Odour-mediated host-seeking behaviour of the Afro-tropical malaria vector *Anopheles gambiae* Giles

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in de landbouw- en milieuwetenschappen
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Cover photograph: The African malaria mosquito *Anopheles gambiae s.s.* feeding on a human arm (copyright Berry Geerligs, DUOTONE, Wageningen, The Netherlands).

Stellingen

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1. Voor een juiste interpretatie van het gastheerzoekgedrag van de Afrikaanse malariavektor *Anopheles gambiae sensu stricto* dient dit bestudeerd te worden binnen een multipartite kontext.

Dit proefschrift.

2. Het nauwkeurig observeren van het gedrag van muggen rond een gastheer kan informatie verschaffen omtrent de origine van kairomonen en eventueel de breedte van het gastheerspectrum.

Dit proefschrift.

3. Het gezegde 'van een mug een olifant maken' is wellicht van toepassing op de interpretatie van de behaalde resultaten met het malaria-vaccin SPf66 in een holo-endemisch gebied in Tanzania.

Alonso, P.L. *et al.*, 1994. *Lancet*, 344, 1175-1181.
D'Alessandro, U. *et al.*, 1995. *Lancet*, 346, 462-467.

4. Het voorstel tot het introduceren van transgene muggen in Afrika typeert het gemak waarmee technologieën die in het Westen de toets der maatschappelijke kritiek niet zouden weerstaan worden toegepast in ontwikkelingslanden.

Hancock, G., 1989. *Lords of Poverty*. Macmillan, London.

5. Aangezien vektor-bestrijding middels het gebruik van pyrethroiden op klamboes niet wezenlijk verschilt van het gebruik van persistente middelen als DDT op wanden is de ontdekking van muggen-resistentie ertegen niet opzienbarend en zal resistentie slechts toenemen.

Vulule, J.M. *et al.*, 1994. *Med. Vet. Entomol.*, 8, 71-75.
Magesa, S.M. *et al.*, 1994. *Bull. Wld. Hlth. Org.*, 72, 737-740.

6. Het voortbestaan van nationale parken in Afrikaanse landen kan slechts dan worden gegarandeerd wanneer de lokale bevolking direkt economisch belang heeft bij de instandhouding ervan.

7. Het vernietigen van enorme hoeveelheden ivoor door de Keniaanse overheid is een zinloze mediastunt; de ban op de handel in ivoor zal het uitsterven van de Afrikaanse olifant (*Loxodonta africana* L.) juist bespoedigen.

Bonner, R., 1993. *At the hand of man, peril and hope for Africa's wildlife*. Simon & Schuster, London.

8. Het toevoegen van een Nederlandstalige samenvatting aan een Engelstalig proefschrift is overbodig.

9. Het schijfje citroen in een glas witbier blijft de indruk wekken gezond bezig te zijn.

10. Werken voor de armen is een snelle manier om rijk te worden.

11. De term 'muggezifter' is sympathiek jegens malaria-entomologen.

Stellingen behorend bij het proefschrift **'Odour-mediated host-seeking behaviour of the Afro-tropical malaria vector *Anopheles gambiae* Giles'**,

Bart G.J. Knols Wageningen, 23 Februari 1996

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Preface

During the days that I was down with malaria deep in the Tanzanian bush, staring at the ceiling during sleepless nights, and hoping that the drugs I had taken would still be effective against the parasites in my body, I experienced what grave effect this disease has on the well-being of man in Africa. The scenes I sometimes witnessed in Tanzanian hospitals are unforgettable and made clear that the outlook for malaria control is grim. No matter how small the contribution of the research presented in this thesis to malaria control would be, these moments have always strongly motivated me, regardless of how difficult the work sometimes was.

Besides this, there have been many people that, through discussions, cooperation and friendship, induced a similar effect in me. For almost two years, I had the pleasure to collaborate with Ruurd de Jong and our mutual enthusiasm for this subject led to some extraordinary findings. Letizia Mattiacci, you were often part of all this and your cheerfulness always created a great atmosphere.

For more than eight years my co-promotor, Dr. Willem Takken, has created endless opportunities for me to become a 'fully grown' medical/veterinary entomologist. Willem, through your efforts I got my first job in Zambia and subsequently the opportunity to do a PhD for which I will always be most grateful.

I sincerely thank Prof. Joop van Lenteren, first of all for accepting to be my promotor. Joop, our contacts were always brief but fruitful, and it was great that you found time to see our work in the Kilombero valley last year.

Various people provided invaluable assistance during my laboratory work in Wageningen. Leo Koopman, Frans van Aggelen and André Gidding conscientiously reared (and fed!) mosquitoes. Ineke Buunk, your help in organising international travel, visa, and filling forms that were abracadabra to me will always be highly appreciated. Ans Klunder and Truus de Vries are also acknowledged for their secretarial help. Gerrit van den Brink, Gerard Schuurman, Barend Tollenaar and Gert Nieuwboer displayed some excellent craftsmanship in constructing the windtunnel and electric nets. Piet Kostense made some brilliant drawings of some of these gadgets. My sincere gratitude goes to all other OBP staff for their support in whatever way during my stay at the Binnenhaven.

Of the scientific staff, some people were more directly involved in my work. Richard Stouthamer often had useful suggestions to improve the quality of manuscripts and Joop van Loon had a most valuable input in chapter 6 of this thesis. More recently Jocelijn Meijerink and Marieta Braks have had significant and stimulating inputs in the line of research and atmosphere in the 'mosquito group'. During the past three years several MSc students participated in my work and have made major contributions to it, for which I am most grateful. A special word of thanks to all other scientific staff, in particular PhD students, for being excellent company on those typical Friday afternoons from 17.00 hrs onwards.

My research was part of a European Community funded project and was linked to research groups in England, Italy and Tanzania. Drs. Alan Cork, Rosemary Robinson and David Hall of the Natural Resources Institute, Chatham, are gratefully acknowledged for their hospitality and fruitful collaboration. Alan, your work has been an enormous contribution to this thesis and despite your incredible workload you were always there to do chemical analyses or discuss results. Useful discussions were held with Drs. John Brady, Gabriella Gibson and Carlo Costantini of Imperial College, London, and Prof. Mario Coluzzi of the Instituto di Parassitologia, Rome, during our annual meetings. Dr. Alessandra della Torre from the latter institute had a major input in chapter 10 by doing PCR analyses on hundreds of mosquitoes. Drs. Barend Mons and Marc de Bruyker coordinated the project at EC headquarters in Brussels and are thanked for providing institutional support and guidelines.

In Tanzania all research was carried out in collaboration with the National Institute for Medical Research. First and foremost I want to thank the Director General Prof. Wen Kilama for allowing me to do research at the institute's stations in Muheza and Ifakara. In Muheza the former Director Dr. Yohanna Matola and in Ifakara the Director Dr. Andrew Kitua are acknowledged for their hospitality and support. Dr. Edith Lyimo and Ikupa Akim played a significant role in making me (and Leonard) feel at home in Ifakara. Thank you both for your hospitality during the many times we gate-crashed hungry and exhausted. Many of the staff of the Ifakara centre and Ubwari field station (Muheza) contributed in one or the other way to our work or social well-being for which you are all thanked. Special thanks to Dr. K. Njunwa, head of Ubwari field station and Tony and Dorothy Wilkes. Tony, your enthusiasm was simply contagious, and I loved listening to the old stories from The Gambia. In Muheza I also met with Anita Rønn and Jacob Knudsen and I am grateful that our friendship continues to date. Derek and Arabel Charlwood are thanked for letting me stay in my own house after my return from Tanzania, during which time I thoroughly enjoyed your company and many games of tennis.

And then Leonard Mboera! Dear Leonard, it is hard to express my gratitude on paper, for the time we spent in Namawala last year. Our devotion was clearly reflected in the names we were given by the people from Namawala village: 'Pwagu na Pwaguzi', and I think we should be proud of this. Your friendship and sense of humour made it so much easier to work in such an isolated area. Japhert Kihonda, Simon Mtenga, Hamidu Katumbo, Ahmed Mushahara and Mselenge all worked hard to reach our goals and improved my Kiswahili enormously.

Even though periods in Wageningen were often brief, there is a group of people that are very special to me and without mentioning names (I don't consider it necessary), I want to express my sincerest appreciation for the love and friendship you offered me over the last ten years! Special thanks to Han Wiskerke for letting me use his laserprinter on many occasions.

Marie-Thérèse and Harry, you are the people that made all this possible for me; friends often say that I should consider myself lucky with parents like you and I believe they are right!

Dearest Ingeborg, people often acknowledge the mental support they received from their partners during the final stages of writing a thesis. In our case this couldn't happen due to your work in Costa Rica. Though sometimes difficult I have always been proud that you too chose to work abroad. At long last you're back, and even though I think that years of separation have created an enormously strong bond between us, I am happy that this separation has ended and look forward to our future life in Africa (without you being treated in a hospital on Zanzibar for Fansidar-resistant malaria again!!).

For Marie-Thérèse and Harry,
and for Ingeborg

1

General introduction

1.1. The evolution and ecology of haematophagy by Dipteran insects

1.1.1. The evolution of haematophagy

The majority of species on our planet are parasites (Price, 1980), depending for their survival, reproduction and/or nutrition on the presence of host species which, per definition, have a decreased fitness due to the influence of the parasite. Within the order of the Diptera some 11.000 species (9% of the total number described) belong to families in which the evolution towards adult parasitism in the form of haematophagy, through structural and physiological adaptations of the insect, has occurred (Crosskey, 1993). If for one-tenth of the Dipteran insects the evolution towards this trait was successful than this merits the study of two basic questions: a) how did haematophagy develop within this group of insects and b) why is haematophagy an evolutionary successful strategy. The first question, the development of the blood-feeding habit, is difficult to answer due to the scarcity of fossilised material, but has been suggested to have taken place along two evolutionary routes (Lehane, 1991; Waage, 1979): a) ***The prolonged close association with vertebrates***, and b) ***Morphological pre-adaptation for piercing the skin surface***. The prolonged association theory is based on the utilisation of organic matter produced by hosts (faeces, feathers, hair, etc.), particularly at nesting sites. Efficient use of these sources required the insects to become associated with the host. Eventually, insects mounted the fur and feathers of the hosts and thus encountered an efficient mode of transmission between their nesting sites and other individuals. The increased reliance on and specialisation towards the use of animal products, combined with the chewing mouthparts of groups such as the Mallophaga, which sometimes break the dermis and consume blood, furthered the development of the trait. The second route of evolution focuses on morphological pre-adaptations for piercing surfaces (skin, plant tissue, insect cuticula). The development of entomophagy, which required physiological adaptations (protein digesting enzymes) may have preceded haematophagy and facilitated development of the latter feeding strategy. Some mosquito species, when offered larvae can pierce the cuticula and suck body fluids which can even result in the production of viable eggs (Harris *et al.*, 1969). Others have argued that mouthparts for piercing plant tissues could have adapted to piercing the skin of vertebrates (Mattingley, 1965).

The second question merely centres on the nutritional and reproductive value of blood compared to that of other food sources such as plant material. This has been studied for various Dipteran families, some of which have facultative haematophagous species (such as biting midges (Ceratopogonidae)), and indeed indicates that survival and fertility is higher for insects offered blood than those maintained on carbohydrates only (Clements, 1992). Herbivorous insects normally require extended periods of feeding in order to accumulate enough protein for oogenesis, but obtain sufficient nutrients for survival (flight energy etc.) during this process. Blood provides a protein-rich source, and a single blood meal can provide sufficient protein to initiate oogenesis in mosquitoes (Culicidae). The latter group, along with other families such as the blackflies (Simuliidae) still utilise floral nectar sources to supplement their diet. In the absence of hosts, mosquitoes and blackflies can thus survive on carbohydrates and some species even manage to produce (limited) offspring without the necessity of obtaining a blood meal, a phenomenon called autogeny (O'Meara, 1985; Linley, 1983). It seems thus that the utilisation of blood is an effective means to obtain protein for oogenesis, but if the continuous availability of hosts cannot be safeguarded, than survival and reproduction should remain possible using other food sources. Tsetse flies (Glossinidae) have developed the blood-feeding habit to the extreme. Both sexes are obligatory haematophagous and blood provides the only energy source as well as the nutrients for larval development (Buxton, 1955). This habit resulted in a strong dependence on the presence of hosts for survival, and the first tsetse control methods therefore aimed at eliminating host animals in order to starve/kill flies and thus stop transmission of trypanosomiasis (Jordan, 1986).

1.1.2. Host-parasite associations

The prolonged association theory suggests that the dependency of the insect on the presence of the host would favour the completion of the insect's entire life-cycle on the host. This is what is seen in lice (Anoplura), in which both nymphal and adult stages live on blood (Ibarra, 1993). However, within the Diptera, three types of associations occur (Lehane, 1991). ***Permanent***

ectoparasites have a similar association with the host as the lice, completing the entire life-cycle on the host, of which several (sometimes wing-less) species are found in the ked/louse fly family (Hippoboscidae). ***Periodic ectoparasites*** spend a longer period of time on the host than necessary for obtaining a blood meal but nevertheless spend considerable periods away from it. ***Temporary ectoparasites*** are considered to be largely free-living insects which only visit the host for long enough to take a blood meal. Most Dipteran families of medical and veterinary importance belong to this category (Phlebotominae, Culicidae, Glossinidae, Simuliidae, Ceratopogonidae, Tabanidae and Stomoxyinae). This might not be surprising since effective transmission of disease can only occur if different hosts are visited, and the chance to encounter different hosts is maximised if the time between subsequent feeds is long. The remainder of this chapter will focus primarily on these temporary ectoparasites.

1.1.3. The host-seeking process

Temporary and periodic ectoparasitic Diptera are thus faced with the problem that they need to locate a suitable host, often more than once during adult life, in order to obtain a blood meal. This problem is solved by displaying a type of behaviour which has been termed host-seeking, host-finding or host-location, and although these terms are sometimes used in slightly different ways I have used them indiscriminately throughout this thesis. In the broadest sense, host-seeking could be defined as any kind of behaviour displayed by the insect that increases the chance to encounter a suitable host, followed by bloodfeeding. This broad definition encompasses two types of behaviour, the first of which is a search behaviour that increases the probability of encountering stimuli signifying the presence of the host, called 'appetitive search' (Sutcliffe, 1987). The second behaviour is a series of manoeuvres that allow the insect to orient towards the source of these signals. This latter behaviour again falls apart in two phases: activation and orientation. Activation is not in itself a behaviour but is the release of a behavioural package (the attraction phase) upon detecting host-derived stimuli, hence terminating the appetitive search behaviour. Orientation is the process of active host-location through which the insect comes in the immediate vicinity of the host. Thereafter it will land on the host and commence bloodfeeding. For most haematophagous Diptera this classification is, with only minor differences, used to fractionate and describe the host-seeking process (Clements, 1963 (Culicidae); Hocking, 1971 (several families); Sutcliffe, 1986 (Simuliidae), 1987 (several families); Lehane, 1991 (several families), Willemse & Takken, 1994, (Glossinidae), see Fig. 1).

