to a choice test. Mosquitoes subsequently attracted to odours in the tents were trapped in the collecting cages placed within the trap chambers. The entire experimental set-up was housed in a screenhouse at ICIPE, Mbita Point.

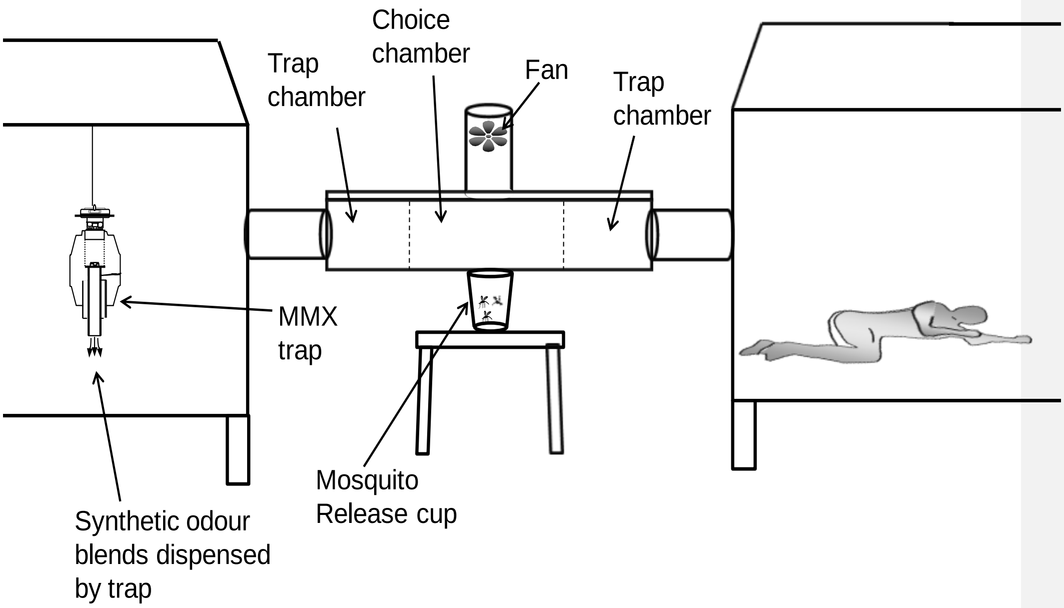

Figure 3: Schematic drawing of the dual-port olfactometer connected to two tents showing how synthetic odour blends were compared to human emanations.

Plate 2: Pictorial view of the experimental apparatus used to study the response of *An. gambiae* to human emanations and synthetic odours augmented with physical cues. A, wooden framework of the tents; B, tents' framework covered with thick white cotton cloth; C, opaque polythene paper (PCV) overlying the white cotton cloth tent covers; and D, the dual-port olfactometer connected to the two tents.

3.3 Behavioural stimuli and their delivery

Behavioural stimuli consisted of synthetic odour blends, total body emanations and foot odours. An MMX® trap (Mosquito magnet model- X) was used to dispense synthetic odours.

3.3.1 Synthetic odour blends

Two synthetic odour blends namely, standard blend and blend 1,were tested for attractiveness to *An. gambiae* in comparison to human emanations. The standard blend consisted of CO_2 (500ml/min), ammonia (2.5%) and distilled H_2O. Blend 1 consisted of the eight aliphatic carboxylic acids: propionic acid (C3; 0.1%), butyric acid (C4; 1%), valeric acid (C5; 0.01%), 3-methylbutanoic acid (3mC4; 0.001%), heptanoic acid (C7; 0.01%), octanoic acid (C8; 0.01%), myristic acid (C14; 0.01%) and lactic acid (85%), plus ammonia (2.5%), distilled water and carbon dioxide (500ml/min). Carbon dioxide was released at a rate of 500ml min^{-1} from a pressurized gas cylinder controlled by a flowmeter (Glass Precision Engineering Ltd, UK). All chemicals, except carbon dioxide, were purchased from the Sigma-Aldrich chemical company (Chemie GmbH, Germany). The industrial grade carbon dioxide was purchased from Carbacid Investments Ltd, Kenya.

