Differential attractiveness of humans to the African malaria vector *Anopheles gambiae* Giles

Effects of host characteristics and parasite infection

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Proefschrift
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Differential attractiveness of humans to the African malaria vector *Anopheles gambiae* Giles: Effects of host characteristics and parasite infection

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Propositions[1]

1. Total body emanations, breath components in particular (but not carbon dioxide *per se*), are responsible for causing variability in human attractiveness to the African malaria vector *Anopheles gambiae* Giles *sensu stricto*.

   This thesis.

   Brady, J., Costantini, C., Sagnon, N., Gibson, G. & Coluzzi, M., 1997, *Annals of Tropical Medicine & Parasitology*, **91** (Suppl. 1), 121-122.

2. Although mosquitoes have an intrinsic ability to locate their blood meal hosts, *Plasmodium falciparum* is capable of mediating the attraction of *Anopheles gambiae* to humans.

   This thesis.

3. Historically successful mosquito vector control methods, such as environmental management and systematic larviciding, are a better option than adult control measures against African malaria vectors.

   Killeen, G.F., Fillinger, U., Kiche, I., Gouagna, L.C. & Knols, B.G.J., 2002, *The Lancet Infectious Diseases* **2**, 618 - 627.

4. Mosquito-proofing of houses should be a central plank of malaria control activities in the tropics yet it is regrettably not part of the Roll Back Malaria (RBM) initiative.

   Lindsay, S.W., Emerson, P.M. & Charlwood, J.D., 2002, *Trends in Parasitology* **18**, 510 - 514.

   WHO, RBM infosheet No. 2, March 2002.

5. Aid given to developing countries cannot remain channelled to its specific aims without food security among target communities.

6. If the world's natural resources can be channelled to a central pool from which all countries can draw equally, human conflicts could possibly be reduced and the much desired goal of health for all achieved.

7. Communication and interaction between people is influenced more by what one person thinks that the other thinks of them rather than what the person really thinks of the other.

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[1] Propositions accompanying the Ph.D. thesis "Differential attractiveness of humans to the African malaria vector *Anopheles gambiae* Giles: Effects of host characteristics and parasite infection" [W.R. Mukabana, 2002].

Table of contents

Preface

The events which led to the beginning of the work reported herein unfolded in a miraculous fashion leading me out of eminent trouble. During that time I had turned down a Ph.D. position offered at the Kenya Medical Research Institute (KEMRI) because the scientist who was to be my supervisor would leave soon. I took up the job of graduate assistant at the University of Nairobi instead. It was not clear to me that the new job was a non-renewable one year contract that entailed showing evidence of registration as a Ph.D. candidate before upgrading to tutorial fellow, the next step in a staff development process. This realisation is what sowed the first seed of the research activities reported in this thesis.

With this background information, I consulted Dr. Willem Takken, a specialist in the new subject area in which I had become interested. Together, we agreed to draft a research proposal seeking for funds from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). Being far away, Willem asked his former Ph.D. student Dr. Bart G.J. Knols to oversee the proposal development and become the principal investigator. Bart had recently assumed the position of Scientist at the International Centre of Insect Physiology and Ecology (ICIPE) in Kenya. Meanwhile, Willem applied for a Ph.D. fellowship from the executive board of "Wageningen Agricultural University" (WAU) on my behalf. The fellowship was awarded and the research proposal submitted to WHO-TDR concurrently. Thus, on August 19, 1998 I travelled to Wageningen to start the first phase of a sandwich Ph.D. construction. After three months of stay Bart was informed by WHO that the research proposal had been selected for funding. Accordingly, on March 25, 1999, just after I had completed phase one of the sandwich Ph.D., I moved to ICIPE - Mbita Point Research and Training Centre (MPR&TC) as a Dissertation Research Internship Programme (DRIP) scholar, to start the research phase of the studies. I worked at MPR&TC until April 28, 2002, after which I returned to 'Wageningen University and Research Centre' (WURC) to compile this thesis.

When I first arrived at WAU I felt as though the tissue matrix that hold my heart in place had become loose. I doubted that I would be able to produce a book such as the one that you are now reading, especially so as I read those that had been written by former students. Bart and Willem, this became possible through your efforts. Accept my sincere gratitude for all the trouble that you took to ensure that I moved to the next step as the events unfolded. Your support throughout the study period is highly appreciated. Willem! Passively but surely, as I worked under your guidance, I imbibed the word 'NO'. Never again did I take what was written by others for gospel truth. According to you nothing was to be taken as being obvious. Your never ending questions such as (1) did you also run the grammar/spelling check?; (2) how would you put it yourself?; (3) but have you seen the work of ...?; (4) how would you explain this difference? etc all did a great

deal to improve my debating skills plus the quality of my work and thinking. For you Bart, I saw more than just a supervisor. On the one hand, you gave me all that I needed for the work and went ahead to support me whenever I sought for that which was necessary yet far for my ability to reach. On the other, you were keen to see that I could independently and comfortably perform that which you taught me, a sign that you cared the quality of the product that you would produce. My earlier impression of you as a caring and committed person was affirmed when you took time to come and work besides me as I wrote parts of this thesis. Further, I will live to remember the 'academic titbits' which we exchanged through e-mail, sometimes up to five times in a day, while I wrote parts of this thesis. Most important, you were very friendly. Receive my earnest appreciation for all these.

Within the list of my mentors was a man known as Prof. Joop van Lenteren. Joop! Allow me to call you *ajuoga* (a word used in the Luo language - of western Kenya - to formally refer to a person with very advanced professional skill yet literally meaning a magician). You and I only met on few occasions. However, when I 'smoothened out' my work ready for you to read and thinking that it would sail through your hands much the same as licks of ice cream sail down the throat, you singled out mistakes instantaneously. Your comments greatly contributed towards putting the research ideas presented in this thesis in a subject-wide context.

A couple of other people were useful along the course of the studies. Foremost was the Irish biologist known as Dr. Gerry F. Killeen. Gerry! I remember with great sorrow and sadness how I did not discover and use the gold that there is in your grey matter while we were together at MPR&TC. Please accept my sincere apologies for this. However, I gained a lot when I finally discovered this after I had returned to WURC. You went out of your way to ensure that I did things in a better way. Together with Richard Coe you did a great deal to improve my ability of understanding statistical methods of data analysis. Behind your backs there was Renate Smallegange who played a role reminiscent of that of a 'spiker' in a game of volley ball - she brought home the missing links in my understanding of statistics and taught me how to use Genstat.

Of the many people at MPR&TC who I remember dearly, miss to be apart with and who are worth acknowledging, I wish to single out Mr. Christian Abuya alias *the pentagon*. Dear Christian, you are the only young man of a kind that I have ever met. Your good sense of humour always made my days and, in fact, dissolved my sensitivity to nicknames. I did not mind anymore when you called me *Luanda Magere* (a legend among the ethnic *Luo* who could only be restrained by attacking his shadow but not the physical self). Readers! Christian Abuya volunteered to participate in two-thirds of the experiments reported in this thesis.

At the Laboratory of Entomology (WURC), B. Koopmanschap, R. Klaarsen, E.J., Scholte, S. Koenraadt, K. Stuke and many more all remembered to pass on the ball in time. Your contributions together with those of colleagues at MPR&TC including E. Mathenge, L. Sumba, B. Oketch, A. Seyoum, B. Njiru, L. Gouagna, U.

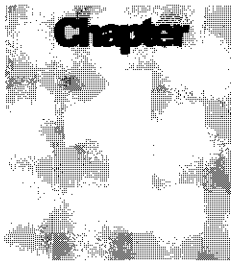
Fillinger and A. Manrakhan provided many finishing opportunities enabling Richard Mukabana, being the centre forward, to score nine goals in the Ph.D thesis league.

I also wish to acknowledge the contributions that were input by various institutions. WURC is thanked for registering, financing, hosting and supervising the Ph.D. studies. ICIPE is thanked for hosting, financing, supervising and providing logical support through its capacity building program. WHO/UNDP/World Bank special programme for Research and Training in Tropical Diseases (TDR) is thanked for financing the studies. The University of Nairobi is thanked for providing pocket money. The Kenya Medical Research Institute (KEMRI) is thanked for providing ethical clearance. The National Institutes of Health (USA) is thanked both for sustaining a large scientific framework in which I performed this work and for funding the installation of a very small aperture terminal (VSAT) that availed e-mail and internet services. The services rendered to me by the MIMCom network have significantly enhanced the speed of access to scientific information and collaboration with colleagues across the globe, in spite of being located in an isolated part of Africa.

More thanks go to my dear parents Dr. John Wesamba Muchere and Mrs. Elizabeth Andawa Muchere. *Papa*! I now see what far a journey you went so as to gain that title - congratulations!!! *Mama*! All these stem from your determination - as a young boy I heard you say that a good education was inevitable for your children.

The last, but not the least, of my acknowledgements go to my dear wife and children. Jacqueline! Thank you for accepting to support it virtually single-handed while I stayed away toiling towards this goal. As for you Sheila, Linda and Emmanuel, let this book be your pace setter. It is merely a miniature of a product that is limited by today's technology but yet the legacy which I leave behind to you. Get your copies from me when you become of age.

For my dear brother Titus N. Muchere whose sight deteriorated to total blindness after high school.



General Introduction

Knowledge on host selection and blood uptake among humans by female mosquitoes is an important element in predicting the transmission dynamics of malaria and other mosquito-borne diseases. In this thesis, molecular-genetic and chemical-ecological tools are used to unravel the principal factors that cause differential attractiveness of humans to mosquitoes. Studies of how host characteristics, particularly olfactory and physical stimuli, but also infection with the malaria parasite *Plasmodium falciparum*, affect the attractiveness of humans to the African malaria vector *Anopheles gambiae* are presented.

1.1. The intricate web of *Anopheles - Homo - Plasmodium* interactions

Malaria in man is caused by four species of protozoan parasites[1] belonging to the genus *Plasmodium*. *Plasmodium* parasites exhibit a complex life cycle involving development through two host[2] species: an insect vector[3] (definitive host[4]) and a vertebrate species (intermediate host[5]). Humans serve as intermediate hosts for several *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) whilst female mosquitoes of the genus *Anopheles* serve as the definitive hosts (Figure 1). *Plasmodium* parasites are transmitted between humans through the periodic blood-feeding behaviour of female *Anopheles* mosquitoes. Thus, humans also serve as hosts for female anophelines which, in turn, constitute (temporary) ectoparasites[6].

The *Plasmodium* parasite initiates its sexual cycle of development within a freshly ingested blood meal present inside the mosquito midgut, when male and female gametocytes shed their erythrocyte membranes. Exflagellation, fertilisation and ookinete formation take place thereafter. Motile ookinetes penetrate the midgut wall and peritrophic membrane, come to lie under the basal lamina and oocysts are formed. Sporozoites develop within the oocyst, mature, and upon rupturing of the oocyst, are released into the haemocoel and invade the salivary

[1]Parasite: an organism that lives in or on another organism of different species from which it derives nutrients or shelter. [2]Host: an organism on or in which a parasite lives. [3]Vector: an agent, such as an insect, capable of mechanically or biologically transferring a pathogen from one organism to another. [4]Definitive host: the host in which a parasite reproduces sexually. [5]Intermediate host: the host in which a parasite multiplies asexually. [6]Ectoparasite: a parasite that lives exterior to its host.

glands. Saliva containing sporozoites is injected into the blood stream of humans by the female mosquito when she feeds next (Figure 1). The development in mosquitoes from gametocytes to sporozoites generally depends on ambient temperature but generally lasts between 10 and 20 days (Gilles, 1993), during which period the mosquito continues to blood-feed and reproduce.

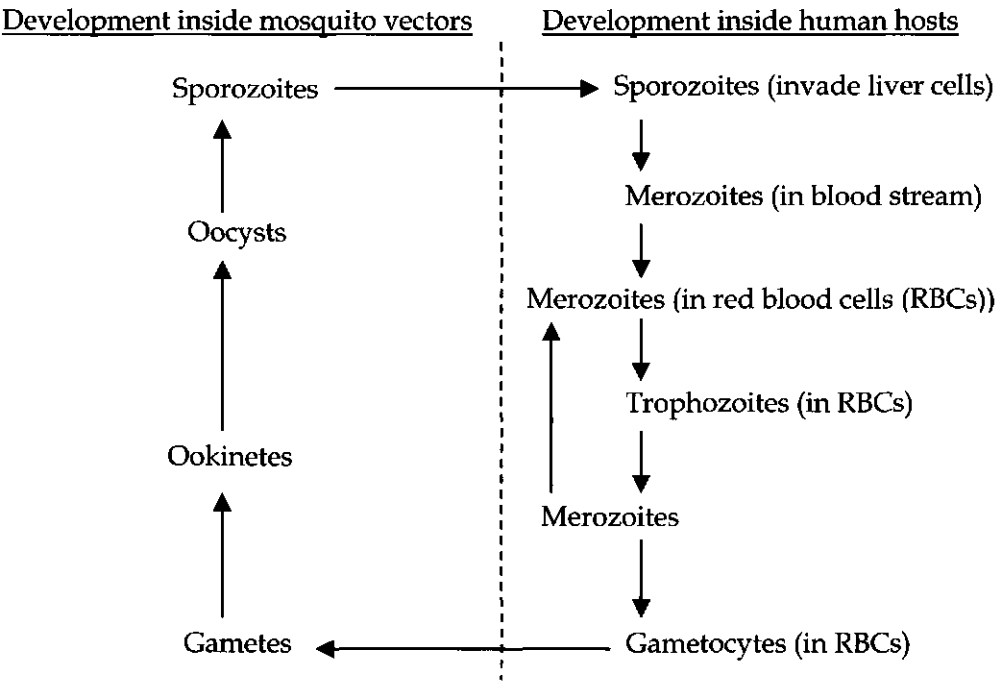


Figure 1. *Schematic representation of the malaria parasite's life cycle showing stages of development within mosquito and human hosts.*

Injected sporozoites enter liver cells (hepatocytes) where they reproduce asexually and cause the cells to burst (exoerythrocytic schizogony) and release merozoites into the blood stream. Merozoites invade red blood cells (RBCs) where they multiply and grow into trophozoites. The infected RBCs burst (erythrocytic schyzogony) releasing more merozoites. The new merozoites re-invade RBCs and the cycle is continued. This process destroys significant numbers of RBCs and causes the paroxysms ('chills and fever') characteristic of malaria infections. Some merozoites differentiate into male and female gametocytes, which are ingested by female *Anopheles* mosquitoes as they take blood meals. Development within the mosquito (sporogonic phase) starts and the cycle carries on (Figure 1). The process leading to the acquisition of a blood meal from humans is complex and is actually an amalgamation of responses to internal and external stimuli in a series of behaviours broadly referred to as host seeking.

1.2. Mosquito host-seeking behaviour

The process of host seeking in mosquitoes and other haematophagous insects is a complex integration of a sequence of responses to host-derived stimuli (Sutcliffe, 1987). The typical pattern of responses is as follows: activation, long-range attraction, short-range attraction, landing, arrestment, probing, biting and engorgement. These series of behaviours are not fixed and are controlled by different sensory stimuli that represent the host to the host-seeking insect (Figure 2). At long distances the process is mediated by olfactory cues whereas visual and physical cues are important in the close vicinity of the host (Gillies & Wilkes, 1969). Long distance olfactory information includes volatile chemicals of the skin and breath produced by the potential host (Takken, 1991). Carbon dioxide is active at medium range and body heat and moisture at short-range (Gillies & Wilkes, 1969). Deriving from this hierarchy of attractive stimuli, and using the distance ranges reported by Knols & Meijerink (1997), it can be hypothesised that olfactory cues, carbon dioxide and physical factors influence variability in human attractiveness to mosquitoes when individuals are located at distances > 20 metres, between 2 and 20 metres and at 0 to 2 metres, respectively.

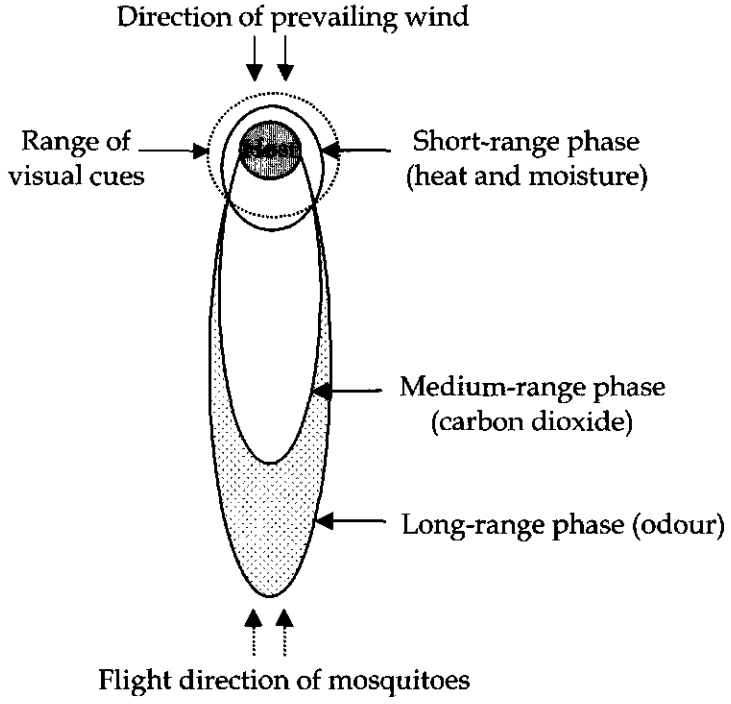


Figure 2. *Sketch showing how different host stimuli are presented to approaching mosquitoes. The host and the direction of movement of mosquitoes and wind are shown (redrawn after Gillies & Wilkes, 1969 and modified after Costantini et al., 1999).*

Certainly, the lack of an apparent relationship between an individuals' attractiveness as measured by the number of mosquitoes entering houses and by the number feeding implies that different host factors are responsible for house entry and blood feeding (Lindsay *et al.*, 1993). Laboratory studies indicate that odour (from the human palm) is responsible for "long distance" attraction of *Aedes aegypti* (Khan *et al.*, 1966). Also, results from field studies suggest that human odour influences long-range attraction of *An. gambiae s.l.*, *An. funestus* and *Culex quinquefasciatus* (Knols *et al.*, 1995). Evidence of the orientation effects of carbon dioxide in the medium-range phase of host seeking has recently been strengthened by studies that evaluated the variability in human attractiveness to *Simulium* species (Schofield & Sutcliffe, 1996) and *An. gambiae* and *An. funestus* (Brady *et al.*, 1997). However, the role of carbon dioxide is thought to be one of activation, entailing eliciting take-off and sustaining flight (Gillies, 1980; Dekker *et al.*, 2001). Physical cues e.g. body heat and moisture are active in the vicinity of the host (Laarman, 1955; Smart & Brown, 1955; Schofield & Sutcliffe, 1997). Despite that chemical and physical factors have been shown to affect mosquito host-seeking behaviour, their effects on differential attractiveness of humans to mosquitoes are not yet well understood.

1.3. Factors causing differential attractiveness of humans to mosquitoes

It has frequently been reported that humans differ in their degree of attractiveness to mosquitoes. This type of behaviour is thought to result from many different factors (Burkot, 1988), yet the principal causes that make certain individuals to be more preferred than others are not well understood. Host age, size, sex, race, pregnancy status, blood group status, parasite infection status and body odour constitute the factors that have been investigated as possible causes of differential mosquito attack rates.

1.3.1. Age

A preference for *An. gambiae* to feed on adults rather than children is known (Thomas, 1951; Carnevale *et al.*, 1978; Boreham *et al.*, 1978; Bryan & Smalley, 1978; Port *et al.*, 1980). This seems also to be the case with *An. farauti* (Spencer, 1967), *An. albimanus*, *An. aquasalis* and *An. bellator* (Muirhead-Thomson, 1951). In contrast, random feeding irrespective of age has been reported for *An. gambiae s.l.* (Smith, 1956) and *An. funestus* (Clyde & Shute, 1958) and the preference for feeding on some children rather than adults has been demonstrated among members of the *An. punctulatus* complex (Burkot *et al.*, 1988). Although the proportion of feeds upon an individual in a group can be associated with the proportion of the total surface area or weight of the group contributed by that individual, so rendering adults to be preferred more than children (Port *et al.*, 1980), the discrepancies noted here suggest that variability in human attractiveness to host-seeking mosquitoes is influenced by additional factors.

1.3.2. Sex and race

Very little is known about the effect of sex and race on the attractiveness of humans to host-seeking mosquitoes. *An. gambiae s.l.* and *An. funestus* have been shown to prefer biting men rather than women (Clyde & Shute, 1958) as has been reported for *Ae. aegypti* (Gilbert *et al.*, 1966; Rahm, 1956). On the other hand, although the hands of Africans were shown to be significantly more attractive to *Ae. aegypti* than those of Asians, and both were significantly more attractive than those of Caucasians, these differences were caused by skin hue but not race (Smart & Brown, 1955). Dark-skinned Caucasians were significantly more attractive to the mosquitoes than light-skinned counterparts. Recent studies have found no racial differences in the attraction of *Ae. aegypti* and *An. quadrimaculatus* to substances obtained from the skin of humans (Schreck *et al.*, 1990).

1.3.3. Diet

The effect of diet on attraction of mosquitoes to humans is largely mythical. Only one recent study has reported increased landing rates of proboscis-amputated *Ae. albopictus* mosquitoes on the skin of individuals who had ingested alcohol (Shirai *et al.*, 2002). This phenomenon was not attributed to skin temperature or carbon dioxide output rates.

1.3.4. Pregnancy status

Pregnant women have a unique susceptibility to malaria infection as opposed to non-pregnant counterparts. This is not so much because of the immunosuppression of pregnancy (Weinberg, 1984), but the preferential adherence of a sub-population of malaria parasites on placental trophoblastic villi (Fried & Duffy, 1996). This coupled to the deleterious effects of malaria in pregnancy [e.g. still births, low-birth weight babies, premature delivery and severe maternal anaemia (McGregor, 1984; Menendez, 1995)] and the increased vulnerability of pregnant women to malaria caused by *P. falciparum* (Espinosa *et al.*, 2000), has raised concern as to whether malaria mosquitoes feed on pregnant women preferentially. Indeed, recent evidence suggests that pregnant women are more attractive to host-seeking *An. gambiae*, the world's most efficient malaria vector, than non-pregnant counterparts (Lindsay *et al.*, 2000; Ansell *et al.*, 2002). This preference has been attributed to the physiological (i.e. increased volume of exhaled breath and elevated skin temperature) and behavioural changes taking place during pregnancy (Lindsay *et al.*, 2000).

1.3.5. Blood group status

It was initially thought that the mosquitoes *An. gambiae* (Wood *et al.*, 1972; Wood, 1974) and *Ae. aegypti* (Wood, 1976) selected their preferred human hosts in accordance to ABO blood group status, O blood group individuals being fed upon preferentially. However, later studies showed decisively that ABO blood group status had no effect on host choice (Thornton *et al.*, 1976).

1.3.6. Parasite infection status

Since mosquito host seeking is an odour-mediated process (Takken, 1991; Takken & Knols, 1999) and disease in humans can result in changes in body odour (Penn & Potts, 1998) it is possible that parasitic infections can affect the process of host selection by mosquitoes. Rodent malaria models have shown that mosquito engorgement success is higher on ailing hosts as these are more tolerant to bites (Day *et al.*, 1983; Day & Edman, 1983; 1984). Interestingly, host tolerance, mosquito engorgement success and peak gametocyte infectivity were also shown to be correlated (Day & Edman, 1983), a phenomenon that predicts doom in terms of the transmission of human malaria. Uninfected rodents were seen to display extensive anti-mosquito behaviour that either eliminated or markedly reduced mosquito engorgement success (Day *et al.*, 1983; Day & Edman, 1983; 1984). Host-defensive behaviour usually results in interrupted feeding which can, in turn, have adverse consequences in terms of disease transmission, parasite evolution and vector ecology (Davies, 1990). Fortunately for the mosquito, the biting cycle of many anophelines precludes active defensive behaviour of their human hosts by attacking while the hosts are asleep, so probably equally tolerant to attacks (Charlwood *et al.*, 1995). This might be the reason why studies based on blood meal analysis have found no preferential selection of malaria- or *Wuchereria bancrofti*-infected individuals against un-infected counterparts by *An. punctulatus* complex mosquitoes (Burkot *et al.*, 1989).

However, host defence may not be enough reason to explain the differences in feeding success observed between infected and uninfected hosts. Studies in which volunteers collected *Simulium* species as they landed to bite found no preference for biting *Onchocerca volvulus*-infected or uninfected individuals (Kruppa & Burchard, 1999). Recent evidence suggests that parasite infection affects the host-selection behaviour of blood-foraging insects through olfactory stimulation (O'Shea, 2002). Furthermore, since phlebotomines prefer to bite on *Leishmania*-infected sites of animals (Coleman & Edman, 1988), other factors might be responsible for the increased attractiveness of infected individuals.

1.3.7. Odour

There is ample evidence to suggest that odour influences the choice of a particular individual by host-seeking insects. The sandfly *Lutzomyia longipalpis* is attracted to hand odour of different humans at significantly different rates (Hamilton & Ramsoondar, 1994) and attraction of *Simulium* species to total human emanations varies depending on the individual source (Schofield & Sutcliffe, 1996). Attractiveness of human arm and hand odour to *An. stephensi* (Brouwer, 1960), *Ae. aegypti* (Khan *et al.*, 1966; Mayer & James, 1969) and both *Ae. aegypti* and *An. quadrimaculatus* (Schreck *et al.*, 1990) has been shown to vary between individuals. The response of *An. gambiae* to total human emanations (Chapter 4), including those from which the body heat component has been excluded (Costantini *et al.*, 1993) also varies between individuals (Brady *et al.*, 1997; Costantini *et al.*, 1999).

Differences in the attractiveness of humans to haematophagous insects are thought to be breath-induced as removal of exhaled air from total emanations (Schofield & Sutcliffe, 1996) or artificially standardising outputs of carbon dioxide (Brady *et al*, 1997), a major constituent of breath, has been seen to eliminate the differential attraction of humans to black flies and mosquitoes, respectively.

1.3.8. Epidemiology and host selection

The relationship between host characteristics and their resultant attractiveness to infectious, host-seeking mosquitoes is an important prerequisite for understanding the epidemiology of malaria transmission. However, sometimes mosquitoes prefer to bite certain individuals for reasons that are unrelated to the presence or absence of malaria. For example, villagers living close to a mosquito breeding site may be exposed to biting rates which are relatively high for the village (Smith *et al.*, 1995; Thompson *et al.*, 1997). Such non-homogeneous selection tends to increase the basic reproductive rate of the disease either with an increase or decrease in disease prevalence rates depending on the degree of contact between susceptible and infective individuals (Dye & Hasibeder, 1986).

The time needed for development and the duration of circulation of infective gametocytes in human hosts has an important impact on the proportion of biting anophelines that become infected. Clearly, if mosquitoes prefer to feed on infectious people, survive long enough for the parasite to develop to its infective stage, and bite susceptible (but uninfected) people for their subsequent meals, transmission rates will be high, and vice versa (Burkot, 1988). Therefore, even if gametocytaemia can increase the attractiveness of humans to anophelines (in a manner similar to that in rodent malaria models), its impact on disease prevalence will depend on the degree of contact between susceptible mosquitoes and infective humans. However, transmission efficiency may be modified by the amount of blood ingested, prevalence of male and female gametocytes, presence of transmission-blocking antibodies (Ponnudurai *et al.*, 1987), availability and usage of antimalarials, and the degree of multiple feeding. A significant degree of multiple feeding can enhance the chances of a mosquito acquiring or transmitting parasites (Koella *et al.*, 1998).

Infection of mosquito salivary glands by sporozoites is thought to induce lesions which impair the mosquitoes' ability to locate blood (Rossignol *et al.*, 1984), thus resulting in increased biting (Rossignol *et al.*, 1986), probing (Wekesa *et al.*, 1992) and multiple feeding rates (Koella *et al.*, 1998). These behaviours increase the risk of parasite acquisition and/or transmission (Davies, 1990). However, because parasite infection reduces mosquito fecundity (Rossignol *et al.*, 1986; Hogg and Hurd, 1995), this risk may be partly countered. Furthermore, as probing and injection of saliva do not necessarily result in the deposition of sporozoites (Ponnudurai *et al.*, 1991) or ingestion of blood (Ribeiro, 1987), models that predict the parasites' ability to manipulate their hosts so as to enhance their own transmission (Ribeiro *et al.*, 1985) should be interpreted with caution.

Mosquitoes with low sporozoite loads might account, in part, for the discrepancy in malaria inoculation rates estimated parasitologically and entomologically. Mosquitoes may have low infections either because of the loss of sporozoites over time and repeated feedings or transmission-blocking immunity in hosts that might render none or only few gametocytes available for infecting mosquito vectors. Discrepancies may also arise from the presence of degenerate sporozoites, inaccuracy in measuring anopheline attack rates, and failure to appreciate the importance of non-homogenous host selection by the anophelines. Measurements of the effect of host characteristics and parasite infection on biting heterogeneity are therefore useful in assessing the effectiveness of disease transmission. For example, although children under 10 years of age are thought to represent the main reservoir of malarial infection to mosquitoes (Githeko *et al.*, 1992), high transmission rates may result if adults are fed upon preferentially, particularly in hypoendemic settings (Muirhead-Thomson, 1998).

Much as knowledge on the epidemiology of host selection between humans needs to be addressed urgently, progress has partly been hindered by the limited availability and distribution of appropriate tools for doing so. Furthermore, the apparent absence of an efficient and standard method of sampling mosquitoes has meant that results cannot be compared properly.

1.4. Methods for measuring differential attractiveness of humans to mosquitoes

In some mosquitoes, there is a strong, innate preference for feeding on humans. The few African species that exhibit this so-called anthropophilic behaviour e.g. *An. gambiae s.s.*, *An. funestus*, *Cx. quinquefasciatus* and *Ae. aegypti* are all important vectors of parasitic diseases. Henceforth, in this thesis, host preference will be discussed from the perspective of host selection at the intraspecific (i.e. human) level. As discussed above, well-known differences in attractiveness of humans to mosquitoes have been reported. Perhaps host-seeking mosquitoes are able to express host preferences at any stage of the host-seeking process. The ability to demonstrate the existence of between-person differences in relative attractiveness using sampling methods that collect insects while they are at different stages of the host-seeking process attests to this (Costantini *et al.*, 1999). What is more, host seeking costs time and energy and can be risky, especially when done at close time intervals (Davies, 1990), so it is likely that natural selection promotes early expression of host preferences. The methods that have been used to measure mosquito host preferences among humans are outlined below. Their suitability in executing this aim is further debated upon in the discussion and conclusions section.

1.4.1. Catches off bait

This method collects mosquitoes while they land to bite or while they are in the process of biting a human or animal host (WHO, 1975). This is referred to as the man landing catch (MLC) when humans pose as bait (Figure 3). MLC is carried out in one of two ways: (1) collectors act as bait by sitting and catching mosquitoes off their exposed legs using suction tubes or test tubes or (2) the collectors catch mosquitoes off a volunteer who acts as a bait. The numbers of mosquitoes collected are taken as a measure of the degree of attractiveness of bait persons to the mosquitoes. Much as MLC can be a useful tool in determining differences in human attractiveness to mosquitoes, it is labour intensive and time consuming and hence expensive. It may also be unreliable because (1) the presence and activity of an observer may inadvertently alter the behaviour of the bait and mosquitoes, (2) it is dependent on the volunteers' ability to catch mosquitoes (Lindsay *et al.*, 1993) and (3) the volunteers may not be equally alert during the collections (Rubio-Palis, 1995). In recent years, the method has become ethically unacceptable due to the risk of exposure to mosquito-borne diseases, which have developed resistance against previously effective drugs (Trape, 2001). The latter is the very reason why WHO is becoming reluctant to fund studies using MLC as a sampling tool.