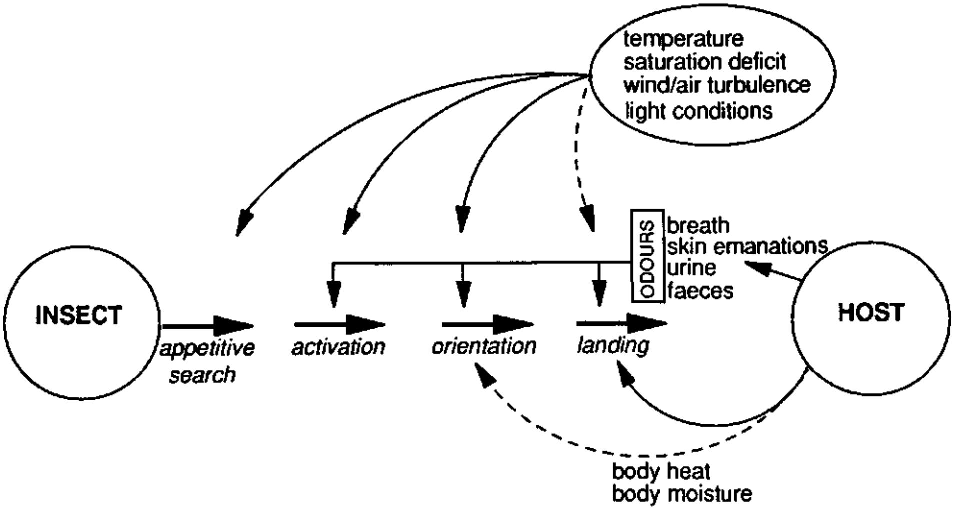

Figure 1. *The different phases of the host-seeking process of haematophagous insects (in italics) and environmental/host-related factors influencing these (modified after Takken, 1996).*

Newly emerged adults usually have a delay period prior to taking their first blood meal. It has been suggested that during this period the reproductive system undergoes maturation (Lehane, 1991). Thereafter the insect becomes receptive to host-related cues, depending on endogenous rhythms and 'hunger', and the interaction of these factors determines when appetitive search behaviour starts. Vertebrate hosts produce three classes of stimuli which are used by haematophagous insects during host-seeking (Fig. 1): ***Chemical (olfactory), Physical (moisture, heat), and Visual (colour, shape, contrast) stimuli.***

Chemical stimuli

Vertebrates produce a great number of volatiles that originate from their skin, breath and excretory products (faeces, urine)(Albone, 1984; Sastry *et al.*, 1980). The odours disperse in the environment and their detectability is a function of the rate of molecule emission, the release area, the distance between the insect and the odour source, wind speed, turbulence and contrast against background odours (e.g. Murlis & Jones, 1981). With regard to blood-sucking insects chemical stimuli are normally divided into two groups: carbon dioxide and (other) body odours.

Carbon dioxide. It is generally accepted that carbon dioxide is involved in both the activation and orientation of virtually all blood-sucking insects. It is secreted by the skin of hosts, but major emissions occur in exhaled breath. An adult human excretes *ca.* 300 ml min^{-1}, at a concentration of 4.5%. Since background levels vary between 0.03-0.04% this implies that the concentration remains above atmospheric levels until diluted by a factor of about 100. For mosquitoes, the active range of carbon dioxide emitted by a single human was estimated to be *ca.* 15 m (Gillies, 1980). However, the diffusion of carbon dioxide in the environment is not constant in space and time, and it is more likely that small pockets of relatively undiluted carbon dioxide extend this active range (e.g. Murlis, 1986). Evidence exists that it is the change in concentration rather than the level of carbon dioxide which is the important factor eliciting behavioural responses, for instance for mosquitoes (Wright & Kellogg, 1962). The activating and/or attracting effect of carbon dioxide for various families has been reviewed by Gillies (1980), Sutcliffe (1987) and Takken (1991).

The use of carbon dioxide in the host-seeking process of most haematophagous Diptera raises two interesting questions. Why is carbon dioxide the 'chosen' chemical and what is the relative importance of this compound for the various blood-sucking insects. Both questions relate to an important step taken while host-seeking: selection of the host (both at species and individual level). Most blood-sucking Diptera have a limited host range (Hocking, 1971, see also section 1.1.4.), and the development of a limited and/or fixed host range was probably preceded by more opportunistic feeding. Carbon dioxide is a host-emitted volatile produced in relatively large quantities. It is released by all vertebrates and is thus an important stimulus in terms of signifying the presence of a potential host. It is likely, therefore, that this chemical played a major role in the evolution of haematophagy. However, the evolution towards feeding on a restricted number of host species demanded selective responses to (species-) specific odours. It is concluded that carbon dioxide, due to its relatively high concentration in exhaled breath, and being an indicator for a (living) blood host has played a crucial role in the development of haematophagy. Its role as a kairomone for species with limited or advanced host preference, and its interaction with other (species-specific) kairomones probably varies, depending on the level of host-preference.

Body odours (other than carbon dioxide). To date, few chemicals other than carbon dioxide have been incriminated as behaviourally active for blood-sucking Diptera. Extensive studies on these kairomones have been undertaken for two Dipteran families only, mosquitoes (Culicidae) and tsetse flies (Glossinidae). Olfaction in host-seeking behaviour of these families has recently been reviewed by Takken (1991) and Willemse & Takken (1994) respectively. A compound which has shown behavioural activity for various blood-sucking Diptera is 1-octen-3-ol (hereafter octenol). This compound was originally identified from ox breath (Hall *et al.*, 1984) and has since been shown to elicit behavioural responses from Glossinidae (Vale & Hall, 1985), Culicidae (Takken & Kline, 1989; Kline, 1994), Ceratopogonidae (Kline *et al.*, 1994), and Tabanidae (French & Kline, 1989). Electrophysiological responses have been recorded from Stomoxyinae (Warnes & Finlayson, 1986). It is interesting to observe that octenol, yet another breath component, elicits responses from a wide variety of blood-sucking Diptera. Other attractants identified for tsetse include acetone, butanone and simple phenols. All of these compounds are behaviourally active under field conditions (see Willemse & Takken, 1994). Compounds identified as attractants for mosquitoes are discussed in section 1.2.

In the natural situation the insect perceives several chemical stimuli at the same time. Insects in general have developed distinctive responses to mixtures of host-derived stimuli, and combinations of different compounds (or blends) can function as 'fingerprints' for the identification of a host species from a distance. An interesting aspect of the interaction of various compounds is that in some cases their effect is greater than the sum of both effects separately, a phenomenon called synergism. Torr (1990) proposed a synergism for tsetse flies, between acetone (another breath compound!) and carbon dioxide, for inducing upwind anemotaxis. Synergism between octenol and carbon dioxide has been reported for several mosquito species (Takken & Kline, 1989).

Physical stimuli (moisture, heat)

Homeothermic blood hosts present heat as a stimulus to the approaching insect. Two types of heat stimuli exist: radiant and convective heat (Clark & Edholm, 1985). Both types of heat, per definition, can act within short distance of the host only. The effect of these stimuli have been most extensively studied in relation to mosquito behaviour and are discussed in section 1.2.

Visual stimuli

The visual ecology of biting Diptera has been reviewed by Allan *et al.* (1987). For tsetse flies, shape, size, orientation, brightness, contrast, movement and colour have been shown to affect responses to hosts or artificial baits (see Colvin & Gibson, 1992). These visual stimuli influence close-range behaviour and landing responses. Mobile baits have been shown to attract up to 16 times as many *G. morsitans* than stationary ones (Brady, 1972). Orientation to and landing on artificial targets of different colours depends upon various factors, such as tsetse species, the bait type, and the arrangement and brightness of colour on the bait (i.e. Green, 1986). Other diurnal biting Diptera such as tabanids and simuliids respond to similar visual features of baits (Browne & Bennett, 1980; Thompson, 1976). Strong responses of insects to targets showing strong intensity contrast with the background probably reflect the natural situation whereby low-intensity (homeothermic) hosts appear dark in contrast to high intensity vegetation (Allan *et al.*, 1987). Visual responses of nocturnal insects are much less understood but will probably play a much reduced role in the location and recognition of a host.

1.1.4. Pre-requisites for successful haematophagy

Successful host-location by temporary ectoparasites is more critical for obligatory haematophagous insects (e.g. Glossinidae, depending on hosts both for survival and reproduction) than for haematophagous insects requiring a blood meal for reproductive purposes only (e.g. Culicidae, Tabanidae, Simuliidae, which can survive on carbohydrates if hosts are not immediately available). In turn successful location of hosts is more important to the latter group than for facultative autogenous insects (e.g. various Culicidae or Ceratopogonidae, which can produce (limited) offspring in the absence of hosts). The first pre-requisite for successful haematophagy is thus the effective location of the host (sections 1.1.3. and 1.2.).

A second factor influencing successful haematophagy is the quality of the blood meal in terms of nutrition or suitability for egg production. Blood, in particular the amino acid composition, varies between vertebrates and affects utilisation by the insect. Longevity of the tsetse fly *Glossina morsitans morsitans* increased significantly when fed on rabbits rather than on goats (Jordan & Curtis, 1972), whereas for several mosquito species fertility varies when offered different hosts (Briegel, 1985; Clements, 1992). These differences in blood quality would favour development of specialisation towards optimal use of a certain type of blood, and thus host-preference. Indeed, most haematophagous Diptera have a restricted host range (Hocking, 1971). However, it has been observed that even anthropophilic mosquito species utilise nitrogen from human blood less efficiently than that from rat or guinea-pig blood (Briegel, 1985; Briegel, 1990). Clements (1992) generalises by saying that mosquito fertility is (a) higher on the blood of certain host species than others, (b) not greatly affected by whether the erythrocytes are nucleate or anucleate, and (c) lower on human blood than on that of most other host species. Thus host-preference plays an important role in successful haematophagy although it can not explain advanced anthropophily of some mosquito species.

A third prerequisite for successful haematophagy is the circumvention of host-defense. After successful location of the host it becomes of utmost importance to survive the act of feeding. Host-defense can result in the interruption of feeding by mosquitoes on birds and thus affect the fertility or even the initiation of oogenesis (Edman & Kale, 1971). However, Charlwood *et al.* (1995) recently demonstrated density-independent feeding success by *Anopheles* mosquitoes on man. Man is apparently a 'safe' host to feed on and this might thus affect the tradeoff between high fertility through feeding on a defensive host versus lower fertility of blood from a non-defensive host.

1.2. Host-seeking behaviour of mosquitoes (Diptera: Culicidae)

At the beginning of this century, soon after it was discovered that mosquitoes are vectors of major human diseases (see section 1.4.), detailed studies were undertaken on the biology of these insects. Since not all anopheline mosquitoes were found to be vectors of disease it was realised that malaria control could only be economical if the biology of mosquitoes was fully understood. Early this century, for instance, Watson (1911) freed a Malaysian lowland area from malaria by focusing on control of the principle malaria vector (*An. umbrosus*) after studies on its ecology revealed it to be the main parasite carrier. This strategy became later known as 'species sanitation' and became widely practised in the former Netherlands Indies (Takken & Knols, 1990a). These studies would probably have continued were it not for the discovery of the insecticidal power of DDT during the second world war. The enormous successes in combating vector-borne disease with this insecticide led to a rapid decline in the scale of interest for and/or funding of studies focusing on mosquito ecology. However, even though DDT is still the most widely used insecticide in public health practice, the majority of important malaria vectors have become resistant to this compound, especially over the last two decades (Gilles & Warrell, 1993). Since then renewed interest in mosquito ecology has accumulated a lot of information of which the following briefly reviews aspects related to host-seeking behaviour.

The terminology used to define characteristics of mosquitoes and host-seeking behaviour are shown in Table 1. Together with host-seeking activity patterns (i.e. diurnal, nocturnal or crepuscular), these terms can describe some important aspects of a species' behaviour. Fig. 2 shows how a sleeping host presents itself to an endophagic, endophilic, anthropophilic mosquito such as *An. gambiae*, an important malaria vector in Africa. The four categories of host-related stimuli presented to the mosquito are indicated in this figure. The

Table 1. *The terminology used to define characteristics of mosquitoes and host-seeking behaviour.*

Place of feeding	Exophagic	Mainly outdoors
	Endophagic	Mainly indoors
Completion of gonotrophic cycle	Exophilic	Mainly outdoors
	Endophilic	Mainly indoors
Host preference	Opportunistic	A wide variety of hosts
	Ornithophilic	Mainly bird-feeding
	Zoophilic	Mainly mammal-feeding (excluding man)
	Anthropophilic	Mainly feeding on man

distance over which the various stimuli act have also been indicated according to Gillies & Wilkes (1969). Long-distance orientation is governed entirely by body odour other than carbon dioxide. Medium-distance orientation starts when the mosquito not only perceives body odour, but in addition a carbon dioxide concentration above threshold level. It ends when the mosquito approaches the host (in this case upon entering the house, which has a slightly increased temperature and humidity besides odour and carbon dioxide). In the vicinity of the host (short-distance orientation) the mosquito perceives body odour, carbon dioxide, increased moisture levels, convective heat, and visual cues. This way of defining the various steps in orientation to the host is thus entirely based on the distance from the host over which the stimulus concentration remains above background and can thus be detected by the mosquito. Bertram & McGregor (1956) used an experimental hut, occupied by

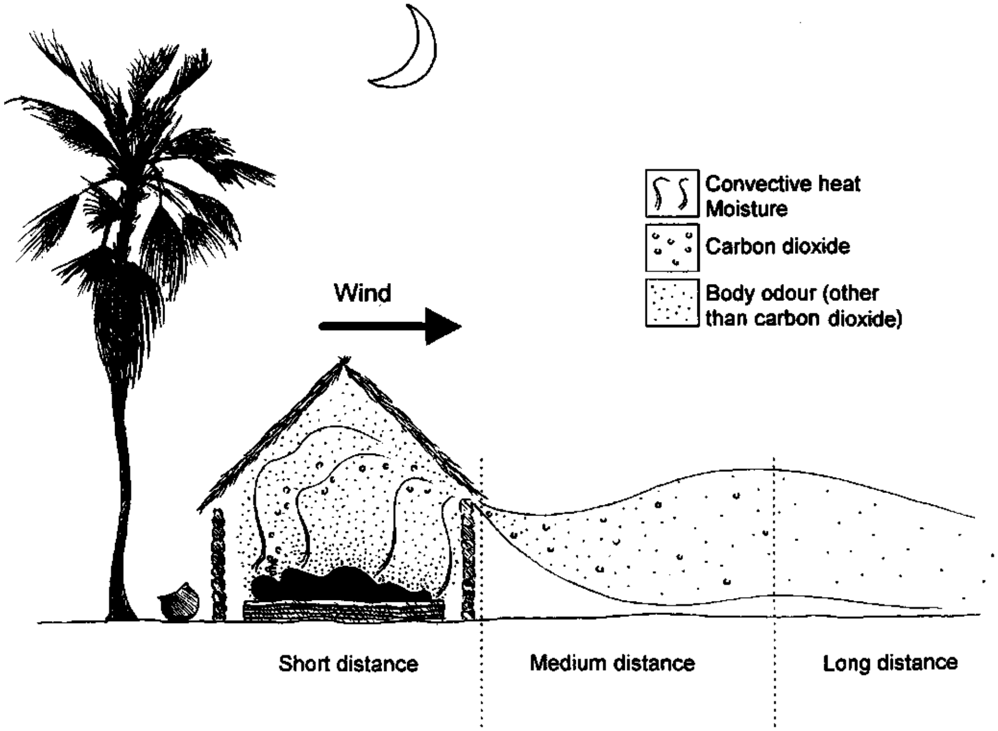

Figure 2. How a sleeping host presents itself to host-seeking An. gambiae. Indicated are the various stimuli to which mosquitoes respond (top) and distance related phases in host-seeking behaviour (bottom) (modified after Gillies, 1988).

a volunteer, with two entrance traps on opposite sides of the hut that were aligned with the general wind direction and showed that catches in the downwind trap were significantly higher than those from the upwind trap. They concluded that smell and other airborne products of the host created a down-wind stimulus of attraction which directed the mosquitoes to the downwind trap of the hut.

The active ranges of the various stimuli is different for various mosquito species as was clearly shown by Gillies & Wilkes (1969, 1970, 1972) and Edman (1979). In the Gambia it was shown that various culicine and anopheline species were attracted to calf baits, or an equivalent of carbon dioxide expired by them, from distances between 18 and 37 m. The range of attraction of both odours was similar for these species. However, even though the range of attraction of carbon dioxide to *An. melas* was similar to that of the other species, the range was extended significantly for the calf baits (up to 73 m), which clearly indicates that odour other than carbon dioxide caused the responses at those distances from the host (Gillies & Wilkes, 1969).