Strips of nylon sock made from 90% polyamide and 10% spandex, measuring 26 cm in length and 1cm wide were dipped in separate one millilitre volumes of chemical constituents' characteristic of a specific blend. Individual strips were dipped in one chemical only. The standard blend consisted of two strips, one soaked in one millimetre of ammonia (2.5%) and another soaked in one millimetre of distilled water. Blend 1 consisted of ten strips, of which eight were individually soaked in the eight carboxylic acids, one in ammonia (2.5%), and the other in distilled water. Soaked strips were placed on a rack at room temperature for five hours to dry prior to experiments. This was to ensure that the chemicals soaked in the strips did not drip and contaminate the experimental set-up or cross-contaminate with chemicals on other strips. The strips were then tied together and placed in the attractant tube of the MMX® trap. The trap was then placed in a tent and connected to an electric power source. An air stream blew over the strips thus delivering odour-laden air at the bottom of the trap into the tent.

The synthetic odours were dispensed by an MMX® trap (American Biophysics Corporation, RI, USA) made up of an oval-shaped plastic container which served as the collection container (Figure 4). The container encircled two tubes; a collection tube and an attractant tube. The thin attractant tube was encircled by the shorter collection tube. At the top of the collection container was a black hood that consisted of an exhaust fan and a small hole for carbon dioxide supply. The trap had two fans, a bigger more powerful fan, named exhaust fan that exhausted air, and a smaller, less powerful counterpart, called the attractant plume fan that drew in air. The bigger fan was disabled such that its power supply was cut off which made the apparatus an odour-dispensing device rather than one that trapped insects. The MMX® trap was tied at the centre of the tent and hung approximately 15 centimetres from the bed sheet surface.

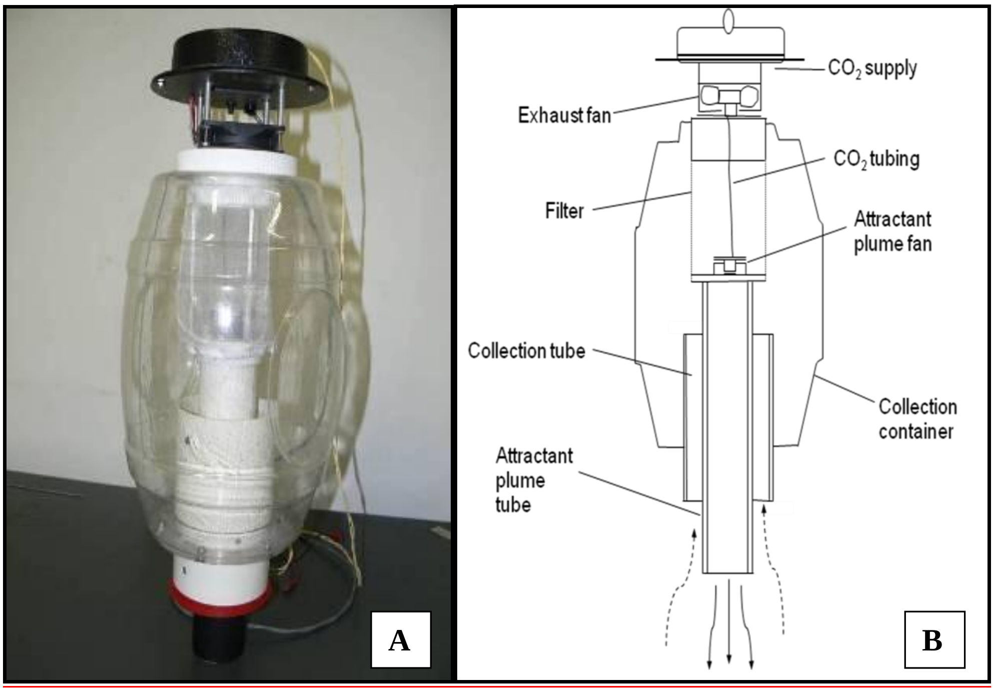

Figure 4: Picture (panel A) and illustration (panel B) of the MMX® trap used to dispense synthetic odour blends and foot odours.

3.3.2 Human odours

Two types of natural stimuli were used in the experiments. These included total body emanations of human volunteers and the volunteers' foot odours adsorbed onto cotton socks for 12 hours.

3.3.2.1 Total body emanations

Two male volunteers aged 32 and 33 years, namely, Person Highly Attractive (person HA) and Person Least Attractive (person LA) were recruited in the study to serve as natural sources of host seeking cues. The volunteers had previously been ranked for attractiveness to *An. gambiae* mosquitoes together with seven other male Kenyans using a similar olfactometer. From that ranking, the two individuals were shown to be less and highly attractive to female *An. gambiae* mosquitoes (Mukabana *et al*., 2002). The persons' bathed with non-perfumed bar soap (Menengai Oil Refineries Limited, Kenya) half an hour before starting experiments. The volunteers wore short trousers during experiments to allow free dissemination of body emanations. Malaria infection status of the volunteers was tested daily through microscopic examination of thick and thin smears of finger-pricked blood stained with Giemsa.