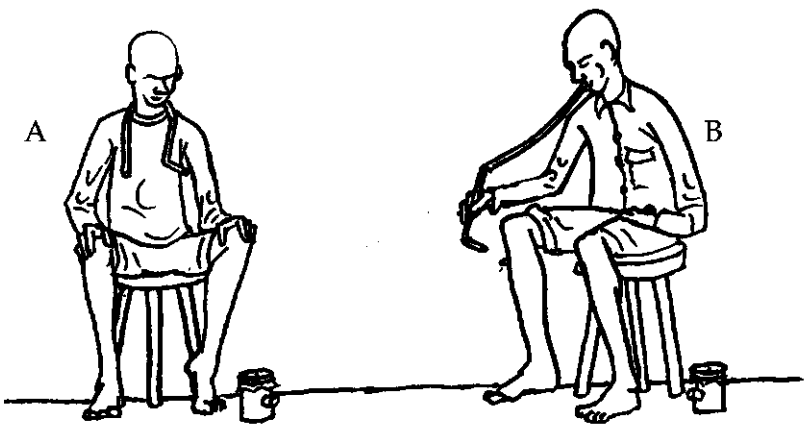


Figure 3. *The man landing catch. Human subjects waiting (A) or collecting (B) mosquitoes off their bare legs (and feet). The number of mosquitoes collected by each individual is taken as a measure of their relative attractiveness (drawing kindly provided by E.J. Scholte).*

1.4.2. Experimental huts, spray catches and resting collections

Host-seeking mosquitoes can also be sampled using specially constructed experimental huts (Figure 4). These resemble the local huts found in disease-endemic areas but may be modified so as to ensure the collection of dead or live mosquitoes by building ant-proof moats around them (WHO, 1975). Mosquitoes are normally collected from such huts using different methods e.g. window traps,

hand collection (with suction tubes) or spray sheet collection (in which indoor resting mosquitoes are collected on white sheets after knock-down spraying with a pyrethrum solution). If experimental huts, built at desirable distances from one another, are occupied by one person each, the number of mosquitoes that enter can be regarded as having been attracted by the sleeper and can be used as a means to measure an individual's attractiveness to mosquitoes (Ribbands, 1950; Lindsay *et al.*, 1993).

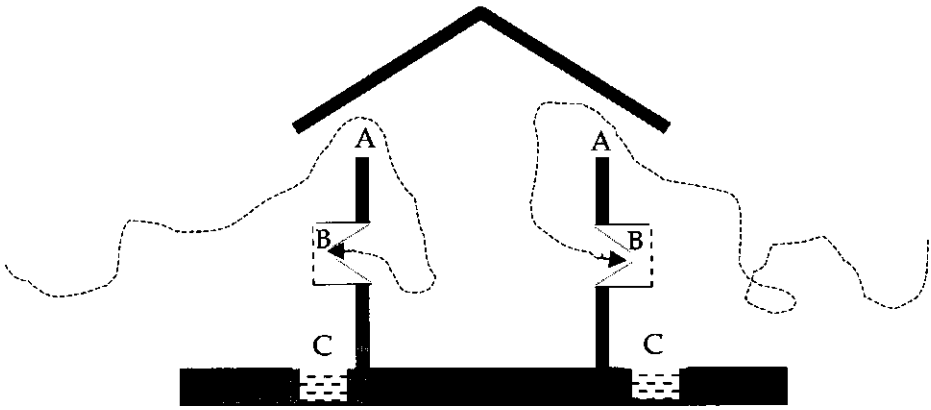


Figure 4. *Sketch of an experimental hut showing eaves-space (A), window (exit) traps (B), moats (C) and mosquito flight paths (redrawn after Gokool* et al., *1992).*

1.4.3. Trap collections and olfaction-based bioassays

Although many trap designs have been developed for sampling mosquitoes (WHO, 1975; Service, 1993), only the bednet trap, or modifications thereof, has been useful in assessing differential attractiveness of humans to host-seeking mosquitoes (Boreham *et al.*, 1978; Port *et al.*, 1980; Ansell *et al.*, 2000; Ansell *et al.*, 2002). Various types of odour-baited entry traps that could be used for similar reasons have recently been developed. One of these is still undergoing field trials (Figure 5) (Mathenge *et al.*, 2002) while two others (Costantini *et al.*, 1993; Knols *et al.*, 1995) have been used in different field set-ups to assess variability in human attractiveness to mosquitoes (Knols *et al.*, 1995; Brady *et al.*, 1997; Costantini *et al.*, 1999). In principle, individual humans occupy separate traps and the number of mosquitoes trapped in response to the odour emanating from the traps compared. Other researchers have studied variability in human attractiveness on a much smaller scale by evaluating mosquito behavioural responses to arm and/or hand odour in laboratory-based olfactometers (Brouwer, 1960; Mayer & James, 1969) or cages (Khan *et al.*, 1966; Maibach *et al.*, 1966; Schreck *et al.*, 1990).

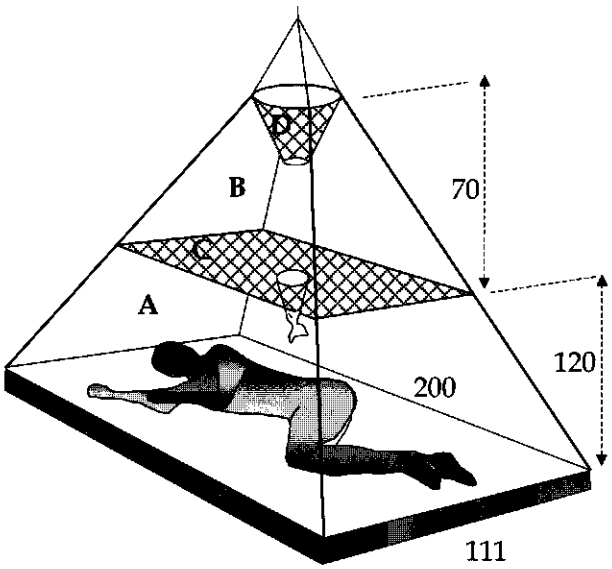


***Figure** 5. Example of an exposure-free bednet trap showing a human subject lying in a bedding compartment (A) separated from a trap chamber (B) with a panel of mosquito net (C). Mosquitoes enter through the conical invagination at the top (D) and are caught in the trap chamber. Dimensions are in centimetres (Mathenge et al., 2002).*

1.4.4. Blood typing

The analysis of blood meals of engorged mosquitoes that are found resting inside human houses has also been used for studying host preferences. Blood typing uses ABO blood groups (Bryan & Smalley, 1978) or serum protein haptoglobins (Hps) (Boreham & Lenahan, 1976; Boreham *et al.*, 1979) as markers for assigning blood meals to potential human host sources. The ABO system relies upon direct or indirect agglutination of A, B and O blood group antigens from mosquito blood meals using anti-A, anti-B or anti-H sera. Haptoglobin typing entails detecting electrophoretic variants of certain serum blood proteins (haptoglobins or Hp) from mosquito blood meals. Three of these proteins Hp 1.1, Hp 1.2 and Hp 2.2 are most common. Phenotypes identified from the blood meals are compared to those of a target population so as to determine the blood meal hosts. Methods based on blood typing are consistent but their reliability is limited by low degrees of polymorphism. Haptoglobin typing is further limited by the fact that some individuals lack Hp genotypes (i.e. they ahaptoglobinaemia with 0.0 Hp types). Furthermore, haptoglobin markers display some inherent inspecificity e.g. the Hp types 1.1 and 2.1 cannot be distinguished from one another if both abound in a single blood meal. Similarly, ABO blood group phenotypes can mask each other as a result of cross-reactions, these are common for blood meals of which digestion has reached an advanced stage.

1.4.5. Differential leukocyte count (or differential blood count)

This method relies on enumerating certain types of white blood cells (leukocytes) (eosinophils, polymorphonucleates and mononucleates) and comparing their numbers in human finger-prick blood samples and mosquito blood meal smears so as to determine host sources of the blood meals. In usual medical practice, 100 leukocytes are counted and classified under the microscope or by electronic apparatus so that the results are expressed as percentages of the total number of leukocytes and absolute numbers per litre of blood. For field purposes it is desirable that (1) finger-prick blood is collected as close as possible to mosquito feeding time and (2) the mosquitoes are sampled subsequent to their optimal feeding time (Clyde & Shute, 1955). This method has only been used in one field study (Clyde & Shute, 1958).

1.4.6. DNA fingerprinting and profiling

Recent advances in molecular genetics have led to the discovery of a large number of DNA-based markers, some of which have been found useful for individual identification in forensic medicine (Gill *et al.*, 1985; Jeffreys, 1992). Most notable are heritable polymorphisms characteristic of loci containing tandem arrays of short, repeating DNA sequences (Bell *et al.*, 1982; Tauz, 1989; Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987; Hearne *et al.*, 1992). Such loci are very informative due to their high degree of polymorphism (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987; Hearne *et al.*, 1992; Edwards *et al.*, 1991). In order to identify insect blood meal sources using such markers, DNA has to be isolated from the blood meals and the genetic patterns generated therefrom matched with those of DNA isolated from tissues (blood, saliva, buccal cells etc) of potential vertebrate hosts (Mukabana *et al.*, 2002).

The suitability of measuring differential attractiveness of humans to host-seeking mosquitoes using the methods listed above varies depending, among other things, on the behaviour of the mosquito species in the locality where sampling is done. Methods based on blood meal analysis are more suitable for use on mosquitoes which feed and rest inside human habitations whereas the rest, notably catches off bait and trap collections, could be used to target both indoor and outdoor feeding/resting mosquitoes. In most African villages *An. gambiae*, which exhibits remarkable indoor resting/feeding behaviours, is the main mosquito species responsible for transmitting malaria throughout the year.

1.5. The *Anopheles gambiae* complex

For a long time mosquitoes of the *An. gambiae* complex (Figure 6) were thought to consist of six morphologically indistinguishable species with five of them transmitting human malaria (Service, 1985). *An. gambiae* [the nominal taxon, also referred to as *An. gambiae sensu stricto* (*s.s.*)] and *An. arabiensis* are the most important vector species in the complex (White, 1974). The occurrence of 80% of

the world's malaria in tropical Africa (WHO, 1993) is due to human biting habits coupled with longevity of these two species (Collins, 1994), plus *An. funestus* (a species not within the *An. gambiae* complex). Distinct chromosomal "forms" of *An. gambiae*, which are strongly associated with specific habitats, exist in West Africa. Three of these so-called ecophenotypes (i.e. BAMAKO, MOPTI, and SAVANNAH) have been found to occur in sympatry at numerous sites (Coluzzi *et al.*, 1979). Two other members of the complex namely *An. melas* in West Africa and *An. merus* in East Africa are localised vectors depending on their levels of contact with people. Of the other sibling species, *An. bwambae* is responsible for localised malaria transmission among the Bambute pygmies of Bwamba in Uganda whereas *An. quadriannulatus* is not a vector. The Ethiopian population of *An. quadriannulatus* was recently recognised as being distinct (from its south African counterpart) and is designated *An. quadriannulatus* species B (Hunt *et al.*, 1998).

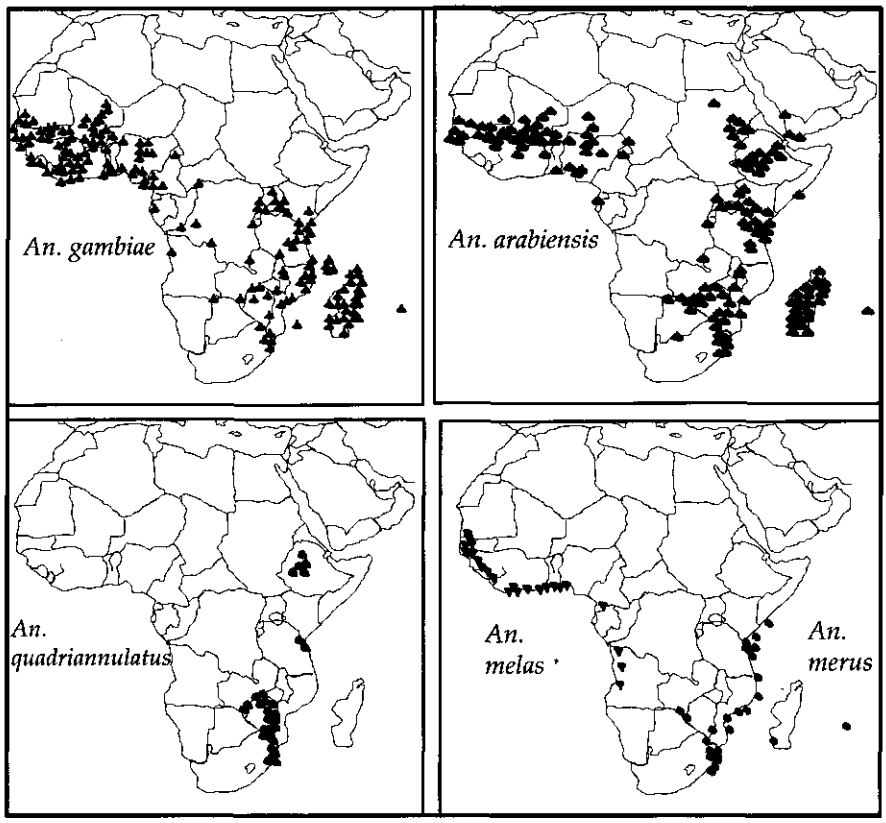


Figure 6. *Map of Africa showing distribution of* Anopheles gambiae *complex mosquitoes.* Anopheles melas *and* Anopheles merus *appear on the west and east coasts, respectively (modified after Coetzee* et al., *2000).*

1.6. Problem definition and research objectives

The preferential selection of human hosts expressed by malaria mosquitoes is an important consideration in modelling the transmission dynamics of the disease (Dye & Hasibeder, 1986). Predictively, high transmission rates can occur if susceptible mosquitoes would prefer to bite infective humans, become infected, survive long enough for the parasites to develop to infective stages and select susceptible humans in order to obtain their subsequent meals (Burkot, 1988). However, since *Plasmodium* parasites would cause damage to their mosquito vectors in various ways (Ferguson & Read, 2002), it would be advantageous for the mosquitoes to develop and/or perpetuate strategies that would enhance their fitness (Kelly, 2001). Thus, avoidance of feeding on humans harbouring the infective gametocyte stages would be a good strategy. Nonetheless, strategies geared towards selecting *Plasmodium*-uninfected individuals would need to be refined such that hosts with reduced defensive behaviour or with blood of high nutritional value etc are favoured as the preferential selection of individuals with these qualities would contribute towards the mosquitoes' longevity and fecundity, respectively, and thus their overall fitness (Figure 7).

However, much as the aforementioned strategies appear ecologically and evolutionarily sound, the principal causes that make certain individuals to be more preferred than others by mosquito vectors remain unknown. From a practical point of view, knowledge of the causes of selective biting between humans by mosquito vectors would (1) improve our understanding of the epidemiology of disease transmission and (2) be used in planning and/or implementing disease control strategies so as to avert epidemics e.g. by directing intervention measures at high risk groups or individuals. Furthermore, much as olfaction is responsible for guiding mosquitoes to their blood-meal hosts (Takken, 1991; Takken & Knols, 1999) and diseases are known to alter human olfactory characteristics (Penn & Potts, 1998), no good quantitative information exists on the presumed positive correlation between olfaction or infection status and human attractiveness to host-seeking mosquitoes. Although humans have been described to have distinctive and specific body odour (Stoddart, 1990) it is not precisely known if this is correlated to variability in their attractiveness to mosquitoes and hence to the dynamics of malaria transmission. However, if the semiochemicals responsible for attracting and/or causing differential attractiveness of humans to mosquitoes can be identified, endeavours that champion mass trapping (Day & Sjogren, 1994) as an additional tool in efforts of integrated vector management (Utzinger *et al.*, 2002), would be boosted.

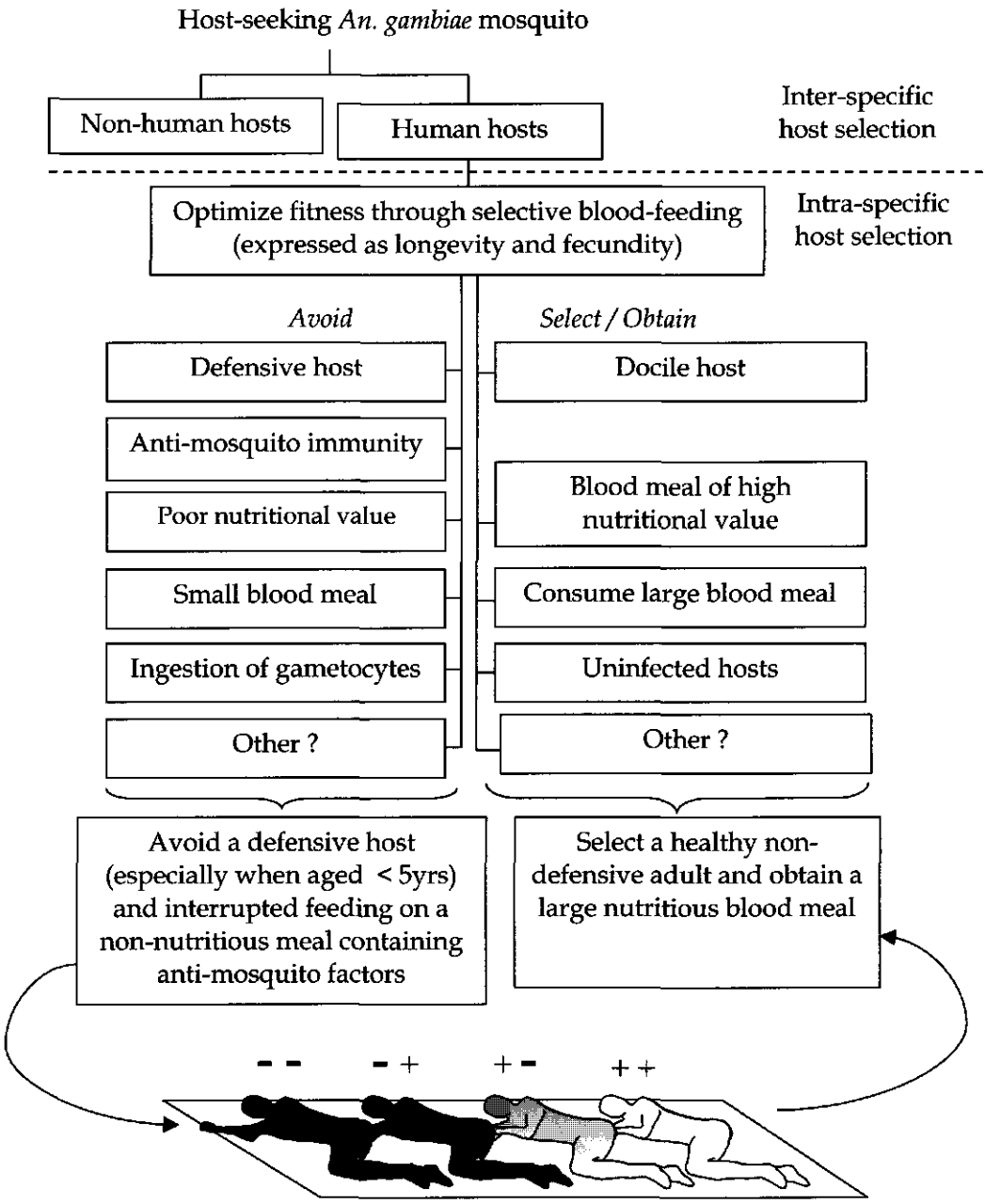


Figure 7. *Overview of factors that are involved in optimal host-selection behaviour of malaria vectors. The + and - signs depict the presumed levels of selection and avoidance of human hosts, respectively.*

The research presented in this thesis consisted of four major objectives*

- To develop an olfaction-based bioassay and use it to verify if variability in human attractiveness to mosquitoes has a physico-chemical basis.
- To investigate how breath and body odour contribute to and possibly interact with the attractiveness of humans to *An. gambiae*.
- To investigate the effect of body heat and moisture on the short-range attractiveness of humans to *An. gambiae*.
- To investigate whether infection with malaria parasites affects the attractiveness of humans to *An. gambiae*.

1.8. Outline of the thesis

Chapter 1. This is the introductory chapter. It reviews the general literature, defines the research questions and gives the justification of the research, stating the objectives.

Chapter 2. States the molecular genetic markers that have hitherto been used to analyse arthropod blood meals. The scientific background upon which DNA-based markers derive is explained and a listing of factors that affect the success of analysing arthropod blood meals using these markers given. Variations in nuclear and mitochondrial DNA markers as used in the identification of individuals fed upon by haematophagous arthropods are described and recommendations for the potential of their improved performance for field application highlighted. The use of DNA markers for identifying specific hosts of blood-feeding arthropods is also highlighted. The sensitivity and specificity of molecular genetic markers in relation to the classical ABO and haptoglobin blood typing techniques is discussed.

Chapter 3. Presents a research example in which forensic techniques borrowed from the Federal Bureau of Investigation (FBI), USA, were used to evaluate the effect of blood meal size and extent of digestion on the ability to identify human DNA from mosquito blood meals. A relationship was established between extent of blood meal digestion and the success probability of obtaining positive reactions. The results are discussed in relation to the time profile observed in the disappearance of blood protein from the midgut of engorged mosquitoes and the need of adhering to a well-defined sampling scheme in order to improve the performance of molecular genetic markers for typing mosquito blood meals.

Chapter 4. A new olfactometer that accommodates complete human beings as sources of host-seeking stimuli was designed, developed and tested. The olfactometer was evaluated by recruiting nine Kenyan human males and ranking them for attractiveness to mosquitoes based on mosquito responses to their total body emanations (body odour, heat and moisture). The influence of total body emanations on between-person differences in relative attractiveness to the

* These investigations received financial support from the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR, project ID N$_o$. 980692) and Research Training Grants (RTG, project ID N$_o$. A10370).

mosquitoes was established and the effect of residual stimuli on mosquito orientation behaviour studied.

Chapter 5. The tent olfactometer developed in chapter 4 was used to investigate how breath and body odour contribute to and possibly interact with the attractiveness of humans to the malaria mosquito *An. gambiae*. The effects of breath and body odour on mosquito attraction were analysed and an additional cause for between-person differences in relative attractiveness proposed.

Chapter 6. The role of body heat and moisture on the relative attractiveness of humans to the malaria mosquito *An. gambiae* was examined. The mosquitoes preferred individuals whose total body emanations were $\geq 0.5^{o}C$ warmer than those of their counterparts or those whose total body emanations had a lower relative humidity (if the temperature difference was $< 0.5^{o}C$). These findings were interpreted in terms of the importance of temperature and humidity in the short-range attraction of *An. gambiae* to human hosts.

Chapter 7. A rare window of opportunity enabled to investigate the effect of human infection with malaria parasites (*P. falciparum*) on between-person differences in relative attractiveness to the malaria mosquito *An. gambiae*. The relative attractiveness of two persons, one of whom always attracted more mosquitoes than the other (when the two were uninfected), changed when the 'putatively' more attractive individual displayed *P. falciparum* clinical malaria symptoms (fever and profuse sweating). These findings provide new and alternative evidence, within the *Anopheles-Homo-Plasmodium* system, that the malaria parasite *P. falciparum* manipulates the olfactory signals produced by the human host.

Chapter 8. The effect of *P. falciparum* parasitaemia on the attractiveness of humans to two Afrotropical malaria mosquito species (*An. gambiae* and *An. funestus*) was investigated in the field. It was observed, for the first time, that the malaria parasite *P. falciparum* is capable of manipulating the characteristics of its vertebrate hosts in such a way as to enhance its own transmission. This strategy is plausible because, from an evolutionary standpoint, it would be best for the parasite to modify those characteristics of the host that affect host selection in such a way that insect vectors prefer to feed on infected individuals.

Chapter 9. This is the concluding chapter. It discusses some questions that have remained unanswered despite the efforts that have been put in investigating the reasons why mosquitoes select certain humans more readily than others. Recommendations for future work are given and the conclusions of the current research activities listed.

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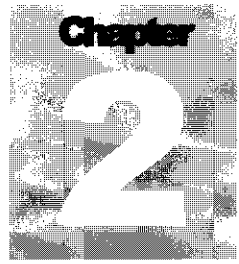
Part I

Characterisation of mosquito blood meals using DNA markers



Analysis of arthropod blood meals using molecular genetic markers[1]

Abstract: Little is known about the transmission dynamics of human malaria and other vector-borne diseases partly because of the limited availability and distribution of appropriate tools for quantifying human-mosquito contact rates. Recent developments in molecular biology have allowed a significant increase in the efficacy and reliability of blood meal identification and DNA-based molecular markers are now being harnessed for typing arthropod blood meals. The extent to which these markers have been used for analysis of mosquito blood meals and the potential they might have for the future is discussed. The contributions that the advent of PCR has made are also examined.

Introduction

The distribution of bites by *Anopheles* mosquitoes on humans is an important element in modelling the transmission of malaria (Dye & Hasibeder, 1986). For example, the basic reproductive rate of malaria and malaria-specific morbidity and mortality could be increased if non-infected mosquitoes prefer to feed on persons carrying infective parasite stages, or if infected mosquitoes prefer to feed on persons at a high risk of malaria (e.g. children and pregnant women). Technical obstacles have hitherto prevented the precise identification of humans from whom mosquitoes have obtained a blood meal. The correct identification of individual human hosts of mosquitoes is important for several reasons: (1) it might reveal the nature of host preference; (2) it might explain why some individuals are more prone to insect bites than others; and (3) it can help to understand whether parasite carriers manipulate the host-seeking behaviour of their vectors. This knowledge can help in developing effective disease control strategies (e.g. distribution of drugs and insecticides according to the risk of infection), thereby helping to decrease the basic reproductive rate of malaria (Dye, 1990), or taking into account patterns for the potential spread of vector-borne diseases within and among communities. The details of mosquito blood-feeding patterns can also help to assess the efficacy and

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effectiveness of various control or surveillance tools (e.g. repellents, bednets and traps), and to estimate the degree of coverage needed for various malaria vaccines when developed (Holloran *et al.*, 1989).

An effective way of identifying species and individuals fed upon by blood-feeding arthropods is the analysis of blood meals imbibed by the arthropods. This analysis has traditionally been done using immunological methods (Tempelis, 1975; Washino & Tempelis, 1983; Pant, 1987), which mainly detect species-specific targets, but might not distinguish between blood meals obtained from similar or closely related host species. However, although immunological analysis based on ABO blood groups and serum haptoglobins can differentiate human individuals fed upon by mosquitoes (Boreham & Lenahan, 1976; Bryan & Smalley, 1978), the degree of polymorphism of ABO blood group and haptoglobin markers is low with only four and three genotypes distinguished, respectively. Arthropod blood meal sources might be identified with greater specificity and sensitivity using modern molecular genetic methods.

DNA-based identification of blood meal sources

Blood meals of arthropods of medical and veterinary importance contain a large variety of cells ingested from vertebrate hosts. Although most cells in blood from birds and reptiles contain nuclei and mitochondria, the erythrocytes of mammals do not. Nevertheless, in mammalian blood, leukocytes provide adequate genetic material in the form of DNA. Although many types of DNA sequence variations exist between species and individual organisms (Rees & Jones, 1972; Watson *et al.*, 1998), only those that have been applied for blood meal analysis are discussed here. These include minisatellites[2] or variable number tandem repeats (VNTRs), and microsatellites[3] or short tandem repeats (STRs). Both types occur abundantly in most eukaryote genomes and have found good application for individual identification in forensic medicine (Gill *et al.*, 1985; Jeffreys, 1992; Schneider & Martin, 2001). Variability at these marker loci results from differences in the number of tandem repeats of a short DNA sequence (Bell *et al.*, 1982; Tauz, 1989), therefore alleles differ in length by an integral number of repeat units. These loci are highly polymorphic (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987; Hearne *et al*, 1992; Edwards *et al.*, 1991); are stably inherited, segregating in a Mendelian fashion (Tauz, 1989; Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987; Hearne *et al*, 1992); can be amplified *in vitro* by PCR (Tauz, 1989; Jeffreys *et al.*, 1988; Weber & May, 1989); and have a high mean heterozygosity[4] (Nakamura *et al.*, 1987; Edwards *et al.*, 1991; Kimpton *et al.*, 1992; Litt & Lutty, 1989) that render them highly informative genetic markers. These markers have been used successfully to identify blood meals of

[2] Minisatellites: > 5 base pair, tandemly repeated DNA segments varying in length according to the number of repeat units and which are mainly located near chromosome ends.

[3] Microsatellites: tandemly repeated 2-5 base pair DNA segments varying in length according to the number of repeat units, which are distributed widely throughout the genome

[4] Heterozygosity: The presence of different forms of a gene at one or more positions on a pair of chromosomes containing the same linear gene sequences, each derived from a separate parent.

mosquitoes (Coulson *et al.*, 1990; Gokool *et al.*, 1992; 1993; Koella *et al.*, 1998; Kreike & Kampfer, 1999; Chow-Shaffer *et al.*, 2000; Ansell *et al.*, 2000; Mukabana *et al.*, 2002), crab lice (Replogle *et al.*, 1994; Lord *et al.*, 1998) and tsetse (Torr *et al.*, 2001).

Factors affecting analysis of arthropod blood meals

To generate reproducible fingerprints from arthropod blood meals, sufficient amounts of intact, high molecular mass DNA must be isolated from the blood meals and analysed using highly specific markers that can detect vertebrate host DNA. However, this capacity is affected by a variety of factors such as the conditions under which the engorged arthropods or their blood meals are stored, and the extent to which the blood meals have been digested. For example, a probe based on human placental DNA allowed detection of good quality DNA in *An. gambiae* that had taken blood meals of variable sizes and were freeze-killed 15 hours after feeding, subsequent to storage, as whole insects, at -70°C or in 100% isopropanol at room temperature, for >14 days (Coulson *et al.*, 1990). By contrast, such DNA was rarely detected in blood meals of mosquitoes that were similarly treated, but where the insects were stored by desiccation on silica gel at room temperature (Coulson *et al.*, 1990). Although this implies that host DNA degrades much faster when blood-fed mosquitoes are stored dry at room temperature, there is conflicting evidence on the effect of desiccation on the ability to analyze blood meal DNA extracts. Dot blot hybridisation with a biotin-labelled probe detected intact human DNA in blood meals of *Ae. aegypti* and *An. sinensis* killed 24 hours after feeding, and stored as whole insects for ~1 year at room temperature (Sato *et al.*, 1992). This discrepancy can be explained by the possibility that eggs, which are usually well developed after 24 hours of blood meal digestion, contain biotin, so false positives can be observed following the detection of endogenous biotin. The absence of a significant relationship between blood meal size (or amount of DNA contained therein) and success of analysis (Coulson *et al.*, 1990) has also been demonstrated among field populations of *An. gambiae* (Gokool *et al.* 1992; 1993), *Ae. aegypti* (Chow-Shaffer *et al.*, 2000) and other unspecified mosquito species (Kreike & Kampfer, 1999).

The successful typing of blood meals of wild-caught arthropods requires adherence to at least three factors. First, the preservation of engorged arthropods or their blood meals must be done appropriately to avoid degeneration of the blood meals. It has been shown that storage of blood meals on filter paper either as dry smears at 4°C (Ansell *et al.*, 2000), or as smears immersed in lysis buffer at 25-30°C, often results in high yields of human DNA, whereas storage of whole mosquitoes in lysis buffer gives non-specific banding patterns (Chow-Shaffer *et al.*, 2000). Second, the concentration of DNA extracted from a blood meal needs to be determined, so that the optimum amount of DNA required for PCR is used. Analysis at one human minisatellite locus either gave no detectable products or produced non-specific banding patterns when <1 ng or >10 ng DNA was used for

analysis, respectively, whereas 1-5 ng DNA usually produced the expected bands (Chow-Shaffer *et al.*, 2000). Third, field mosquitoes should be collected soon enough after they have obtained a blood meal because there is a significant negative relationship between the time since ingestion and the success of analysis (Mukabana *et al.*, 2002). This could be achieved through using appropriate sampling methods (e.g. well designed bednet and exit traps that can be quickly removed and replaced at selected time intervals during the sampling process), allowing for preservation of specimens before blood meals become adversely degraded. It would be useful to know, beforehand, whether a field-collected mosquito has a blood meal that falls into the optimum range for successful analysis. Thus, further studies describing the efficacy of typing blood meals of insects with different abdominal appearances (e.g. fully fed, half-gravid and sub-gravid) would be helpful.

Individual hosts of blood-feeding arthropods

The identification of individuals fed upon by haematophagous insects using molecular genetic techniques has been based on variations in nuclear and mitochondrial DNA (mtDNA) sequences.

Identification by nuclear DNA sequences

The nuclear genome comprises specific coding regions (*genes*) in which there are non-coding sequences (*introns*) that contain many minisatellite and microsatellite loci. Analysis of hypervariable marker loci may involve restriction enzyme digestion of DNA targets [Restriction Fragment Length Polymorphism (RFLP[5]) analysis] and/or analysis of Amplified Fragment Length Polymorphisms (AFLPs[6]). Several workers have investigated the use of PCR for arthropod blood meal analysis. The pioneering study generated human DNA patterns from blood meals of *An. gambiae* digested for up to 15 hours (Coulson *et al.*, 1990). This sensitivity is lower than that seen with RFLP analysis of raw DNA, where a locus-specific minisatellite probe enabled detection of DNA profiles[7] from blood meals digested for up to 25 hours (Coulson *et al.*, 1990) (Table 1). The low sensitivity was attributed to the use of an unsuitable DNA marker because, whereas it was shown that ~10 ng human DNA can routinely be isolated from a single fully fed *An. gambiae* mosquito (Coulson *et al.*, 1990), at least 50 ng of relatively intact DNA is required for typing with locus-specific minisatellite probes (Saiki *et al.*, 1985) and 0.1-1 µg DNA for analysis with multi-locus DNA fingerprint[8] probes (Jeffreys *et al.*,

[5] RFLP analysis: The cutting of genomic DNA with an enzyme (restriction endonuclease), followed by electrophoretic separation of variable-length DNA fragments before detection for example by southern blot analysis.