How mosquitoes, especially nocturnal species, orient themselves towards a host is not clearly understood. Generally mosquitoes fly near groundlevel where windspeeds are lower. Furthermore, flying nearer to the ground enables the insect to resolve finer detail in the visual field. Gillies & Wilkes (1981) recorded flight speeds of up to 1.8 m sec^{-1} for some West African *Anopheles* and *Mansonia* species. A common mechanism used by insects to orient towards a distant odour source is optomotor-steered positive anemotaxis (Kennedy, 1983; 1986). Airspeed is detected by the image flow along the body axis of the insect. Deviation from straight upwind flight will result in its ground track not being aligned with its body axis which is compensated for by upwind turning (Kennedy, 1939; David, 1982). This flight mechanism has been demonstrated for *Ae. aegypti*, a diurnal species, but it is less clear how nocturnal species accomplish in-flight orientation in the absence of visual cues (Allan, 1994). Gillett (1979) suggested that non-visual in-flight orientation might be achieved by flying low, where the vertical gradient of the wind speed is at its steepest, and frequently 'dipping' even lower thus detecting the wind direction by feeling the wind 'shear' mechanically at each dip. However, in complete darkness mosquitoes fail to orient towards an odour source (G. Gibson, *pers. comm.*) and it is therefore likely that vision does play a role in in-flight orientation. Thus certain landmarks such as trees and houses, illuminated by moonlight, can provide visual information for the mosquito (Allan *et al.*, 1987). How mosquitoes exploit an odour plume, i.e. what flight mechanism is used to orient towards the odour source is also unknown. Daykin *et al.* (1965) observed that *Ae. aegypti,* upon entering a plume of host odour did not make a turn but made a sharp turn upon leaving the plume, most likely to re-establish contact with it, a mechanism which has also been observed for tsetse flies (Gibson & Brady, 1988).

Olfaction in mosquito host-seeking behaviour

As for all haematophagous Diptera, host odours are normally classified in two groups: carbon dioxide and body odours (other than carbon dioxide). Chemosensitive neurosensilla used for host-seeking in mosquitoes are located on the antennae and maxillary palps (McIver, 1982). Receptor systems mediating host-seeking behaviour have recently been reviewed by Davis & Bowen, 1994). Specific carbon dioxide receptors are located on the maxillary palps (Kellogg, 1970; Omer & Gillies, 1971), whereas receptors sensitive to other odours are found on the antennae.

Carbon dioxide. Carbon dioxide was first reported as a mosquito attractant by Rudolfs (1922). Various *Aedes* species responded to carbon dioxide in the field (Reeves, 1951), and landing responses were observed when carbon dioxide was emitted from the head of a heated dummy (Brown, 1951). Clements (1963) reviewed the role of carbon dioxide in mosquito host-seeking and concluded that it causes activation, orientation and landing in the laboratory but that it remained to be determined whether these effects also occur in the field. Laarman (1955) demonstrated the attractive effect of human breath to *An. atroparvus* in a laboratory olfactometer, and could later attribute this to the effect of carbon dioxide only. Filtering carbon dioxide from exhaled human breath resulted in significantly fewer mosquitoes approaching a host, although the number attempting to feed once in the vicinity of the host was not reduced (Snow, 1970). Gillies' review (1980) describes carbon dioxide as an 'attractant' in the presence of moving air currents and as an 'activator' in the absence thereof. Moreover it is suggested that, in the absence of other body odours, sustained flights only occur in response to intermittent pulses of carbon dioxide (Omer, 1979). Bowen (1991) later argued that not the level but the change in concentration of the gas is the important factor eliciting behavioural responses. In the field odours are likely to be perceived intermittently. Mammals release breath in pulses and wind turbulence will ensure the odour plume is continuously mixed and diluted in an irregular fashion (Murlis *et al.*, 1992).

Carbon dioxide is known to influence responses to other host-related cues. Burgess (1959) showed that *Ae. aegypti* would only respond to warm, moistened air in an olfactometer after a few millilitres of carbon dioxide had been injected into the cage. Laarman (1955) observed that responses by *An. atroparvus* to an airstream 1-3°C warmer than ambient were only elicited in the presence of carbon dioxide. The majority of mosquitoes would then alight in front of the airstream containing warm air instead of the airstream containing the carbon dioxide. In the field it was observed that visual stimuli (i.e. dummies) can divert mosquitoes from following an odour plume of carbon dioxide (Bellamy & Reeves, 1952). More interesting are the interactions between certain odours and carbon dioxide. There is a spectrum of responses which can be seen to these dual stimuli, ranging from synergism (whereby the two stimuli give an overall reaction greater than the sum of the two stimuli given separately) to an interaction where one stimulus 'primes' the insect to respond to the second which, if given alone, has no effect (Laarman, 1958; Bos & Laarman, 1975; Bar-Zeev *et al.*, 1977). The attractive effect of L-lactic acid to *Ae. aegypti*, in olfactometer experiments, could only be demonstrated if carbon dioxide was offered simultaneously (Acree *et al.*, 1968). Vickery *et al.* (1966) found a synergistic effect when carbon dioxide was released in combination with a chicken as a bait. More recently field experiments have shown a synergistic effect of carbon dioxide and octenol for various mosquito species, most notably for *Ae. taeniorhynchus* (Takken & Kline, 1989). Knols & Mboera (unpublished), in Tanzania, found doubling of catches of *Mansonia africana* when octenol was added to carbon dioxide, though catches were not significantly different from those of carbon dioxide only.

Body odours (other than carbon dioxide). A multitude of studies have examined body odours (particularly of man) for their attractiveness to mosquitoes, in particular *Ae. aegypti*. Volatile substances produced by man originate from expired air, skin emanations, urine and faeces.

Human breath has been reported attractive to *Anopheles* mosquitoes (Mer *et al.*, 1947; Laarman, 1955) and for *Aedes aegypti* (Khan & Maibach, 1972). Krotozynski *et al.* (1977) identified 102 organic compounds of endogenous and exogenous origin in human breath, obtained from a group of 28 carefully selected healthy individuals. Carbon dioxide is by far the most abundant compound, and 97% of the other chemicals identified have a mean concentration between 0.06 and 9.5 ng/l. Acetone, isoprene and acetonitrile, with concentrations of respectively 120, 33 and 24 ng/l account for 51% of the mean organic contents. Depending on the resident microflora of the mouth cavity a number of compounds of bacterial origin can be found in breath, such as dimethylsulphide or methanethiol (Tonzetich, 1977). Laarman (1955) could not find an additional effect of compounds other than carbon dioxide in human breath on responses of *An. atroparvus* in olfactometer experiments. The same was found for *An. gambiae* by Healy & Copland (1995). In higher vertebrates gaseous exchanges between the body and the environment occur in the lungs, and therefore volatile substances in blood (other than carbon dioxide) are present in expired air. Therefore various workers have examined the effect of host blood on mosquito responses. Laarman (1955) demonstrated an attractive effect of fresh rabbit blood for *An. atroparvus* and suggested that these compounds might be present in expired air. Roessler (1961) found attraction of *Ae. aegypti* to bovine blood, whereas Müller (1968) demonstrated attraction of the same mosquito to pig blood.

Skin emanations. In comparison with other mammals (even primates), human skin is truly unique for a variety of reasons. First of all man is practically hairless, but more interestingly, the glands that nurtured hair earlier in hominid evolution, have remained. Secondly man, the hairless ape, has more sebaceous glands than almost any other species of mammal (Montagna & Parakkal, 1974). Cooling of the body takes place by excreting a thin watery fluid onto the skin surface from where it evaporates. Eccrine sweat glands are involved in this process and are distributed over the entire body surface, but are most abundant on the palms of the hands and soles of the feet. Eccrine sweat consists of an aqueous solution of inorganic salts and amino acids which has no significant odour to the human nose (Labows, 1979). However, the breakdown of sulphur amino acids in eccrine sweat by *Brevibacterium* spp. has been suggested to be a source of odour (Jackman, 1982). *Brevibacterium* spp. have been reported to produce methanethiol, a pungent sulphur compound which is metabolized from L-methionine (Sharpe *et al.*, 1977; Ferchichi *et al.*, 1985). The third class of glands are the apocrine glands, which occur in high densities in the axillae, the suprapubic region, the circumanal region and perineum, face, scalp, and the umbilical region of the abdomen. It is noteworthy that these parts have retained substantial growths of hair. In terms of numbers and sizes of sebaceous and apocrine glands, man has been considered by far the most highly scented ape of all (Stoddart, 1990).

Sebaceous glands produce a thick oily secretion (sebum) which is unpigmented. The secretory duct (to the skin surface) contains mainly lipids but also cellular detritus and epidermal cell flakes. *Corynebacterium* and *Pityrosporum* microorganisms reside in the duct and their lipolytic enzymes are responsible for the breakdown of triglycerides to free glycerol. The various steps in which this process takes place result in the production of free fatty acids, compounds not normally encountered in internal tissues (Nicolaides, 1965). Small amounts of squalene, lanosterol, dihydrocholesterol, lathosterol, 7-dihydrocholesterol, and 17-ketosteroids have also been identified. Table 2 shows the composition of human skin surface lipid and sebum (after Nicolaides, 1974). From this table it can be seen that triglycerides are by far the most abundant compounds in sebum, and that microbial breakdown results in the production of free fatty acids, which constitute a quarter of the skin surface lipid. It is interesting to note that sterol esters, wax esters and squalene do not vary appreciably between different individuals, but large differences have been observed in the degree of hydrolysis of triglycerides to free fatty acids

Table 2. *The chemical composition of human skin surface lipid and sebum (in %).*

	Surface lipid	Sebum
Free fatty acids	25	0
Squalene (hydrocarbon)	10	12
Sterol esters	2.5	<1
Wax esters	22	23
Triglycerides	25	60
Mono- and Diglycerides	10	0
Free sterols	1.5	0

(after Nicolaides, 1974)

(Downing *et al.*, 1969). Puhvel *et al.* (1975) ascribed this to the pH of the skin and the influence this has on the metabolic activity of the resident microflora. Of the free fatty acids, two thirds are accounted for by over 200 different fatty acids that are present in trace amounts only. The other third is composed of the commonly occurring sebaceous acids tertadecanoic, hexadecanoic, octadecanoic, 9-octadecenoic and 9,12-octadecadienoic. It has been suggested that the huge number of acids, which generally are odorous, contribute to our distinctive olfactory signature (Nicolaides & Apon, 1977). The production of sebum is under hormonal control, and sebum production increases during sexual maturity (Montagna & Parakkal, 1974).

Whereas sebaceous glands are distributed over the entire body, apocrine glands are restricted to fewer sites. The high density of apocrine glands in the axillae of man, and the odours emanating from this site have received major attention by the perfume industry, with the main aim to quench these odours (e.g. Labows, 1979; Kanda *et al.*, 1993; Baydar *et al.*, 1992). Apocrine sweat, when freshly excreted, is odourless and sterile, and the activity of the microflora resident in the axilla is responsible for axillary odour (Shelley *et al.*, 1953; Labows *et al.*, 1982; Rennie *et al.*, 1990, 1991). Aerobic coryneform bacteria are currently thought to be responsible for the production of these odours. Axillary odour can be separated into at least three components: the short chain fatty acids, such as propionic, butanoic and 4-methyl butanoic acids, providing the 'sweaty' odour; the musk or urine-like odours derived from the 16-androstenes and finally the 'pungent' or 'burnt'-like odour which is derived from higher molecular weight, or less volatile substances (Gower & Ruparelia, 1993).

Urine/faeces. Sastry *et al.* (1980) review the studies on volatiles from human urine and faeces. More than 300 compounds have been identified. Ketones, alcohols, dimethylsulphide are the dominant volatiles. Various volatile acids and androstenes have also been reported. Oxidation and bacterial degradation of fresh urine cause its powerful odour. The odour of human faeces depends heavily on diet, and has been reported to contain offensive odorous compounds such as dimethylsulphide, indole and skatole.

Table 3 reviews the studies on mosquito olfactory responses to human skin emanations, excretory products and chemical substitutes thereof (modified after Takken, 1991). From this table it becomes clear that (a) the majority of studies have focused on the yellow fever mosquito *Ae. aegypti*, (b) various skin emanations have been shown to have an attractive effect to this mosquito, and (c) they originate from different sites of the human body. However, various studies often showed contradictory results, that were probably caused by variations in the experimental setup and/or testing procedures. Lastly it should be considered that few of the compounds tested have shown a demonstrable effect outside the laboratory. A factor which has been neglected in all these studies is the effect of the skin microflora on the production of odours, and this may well be a reason for contradictory results. In those studies where individual compounds were tested it is often not clearly indicated under what climatic conditions they were attractive/repellent. Some compounds have been incriminated as 'not attractive' but it remains unknown what behavioural effect these compounds would have when offered with other stimuli (other compounds, carbon dioxide or physical stimuli). The best results (i.e. consistent results) in the field have been obtained with octenol (Takken & Kline, 1989), although its effect is most pronounced (in the form of synergism) in the presence of carbon dioxide (Kline, 1994). Octenol is the only kairomone (other than carbon dioxide) that has so far found practical application in the field (USA), i.e. for mosquito surveillance (Bonvechio, 1991).

Physical and visual cues influencing host-seeking behaviour of mosquitoes.

Physical and visual cues play a role during the latter part of orientation to the host. Laarman (1955) undertook extensive studies on the role of moisture on responses by *An. atroparvus*. In a small cage dual-port olfactometer he observed strong responses and alighting to a moistened airstream. Mosquitoes showed clear responses to air let over a rabbit, but failed to do so when the same air had been dried. Responses were also absent if the relative humidity of the same air was above 90%. Ambient temperatures were shown to affect responses of *Aedes* mosquitoes to moistened warm dummies in the field (Brown, 1951). Induced eccrine sweating by man, hence increasing moisture levels around the body, have been reported to increase the attractiveness to *Ae. aegypti* (Khan *et al.*, 1969). Clements (1963) concluded that increased moisture levels induce landing responses in mosquitoes. The response of various mosquito species to heat was demonstrated by Howlett (1910). He also showed that convective and not radiant heat is responsible for eliciting mosquito

***Table 3.** Studies on mosquito olfactory responses to human skin emanations, excretory products and chemical substitutes thereof (modified after Takken, 1991).*

Skin product/chemical	Mosquito species	Response[1]	References
Sebum	*Ae. aegypti*	slightly attractive	Rudolfs (1922)
Air led over arm	*Ae. aegypti* *An. quadrimaculatus*	attractive attractive	Willis (1948)
Armpit sweat	*Ae. aegypti*	attractive	Parker (1948)
Sweat	*Ae. aegypti*	attractive/repellent	Brown *et al.* (1951)
Trunk sweat	*An. gambiae* *An. funestus*	attractive	Haddow (1942)
Air led over arm	*An. atroparvus*	attractive	Laarman (1955)
Air led over hand	*An. arabiensis* *Cx. quinquefasciatus*	attractive	Omer (1979)
Armpit sweat Forehead sweat	*Ae. aegypti*	attractive not attractive	Thompson & Brown (1955)
Air led over arm	*An. stephensi*	attractive	Brouwer (1960)
Amino acids: Lysine, Alanine	*Ae. aegypti*	attractive	Brown & Carmichael (1961)
Armpit sweat	*Ae. aegypti*	attractive	Roessler (1961)
Artificial sweat		attractive	
amino acids:			
tyrosine/threonine		attractive	
aspartic/glutamic		attractive	
Whole body sweat	*Ae. aegypti*	attractive	Skinner *et al.* (1965)
Whole body sweat Skin lipids	*Ae. aegypti*	attractive repellent	Maibach *et al.* (1966)
Air led over arm	*Ae. aegypti*	attractive	Mayer & James (1969)
Arm sweat		attractive	Müller (1968)
Trunk sweat		not attractive	
Leg sweat		not attractive	
Lactic acid		attractive	
Formic acid		attractive	
Acetic acid		attractive	
Propionic acid		attractive	
Alanine, Lysine		not attractive	

Table 3 (continued)

Lactic acid	*Ae. aegypti*	attractive +	Acree *et al.* (1968)
Lactic acid	*Ae. aegypti*	attractive +	Smith *et al.* (1970)
Carboxylic acids	*Ae. aegypti*	attractive	Carlson *et al.* (1973)
Lysine Cadaverine Estradiol	*An. stephensi*	attractive attractive attractive	Bos & Laarman (1975)
Lactic acid	*Ae. aegypti*	attractive +	Bar-Zeev *et al.* (1977)
Lactic acid	*An. quadrimaculatus*	attractive +	Price *et al.* (1979)
Lactic acid	*Ae. aegypti*	attractive +	Eiras & Jepson (1991)
1-octen-3-ol	various Aedine and Culicine spp.	attractive +	Takken & Kline (1989)
Urine	*Ae. aegypti*	attractive	Roessler (1961)
Unknown (from skin)	*Ae. aegypti* *An. quadrimaculatus*	attractive	Schreck *et al.* (1981)
Unknown (from skin)	*Ae. aegypti*	attractive +	Eiras & Jepson (1991)

[1] Response: Attractive (response to odour significantly higher than to control odour); not attractive (no difference in response to odour and control); repellent (response to control significantly higher than to odour) + indicates that behavioural responses to the compound(s) are only elicited in the presence of carbon dioxide.

responses. Mer *et al.* (1947) observed probing responses to convection current rising 30 cm above a human head. In this case, however, other stimuli would have been presented to the mosquitoes as well. Laarman (1955) concluded that *An. atroparvus*, when responding to heat sources, were reacting to temperature gradients. The latter author also observed interaction between physical cues and carbon dioxide. Burgess (1959) observed the same, that *Ae. aegypti* stopped responding to convection current when carbon dioxide was no longer admitted to the cage. Sensory physiological studies have shown that mosquitoes are sensitive to very small changes in temperature (0.2°C) and relative humidity (0.01%)(Bowen, 1991).