3.3.2.2 Foot odours

Cotton socks were worn by the volunteers for eight hours during the day and their foot odour adsorbed onto the socks. Foot odours acted as an alternative natural source of host seeking cues. Worn cotton socks were placed in the attractant tube of the MMX® trap and the odours dispensed when the trap was switched on. The foot odours were dispensed in one tent while a human volunteer was present in the second tent. A new set of worn socks was used every night.

3.4 Experimental design

An experimental comparison consisted of two baits. Person LA and person HA served as positive controls while synthetic odour blends and foot odours on their own or when augmented with heat and moisture were treatments. The two baits were alternated between tents to avoid positional bias. Each experimental comparison was conducted over a period of four nights and consisted of eight replicates. Two replicates were carried out each night. After two replicates, baits were alternated between the tents.

3.4.1 Testing functional integrity of dual-port olfactometer

The dual-port olfactometer was calibrated in a series of three experimental comparisons each lasting four nights. The comparisons included an empty tent versus an empty tent, the standard blend versus the standard blend and person HA versus person LA. In each experiment 100 female mosquitoes aged 3 - 6 days old, previously starved for eight hours, were released. Each experiment was conducted over 30-minute time periods each night for 4 consecutive nights. Two replicates were performed each night from 1930 – 2000 hours and 2030 – 2100 hours since those were the times that female *An. gambiae* mosquitoes are known to actively engage in seeking for and biting human hosts (Mathenge *et al.*, 2001). Each comparison consisted of eight replicates. Mosquitoes trapped in the trap chambers were counted after each experiment. In each experiment, 800 mosquitoes were used totalling to 2,400 mosquitoes for the three experimental comparisons. Temperature and relative humidity of tents occupied by person LA and person HA was recorded in all replicates using data loggers (Tinytag®). The average temperature and relative humidity readings were calculated for the tent that each individual occupied.

3.4.2 Comparison of female *An. gambiae* responses to human emanations and synthetic blends

Responses of female *An. gambiae* mosquitoes to synthetic blends in comparison to human emanations were determined over a period of 32 days. The standard blend or blend 1 were dispensed by an MMX® trap placed in one tent while a male volunteer aged 32 or 33 years slept in the second tent. Human odours were obtained from person HA and person LA either by sucking air from tents in which they slept or by using cotton socks worn by them for eight hours. The human subject and the MMX® trap were alternated between the tents to minimize positional or site effects. Six experimental comparisons, which included i) person LA versus an empty tent, ii) LA versus standard blend, iii) person LA versus foot odours, iv) person LA versus Blend 1, v) person HA versus an empty tent, vi) person HA versus standard blend, vii) person HA versus foot odours and viii) person HA versus blend 1, were performed. Each comparison consisted of eight replicates. In each experiment, 800 mosquitoes were used totalling to 6,400 mosquitoes for the six experimental comparisons.

3.4.3 Female *An. gambiae* responses to human emanations and synthetic odour blends augmented with physical cues

Data loggers (Tinytag®) were placed in the tents to record temperature and relative humidity (RH). Temperature and RH was recorded in tents where the volunteers slept. The average temperature and RH was then calculated. For an empty tent, or a tent containing an MMX® trap, the average temperature was 23.2°C and RH was 73%. The average temperature and RH for a tent occupied by person HA was 25.5°C and 80%, respectively. The average temperature and RH for person LA's tent was 24.9°C and 75%, respectively. Each comparison consisted of eight replicates. In each experiment, 800 mosquitoes were used totalling to 14,400 mosquitoes for the eighteen experimental comparisons.

3.4.3.1 Comparison of female *An. gambiae* responses to human emanations and synthetic odour blends augmented with heat

A portable heater (Conrad Electronics®) was used in a tent fitted with an MMX® trap to regulate the temperature to that comparable to the tent occupied by the volunteers. The heater was hung at the centre of the tent. When person LA occupied one tent, the heater regulated the temperature in the other tent (occupied by an MMX® trap) to 24.9^{o} C. In the comparisons involving person HA, the temperature in the opposite tent was raised to 25.5^{o}C. Six experimental comparisons were carried out for a period of 24 days. The six experimental comparisons included i) person LA against standard blend augmented with heat, ii) person LA against foot odours augmented with heat, iii) person LA against Blend 1 augmented with heat, iv) person HA against standard blend augmented with heat, v) person HA against foot odours augmented with heat and iv) person HA against blend 1 augmented with heat.