[6] AFLP analysis: The pre-amplification of DNA targets (typically by PCR) before manual or automated detection of variable-length DNA fragments.

[7] DNA profile: a pattern of DNA bands generated from one or few loci that may distinguish between individuals but that is not necessarily individual-specific.

[8] DNA fingerprint: an individual-specific pattern of DNA bands generated at multiple tandem repetitive loci using a probe constructed of one or more core repeat units.

1985). However, although it has been shown that blood meals of *An. gambiae* may contain much more than 10 ng of human DNA (Chow-Shaffer *et al.*, 2000; Ansell *et al.*, 2000) (Table 1), this amount is still less than what is required for analysis with multilocus DNA fingerprint probes. Thus, the use of minisatellite markers for the analysis of raw cellular DNA in blood meals of wild small-sized arthropods would best be achieved using locus-specific probes. From these studies, it appears that an optimal PCR protocol would need to be developed to facilitate blood meal analysis using multilocus DNA fingerprint probes.

Despite the fact that, for some time now, it has been possible to PCR-amplify and profile human minisatellites from mosquito blood meals (Kreike & Kampfer, 1999, Chow-Shaffer *et al.*, 2000) and from excreta of the human crab louse *Pthirus pubis* (Replogle *at al.*, 1994), microsatellites appear to be more amenable to genetic analysis than minisatellites. Microsatellites can be easily amplified from highly degraded DNA because much smaller length fragments are targeted. So far, microsatellites have been used to analyse blood meals to evaluate; (1) the amount of protection conferred to sleepers by insecticide treated bednets (ITNs) (Gokool *et al.*, 1992; 1993); (2) the effect of *Plasmodium* infection on mosquito blood-feeding behaviour (Koella *et al.*, 1998); and (3) the feeding rates of mosquitoes (Chow-Shaffer *et al.*, 2000; Ansell *et al.*, 2000) and tsetse (Torr *et al.*, 2001) on different host individuals, including a comparison of feeding rates on pregnant versus non-pregnant women (Ansell *et al.*, 2002) (Table 1). However, microsatellite analysis has been used to identify blood meals from closely related individuals (Ansell *et al.*, 2000; Torr *et al.*, 2001), although error rates resulting from misidentification of close relatives can be high when few marker loci are analysed (Chow-Shaffer *et al.*, 2000). Thus, if microsatellite analysis has to be adopted for field studies, a large number of loci needs to be assayed for accuracy of analysis. Indeed, a 9-locus microsatellite system has recently enabled the identification of up to 80% of blood meals of field populations of *Cx. quinquefasciatus* (Michael *et al.*, 2001).

Identification by mitochondrial DNA (mtDNA) sequences

One feature characteristic of all studies reported above is that only nuclear DNA targets were analysed. Although, nuclear DNA sequences generally offer greater potential variation and therefore more informative typing, nuclear DNA sequences are a greater challenge to detect as only two copies of nuclear chromosomes are present in a cell. Despite the fact that earlier attempts to type human nuclear DNA targets from lice engorged with human blood had failed (Replogle *et al.*, 1994), it has recently been possible to extract, amplify and sequence human mtDNA from blood meals of adult crab lice fed on human volunteers (Lord *et al.*, 1998). Unfortunately, the degree of polymorphism of mtDNA is limited by a great sequence economy (Anderson *et al.*, 1981) implying that no great potential exists of using mtDNA polymorphisms for insect blood meal analysis. However, mtDNA sequences occur in high copy numbers per cell and mtDNA typing, therefore, suits situations where extracted DNA is small or significantly degraded

which is likely the case for extracts from mosquito blood meals. In addition, mtDNA is inherited from the mother only, so that in situations where potential host individuals are unwilling to provide control specimens for a direct comparison with a biological sample, any maternally-related volunteer may provide a reference sample.

Specific hosts of blood-feeding arthropods

Apart from determining individuals upon whom blood-feeding arthropods have fed, DNA markers also offer potential in identifying the species origin of blood meals. For example, laboratory tests demonstrated the ability of a biotin-labelled probe to discriminate between DNA from humans, dogs, cattle, pigs, mice and goats with cross-reactions only occurring between human and Japanese monkey DNA (Sato *et al.*, 1992). Field studies using the non-isotopic probe described *An. quadrimaculatus* species A as feeding more on humans than species B and C_1 in a location where the three species occurred in sympatry (Jensen *et al.*, 1996). More precise identification of vertebrate host species fed upon by arthropod vectors of disease can be achieved by probing gene sequences coding for electron transfer proteins (cytochromes) coupled to a haem type b subgroup (cytochrome b). For instance, the use of cytochrome b-specific markers showed that tsetse flies known to have fed on cattle had also taken meals from the African buffalo, *Syncerus caffer* (Torr *et al.*, 2001). Cytochrome b-specific sequences have also been used to type meals of the black fly *Simulium damnosum* (Boakye *et al.*, 1999) and nymphs of the tick *Ixodes ricinus* (Kirstein & Gray, 1996). Human DNA was detected from blood meals of the black flies up to three days after ingestion; those of wild-caught *Glossina palpalis* were identified as taken from domestic pigs (Boakye *et al.*, 1999). Tick blood meals ingested from mice during the larval stage could be detected up to 280 days afterwards during the nymphal stage (Kirstein & Gray, 1996). However, ticks have the capacity to store part of the blood meal in an undigested form inside inclusion bodies (endosomes) contained within midgut epithelial cells. Thus, it is not surprising that host DNA could be detected after many months following blood meal ingestion. In general, the high specificity of detection provided by molecular genetic markers implies that interspecific arthropod host preferences can be characterised with increased precision. Thus, molecular genetic markers should enable: (1) the accurate determination of anthropophilic indices of various arthropod species, and the assessment of the risk of transmission of vector-borne diseases posed to man and other animals; (2) the precise evaluation of the efficacy and effectiveness of control tools aimed at reducing human-vector contact; and (3) the enhanced detection of reservoir hosts of various parasitic pathogens.

Table 1. Molecular genetic markers that have been used to characterise blood meals of haematophagous arthropods.[a]

Marker DNA	Arthropod species	Objectives	Results	References
Human placental	*An. gambiae*	To investigate the effect of storage conditions on the quality of DNA present in mosquito blood meals.	High quality DNA was isolated from blood meals stored at -70°C or in 100% isopropanol, but not under desiccation at room temp.	Coulson *et al.*, 1990
		Use of PCR for enhanced detection of human DNA from mosquito blood meals.	Blood meals digested for 15 hours were successfully analysed	Coulson *et al.*, 1990
		Effect of quantity of host DNA in blood meals on profiling success.	Profiling success was not related to the quantity or purity of DNA.	Gokool *et al.*, 1993
Human Kpn-1 family	*Ae. aegypti* & *An. sinensis*	Sensitivity and specificity of a biotin probe for detection of human DNA in engorged mosquitoes.	DNA was detected in blood meals digested for ≤100 hours, and in those digested for 24 hours stored at room temperature for ~1 year.	Sato *et al.*, 1992
	An. quadrimaculatus	Measuring human blood-feeding rates of sympatric species	Blood-feeding rates of spp. A were higher than of spp. B & C_1	Kirstein & Gray, 1996
HLA and minisatellite	Mosquito species not named	Isolation and characterisation of human DNA from blood meals.	High quality DNA was isolated. Double bloodmeals and those digested for > 26 hours could be typed.	Jensen *et al.*, 1996
Minisatellite	*An. gambiae*	To find out how extent of digestion affects blood meal analysis.	DNA profiles were generated from blood meals digested for 25 hrs. No individual-distinctive patterns were detected.	Coulson *et al.*, 1990 Gokool *et al.*, 1993
Minisatellite and microsatellite	*Pthirus pubis*	To determine the feasibility of PCR for blood meal analysis in pubic lice.	Human genotypes were detected from louse excreta but not blood meals.	Replogle *et al.*, 1994
	Ae. aegypti	To develop a highly specific method of DNA analysis and use it to map mosquito blood-feeding patterns.	Analysis of only few marker loci increases the chances of mis-identifying blood meals obtained from close relatives.	Chow-Shaffer *et al.*, 2000
Microsatellite	*An. gambiae*	Quantification of the degree of protection provided by ITNs.	Mosquitoes fed most on people not sleeping under intact ITNs	Gokool *et al.*, 1992
			Mosquitoes (90%) fed on persons sleeping under intact ITNs	Gokool *et al.*, 1993
	An. gambiae s.l	Study of feeding patterns of *Plasmodium*-infected mosquitoes.	Double feeding was 12% higher among infected mosquitoes.	Koella *et al.*, 1998
	An. gambiae	Assessment of the efficacy of a simple DNA extraction method and quantification of mosquito blood-feeding rates in the field.	~400 ng of human DNA was extracted from blood meals digested for 12 hours. Both single and double meals were identified.	Ansell *et al.*, 2000
	An. gambiae s.s	To find out how extent of digestion affects blood meal analysis.	Typing success reduced with increase in length of digestion time but was independent of bloodmeal size.	Mukabana *et al.*, 2002
	Glossina species	To identify individual-specific hosts of tsetse feeding on cattle	Meals digested for 72 hrs plus double meals could be analysed.	Torr *et al.*, 2001
	An. gambiae s.l	Comparing the relative attractiveness of pregnant and non-pregnant women.	Pregnant women received a higher proportion of bites than non-pregnant counterparts.	Ansell *et al.*, 2002
	Culex quinquefasciatus	Assessment of exposure rates to parasitic diseases in endemic communities using a 9-locus microsatellite system.	Source of human DNA was identified within blood meals of ~ 80 % of mosquitoes. The double feeding rate was 13%.	Michael *et al.*, 2001
mtDNA	*Pthirus pubis*	To assess the power of mtDNA in typing louse blood meals	Individual human hosts of the lice were detected.	Lord *et al.*, 1998
	Simulium damnosum & *Glossina palpalis*	Development of a simple assay for identifying host species.	Host species, including humans, were correctly identified from blackfly blood meals 72 hours after ingestion.	Boakye *et al.*, 1999
	Ixodes ricinus	Assessing the efficacy of the cytochrome b gene marker in discriminating reservoir hosts of *Borrelia burgdorferi* and evaluation of its stability in analysing tick blood meals.	Animals serving both as reservoirs of the spirochaete and tick hosts could be identified up to 280 days after larvae had ingested blood meals.	Kirstein & Gray, 1996

[a]Abbreviations: HLA, human leukocyte antigen; ITNs, insecticide treated nets; *Kpn*-1,(the bacterium) *Klebsiella pneumoniae*; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

General considerations

One drawback inherent with using DNA-based markers for arthropod blood meal analysis rests in the fact that the favourite type of target DNA is located in nuclei that reside inside the relatively less abundant white blood cells of most vertebrate hosts. By contrast, recognition targets of the classical ABO blood group markers are plentiful on surfaces of the more abundant red blood cells. The DNA-based markers that have been used to analyse arthropod blood meals are shown in the Table 1. In the studies listed, DNA marker loci that were analysed following PCR amplification, generated individual-specific patterns from blood meals of *Ae. aegypti* (Chow-Shaffer *et al.*, 2000) and *An. gambiae s.s.* digested for up to a maximum of 32 hours (Mukabana *et al.*, 2002). This sensitivity to digestion is lower than that detected using ABO blood grouping, through which method blood meals of *Ae. aegypti* (Boreham & Lenahan, 1976) and *An. gambiae* (Bryan & Smalley, 1978) were detected after digestion for up to a maximum of 48 and 34 hours, respectively. By contrast to mosquitoes, DNA-based markers have enabled to type blood meals of tsetse (Torr *et al.*, 2001), black flies (Boakye *et al.*, 1999) and ticks (Kirstein & Gray, 1996) digested for much longer. The reason for this difference may be sought in differences in digestive physiology between the arthropods (Lehane, 1991). Mosquitoes ingest blood, on average, at three-day intervals (Clements, 1999), whereas black flies and tsetse take meals at greater intervals. Although molecular genetic tools might not be suitable for characterising blood meals that have undergone extensive digestion, their power lies in the highly polymorphic nature, an attribute that gives them the potential of discriminating between individuals and specific hosts of blood-feeding arthropods definitively.

Although AFLP analysis has mainly been by electrophoresis on agarose and polyacrylamide gels, non-specific amplification products may be confused for true bands, especially when the PCR stringency conditions and enzyme used for primer extension are sub-optimal (Kebelmann-Betzing *et al.*, 1998). Automated capillary electrophoresis instruments and computer software have been developed which can determine the base pair sizes of tandem repetitive amplification products (Lazaruk *et al.*, 1998), enabling more precise resolution of allelic forms. A major advantage of such systems, apart from ease, is that background amplification products could be distinguished from authentic products (Wilson *et al.*, 1995). This system has successfully been used to analyse human mtDNA polymorphisms from blood meals of *Pthirus pubis* (Lord *et al.*, 1998). With such a system available, our ability to generate information on arthropod blood-feeding behaviour in nature may be enhanced and so will our understanding of the epidemiology of vector-borne diseases.

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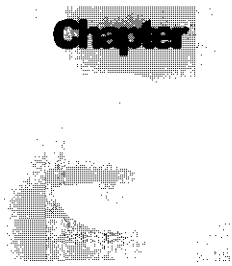

Extent of digestion affects the success of amplifying human DNA from blood meals of *Anopheles gambiae* (Diptera: Culicidae)[1]

Abstract: The success of distinguishing blood meal sources of *Anopheles gambiae* Giles through deoxyribonucleic acid (DNA) profiling was investigated by polymerase chain reaction (PCR) amplification at the TC-11 and VWA human short tandem repeats (STR) loci. Blood meal size and locus had no significant effect on the success of amplifying human DNA from blood meals digested for 0, 8, 16, 24 and 32 hours (P = 0.85 and 0.26 respectively). However, logistic regression found a significant negative relationship between time since ingestion and the success probability of obtaining positive PCR products among meals digested for between eight and 32 hours (P = 0.001). Approximately 80% of fresh blood meals were successfully profiled. After eight hours, the proportion of blood meals that could be successfully profiled decreased slowly with time after ingestion, dropping below 50% after approximately 15 hours. There was no significant difference in the success of amplifying human DNA from blood meals of mosquitoes killed at zero and 8 hours after ingestion (P = 0.272).

Introduction

Arthropod blood meal sources have traditionally been identified by immunological methods (Tempelis, 1975; Washino & Tempelis, 1983; Pant *et al.*, 1987). These methods mainly detect species-specific immunological targets and so are not suitable for distinguishing between meals obtained from members of the same, or closely related, host species. Immunological analyses based on ABO blood grouping and identification of serum haptoglobins in mosquito blood meals can, however, differentiate human individuals upon whom mosquitoes have fed (Boreham & Lenahan, 1976; Bryan & Smalley, 1978). Blood markers are less polymorphic and may lead to false identification especially when used to characterise blood meals of indoor- or outdoor-fed mosquitoes collected outside or inside houses respectively, such mosquitoes may have fed on hosts outside the target population whose phenotypes are usually identified for comparative

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purposes. The ability to characterise insect blood meal sources can be improved tremendously using molecular genetic methods such as hybridization with sequence-specific probes (Sato *et al.*, 1992) and DNA fingerprinting or profiling (Coulson *et al.*, 1990; Gokool *et al.*, 1993; Kreiker & Kampfer, 1999; Chow-Shaffer *et al.*, 2000).

DNA fingerprinting and/or profiling can generate species- or individual-distinctive DNA patterns e.g. through detection of length variations in segments of the genome containing repetitive DNA sequences. The polymorphic fragments may be detected with (Jeffreys *et al.*, 1988) or without (Jeffreys *et al.*, 1985) amplification by the polymerase chain reaction (PCR). These methods have been used successfully to characterise individual host DNA present in blood meals of *An. gambiae* Giles *s. l.* (Diptera: Culicidae) (Coulson *et al.*, 1990) and *Glossina* species (Diptera: Glossinidae) (Torr *et al.*, 2001) and blood meals plus excreta of the human crab louse *Pthirus pubis* (Linnaeus) (Phthiraptera: Phthiridae) (Replogle *et al.*, 1994). The capacity of these methods to identify blood meal sources of wild-collected mosquitoes has also been demonstrated (Gokool *et al.*, 1993; Koella *et al.*, 1998; Chow-Shaffer *et al.*, 2000), albeit with varying levels of success.

The ability to detect mosquito blood meal sources using DNA-based markers is affected by a variety of factors. Amount of DNA extracted from blood meals, conditions under which dead engorged females are stored and lengths of digestion time are important (Coulson *et al.*, 1990; Chow-Shaffer *et al.*, 2000). Although the efficacy of various storage conditions for preserving dead mosquitoes killed after different digestion time lengths has been studied (Coulson *et al.*, 1990; Sato *et al.*, 1992; Kreiker & Kampfer, 1999; Chow-Shaffer *et al.*, 2000), maximum digestion time lengths at which positive reactions are expected are emphasised (Sato *et al.*, 1992; Kreiker & Kampfer, 1999). Knowing how the extent of blood meal digestion affects the probability of obtaining positive amplification reactions could increase the potency of DNA based methods for distinguishing mosquito blood meal sources in the field.

This study evaluated the effect of time since ingestion on the ability to identify mosquito blood meals through DNA profiling. Two tetrameric Short Tandem Repeats (STR) loci were used as markers to differentiate known human individuals upon whom *An. gambiae s.s.* mosquitoes were fed. The proportion of profiling tests that were successful was assessed as a function of post-feeding time intervals. The effect of blood meal size on success of profiling human DNA contained in the mosquito blood meals was also investigated.

Materials and methods

Mosquitoes

Experiments were carried out using *An. gambiae s.s.* mosquitoes which were originally obtained from Suakoko, Liberia (courtesy, Prof. M. Coluzzi, Rome). The mosquitoes were reared under controlled conditions, 27±1°C, 80±5% relative

humidity and 12L: 12D photoperiod. Adult mosquitoes were maintained on 6% glucose solution. Test mosquitoes were starved overnight and supplied only with water on cotton wicks. The mosquitoes had not taken a blood meal prior to the experiments.

The amount of blood taken by a proportion of the mosquitoes was estimated by gravimetric analysis. This method has the disadvantage that it could underestimate blood meal size (Redington & Hockmeyer, 1976) because it does not account for the changes in blood meal size that occur as a result of excretion of water and salts during (pre-diuresis) and after feeding (diuresis). However, the method is simple and non-lethal and as the amount of fluid excreted is directly related to the amount of blood ingested (Nijhout & Carrow, 1978), the overall increase in weight resulting from blood feeding could be used as an indicator of blood meal size. Further, although weight increase by feeding does not take into account the weight lost as a result of diuresis and pre-diuresis, the genetic information of the blood host is contained in the cellular fraction of the blood meal which is retained in the midgut.

Test mosquitoes were immobilised through cold-treatment (4°C, 15 minutes) and weighed individually on a Cahn (C-33) microbalance and then kept in glass tubes. Mosquitoes that became active again were fed on human blood by placing the mesh-covered tubes against the forearm of one of three test persons designated X, Y and Z. Mosquitoes were removed from the arm after voluntary withdrawal of the mouthparts from the skin and feeding had ceased. Fed mosquitoes were reweighed and blood uptake by weight recorded after feeding-associated diuresis had ceased. The effect of meal size on profiling success was estimated after categorising the blood meals as small or large according to whether they were less or greater than the median (1.48 mg), respectively. This helped to minimize the probable error that could occur as a result of estimating blood meal sizes using the gravimetric method.

A few mosquitoes were offered double meals by removing them mid way during feeding from the arm of a first person and transferring them gently to that of a second where they were allowed to feed to repletion. Of these mosquitoes only three were selected including one that was fed on persons X and Y, a second on Y and Z and a third on persons X and Z. It was not our goal to rigorously demonstrate the efficacy of profiling multiple feeds. Forty-four mosquitoes were freeze-killed (-80°C, 5 minutes) immediately after blood feeding and subdivided into five classes with respect to blood meal size (class interval = 0.5 mg). Other blood-fed mosquitoes were held under rearing conditions (27±1°C, 80±5% RH) and freeze-killed in sets of ten at 8, 16, 24 and 32 hours after feeding. Blood meals of all experimental mosquitoes were processed following storage at -80°C for two weeks. Control mosquitoes were males (n = 5) as well as cow-fed (n = 5) and unfed (n = 5) females.

Ethical clearance

Informed consent was obtained from all the three adult human participants. The project was approved by the Kenya National Ethical Review Committee based at the Kenya Medical Research Institute.

Preparation of DNA samples

DNA was isolated from human cheek cells and male mosquitoes; human-fed, cow-fed and unfed female mosquitoes. Isolation was by modification of a phenol extraction procedure in which DNA was precipitated with ethanol (Wetton *et al.*, 1987). Extracted DNA pellets were air-dried and dissolved in 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA).

Amplification of DNA

DNA extracts were PCR-amplified at the Human Tyrosine Hydrolase (TC - 11 or HUMTHO1) (Polymeropoulos *et al.*, 1991) and VWA (HUMVWFA31/A) (Kimpton *et al.*, 1992) genetic loci. Reactions were carried out in 15 µl volumes each containing 3.75 µl of template DNA, 0.36 µl 25 mM $MgCl_2$, 1.5 µl 10 × PCR buffer, 3.1 units *Taq* DNA polymerase, 1.2 µl each of 10 mM dNTPs and 1 pmole of each primer. Amplifications were done on a Techne Unit Progene thermal cycler. DNA was denatured at 95°C for 20 seconds and 94°C for 45 seconds for the VWA and TC-11 loci respectively. For each cycle oligonucleotide primers were annealed to opposite DNA strands at 59°C for 20 seconds (VWA) or 62°C for 20 seconds (TC-11), the annealed primers were extended at 72°C for 20 seconds (both loci). Thermal cycling was carried out for either 30 (VWA) or 27 cycles (TC-11) followed by further extension at 72°C for 5 minutes (both loci). Some attributes of the short tandem repeats loci investigated are shown in Table 1.

Table 1. Chromosomal location and primer sequences of the two human loci amplified from blood meals of *An. gambiae* Giles *s. s.*

Locus	Chromosomal Location	Repeat Sequence	Primer sequence (5' – 3')	References
TC - 11	11p 15-15.5	AATG	GTGGGCTGAAAAGCTCCCGATTAT ATTCAAAGGGTATCTGGGCTCTGG	Edwards *et al.*, 1991
VWA	12p 12-pter	TCTA	CCCTAGTGGATGATAAGAATAATC GGACAGATGATAAATACATAGGATGGATGG	Kimpton *et al.*, 1992

Electrophoresis

PCR-amplified products were separated on high-resolution, horizontal-slab, polyacrylamide gels (Budowle *et al.*, 1993). The gels were about 480 µm thick, backed on Gel Bond™ (FMC Corp., Rockland, Maine, U.S.A). A resolution of 2 - 4 base pairs (bp) can be achieved for fragments between 100 and 500 bp long (Allen

et al., 1989), alleles of loci used in this study are in the 154-178 (TC-11) and 126-166 (VWA) bp size range (Urquhart *et al.*, 1995). Amplified DNA was surface-loaded (in 5-8 µl volumes) on pieces of sample applicator tabs (Pharmacia-LKB) and separation carried out at constant temperature (15°C) on an E-C 1001 isothermally controlled apparatus (E-C, St. Petersburg, FL) set at 600 V, 25 mA and 20 W. A discontinuous buffer system using formate-borate (pH 9.0) as leading and trailing ions, respectively, was employed on 7% (TC-11) or 8% T (VWA) gels crosslinked with 5% C (N, N'-methylene-bisacrylamide). Tris-formate (pH 9.0), 0.12 M with respect to the formate ion, was the gel buffer. Tris-borate (pH 9.0) (0.28M with respect to the borate ion) was contained in paper wicks (1 × 13 cm, Gibco Biotechnology Resource Laboratories, Gaithersburg, Maryland, U.S.A) on the anode and cathode ends of the gel and served as the cathode buffer.

Silver staining

Size separated amplification products were visualised by silver staining (Cairns & Murray, 1994). Stained gels were air-dried overnight and attached to transparency film for storage and handling.

Genetic profiling of mosquito blood meals

Amplified fragment length products generated from mosquito blood meals and human cheek cells were matched to differentiate between the human individuals upon whom mosquitoes fed. Genotypes of the human volunteers were determined using allelic ladders constructed by mixing DNA samples extracted from persons with variable alleles. These helped to distinguish mosquito blood meals obtained from the different human individuals. The approximate molecular weight of the amplification products was determined using a size marker from digested pBR322 DNA.

Statistical analysis

Data were analysed using the Statistical Analysis System (SAS, version 8E) and Statistical Products and Service Solutions (SPSS version 10). Effects of locus and time since ingestion on success of detecting human DNA in the mosquito blood meals were tested by logistic regression using a forward conditional stepwise selection procedure. The influence of blood meal size was analysed in the same way after categorising into large and small blood meals according to whether they were less or greater than the median.

Results

A total of 84 blood meals from *An. gambiae* were PCR-amplified at the TC-11 and VWA short tandem repeats loci. The blood meals were obtained from one of three human volunteers. Only three mosquitoes of those that were offered double feeds were analysed. Although mosquitoes were given the chance to feed until

feeding-associated diuresis had ended, a few did not feed to repletion. As the amount of fluid excreted is directly related to the amount of blood ingested (Nijhout & Carrow, 1978), blood meals were analysed regardless of whether mosquitoes had engorged fully or partially.

Effect of blood meal size on profiling success

The number of blood meals present and successfully amplified were counted at six size classes with respect to their weight (0.01-0.50, 0.51-1.00, 1.01-1.50, 1.51-2.00, 2.01-2.50 and 2.51-3.00 mg). A total of 44 blood meals were evaluated, all from mosquitoes killed immediately after blood feeding. Figure 1 is the relative frequency histogram showing the relationship between blood meal size and amplification success. The smallest and largest blood meals that were successfully amplified at both loci were 0.08 and 2.74 mg, respectively. Logistic regression found that blood meal size and locus did not significantly affect the success of amplifying human DNA from blood meals digested for 0, 8, 16, 24 and 32 hours (P = 0.85 and 0.26 respectively). However, the extend of digestion had a significant effect on ability to yield amplification products (P < 0.001).

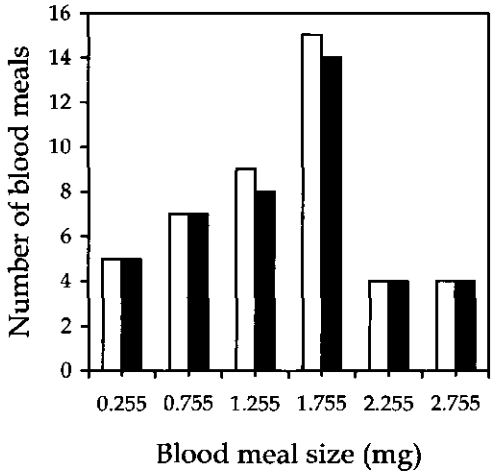

Figure 1. Frequency distribution of *Anopheles gambiae* Giles *sensu stricto* blood meals present (□) and successfully amplified (■) per size class for mosquitoes killed immediately after blood meal ingestion.

Effect of extent of blood meal digestion on profiling success

Analysis by logistic regression of the dependence of profiling success upon time for blood meals between 8 and 32 hours old found a significant negative relationship between time since ingestion and the success probability of obtaining PCR products (P < 0.001). At least half of the blood meals could be successfully profiled from mosquitoes killed within 15 hours of feeding at both TC-11 and VWA human genetic loci. After eight hours the proportion of blood meals which could be successfully profiled decreased slowly with time after ingestion dropping below 50% after approximately 15 hours. There was no significant difference in amplification success between meals of mosquitoes killed at 0 and 8 hours after

ingestion (P = 0.27). The number of blood meals that successfully amplified were 39/44 (89%), 9/10 (90%), 4/10 (40%), 1/10 (10%) and 0/10 (0%) (locus TC-11) and 32/44 (73%), 9/10 (90%), 4/10 (40%), 1/10 (10%) and 1/10 (10%) (locus VWA) for meals digested for 0, 8, 16, 24 and 32 hours respectively (Figure 2). Although slightly more meals were successfully amplified at locus TC-11 (53/84 i.e. 63%) than VWA (45/84 i.e. 54%) multiple regression analysis did not find a significant relationship between locus and amplification success (P = 0.35).

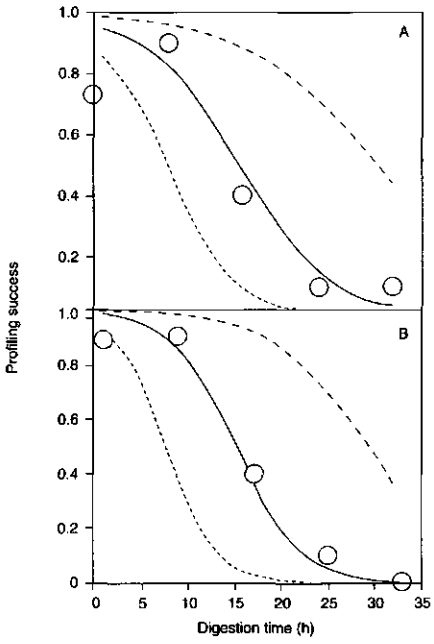

Figure 2. Effect of extent of digestion on success of amplifying human DNA from blood meals of *Anopheles gambiae* Giles *sensu stricto*. Panel A represents locus VWA and panel B locus TC-11. Dashed lines represent upper and lower confidence intervals defined by plus or minus one standard error of both $ß_0$ and $ß_1$. Solid lines describe the fitted logistic relationships between profiling success for each locus and time elapsed since feeding [Logit (profiling success) = $ß_0$ + $ß_1$ time]. Open circles denote observed profiling successes at 0, 8, 16, 24 and 32 hours of digestion.

Genetic profiling of mosquito blood meals

Analysis of blood meals of *An. gambiae* originating from the three test persons detected three distinct alleles at the TC-11 locus and 5 at locus VWA (Table 2). Although most blood meals yielded PCR products on first trial some did so on second or third attempts when template DNA was either doubled or tripled. The

Table 2. TC-11 and VWA locus alleles detected in blood meals of *An. gambiae* fed on persons X, Y and Z. Alleles are numbered from slowest (allele 1 at locus TC-11 and allele 2 at locus VWA) to the fastest migrating (allele 3 at locus TC-11 and allele 8 at locus VWA).

Person	Alleles detected (also see Figure 3)	
	Locus VWA	Locus TC-11
X	5,7	2,3
Y	5,8	1,2
Z	2,4	2,2

TC-11 alleles are shown in Figure 3 (see also Table 2). Persons X and Y were heterozygous (allelic genotypes 2,3 and 1,2 respectively) whilst person Z was homozygous (genotype 2,2). The homozygous allele of person Z allele 2) was also present in person X and Y. In addition, person X and Y had fast (allele 3) and slow migrating (allele 1) alleles, respectively. Both single and multiple blood meals were successfully profiled (Figure 3). Blank and unfed female, male and cow-fed mosquitoes (not shown) yielded no PCR product implying that only human, but not mosquito, DNA patterns were detected in the amplifying specimens.

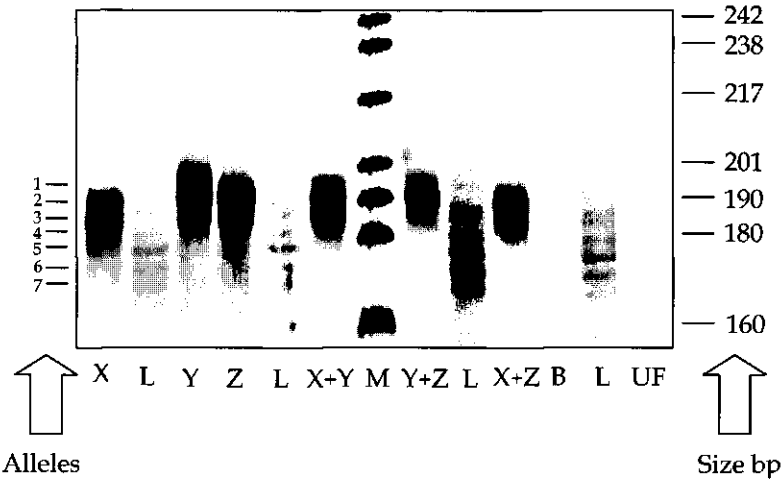

Figure 3. Human TC-11 locus profiles generated from blood meals of *An. gambiae.* Samples include single (X, Y and Z) and multiple meals (X+Y, Y+Z and X+Z) as well as allelic ladders (L) plus blank (B) and unfed female (UF) controls. Lengths (base pairs) of size-separated pBR322 marker DNA are shown (lane marked M). Alleles in the ladder are numbered from slowest (allele 1) to the fastest migrating (allele 7).