Vision plays a role during orientation towards the host (see above), but visual cues from the host affect the short-range behaviour of mosquitoes as well (Allan *et al.*, 1987). The importance of visual cues in host-seeking varies markedly for different species. Noctural endophagic mosquitoes can only use visual information from the (sleeping, immobile) host during the final approach after entering a house. Diurnal exophagic species encounter mobile hosts, and colour plus contrast between host and background play a much bigger role. For some diurnal species it has been reported that the absence of visual cues may affect location of the host. Kalmus & Hocking (1960) reported the inability for various *Aedes* species to locate a human host after he lay down. A similar result was found for African *Eretmapodites* mosquitoes (Haddow, 1956). In daylight, moving hosts are often much more attractive than stationary ones (Haddow, 1956, Gillett, 1972). The range of visual attraction of diurnal species to large targets has been reported to begin between 5-20 m (Bidlingmayer & Hem, 1980; for review see Bidlingmayer, 1994). Landing by mosquitoes on surfaces of different colours has been shown to be affected by reflectivity or brightness of the colour. The lower the reflectivity (i.e. the darker the colour) the more attractive the cloth (Brown, 1951, Clements, 1963). Different sizes and shapes of objects affect the attraction of diurnal species, and a preference for landing on the extremities of these objects has been suggested to be caused by the sharp contrast between these parts and background light (Browne & Bennett, 1981). The exact role of visual cues in host-seeking of nocturnal species remains unclear.

1.3. Ecology of the *Anopheles gambiae* complex

An. gambiae s.l. consists of a complex of at least six sibling species (White, 1974). The history of the complex, and its importance in the understanding of the epidemiology of malaria in different parts of sub-Saharan Africa was recently reviewed by Hunt & Coetzee (1995). The six species occur in different regions of the continent. *An. gambiae s.s.* occurs throughout the region, but is generally more abundant in more humid areas. *An. arabiensis* often occurs sympatrically with *An. gambiae s.s.* but appears to be more drought resistant. *An. quadriannulatus* is found in Ethiopia, Zanzibar and certain parts of South Africa only. *An. bwambae* has only been found in mineral springs of the Semliki forest in Uganda. Two siblings breed in brackish water, *An. melas* along the West African coast, and its East African equivalent

An. merus. (Gillies & Coetzee, 1987). Only the last two siblings can be distinguished on the basis of their morphology, whereas the other four members of the complex can only be identified with cytotaxonomic and biochemical techniques.

The single most important reason for analyzing this species complex is their marked differences in adult behaviour which is directly reflected in their vectorial capacities (Coluzzi, 1992). Innate host preferences have been reported to vary greatly between the siblings. *An. gambiae s.s.* is one of the most anthropophilic mosquito species in the world. Analyses of blood meals from specimens collected in various African countries have shown a strong preference for feeding on man (Davidson & Draper, 1953; White, 1974; Garrett-Jones *et al.*, 1980; Gillies, 1988). This distinct odour-mediated preference for man became clear when *An. gambiae s.s.* was found to pass through a herd of cattle surrounding Fulani nomadic settlements in Nigeria. The mosquitoes apparently ignored the massive quantities of cattle odour and selectively responded to human odour released from the temporal structures (M. Coluzzi, *pers. comm.*, Gillies, 1988). *An. arabiensis* is a more catholic feeder. Throughout its range varying degrees of anthropophily are found. Interestingly, it has been observed that anthropophily is more dominant in West Africa, and that this decreases towards East Africa, with entirely zoophilic populations on Madagascar (M. Coluzzi, *pers. comm.*). This plasticity in feeding preferences bears an impact on vectorial capacity of the two siblings, and sporozoite rates from *An. arabiensis* from East Africa have been found to be only 1/15 th of that found in *An. gambiae s.s.* (White *et al.*, 1972), despite the fact that both siblings are equally susceptible to infection with *Plasmodium* gametocytes (White, 1974). *An. quadriannulatus* is a strongly zoophilic species and not a vector of disease. Both salt-water siblings feed primarily on non-human hosts, but *An. melas* feeds more regularly on man and is thus the more important vector of the two. Host availability will naturally influence these innate preferences, but choice experiments with members of the complex have shown that besides opportunistic factors (host density, accessibility etc.) preferences for feeding on specific hosts are genetically determined (Gillies, 1967; Coluzzi *et al.*, 1977). Within the *An. gambiae* complex these differences have been (uniquely) linked to chromosomal characters as seen on the polytene chromosomes (Coluzzi *et al.*, 1979).

The siblings within the *An. gambiae* complex also vary according to their endo- versus exophagic biting habits and endo- versus exophilic completion of gonotrophic cycles. *An. gambiae s.s.* readily enters houses and after feeding it spends a considerable time (at least the first day of the gonotrophic cycle) inside of them. *An. arabiensis* is again more liberal and feeds on hosts both in- and outdoors. Populations are also known to differ in their tendency to complete the gonotrophic cycle inside structures. Coluzzi *et al.* (1979) observed that the genetic make-up of in- and outdoor feeding populations of *An. arabiensis* differed. It was suggested that different inversion karyotypes of this sibling respond differentially to variations in the saturation deficit of air leaving the house versus outdoor air. Since then more associations between chromosome inversion frequencies and eco-climatic parameters have been demonstrated, also for *An. gambiae s.s.* (Touré *et al.*, 1994). The salt-water siblings have varying degrees of endo- and exophagy but exodus from houses after feeding is generally higher then for *An. gambiae s.s.*. *An. quadriannulatus* is largely exophagic and exophilic although it has been reported as endophilic from the Ethiopian highlands, probably as an adaptation to cold nights.

Female *An. gambiae*, after emerging from the pupae, become active at dusk (the following day), disperse, locate a swarm of males and become inseminated. Jones & Gubbins (1978) demonstrated that insemination, feeding and oviposition affected circadian flight activity, which was thought to exist of a peak at dusk, followed by a second peak 6-10 hrs later (Jones *et al.*, 1972). After insemination, which normally takes place at dusk (Charlwood & Jones, 1979, 1980; Marchand, 1984), this peak of activity is greatly reduced in subsequent days. Blood-feeding suppresses activity for 2-3 days, with an increase in activity prior to oviposition. The biting periods of the members of the *An. gambiae* complex have been studied in various locations and generally show low activity until 22.00 hrs, followed by a gradual increase in biting with a peak between midnight and 04.00 hrs (Gillies, 1957; Gillies & Coetzee, 1987; Lyimo, 1993). Densities of *An. gambiae* mosquitoes vary greatly between localities and seasons, but generally follow rainfall patterns (Gillies, 1954). Longevity of the various siblings again varies according to locality but has been reported to be higher for *An. gambiae s.s.* and *An. merus* than for *An. arabiensis* (Mutero & Birley, 1987). Up to 12 ovipositions have been recorded for *An. gambiae s.s.* from coastal Tanzania (Gillies, 1988). Besides host-preference, longevity directly influences vectorial capacity of the siblings.

Host-seeking behaviour of the *An. gambiae* complex has received remarkably little attention, despite the importance of this group in malaria transmission. Gillies & Wilkes (1969, 1970, 1972) studied outdoor behaviour of *An. melas* in The Gambia, and concluded that odour other than carbon dioxide attracts females from a long distance. Carbon dioxide activates mosquitoes and serves as an attractant provided the stimulus is presented to the mosquito in an irregular fashion. At short range humidity and temperature play an additional role. Omer (1979) arrived at the same conclusion while studying *An. arabiensis* in a windtunnel setup. To date, no kairomones, other than carbon dioxide, have been identified as playing a role in the host-seeking behaviour of any of the members belonging to the *An. gambiae* complex.

The clear-cut host preference for human blood by *An. gambiae s.s.* has raised the question whether all individuals of a population receive the same number of bites by this species (Burkot, 1988). Clear indications that this is not the case have been published, and adults have been reported more attractive than children (Carnevale *et al.*, 1978), which has been suggested to relate to the surface area of the body exposed to bites (Port *et al.*, 1980). Other factors have also been suggested to influence preferential feeding. Wood *et al.* (1972) claimed that ABO blood group status influenced selection of human hosts by *An. gambiae s.s.* but this was later refuted by Thornton *et al.* (1976). The latter authors found increased landing and blood-feeding on a human arm after thermogenically induced sweating. Lindsay (1993) studied attractiveness of *An. gambiae s.l.* to six Gambian men under field conditions and found clear differences between them. Whether this preference for certain individuals within a group is based on physical or chemical features or a combination of both is not known.

It can be concluded that even though *An. gambiae s.s.* is one of the most important malaria vectors in the world, the factors governing its host-seeking behaviour remain largely unknown.

1.4. Mosquitoes as vectors of disease

Following Manson's (1878) observations that filarial *Wuchereria bancrofti* worms developed in *Culex* mosquitoes, a multitude of diseases subsequently became classified as vector-borne. Mosquitoes thus were incriminated as vectors of major human diseases such as malaria, filariasis, yellow fever and dengue. Some interesting accounts of the history of these discoveries (and their discoverers) have been written (Busvine, 1993; Desowitz, 1991), and show that despite tremendous control efforts mosquito-borne disease still has a grave impact on human well-being.

Malaria remains the single most important mosquito-borne disease in the world (Gilles & Warrell, 1993). It occurs in most tropical areas, notably in sub-Saharan Africa, Asia and Latin America. It is estimated that 40% of the world's population, in 102 countries, is at risk from the disease. Some 100-200 million cases occur annually worldwide, of which 90 million in Africa, with 1-2 million deaths (AAAS, 1991; WHO, 1993). Trends in the world malaria situation show that for most African countries this is deteriorating (Gilles & Warrell, 1993). In Tanzania, for instance, the malaria situation is not different from that almost thirty years ago (Clyde, 1967), and in many areas has worsened since then (Kilama & Kihamia, 1991).

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Four species of *Plasmodium* cause malaria in man (*P. ovale*, *P. malariae*, *P. vivax* and *P. falciparum*). In tropical Africa, *P. falciparum* is the most prevalent parasite and is the most malignant form of malaria. In Tanzania it accounts for more than 90% of all infections (Kilama & Kihamia, 1991). Detailed accounts of *Plasmodium* parasite biology and its intricate life-cycle can be found in Gilles & Warren (1993). Two life-cycle phases are recognised: the exogenous sexual phase (sporogeny) which takes place in mosquitoes belonging to the genus *Anopheles* and the endogenous asexual phase (schizogony) with multiplication in the vertebrate host (i.e. man).

Although there are some 422 *Anopheles* mosquito species only 70 are known malaria vectors and of these only about 40 are important (Service, 1993). If a species of *Anopheles* can acquire an infection with *Plasmodium* than three factors determine the vectorial capacity of that vector namely (a) the intrinsic preference for biting a human host (i.e. the degree of anthropophily), (b) longevity of the vector (i.e. the probability of the mosquito surviving through the development period of the malaria parasite), and (c) the size of the vector population (Burkot, 1988). This explains the high endemicity of malaria in major parts of sub-Saharan Africa; it's main vectors *An. gambiae s.s.* and *An. funestus* have a high degree of anthropophily (White, 1974), have a relatively high survival rate and often occur (sympatrically) in high densities (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987). As a consequence sporozoite infections of about 5% are common and high mosquito population densities can result in entomological inoculation rates (i.e. the number of infective bites per person per year) exceeding 1000, but have been reported from Ifakara, Tanzania to be as high as 3000 (Snow & Marsh, 1995).

Though direct evidence is scarce, it is likely that *Plasmodium* parasites affect both the mosquito vector and the host in a way that might enhance transmission. Mice with high parasitaemia became lethargic and were thus more readily fed upon by mosquitoes than uninfected mice. Furthermore, most mosquitoes were observed feeding on these (lethargic) mice two days after peak parasitaemia, at which time gametocyte levels were maximal (Day & Edman, 1983). In contrast, invasion of the mosquito's salivary glands by sporozoites affects secretion of saliva, which results in increased probing (Ribeiro *et al.*, 1985). Increased probing is likely to enhance host defensive responses and may therefore result in multiple feeding on the same, but also on different hosts. Similar parasite induced behavioural changes in host and vector have not been extensively studied for human malarias. Even though clinical malaria induces changes in the host that might increase its attractiveness to mosquitoes (increased body temperature, increased moisture levels, sweating), this does not affect disease transmission since sexual stages are normally absent in the blood of the host when densities of asexual stages are high (Carter & Gwadz, 1980). In Africa asymptomatic parasitaemias are much more common than symptomatic ones (Burkot, 1988), and the contribution of asymptomatic malaria to disease transmission is thus much higher than that of symptomatic malaria. Disease transmission is therefore directly related to the prevalence and infectiousness of gametocytes in asymptomatic parasitaemias. It has been suggested that gametocytes affect the attractiveness of the host. If gametocytaemic episodes increase human attractiveness and susceptibility to anophelines then the efficiency of malaria transmission could be significantly increased. Thus, if human attractiveness is influenced by the circulation of mosquito-infective *Plasmodium* stages than this directly influences the host-seeking process, and most likely the odour-mediated part of it. To date, no studies have been undertaken to verify this. Examples of this phenomenon, however, exist for another vector-borne disease namely leishmaniasis. *Leishmania* infected dogs have been reported to be more attractive to sandflies than uninfected dogs, and sandflies are attracted to those body parts of mice that have cutaneous lesions (Coleman & Edman, 1988). Both parasite-induced behavioural changes in host-seeking by these insects increase the transmission of *Leishmania* parasites.

1.5. Problem definition and research goals

Malaria remains the singlemost important parasitic disease of man in tropical regions of the world. Efforts to control the malaria parasite by chemoprophylactic and/or curative drugs are seriously jeopardized due to widespread resistance (Ter Kuile, 1994). *Anopheles* mosquito vectors are rapidly developing physiological resistance against insecticides used for indoor spraying (WHO, 1986) or on bed nets (Vulule *et al.*, 1994; Magesa *et al.*, 1994). The development and application of vaccines against *Plasmodium* parasites has recently shown to give partial protection to children in Tanzania (Alonso *et al.*, 1994) but will not be available for large-scale use during the coming decade. Physical control methods such as breeding site modification through environmental management and biological control methods such as the application of *Bacillus thuringiensis* can only be applied against certain vector species, depending on breeding site characteristics.

Africa's most important malaria vector, *An. gambiae s.s.*, can not be controlled with these latter methods due to its breeding in a wide variety of semi-permanent waterbodies. Resistance of this sibling species and *An. arabiensis* to DDT and other insecticides has been recorded in several African countries (WHO, 1986). At present the only available vector control technique appears to be the use of impregnated bed nets (Curtis, 1991), although in areas with high perennial transmission these have had little effect on disease prevalence. In general it can be said that the outlook for malaria control is grim, and that the situation in sub-Saharan Africa is rapidly deteriorating due to failure of the tools at hand (see Collins & Paskewitz, 1995).