3.4.3.2 Comparison of female *An. gambiae* responses to human emanations and synthetic odour blends augmented with moisture

Addition of moisture to synthetic blends and foot odours was done by use of a portable humidifier (Honeywell®) with an in-built humidistat. The humidifier was placed on the bed sheet at the centre of the tent. The humidifier regulated the RH in the tent occupied by the MMX® trap to 75% when person LA occupied the other tent. The RH in the tent in which the MMX® trap was placed was augmented to 80% when tested against person HA. Six comparisons were tested for 24 days. The six experiments included i) person LA compared to the standard blend augmented with moisture, ii) person LA compared to his foot odours augmented with moisture, iii) person LA compared to Blend 1 augmented with moisture, iv) person HA versus compared to the standard blend augmented with moisture, v) person HA compared to his foot odours augmented with moisture and iv) person HA compared to blend 1 augmented with moisture.

3.4.3.3 Comparison of female *An. gambiae* responses to human emanations and synthetic odour blends augmented with heat and moisture

A heater (Conrad Electronics®) and humidifier (Honeywell®) were placed side by side at the centre of the tent in which an MMX® trap was placed. This was done to simulate the temperature and RH to that in the tent occupied by the volunteers. The temperature and RH of the tent fitted with an MMX® trap was raised to 24.9°C and 75%, respectively, when compared to person LA. So as to equal heat and moisture content of the tent occupied by person HA, the temperature and RH were regulated to 25.5°C and 80% in the opposite tent containing the MMX® trap. A total of six binary assays were performed for 24 days. The six experimental comparisons included i) person LA versus standard blend augmented with both heat and moisture, ii) person LA versus foot odours augmented with both heat and moisture, iii) person LA versus Blend 1 augmented with both heat and moisture, iv) person HA versus standard blend augmented with both heat and moisture, v) person HA versus foot odours augmented with both heat and moisture and iv) person HA versus blend 1 augmented with both heat and moisture.

3.5 Data management and analysis

In the experiments, mosquitoes were subjected to a choice test, such that they were either attracted to human emanations or other behavioural stimuli consisting of the standard blend, foot odours and blend 1. Thus, behavioural responses of individual mosquitoes were mutually exclusive and obtained through a count process. The proportion of mosquitoes attracted to humans and synthetic odours were estimated through a count process. Factors that potentially affected mosquito responses such as tent and experimental period were categorical. Such data likely to follow a Poisson distribution was authenticated through dispersion tests which examined whether means equalled the variances.

Data analysis was carried out using the General Statistical software (GenStat® Discovery Edition 3). Using a Generalized Linear Model (GLM) the number of mosquitoes attracted to odour baits (persons' emanations, their foot odours or synthetic attractants) were modelled as a proportion of the total number recovered from the choice chamber, release cup and two trap chambers. The effect of date, tent and age of mosquitoes were incorporated into the model. The data were transformed to assume a normal distribution using a logarithm link function (Payne, 1986). The symmetry and functional integrity of the experimental setup was assessed using tent A as a reference when i) an empty tent was compared to an empty tent and ii) when the standard blend was compared against the standard blend. Person HA was the reference in the binary assay where he was compared to person LA. In all other binary assays that assessed relative attraction of humans to synthetic attractants on their own or when augmented with heat, moisture or both, the human subjects posed as baseline references against which the degree of attraction of the mosquitoes to the synthetic attractants was compared.

3.6 Ethical clearance

Institutional ethical clearance was granted by the joint University of Nairobi – Kenyatta National Hospital ethical review committee (protocol approval number P102/7/2004 amended in 2007 and 2008). Ethical clearance was also sought from Kenyatta University before the onset of this research. The volunteers also gave their written consent to participate in the study.

CHAPTER FOUR

RESULTS

4.1 Functional integrity of the dual-port olfactometer

When both tents were left empty, no mosquito was caught in the trapping chambers. No significant differences were found in the number of mosquitoes attracted to the control blend when competed against itself ($P = 0.889$). The relative attractiveness of the volunteers differed significantly ($P < 0.001$). One hundred percent of the mosquitoes were attracted to the person HA while person LA did not attract any mosquito (Table 1). The three calibration experiments confirmed that the dual-port olfactometer was well suited for discriminating mosquito responses to candidate behavioural stimuli.

Table 1: Number of mosquitoes attracted in the absence of human emanations or in the presence of synthetic and natural (human-derived) behavioural stimuli in binary assays.