Discussion

This study demonstrated that extent of digestion but not blood meal size affects the success of amplifying human DNA from blood meals of *An. gambiae s.s.* Since the relationship between blood uptake and mosquito body size was not investigated, it cannot be inferred from these results that the success rate of DNA profiling was associated with mosquito size. The lack of a positive relationship between blood meal size and success of generating PCR products from the mosquito blood meals was not surprising because PCR amplification can be primed from single target molecules (Saiki *et al.*, 1988). Since 0.01 µl of human blood contains approximately 50 nucleated cells (Jeffreys *et al.*, 1988) and one

nucleated human blood cell contains approximately 6 pg DNA (Rees & Jones, 1972) the smallest and largest blood meals which successfully amplified were estimated to contain 2 and 82 ng DNA, respectively. The quantity of DNA could have even been higher because anopheline mosquitoes concentrate host blood cells, which contain the DNA, during feeding by pre-diuresis (Vaughan *et al.*, 1991; Clements, 1992).

The decline in profiling success over time greatly resembles the time profile observed in the disappearance of blood meal protein from midguts of *An. stephensi* (Liston) (Diptera: Culicidae) (Billingsley & Hecker, 1991) and *Ae. aegypti* (Linnaeus) (Diptera: Culicidae) (Briegel & Lea, 1975) which were held at 27°C as done in this study. Billingsley and Hecker (1991) observed a reduction in protein content first at 9 hours after feeding, the decline was rapid between 12 and 24 hours with the midgut protein content reaching less than 20% by 36 hours after feeding. The time profile associated with the degradation of host DNA in the mosquito midgut may thus be similar to that of protein digestion because DNA is contained within cells and packaged with chromatin proteins such as histones. Indeed, the decline in amplification success over time coincides the production pattern of late trypsin and the onset of the most rapid phase of blood meal digestion (Briegel & Lea, 1975; Noriega & Wells, 1999).

Although it was possible to profile blood meals digested for up to 32 hours after feeding, for maximising the proportion of meals that can be successfully profiled it is essential to time mosquito collection exercises such that fed mosquitoes are collected within eight hours of feeding. This emphasises the need for selecting suitable field-sampling methods that would allow quick and effective collection of blood-fed mosquitoes. For example, the use of exit or bednet traps may not only facilitate quick and effective collections but would also allow all-night collections in which trapped blood fed females are aspirated at selected time intervals and preserved before blood meals become adversely degraded (Service, 1977). Besides, if sampling cannot be done at night it is important to collect (and appropriately store) wild-caught mosquitoes at daybreak so that blood meals of mosquitoes that begin to host-seek and subsequently blood-feed in the early evening hours have a higher chance of being profiled successfully.

Even if blood fed mosquitoes are collected early enough and stored appropriately the probability of obtaining positive amplification reactions can still be limited by the potential effect of ambient temperature on the kinetics of blood meal digestion. Under natural conditions mosquitoes often expose to wide variations in environmental temperature, which can affect their metabolism. For example, it was demonstrated that *Ae. aegypti* mosquitoes digested their blood meals twice as fast when kept at 32°C as compared to 22°C (Briegel & Lea, 1975). Conversely, when night-time temperatures in the field are substantially cooler or warmer than the 27°C used in this study, the eight hour period of maximal amplification success may be lengthened or reduced respectively. Further studies describing the efficacy of typing blood meals of mosquitoes with different

abdominal appearances (e.g. fully fed, half gravid and sub gravid) are necessary in order to know beforehand what likelihood a field collected mosquito has of successful analysis.

Despite the recent rapid advances in molecular genetic technology, the usefulness of molecular biological tools for insect blood meal analysis remains under-utilised. The application of these tools for mosquito blood meal analysis needs to be thoroughly evaluated and harnessed for routine field application. A large number of loci suitable for identifying mosquito blood meal sources need to be tested so that closely related individuals within houses or from kin networks within villages can be distinguished with increased precision. Meanwhile, DNA profiling methods have been useful to verify the occurrence of multiple feeding (Ansell *et al.*, 2000; Chow-Shaffer *et al.*, 2000) in malaria-infected mosquitoes (Koella *et al.*, 1998) and to quantify the amount of protection provided by impregnated bednets (Gokool *et al.*, 1992; Gokool *et al.*, 1993). These methods could be further refined so that the extraction of DNA for mosquito species identification, parasite detection and host identification can be combined. By correctly selecting mosquito sampling time it should be possible to increase the reproducibility of DNA-based tests for mosquito blood meal identification as a tool for field entomology and epidemiology.

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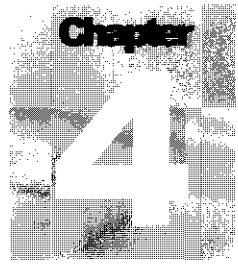
Part II

Host characteristics and differential attractiveness of humans to *Anopheles gambiae*



Host-specific cues cause differential attractiveness of human males to the malaria mosquito *Anopheles gambiae*[1]

Abstract: A new three-port olfactometer that accommodates human beings as sources of host-seeking stimuli was developed and used to study behavioural responses of *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*). Trap catches were significantly affected by the factor person ($P < 0.001$). Nine persons were classified into least, medium and most attractive groups. There was no significant interaction between person and trap ($P = 0.302$) or person and test period ($P = 0.223$). Presence ($P < 0.001$) or absence ($P = 0.949$) of significant differences in the number of mosquitoes caught per trap when tents were simultaneously occupied by one person in each or left empty, respectively, demonstrated that human residuum did not affect behavioural responses of the mosquitoes. It is concluded that variability in human attractiveness to *An. gambiae* arises from individual differences in complete body emanations. The results of this study are used to discern the need for the development of powerful independent mosquito sampling tools.

Introduction

Although there is evidence that some humans are more attractive to host-seeking mosquitoes than others (Lindsay, 1993; Knols *et al.*, 1995) the reasons for this variability are not clearly understood (Ribbands, 1950). Various studies have implicated some *Anopheles* species as preferring to feed on adults rather than children (Spencer, 1967; Muirhead-Thompson, 1951) and on men rather than women (Muirhead-Thompson, 1951). In *An. gambiae* s.l. the preference for feeding on adults rather than children (Thomas, 1951; Boreham *et al.*, 1978; Bryan & Smalley, 1978) has been attributed to size (Carnevale *et al.*, 1978), surface area and weight (Port *et al.*, 1980). In contrast, random feeding irrespective of age (Smith, 1956; Clyde & Shute, 1958) and sex in *An. gambiae s.l.* (Carnevale *et al.*, 1978; Port *et al.*, 1980) and age, height and weight among *An. punctulatus* complex mosquitoes has been reported (Burkot *et al.*, 1988). Some studies have shown that pregnancy

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(Lindsay *et al.*, 2000; Ansell *et al.*, 2002), parasite infection (Day *et al.*, 1983) and ABO blood group status (Wood *et al.*, 1972; Wood, 1974; 1976) influence human attractiveness to mosquitoes while others have refuted the influence of blood group (Thornton *et al.*, 1976; Burkot *et al.*, 1988) and parasite infection (Burkot *et al.*, 1989).

In two of the studies reported above it was postulated that variability in human attractiveness to mosquitoes is related to the amount of exhaled breath and volatile substances released from the skin (Carnevale *et al.*, 1978; Lindsay *et al.*, 2000). These hypotheses are plausible for at least two reasons. First, mammalian semiochemical blends are complex and include indicators of sex, health, age, reproductive status and diet (Brown, 1979). Second, mosquitoes are attracted to human hosts by body emanations (Takken, 1991; Takken & Knols, 1999) and body odour is responsible for > 90% of the attractiveness of humans to *An. gambiae* (Mboera *et al.*, 1997).

It has been hypothesised that body odour can influence the choice by mosquitoes of a particular individual upon encountering a group of human hosts (Knols *et al.*, 1995). Brouwer (1960) showed that odour emanating from hands and forearms caused individual differences of attraction to *An. stephensi* Liston. Khan *et al.* (1966) found that *Aedes aegypti* L. probed on the forearms of certain individuals more than it did on those of others. Schreck *et al.* (1990) reported significant differences in the response of *Ae. aegypti* and *An. quadrimaculatus* May to substances collected from hands of different human individuals. Although these studies provide an alternative reason for variability in human attractiveness to mosquitoes, the odour emanating from hands and forearms is not representative of that emanating from the entire body. Therefore we cannot conclude from these studies that complete body emanations are associated with differences in attractiveness of humans to mosquitoes as substances from different body parts of an individual can elicit significantly different mosquito behavioural responses (Schreck *et al.*, 1990).

Field studies in Burkina Faso demonstrated that differences in human attractiveness to *An. gambiae* and *An. funestus* Giles can be associated with olfactory cues released by the body, in particular carbon dioxide from expired air (Brady *et al.*, 1997). Brady and his colleagues utilised odour-baited entry traps (OBETs) that separated olfactory cues from visual features of the host and its convective or radiant heat (Costantini *et al.*, 1993). The OBETs have the inherent disadvantage of increasing experimental variance because of the varying experimental conditions of the tests (Costantini *et al.*, 1999). In the current study we investigated the effects of complete body emanations including body odour, heat and moisture on differential attractiveness of humans to *An. gambiae*. We sought to (i) develop an olfactometer that accommodates humans as sources of host-seeking stimuli and use it to test whether complete body emanations are associated with variability in attractiveness to mosquitoes; (ii) assess whether attractiveness of humans to mosquitoes can be ranked based on behavioural responses towards complete body emanations; and (iii) find out whether mosquitoes can be attracted to tents which have been previously occupied by humans in response to residual stimuli.

Materials and methods

Mosquitoes

Experiments were conducted using laboratory-reared *An. gambiae* mosquitoes established from specimens collected in Njage village, 70 km from Ifakara, Southeast Tanzania, in 1996. The mosquitoes were reared at ambient temperature and humidity at insectaries of the Mbita Point Research and Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE). Mbita Point is located on the Southern shore of the Winam gulf of Lake Victoria in Nyanza Province, Kenya (00°25' S, 34°13' E). Adult female mosquitoes were routinely offered a human arm to feed upon. Larvae were fed on Tetramin® fish food three times per day. The larvae were reared in plastic pans (25 × 20 × 14 cm) filled with fresh water from Lake Victoria to a depth of 3 cm. Trays contained 100 - 150 larvae. Pupae were collected daily and kept in mesh-covered cages (30 × 30 × 30 cm) containing 6% glucose solution on filter paper wicks. Adult females were used for experiments when 4 - 8 days old and with no prior access to a blood meal.

Experimental set-up

Experiments were conducted using an olfactometer designed to accommodate humans as sources of host-seeking stimuli (Figure 1). The set-up consisted of three tents connected to a collecting system by polyvinyl chloride (PVC) pipes. The collecting system consisted of one choice chamber and three trap chambers, all covered with a single top-lid. The choice chamber opened into mesh-covered collecting cages (12 × 12 × 12 cm), placed inside the trap chambers, through 8-cm (mouth diameter) funnels made of netting mesh. The collecting system, pipes and tents were covered with opaque polythene sheets to exclude visual cues. Tent-ends proximal to the collecting system had stockinette sleeves in which the pipes were fitted. Pipe-ends debouching into trap chambers plus the fan-pipe, at its point of connection on the top lid, were covered with panels of mosquito netting. The set-up was housed inside a large screen house (perimeter, 11 × 6 metres) lined with a mosquito net barrier along the walls on the inside. The roof of the screen house was covered with glass and the sides with mesh (density 90%). A layer of reed mats was placed beneath the roof so as to lower temperatures. The fan pipe was extended through the screenhouse wall, so delivering odour laden air outside the screenhouse.

Mosquito behaviour in the absence of host emanations

The symmetry and neutrality of the olfactometer was tested by releasing mosquitoes and noting the number caught per trap in the absence of host-seeking stimuli. Tests were conducted between 20.00 - 21.00, 22.00 - 23.00 and 24.00 - 01.00 hours. About 100 mosquitoes, starved for 6 hours, were released during each test period.

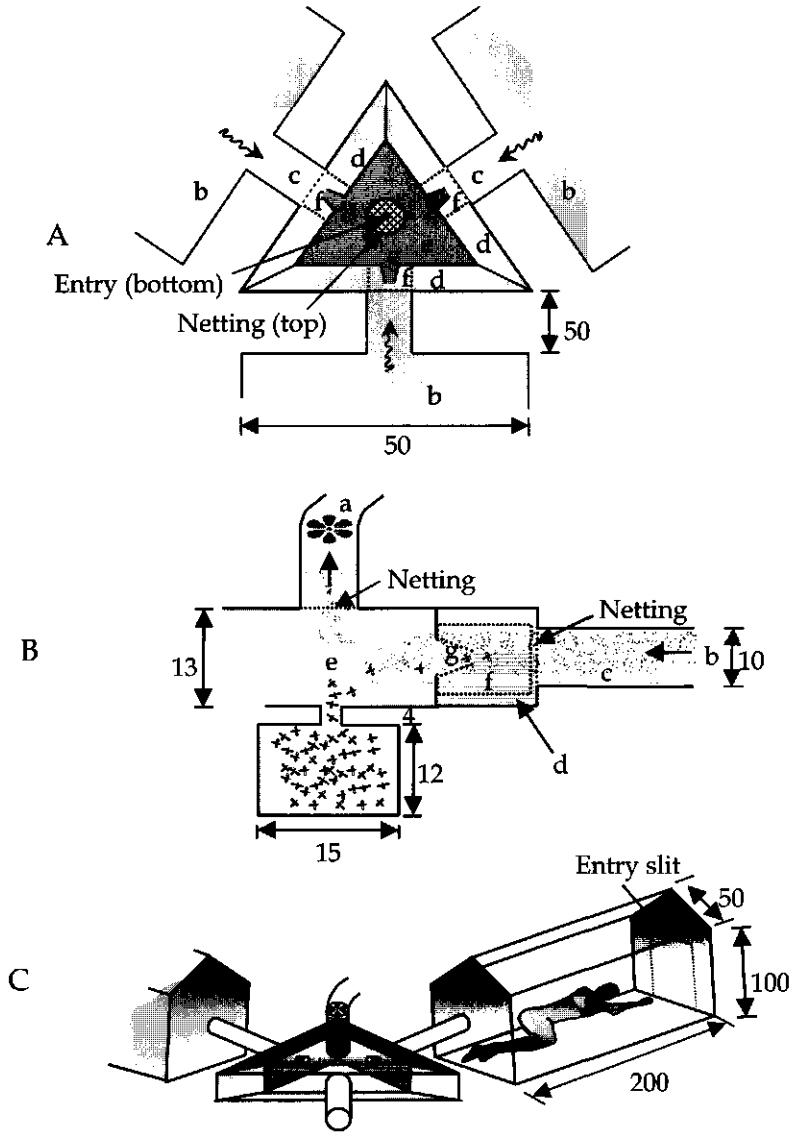

Figure 1. Top (A), cross-section (B) and three-dimension (C) sketches of the experimental set-up. The fan (**a**, see cross section view) drew air (130L/min/per tent) from the tents (**b**) to the outside environment via PVC pipes (**c**), trap chambers (**d**) and a choice chamber (**e**). Each trap chamber contained a collecting cage (**f**) into which an exit trap (**g**) opened. The fan-pipe and the release cup were attached to the choice chamber from the top and underside of the choice chamber, respectively. Mosquitoes emanating from a release cup flew upwind and were trapped in the collecting cages. All mosquitoes were removed from the choice chamber, release cup and collecting cages between experiments. The diagrams are not drawn to scale. Dimensions are in centimetres.

Mosquito behaviour in the presence of human emanations

Nine male humans aged between 18 and 22 years old were selected to participate in experiments designed to establish whether differences in attractiveness to mosquitoes can be discerned based on total body emanations. The persons were designated P_{CAB}, P_{FAG}, P_{FOK}, P_{KMW}, P_{KOK}, P_{MKA}, P_{ROL}, P_{WOK} and P_{WOT}. No special criteria were used to select the persons. Three persons were compared for their attractiveness to the mosquitoes within a single experiment. The attractiveness of each person was compared to that of P_{CAB} at least three times. Three experiments were carried out per night at 20.00 - 21.00, 22.00 - 23.00 and 24.00 - 01.00 hours. Either 50 or 100 female mosquitoes, starved for 6 hours, were used in each experiment. Mosquitoes exiting the release cup into the choice chamber encountered total body emanations including odour, heat and moisture and were caught in trap chambers as they flew upwind towards the stimuli sources i.e. persons present in tents.

The number of mosquitoes caught in each trap chamber was considered as having been attracted by the person sleeping in the adjacent tent. Persons were alternated between tents, completing a Latin square design on each experimental night. The persons shifted with their bedding material. The participants (i) lay with their feet proximal to the collecting system (ii) bathed with non-perfumed soap one hour prior to the first experimental period (iii) did not use perfumes or deodorants during the recruitment period (iv) only wore a pair of short trousers while inside the tents and (v) did not cover themselves with sheets or blankets during the experiments. Smears of their blood were examined microscopically for the presence of malaria parasites after staining with field's A and B stains. Diets of the test persons were not taken into account except prohibiting them from ingesting alcohol, which has recently been shown to affect the attractiveness of humans to mosquitoes (Shirai *et al.*, 2002). However, the diet of the people of Mbita Point is somewhat fixed, *Ugali* (a type pasta made from maize meal) served with green vegetables (mainly collards) is consumed virtually every day for lunch and supper.

Effects of residual odour on mosquito behaviour

The effects of residual human emanations on mosquito behaviour were studied by releasing mosquitoes when all 3 tents of the olfactometer were occupied by one human subject in each (at 20.00 to 20.45 hours), followed by a second release when the tents were unoccupied (21.30 to 22.15 hours). The persons who participated in these experiments were P_{FAG}, P_{MKA} and P_{KOK}, only bedding material were present in the tents during the second test period. Each experiment utilised 100, 6-hour starved female mosquitoes. The human subjects were not shifted between tents. The number of mosquitoes that was trapped during each test period was noted.

Statistical analysis

The number of mosquitoes caught in the presence and absence of host-seeking stimuli with respect to trap, person and test period were analysed by log-linear modelling (Agresti, 1990). Log-linear modelling allowed for differences between traps, days, test periods and persons. Parameter estimates provided an index of attractiveness of each person. Relatively higher estimates corresponded to high degrees of attractiveness and small estimates to low degrees of attractiveness. Parameter estimates were calculated with reference to person P_{CAB} as the standard. Pairwise t-probabilities were calculated to establish the significance of differences in the number of mosquitoes attracted to the participants: ranks of attractiveness were assigned based on this criterion. Data were analysed using the General statistical computer software program (Payne, 1986).

Ethical clearance

Informed consent was obtained from all nine human participants. The project was approved by the Kenya National Ethical Review Committee at the Kenya Medical Research Institute.

Results

Microscopic examination of blood smears detected no malaria parasites in the participants' blood during the experimental period. The experiments were conducted between January and March 2000, a part of the dry season preceding the long rains.

Mosquito behaviour in the absence of human emanations

Experiments in which mosquitoes were released in the absence of humans demonstrated that the olfactometer was symmetrical as entry responses did not differ significantly between traps ($P > 0.05$). The number of mosquitoes caught in trap A were not significantly different from those caught in trap B ($P = 0.808$) or trap C ($P = 0.147$). Of the mosquitoes released only 9.6% (49 of 512) were captured in the trap chambers (Table 1), the rest were either in the choice chamber or release cup. The number of mosquitoes trapped varied from 0 to 9. There was no significant difference in the number of mosquitoes trapped during test period I and II ($P = 0.594$) or test period I and III ($P = 0.147$). There was also no significant interaction between test period and trap ($P = 0.138$).

Mosquito behaviour in the presence of human emanations

Forty six percent (1688 of 3673) of the mosquitoes that were released in the experiments conducted with nine human subjects, three of whom were simultaneously present each time, were recaptured in the collecting cages. The rest were recaptured in the choice chamber or release cup. The experiments were conducted over 16 days encompassing a total of 48 test periods. The model with the

best fit incorporated the factors person and trap and the interactions between (i) person and trap, (ii) person and test period and (iii) day and test period. The number of mosquitoes caught per trap were significantly affected by the factor person ($P < 0.001$). It was possible to classify the persons into high (P_{FAG} and P_{CAB}), medium (P_{KOK}, P_{KMW}, P_{WOT} and P_{ROL}) and low attractiveness (P_{WOK}, P_{FOK} and P_{MKA}) groups. Person P_{FAG} was the most attractive whereas P_{MKA} was the least attractive. Parameter estimates for the persons are shown in Table 2. Although the behavioural responses of the mosquitoes were significantly affected by the factor trap ($P = 0.028$), there was no significant interaction between person and trap ($P = 0.302$) or person and test period ($P = 0.223$).

Table 1. The number of mosquitoes caught per trap in the absence of host-seeking stimuli. N, the number of replicates. n, the total number of mosquitoes trapped per test period also expressed (in parentheses) as a proportion of the total number of mosquitoes released.

Test period	N	Number of mosquitoes trapped (mean)			n (proportion)
		Trap A	Trap B	Trap C	
20.00 – 21.00	2	8	9	3	20 (0.12)[a]
22.00 – 23.00	2	6	8	6	20 (0.12)[a]
24.00 – 01.00	2	3	1	5	9 (0.05)[a]
Total	6	17 (2.833)[a]	18 (3.00)[a]	14 (2.33)[a]	49 (0.096)

Numbers followed by the same letter (between traps or test periods) are not significantly different ($P > 0.05$).

Table 2. The mean number of mosquitoes attracted to each of 9 persons and parameter estimates calculated for the individual persons from the log-linear model. Calculations used person P_{CAB} as the reference standard. Ranks of attractiveness are based on the level of significance of differences in the number of mosquitoes attracted to pairs of persons. N is the number of replicates and s.e. is the standard error of the mean number of mosquitoes attracted by each person.

Person	N	Estimate (ß)	Mean catch ± s.e.	Rank of attractiveness
P_{CAB}	21	0	20.14 ± 3.17 a	1
P_{FAG}	15	0.030	18.20 ± 3.65 a	1
P_{KMW}	21	- 0.667	11.95 ± 1.83 b	2
P_{KOK}	21	- 0.651	11.33 ± 1.72 b	2
P_{ROL}	12	- 0.843	9.92 ± 1.55 b	2
P_{WOT}	9	- 0.711	9.67 ± 3.54 b	2
P_{WOK}	18	- 1.155	6.78 ± 1.01 c	3
P_{MKA}	15	- 1.204	6.73 ± 1.41 c	3
P_{FOK}	12	- 1.193	6.17 ± 1.12 c	3

Numbers not followed by the same letter are significantly different ($P < 0.05$)

Effects of residual odour on mosquito behaviour

A significant effect of treatment was demonstrated when tents were occupied by one subject in each ($P < 0.001$). These differences were attributed to the factor person as participants did not shift between tents. P_{FAG} attracted 2.7 and 5.6 times as many mosquitoes as P_{KOK} ($P < 0.001$) and P_{MKA} ($P < 0.001$), respectively. The effect of trap was not significant in the subsequent test period when the tents were empty ($P = 0.949$). Trap A had 1.1 and 1.0 times as many mosquitoes as trap B ($P = 0.869$) and C ($P = 0.873$), respectively. Mosquito catches in the presence and absence of human subjects are shown in Table 3.

Table 3. The number of mosquitoes caught per trap in the presence (test period I, 20.00 - 20.45 hours) and absence (test period II, 21.30 - 22.15 hours) of host-seeking stimuli. Traps A, B and C were linked to tents occupied by persons P_{FAG}, P_{MKA} and P_{KOK}, respectively. n, the total number of mosquitoes trapped per test period also expressed (in parentheses) as a proportion of the total number of mosquitoes released.

Day	Test Period	Number of mosquitoes trapped			n (proportion)
		Trap A	Trap B	Trap C	
1	I	36	10	12	58 (0.55)
	II	2	4	2	8 (0.09)
2	I	33	3	8	44 (0.49)
	II	8	8	8	24 (0.25)
3	I	49	9	14	72 (0.72)
	II	5	2	5	12 (0.13)
4	I	27	4	20	51 (0.53)
	II	4	4	5	13 (0.13)
Total	I	145 (0.64)[a]	26 (0.12)[b]	54 (0.24)[c]	225 (0.57)
	II	20 (0.34)[a]	18 (0.31)[a]	20 (0.34)[a]	58 (0.15)

Numbers not followed by the same letter in the same row are significantly different ($P < 0.001$).

Discussion

The tent olfactometer developed in the current studies demonstrated the ability to discern differences in human attractiveness to mosquitoes based on complete body emanations. The set-up barred the potential interference of host irritability and defensive behaviour on the mosquito responses. Interestingly, the mosquitoes preferred certain individuals despite being presented with emanations of three persons simultaneously. This demonstrates the great discriminatory power exhibited by the mosquitoes in finding their blood-meal hosts. This capacity may be intrinsic as *An. gambiae* has been found to bite humans selectively in settings where

mixing of attractant stimuli is inevitable e.g. inside shared bedrooms or houses (Boreham *et al.*, 1978; Bryan & Smalley, 1978; Port *et al.*, 1980). However, we have no proper reasons to explain the basis of this high discriminatory power; follow-up experiments found that this was not related to reproductive fitness (W.R.M., Unpublished data).

Despite that there seems to be no immediate biological reason for the mosquitoes being more readily attracted by certain individuals, it is certain that they did so in response to factors present in the persons' total body emanations which included odour, heat and moisture. Variability in the attraction of black flies (Schofield & Sutcliffe, 1996) and the mosquitoes *An. gambiae* and *An. funestus* (Brady *et al.*, 1997) to body emanations has been attributed to differences in carbon dioxide (CO_2) output rates. Carbon dioxide is thought to elicit take-off or sustain flight (Gillies, 1980) in the short- and medium-range phases of host location (Gillies and Wilkes, 1969). However, CO_2 may not be a good kairomone for *An. gambiae* because it is not host-specific (Knols *et al.*, 1997). In fact, *An. gambiae* is preferentially attracted to human body odour in disfavour of CO_2 (Mboera *et al.*, 1997). In wind tunnel studies, carbon dioxide has been shown to cause an inhibitory or neutral effect on the behaviour of *An. gambiae* (Takken *et al.*, 1997; Dekker *et al.*, 2001).

The cues that impact greatly on mosquito orientation in the close vicinity of the host comprise body heat and moisture (Gillies and Wilkes, 1969). These factors may explain the differences in human attractiveness currently reported as mosquitoes were released ~1 m downwind of the participants inside tents. Black flies tend to bite man at rates that are partially related to inter-individual variation between skin and ambient temperatures (Schofield & Sutcliffe, 1997). Nonetheless, the role of olfaction in the short-range attraction of mosquitoes cannot be discounted (Takken, 1991).

Even if residual human effluvia may have been left behind after participants exited the tents, such cues did not elicit positive mosquito behavioural responses. This result corroborates that of Braks (1999) who found no preferential attraction of *An. gambiae s.l.* to an air stream exhausted from a tent containing unwashed clothing and bedding material. However, although there have been higher catches of *An. gambiae s.l* in huts containing worn clothing as opposed to empty ones (Haddow, 1942), there are only few field reports of attraction of *An. gambiae* to residual stimuli. Accordingly, Braks (1999) suggested that the kairomones that induce behavioural responses in *An. gambiae* may be highly volatile being produced continuously from a living host but lost rapidly from worn clothing. Ecologically, it is beneficial for a blood-feeding insect to pursue stimuli that signify the physical presence of a host rather than those that do not. Recently, Braks *et al.* (2001) found that the residual effect of human sweat was lost after as few as 20 minutes. It remains interesting though that in our set-up (Table 3) sometimes more mosquitoes were caught in tent B and C during the second (no human emanations present) than in the first (human emanations present) test periods. This shows that the number of mosquitoes caught by the most attractive individual had an effect on

those caught by the other participants. However, the absence of significant differences in the number of mosquitoes caught between traps in the second test period implies that there were no individual-specific residual odour effects on the number of mosquitoes caught between tents.

The olfactometer developed in the current study could be used in various ways in studies of insect behaviour. First, the system can be manipulated so as to separate between components of total body emanations allowing to study the effect of major fractions on insect behaviour (see Chapter 5). Second, as differences in attractiveness quantified based on man landing catches (MLC) apparently resemble those measured using odour baited entry traps (Brady *et al.*, 1997), the set up may serve to replace MLC as its working principle is very similar to that of OBETs (Costantini *et al.*, 1993). However, care needs to be taken as the degree of attractiveness could differ depending on whether measurements are based on cues involved in short- or long-range orientation (Costantini *et al.*, 1999; Schofield & Sutcliffe, 1996; 1997). Third, the olfactometer offers a unique opportunity where effects of parasite infection and pregnancy on attractiveness can be examined with minimal ethical concerns. Fourth, the olfactometer offers the possibility to trap volatiles from individuals while at the same time conducting behavioural assays. In this way chemical bases responsible for differential attractiveness of humans to mosquitoes can be applauded more confidently.

The determination of olfactory bases responsible for variability in human attractiveness to mosquitoes will have several applications in terms of malaria control. First, once factors associated with increased attractiveness of humans have been identified it should be possible to develop traps that are suitable for use in mosquito surveillance. For example, the development of an odour-baited trap that utilises human-specific infochemicals has the potential advantage of sampling mosquitoes that are reflective of the true host-seeking population. This can, in turn, enable more precise estimation of important parameters such as the entomological inoculation rate without putting humans at the risk of infection posed through performing man landing catches. Second, identified semiochemicals could also be used as lures in mass trapping as a means of vector control (Day & Sjogren, 1994). Third, the attractant compounds identified may be applied on hosts that are less desirable to mosquitoes so as to deviate host-seeking mosquitoes away from humans (Costantini *et al.*, 1999).

In conclusion, the results of this study provide additional evidence that variability in attractiveness of humans to *An. gambiae* exists and can be associated to differences in the composition of complete body emanations encompassing body odour, heat and moisture. Although the exact component(s) responsible for the variability in attractiveness remain unknown, the efficacy of sampling host-seeking mosquitoes using total body emanations in human baited traps is already being exploited (Costantini *et al.*, 1993; Mathenge *et al.*, 2002). However, the aim of developing odour-baited traps for mosquito control will remain unachieved until key factors associated with increased attractiveness to host-seeking mosquitoes

have been identified and harnessed for use in trapping devices. Only then shall the potential of using information gained in studies of the chemical ecology of mosquito host-seeking behaviour be tapped in strategies aimed at controlling mosquito vectors through an integrated approach.

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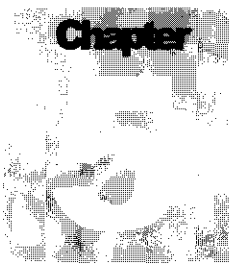

The role of breath and body odour in the relative attractiveness of humans to *Anopheles gambiae*[1]

Abstract: The contribution and possible interaction of breath and body odour in the attraction of *Anopheles gambiae* to humans was investigated by conducting dual choice tests using a tent olfactometer. Experiments conducted with one human subject (person PI) as bait found his body odour and total emanations (breath plus body odour) to collect more mosquitoes than a negative control (empty tent) ($P = 0.005$ and < 0.001, respectively). His body odour and the control collected more mosquitoes than his breath ($P < 0.001$ and 0.034, respectively). Total emanations of PI attracted more mosquitoes above those of another person designated PII ($P = 0.021$) whereas breath of PII attracted more mosquitoes than breath of PI ($P < 0.001$). The attractiveness of body odour of PI versus body odour of PII did not differ ($P = 0.346$). Body odour from either individual was consistently more attractive than total emanations from the other ($P = 0.001$). We conclude that breath, although known to contain attractive semiochemicals like carbon dioxide, may also contain compounds that inhibit attraction and may thus serve as an important contributor to between-person differences in relative attractiveness to this malaria vector.

Introduction

Host-seeking mosquitoes utilise a combination of visual, physical and chemical cues to locate their blood meal hosts (Takken, 1991, Takken & Knols, 1999). Vision is much more important among diurnally active mosquitoes (Allan *et al.*, 1987) whereas physical and olfactory cues are presumably the dominant cues for nocturnal species. Many species of blood-feeding insects, even those in the same species, are attracted to varying extents by individual hosts. The sandfly *Lutzomyia longipalpis* is attracted to hand odour of different humans at significantly different rates (Hamilton & Ramsoondar, 1994) and attraction of *Simulium* species to total human emanations varies depending on the individual person used as the source (Schofield & Sutcliffe, 1996). Attractiveness of human arm and hand odour to

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Anopheles stephensi (Brouwer, 1960), *Aedes aegypti* (Khan *et al.*, 1966; Mayer & James, 1969; Schreck *et al* 1990) and *An. quadrimaculatus* (Schreck *et al.*, 1990) has been shown to vary substantially between individuals. The response of members of the *An. gambiae* complex to individual humans differs considerably (Knols *et al.*, 1995, Lindsay *et al.*, 2000), and such between-host differences are also seen in traps baited with total body emanations, including those from which the body heat component has been excluded (Costantini *et al.*, 1993; Brady *et al.*, 1997). Recently it was shown that removal of exhaled air from total emanations (Schofield & Sutcliffe, 1996) or artificially standardising carbon dioxide outputs (Brady *et al*, 1997) eliminates differential attraction of humans to blackflies and mosquitoes, respectively. We therefore used a dual-choice trap olfactometer to investigate how breath and body odour contribute to and might possibly interact in the attractiveness of humans to *An. gambiae*, one of the most highly anthropophilic, abundant and efficient vectors of malaria in Africa.