The control of African trypanosomiases in livestock has experienced similar set-backs. The most commonly used trypanocidal drugs are in many areas no longer effective due to resistance and cross-resistance between chemically related compounds (Jordan, 1986). Traditional vector control methods such as the use of fixed-wing or groundspraying of persistent insecticides are no longer cost-effective, bear significant impact on the environment, and are logistically complex. A dramatic change in the control of tsetse vectors of trypanosomiasis has occurred due to a revolution in the understanding of fly biology, in particular how it locates a host. Initial separation of the cues that may be responsible for attracting tsetse to a cow showed that >90% of the attraction was due to chemical cues (Vale, 1974). Identification of the compounds responsible for the attraction of flies to a host might thus be used to lure flies from considerable distances to artificial bait systems, which were subsequently developed. Gaschromatography/Mass spectrometry and gaschromatography linked to electroantennogram setups (EAG) were used to identify physiologically active compounds which were then tested for attractiveness in the field (Hall *et al.*, 1984, 1990; Bursell *et al.*, 1988). Simple traps and insecticide impregnated targets that kill flies upon entering or contacting it respectively, were developed simultaneously. Thus, in less than two decades, an entirely new technology to control tsetse flies, based upon thorough knowledge and subsequent exploitation of its natural host-seeking behaviour, became available and is now applied in various African countries (Wall & Langley, 1991; Colvin & Gibson, 1992; Vale, 1993; Willemse & Takken, 1994). These successes have shown that baits offer the prospect of being a promising approach to controlling pests of man and livestock.

It is with these successes in mind that interest has arisen in the host-seeking behaviour of mosquitoes (Torr, 1994). Ample evidence exists that mosquitoes also use olfactory cues to orient towards hosts (Laarman, 1955; Bertram & McGregor, 1956; Gillies & Wilkes 1969, 1970, 1972). However, little is known about the odour-mediated host-seeking behaviour of the important malaria vector *An. gambiae s.s.*, but furthering the knowledge of this process might ultimately result in the development of new control strategies analogous to those for tsetse flies. In addition, the development of bait systems might provide important tools for monitoring vector populations and thus aid effective epidemiological modelling of disease.

Initial studies on the flight behaviour of individual *An. gambiae s.s.* in a windtunnel showed that mosquito responses to human odour can be studied in the laboratory (Takken & Knols, 1990b). Nevertheless, tracking of individual mosquito flight paths proved laborious and translation of the results to levels of attractiveness difficult. An important improvement in the identification of behaviourally active odours would thus be the development of a laboratory based bioassay that would allow rapid and reliable screening of candidate compounds.

Carbon dioxide, having been identified as an important kairomone in the attraction of many mosquito species (Gillies, 1980) has been shown to affect *An. gambiae s.s.* responses (Takken & Knols, 1990b). It remains unclear to what extent this compound affects mosquito behaviour in the field, and whether or not it interacts with other body odours. It is envisaged, however, that due to its anthropophilic behaviour, the role of carbon dioxide will be limited, it merely signifying the presence of a vertebrate host rather than man. Other human-specific odours will most likely play a more dominant role in host-seeking by this species (providing reliable cues, see Vet & Dicke, 1992).

The reported differential attractiveness of humans to *An. gambiae s.s.* may be olfactory based and implies that humans produce varying amounts of kairomones used by this species while selecting a host. The identification of odour complexes or individual components thereof might reveal the origin of these preferences.

At present no sampling tools for examining the response of *An. gambiae s.s.* to odours in outdoor situations are available. Evaluation of laboratory identified kairomones under field conditions therefore requires the development of sampling devices for outdoor use.

In summary, the specific objectives of the work presented in this thesis were[1]:

1. to investigate the importance of chemical cues in the host-seeking behaviour of *An. gambiae s.s.*.

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[1] The research presented in this thesis was part of a European Community funded project titled 'Behavioural studies on malaria vectors', which was executed in collaboration with the Natural Resources Institute (NRI) and Imperial College (IC) in the United Kingdom, the Instituto di Parassitologia in Rome, Italy, and the National Institute for Medical Research (NIMR) in Muheza and Ifakara, Tanzania.

2. to develop tools for studying these chemical cues, in particular carbon dioxide and human odours, both under laboratory and field conditions.

3. to identify odour complexes/individual kairomones that influence the host-seeking behaviour of *An. gambiae s.s.*.

4. to conduct studies on odour-mediated host-seeking behaviour of *An. gambiae s.l.* in order to determine the potential for using the laboratory-identified kairomones as attractants under field conditions.

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Part I:

Selection of biting sites by mosquitoes on man: a new approach to kairomone identification

2

Selection of biting sites on man by four mosquito vectors (Diptera: Culicidae) of disease[1]

ABSTRACT - While searching for blood, female mosquitoes pass through a behavioural process involving responses to visual, physical and chemical properties of the host. Temperature and moisture are thought to dominate mosquito orientation near the host. In this study the selection of biting sites on a naked, motionless, human host by *Anopheles atroparvus* Van Thiel, *An. gambiae s.s.* Giles, *Aedes aegypti* L. and *Culex quinquefasciatus* Say was observed. Only the latter species did not show a preference for biting a specific region of the body. *An. atroparvus* and *Ae. aegypti* preferentially bit the head and shoulder regions whereas *An. gambiae s.s.* showed a strong preference for biting the feet and ankles. These preferential biting patterns correlated with particular combinations of skin temperature and eccrine sweat gland density. Direct observations on the behaviour of both anopheline species around the host suggested that host odour might influence this process. Modification of the host's odour profile by removing exhaled breath and washing feet for *An. atroparvus* and *An. gambiae s.s.* respectively, resulted in significant changes of these preferences, and both species were diverted to other body regions. Since biting no longer correlated in the same way with skin temperature and eccrine sweat gland density, initial preferences for biting specific regions on a normal host could thus be attributed to the influence of host odour. The results demonstrate the significance of

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odours in the selection of biting sites for these two malaria vectors. These studies can therefore assist in revealing the origin of chemical cues involved in host-location. The differences in selection of biting sites may also reflect differences in the range of hosts fed upon and are therefore discussed within the general framework of host-seeking by mosquitoes.

Introduction

Many investigations have underlined the influence of heat, moisture and, to a lesser extent, carbon dioxide on mosquito behaviour in the close vicinity of the host (Gillies, 1980; Hocking, 1971; Wright & Kellogg, 1962). These factors are so dominant that odours are believed to be involved only at those distances where heat and moisture are not detectable (Clements, 1963; Laarman, 1955; Gillies & Wilkes, 1972). However, experimental evidence to confirm this distance-related action of chemotaxis is lacking, whereas insight in mosquito olfaction at close proximity to the host would provide valuable information about odours involved in host-seeking.

Since heat and moisture are reported to govern biting behaviour of mosquitoes (Clements, 1963; Gillies & Wilkes, 1972; Friend & Smith, 1977; Burgess, 1959) one expects the choice of biting sites on a host to be determined by local variations in body temperature and/or humidity. Induced eccrine sweating, for example, increases the attractiveness of man to mosquitoes (Khan *et al.*, 1969). A preference for hands above forearms has been demonstrated for several mosquito species (Thornton *et al.*, 1976; Rahm, 1956; Freyvogel, 1961) but it remains unclear whether this is solely caused by differences in skin temperature and humidity (Thornton *et al.*, 1976). Few and inconclusive reports of selection of biting sites by mosquitoes on man exist. Haddow (1945) found *Aedes simpsoni* mainly biting on the heads of naked individuals. Testing of the subjects in standing, sitting and laying positions gave similar results and therefore excludes the influence of height. In contrast, biting of the mosquito *Eretmapodites chrysogaster* is confined to a narrow band above ground level, between ankles and knees of a standing person, and apparently not influenced by body heat and moisture since biting occurred all over the body when this person was laying (Haddow, 1956). Also *Culex pipiens fatigans* bites mainly on the lower half of the body (Self *et al.*, 1969). These scanty records of biting site preferences indicate that several mosquito species use different cues to select biting sites.

Differences in biting site selection may reflect different host-seeking strategies, probably linked with host selection. *Er. chrysogaster*, for example, does not actively search for a bloodmeal but waits until a host enters its visual range (Haddow, 1956), a strategy based on visual host-finding which supposedly requires a broad host spectrum. Opportunistic feeders can also orient towards commonly produced chemical cues, such as carbon dioxide, whereas specialists need host-specific information. Visual cues play an important role in host-seeking by *Ae. aegypti* (Gillett, 1972b) but its innate preference for biting man requires responses to human-specific olfactory cues.

In this chapter a study on the selection of biting sites by four mosquito species with different behavioural traits on the same adult male volunteer under controlled laboratory conditions is reported, in an attempt to elucidate which factors govern this process. *An. atroparvus*, is a nocturnal opportunistic bloodfeeder (Swellengrebel & De Buck, 1938), *An. gambiae s.s.* is nocturnal and highly anthropophilic (White, 1974; Garrett-Jones *et al.*, 1980), *Ae. aegypti* is diurnal and anthropophilic (Gillett, 1972a) and *Cx. quinquefaciatus* is crepuscular/nocturnal and anthropophilic (White, 1971).

Materials and Methods

Mosquitoes. Mosquito strains originated from Leiden, The Netherlands (*An. atroparvus*), Moshi, Tanzania (*An. gambiae s.s.*), Muheza, Tanzania (*Cx. quinquefasciatus*), and *Ae. aegypti* was obtained from a strain maintained at the RIVM (Bilthoven, The Netherlands). Adults were kept in 30x30x30 cm gauze cages, fed on 6% glucose solution and offered blood (rat for *An. atroparvus*, human arm for the other species) twice weekly. Eggs (laid on moist filter paper) were allowed to emerge in water trays and larvae were fed on Tetramin$^{(r)}$ fishfood. Pupae were collected from the trays daily and placed inside cages. Females used in the experiments were 5-10 days old and had not been given the opportunity to feed on blood. They were starved the night before the experiments by putting them individually into glass vials, closed with a water-moistened plug of cotton wool.

Experimental procedures. In a climate-controlled room, the test person (male Caucasian, 27 years, not bathed for at least 9 hours) was seated on a stool (height 34 cm) inside a cubic bed net (190x190x190 cm; meshwidth 0.6 mm, see Fig. 1). He was only wearing tight underwear, and sitting in an upright stationary position with hands resting on the knees and feet on the floor. Mosquitoes were released through a small hole in the net (at 95 cm from the side and 27 cm from the top) and were tested individually by allowing them to bite within three minutes, after which non-responders were discarded. The site of biting was confirmed by a second person, entering the bed net and removing the mosquito, after probing was felt or three minutes had passed. Every after ten mosquitoes had been tested the subject changed position, from facing the release point (head at 140 cm) to backing it.

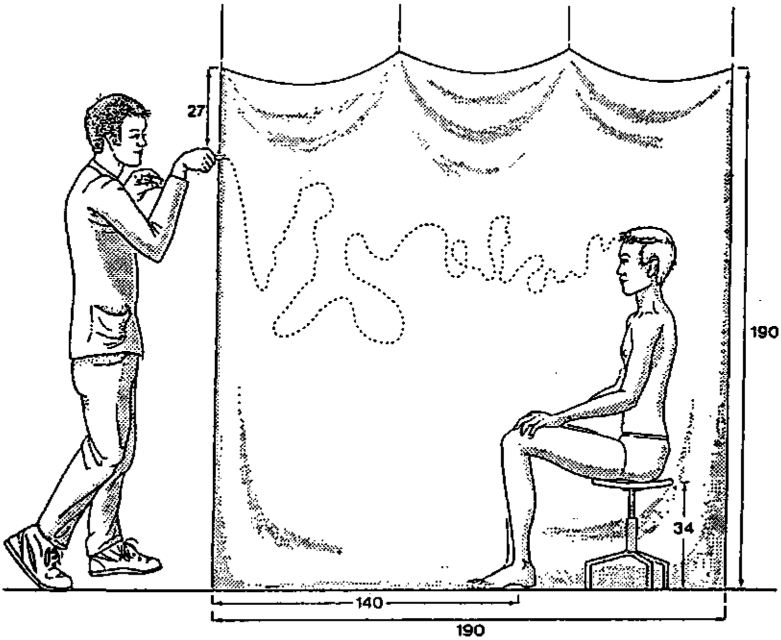

Figure 1. The experimental setup, showing the position of the 'host' inside a bed net into which mosquitoes were released individually by a second person (standing). Dimensions in cm, more details see text.

Temperature inside the room was 27±2°C, RH was 60-70%, and illumination intensity in the bed net was below 20 Lux, except for *Ae. aegypti*, which, because of its diurnal host-seeking was tested with increased light intensity (not measured). Possible day effects, for example caused by variations in diet or physical condition of the test person, were eliminated by testing, as much as possible, all species each day for several days. This procedure also excluded the possibility that preferences were 'induced' by previous biting and marking, since bathing took place in between test days. Circa hundred mosquitoes per

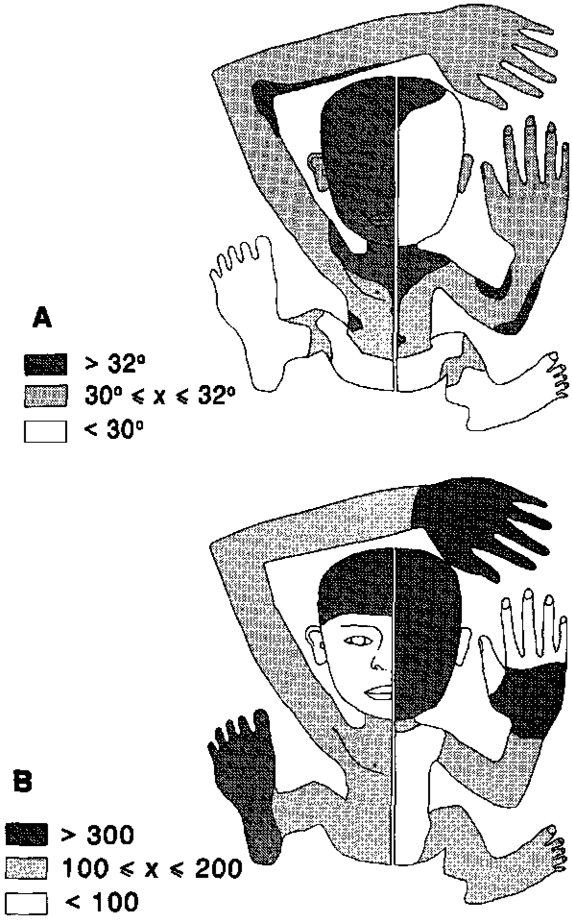

Figure 2. *(A) Distribution of skin temperatures and (B) eccrine sweat gland densities on the human body (after Clark & Edholm, 1985 (A) and Marples, 1969 (B)).*

species were tested individually and their biting sites recorded.

Data analysis. We analysed the biting site distributions by comparing relative skin surface areas of various body parts (Clarke & Edholm, 1985) with received and expected number of bites using a G-test (Sokal & Rohlf, 1981). Since skin temperature and humidity are believed to be the main, if not the only factors determining short-range host-seeking behaviour of most mosquito species, we correlated the biting site distributions with these skin properties. The body was divided into three temperature (Fig. 2A, Clarke & Edholm, 1985) and three humidity regions (Fig. 2B). Since the main discharge of water by the skin is effected by eccrine sweat glands we used their apparent densities to assess humidity levels above the skin (Marples, 1969).

Results and Discussion

The choice of biting sites for *Ae. aegypti* and *Cx. quinquefasciatus* is shown in Fig. 3, and for *An. atroparvus* and *An. gambiae s.s.* in Fig. 4A and Fig 5A respectively. The distribution of bites on various body parts is shown in Table 1.