Behavioural stimuli		N	Replicates	Mosquito responses		P value	Total responses (%)			
Reference stimuli	Other stimuli			Reference stimuli	Other stimuli		c	t	Net attraction	Non-responsive (%)
Tent A (empty)	Tent B (empty)	800	8	0	0	1.000	70	0	0	30
Standard Blend	Standard Blend	800	8	25	26	0.889	60	6	10	34
Person HA	Person LA	800	8	0	99	0.001	55	12	13	33

N refers to the sum of mosquitoes subjected to a choice test per comparison; P values indicate the level of statistical difference between pairs of contrasting stimuli specifically the reference stimulus versus other stimulus (c) refers to the total number of mosquitoes collected in the choice chamber; (t) indicates the total number of mosquitoes collected from the two trap chambers; Net attraction refers to the percentage of mosquitoes that were attracted to both reference and other stimuli from the total number of mosquitoes recovered in (c) and (t); Non-responsive mosquitoes indicates the percentage of mosquitoes that were left in the release cup.

4.2 Comparison of female *An. gambiae* responses to human emanations and synthetic blends

Significantly more mosquitoes responded to person LA than an empty tent ($P < 0.001$). Similar results were obtained when person HA was tested against empty tents ($P < 0.001$). A higher proportion of mosquitoes was attracted to person LA when compared to the standard blend that consisted of ammonia, distilled water and carbon dioxide ($P < 0.001$). Similarly, significantly more mosquitoes responded to person HA than the standard blend ($P < 0.001$) (Table 2).

The proportion of mosquitoes attracted to person LA when tested against his foot odours did not differ significantly ($P = 0.163$). On the contrary, person HA attracted significantly more mosquitoes when compared to his foot odours ($P = 0.001$).When person LA and blend 1(that consisted of seven carboxylic acids, lactic acid, ammonia, distilled water and carbon dioxide) were compared, they appeared equally attractive to mosquitoes ($P = 0.417$). However, this was not the case when person HA was compared to blend 1 as a significantly higher proportion of mosquitoes were attracted to person HA than to blend 1 ($P = 0.001$) (Table 2).

Table 2: Attraction of *Anopheles gambiae* to human emanations versus synthetic odour blends and foot odours

Behavioural stimuli		N	Replicates	Mosquito responses		P value	Total responses (%)			
Person	Treatment			Person	Treatment		c	t	Net attraction	Non-responsive (%)
LA	Empty tent	800	8	30	6	0.001	59	4	7	37
LA	Standard Blend	800	8	14	8	0.001	64	2	4	34
LA	Blend 1	800	8	39	40	0.417	59	10	14	31
LA	Foot odours	800	8	86	53	0.613	61	17	16	22
HA	Empty tent	800	8	145	10	0.001	51	19	28	30
HA	Standard Blend	800	8	98	23	0.001	42	13	27	45
HA	Blend 1	800	8	150	5	0.001	51	19	28	30
HA	Foot odours	800	8	186	17	0.001	45	25	36	30

4.3 Comparison of female *An. gambiae* responses to human emanations and synthetic odour blends augmented with heat

Augmentation of the standard blend, foot odours and blend 1 with heat at 24.9°C and 25.5°C did not enhance attractiveness of these behavioural stimuli to *An. gambiae* mosquitoes. When tested against each other, more mosquitoes responded to person LA than to the standard blend ($P < 0.001$) augmented with heat (24.9°C). Person HA on the other hand attracted a significantly higher proportion of mosquitoes when compared to the standard blend ($P < 0.001$) augmented with heat (25.5°C) (Table 3).

There were no significant differences between the number of mosquitoes attracted to person LA and his foot odours ($P = 0.650$) augmented with heat (24.9°C). On the other hand, significantly more mosquitoes were attracted to person HA in comparison to his foot odours ($P < 0.001$) augmented with heat (25.5°C) (Table 3).

The augmentation of blend 1 (eight carboxylic acids, ammonia, and distilled water) with heat did not increase its attractiveness to *An. gambiae* mosquitoes. Person LA attracted a higher proportion of mosquitoes compared to blend 1 ($P = 0.016$) augmented with heat (24.9°C). Similarly, there was a significantly higher number of mosquitoes attracted to person HA when blend 1 ($P < 0.001$) augmented with heat (25.5°C) was the alternative choice (Table 3).

Table 3: Responses of *Anopheles gambiae* to human emanations versus synthetic odour blends and foot odours augmented with heat.