Materials and methods

Mosquitoes

Experiments were conducted using the Ifakara strain of laboratory-reared *An. gambiae* s.s., originally colonized from wild-caught gravid females in Njage, South-east Tanzania, in 1996. The mosquito larvae were reared under ambient temperature and light conditions in screen house insectaries of the Mbita Point Research and Training Centre of the International Centre of Insect Physiology and Ecology (00°25'S, 34°13'E). The larvae were reared using fresh water from Lake Victoria and were fed on Tetramin® fish food three times per day. Pupae were collected from rearing trays and transferred to an adult insectary where they were kept in mesh-covered 30 cm cubic cages in which 6% glucose solution soaked in rolls of filter-paper was provided. The colony was maintained by routinely offering a human arm to feed upon. Adult females with no prior access to blood were used for experiments when they were 4 - 8 days old and were transferred from the holding cages into release cups 6 hours before the onset of experiments. Only water-wet cotton wool pads were provided on the mesh-topped open ends of the release cups.

Experimental set-up

Experiments were conducted using two arms of a previously described (Chapter 4) three-arm tent olfactometer (Figure 1), within a large semi-field screenhouse where the ambient atmospheric conditions were not controlled (Chapter 4). Approximately 100 mosquitoes (the exact number was recorded for each experimental release), released into a choice chamber located ~1 metre away from the participants, were used for each experiment.

Human subjects

Two healthy African males designated person I (PI) and person II (PII) were recruited to participate in the experiments. PI was aged nineteen years (weight, 80 kg; height, 1.80 m) and PII was aged 22 years (weight, 79 kg, height 1.85 m). The participants only wore short trousers at the time of the experiment and bathed with un-perfumed soap one hour before starting the first experiment of a night. No attempt was made to control their daily diet except prohibiting them from ingesting alcohol, a factor that has recently been shown to influence the relative attractiveness of humans to *Aedes albopictus* (Shirai *et al.*, 2002). However, the diet of the people of Mbita Point is somewhat fixed, *Ugali* (a pasta made from maize meal) served with green vegetables (mainly collards) or fish is consumed virtually every day. The participants' malaria infection status was tested daily by microscopic examination of thin and thick smears of finger-prick blood stained with Giemsa. Previous experiments (Chapter 4) had found person I to be significantly more attractive to the mosquitoes than person II (person I and II were designated P_{CAB} and P_{WOK} in the previous experiments).

Attraction of An. gambiae to total emanations, body odour and breath

Person I was recruited to assess the attraction of mosquitoes to his total body emanations, breath and body odour. Here, total emanations refer to breath plus all volatile discharges of the skin; body odour refers to volatiles discharged solely from the skin. Breath and body odour were separated using a one-way breathing valve (Harvard-Douglas®). The participant wore the breathing valve by the mouth-piece and clipped a sprung nose clip on his nose so that he inhaled and exhaled air through the mouth only. Air was breathed in from within the confines of the screen house via a polyvinyl chloride (PVC) pipe and breathed out via a bendable, corrugated PVC pipe. The breath was discharged to a destination dictated by the needs of each experiment (Figure 1). Separated breath was either recombined with body odour to reconstitute the total body emanations (I), diverted to the other tent exhaust (II), or vented from the apparatus completely (III).

The first of these three alternative arrangements (I) allowed the attractiveness of total body emanations to be compared with a (negative) control tent lacking a bait host or body emanations. The second and third of these three arrangements allowed the attractiveness of body odour to be compared with breath (II) and with a control tent lacking a bait host or body emanations (III). The attractiveness of breath, compared with an empty tent was also assessed: the participant sat outside the tents but breathed into one of the tent exhausts (IV). Experiments were conducted over 30-minute test periods, at 19.30 - 20.00 and 20.30 - 21.00 hours, following each of which all mosquitoes were removed from the apparatus and the number trapped counted. Each of the four possible arrangements were repeated 8 or 16 times with the human bait host switching between the two tents so that half of the experiments were conducted with the human subject in the two alternative tents (Table 1).

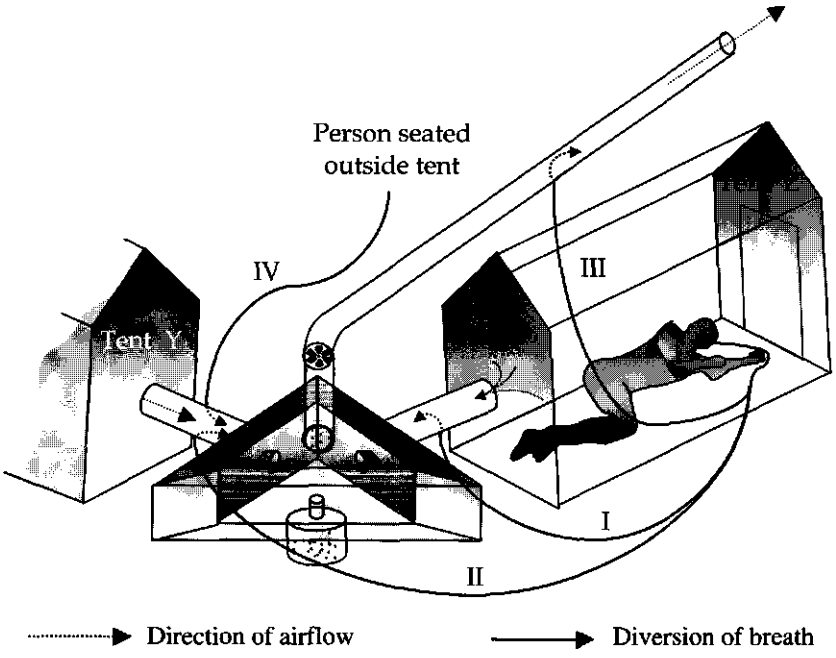


Figure 1. Apparatus used to study the attraction of *An. gambiae* to human breath, body odour and total emanations. Breath exhaled by a participant present in one tent was separated from body odour using a one way breathing valve and diverted to the exhaust of the same (I) or separate tent (II), or vented out (III). Alternatively, the participant sat outside the tents and his breath was diverted to one of the tent exhausts (IV).

Table 1. Experimental design showing location of the human subject (person I) and place of discharge of his breath in experiments evaluating the attraction of mosquitoes to breath (BR), body odour (BO) and total emanations (TE).

Treatment (arrangement)	Location of human subject	Diversion of breath
1a. TE versus control	Tent Y	To the exhaust of tent Y
1b. TE versus control (I)	Tent Z	To the exhaust of tent Z
2a. BO versus BR	Tent Y	To the exhaust of tent Z
2b. BO versus BR (II)	Tent Z	To the exhaust of tent Y
3a. BO versus a control	Tent Y	To the main vent
3b. BO versus a control (III)	Tent Z	To the main vent
4a. BR versus a control (IV)	Neither tent Y nor Z	To the exhaust of tent Y
4b. BR versus a control	Neither tent Y nor Z	To the exhaust of tent Z

The role of breath and body odour in between-person differences in relative attractiveness to An. gambiae

Behavioural responses of mosquitoes as a result of simultaneous exposure to emanations originating from two human subjects were assessed so as to determine their relative attractiveness when breath was included or excluded from their total emanations. Breath was removed from the apparatus using a one-way breathing valve as shown in path III (Figure 1). Inclusion of breath did not involve re-direction as shown in path I, the human subject simply occupied the tent without using the breathing valve. The following choice tests were carried out: (i) total emanations of person I versus total emanations of person II (experiment 1), (ii) breath of person I versus breath of person II (experiment 2), (iii) body odour of person I versus body odour of person II (experiment 3), (iv) body odour of person I versus total emanations of person II (experiment 4) or (v) body odour of person II versus total emanations of person I (experiment 5) (Table 2). Again, the term total emanations refers to breath plus all volatile discharges of the skin; and body odour refers to volatiles discharged solely from the skin. Experiment 2 was conducted with both participants outside the tents, each of them used a breathing valve in order to direct his breath to a separate tent exhaust. The number of mosquitoes trapped in all comparisons as a result of responses to stimuli originating from person I or II were counted and noted. Experiments were conducted thrice per night: at 19.30 - 20.00, 20.30 - 21.00 and 21.30 - 22.00 hours. Each of the five experiments was repeated 12 or 18 times with the two human subjects switching between the two tents so that half of the tests were conducted with each subject in the two alternative tents. The number of replicates conducted for each experiment are shown in Figure 3.

Table 2. Experimental design showing the type of emanations discharged by each of two test subjects in experiments evaluating the contribution of breath and body odour to between-person differences in relative attractiveness to the malaria mosquito *An. gambiae.*

Experiment	Type of emanation discharged	
	Person I (PI)	Person II (PII)
1	Total emanations (TE)	Total emanations (TE)
2	Breath (BR)	Breath (BR)
3	Body odour (BO)	Body odour (BO)
4	Body odour (BO)	Total emanations (TE)
5	Total emanations (TE)	Body odour (BO)

Statistical analysis

Relative attractiveness was calculated as the number of mosquitoes trapped by the emanations of PI divided by the sum of the number trapped by PI and PII. Thus relative attractiveness of greater than 0.5 indicates greater attractiveness of person PI whereas relative attractiveness of less than 0.5 indicates greater attractiveness of PII. Non-parametric statistical methods were used for analysis because of their robustness and flexibility. The significance of differences in attractiveness between the baits in the two traps in each experiment was assessed by Kendall's W test for related samples, comparing the catches of person PI with those of person PII in the same experiment. The significance of changes in relative attractiveness between experiments was assessed by the Kruskal-Wallis H test for independent samples, comparing the relative attractiveness estimates from repetitions of the same experiments with those of another. Analysis of the attractiveness of body odour of person I versus his breath or the control as well as the attractiveness of his total emanations or breath versus the control followed the same procedures. Data were analysed using the Statistical Products and Service Solutions (SPSS, version 10.0) software program.

Results

Experiments were carried out over a total of forty-eight nights, 24 nights for experiments involving the one human subject (PI) and 24 nights for experiments concerning the two participants (PI and PII). None of the participants presented with malaria parasites over the duration of the study.

Attraction of **An. gambiae** *to total emanations, body odour and breath*

The attractiveness of body odour of person I versus his breath or a control as well as the attractiveness of his total emanations or breath versus a control are shown in Figure 2. His body odour ($P = 0.005$) and total emanations ($P < 0.001$) were significantly more attractive than a control (empty tent) as was his body odour ($P < 0.001$) and the control ($P = 0.034$) over breath.

The role of breath and body odour in between-person differences in relative attractiveness

The between-person differences in relative attractiveness which were measured with respect to person I (i.e. the number of mosquitoes trapped by emanations of person I divided by the sum of the number of mosquitoes trapped by emanations of person I and person II) following inclusion or exclusion of breath or body odour from their total emanations are shown in Figure 3. Person I was more attractive than person II based on mosquito responses to their total emanations ($P = 0.021$) whereas person II was more attractive than person I based on responses to their breath ($P < 0.001$). There was no significant difference in the attractiveness of the two persons based on responses to their body odour ($P = 0.346$). Body odour of

person I was more attractive than total emanations of person II (P = 0.001) and body odour of person II was more attractive than total emanations of person I (P = 0.001). Thus, total emanations without breath from either individual was consistently more attractive than total emanations from the other. Comparisons of the relative attractiveness of the study subjects between experiments (lower section of Figure 3) were all significant except between experiment 1 and experiment 3 (P = 0.253).

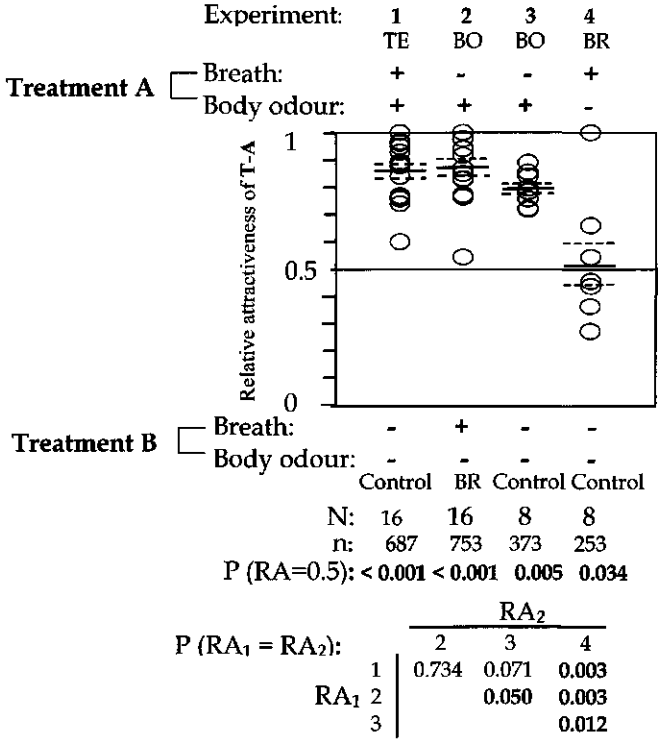
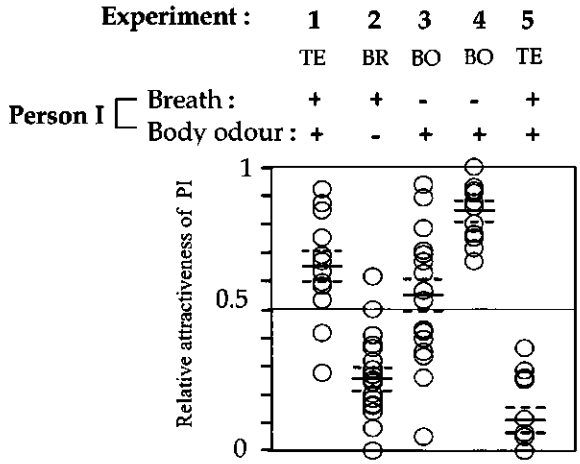


Figure 2. Relative attractiveness of total emanations (TE) of person I versus a control (experiment 1), his body odour (BO) versus his breath (BR) (experiment 2), his body odour versus a control (experiment 3) and his breath versus a control (experiment 4). N, number of replicates and n, the total number of mosquitoes collected by both treatments in each experiment. P, the statistical significance of (i) differences between the catches of treatment A (T-A) and treatment B (RA = 0.5) or (ii) the change in the relative attractiveness of treatment A between different experiments ($RA_1 = RA_2$). The solid and dotted horizontal lines depict the mean and standard error of the relative attractiveness of treatment A, respectively.



$P(RA_1 = RA_2)$:

RA_1 \ RA_2	2	3	4	5
1	<0.001	0.253	0.006	<0.001
2		<0.001	<0.001	<0.017
3			<0.001	<0.001
4				<0.001

Figure 3. Relative attractiveness (RA) of person I in choice experiments evaluating mosquito responses to total emanations (TE) of person I (PI) versus total emanations of person II (experiment 1), breath (BR) of person I versus breath of person II (experiment 2), body odour (BO) of person I versus body odour of person II (experiment 3), body odour of person I versus total emanations of person II (experiment 4) and total emanations of person I versus body odour of person II (experiment 5). N, number of replicates and n, the total number of mosquitoes collected from both treatments in each experiment. P, the statistical significance of (i) differences between the catches of person I and person II (RA=0.5) or (ii) the change in the relative attractiveness of person I between different experiments ($RA_1 = RA_2$). The solid and dotted horizontal lines depict the mean and standard error of the relative attractiveness of person I, respectively.

Discussion

The attraction of *An. gambiae* as assessed through choice experiments among all possible dual comparisons of total emanations, body odour, breath and a control done using one human subject demonstrated an inhibitory effect of breath and an attractant effect of body odour and total emanations. Whereas the inhibitory effect of breath was not known previously, total emanations have been shown to be responsible for over 90% of the attractiveness of humans to *An. gambiae s.l.* (Mboera *et al.*, 1997).

Surprisingly, there was no difference in the number of mosquitoes attracted by two persons, who were otherwise consistently different in their attractiveness, when breath was simultaneously excluded from their total emanations. Since host seeking is modulated by olfactory cues (Takken, 1991), and *An. gambiae* prefers human rather than other vertebrate-host cues/odours (Costantini *et al.*, 1998; Pates *et al.*, 2001), regardless of a person's degree of attractiveness, we suggest that breath is a key factor responsible for variability in human attractiveness to *An. gambiae.* The current findings are consistent with observations in other blood-feeding insects: removal of breath from total emanations has been shown to eliminate individual differences of attractiveness of humans to *Simulium* species (Schofield & Sutcliffe, 1996) and artificially standardising outputs of carbon dioxide, a major component of breath, has been shown to equalise human attractiveness to *An. gambiae s.l.* and *An. funestus* (Brady *et al.*, 1997).

The possibility of an interaction between components of breath and body odour is also plausible as the attractiveness of person I, who was significantly more attractive than person II based on responses to their total emanations, was reversed when mosquitoes were allowed to make choices between their breath. This suggestion is supported by some laboratory experiments. Homogenous plumes containing skin odour have been shown to result in trap entry whereas homogenous plumes containing carbon dioxide, a major constituent of breath, have been shown to reduce trap catches of *An. gambiae* and *Ae. aegypti* (Dekker *et al.*, 2001). Furthermore, a homogenous plume containing skin odour and carbon dioxide also reduced trap catches. Thus, the inhibitory effect of breath seen in the current studies may have been caused by carbon dioxide.

Nevertheless, the between-person differences in relative attractiveness to *An. gambiae* seen in this study cannot be solely explained by carbon dioxide output rates. The number of mosquitoes attracted to materials obtained from human hands and arms tend to differ significantly (Brouwer, 1960; Khan *et al.*, 1966; Schreck *et al.*, 1990) even though carbon dioxide output rates from these body parts are scarcely different (Carlson *et al.*, 1992). Also, standardising the concentration of carbon dioxide does not always equalise human attractiveness to *An. gambiae* (Costantini *et al.*, 1999). Furthermore, it was shown, in contrast to our results, that reducing the amount of carbon dioxide exhaled by an individual by up to 95% caused a significant decrease in the number of *An. gambiae* complex mosquitoes attracted to

that individual compared to those attracted by a counterpart (Snow, 1970). However, most of these mosquitoes were *An. melas*, a subspecies within the *An. gambiae* complex. The cues that impact greatly on mosquito orientation in the vicinity of the host comprise body heat and moisture (Gillies & Wilkes, 1969). These factors may have contributed to the between-person differences in relative attractiveness that are currently reported as mosquitoes were released ~1 m away from the participants.

It can be concluded that factors present in breath have an inhibitory effect against *An. gambiae* and contribute to between-person differences in relative attractiveness. This (inhibition) effect could be allomonal as the end result is beneficial to the emitter (human being), but neutral to the receiver (the mosquito). Unfortunately, whereas factors responsible for the allomonal effects are unknown, it is interesting to contrast our observations with those who have noted that carbon dioxide, a major component of breath, is a potent activator for *An. gambiae* (Gillies, 1980). It is also interesting to note that the orientation behaviour of such a specialised human-feeding mosquito as *An. gambiae* is inhibited by breath and tends to bite overwhelmingly upon the ankles of its chosen host (De Jong & Knols, 1995). We suggest that the avoidance of human breath and the attraction to such extremities as the feet and ankles may represent a mechanism that facilitates successful, undetected feeding of *An. gambiae* upon their favoured human hosts.

Acknowledgements

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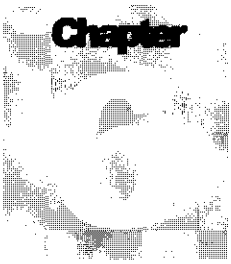

The role of body heat and moisture in the attractiveness of humans to *Anopheles gambiae*[1]

Abstract: The effects of body heat and moisture on the relative attractiveness of humans to *Anopheles gambiae* at short range was investigated using a two-arm olfactometer in which two persons (A and B) were presented as separate baits. Total emanations (breath plus body odour) of A attracted more mosquitoes than total emanations of B ($P = 0.008$), the trap catches being significantly affected by temperature ($P < 0.001$), relative humidity (RH) ($P < 0.001$) and the interaction between person and these physical stimuli ($P < 0.001$). The attractiveness of the persons' body odour (total emanations minus breath) did not differ significantly ($P = 0.132$) and the trap catches were not affected by temperature ($P = 0.15$) or RH (0.751). Body odour of A attracted more mosquitoes than total emanations of B ($P = 0.007$) and the trap catches were significantly affected by RH ($P < 0.011$) and the interaction between temperature and RH ($P < 0.001$). Body odour of B attracted more mosquitoes than total emanations of A ($P = 0.001$) and the trap catches were mainly affected by RH ($P < 0.001$) and the interaction between person and RH ($P < 0.001$). These findings suggest that body heat and moisture are important physical stimuli affecting host selection at short-range.

Introduction

Mosquito host seeking entails a series of responses to internal and external stimuli in a chain of events whose outcome is an increased probability of encountering the host. At long distances the process is mediated by olfactory cues whereas visual and physical cues are important in the vicinity of the host (Gillies & Wilkes, 1969). Long distance cues include volatile chemicals of the skin and breath, and waste products produced by the potential host (Takken, 1991). Carbon dioxide is active in the medium-range phase (Gillies, 1980) and body heat and moisture in the final short-range phase (Gillies & Wilkes, 1969). Based on this hierarchy of attractive stimuli and sequence in which they are encountered, it can be argued that differences in human attractiveness to mosquitoes are influenced by different factors at various distances from the host.

[1] This chapter will be published as: Mukabana, W.R., Takken, W. & Knols, B.G.J., The role of body heat and moisture in the relative attractiveness of humans to *Anopheles gambiae*, Submitted.

The lack of an apparent relationship between an individuals' attractiveness as measured by the number of mosquitoes entering houses (located ~12 metres apart) and by the number feeding implies that different host factors are, indeed, responsible for house entry and blood feeding (Lindsay *et al.*, 1993). Laboratory studies indicate that odour (from the human hand) is responsible for long-distance attraction of *Aedes aegypti* L. (Khan *et al.*, 1966) and results from field studies suggest that human odour influences variability in human attractiveness to *An. gambiae s.l.*, *An. funestus* Giles and *Culex quinquefasciatus* Say at long-range distances (Knols *et al.*, 1995). There is also evidence that variability in attraction of *Simulium* species (Schofield & Sutcliffe, 1996) and *An. gambiae* and *An. funestus* (Brady *et al.*, 1997) to humans located 2 - 20 metres apart from each other is influenced by the output rate of carbon dioxide, a major constituent of breath.

It is not clear to what extent, at short-range distances, mosquito host selection is influenced by differences in body heat and moisture. Earlier studies on *Aedes* species suggest that the effects of heat and moisture vary within certain limits. Moisture was found to be a major attractant when the ambient temperature exceeded 15.5°C whereas warmth was the major one when the temperature was less than 15.5°C (Brown, 1951). Warmer objects were described to be more attractive than cooler ones up to 43°C, attractiveness was reversed beyond 49°C (Peterson & Brown, 1951). Whereas hands of warm-skinned individuals were found to be more attractive to *Ae. aegypti* than those of cool-skinned individuals, those with a lower moisture output were always more attractive when the temperature difference between the two was less than 0.5°C (Smart & Brown, 1955). A recent study has shown that temperature affects variability in human attractiveness to black flies (Schofield & Sutcliffe, 1997). All these studies demonstrate that heat and moisture influence mosquito behaviour close to the target, but it remains unknown whether or not these factors contribute to differential attractiveness of humans to mosquitoes.

Recently, we showed that differences in human attractiveness to *An. gambiae* can be attributed to a person's emanations encompassing body odour, heat and moisture (Chapter 4). In follow-up studies, we investigated how breath and body odour contribute to and possibly affect the attractiveness of humans to the mosquitoes (Chapter 5). In the latter, we showed that differences in human attractiveness to mosquitoes were lost when breath was simultaneously eliminated from total emanations of (two) competing individuals. It was also shown that elimination of breath from an individual's total body emanations rendered that individual more attractive than a competitor whose total emanations were presented to the mosquitoes. These findings contrasted with what is known about odour-mediated mosquito host-seeking behaviour, where the general opinion is that body odour is responsible for causing differential attraction of mosquitoes to humans. In our studies, we hypothesised that variations in physical cues may have influenced the observed differences in human attractiveness, as the mosquitoes were released ~1 metre downwind of the participants. Thus, in this study, a pair of

volunteers was recruited and a selection of the previous tests repeated so as to (i) verify the previous findings and (ii) establish to what extent body heat and moisture contributed to the behavioural responses and patterns of differential attractiveness observed.

Materials and methods

Mosquitoes

Experiments were conducted using the Ifakara strain of laboratory-reared *An. gambiae* s.s., originally colonised from wild-caught gravid females in Njage, South-east Tanzania, in 1996. The mosquito larvae were reared under ambient temperature and light conditions in screen house insectaries of the Mbita Point Research and Training Centre (MPR&TC) of the International Centre of Insect Physiology and Ecology (00°25'S, 34°13'E). The larvae were reared using water from Lake Victoria and were fed on Tetramin® fish food three times per day. Pupae were collected from rearing trays and transferred to an adult insectary where they were kept in mesh-covered 30 cm cubic cages in which 6% glucose solution soaked in rolls of filter paper was provided. The colony was maintained by routinely offering a human arm to feed upon. Adult females with no prior access to blood were used for experiments when they were 4 - 8 days old and were transferred from the holding cages into release cups 6 hours before the onset of experiments. Only water-wet cotton wool pads were provided on the mesh-topped open ends of the release cups.

Human subjects

Two healthy African males designated person A and B were recruited to participate in the experiments. Person A was aged 22 years (weight, 60 kg; height, 1.75 m) and person B was aged 18 years (weight, 72 kg, height 1.82 m). The participants only wore pairs of short trousers for the duration of the individual experiments and bathed with non-perfumed soap one hour before starting the first experiment of a night. Their malaria infection status was tested on each experimental day by microscopic examination of thin and thick smears of finger-pricked blood stained with Giemsa. The temperature and relative humidity of the air stream associated with their emanations was measured. Their diet was not controlled except that they were prohibited from ingesting alcohol, a factor that has recently been shown to influence the relative attractiveness of humans to *Aedes albopictus* (Shirai *et al.*, 2002). However, the diet of the people of Mbita Point is somewhat fixed, *Ugali* (a pasta made from maize meal) served with green vegetables (mainly collards) is consumed virtually every day. Previous experiments (Chapter 4) demonstrated that person A was significantly more attractive to *An. gambiae* than person B.

Experimental design

Experiments were conducted with a tent olfactometer as described previously (Chapter 4 & 5). Experimental mosquitoes were allowed to make a choice between the following test stimuli: (i) total emanations of person A versus total emanations of person B (experiment 1), (ii) body odour of person A versus body odour of person B (experiment 2), (iii) total emanations of person A versus body odour of person B (experiment 3) and (iv) body odour of person A versus total emanations of person B (experiment 4). The various experimental combinations are summarised in Table 1. In this context total emanations refers to breath plus all volatile discharges of the skin, and body odour refers to volatiles discharged solely from the skin. The number of mosquitoes attracted to the discharges of each person in each experiment were recorded. The experiments were conducted over 30-minute periods: at 19.30 - 20.00, 20.30 - 21.00 and 21.30 - 22.00 hours. The mosquitoes were counted and removed from the apparatus after each experimental period. The two human subjects were alternated between the two tents each time.

Table 1. Experimental design showing the emanations of person A and person B offered to mosquitoes in choice experiments evaluating the role of body heat and moisture in the short-range attractiveness of two humans.

Experiment	Type of emanations offered	
	Person A	Person B
1	Total emanations (TE)	Total emanations (TE)
2	Body odour (BO)	Body odour (BO)
3	Total emanations (TE)	Body odour (BO)
4	Body odour (BO)	Total emanations (TE)

Temperature and relative humidity records

A set of data loggers (HOBO®) was used (in all experiments) to record temperature (T) and relative humidity (RH) of the air streams as they passed from the occupied tents to the central part of the olfactometer. The data loggers were located inside the tent exhausts and were programmed to record T/RH at 1 min intervals. The temperature and relative humidity records were averaged for each experimental period and correlated to mosquito catches.

Statistical analysis

Relative attractiveness was calculated by dividing the number of mosquitoes trapped by emanations of person A by the sum of the number trapped by emanations of person A and person B. The significance of differences in attractiveness between the baits in the two traps in each experiment was assessed by Kendall's W test for related samples, comparing the catches of person A with those of person B in the same experiment. The significance of changes in relative attractiveness between experiments was assessed by the Kruskal-Wallis H test for independent samples, comparing the relative attractiveness estimates from repetitions of the same experiments with those of another. The effects of temperature and relative humidity on the number of mosquitoes caught was analysed by the Generalised Linear Model of binomial data, using a logit link function. Data were analysed either by Statistical Products and Service Solutions (SPSS, version 10.0) or the General Statistics analysis software (GenStat® for Windows, 5th Edition).

Results

Experiments were carried out over a total of eighteen nights incorporating 54 choice tests. Neither of the participants were found to have malaria parasites over the duration of the study. There were significant differences in the number of mosquitoes attracted to the persons' total emanations ($P = 0.008$), person A being more attractive than person B. The attractiveness of body odour of person A and B did not differ significantly ($P = 0.132$). Body odour of person A attracted more mosquitoes than total emanations of person B ($P = 0.007$) and body odour of person B attracted more mosquitoes than total emanations of person A ($P = 0.011$). Mosquito catches of each of the two human subjects are depicted in Figure 1(i).

Mosquito responses to total emanations of person A versus total emanations of person B were significantly affected by temperature ($P < 0.001$), relative humidity (RH) ($P < 0.001$) and the interaction between person, temperature and RH ($P < 0.001$). However, mosquito responses to the persons' body odour were not affected by temperature ($P = 0.15$) or RH ($P = 0.751$) but by the interaction between person and RH ($P < 0.001$) and RH and temperature ($P = 0.004$). Mosquito responses to body odour of person A versus total emanations of person B were significantly affected by RH ($P < 0.001$) and the interactions between temperature and RH ($P < 0.001$) and temperature, RH and person ($P = 0.002$). Mosquito responses between body odour of person B versus total emanations of person A were significantly affected by RH ($P < 0.001$), temperature ($P = 0.004$) and the interactions between person and RH ($P < 0.001$) and person and temperature ($P = 0.024$).

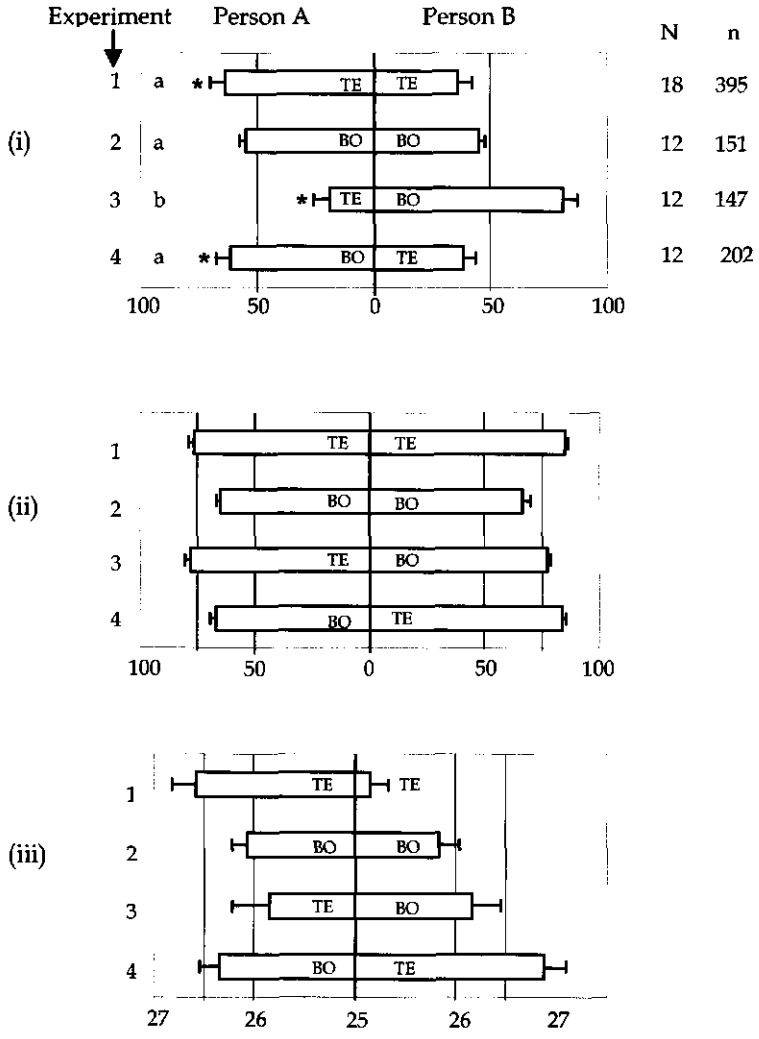

Figure 1. Percent mosquito catches (i), relative humidity (ii) and temperature (iii, °C) recorded simultaneously in experiments evaluating mosquito behavioural responses to total emanations (TE) of person A versus TE of person B (experiment 1), body odour (BO) of person A versus BO of person B (experiment 2), TE of person A versus BO of person B (experiment 3) and BO of person A versus TE of person B (experiment 4). N is the number of replicates and n the total number of mosquitoes attracted to emanations of person A and B. Asterisks depict significant differences in mosquito catches between emanations of person A and B within experiments ($P \leq 0.011$). Similar letters depict no significant differences in relative attractiveness between experiments.