Table 1. *Distribution of biting sites by four mosquito species on various body parts of the same human host.*

		Body part				
	N[a]	Head[b]	Trunk	Arms	Legs	G-test[c]
Ae. aegypti	105	24	39	16	26	$P<0.001$
Cx. quinquefasciatus	101	13	32	26	30	n.s.
An. atroparvus	100	50	15	17	18	$P<0.001$
An. gambiae s.s.	100	1	16	7	76	$P<0.001$
Skin surface (as % of total)		9	32	19	40	

[a] Total number of bites recorded

[b] Head includes neck region

[c] G-test of goodness of fit (Sokal & Rohlf, 1981) with expected number of bites based on shown relative skin surfaces (as % of total body surface). n.s.: not significant.

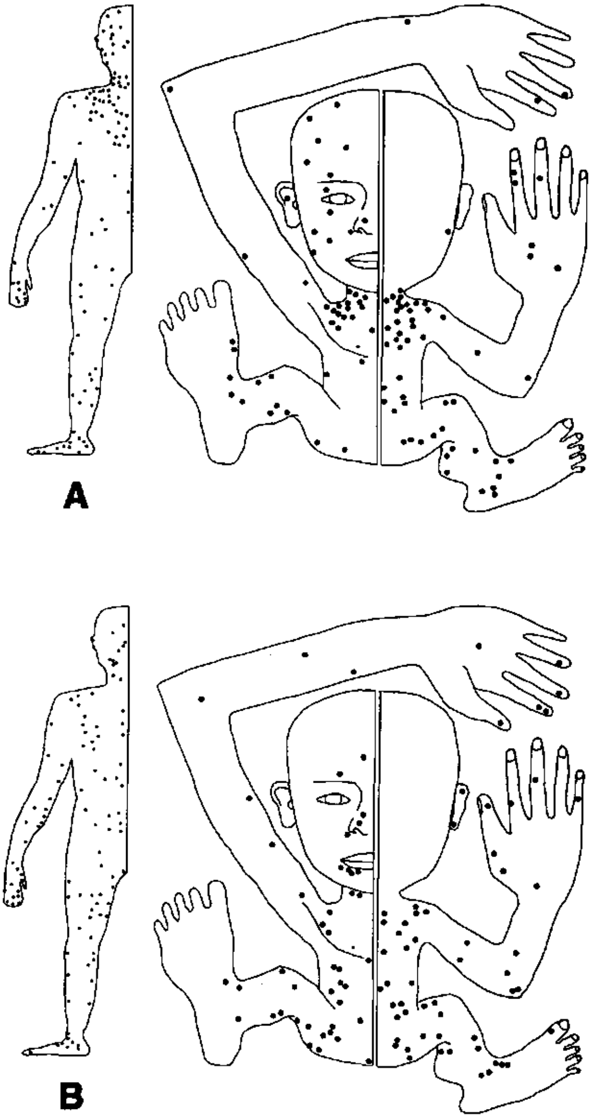

***Figure** 3. Distribution of biting sites of* Ae. aegypti *(A, $n=105$) and* Cx. quinquefasciatus *(B, $n=101$), on the same human host. All bites are superimposed on a half silhouette (left) and front/back of the body (right).*

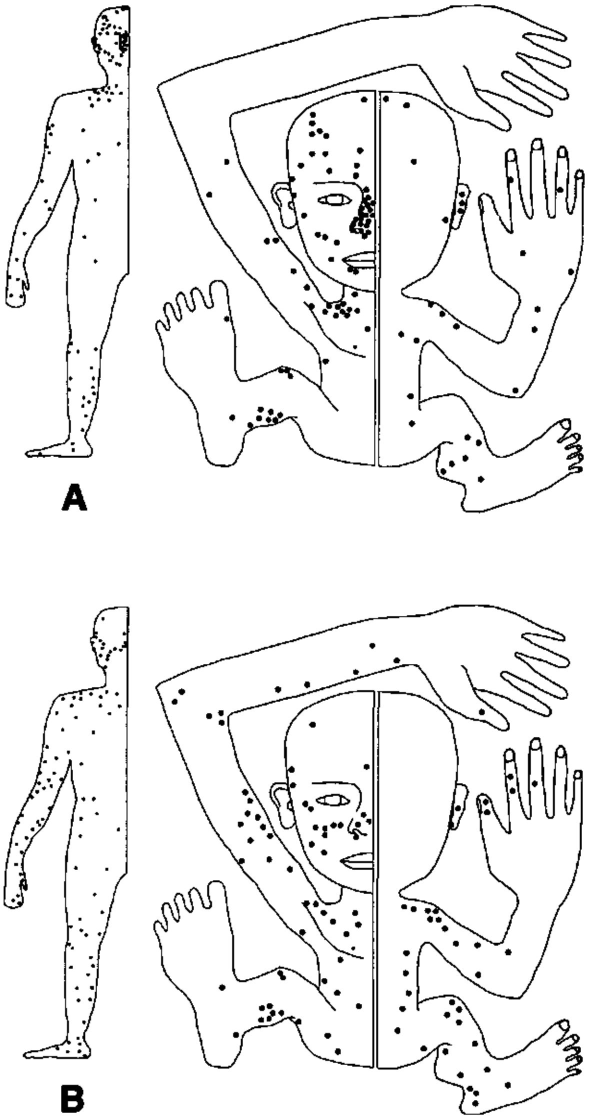

Figure 4. *Distribution of biting sites of* An. atroparvus *on the same human host, before (A) and after breath removal (B). All bites are superimposed on a half silhouette (left) and front/back of the body (right).*

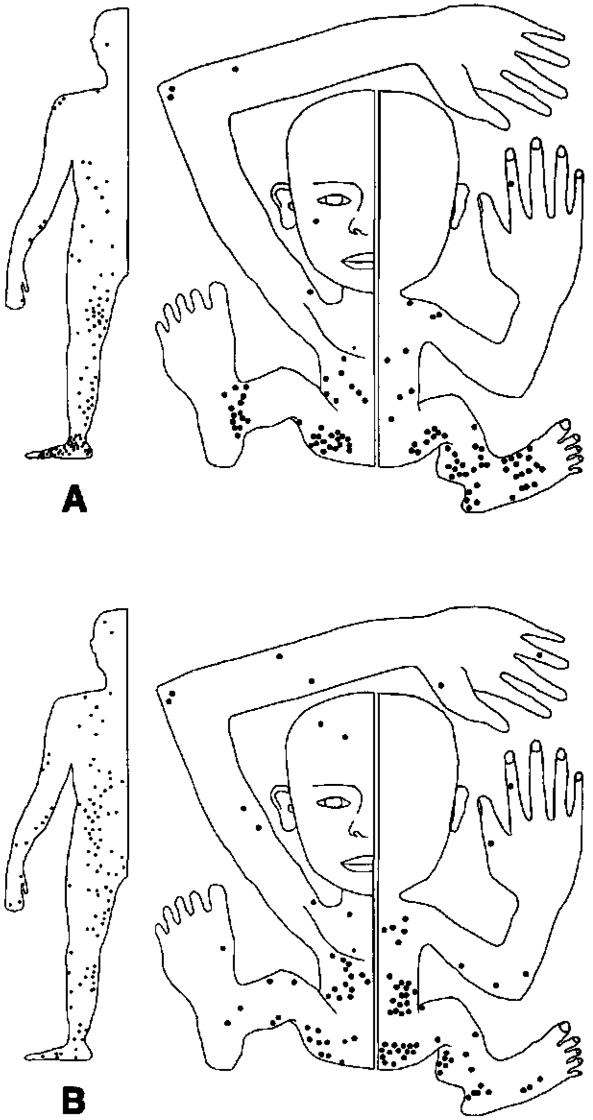

Figure 5. *Distribution of biting sites of* An. gambiae s.s. *on the same human host, before (A) and after washing the feet and ankles with a bactericidal soap (B). All bites are superimposed on a half silhouette (left) and front/back of the body (right).*

Whether or not the human subject was facing the release point did not influence the biting patterns. The data clearly show that biting is non-random and that both *Ae. aegypti* and *An. atroparvus* strongly preferred to bite the head/shoulder region ($P < 0.001$). *An. gambiae s.s.*, on the other hand, showed a significant preference ($P < 0.001$) for the foot region. The biting pattern for *Cx. quinquefasciatus* did not differ from that expected for random biting. For all four species correlations exist with particular combinations of skin temperature and eccrine sweat gland density (Table 2A). Further analysis (Table 2B) shows that for *Cx. quinquefasciatus* no correlation exists with skin temperature but the preference for biting body parts with low densities of eccrine sweat glands was significant. The other three species also preferred regions with specific temperatures or eccrine sweat gland density.

Even though these correlations exist for all four species it was observed that the way they approached the host might be influenced by other factors as well, and it was decided to determine whether body odour plays a role in the selection of biting sites by both anopheline species. These mosquito species showed a typical behaviour prior to biting. *An. atroparvus* appeared to be responding to exhaled breath by a sudden reduction of flight speed followed by orientation towards the head. Perception of exhaled breath, therefore, seemed to influence the biting site selection of this species. In olfactometer experiments Laarman (1955) indeed showed that human breath is attractive for *An. atroparvus.* On the other hand, *An. gambiae s.s.* was often seen approaching the host in the head region, apparently not responding to breath, and gradually moving downwards to the feet. This species seemed to orient itself by using convection currents along the host (Lewis *et al.*, 1969; Clark & Toy, 1975), a phenomenon described earlier for other species (Wright & Kellogg, 1962; Peterson & Brown, 1952). Odours emanating from the foot region, carried by these convection currents, might have guided *An. gambiae s.s.* to this particular odour source. Both species apparently were using other cues than only skin temperature and/or humidity at short range, i.e. breath for *An. atroparvus* and foot odour for *An. gambiae s.s.*. If correct, this hypothesis involves the role of odours near the host while biting site distributions on the body provide important clues as to which chemical factors govern the host-seeking process.

In order to verify this hypothesis we altered the host for *An. atroparvus* by removing exhaled breath from the room with a Harvard Douglas(r) one-way breathing valve, connected to polythene lay-flat tubing. In case breath does not

Table 2. Distribution of mosquito bites for combinations of skin temperature and eccrine sweat gland density (A) and both factors separately (B).

A

Skin temperature[1]	Low			Medium			High			
Eccrine sweat gland density[2]	Low	Medium	High	Low	Medium	High	Low	Medium	High	G-test[3]
Ae. aegypti	3	15	6	12	12	5	24	23	5	P<0.001
Cx. quinquefasciatus	4	22	1	16	26	8	17	6	1	P<0.001
An. atroparvus	1	14	2	8	10	3	35	14	13	P<0.001
An. gambiae	1	64	7	8	14	0	3	3	0	P<0.001
Expected[3]	5	28	4	9	33	3	6	7	5	

B

	Skin temperature[1]				Eccrine sweat gland density[2]			
	Low	Medium	High	G-test[3]	Low	Medium	High	G-test[3]
Ae. aegypti	23	29	53	P<0.001	39	50	16	P<0.001
Cx. quinquefasciatus	27	50	24	n.s.	36	55	10	P<0.001
An. atroparvus	17	21	62	P<0.001	44	38	18	P<0.001
An. gambiae s.s.	72	22	6	P<0.001	12	81	7	P<0.05
Expected[3]	37	45	18		20	68	12	

[1] Categorised after Clark & Edholm, 1985. Low, <30°C; Medium, 30°-32°C; High, >32°C.

[2] Categorised after Marples, 1969. Low, <100 glands cm^{-2}; Medium, 100-300 glands cm^{-2}; High, >300 glands cm^{-2}.

[3] G-test of goodness of fit with expected frequencies based on hypotheses extrinsic to the sampled data. n.s.: not significant.

Table 3. *Distribution of mosquito bites on various body parts (A), for combinations of skin temperature and eccrine sweat gland density (B) and both factors separately (C) after removal of breath (*An. atroparvus*) or washing of the feet (*An. gambiae s.s.*).*

A

Body part	Head[1]	Trunk	Arms	Legs	G-test[2]
An. atroparvus	19	16	39	26	P < 0.001
An. gambiae s.s.	2	48	14	36	P < 0.001

B

Skin temperature[3]	Low			Medium			High			
Eccrine sweat gland density[4]	Low	Medium	High	Low	Medium	High	Low	Medium	High	G-test[2]
An. atroparvus	1	18	2	13	33	1	18	11	3	P < 0.001
An. gambiae s.s.	14	26	2	17	27	2	5	5	2	P < 0.001

C

	Skin temperature[3]				Eccrine sweat gland density[4]			
	Low	Medium	High	G-test[2]	Low	Medium	High	G-test[2]
An. atroparvus	21	47	32	P < 0.001	32	62	6	P < 0.001
An. gambiae s.s.	42	46	12	P < 0.001	36	58	6	P < 0.001

[1] Head includes neck region.

[2] G-test of goodness of fit with expected frequencies based on observed data from Table 1 and 2.

[3,4] see Table 2.

play a role in the mosquito's behaviour close to the body we expect to find a similar biting pattern as found before. However, the results (Fig. 4B and Table 3), show a dramatic change in the choice of biting sites. Biting no longer correlates in the same way with skin temperature and eccrine sweat gland density. The strong preference of *An. atroparvus* for landing and probing on the head can thus be explained by the attractiveness of breath for this species. It responds well to carbon dioxide (Laarman, 1955; Van Thiel, 1947), an important constituent of breath, and a reliable host related cue for opportunistic bloodfeeders.

For *An. gambiae* we changed the host's odour profile by washing feet and ankles with Unicura(r) non-perfumed medical soap containing a bactericidal agent (2,4,4'-trichloro-2' hydroxydiphenyl ether (1%)). Washing took place at hourly intervals in a warm soap-water solution for a few minutes, followed by rinsing in warm water and drying with a towel. There is no evidence for repellency of this soap. On the contrary, strong attraction occurred towards a humidified bar of this soap placed on top of a cage, inducing landing and fierce probing responses of females. A dry bar of soap, however, did not elicit any responses. GC-MS analyses of foot odour after washing failed to detect soap residues, but, instead, showed a marked reduction in the overall amount of volatiles emanating from the subject's feet (E.J. Van der Meent, *pers. comm.*). The washing resulted in a remarkable change in the biting site distribution (Fig. 5B, Table 3) when tested for skin surface areas, skin temperature and sweat gland distribution. The fact that we were able to divert *An. gambiae* from biting the feet and ankles to other body regions so dramatically reveals the importance of odours emanating from this region in the biting site selection of this species. Since *An. gambiae* is highly anthropophilic we expect these to be human-specific.

We did not attempt to elucidate the factors that caused *Ae. aegypti* to bite the head and shoulder region. In similar experiments, whereby breath was removed, it was observed that the biting pattern of *Ae. aegypti* was not different from that in the presence of breath, which indicates that the preference for biting this body part was not mediated by exhaled breath (A. de Bever & S. van der Laak, *pers. comm.*). It's preference for biting the warmer parts of the body could have resulted in the observed biting site distribution. Alternatively, this preference may have been caused by visual responses to the head of the volunteer. Nevertheless it is interesting to note that the head and shoulder region harbour high densities of anaerobic *Propionibacterium* spp. (Noble, 1982), which produce a wide variety of carboxylic acids to which *Ae. aegypti* is known to be attracted (Carlson *et al.*, 1973). The biting pattern by *Cx. quinquefasciatus* did not seem to be influenced by exhaled breath, which supports findings by Service (1993) that carbon dioxide exerts limited attraction of this species. Other factors might govern its selection of biting sites, for instance odours which emanate from the entire body surface.

This work shows that different mosquito species exhibit different biting site preferences on one and the same human host. It clearly demonstrates that this process is not entirely governed by skin temperature, skin humidity and vision, but also by body odours. Moreover, biting site distributions on the body give important information about production sites of kairomones, and thus of their potential composition, used in host-location and host-selection by mosquito species. Knowledge about these chemical cues opens up possibilities for the development of odour-baited traps, which will find wide application in epidemiological studies of mosquito borne disease and its control.

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3

Influence of human breath on selection of biting sites by the malaria mosquito *Anopheles albimanus* Wiedemann (Diptera: Culicidae)[1]

ABSTRACT - The selection of biting sites by *Anopheles albimanus* Wiedemann on a human host was studied in the laboratory. The perception of exhaled breath guided the mosquitoes towards the head region, and was associated with a preference ($P < 0.001$) for biting this part of the body. Subsequent removal of breath resulted in a strong reduction of the number of bites on the head (from 49% to 20%). The significance of these findings in relation to host-location is discussed.