Behavioural stimuli		N	Replicates	Mosquito responses		P value	Total responses (%)			
Person	Treatment			Person	Treatment		c	t	Net attraction	Non-responsive (%)
LA	Standard Blend + Heat	800	8	97	27	0.001	54	16	22	30
LA	Blend 1 + Heat	800	8	65	40	0.016	50	13	21	37
LA	Foot odours + Heat	800	8	41	37	0.650	62	9	13	29
HA	Standard Blend + Heat	800	8	214	0	0.001	55	26	33	12
HA	Blend 1 + Heat	800	8	173	1	0.001	49	22	31	29
HA	Foot odours + Heat	800	8	107	3	0.001	61	14	19	25

4.4 Comparison of female *An. gambiae* responses to human emanations and synthetic odour blends augmented with moisture

There was a reduction in the number of mosquitoes attracted to the standard blend, foot odours and blend 1 when these were augmented with moisture. The tent in which person LA slept attracted significantly more mosquitoes than the tent baited with the standard blend ($P < 0.001$) augmented with moisture (75%). Similarly, mosquitoes showed an absolute preference for person HA over the standard blend ($P < 0.001$) augmented with moisture (80%) (Table 4).

Significant differences were found between the number of mosquitoes attracted to person LA and his foot odours ($P < 0.001$) augmented with moisture (75%). Person HA attracted a significantly higher proportion of mosquitoes compared to his foot odours ($P < 0.001$) augmented with moisture (80%) (Table 4).

Relative to blend 1 augmented with moisture (75%), person LA ($P<0.001$) was significantly attractive to *An. gambiae*. No mosquitoes responded to blend 1 augmented with moisture (80%) when tested against person HA ($P < 0.001$). The results indicate that moisture as a physical cue at 75% and 80% does not enhance attractiveness of odour baits (Table 4).

Table 4: Proportions of *Anopheles gambiae* mosquitoes attracted to humans compared to odours of synthetic and natural origin augmented with moisture.

Behavioural stimuli		N	Replicates	Mosquito responses		P value	Total responses (%)			
Person	Treatment			Person	Treatment		c	t	Net attraction	Non-responsive (%)
LA	Standard Blend + Moisture	800	8	228	1	0.001	54	27	34	19
LA	Blend 1 + Moisture	800	8	137	2	0.001	61	17	22	22
LA	Foot odours + Moisture	800	8	102	2	0.001	61	13	18	26
HA	Standard Blend + Moisture	800	8	171	0	0.001	55	21	28	24
HA	Blend 1 + Moisture	800	8	153	0	0.001	61	19	24	20
HA	Foot odours + Moisture	800	8	152	1	0.001	50	19	28	31

4.5 Comparison of female *An. gambiae* responses to human emanations and synthetic odour blends augmented with heat and moisture

The attractiveness of the standard blend, foot odours and blend 1 did not increase with the addition of both heat and moisture. These results revealed that the mosquitoes made an absolute choice of person LA when compared to the standard blend ($P < 0.001$) augmented with both heat (24.9^{o}C) and moisture (75%). Likewise, a high number of mosquitoes responded to person HA when tested against the standard blend ($P < 0.001$) augmented with heat (25.5^{o}C) and moisture (80%) (Table 5).

When competed against each other, person LA attracted a higher proportion of mosquitoes unlike his foot odours ($P < 0.001$) augmented with both heat and (24.9^{o}C) and moisture (75%). Similar results were obtained when person HA was compared to his foot odours ($P < 0.001$) augmented with both heat (25.5^{o}C) and moisture (80%) (Table 5).

No significant differences were found between the number of mosquitoes attracted to person LA and blend 1 ($P = 0.416$) augmented with heat (24.9^{o}C) and moisture (75%). In contrast, person HA attracted a significantly higher proportion of mosquitoes when compared to blend 1 ($P < 0.001$) augmented with heat (25.5^{o}C) and moisture (80%) (Table 5).

Table 5: Attraction of *Anopheles gambiae* to humans versus odours of synthetic and natural origin augmented with heat plus moisture.