Figure 1 shows that emanations of the person associated with higher mosquito catches (regardless of whether the catches differ significantly or not) either had a higher temperature (experiment 1, 2 and 3), lower RH (experiment 1, 2, 3 and 4) or both a higher temperature and lower RH (experiment 1, 2 and 3). However, since the values of Figure 1 are amalgamated for many experiments, we decided to inspect temperature and relative humidity values for individual pair-wise comparisons so as to find out how firm these criteria held and if possible come up with a rule of scoring for variability in human attractiveness to the mosquitoes. The rule turned out to be as follows: if the temperature difference between the emanations of person A and B was equal to or greater than 0.5°C, the person with warmer emanations had higher mosquito catches. However, if the temperature difference between the emanations of person A and B was less than 0.5°C, the person with emanations of a lower RH had higher mosquito catches. The predictive value of this rule as applied to all repetitions in the four experiments is presented in Table 2.

Table 2. Number of replicates which passed, failed or neither passed nor failed (i.e. mosquito catches were equal) a rule predicting the outcome of mosquito behavioural responses towards emanations of person A and B. N depicts the total number of replicates done in each experimental comparison.

Experiment	N	Rule passed	Rule failed	Neither (i.e. equal catches)
1	18	14	3	1
2	12	6	5*	1
3	12	9	1	2
4	12	7	4*	1
Total	54	34	13	5

*in all these cases the emanations that attracted a higher number of mosquitoes were those with lower RH levels (regardless of whether the difference in temperature was equal, less or more than0.5°C).

The rule was most predictive in experiment 1 (78% i.e. 14/18) then followed by 3 (75%), 4 (58%) and 2 (50%), respectively. The low predictability of this rule in experiment 2, in which breath from either person was excluded and where mosquito catches did not differ significantly, implies that the effects of temperature and relative humidity on mosquito attraction interact with yet another factor, most likely present in breath. Since the most natural presentation of humans to mosquitoes is depicted by experiment 1, it is likely that this rule might apply under natural conditions of mosquito host seeking.

Discussion

This study demonstrated that the malaria mosquito *An. gambiae* is preferentially attracted to the individual whose emanations were either ≥ 0.5°C warmer or which had a lower relative humidity if the temperature difference between emanations of the two human subjects was less than 0.5°C. Also, the number of mosquitoes attracted were significantly affected by the interactions between person and temperature, person and relative humidity or temperature and relative humidity of the body emanations. These results demonstrate that temperature and humidity affect the relative attractiveness of humans to *An. gambiae* in the short range phase of host seeking.

That *An. gambiae* is attracted more significantly towards the individual whose body emanations are ≥ 0.5°C warmer than those of another agrees with other studies. Mayer & James (1968) found that *Ae. aegypti* responded significantly more towards an un-rinsed human arm which was 5.4°C warmer than one which was rinsed in water or acetone. The black fly *Simulium venustum* was found to bite persons whose skin temperature was 14-17°C higher than the ambient environmental temperature at significantly higher rates than those whose skin temperature differed less (11-14°C) from ambient levels (Schofield & Sutcliffe, 1997). However, although hands of warm-skinned individuals were shown to be more attractive to *Ae. aegypti* than those of cool-skinned counterparts, artificially-warmed hands were less attractive than un-warmed hands and hands with a lower moisture output were more attractive when the temperature difference between two hands was less than 0.5°C (Smart & Brown, 1955).

Probably, heat does not necessarily elicit an attraction response to mosquitoes but acts in synergism with other factors or boosts mosquito behavioural responses by increasing the volatility of attractants. Laboratory studies have shown that although heat activates *Ae. aegypti*, landing, probing or flight in close vicinity of a target are induced when moisture (Khan & Maibach, 1971), carbon dioxide or both (Khan & Maibach, 1966) are provided in addition. Also, whereas *Ae. aegypti* has been seen not to prefer the warmer option when offered the choice between two otherwise similar air streams, the warmer air stream is preferred over the cooler one when the two are moisturised to similar levels, more humidified air currents being preferred more by water-deprived than by water-satiated mosquitoes (Bar-Zeev *et al.*, 1977). Our results demonstrate that the effects of temperature and relative humidity interact with some breath component(s). This is plausible since, in fact, most of the moisture released from the human body is in breath, being a by product of aerobic respiration. It is therefore likely that certain breath components interact with temperature and relative humidity and hence influence mosquito host-seeking behaviour. However, this inference contrasts with those who have suggested that relative humidity interacts with skin odour in influencing the mosquito host-seeking process (Takken *et al.*, 1997).

From an ecological standpoint there would be at least one advantage for mosquitoes to obtain their blood meals from hosts with higher body temperatures. The human thermoregulatory system reacts to high body temperatures, among other ways, by dilating veins. Vasodilation increases peripheral circulation, an adaptation geared towards dissipating excess heat. It can be hypothesised that feeding mosquitoes would engorge more rapidly at high body temperatures as these decrease the viscosity of blood (Chien, 1966). Indeed, there is evidence that *Ae. aegypti* doubles her blood-feeding rate when the skin temperature of her human hosts is raised from 29°C to 36.2°C (Grossman & Pappas, 1991). Rapid engorgement reduces the danger of death which is likely to occur as a result of host-defensive behaviour (Daniel & Kingsolver, 1983). However, it is ironical that mosquitoes, in some cases, prefer to feed on hosts with lower body temperatures (Day & Edman, 1984).

Preferential selection of hosts with higher emission rates of body heat may be associated with important epidemiological consequences. First, yellow fever viraemia in primates, which is typically accompanied by a rise in body temperature, lasts for 2 to 4 days and is followed by life-long immunity. It is likely that preferential feeding by arboreal mosquitoes during the febrile period would enhance the transmission of the virus (Gillett & Connor, 1976). Second, accumulating evidence suggests that pregnant women are more attractive to host-seeking *An. gambiae* than non-pregnant counterparts (Lindsay *et al.*, 2000; Ansell *et al.*, 2002) by virtue of physiological changes associated with increased body temperature (Lindsay *et al.*, 2000). This preponderance, coupled with the increased risk of malaria infection (during pregnancy) which occurs as a result of preferential adherence of *P. falciparum* parasites on sites on placental trophoblastic villi (Fried & Duffy, 1996), could aggravate sequelae such as still births, low-birth weight babies, premature delivery and severe maternal anaemia (McGregor, 1984).

In conclusion, this study has demonstrated that temperature and moisture affect mosquito host-seeking behaviour during the short-range phase. These results give important leads towards optimising the trapping efficiency of odour baits once they become available for field application since a short-range attractant will be most crucial for inducing effective trap entry behaviour. For example, raising temperature has been shown to increase the attraction of several mosquito species to handled rather than (clean) un-handled glass petri dishes (Schreck *et al*, 1990) and warming attractant material present inside trapping devices has been shown to increase mosquito catches (Kline & Lemire, 1995). If the catch efficiency of the long-awaited odour-baited traps can be optimised then the aim of controlling mosquitoes by removal trapping (Day & Sjogren, 1994) might become a useful component within an integrated approach for malaria control.

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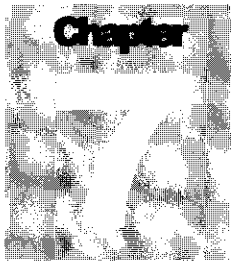
Part III

Parasite infection and differential attractiveness of humans to *Anopheles gambiae*



Do clinical malaria symptoms suppress the attractiveness of humans to mosquitoes?[1]

Abstract: The relative attractiveness of two human subjects to the malaria mosquito *Anopheles gambiae* Giles was measured over seven consecutive nights, including periods when they were both uninfected or alternately displaying *Plasmodium falciparum* parasitaemia and clinical symptoms. A series of 20 separate experiments conducted when the two subjects, PI and PII, were uninfected before and after the seven day trial period found that PI was consistently more attractive than PII ($P < 0.001$). However, on the one night that PI suffered clinical symptoms of *P. falciparum* infection (fever and profuse sweating) he became less attractive than PII and this difference approached significance ($P=0.083$). Furthermore, his relative attractiveness when suffering clinical malaria differed significantly from all other nights of the seven day trial period ($P=0.050$) and all nights before ($P=0.014$) and afterwards ($P=0.009$). The relative attractiveness of the two individuals returned to baseline levels over the following two days, during which no symptoms were observed but trophozoites were readily detected in the blood of PI. Immediately after PI no longer exhibited clinical symptoms or patent parasitaemia, PII developed clinical malaria symptoms (chills) and then patent *P. falciparum* trophozoite infection but this did not cause an obvious effect in the relative attractiveness of the two subjects, probably because of the lower baseline relative attractiveness of PII. These results provide the first evidence that malaria infection in humans may modulate their attractiveness to potential vector mosquitoes.

Introduction

The question of whether or not medically important blood parasites enhance their own transmission by manipulating the host-seeking behaviour of their insect vectors through physical or chemical signalling by vertebrate hosts remains largely unresolved. Although there is evidence to suggest that the parasites might be doing so, examples mainly derive from rodent models (Day & Edman, 1983; Day *et al.*, 1983; Day & Edman, 1984; Coleman & Edman, 1988). These examples cannot be

[1] This chapter will be published as: Mukabana, W.R., Takken, W., Killeen, G.F. & Knols, B.G.J., Clinical malaria symptoms reduce attractiveness of humans to mosquitoes. Submitted.

directly extrapolated to explain the *Anopheles-Homo-Plasmodium* interaction because, unlike sleeping humans, rodents display extensive defensive behavioural responses against attacking mosquitoes, the reason why the infected, lethargic rodents were bitten preferentially (Day & Edman, 1983; Day & Edman, 1984; Day *et al.*, 1983). Accordingly, it may not be surprising that no cases of preferential selection of parasite-infected humans by medically important insects are known. In fact, studies have, so far, found no preference by mosquitoes or black flies for biting humans with malaria, filariasis (Burkot *et al.*, 1989) or onchocerciasis (Kruppa & Burchard, 1999), compared with uninfected counterparts, respectively.

Nevertheless, because host seeking is mediated by physico-chemical stimuli (Gillies & Wilkes, 1969; Sutcliffe, 1987; Takken, 1991; Takken & Knols, 1999) that are known to vary according to infection status (Penn & Potts, 1998), it is tempting to speculate that mosquitoes respond differently to stimuli originating from malaria-infected individuals. Analysis by gas chromatography has recently implicated a chemical basis for the higher attractiveness of *Leishmania infantum*-infected hamsters, compared with non-infected counterparts, to female *Lutzomyia longipalpis* sand flies based on behavioural bioassays in a wind tunnel (O'Shea *et al.*, 2002). Here we describe how a rare window of opportunity enabled us to investigate the effect of clinical symptoms and parasitaemia due to *P. falciparum* on variability in the attractiveness of two human subjects to the malaria vector mosquito *An. gambiae* using a tent olfactometer.

Materials and methods

Mosquitoes

Experiments were conducted using the Ifakara strain of laboratory-reared *An. gambiae s.s.*, originally colonized from wild-caught gravid females in Njage, south-east Tanzania, in 1996. The mosquito larvae were reared under ambient temperature and light conditions in screen house insectaries at the Mbita Point Research and Training Centre (MPR&TC) of the International Centre of Insect Physiology and Ecology (00°25'S, 34°13'E). The larvae were reared using water from Lake Victoria and were fed on Tetramin® fish food three times per day. Pupae were collected from rearing trays and transferred to an adult insectary where they were kept in mesh-covered 30 cm cubic cages in which 6% glucose solution soaked in rolls of filter-paper was provided. The colony was maintained by routinely offering a human arm to feed upon. Adult females with no prior access to blood were used for experiments when they were 4 - 8 days old and were transferred from the holding cages into release cups 6 hours before the onset of experiments. Only water-wet cotton wool pads were made available for mosquitoes in the experimental system and were provided on the mesh-topped ends of the release cups.

Human subjects, experimental set-up and design

Two healthy African males designated person I (PI) and person II (PII) were recruited to participate in experiments that had been initially designed to investigate how breath and body odour contribute to and possibly affect the attractiveness of humans to *An. gambiae*. PI was aged nineteen years (weight, 80 kg; height, 1.80 m) and PII was aged 22 years (weight, 79 kg, height 1.85 m). The malaria infection status of both individuals was examined daily so as to take note of infection as a potential source of variation. Efforts to achieve the initial study objective (i.e. to investigate how breath and body odour contribute to and possibly affect the attractiveness of humans to *An. gambiae*) were confounded when the two participants alternately succumbed to *P. falciparum* malaria. The experimental manipulations and data collected during that first phase of tests designed to accomplish the initial objective were thus extracted and analyzed separately for the current report.

The experiments were conducted using two arms of a three-arm tent olfactometer (Chapter 4) as previously described (Chapter 5). The apparatus was set-up such that experimental mosquitoes were trapped before reaching the human subjects who were present inside tents located at the upwind end. Approximately 100 mosquitoes (the exact number was recorded for each experimental release) were used per experiment. The mosquitoes were released into a choice chamber situated ~1 metre away from the occupied tents. They were then allowed 30 minutes in which to choose either of the two human baits by flying into, and becoming trapped in cages that were adjacent to the two pipes, separately carrying their body emanations. Total body emanations included the persons' breath, body odour, heat and moisture. The subjects only wore short trousers during the experiments, bathed with un-perfumed soap one hour before starting and no attempt was made to control their daily diet except prohibiting them from drinking alcohol, which may affect the attractiveness of humans to mosquitoes (Shirai *et al.*, 2002). However, the diet of the people of Mbita Point is somewhat consistent. *Ugali* (a thick paste made from maize meal) served with green vegetables (mainly collards) and/or fish is consumed virtually every day. Three experiments were conducted per night: at 19.30 – 20.00, 20.30 – 21.00 and 21.30 – 22.00 hours. The two human subjects switched between the two alternative tents each night.

Examination for malaria parasites and drug treatment

The participants' malaria infection status was tested daily through microscopic examination of thin and thick smears of finger-pricked blood stained with Giemsa. Blood samples were taken and smears prepared between 19.00 and 19.15 hours pending final processing on the next day (08.00 – 10.00 hours). The blood smears were interpreted as negative only after examination at × 1000 magnification of at least ten fields. Examination was done by a qualified technician who performed similar work at the local private and institutional health facilities as part of his daily routine. A second blood smear was taken the next day (between

10.00 and 11.00 hours) whenever the first smear was negative but when infection with malaria parasites was suspected. Participants were treated with a single dose of sulfadoxine pyrimethamine (FANSIDAR®) whenever they were found to be positive with malaria parasites.

Ethical considerations

Written informed consent was obtained from both human subjects prior to starting the investigations. Transport (institutional ambulance) was provided to ferry the participants to and from the experimental site whenever they had bouts of malaria. Clinical malaria symptoms were assumed to be genuine, so chemotherapy was administered before confirming parasitaemia. The project was approved by the Kenya National Ethical Review Committee based at the Kenya Medical Research Institute (KEMRI/7/3/1).

Statistical analysis

Relative attractiveness was calculated as the number of mosquitoes trapped by the emanations of PI divided by the sum of the number trapped by the emanations of PI and PII. Thus relative attractiveness of greater than 0.5 indicates greater attractiveness of person PI whereas relative attractiveness of less than 0.5 indicates greater attractiveness of PII. Non-parametric statistical methods were used for analysis because of their robustness and flexibility. The significance of differences in attractiveness between the baits in the two traps in each experiment was assessed by Kendall's W test for related samples, comparing the catches of person PI with those of person PII in the same day (1-7) or days (period A and B). The significance of changes in relative attractiveness between experiments was assessed by the Kruskal-Wallis H test for independent samples, comparing the relative attractiveness estimates from repetitions of the same experiments with those of another. In all cases the number of repetitions or releases for each experiment is denoted by N, whereas the total number of mosquitoes which were actually trapped by either human bait is denoted by n. Data were analysed by the Statistical Products and Service Solutions (SPSS, version 10.0) software package.

Results

Twenty-one experiments were conducted over seven consecutive experimental nights when the participants underwent disease-free phases concurrently and trophozoite/clinical malaria symptom phases alternately. Details of the persons' malaria infection status during the study period are shown in Table 1. Clinical symptoms either presented as fever and profuse sweating (person PI) or chills (person PII). In both cases the infections were due to *P. falciparum*. In PI, clinical symptoms (fever and profuse sweating) were reported during all the three test periods on day 2 but in PII, clinical symptoms (chills) were only reported on one occasion. Clinical symptoms were not accompanied by detectable parasitaemia

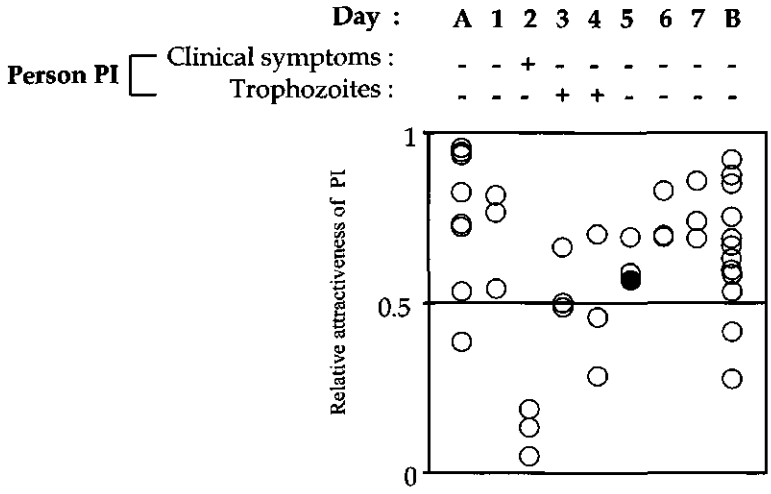
in either case. Drug (sulfadoxine pyrimethamine or FANSIDAR®) administration cleared parasites in PI and PII to microscopically undetectable levels after 2 and 1 day, respectively.

Table 1. Daily malaria infection status of two persons (PI and PII) during studies of the effect of infection with malaria parasites on between-person differences in relative attractiveness to the malaria mosquito *An. gambiae*

Day	Malaria infection status	
	Person PI	Person PII
1	No parasites	No parasites
2	Clinical symptoms, no parasites*	No parasites
3	Trophozoites	No parasites
4	Trophozoites	No parasites
5	No parasites	Clinical symptoms, no parasites**
6	No parasites	Trophozoites
7	No parasites	No parasites

*characterised by fever and profuse sweating
**characterised by chills and experienced once only (21.30 - 22.00 hours)

The between-person differences in relative attractiveness are shown in Figure 1. The seven consecutive experimental nights are numbered 1 to 7, A and B denote experiments that were conducted 2 to 5 days before and 770 to 773 days after the core seven days, respectively, when both human subjects were free from malaria. It is clear that, although person PI was, on all occasions, more attractive to the mosquitoes when both were not infected, clinical malaria symptoms (fever and profuse sweating) suppressed his degree of attractiveness relative to person PII, resulting in a reversal of their usual ranking and the greater attractiveness of person II approaching significance ($P = 0.083$). Furthermore, the relative attractiveness of PI, when suffering clinical malaria on day 2, differed significantly from all other nights of the seven day trial period ($P = 0.050$) and all nights before ($P = 0.014$) and afterwards ($P = 0.009$) (Figure 1). The relative attractiveness of the two individuals returned to baseline levels over the following two days, during which no symptoms were observed but trophozoites were readily detected in the blood of PI. Immediately after PI no longer exhibited clinical symptoms or patent parasitemia, PII developed clinical malaria symptoms (chills) and then patent *P. falciparum* trophozoite infection but these did not cause an obvious effect in the relative attractiveness of the two subjects, probably because of the lower baseline relative attractiveness of PII.



$P(RA_1 = RA_2)$:

RA_1 \ RA_2	1	2	3	4	5	6	7	B
A	0.68	**0.01**	0.10	0.07	0.22	0.54	0.84	0.22
1		**0.05**	0.13	0.13	0.51	0.83	0.83	0.67
2			**0.05**	**0.05**	**0.05**	**0.05**	**0.05**	**0.009**
3				0.51	0.28	**0.05**	**0.05**	0.31
4					0.51	0.28	0.13	0.31
5						0.08	0.13	0.67
6							0.84	0.31
7								0.31

Figure 1. Relative attractiveness of person PI and PII to *An. gambiae* during periods when both were uninfected (A, B, day 1 and day 7) or alternately displaying clinical *P. falciparum* malaria symptoms (day 2, PI; day 5, PII) or trophozoites (day 3 and 4, PI; day 6, PII). The closed circle represents the only time when PII exhibited clinical malaria symptoms. N, number of replicates and n, the total number of mosquitoes used in the experiments. A and B represent experiments conducted 2-5 days before and 770-773 days after the seven core experimental nights (1-7), respectively. P (RA = 0.5) represents the significance of differences in relative attractiveness (RA) between PI and PII in the same day (1-7) or days (periods A and B). P ($RA_1 = RA_2$) represents the changes in relative attractiveness of the test subjects between experiments e.g. the relative attractiveness of the test subjects between day 2 and period B was significantly different (P = 0.009). Plus and minus signs represent the expression (+) or non-expression (-), by person PI and PII, of *P. falciparum* clinical malaria symptoms and/or trophozoites.

Discussion

This study demonstrated, for the first time, that infection with *P. faciparum* alters between-person differences in relative attractiveness to a species of malaria vector mosquito. Clinical malaria symptoms (fever and profuse sweating) suppressed the relative attractiveness of a human subject who had been otherwise consistently more attractive to *An. gambiae* when compared to a specific counterpart in choice experiments. These findings support the general expectation that malaria parasites alter the attractiveness of their human hosts to mosquito vectors (Morell, 1997).

The observed reduction of person PI's relative attractiveness to host-seeking mosquitoes as a result of fever and profuse sweating is somewhat surprising as induced thermogenic sweating, in the absence of disease, has been shown to increase landing and engorgement rates by *An. gambiae* on humans (Thornton *et al.*, 1976). Olfactometer studies have, however, demonstrated that incubated human sweat but not fresh sweat attracts the malaria mosquito *An. gambiae* (Braks *et al.*, 1999). In contrast to our studies, the preferential feeding of *Cx. pipiens* mosquitoes on Rift Valley Fever virus infected-lambs, compared with uninfected ones, was demonstrated as being positively correlated with body temperature (Turell *et al.*, 1984). Nevertheless, there have been reports that suggest that fever can reduce the attractiveness of humans to host-seeking mosquitoes. In one field study it was observed that although the total number of *An. gambiae* complex mosquitoes that fed on a mother and a child pair during a 10-day period when both were healthy and sleeping under a bednet trap was 40 versus 2, these numbers reversed to 3 versus 23, respectively, during a subsequent 4-day period when the mother had a fever (Bryan & Smalley, 1978). Despite that these observations were made in a malaria-endemic area of The Gambia, the fever was said to be of unknown origin, no tests were done to confirm the presence of malaria parasites.

The observation that no similar suppression of attractiveness was associated with chills might be considered as somewhat surprising because attraction of blood sucking insects to uninfected individuals has generally been associated with temperature increments (Schreck *et al.*, 1990; Schofield & Sutcliffe, 1997). However, since PII was normally always the less attractive person, these effects might have had little influence on the clear preference of the test mosquitoes for PI. Furthermore, this subject suffered from chills only during one of the experiments conducted that night (filled circle in Figure 1), so the period of symptomatic illness was not as prolonged as that experienced by PI. Day and Edman (1984) demonstrated peak biting by *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi* on *P. berghei* and *P. chabaudi*-infected mice during periods of hypothermia. Likewise, studies on selection of biting sites on man found that *An. gambiae* preferred to bite body parts with lower (<30°C) skin temperature (De Jong & Knols, 1995).

Our results have two implications i.e. that (1) body temperature alone cannot explain differences in the attractiveness of humans to disease vectors and (2) host

defensive behaviour alone cannot explain the increased attractiveness of *Plasmodium*-infected individuals to foraging mosquitoes, as has been implied in other host-vector combinations (Day *et al.*, 1983). As experimental mosquitoes were trapped along their flight path before reaching the human subjects, who were located further upwind, the influence of host-defensive behaviour on the mosquitoes' behavioural responses was eliminated. Interestingly, it seems that factors other than temperature were responsible for deviating mosquitoes away when clinical malaria symptoms were displayed by PI, who was otherwise consistently more attractive in the absence of infection. There is evidence that parasitic diseases can alter body odour (Penn & Potts, 1998) and that differential attractiveness of infected and healthy individuals may have a chemical basis (O'Shea *et al.*, 2002).

These results constitute the first evidence that *Plasmodium* infection in humans can indeed alter the attractiveness of individuals to host-seeking mosquitoes. Since no observations were made when the participants had gametocyte stages, which are infectious to susceptible mosquito vectors, we cannot extrapolate our results in terms of disease transmission. Ecologically, however, it may be suggested that the malaria parasite *P. falciparum* has evolved a mechanism of keeping its vectors away until it is ready to be carried to a new host and it is thus evolutionarily more advanced than its intermediate and definitive hosts. However, avoidance of clinically ill individuals can also be viewed as a strategy of *An. gambiae* to avoid unhealthy individuals who may, however, be signalling defensiveness (Kelly, 2001).

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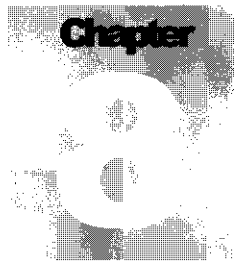

Plasmodium falciparum parasitaemia enhances the attractiveness of humans to *Anopheles gambiae*[1]

Abstract: The effect of infection with *Plasmodium falciparum* on the attractiveness of two human subjects to the African malaria vector *An. gambiae* was studied in a malaria-endemic area of western Kenya. Mosquitoes attracted to the subjects were actively collected using the man landing catch (MLC) or passively sampled by bednet traps (BNT). Collections were carried out during the dry and wet seasons at the start of 2002. MLC and BNT collections during the dry season found no significant differences in the probability of person Z being bitten (P = 0.433 and 0.102, respectively), between periods when he was infected and uninfected. With respect to collections of person X during the dry season, MLC found a nearly significant but lower (P = 0.081) and BNT found a significant but higher (P = 0.032) probability of attracting mosquitoes when infected. Wet season collections with MLC found probabilities approaching significance that person X (P = 0.057) and Z (P = 0.078) would attract more mosquitoes when they were infected. BNT collections of the wet season found significantly higher probabilities of person X (P = 0.028) and Z (P = 0.004) attracting more mosquitoes when they were infected. These findings suggest that infection of humans with the malaria parasite *P. falciparum* may modulate their attractiveness to potential vector mosquitoes in such a way as to enhance its own transmission.

Introduction

Malaria parasites are thought to manipulate their mosquito and vertebrate hosts so as to enhance their own transmission (Morell, 1997; Takken & Knols, 1999). In the mosquito they accomplish this in various ways. Pathology of salivary glands as a result of infection with *Plasmodium* sporozoites lowers the secretion of salivary apyrase (Rossignol *et al.*, 1984), an enzyme that facilitates the ability of mosquitoes to locate blood (Ribeiro *et al.*, 1984; 1985a). Insufficient amounts of salivary apyrase impair the ability of mosquitoes to locate blood during probing

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and so increase vector-host contact. These two factors promote interrupted feeding by stimulating host defence (Waage & Nondo, 1982) in response to irritation (Gillet, 1967) and increasing the chance that the mosquito desists from feeding (Ribeiro *et al.*, 1985b) and recommences elsewhere. Thus, the chances of disease transmission are enhanced (Davies, 1990). Infection of mosquitoes with *Plasmodium* parasites is also associated with other transmission-enhancing behaviours such as increased biting (Rossignol *et al.*, 1986), probing (Wekesa *et al.*, 1992) and multiple feeding rates (Koella *et al.*, 1998). However, parasites may have a negative effect on their own transmission by reducing the survival and reproductive fitness of their mosquito hosts (Anderson *et al.*, 2000; Hogg & Hurd, 1995). Nevertheless, in spite of the damage that may be caused to insect vectors (Ferguson & Read, 2002), *Plasmodium* species ought to limit their negative effects as part of the best evolutionary strategy.

Exactly how parasites manipulate their vertebrate hosts so as to enhance their own transmission is hitherto not well understood. Rodent malaria models suggest that parasites do so by increasing the attractiveness of hosts to mosquitoes when the infective gametocytes are in circulation (Day & Edman, 1983; Day *et al.*, 1983; Day & Edman, 1984). Rodents infected with *Leishmania* have also been shown to be more attractive to biting sand flies than uninfected ones (Coleman *et al.*, 1988; O'Shea *et al.*, 2002). Much as the increased attractiveness of infected rodents to mosquitoes has been mainly explained in terms of reduced defensive behaviour (Day & Edman, 1983; Day *et al.*, 1983; Day & Edman, 1984), other factors might also be involved as even infected rodents are bitten by sand flies at specific sites i.e. on lesions where the chances of picking up parasites are higher (Coleman *et al.*, 1988). Recent evidence has suggested that olfaction may be one of the other factors, at least among sand flies feeding on infected hamsters (O'Shea *et al.*, 2002) and possibly malaria vectors feeding on humans (H. Hurd, personal communication).

Regardless of this background information, there still is a dearth of information about how human-invading parasites e.g. *Plasmodium, Leishmania,* filarial worms etc might be enhancing their own transmission. Two studies have, so far, found no preferential selection of malaria-/filariasis (*Wuchereria bancrofti*)- and ochocerciasis-infected humans by mosquitoes (Burkot *et al.*, 1989) and black flies (Kruppa & Burchard, 1999), respectively. However, even if this phenomenon was absent for members of the *Anopheles punctulatus* complex (Burkot *et al.*, 1989), it may be there among other mosquito species because human attractiveness to mosquitoes is species-specific (Curtis *et al.*, 1987; Knols *et al.*, 1996). In evolutionary terms it would be best for the parasite to modify host characteristics in a way that mosquito vectors preferentially select infective humans for a blood meal. The current study investigated the effect of infection with *P. falciparum* on the attractiveness of humans to *An. gambiae s.l.*

Materials and methods

Study area

The study was conducted in Lwanda, a village located on the southern shore of the Winam Gulf of Lake Victoria in Suba District, Kenya (Figure 1) where *An. gambiae, An. arabiensis* Patton and *An. funestus* Giles are the most important malaria vectors. The non-malaria mosquito *Cx. quinquefasciatus* Say is also abundant. Numerous hoof prints of cattle and night-grazing hippopotami provide excellent mosquito breeding sites on a low-lying plain that is continuous with the lakeshore. Fishing and livestock keeping are the main occupation of the local inhabitants. Cattle, goats, chicken, dogs, cats and a few sheep constitute the domestic animal population. Maize, millet and sorghum, but no cash crops are cultivated at subsistence level. Most houses in Lwanda are constructed of wood and mud with corrugated iron or thatched roofs. Eaves, about one foot in size, increase ventilation in the houses and form the predominant entry points for mosquitoes (Snow, 1987; Lindsay & Snow, 1988).