Introduction

In the process of host-location, bloodfeeding mosquitoes orient by means of various host-related stimuli, i.e. visual, physical and chemical cues (Clements, 1963; Gillies, 1980; Takken, 1991). For nocturnally active mosquito species, the long-distance orientation behaviour is thought to be governed by olfaction (Gillies & Wilkes, 1972). It has long been assumed that in the vicinity of the host, body heat and humidity dominate this process (Burgess, 1959; Friend & Smith, 1977) but De Jong & Knols (1995) demonstrated that body odours play an important additional role in the selection of biting sites by mosquitoes. It was shown, that both *Anopheles atroparvus* van Thiel and *An. gambiae* Giles *s.s.* have a significant preference for biting on a particular site of the human body, the head and feet, respectively. By altering the host's odour profile through breath removal or washing of the feet, both species were diverted from biting the previously preferred sites to other parts of the body. Thus, an analysis of how biting sites are selected by mosquitoes provides important information

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about the origin of cues involved in host-location. Moreover, selection of biting sites might be an indication of the range of hosts fed upon. Biting of the opportunistic species *An. atroparvus* (Swellengrebel & De Buck, 1938), for example, is strongly influenced by breath, while its major component carbon dioxide is a known attractant for this species (Van Thiel & Weurman, 1947) and represents a reliable kairomone for opportunistic feeders.

Anopheles albimanus, an important malaria vector in Mexico and Central America (Ramsey *et al.*, 1986), is reported to be opportunistic (Breeland, 1972; Loyola *et al.*, 1993). On the basis of bloodmeal analyses Loyola *et al.* (1993) found high forage ratios for bovines and equines but also reported bloodfeeding on humans, pigs and chickens. Carbon dioxide could well play a role in its host-location because Wilton (1975) found a 2.2 fold increase in *An. albimanus* catch when CDC traps were baited with dry ice. Therefore, we expect that the selection of biting sites by this species is similar to that of *An. atroparvus*. Here we report on the selection of biting sites by *An. albimanus* on human bait in an attempt to determine whether breath indeed plays a role in the host-location process.

Materials and Methods

Mosquitoes: The *An. albimanus* strain used originated from El Salvador. Adults were kept in gauze covered cages (38 x 39 x 46 cm) at 27° C and RH ca. 80%, and fed on cotton wool soaked with a 10% glucose solution. Experimental females were 2-3 days old, had not received a bloodmeal, and were selected for experimental use with an apparatus as described by Posey & Schreck (1981). These females were then transferred individually into glass vials using a suction tube where they were kept prior to testing.

Experimental design: A male Caucasian volunteer (28 yr) was seated on a 36 cm high stool inside a rectangular bed net (80 x 200 x 145 cm; mesh width 2 mm), wearing tight underwear only (see Fig. 1 in chapter 2). He sat upright, hands on the knees and feet on the floor, and facing the release point (130 cm above floor, in the short side of the net) for the mosquitoes. The head of the subject was thus at 150 cm from the release point. Mosquitoes were released individually by a second person (outside the bednet) and after probing was felt, their biting sites were recorded and the mosquito removed from the net. This was repeated until 100 bites were scored. Mosquitoes not biting within a 3 min. period were removed and discarded in the analyses. Temperature in the room was 27° C and RH ca. 80%. In a second experiment, to assess the importance of breath in the selection of biting sites, breath was removed from the bed net by exhaling into 60 cm long plastic tubing (internal diameter 2 cm) and the nostrils were closed by a nose-clip. Both treatments were completed in three test days. The distribution of biting sites was analyzed by comparing relative skin surface areas of various body parts (Clarke & Edholm, 1985) with received and expected number of bites using a G-test (Sokal & Rohlf, 1981).

Results and Discussion

After release, the mosquitoes responded rapidly to the presence of the host and all but 7% bit within the 3 min. experimental period. It was clearly visible that the mosquitoes reduced their flight speed when approaching the frontal part of the face, most likely upon entering the cone of exhaled breath. This could have resulted in the non-random biting site distribution as shown in Fig. 1A and Table 1. Up to 5.4 times the expected number of bites were recorded on the head, predominantly on the face. However, the influence of other factors, i.e. vision, skin temperature and skin humidity can not be excluded. In order to determine whether correlations between selection of

Table 1. Distribution of Anopheles albimanus *bites on various body parts.*

	Body part				
Host	Head[1]	Trunk	Arms	Legs	G-test[3]
Normal	49	14	28	9	a
Modified (breath removed)	20	33	31	16	b
Expected[2]	9	32	19	40	c

[1] Head includes neck region.

[2] Relative skin surface (after Clarke & Edholm, 1985) expressed as expected number of bites.

[3] G-test of goodness of fit (Sokal & Rohlf, 1981). Treatments not followed by the same letter are different at $P < 0.01$.

biting sites and these skin properties exist, we projected the bites on categorised skin temperature and/or eccrine sweat gland density patterns on the body (Fig 1B).

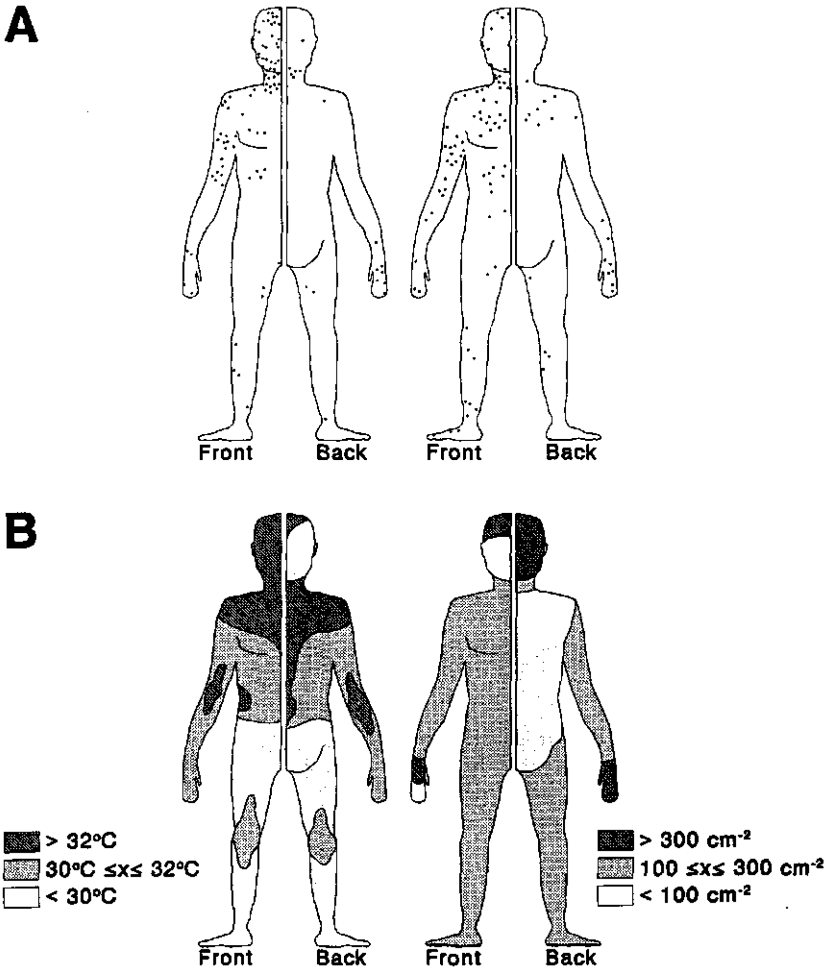

Figure 1. *(A) Distribution of* Anopheles albimanus *bites on a human host before (left, n=100) and after breath removal (right, n=100). All bites on front and back are superimposed on half silhouettes. (B) Categorised skin temperatures (left, after Clarke & Edholm, 1985) and eccrine sweat gland densities (right, after Marples, 1969).*

***Table** 2. Distribution of* Anopheles albimanus *bites for combinations of skin temperature and eccrine sweat gland density (A), and both factors separately (B), before (Normal) and after breath removal (Modified).*

(A)

Skin temperature[3]	Low			Medium			High			
Eccrine sweat gland density[4]	Low	Medium	High	Low	Medium	High	Low	Medium	High	G-test[2]
Normal	0	9	5	4	20	6	18	26	12	a
Modified	0	11	0	4	29	7	13	32	4	b
Expected[1]	5	28	4	9	33	3	6	7	5	c

(B)

	Skin temperature[3]				Eccrine sweat gland density[4]			
Host	Low	Medium	High	G-test	Low	Medium	High	G-test[2]
Normal	14	30	56	a	22	58	20	a
Modified	11	40	49	a	17	72	11	b
Expected[2]	37	45	18	b	20	68	12	b

[1,2] See Table 1.

[3] Categorised (after Clarke & Edholm 1985). Low, $<30^{\circ}$C; Medium, $30^{\circ} \leq x \leq 32^{\circ}$C; High, $>32^{\circ}$C.

[4] Categorised (after Marples 1969). Low, <100 glands cm^{-2}; Medium, $100 \leq x \leq 300$ glands cm^{-2}; High, >300 glands cm^{-2}.

Since the main discharge of water by the skin is effected by eccrine sweat glands we used their apparent densities (Fig. 1B) to assess humidity levels above the skin. As shown in Table 2 correlations indeed exist, especially with the warmer parts of the body and to a lesser extend with skin surfaces excreting relatively large amounts of water.

In previous work (De Jong & Knols, 1995) we found similar patterns for selection of biting sites selection by *An. atroparvus*, which also occurred on the head and correlated with elevated skin temperature. However, removal of exhaled breath drastically changed the selection of biting sites of this species, the number of bites on the head dropping from 50 to 19% ($P<0.001$). Furthermore, biting no longer correlated with skin temperature and humidity as before. On this basis it was concluded that breath dominates the selection of biting sites for *An. atroparvus*. Laarman (1955), in olfactometer experiments already showed the attractiveness of breath, and in particular of its CO_2 content.

In order to verify our hypothesis that biting of *An. albimanus* is also influenced by breath, we conducted a similar experiment where breath was removed from the bednet. The results are shown in Table 1 and Fig. 1A. A significant reduction ($P<0.001$) of the number of bites on the head was found. Mosquitoes still preferred to bite the warmer parts of the body, but the correlation with eccrine sweat gland densities disappeared. This shows that in these tests breath therefore influenced selection of biting sites, thus confirming its role in the host-location of this mosquito species.

We also observed some interesting changes in orientation behaviour. Mosquitoes generally flew closer to the ground and were sometimes seen flying very close to the body without immediate landing, resulting in delayed biting. Another indication of the importance of breath is that a few mosquitoes that escaped during the experiments were seen hovering outside the bed net near the exhaust of the plastic tube used for removing breath. The apparent difficulty in locating the host when breath was removed was also expressed in the slightly higher percentage of non-responding mosquitoes (12%).

These results support our theory that breath, and probably its CO_2 content, influences the selection of biting sites of generalistic bloodfeeders, like *An. albimanus*. However, we cannot exclude other chemicals commonly found in animals, e.g. acetone (Krotozynski, 1977), also present in human breath, as responsible for guiding this species to its hosts. Nevertheless, our results made clear that with regard to the selection of biting sites for *An. albimanus* these compounds are to be found in human breath.

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Part II:

Odour-mediated host-seeking behaviour and kairomone identification: a laboratory approach

4

A trapping system for testing olfactory responses of the malaria mosquito *Anopheles gambiae s.s.* Giles (Diptera: Culicidae) in a windtunnel[1]

ABSTRACT - A new dual-port trapping system for *Anopheles gambiae sensu stricto* Giles (Diptera: Culicidae) for use in a windtunnel is described. Its design proves efficient in preventing mosquitoes from escaping and allows easy testing of large numbers of insects. Traps baited with carbon dioxide at a human equivalent caught significantly ($P<0.001$) more mosquitoes than control traps. However, additional chemicals are believed to govern host-location for this anthropophilic species and the system presented here is a useful tool for the identification of the kairomones involved in this process.

Introduction

Odours are thought to play a major role in host-location by blood-sucking mosquitoes (Clements, 1963; Sutcliffe, 1987; Takken, 1991). One of the best studied mosquito species is the yellow fever mosquito *Aedes aegypti* (L.) for which several chemicals have been identified as being attractive (Acree *et al.*, 1968; Carlson *et al.*, 1973). Very few laboratory studies have focused on malaria-transmitting *Anopheles* spp. (Bos & Laarman, 1975; Price *et al.*, 1979), probably because of their nocturnal biting habits and difficulty of rearing. However, knowledge about the chemical cues leading these mosquitoes to their hosts is important for the development of odour-baited traps for mosquito surveillance and/or control.

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[1] Published in a slightly different form as: **Knols, B.G.J.**, **De Jong, R.** & **Takken, W.** (1994). Trapping system for testing olfactory responses of the malaria mosquito *Anopheles gambiae* in a windtunnel. *Medical and Veterinary Entomology*, **8**, 386-388.

Windtunnels have proven to be useful in testing behavioural responses of mosquitoes towards odours. They can be used for simple and reliable screening experiments with mosquitoes based on mass-release (Mayer & James, 1969; Omer, 1979; Eiras & Jepson, 1991; Schreck *et al.*, 1967). Most of these tunnels are equipped with a dual-port system whereby mosquitoes can choose between two different stimuli. So far no effective system is available for studying behavioural responses of *An. gambiae s.s.*, Africa's most notorious malaria vector. In conjunction with the development of odour-baited traps for field studies (Costantini *et al.*, 1993) we now present a trapping system for *An. gambiae s.s.*, suitable for screening the importance of odours, in a windtunnel.

Materials and Methods

Windtunnel: The windtunnel (Fig. 1A) consists of a flight chamber (160 x60x60 cm) connected to a climate-control system. The flight chamber is made of transparent lexane with a white trespa floor. The outside of the lexane walls is partially covered with white plastic to prevent visual disturbance of the mosquitoes. Conditioned air enters the chamber through two ports in a white trespa board and leaves it via a stainless steel mesh gauze (meshwidth 1 mm) at the downwind side of the tunnel.

The flight chamber is illuminated by nine 40 Watt light bulbs placed on top of the tunnel. Light is diffused through a 3 mm layer of opaque white acrylate and filter paper which reduced its intensity to 6.3±1.4 Lux in the chamber. No other illumination is present in the room.

Outside air is drawn by a Fischbach(r) stepless speed-controllable ventilator and filtered through a glasswool Camfil(r) HI-FLO-95 filter. A Regin(r) thermostat controlled heating system regulates the air temperature to 27±0.5 °C. The air is subsequently moistened to 60±5% RH by a feed-back controlled steam injection system. Before entering the flight chamber the air is purified by passing a 1 cm layer of activated charcoal.