Behavioural stimuli		N	Replicates	Mosquito responses		P value	Total responses (%)			
Person	Treatment			Person	Treatment		c	t	Net attraction	Non-responsive (%)
LA	Standard Blend + Heat + Moisture	800	8	100	0	0.001	55	13	19	32
LA	Blend 1 + Heat + Moisture	800	8	10	14	0.416	76	3	4	21
LA	Foot odours + Heat + Moisture	800	8	55	13	0.001	68	9	11	23
HA	Standard Blend + Heat + Moisture	800	8	84	1	0.001	74	11	13	15
HA	Blend 1 + Heat + Moisture	800	8	168	1	0.001	63	21	25	16
HA	Foot odours + Heat + Moisture	800	8	48	0	0.001	73	6	8	21

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Experiments to calibrate the dual-port olfactometer used in this research work have shown that there was no bias in the response of *An. gambiae* mosquitoes to the arms of the dual-port olfactometer. No mosquitoes were attracted to any of the tents when both were empty. The fact that there were no differences in the proportion of mosquitoes trapped when the two tents were baited with the standard blend (consisting of ammonia, distilled water and carbon dioxide), confirms that the mosquito behavioural responses were symmetrical. Results from this work showed that there were differences in relative attractiveness between the two human subjects. This is in conformity with previous studies where a similar discriminatory system was used (Mukabana *et al*., 2002, 2004; Lacroix *et al*., 2006) to study behavioural responses of *An. gambiae* to human beings in Western Kenya.

The importance of chemical cues in mediating the host-seeking behaviour of *An. gambiae* at the short range of attraction under semi-field situations was demonstrated in this study. This was supported by the high proportion of mosquitoes attracted to the volunteers, one less and the other highly attractive to *An. gambiae*, when compared to the empty tents or the standard blend. *Anopheles gambiae* prefers to feed on humans rather than other animal hosts (White, 1974; Gillies and Coetzee, 1987; Pates *et al*., 2001). Relatively more mosquitoes were attracted by the human subjects. These findings support earlier reports in which Mboera *et al.* (1997) reported *An. gambiae* preference for human odour over carbon dioxide in tents. Failure of the standard blend (carbon dioxide (released at 500 ml/min), ammonia (2.5%) and distilled water) to attract more or as many mosquitoes as a human may be attributed to three reasons. Firstly, its limited chemical composition relative to the large diversity of compounds emanating from humans. Studies have identified approximately 350 chemical compounds in human volatiles (Krotozynski *et al*., 1977; Bernier *et al*., 2000). Secondly, the blend lacks constituents active in the short range phase of attraction as limited by the experimental setup. Lastly, it lacks compounds distinctive and specific of human odour (Stoddart, 1990). These reasons might explain why the total proportion of mosquitoes attracted was low when the volunteers were compared with the standard blend and high when they were compared to their foot odours.

When compared with Blend 1 or his foot odours, mosquitoes strongly responded to the highly attractive person. On the other hand, the proportion of mosquitoes attracted to the less attractive person versus blend 1 or his foot odours did not differ. This implied that the less attractive person, his foot odours and Blend 1 are similar in attractiveness to *An. gambiae* mosquitoes. This shows the potential of Blend 1 as a mosquito attractant that can be used to lure adult *An. gambiae* mosquitoes. Majority of the components of Blend 1 have been shown to be highly attractive to mosquitoes under laboratory (Knols *et al*., 1997; Braks *et al.*, 2001; Smallegange *et al*., 2005) and semi-field conditions (Njiru *et al.*, 2006).

There was no statistical difference between the numbers of mosquitoes attracted to the least attractive person versus his foot odours (P = 0.163). This result indicates that his degree of attractiveness to mosquitoes is dictated by foot odours. Human foot odour has been reported to be attractive to *An. gambiae* (Njiru *et al*., 2006; Schmied *et al*., 2008) under semi-field conditions. Further studies have also shown that this mosquito species has a distinct preference for biting human legs and feet (De Jong and Knols, 1995; Dekker *et al*., 1998). Total body emanations of the highly attractive person attracted significantly more mosquitoes than his foot odours (P = 0.001). This finding suggests that attractive odours abound in other parts of his body and supports findings by Qiu *et al*. (2006) and Braks *et al*. (1997) who reported attraction of *An. gambiae* to material obtained from body parts other than feet specifically hands, forehead and trunk region.

In this study, experiments on the effect of heat and moisture on the response of *An. gambiae* mosquitoes to synthetic odour blends have shown that, heat and moisture do not significantly affect attraction of this mosquito to synthetic odour blends. This was supported by high attraction of mosquitoes to the highly attractive individual (≥97%) than the contrasting behavioural stimuli (standard blend, Blend 1 or his foot odours) augmented with heat or moisture (P = 0.001). The highly attractive person also attracted far more mosquitoes than contrasting behavioural stimuli augmented with both heat and moisture. These observations revealed that the highly attractive individual had perfect combinations of both physical and chemical cues. These two cues have been reported by Sutcliffe (1987), Takken (1991) and Takken and Knols (1999), to be responsible for attraction of host-seeking mosquitoes to humans.