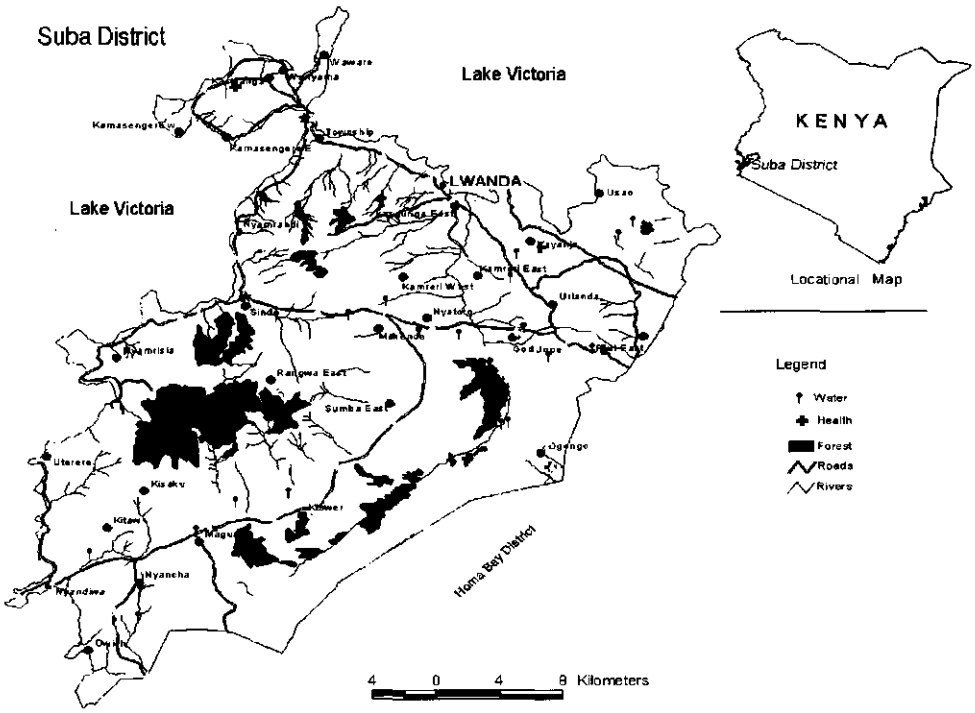

Figure 1. Map showing location of Lwanda village in relation to Suba District, Kenya.

The village experiences two rainy seasons: between April-June and September-October. Mosquito breeding grounds proliferate and mosquito populations rapidly increase in size during the rainy seasons. [The experiments reported herein were conducted during parts of the dry (January - mid March) and wet seasons (April - May) in 2002]. Malaria caused by *P. falciparum* is endemic in the study area, with high prevalence rates among young children. Because of acquired immunity, it is presumed that adults (> 15 years of age) are no longer at risk of severe malaria, although they may occasionally suffer from clinical disease. Malaria prevalence levels in the area are more consistent with mesoendemicity with entomological inoculation rates of less than 10 infectious bites per year, than with hyperendemicity and high transmission levels (Beier *et al.*, 1999). Infection fluctuates considerably in response to rainfall, consistent with models of seasonal transmission, which is quite different from the stable situation observed in holoendemic areas where transmission and infection remain in a relatively constant equilibrium. Thus, the malaria situation in the study area is less endemic than has been previously thought (Mutero *et al.*, 1998).

Human participants

Two male human subjects volunteered to participate in the experiments. They were 22-year old fraternal twins, both of whom were fishermen and had stayed in the study area throughout their life. Blood-smears of both subjects (designated X and Z) were collected by finger prick with monolet lancets on each experimental night (19.45 hours), and examined for the presence of malaria parasites using a light microscope (see Chapter 7). Their body temperature (T_b) was measured twice on every experimental night, between 19.50 - 20.00 hours and 22.00 - 22.10 hours. T_b was measured from beneath the tongue using an electronic clinical thermometer.

Ethical considerations

The participants were interviewed about previous infections with malaria, their knowledge on the ability of mosquitoes to transmit malaria and the dangers of exposure to malaria parasites. The participants were taught about the possible early warning signs of malaria e.g. fever, sweating, chills, vomiting etc. The participants' consent to participate in the study was then sought and an informed consent form signed voluntarily, after being verbally translated into their ethnic language (*Luo*) by the experimenter. The participants were interviewed as often as possible whether they experienced any of the malaria symptoms. Malaria drugs (Sulfadoxine-pyrimethamine i.e. FANSIDAR®) were availed so that prompt treatment could be effected upon suspecting infection with malaria parasites. The Kenya National Ethical Review Committee located at the Kenya Medical Research Institute (protocol approval form KEMRI/7/3/1) provided ethical clearance for this study.

Collection of mosquitoes

Host-seeking mosquitoes attracted to each of the two persons were collected with the man landing catch (MLC) and bednet traps (BNTs). Bednet traps were made by punching six 5 × 5 cm holes (50 cm apart from each other) in such a way that they lay ~20 cm above the bedding material when the net was tucked in. The participants sat or slept on beds situated 2-m apart and sampled mosquitoes by the MLC or BNT, respectively. MLC was performed with the participants' trousers folded to knee-high level. Sampling either progressed for two (20.00 - 22.00 hours, MLC) or ~ 8 hours (22.10 - 06.00 hours, BNT). This arrangement matched the daily activity patterns of the adult local inhabitants of Lwanda who are typically out of bed until about 22.00 hours and who remain in bed until about 06.00 hours. Mosquitoes entering the BNTs were collected the next morning (06.00 and 06.30 hours) using suction tubes. All experiments were carried out inside a local hut constructed of mud, wood and thatch, and which only contained two beds with bedding material (Figure 2). *An. gambiae* complex mosquitoes were distinguished from other mosquito species morphologically.

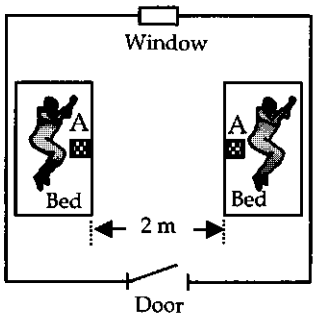

Figure 2. Sketch showing the experimental set-up. The human subjects either sat on the beds (sites marked A) to actively perform a man landing catch (20.00 - 22.00 hours) or slept on the beds (22.10 - 06.00 hours) to passively collect mosquitoes using bed net traps. The participants were trained on how to carry out the man landing catch for a total of 10 evenings.

Examination for malaria parasites

Thick and thin blood smears prepared from finger prick blood of the two human subjects were examined for malaria parasites after staining with Giemsa. Since we lacked equipment for quantifying parasitaemia in terms of numbers present per microlitre of blood, infection levels were assigned semi-quantatively using the 'plus' system. Hence, the presence of 1-10 parasites per 100 thick-film fields was scored as one plus (+), 11-100 parasites per 100 thick-film fields as two plus (+ +), 1-10 parasites per one thick-film field as three plus (+ + +) and the presence of over 10 parasites per one thick-film field as four plus (+ + + +).

Statistical analysis

Given the epidemiological situation of malaria in the study area, where the number of biting mosquitoes and the chance of being malaria positive increase with the onset of the rains, it was very likely that infection with malaria parasites would coincide with higher numbers of biting mosquitoes. It was also possible that test subjects would become infected concurrently. These factors would inevitably influence our main objective of investigating if being malaria positive is associated with higher numbers of attracted mosquitoes. So, we resorted to calculating and testing the significance of the probability of *An. gambiae* biting tests subjects between periods when they were *P. falciparum*-infected and uninfected. The two body temperature (T_b) readings taken of each individual before and after MLC were averaged and the difference (in T_b) between the two subjects calculated. A Generalised Linear Model for binomial data, using a canonical link function (ln) was adopted for the analysis. Factors analysed included site, malaria infection status and between-person difference in T_b. Data collected by MLC and BNT were treated separately. Those collected during the dry and wet seasons were also treated separately as it is possible that the proportions of sibling species differed between the two seasons (White, 1972). Data were analysed using the General statistical software program (Genstat® for windows, 5th Edition).

Results

A total of 2858 *An. gambiae s.l.* mosquitoes were collected, 1398 by the man landing catch (MLC) and 1460 by the bed net trap (BNT). The MLC and BNT collections were carried out for 91 and 95 days, respectively. *An. funestus* was also collected, but given the focus of this thesis on *An. gambiae*, these data have not been included in this chapter. More mosquitoes were collected in the wet than in the dry season. The temporal distribution of the number of mosquitoes collected by the BNT and MLC, and the infection status of the human subjects (person X and Z) during the dry and wet seasons are shown in Figure 3. Both trophozoites (+ and ++) and gametocytes (+) of *P. falciparum* were observed in blood smears of the participants. Gametocytes were observed once for person X and thrice for person Z. Malaria parasitaemia in the two study subjects was mostly observed concurrently and more infections were detected in the wet than in the dry season. No cases of clinical malaria were reported of the two human subjects throughout the entire period of the study.

Effects of infection with **P. falciparum** *on attractiveness to* **An. gambiae**

A general higher probability of individuals being bitten by *An. gambiae* when they were *P. falciparum*-infected was demonstrated, except for dry-season MLC collections which showed a lower near-significant (P = 0.081) or non-significant (P = 0.433) probability of person X and Z being bitten, respectively, when they were infected (Table 1). A higher near-significant probability of *An. gambiae* biting person

X (P = 0.057) and Z (P = 0.078) when they were infected was observed of MLC collections of the wet season. Analysis of BNT collections found a high and significant probability of person X being bitten more when infected in both the dry (P = 0.032) and wet seasons (P = 0.028). The probability of person Z being bitten when infected was significantly higher in the wet (P = 0.004) but not dry season (P = 0.102) as calculated based on BNT collections. As malaria parasitaemia in the two human subjects were generally exhibited concurrently, it was not possible to analyse the effect of infection on the relative attractiveness of the participants. Site effects and the effect of differences in body temperature were not significant.

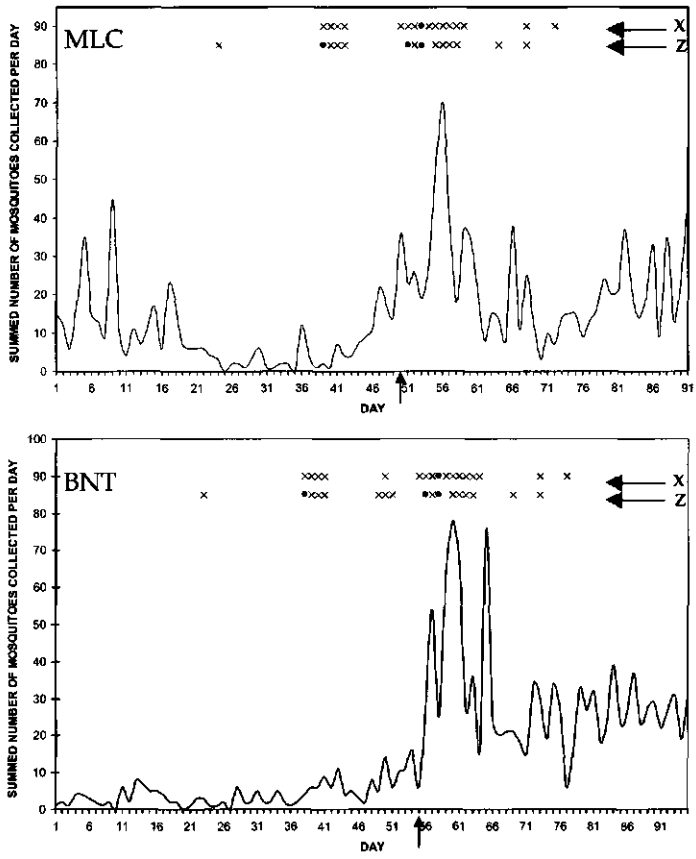

Figure 3. Temporal distribution of *An. gambiae* collected by the man landing catch (MLC) and bednet trap (BNT) during the dry and wet seasons. The infection status of person X and Z, manifested as trophozoites (×) or both trophozoites and gametocytes (•) is also shown. Arrows denote the beginning of the wet season. There were breaks (not shown) of 14 (MLC) and 8 days (BNT) between the end and the beginning of the dry and wet season collections, respectively.

Table 1. t probabilities and beta (β) values showing the probability of *Anopheles gambiae* preferring to bite person X and Z when they were infected with *Plasmodium falciparum* as compared to when they were uninfected for both trapping methods (man landing catch and bednet trap) and collection seasons (dry and wet).

Season	Sampling method	Person	Beta (β)	t probability
Dry	MLC	X	-1.238	0.081
		Z	- 0.80	0.433
	BNT	X	0.804	0.032
		Z	0.588	0.102
Wet	MLC	X	0.253	0.057
		Z	0.251	0.078
	BNT	X	0.120	0.028
		Z	0.358	0.004

MLC and BNT collections found no significant differences in the number *of An. gambiae* attracted to person X and Z when they were infected with *P. falciparum* in both the dry and wet season. This was also the case with MLC collections of person X and Z during the dry ($P = 0.140$) and wet seasons ($P = 0.607$), when both were uninfected. There were significant differences in the number of *An. gambiae* collected with BNT by the two persons during both the dry ($P = 0.029$) and wet season ($P < 0.001$), when both were uninfected, probably signifying method-related differences in attractiveness (Costantini *et al.*, 1999).

Discussion

This study, with the exception of dry season collections, which were not consistent, demonstrated a higher probability of *An. gambiae* being attracted to person X and Z when they were infected with *P. falciparum* as compared to when they were not infected. Malaria parasitaemia in the two human subjects was generally exhibited concurrently. The low mosquito densities in the dry season may have led to erratic, subjective collections thus bringing about the inconsistencies in the results. In addition, differences in the proportions of sibling species of *An. gambiae* complex mosquitoes, which are known to differ in their degrees of anthropophily, between the dry and wet seasons may have also added to this result. Opportunistic *An. arabiensis* is more common in the dry season whereas the highly anthropophilic *An. gambiae s.s.* predominates in the wet season (White, 1972).

Although the idea that malaria parasites might enhance their own transmission by increasing the attractiveness of humans to host-seeking mosquitoes has been speculated upon by many ecologists (Knols & Meijerink, 1997; Braks *et al.*, 1999; Takken & Knols, 1999), the current findings are the first to attest to this. However, it defeats logic that the parasitaemia observed in the

current studies was mostly presented as trophozoites, which do not infect mosquitoes. Because gametocytes were not seen on consecutive days and participants were not given medication (to wipe out presumptive trophozoites), it is likely that false negatives marred our results. So, gametocytes may have been missed especially on days superseding those when they were observed. Also, since mature *P. falciparum* gametocytes are produced at each schizogony and are released in the peripheral circulation some nine days later (Gautret & Motard, 1999), it is likely that the presence of trophozoites and gametocytes in the peripheral circulation coincided. Besides, conventional microscopy is less sensitive for detecting malaria parasites as compared to PCR (Snounou *et al.*, 1993). Should infectivity of mosquitoes be positively correlated to gametocyte density, the possible higher mosquito mortality rates resulting from high oocyst loads would not be evolutionarily advantageous for the parasite. Thus, it would probably be advantageous if low gametocyte densities would result in increased human attractiveness to mosquitoes. Indeed, studies show that peak *P. falciparum* periodicity (Magesa *et al.*, 2000) does not coincide with peak biting times of *An. gambiae* (Magesa *et al.*, 1991).

Since the infections observed in the current study were asymptomatic (thus there was no amplification of physical cues e.g. through fever and profuse sweating) it can be inferred that the cause of differences in the attractiveness of the tests persons between periods when they were *P. falciparum*-infected and uninfected was olfactory. It is known that mosquito host seeking is odour-mediated (Takken, 1991; Takken & Knols, 1999) and that human diseases can result in changes in body odour (Penn & Potts, 1998). In fact, a recent study indicates that the chemical profiles of body odour of *Leishmania infantum*-infected and uninfected hamsters differ and that *Lutzomyia longipalpis* females preferentially respond to entrained odour of the infected hamsters (O'Shea *et al.*, 2002). The potential role of defensive behaviour in influencing our results is discounted by the fact that the test subjects were actively involved in MLC and were fast asleep when (passively) collecting mosquitoes with BNT.

If it is true that *An. gambiae* avoids *P. falciparum*-infected humans when they are clinically ill (Chapter 7) but prefers to feed upon them when they are parasitaemic, then at least one evolutionary consequence of this behaviour is implied i.e. that *P. falciparum* has a selective advantage over *An. gambiae*, being able to signal this mosquito when it is ready to move to the next stage of its life cycle and possibly get transmitted to new vertebrate hosts.

In conclusion, this study demonstrates that under the experimental conditions, infection with *P. falciparum* increases the attractiveness of humans to *An. gambiae*. However, it is realised that the data is based on two individuals only and with relatively few moments of parasitaemia compared to moments when parasites were absent. Thus, more days with parasites present would be required to ascertain these results. Also, it would be interesting to study the attractiveness of individuals when one presents with parasites and the other not. This was not

possible in the present study. However, the use of a twin pair is a unique opportunity that served to eliminate possible confounding factors e.g. the potential effects of genetics on odour profiles. Nevertheless, the data show a clear effect of *P. falciparum* parasites on the attraction of *An. gambiae* to human hosts. These findings coupled to others where it has been shown that *An. gambiae* is preferentially attracted to pregnant women than to non-pregnant counterparts (Lindsay *et al.*, 2000; Ansell *et al.*, 2002) demonstrate an evolutionary advanced system that has far-reaching consequences in terms of malaria transmission. In this respect it should be confirmed whether the higher probability of persons being bitten when infected, as reported here, is related to the presence of gametocytes.

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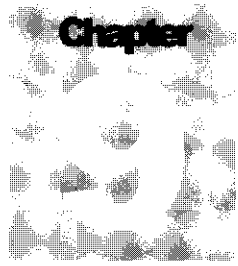

General discussion and conclusions

A large number of studies have investigated the reasons why host-seeking mosquitoes select certain humans more readily than others. Some of these studies have given contradicting results and either left some key questions unanswered or the importance of some schools of thought questioned. Remaining questions include the following: (1) Is it important to know why host-seeking mosquitoes bite certain humans more readily than others? (2) How can we tell whether a person is more or less attractive to host-seeking mosquitoes than another person? (3) Why are causes of selective biting of humans by mosquitoes not yet well understood? (4) Do parasites affect the host-seeking behaviour of their insect vectors? (5) How do evolutionary processes related to host-seeking behaviour affect the fitness of mosquito vectors? These questions and a myriad of others still ponder the medical entomologist. I have attempted to debate upon each of them, linking to the findings reported in this thesis wherever it is applicable.

1. Is it important to know why host-seeking mosquitoes bite certain humans more readily than others?

The simple answer is yes, which I explain below.

Control of vector borne diseases

Efforts aimed at combating insect vectors of disease are either targeted at the community (e.g. larval control, transgenic vectors etc.) or individual/household level (e.g. insecticide-treated bednets, vaccines, chemotherapy/chemoprophylaxis etc). Scarce resources would be utilised best if control measures targeted at individuals would prioritise on persons who are at a high risk of being infected (e.g. those preferred for biting by disease vectors) or those that pose as reservoirs of stages of the parasite that are infective to mosquito vectors. Likewise, the planning of strategies aimed at managing epidemics would become more effective if the patterns of man-vector contact are taken into account. The spill over of this information would be useful in the management of insects that constitute a biting nuisance to humans i.e. people who regard themselves to be at a high risk of being bitten by insects would plan in space and time when and/or whether to visit certain places.

Epidemiology of vector-borne diseases

The distribution of bites by insect vectors among humans is an important prerequisite for understanding the epidemiology of disease transmission. For example, the transmission of malaria between humans requires at least two man-mosquito vector contacts: (1) a susceptible female *Anopheles* mosquito must pick up *Plasmodium* parasites from an infective person and (2) an infective female *Anopheles* mosquito must pass on *Plasmodium* parasites to another human. Models predict an increase in the basic reproductive rate of infection (R_o) if susceptible mosquitoes prefer to bite infective humans (Kingsolver, 1987) or if the variance in the rate of contact between susceptible and infectious individuals increases (Dye & Hasibeder, 1986). Besides, the multiple feeding tendencies of *Plasmodium*-infected mosquitoes (Koella *et al.*, 1998) plus the effects of re-distribution of bites as a result of interrupted feeding (Davies, 1990), attest to the importance of knowledge on host selection and biting preference for understanding the epidemiology of malaria and other vector-borne diseases.

Development of odour-baited traps and devices

Blood-feeding insects are attracted to vertebrate hosts in response to visual, physical and chemical stimuli (Allan *et al.*, 1987; Sutcliffe, 1987; Takken, 1991; Takken & Knols, 1999; Gibson & Torr, 1999). This together with the fact that individuals differ with respect to their body odour (Stoddart, 1990) has prompted speculation that body odour is responsible for differential attractiveness of humans to mosquitoes (Brouwer, 1960; Knols *et al.*, 1995; Chapter 4). Thus, identification of odour molecules that render individuals more attractive to mosquitoes may trigger the development of odour-baited traps and devices. Such traps could be used for sampling or mass trapping as an additional tool for control (Day & Sjogren, 1994) as has already been demonstrated with human baited traps (Costantini *et al.*, 1993; Knols *et al.*, 1995; Mathenge *et al.*, 2002). Since body temperature (Schofield & Sutcliffe, 1997), breath (Schofield & Sutcliffe, 1996; Chapter 5) and carbon dioxide (Brady *et al.*, 1997) have been shown to contribute to differential attractiveness of humans to blood-feeding insects the effects of these parameters also need to be refined so as to augment the trapping potential of body odour. Traps with maximal catch efficiency might then be used in or around houses in order to deviate endophagic insects from biting house occupants.

Insect repellents

Although many repellents are currently playing an additional role in the control and prevention of vector-borne diseases, their advanced performance has been limited by inadequate understanding of insect olfactory responses. Perhaps, it is initially important to understand the chemical basis of why certain humans are less preferred than others by insect vectors of disease. The use of chemicals so identified would help to mask the "correct" odour profile, so deviating insect vectors away from preferred hosts (Costantini *et al.*, 1999).

2. How can we tell whether one person is more/less attractive to host-seeking mosquitoes than another person?

No criteria have been outlined for this purpose yet. Nonetheless, a few insights might give some clues. In chapter 5 it was demonstrated that breath is an important contributor to between-person differences in relative attractiveness as the attractiveness of human subjects was seen to equal out when breath was simultaneously excluded from their total body emanations. This was also the case in studies on black flies (Schofield & Sutcliffe, 1996). It was also demonstrated (chapter 5) that eliminating breath from total body emanations of a human subject rendered him significantly more attractive to the mosquitoes than a counterpart who had his total body emanations intact. However, this does not imply that individuals with lower breath output rates are more attractive to host-seeking mosquitoes. Rather, the amount of potential allomone(s) that is exhaled is what might determine a person's degree of relative attractiveness to host-seeking mosquitoes. However, although it has been suggested that breath contains an active ingredient i.e. carbon dioxide that renders people equally attractive when they exhale it at similar concentrations (Brady *et al.*, 1997), this thinking has waned amid the inability to replicate these results (Costantini *et al.*, 1999).

Much as breath may be an important contributor to between-person differences in relative attractiveness, it should be realised that differences in the relative attractiveness of humans may vary depending on the mosquito species. One individual can be more attractive than another with respect to one mosquito species but less attractive with respect to a different species (Curtis *et al.*, 1987; Knols *et al.*, 1996). Thus, persons who claim that they are always "liked" by mosquitoes, without taking note of the species, might be thinking subjectively. Also, a distinction should be made between anthropophilic and zoophilic mosquitoes. As body odour accounts for approximately 90% of the anthropophilic mosquito *An. gambiae*'s attraction to humans (Mboera *et al.*, 1997), it is likely that an isolated individual can receive strong attacks without necessarily being high ranking in terms of his relative attractiveness to host-seeking mosquitoes.

It should also be realised that an individual's degree of attractiveness can vary depending on transient phenomena. An example of this is provided in chapter 7 where consistent between-person differences in relative attractiveness to *An. gambiae* changed when the person who had been consistently more attractive displayed clinical symptoms of malaria caused by *P. falciparum*. Recent reports indicate that human attractiveness to mosquitoes can also be influenced by diet. Proboscis-amputated *Aedes albopictus* mosquitoes were seen to prefer landing on individuals who had ingested alcohol (Shirai *et al.*, 2002b). The involvement of these transient factors in influencing human attractiveness to mosquitoes shows that it is difficult to predict whether one is more or less attractive to host-seeking mosquitoes. In conclusion, although the difference in attractiveness between

human individuals is presumably odour-based, current knowledge does not allow the use of this information to predict the 'degree' of a person's attractiveness.

3. Why are causes of selective biting of humans by mosquitoes not yet well understood?

Before attempting to answer this question one brain teaser is worth taking: who would a nocturnally-active host-seeking mosquito select when given the choice between two stationary human subjects of different age or sex, size, race, pregnancy status, blood group status, parasite infection status etc present inside separate, see-through glass boxes placed besides one another? In other words, do these factors directly affect the number of mosquitoes attracted to human hosts? A best bet, in view of the understanding that mosquito host-seeking behaviour is modulated by olfactory cues (Takken, 1991; Takken & Knols, 1999) and that different cues are active at different distances away from the host (Gillies & Wilkes, 1969), is that preferential selection would not occur. My expectation is that the glass would screen off the subjects' host characteristics i.e. their body odour, heat and moisture, so precluding the mosquitoes from engaging in activation and orientation behaviour.

Although this prognosis might be interpreted to mean that preferential selection with respect to the listed characteristics would be displayed if the hosts were not concealed, it should be remembered that individuals of the same sex (Lindsay *et al.*, 1993), age (Knols *et al.*, 1995), race (Schreck *et al.*, 1990), blood group status (Thornton *et al.*, 1976) etc can differ in their attractiveness to host-seeking mosquitoes (see also Chapter 4). Furthermore, mosquitoes that have located suitable hosts bite on selected body sites as dictated by convection currents (Dekker *et al.*, 1998) and skin odour/emanations (Knols *et al.*, 1994; De Jong & Knols, 1995; Shirai *et al.*, 2002a). Thus, although preferential selection might be demonstrated, reproduction status, health status, anthropological and somatotypic attributes of hosts cannot fully account for differences in human attractiveness to mosquitoes. Accordingly, causes of selective biting of humans by host-seeking mosquitoes cannot be thoroughly understood if this paradigm is not appreciated.

More insights towards solving the core question (i.e. why are causes of selective biting of humans by mosquitoes not yet well understood?) can be gained by inspecting Table 1. Studies investigating the effect of host age on attractiveness have either used blood typing (ABO blood groups, haptoglobins and blood counts) or catches off (human) bait to measure intra-specific host preferences. Whereas studies using ABO blood groups and/or haptoglobins to assess feeding preferences of *An. gambiae* yielded similar results (Boreham *et al.*, 1978; Bryan & Smalley, 1978; Port *et al.*, 1980), those based on catches off bait did not. In Tanzania this mosquito was seen to bite adults and children randomly (Smith, 1956) while in Sierra Leone it either preferred adults (large families) or bit randomly (small families) (Thomas, 1951). Studies that investigated the effect of sex showed

consistently, irrespective of the mosquito species, that male humans were preferred compared to females (Rahm, 1956; Gilbert *et al.*, 1966; Carnevale *et al.*, 1978) while those addressing the effect of pregnancy status found *An. gambiae* as preferring pregnant women (Lindsay *et al.*, 2000; Ansell *et al.*, 2002). Analysis of blood meals through typing A, B and O blood group phenotypes found *An. gambiae* to either prefer blood group O individuals (Wood *et al.*, 1972; Wood, 1974; 1976) or bite randomly irrespective of the blood group type (Thornton *et al.*, 1976).

These examples demonstrate that the method used to measure host attractiveness can influence the outcome of the results. Another look at Table 1 reveals that the various sampling methods target mosquitoes when they are at different stages in the host-seeking process. Since mosquitoes approaching their hosts use different cues when they are at different stages and/or distances away from the host (Gillies & Wilkes, 1969), it is unlikely that the measures of attractiveness reported using the different sampling methods are comparable. Clearly, mosquitoes collected after obtaining a blood meal have positively responded to most host cues along the general sequence. This sign of avidity, accomplished against the costs and risks associated with host location, is unlikely to associate with preferential host selection. Besides, since, for example, heavily infected (Lyimo *et al.*, 1992) and small-sized mosquitoes may die off before reaching their blood meal hosts (Takken *et al.*, 1998) it can be inferred that samples collected post-engorgement might not be representative (Costantini *et al.*, 1993). On the other hand, the authenticity of using catches off human bait as a method of sampling adult mosquitoes is questionable (Service, 1977). Nonetheless, the analysis of blood meals for the purpose of determining mosquito host sources cannot be regarded as being superfluous. This approach can be used superbly to study multiple blood-feeding rates of mosquitoes under natural settings (Koella *et al.*, 1998) and to assess the efficacy and effectiveness of control tools such as insecticide treated bed nets (Gokool *et al.*, 1992; 1993) and mosquito repellents.

Mosquito behavioural responses may also vary depending on the geographical origin of species. For example, whereas populations of *An. arabiensis* in East Africa are less anthropophilic than those in West Africa, those in Madagascar are mainly zoophilic (Ralisoa-Randrianasolo & Coluzzi, 1987; Duchemin *et al.*, 2001). It may also be that the different geographical populations are actually distinct taxonomic entities pending discovery. The Ethiopian population of *An. quadriannulatus* was recently described to be a distinct species (Hunt *et al.*, 1998) after having been considered similar with its South African namesake for a long time. A new PCR assay now distinguishes the new species separately from other siblings of the *An. gambiae* complex (Fettene *et al.*, 2002). Furthermore, debate is ongoing as to whether incipient species of *An. gambiae s.s* from west Africa might be reproductively isolated populations (Coetzee *et al.*, 2000).

Table 1. Previous investigations of between-person differences in relative attractiveness to host-seeking mosquitoes. The host factor investigated, the mosquito species studied, the place where the studies were conducted, the method used to measure preferential selection (method), the method used to sample mosquitoes (sampling), the event in the host-seeking process on which the score of host preference was based (host seeking) and results of the investigations are shown.

Factor	Mosquito species	Place	Method	Sampling	Host-seeking	Result	Reference(s)
Age	*An. gambiae*	Sierra Leone	Catches off bait	MLC[1]	Landing/biting	Adults preferred over children (in large families) Random biting (in small families)	Thomas, 1951
Age	*An. albimanus, An. aquasalis, An. bellator*	West Indies	Catches off bait	MLC[1]	Landing/biting	Adults preferred over children	Muirhead-Thomson, 1951
Age	*An. gambiae*	Tanzania	Catches off bait	MLC[1]	Landing/biting	No difference in attractiveness	Smith, 1956
Age	*An. gambiae, An. funestus*	Tanzania	Leukocyte counts	Not stated	Engorgement	Feeding occurred randomly	Clyde & Shute, 1958
Age	*An. farauti, An. punctulatus*	PNG	Catches off bait	MLC[1]	Landing/biting	Adults preferred over children	Spencer, 1967
Age	*An. gambiae*	Congo	Catches off bait	MLC[1]	Landing/biting	Adults preferred over children	Carnevale *et al.*, 1978
Age	*An. gambiae, Culex quinquefasciatus*	Kenya	Hp types	BNT	Engorgement	Adults preferred over children	Boreham et al., 1978
Age	*An. gambiae*	Gambia	Blood group	BNT	Engorgement	Adults preferred over children	Bryan & Smalley, 1978
Age	*An. gambiae*	Gambia	Hp/blood group	BNT	Engorgement	Adults preferred over children	Port *et al.*, 1980[a]
Age	*An. punctulatus*	PNG	Blood group	BNT & RC	Blood feeding	Random, but some children preferred over adults	Burkot *et al.*, 1988
Sex	*Aedes aegypti*	Laboratory	Landings on arm/hand	-	Landing/biting	Males preferred over females	Rahm, 1956
Sex	*Ae. aegypti*	Laboratory	????	-	????	Males preferred over females	Gilbert *et al.*, 1966
Sex	*An. gambiae*	Congo	Catches off bait	MLC[1]	Landing/biting	Males preferred over females	Carnevale *et al.*, 1978
Race/hue	*Ae. aegypti*	Laboratory	Landings on arm/hand	-	Landing	No race effect but dark-skinned persons preferred	Smart & Brown, 1955
Diet	*Ae. albopictus*	Laboratory	Landings on arm/hand	-	Landing	Ingestion of alcohol increased mosquito attraction	Shirai *et al.*, 2002b
Pregnancy	*An. gambiae, Mansonia* spp	Gambia	Number present inside hut	Not clear	Orientation	Pregnant women preferred over non-pregnant ones	Lindsay *et al.*, 2000

Table 1 continued

Pregnancy	*An. gambiae*	Gambia	DNA profiling	BNT	Engorgement	Pregnant women preferred over non-pregnant ones	Ansell *et al.*, 2002
Blood group	*An. gambiae, Ae. aegypti*	Laboratory	Blood group	-	Engorgement	Blood group O individuals preferred	Wood *et al.*, 1972; Wood, 1974; 1976
Blood group	*An. gambiae*	Laboratory	Landings on arm / hand, blood group	-	Landing, engorgement	No preferences according to blood group status	Thornton *et al.*, 1976
Infection	*An. punctulatus*	PNG	Blood typing	RC	Engorgement	Random biting for malaria/ filaria-infected and un-infected persons	Burkot *et al.*, 1989
Odour	*An. stephensi*	Laboratory	Number of probes	Olfactometer	Probing	Consistent attraction differences between persons' arm/hand odour seen	Brouwer, 1960
Odour	*Ae. aegypti*	Laboratory	Trap catches	Olfactometer	Orientation	Consistent attraction differences between persons' arm/hand odour seen	Mayer & James, 1969
Odour	*Ae.aegypti, An. quadrimaculatus*	Laboratory	Number of landings / probes	-	Landing, probing	Consistent attraction differences in material from hands of different persons seen	Schreck *et al.*, 1990
Odour	*An. gambiae*	Burkina Faso	Number trapped	OBETs	Orientation	Consistent between-person attraction differences seen	Brady *et al.*, 1997
Odour	*An. gambiae*	Kenya	Number trapped	Olfactometer	Orientation	Consistent between-person attraction differences seen	Chapter 4.
General	*An. melas*	Sierra Leone	Number inside hut	PSC	Orientation/ feeding	Inconsistent between-person attraction differences seen	Ribbands, 1950
General	*Ae. aegypti*	Laboratory	Probing time, number of bites	-	Probing, biting	Attraction differences seen based on probing time, not number of bites	Khan *et al.*, 1966; Maibach *et al.*, 1966
General	*An. coustani, Mansonia spp, Cx. quinquefasciatus*	Tanzania	Catches off bait	MLC[2]	Landing/biting	Species-specific between-person differences in relative attractiveness reported	Curtis *et al.*, 1987
General	*An. gambiae s.l*	Gambia	Number in hut / with human blood	Trap/RC, ELISA	Orientation, engorgement	Consistent between-person attraction differences found	Lindsay *et al.*, 1993
General	*An. nuneztovari, An. marajoara, An. triannulatus*	Venezuela	Catches off bait	MLC[2]	Landing/biting	No between-person differences in relative attractiveness	Rubio-Palis, 1995
General	*An. gambiae, An. funestus, cx. quinquefasciatus*	Tanzania	Number trapped	Trap/RC	Orientation	Consistent species-specific between-person differences in relative attractiveness reported	Knols *et al.*, 1995

[a]age effects were attributed to differences in body mass i.e adults were preferred over children because of large size; blood group, A, B or O blood group status; BNT, bednet trap; ELISA, enzyme linked immunosorbent assay; Hp, haptoglobin genotypes; MLC[1], man landing catch done with one person as bait and another as collector; MLC[2], man landing catch done by the bait person; OBET, odour baited entry trap; PNG, Papua New Guinea; PSC, pyrethrum spray catches; RC, resting collection.