Augmenting Blend 1 with heat or moisture did not increase its attractiveness over the less attractive individual. Adding both heat and moisture to Blend 1 and comparing it to the less attractive individual rendered it equally attractive to *An. gambiae*. These results were consistent with earlier works of Bar-Zeev *et al*., (1977) who demonstrated that heat and moisture have a synergistic effect on the attraction of mosquitoes to odour baits. Other studies have also confirmed that human skin odour and relative humidity interact in the host-seeking process of *An. gambiae* (Takken *et al*., 1997).

The less attractive individual remained as attractive as his foot odours augmented with heat. Previous studies by Khan and Maibach (1966) and Khan *et al*. (1966) suggested that heat acts synergistically with odour baits increasing their attraction to mosquitoes. Kline and Lemire (1995) reported that addition of heat to odour baits increased catches of mosquitoes in the field. The less attractive individual was more attractive against his foot odours augmented either with moisture alone or with heat plus moisture. The attractiveness of an odour bait augmented with heat can be reduced by the presence of moisture (Bar-Zeev *et al*., 1977). The result of the less attractive individual attracting more mosquitoes than his foot odours augmented with both heat and moisture was in accordance with the findings of a study reported by Bar-Zeev *et al*. (1977). These authors reported that the presence of moisture can reduce the attraction of mosquitoes to an odour bait. Mosquitoes used in this study were starved for eight hours during which period they were kept hydrated using water on cotton wicks. According to Bar-Zeev *et al*., (1977), water-satiated mosquitoes, unlike thirsty counterparts, are known to avoid environments with high relative humidity. This potentially explains why the volunteers consistently attracted more mosquitoes over odour baits augmented with moisture.

Heat and moisture did not significantly increase attraction of mosquitoes to the candidate odour baits. This could be attributed to temperature and relative humidity fluctuations specifically 25-27^{o}C and 75-85%, respectively that were recorded during the experiments. Small differences in the levels of these parameters can be associated with major differences in mosquito behavioural responses (Smart and Brown, 1955). This could have caused the failure of both heat and moisture to significantly increase attraction of mosquitoes to the contrasting behavioural stimuli. *Anopheles gambiae*, being highly anthropophlic (White, 1974; Gillies and Coetzee, 1987; Pates *et al*., 2001), has evolved to respond to human-specific rather than generalist cues. Ideally, heat and moisture, being host-unspecific, should, in interaction with other host-specific cues, at short range (Takken *et al*., 1997; Costantini *et al*., 1999), be indicative of the physical presence of hosts.

Of the three sources of behavioural stimuli tested, Blend 1 emerged as the most potent mosquito attractant. The proportion of mosquitoes attracted to it equalled those attracted to the less attractive human subject when the blend was used on its own or when it was augmented with heat plus moisture. Except in one case reported by Murphy *et al*. (2001), the key components of Blend 1 have been shown by Knols *et al*. (1997) and Smallegange *et al*. (2005) to be highly attractive to mosquitoes under laboratory conditions. Studies conducted in Tanzania using components of Blend 1 have found a similar trend in both field and semi-field conditions (Okumu *et al*., 2010). Evidence from field studies has shown that *An. gambiae* is strongly attracted to natural human odours (Costantini *et al*., 1996; Mboera *et al*., 1997), and from this study the development of traps baited with highly attractive synthetic blends simulating human odours appears realistic.

5.2 Conclusions

1. Human emanations, Blend 1 (consisting of eight carboxylic acids, ammonia, distilled water and carbon dioxide) and foot odours are attractive to *An. gambiae* mosquitoes signifying that chemical cues present important signals mediating *An. gambiae* host-seeking behaviour.
2. Blend 1 emerged as the most potent attractant of *An. gambiae* out of the three behavioural stimuli tested.
3. Heat or moisture or both did not significantly influence the relative attractiveness of synthetic odours and foot odours to *An. gambiae* mosquitoes.

5.3 Recommendations

1. One human volunteer of pre-determined average attractiveness should be used as a positive control in studies comparing attractiveness of synthetic odours and human emanations. This is because of the variation in attractiveness of humans to mosquitoes.
2. Studies should be conducted on chemicals that can be added to Blend 1 to increase its attractiveness to *An. gambiae* mosquitoes.
3. Field studies should be carried out using Blend 1 as an odour bait to capture *An. gambiae* mosquitoes.

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