From these examples it can be realised that although mammalian semiochemicals include indicators of sex, health, age, reproductive status and diet (Brown, 1979; Penn & Potts, 1998), these factors cannot be tagged to variability in human attractiveness to mosquitoes. The individual differences in body odour profiles, which are known to exist between persons (Stoddart, 1990), seem to be more important. Yet another look at Table 1 makes it clear that a reliable result can be expected if variability in human attractiveness to mosquitoes is assessed using a chemical-ecological approach. However, the effect of physical cues in causing between-person differences in relative attractiveness at short-range cannot be disregarded (Chapter 6). Degrees of human attractiveness to haematophagous insects can vary depending on whether assessments are made based on short- or long-range active cues (Schofield & Sutcliffe, 1996; 1997; Costantini *et al.*, 1999).

4. Do parasites affect the host-seeking behaviour of their insect vectors?

The question of whether or not medically important parasites affect the host-seeking behaviour of their insect vectors is still not clear. Although there is evidence to suggest that diseases alter human olfactory characteristics (Penn & Potts, 1998), the effect of this on the host-seeking behaviour of disease vectors remains unknown. Studies have found no preference by *An. puntulatus* complex mosquitoes and simuliids for biting malaria-/filariasis- (Burkot *et al.*, 1989) and *Onchocerca volvulus*-infected humans against uninfected counterparts (Kruppa & Burchard, 1999), respectively. In evolutionary terms, it would be best for vector-borne parasites to modify the olfactory characteristics of their vertebrate hosts so that they are preferentially fed upon by insect vectors at a time when the parasites are most infective to the insects, and therefore ready to proceed to the next step in their life cycles. However, even though this was not demonstrated among mosquitoes of the *An. punctulatus* complex (Burkot *et al.*, 1989), this phenomenon may be there among other species since differences in the attractiveness of humans to host-seeking mosquitoes are apparently species-specific (Curtis *et al.*, 1987; Knols *et al.*, 1996).

Studies deriving from rodent malaria models have demonstrated that host-seeking mosquitoes prefer to feed on infected individuals because these display reduced defensive behaviour (Day & Edman, 1983; Day *et al.*, 1983; Day & Edman, 1984). However, since many nocturnal mosquito species bite at times when their human hosts are asleep, it is unlikely that defensive behaviour alone can explain the ecological hypothesis that infected humans might be more attractive to host-seeking mosquitoes than uninfected counterparts (Knols & Meijerink, 1997; Takken & Knols, 1999). Furthermore, since insects attacking infected rodents feed on specific sites i.e. lesions (Coleman *et al.*, 1988), it is likely that some other factors, more so olfactory (O'Shea *et al.*, 2002), are responsible for guiding insect vectors to infected individuals. The studies reported in this thesis constitute the first evidence that malaria parasites influence the host-seeking behaviour of their insect vectors

by signalling through vertebrate hosts. Seemingly, host-seeking mosquitoes avoid human hosts during clinical malaria episodes (Chapter 7) and feed on them preferentially when they are parasitaemic (Chapter 8).

5. How do evolutionary processes related to host-seeking affect the fitness of mosquito vectors?

The many factors that might cause *An. gambiae* to avoid or select certain humans as their blood meal hosts were proposed in Chapter 1 (section on problem definition and research objectives). Of all those, this thesis directly answers one, although it gives negative evidence. Contrary to my expectations, *An. gambiae* was attracted in greater numbers to humans when they were parasitaemic and possibly harbouring infective *P. falciparum* gametocytes (Chapter 8). Although this is advantageous for the parasite, it has negative effects on mosquito vectors as these are harmed by malaria parasites in many different ways (Ferguson & Read, 2002). Thus, it is likely that the malaria parasite *P. falciparum* is more evolutionarily advanced than its mosquito vector *An. gambiae*. Perhaps the r-selection reproductive strategy is one way through which the mosquitoes have adapted to deal with this selective disadvantage.

An. gambiae was also found to avoid an otherwise highly attractive individual when he presented with clinical symptoms (fever and profuse sweating) of malaria caused by *P. falciparum* (Chapter 7). Malarial fever is usually associated with the rupturing of infected red blood cells when a new brood of parasites that are not infective to mosquitoes (i.e. merozoites) is produced asexually (Chapter 1). From an evolutionary point of view it does not benefit the parasite to signal mosquito vectors so that they go foraging for blood of infected individuals at a time when merozoites are predominant. Given the potentially enormous losses to the host's immunity, it would be reasonable for parasites to minimise additional losses in terms of their population density so that more gametocytes are developed.

Avoidance of individuals with clinical malaria symptoms is probably also advantageous to the mosquito and can be interpreted to mean that *An. gambiae* has co-evolved with humans for long. Although merozoites are unlikely to harm mosquito vectors, nobody knows whether this is the case. My expectation is that merozoites may possibly lower the quality of the blood meal. Mosquito fecundity may even be reduced when females feed on infected, but non-infective, blood. In terms of co-evolution between man and *An. gambiae*, one often hears people say, after they have recovered from bouts of malaria, that "that was not me". I add to say that *An. gambiae* keeps away until it hears one say "now I am Ok". This information is most probably communicated through physico-chemical signals, a sign that *An. gambiae* has lived with man for long enough that it knows when he oscillates between healthy and ill health status. As proposed in Chapter 1, it would be a sign of fitness for *An. gambiae* to select a blood meal of high nutritional value, here interpreted to mean one from a healthy individual.

More evolutionary implications, besides those initially proposed as being ideal for biologically fit *An. gambiae*, are, however, implied by the findings reported in this thesis. That *An. gambiae* undergoes orientation towards human body odour in disfavour of breath (Chapter 5) is further evidence that this mosquito has evolved alongside man for long. It would be less advantageous for this mosquito to prefer human breath against body odour as breath is mainly composed of carbon dioxide, heat and moisture. None of these factors are distinctive of her (*An. gambiae*'s) preferred human hosts. It has been argued that carbon dioxide is not a good kairomone for *An. gambiae* because it is not host-specific (Knols *et al.*, 1997). Being highly anthropophilic, it would be logical for *An. gambiae* to select for mechanisms that would detect her human hosts distinctively. Such a mechanism probably exists as it has been shown that body odour accounts for > 90% of the attraction of *An. gambiae* to humans (Mboera *et al.*, 1997).

The preference for warmer individuals (Chapter 6) probably highlights a general strategy utilised by many species to differentiate between endothermic and ectothermic animals. Furthermore, given that an engorging mosquito exposes herself to an increased risk of death by irritating her host (Gillet, 1967), it is reasonable for *An. gambiae* to feed on warmer individuals as this ensures rapid engorgement (Grossman & Pappas, 1991). Besides, this might be an adaptation towards saving energy and time since warmth is often associated with the physical presence of a host. It is still not known whether this selective behaviour may also be coupled to reproductive fitness i.e. preferential selection of individuals whose blood supports the development of more eggs thus leading to a higher clutch size and possibly more surviving offspring. My own unpublished data and those of others (R. Anderson, personal communication) do not provide positive evidence for this. All these factors need further investigation.

In Chapter 1 I proposed that *An. gambiae* would optimise her fitness, among other ways, by avoiding defensive hosts and selecting docile ones. Her nocturnal feeding habits and the fact that she prefers to bite the (less sensitive) foot and leg region (De Jong & Knols, 1995) precludes her human hosts from engaging in active defence. Ecologically, one would expect that man may have developed passive defence mechanisms against this mosquito e.g. through the composition of his body emanations (Kelly, 2001). Thus, humans might be releasing compounds that are beneficial to themselves (i.e. those that keep mosquitoes away) but which are harmless to mosquitoes. This allomonal effect is probably associated with human breath (Chapter 5). However, the fact that *An. gambiae* prefers humans against other animals implies selection by this mosquito for feeding on a relatively less defensive host, a factor manifested by the absence of a complete cover of skin hair, a would be passive defence barrier against attacking mosquitoes.

The following conclusions can be drawn from the research objectives that were formulated at the beginning of the studies reported herein:

Objective 1: To develop an olfaction-based bioassay and use it to verify if variability in human attractiveness to mosquitoes has a physico-chemical basis

- variability in human attractiveness to host-seeking *An. gambiae* exists and can be quantified based on differences in the composition of total body emanations including body odour, heat and moisture (*Chapter 4*).
- no residual effects that are attractive to *An gambiae* are left behind when humans vacate locations where they have occupied temporarily *(Chapter 4).*

Objective 2: To investigate how breath and body odour contribute to and possibly interact with the attractiveness of humans to *An. gambiae*

- human breath seemingly inhibits the response of *An. gambiae* towards her blood meal hosts (*Chapter 5 & 6).*
- human breath is an important contributor to between-person differences in relative attractiveness to *An. gambiae (Chapter 5 & 6).*
- body odour is chiefly responsible for the attraction of *An. gambiae* to humans (*Chapter 5*).

Objective 3: To investigate the effect of body heat and moisture on the short-range attractiveness of humans to *An. gambiae*

- *An. gambiae,* to a good extend, prefers humans who are warmer but who have low moisture output rates (*Chapter 6*)
- body heat and moisture interact in the short-range attractiveness of humans to the malaria mosquito *An. gambiae* (*Chapter 6*)

Objective 4: To investigate whether infection with malaria parasites affects the attractiveness of humans to *An. gambiae*

- clinical symptoms (fever and profuse sweating) due to infection with *P. falciparum* suppress the relative attractiveness of humans to the malaria mosquito *An. gambiae* (*Chapter 7*)
- being positive with *P. falciparum* is likely associated with increased attractiveness of humans to *An. gambiae* (*Chapter 8*)

Objective 5 (minor objective): To evaluate the effect of blood meal size and extent of digestion on the success of amplifying human DNA from blood meals of *An. gambiae*

- blood meal size has no significant effect on the success probability of amplifying human DNA from blood meals of *An. gambiae* (*Chapter 3*)
- digestion has a significant negative effect on the success probability of amplifying human DNA from blood meals of *An. gambiae* (*Chapter 3*).

- there is no significant difference in the success of amplifying human DNA between blood meals of *An. gambiae* digested for 0 and 8 hours (at 27±1°C and 80±5% RH) (*Chapter 3*).

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Summary

The results of a series of studies designed to understand the principal factors that determine the differential attractiveness of humans to the malaria vector *Anopheles gambiae* are described in this thesis. Specific attention was paid to the role of body emanations and infection (of humans) with the malaria parasite *Plasmodium falciparum*. The main findings of these studies are summarised in the following sections.

Differential attractiveness of humans to *Anopheles gambiae* (Chapter 1)

Although it has frequently been reported that human beings differ in their degree of attractiveness to mosquitoes, the principal causes that make certain individuals to be preferred more than others are not well known. This gap in knowledge has hindered the understanding of the transmission dynamics of malaria and other mosquito-borne diseases. From an epidemiological point of view, high malaria transmission rates are expected if mosquito vectors preferentially select infective humans for a blood meal, become infected, survive long enough for the parasites to develop to infective stages and proceed to bite uninfected individuals selectively. In terms of fitness, mosquito vectors would be better off if they select hosts that (1) are docile and less defensive so as to minimise feeding-associated risks of mortality, (2) have blood of a high nutritional value, (3) are free of (gametocyte) parasites, healthy and (4) have no anti-mosquito immunity. The answers to these epidemiological and fitness factors are still lacking. Furthermore, since host seeking is odour-mediated, the identification of chemical compounds responsible for attracting mosquitoes to their vertebrate hosts would help in developing traps that are useful for vector surveillance and control. Research in this area is rapidly advancing but has not produced tools for field application. It is felt that more research effort is needed so that new approaches towards understanding and combating disease vectors can be developed.

Characterisation of mosquito blood meals using DNA markers (Chapters 2 & 3)

The analysis of arthropod blood meals using molecular genetic markers was reviewed and forensic techniques borrowed from the Federal Bureau of Investigation (FBI), USA, used to evaluate the effect of blood meal size and extent of digestion on the ability to identify human DNA extracted from blood meals of *Anopheles gambiae*. The review recommended that proper and appropriate storage, determination of the concentration of host DNA and collection within few hours after ingestion are important parameters for improving the success of identifying blood meal sources of field-collected mosquitoes. Further, microsatellite markers were highlighted as being more appropriate than minisatellites in analysing blood

meals that have been highly degraded e.g. through prolonged digestion. Also, mitochondrial DNA targets were recommended to be better than nuclear DNA targets for analysing blood meals that have been highly degraded. Blood meal size and (microsatellite) locus (analysed) were shown not to affect the success of amplifying human DNA extracted from blood meals of *An. gambiae* after having been digested for 0, 8, 16, 24 and 32 hours. However, a significant negative relationship between the time since ingestion and the success probability of obtaining positive PCR reactions among blood meals digested for between eight and 32 hours was demonstrated. There was no significant difference in the success probability of amplifying human DNA from blood meals of mosquitoes killed at zero and 8 hours after ingestion. The research demonstrated that not the quality of ingested blood, but the time since ingestion determined the success of blood meal analysis.

Host characteristics and differential attractiveness of humans to *An. gambiae* (Chapter 4, 5 & 6)

A tent olfactometer that accommodates complete humans as sources of host-seeking stimuli was designed, developed and tested. The olfactometer was used to study (1) differential attractiveness of humans to host-seeking *An. gambiae* and (2) how the differences, so elicited, are affected by human breath, body odour, heat and moisture. Nine human subjects were successfully ranked for their attractiveness to the mosquitoes based on (mosquito) responses to their complete body emanations encompassing body odour, heat and moisture. The nine subjects were classified into least (3 persons), medium (4 persons) and most attractive groups (2 persons). Breath was shown to reduce mosquito responses, whereas body odour was highly attractive. Breath was also shown to be an important contributor to between-person differences in relative attractiveness to *An. gambiae*. Whereas differential attractiveness of two human subjects for the mosquitoes could be demonstrated based on their total body emanations (breath plus body odour), the attractiveness of the two subjects did not differ significantly based on body odour alone. Body odour from either individual was consistently more attractive than total emanations from the other. The same results were obtained with another pair of individuals. It was concluded that breath, although known to contain attractive semiochemicals like carbon dioxide, may also contain compounds that inhibit attraction and may thus serve as an important contributor to between-person differences in relative attractiveness to this important malaria vector. The inhibitory effect of breath was postulated to be allomonal as it benefits the emitter (human being) but does not harm the recipient (mosquito vector). Body heat and moisture were shown to have significant effects on the attraction of *An. gambiae* to humans. In general, *An. gambiae* was more attracted to the individual whose body emanations were warmer but less moist than those of an opposing counterpart, in choice experiments. It was concluded that body heat and moisture influence host-

selection by *An. gambiae* at short range and that their effect is probably achieved through interaction with breath components.

Parasite infection and differential attractiveness of humans to *An. gambiae* (Chapter 7 & 8)

A rare window of opportunity allowed for the investigation of the effect of clinical symptoms and parasitaemia due to *Plasmodium falciparum* on variability in human attractiveness to the malaria mosquito *An. gambiae* using the tent olfactometer previously developed. The relative attractiveness of an individual who was always more attractive than a specific counterpart (when both were uninfected) was suppressed when he (the 'putatively' more attractive individual) displayed clinical symptoms (fever and profuse sweating) of malaria caused by *P. falciparum*. This finding provided both new and alternative evidence, within the intricate web of *Anopheles-Homo-Plasmodium* interactions, that the malaria parasite *P. falciparum* influences the olfactory signals produced by human hosts. Field studies, in which a twin pair of male humans was recruited as a follow-up to the olfactometer study, found that being positive with malaria parasites (trophozoites and possibly gametocytes of *P. falciparum*) is associated with higher numbers of attracted mosquitoes (*An. gambiae sensu lato*). This effect was not a consequence of the time of the year and the higher number of mosquitoes present in that period. It was recommended that further investigations be carried out. If these results are confirmed to be true, this work will constitute the first evidence that *P. falciparum* is capable of enhancing its own transmission by manipulating the physico-chemical characteristics of its vertebrate hosts in such a way that infected individuals are preferentially selected as blood meal sources by host-seeking malaria vectors.

What questions remain unanswered (Chapter 9)

Much as this thesis has provided some answers to why humans differ in their degrees of attractiveness to mosquitoes (*An. gambiae*), many more questions remain unanswered. The key questions in point include the following: (1) How can we tell whether one is more or less attractive to host-seeking mosquitoes? (2) How do parasites affect the host-seeking behaviour of their insect vectors? and (3) How do evolutionary processes related to host-seeking behaviour affect the fitness of mosquito vectors? The answers to these questions and those to a myriad of others are still waiting to be resolved.

Samenvatting

Dit proefschrift beschrijft de resultaten van een onderzoek naar de belangrijkste factoren welke verschillen tussen mensen in hun aantrekkelijkheid voor de malariavector *Anopheles gambiae* bepalen. De rol van lichaamsgeuren en van infectie met de humane malariaparasiet *Plasmodium falciparum* zijn specifiek bestudeerd. De belangrijkste bevindingen van het onderzoek worden hieronder samengevat.

Variatie in aantrekkelijkheid van mensen voor *Anopheles gambiae* (Hoofdstuk 1)

Hoewel vaak gerapporteerd is dat mensen verschillen in de mate van aantrekkelijkheid voor muggen, zijn de voornaamste oorzaken hiervoor nog niet bekend. Dit gebrek aan kennis vormt een belemmering om de dynamiek van malariatransmissie and andere door muggen overgedragen vectorziekten goed te begrijpen. Epidemiologisch gezien wordt een hoge graad van transmissie verwacht als muggen a) bij voorkeur een bloedmaaltijd nemen bij geïnfecteerde mensen, b) zelf geïnfecteerd raken met de malariaparasiet, c) voldoende lang overleven om de parasiet tot ontwikkeling te laten komen en d) vervolgens bij voorkeur gezonde mensen steken. Vanuit het oogpunt van fitness zijn muggen beter af bij een voorkeur voor een (menselijke) gastheer e) die in rust is en geringe zelfverdediging toepast, f) wiens bloed een hoge voedingswaarde heeft, g) vrij is van malaria parasieten en h) (nog) geen immuniteit tegen muggen ontwikkeld heeft. Diepgaande kennis over deze epidemiologische en fitness factoren is nog niet beschikbaar. Omdat het zoeken naar een gastheer in sterke mate bepaald wordt door vluchtige signaalstoffen, zal de identificatie van chemische stoffen die de aantrekking van muggen naar hun vertebraat gastheren bepalen, helpen bij de ontwikkeling van geurvallen welke ingezet kunnen worden bij de meting van vector dichtheden en bestrijding. Onderzoek op dit terrein ontwikkelt zich snel maar heeft nog niet geleid tot middelen welke in het veld gebruikt worden. Een extra onderzoeksinspanning is nodig om een nieuwe aanpak voor kennis en bestrijding van vectoren te ontwikkelen.

Typering van muggenbloedmaaltijden met DNA technieken (Hoofstukken 2 & 3)

Een overzicht wordt gegeven van de analyse van bloedmaaltijden van arthropoden met behulp van moleculair genetische technieken. Forensische technieken, overgenomen van het Federal Bureau of Investigation (FBI) in de Verenigde Staten, zijn gebruikt om vast te stellen in hoeverre de grootte van bloedmaaltijden en de mate van vertering een effect hebben op de succesvolle typering van humaan DNA afkomstig uit bloedmaaltijden van *Anopheles gambiae*. Het literatuuroverzicht beveelt aan dat correcte bewaarmethoden van bloed, de

bepaling van de concentratie van gastheer DNA en het verzamelen van bloedmaaltijden binnen enkele uren na ingestie, belangrijke aspecten zijn om een succesvolle typering van bloedmaaltijden uit muggen mogelijk te maken. Microsatelliet merkers blijken beter geschikt voor de typering van bloed dan minisatelliet merkers, indien het bloed reeds afgebroken is bijvoorbeeld door langdurige vertering in de middendarm. Voor de typering van bloedmaaltijden die al in hoge mate verteerd zijn is mitochondriaal DNA een betere doelgroep dan kern DNA. De grootte van de bloedmaaltijd en de plaats van de microsatelliet binnen het DNA bleken niet bepalend te zijn voor een succesvolle amplificatie van menselijk DNA afkomstig uit bloedmaaltijden van *Anopheles gambiae* na vertering van 0, 8, 16, 24 en 32 uur. Een significant negatief verband werd vastgesteld tussen de tijd verstreken tussen ingestie van bloed door de mug en de kans op een geslaagde PCR reactie over een traject van 8 tot 32 uur na ingestie. Het onderzoek toonde aan dat niet de kwaliteit van het opgenomen bloed, maar de tijd verstreken na ingestie bepalend was voor een succesvolle typering van het bloed.

Gastheer eigenschappen en variatie in aantrekkelijkheid van mensen voor *Anopheles gambiae* (Hoofstukken 4, 5 & 6)

Een tent olfactometer, geschikt om mensen als bron van gastheerstimuli te gebruiken, is ontworpen, ontwikkeld en getest. De olfactometer is gebruikt om de verschillen in aantrekkelijkheid bij mensen voor de mug *Anopheles gambiae* te onderzoeken en om vast te stellen of deze verschillen veroorzaakt worden door adem, huidgeur, lichaamswarmte en/of -vocht. Negen mannen werden gerangschikt op basis van hun aantrekkelijkheid voor de muggen op basis van het aantal muggen dat reageerde op complete lichaamsgeur, inclusief adem, warmte en vocht. De mannen werden op basis van het onderzoek geclassificeerd als minst aantrekkelijk (3 personen), matig aantrekkelijk (4 personen) en meest aantrekkelijk (2 personen). Adem verlaagde de aantrekking van de muggen, terwijl de geur van de huid zeer aantrekkelijk was. Adem veroorzaakte ook het verschil tussen personen in mate van aantrekkelijkheid voor de muggen. Terwijl de gevonden verschillen tussen twee mannen terug te voeren waren op de complete lichaamsgeuren, bleken deze verschillen niet langer significant te zijn indien de reactie van de muggen op de geuren van de huid met elkaar vergeleken werd. De huidgeur van de een bleek consistent sterker attractief dan de complete lichaamsgeur (adem + huidgeur) van de ander. Vergelijkbare resultaten werden verkregen met een ander paar mannen. Uit deze resultaten wordt geconcludeerd dat adem signaalstoffen bevat welke attractie verlagen, ondanks het feit dat adem ook sterk aantrekkelijke stoffen bevat zoals o.a. koolzuur. Adem kan dus een belangrijke bijdrage leveren aan het verschil in attractie tussen mensen bij *Anopheles gambiae*. Het attractie-verlagend effect van adem is terug te voeren op een allomoon, omdat het de producent (de mens) bevoordeelt zonder de ontvanger (de mug) te benadelen. Lichaamswarmte en vocht bleken een significant positief effect

te hebben op de aantrekkelijkheid van mensen voor *An. gambiae*. In een keuze experiment bleek de mug meer aangetrokken te worden tot een persoon met een hogere lichaamstemperatuur, maar lagere vochtafgifte, dan een ander persoon. Hieruit wordt geconcludeerd dat lichaamswarmte en vocht de gastheerkeuze van *An. gambiae* beïnvloeden op de korte afstand, and dat dit effect waarschijnlijk wordt veroorzaakt door interactie met stoffen uit de adem.

Parasitaire infectie en verschillen in aantrekkelijkheid van mensen voor *An. gambiae* (Hoofdstukken 7 & 8)

Een unieke gelegenheid maakte het mogelijk om het effect van klinische malaria symptomen en van parasitemie veroorzaakt door *Plasmodium falciparum* op de variatie van mensen in aantrekkelijkheid voor malariamuggen te bestuderen. Dit onderzoek werd uitgevoerd met *An. gambiae* in de tent olfactometer (zie hfst 4-6). De relatieve aantrekkelijkheid van een persoon die altijd meer muggen aantrok dan een tweede persoon (in situaties waarbij beiden niet besmet waren) was significant verlaagd tijdens een moment van klinische symptomen (koorts en sterk zweten) van malaria door *Plasmodium falciparum*. De resultaten van dit onderzoek leveren nieuw bewijs, binnen het gecompliceerde weefsel van *Anopheles-Homo-Plasmodium* interacties, dat de malariaparasiet *P. falciparum* de afgifte van olfactorische signaalstoffen van de gastheer beïnvloedt. In een hierop volgende veldstudie, waarvoor een mannelijke humane tweeling werd gerekruteerd, is gevonden dat de aanwezigheid van malaria parasieten (trophozoieten en mogelijk ook gametocyten van *P. falciparum*) was geassocieerd met attractie van een groter aantal muggen (*An. gambiae sensu lato*) in vergelijking tot parasiet-vrije episoden. Dit effect werd niet veroorzaakt door de seizoensinvloeden of een 'hoge' muggendichtheid tijdens de perioden van parasitemie. Het wordt aanbevolen om dit onderzoek nader voort te zetten. Indien deze resultaten bevestigd kunnen worden, is dit het eerste bewijs dat *P. falciparum* zijn eigen transmissie kan beïnvloeden door manipulatie van fysisch-chemische eigenschappen van de vertebraat gastheer zodat besmette mensen bij voorkeur geselecteerd worden als voedselbron door malaria vectoren op zoek naar een gastheer.

Welke vragen blijven onbeantwoord? (Hoofdstuk 9)

Hoewel in deze dissertatie vragen beantwoord worden die mede verklaren waarom mensen verschillen in mate van aantrekkelijkheid voor muggen (*An. gambiae*), blijven nog vele vragen onbeantwoord. Enkele belangrijke vragen zijn 1) Hoe kan men weten of men aantrekkelijk is voor steekmuggen of niet? 2) Op welke wijze beïnvloeden (malaria) parasieten het gastheer zoekgedrag van hun vectoren? 3) Op welke manier wordt de fitness van muggen beïnvloed door evolutionaire processen welke het gastheerzoekgedrag bepalen? Antwoorden op deze en op talloze andere (gerelateerde) vragen moeten nog opgelost worden.

Curriculum vitae

Richard Mukabana[1] was born on 12 July, 1970 in Kericho District, Kenya. He started formal education at Mumias Boys' Boarding Primary School (Mumias District) where he attained a Certificate of Primary Education (C.P.E.) in 1982. He proceeded to Kolanya Boys' High School (Teso District) for his ordinary level of education ('O' level) and obtained a Kenya Certificate of Education (K.C.E) in 1986. He furthered on to Nyabondo High School (Kisumu District) for his advanced level of education ('A' level) and obtained a Kenya Advanced Certificate of Education in 1988. In 1989 he attended a pre-university course in military training and joined Egerton University (Nakuru District) for a B.Sc. degree (Botany and Zoology) which he completed in 1992. The Catholic Church diocese of Meru employed him as a teacher at Karamani Boys' Secondary School (Tharaka-Nithi District) in 1993. He quit and went on to study for a M.Sc. degree (Medical Parasitology) at the University of Nairobi. Between 1995 and 1997 he worked as a research assistant/MSc. project student with the USA Centres for Disease Control and Prevention (CDC) based at the Kenya Medical Research Institute (KEMRI) in Nairobi. There, he studied the population genetics of the African malaria mosquito *Anopheles gambiae* Giles *sensu stricto* using microsatellites. He obtained his M.Sc. degree in 1997 and joined the Department of Zoology (University of Nairobi) as a graduate assistant. He became a Tutorial Fellow in 1998. In the same year he (1) served as a WHO temporary advisor in a course on population genetics of African malaria vectors at the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi and (2) obtained a sandwich Ph.D. scholarship to study at 'Wageningen Agricultural University'in The Netherlands. He returned to Kenya in 1999 and became a Dissertation Research Internship Program (DRIP) scholar at ICIPE. He worked at ICIPE-Mbita Point Research and Training Centre (MPR&TC) (Suba District) until April 28, 2002 and then returned to 'Wageningen University & Research Centre' (May 3, 2002) to write and compile his Ph.D. thesis. In between, in 2001, he participated in the prestigious Colorado State University-organised "Biology of Disease Vectors" summer course at the Institute of Parasitology, University of South Bohemia, České Budějovice, Czech Republic, and in a Case Western Reserve University-organised course on "Emerging and Re-emerging Infections" at the Kenya College of Communications Technology in Nairobi. Again, in 2001, he won a UNDP/World Bank/WHO Research Training Grant (RTG) (project ID. No. A10370) through ICIPE. He is married to Jacqueline E. Oduke with three children: Sheila, Linda and Emmanuel.

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List of Publications

Kamau, L., *Mukabana, W.R.*, Hawley, W.A., Lehmann, T., Irungu, L.W., Orago, A.A. & Collins, F.H., 1999, Analysis of genetic variability in *Anopheles arabiensis* and *Anopheles gambiae* using microsatellite loci. *Insect Molecular Biology*, **8**: 287-297.

Knols, B.G.J., Njiru, B.N., Mathenge, E.M., *Mukabana, W.R.*, Beier, J.C. & Killeen, G.F. A greenhouse-based simulation of a natural *Anopheles gambiae* (Diptera: Culicidae) ecosystem in Western Kenya. To be submitted.

Knols, B.G.J., Njiru, B.N., *Mukabana, R.W.*, Mathenge, E.M. & Killeen, G.F. Contained semi-field environments for ecological studies on transgenic African malaria vectors. In: *Ecology of Transgenic Mosquitoes* (Scott, T.W. & Takken, W., eds.). In press.

Mukabana, W.R., Takken, W., Seda, P., Killeen, G.F., Hawley, W.A. & Knols, B.G.J., 2002, Extent of digestion affects the success of amplifying human DNA from blood meals of *Anopheles gambiae* (Diptera: Culicidae). *Bulletin of Entomological Research*, **92**, 233-239.

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Mukabana, W.R., Takken, W., Killeen, G.F. & Knols, B.G.J., 2002, The role of breath and body odour in the attractiveness of humans to *Anopheles gambiae*. Submitted.

Mukabana, W.R., Takken, W., Killeen, G.F. & Knols, B.G.J., 2002, The role of body heat and moisture in the relative attractiveness of humans to *Anopheles gambiae*. Submitted.

Mukabana, W.R., Takken, W., Killeen, G.F. & Knols, B.G.J., Clinical malaria symptoms reduce attractiveness of humans to mosquitoes. Submitted

Mukabana, W.R., Takken, W., Njiru, B.N. & Knols, B.G.J., *Plasmodium falciparum* parasitaemia enhances the attractiveness of humans to African malaria vectors. To be submitted.

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