The ports of the flight chamber are linked to trapping devices (Fig. 1B). These are made of glass containers through which the conditioned airstream enters the flight chamber. The windspeed through these containers is fine-regulated to 20 cm/s by adjusting a diaphragm-like opening in side of the tunnel. A ring covered with gauze (meshwidth 1 mm), both stainless steel, prevents trapped mosquitoes from easily returning into the chamber. The

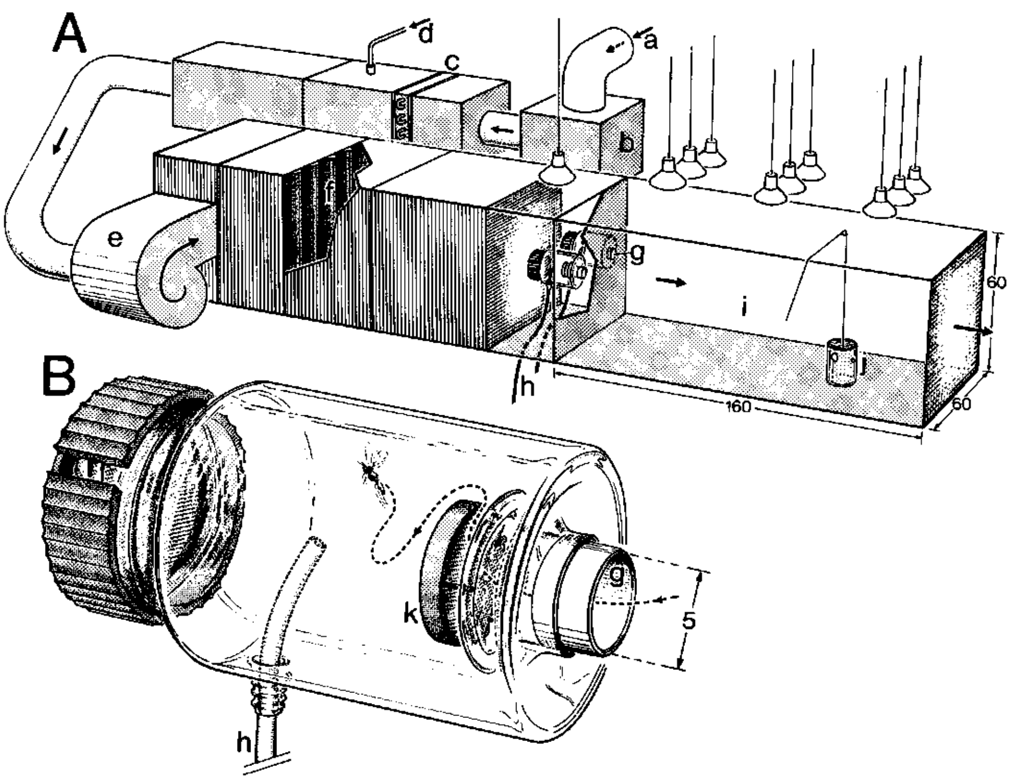

Figure 1. *Schematic drawing of the windtunnel (A) and close-up of a trap (B). Outside air is drawn in (a) by a ventilator (e), filtered (b), heated (c), moistened by steam injection (d) and cleaned by activated charcoal (f). It then enters the flight chamber (i) via two ports (g) to which traps are connected. Test odours are pumped into the traps through teflon tubing (h) and mix with the conditioned air. Mosquitoes are released from containers (j) by external control. A gauze covered ring (k) prevents their escape from a trap. All dimensions are in cm.*

upwind side of the traps are closed by stainless steel gauze (meshwidth 1.8 mm).The light intensity in the traps is equal to that in the flight chamber.

Volatile chemicals, stored in 100 l Tedlar(r) gas-bags, are pumped through teflon tubing (5 mm width) into the traps by two S-200 Du Pont(r) constant flow sampler pumps at a rate of 230 ml/min..

Mosquitoes: The *Anopheles gambiae s.s.* strain originated from Moshi, Tanzania and was colonized in a climate controlled room (28±1 °C, 80±5% RH, LD 12:12). Adult mosquitoes were kept in gauze cages (30x30x30 cm) and fed on glucose 6% solution. Females were offered a human arm twice weekly for egg production. Eggs were laid on wet filter paper and larvae were reared in plastic trays. Larvae were fed on Tetramin(r) fishfood. Females used in the experiments were 5-8 days old, had not received a bloodmeal, and were deprived of sugar the night before testing.

Experimental procedures: Each test day consisted of four test periods of 20 minutes. During one test period 50 females were simultaneously released from a container. This container was placed in the flight chamber (Fig. 1A) and could be opened from the outside of the tunnel. New traps were used for each test period and the port at which the stimulus was applied changed. Fifty additional mosquitoes were subsequently introduced. Trap catches were counted afterwards and mosquitoes which did not leave the containers during the tests were discarded. Catches were subjected to chi-square testing.

In order to test the symmetry of the system we offered clean conditioned air in both traps. As a test stimulus we used carbon dioxide which has been shown to play a role in the host location process of many haematophagous insects (Hocking, 1971; Gillies, 1980; Sutcliffe, 1987). However, to our knowledge, no evidence exists of this compound eliciting behavioural responses in *An. gambiae s.s.*. We therefore investigated whether carbon dioxide at a human equivalent (4.5% in clean air) increases trap catches. The gas was stored in Tedlar(r) bags and pumped at 230 ml/min as described above.

Results

Table 1 shows that results obtained with clean air pumped in both traps did not indicate a preference for either the left or right trap. Even though

Table 1. *Numbers of mosquitoes caught in traps placed on the left or right side of the tunnel on three different days (A,B,C). Clean conditioned air was pumped into both traps.*

A

Test Period	Clean air (Left)	Clean air (Right)	n	X^2
1	1	0	47	
2	2	7	95	
3	9	5	133	
4	7	4	168	
Total	19	16	192	0.3^{ns}

B

Test Period	Clean air (Left)	Clean air (Right)	n	X^2
1	15	16	49	
2	20	33	66	
3	23	18	62	
4	26	16	71	
Total	84	83	196	0^{ns}

C

Test Period	Clean air (Left)	Clean air (Right)	n	X^2
1	2	6	48	
2	7	18	89	
3	25	11	113	
4	16	21	127	
Total	50	56	196	0.3^{ns}

ns: not significant

n: Cumulative number of mosquitoes in flight chamber during test period(s).

catches within days were not significantly different we observed marked differences between the total catch sizes, ranging between 18 and 85 % of the total number released.

When CO_2 was used as a bait this increased catches about four-fold when compared to the control trap (Table 2). For two test days, the total catches were 70 and 87 % respectively. Escapes from the traps were rarely observed, the majority of the mosquitoes landed and remained on the gauze at the upwind side of the traps.

Table 2. *Numbers of mosquitoes caught in traps into which carbon dioxide or clean conditioned air was pumped. Data shown for two test days (A,B).*

A

Test Period	Clean air	CO_2 (4.5%)	n	X^2
1	6	24	48	
2	5	21	65	
3	11	28	88	
4	3	33	92	
Total	25	106	187	71.7*

B

Test Period	Clean air	CO_2 (4.5%)	n	X^2
1	6	32	50	
2	11	37	61	
3	9	21	63	
4	8	48	82	
Total	34	138	198	62.9*

*: $P << 0.001$

n: Cumulative number of mosquitoes in flight chamber during test period(s).

Discussion

Our results clearly demonstrate that the above described trapping system can successfully be used for studying behavioural responses of *An. gambiae s.s.* to volatile chemical stimuli. Mosquitoes did not show a preference for either of the traps when baited with clean air, while the presence of carbon dioxide in one of these significantly increased the catch compared to the control. Interestingly, an important variation in the totals caught exists between the various experimental days (Table 1), demonstrating that results can only be analyzed within days but not between days. These variations can not yet be explained but evidence exists that uncontrollable factors, e.g. barometric pressure, influence insect behaviour (Steinberg *et al.*, 1992). Recent studies on the influence of climatic factors on trap-entry responses of *An. gambiae s.s.* indicated that fluctuations in the relative humidity of the air entering the flight chamber might partially be responsible for this (H. Otten, unpublished data). Even though these variations are important the percentages of mosquitoes caught are high, enabling us to rapidly make statistically reliable comparisons between treatments.

The data show that *An. gambiae s.s.* responds well to 4.5% carbon dioxide. This carbon dioxide was pumped at 230 ml min^{-1} into the traps, and diluted in conditioned air before entering the flight chamber. If the dilution would be complete than the concentration would have dropped to 0.01%, a concentration lower than background levels (Richards, 1952). The clear responses of mosquitoes suggest that this dilution was incomplete and that small 'pockets' of concentrations higher than background were perceived and caused the attraction. This incomplete mixing of odour and the way insects perceive and subsequently orient towards its source (see Murlis *et al.*, 1992) was found to induce sustained flight for other mosquito species in windtunnels (Omer & Gillies, 1971; Omer, 1979). Recently Healy & Copland (1995) described an activating and orienting response of the same *An. gambiae* strain to carbon dioxide levels 0.01% above background.

In field studies Snow (1970) found a marked reduction in the number of *An. gambiae s.l.* attracted to a human host when carbon dioxide was filtered from the breath, indicating that this compound plays an important role in host location for this species. Unfortunately it is not known what percentage of these mosquitoes were *An. gambiae s.s.*. In contrast to some other species within the

Anopheles gambiae complex, *An. gambiae s.s.* is highly anthropophilic (White, 1974). Therefore it is expected that odours other than carbon dioxide are also involved in guiding this species to its host. The system described in this paper can be an important instrument in the identification of the kairomones involved in host location of this important malaria vector.

Acknowledgments

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5

Olfactory responses of female *Anopheles gambiae s.s.* Giles (Diptera: Culicidae) to human breath or Limburger cheese in a windtunnel bioassay[1]

ABSTRACT - The response of female *Anopheles gambiae s.s.* towards human breath was studied in a dual-port windtunnel bioassay. The number of mosquitoes attracted to whole breath was not significantly different from that attracted to a concentration of carbon dioxide similar to that in breath only, indicating the limited role of breath compounds other than carbon dioxide in host-seeking by this species. Neither did whole breath attract more mosquitoes than a control treatment (clean air), and showed that carbon dioxide concentrations ≤3.8% do not have an attractive effect in this set-up. Since foot odour was shown to play a role in the selection of biting sites by this species we decided to study responses towards Limburger cheese headspace, to the human nose reminiscent of foot odour. Both East and West African *An. gambiae s.s.* showed highly significant ($P < < 0.001$) attraction to small quantities of this cheese. GC and GC-MS analyses of the acid fractions of Limburger cheese, human sweat and foot scrapings showed strong similarities in aliphatic fatty acid content and composition. Attraction of this highly anthropophilic mosquito to Limburger cheese, a bait of non-human origin, which obtains its odour through microbial processes, strongly suggests that *An. gambiae s.s.* responds to compounds of bacterial origin while searching for man, its preferred host. Limburger cheese odour is the first host-seeking attractant, other than carbon dioxide, reported for

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this important malaria vector and its discovery will aid the development of odour-baited traps for mosquito surveillance and control in Africa.

Introduction

During its lifetime, a female *An. gambiae* mosquito exhibits three important behaviours that require source-location from a distance. Mating behaviour takes place 1-2 days after emerging from the pupa, and requires the female to effectively locate a swarm of males (Charlwood & Jones, 1980; Marchand, 1984). Blood- and sugar feeding sometimes occur prior to mating but dominate the remainder of its life (Clements, 1992). Sugar feeding satisfies energy requirements of both sexes and nectar has been reported to be the major source of carbohydrate (reviewed by Yuval (1992) and Clements (1992)). Distance location of floral nectar sources is odour-mediated (Jepson & Healy, 1988) and implies that females can distinguish between plant odours and kairomones emitted by potential blood hosts. The latter group of semiochemicals plays a role during the host-seeking process, which, depending on the length of the gonotrophic cycle, takes place every 3-4 days.

Though evidence from field studies is limited, sugar feeding is primarily an opportunistic activity (Yuval, 1992) and differs therefore markedly from the selection expressed in the choice of blood hosts, which, more often than not, is preferential. Within the *An. gambiae* complex, which consists of six sibling species, *An. gambiae s.s.* is strongly anthropophilic (Garrett-Jones *et al.*, 1980), *An. quadriannulatus* entirely zoophilic and *An. arabiensis* expresses intermediate levels in different geographical regions (White, 1974). Anthropophilic strains of this latter species have been found in Burkina Faso and Mali, whereas populations from Madagascar seldom feed on man (M. Coluzzi, *pers. comm.*). Even though the availability of different host species will, to a certain extent, determine the range of hosts fed upon, these preferences seem to be genetically determined, and in fact have limited the distribution of *An. gambiae s.s.* to that of man in Africa (Coluzzi *et al.*, 1979; Gillies, 1988; Hunt & Coetzee, 1995).

A preference for feeding on particular host species will be reflected in the host-seeking strategy of mosquito species. Truly opportunistic species such as the diurnal *Eratmopodites chrysogaster* (Haddow, 1956) have adopted a 'sit-and-wait' strategy and any host species entering its field of vision may provide a blood meal. Nocturnal opportunistic species can use common and abundantly available host odours such as carbon dioxide to locate potential hosts. However, if the association between mosquito species and host becomes stronger, than the role of species-specific kairomones during host-seeking will increase.

Most haematophagous arthropods use carbon dioxide while host-seeking (Gillies, 1980; Sutcliffe, 1987; Bowen, 1991; Takken, 1991). It is a major constituent of exhaled air and human breath has been reported attractive for *Anopheles* spp. (Mer *et al.*, 1947; Laarman, 1955; Knols *et al.*, 1994b; De Jong & Knols, 1995) and for *Aedes aegypti* (Khan & Maibach, 1972). A carbon dioxide concentration similar to that in human breath (4.5% or *ca.*300 ml min^{-1}) is attractive for *An. gambiae s.s.* in our windtunnel bioassay (Knols *et al.*, 1994a, see chapter 4). In The Gambia, Snow (1970) found a significant reduction in the number of *An. gambiae s.l.* approaching a human from which the carbon dioxide had been filtered, which indicates that also in the field carbon dioxide influences host-seeking behaviour. However, the soda-lime used to absorb the carbon dioxide is known to filter other compounds as well (A. Cork, *pers. comm.*) and Snow's conclusions therefore need to be interpreted with care. We decided to investigate whether chemicals present in exhaled human breath, other than carbon dioxide, might play a role in the attraction of *An.gambiae s.s.*.

Biting of *An. gambiae s.s.* on a naked motionless human host preferentially occurs on feet and ankles. Washing of this region with a bactericidal soap diverts *An. gambiae s.s.* from biting this region to other parts of the body, suggesting that foot odours play a role in the host-location of this species (De Jong & Knols, 1995, see also chapter 2). The typical and distinct smell of human feet is caused by unique combinations of the resident skin microflora and their environment. It has frequently been described as 'cheesy' (Noble, 1982; Tachibana, 1976; Jackman, 1982; Marshall, 1988), and also the Dutch word 'tenenkaas' (literally 'toes-cheese') refers to its strong resemblance to cheese odour (Sterkenburg & Pijnenburg, 1984). This similarity may not be purely coincidental since bacteria also play an essential role in cheese production and contribute to their flavours (Cogan & Daly, 1987). It has even been suggested that certain bacteria involved in cheese production originate from human skin (Sharpe *et al.*, 1976) and therefore that 'cheese smells of feet rather than the reverse'(Jackman, 1982). This anthropomorphic cheese-foot odour analogy made us decide to test the response of *An. gambiae s.s.* to

Limburger cheese, famous for its strong 'foot' odour, in our windtunnel bioassay.

Materials and Methods

Experimental mosquitoes. The *An. gambiae s.s.* strains originated from Moshi, Tanzania (courtesy Prof. C.F. Curtis) and Suakoko, Liberia (courtesy Prof. M. Coluzzi). Adults were kept in 30 cm cubic gauze cages, fed on 6% glucose solution and offered human blood by inserting an arm into a cage for 10 minutes twice weekly. Oviposition took place on wet filter paper after which the larvae were allowed to emerge in water trays and were fed on Tetramin^(r) fishfood. Pupae were collected from the trays daily and placed inside adult cages. The females used in the windtunnel bioassays were 5-8 days old and had not been given the opportunity to feed on blood. Four groups of fifty females were kept in cylindrical plexiglass containers (height 10 cm, diameter 6 cm) the night before testing and were given access to water-soaked cotton wool placed on top of the containers.

Windtunnel bioassay. The windtunnel bioassay consists of a rectangular flight chamber (160 x 60 x 60 cm) connected to a climate control system. Conditioned air (27 ± 1^0C, RH 60 ± 5%, 20 cm sec^{-1}) enters the chamber through two ports (diameter 5 cm, horizontally aligned and 30 cm apart) which are linked to trapping devices. These consist of glass containers with a non-return device to prevent escapes from the traps. Experiments were conducted under low-light conditions (*ca.* 6 Lux) during simulated host-seeking activity peaks (between 02.00 and 06.00 am, see Lyimo, 1993). For a detailed description of the apparatus see Knols *et al.* (1994a), and chapter 4.

Test-odours and experimental procedures. Breath samples from three resting male Caucasian volunteers (aged 27, 38 and 50 years) were collected in Tedlar^(r) 100 l gas bags and analyzed for their carbon dioxide concentrations by using an infrared CO_2 gas analyser. A similar bag was then filled with cleaned and moistened air and adjusted to a similar carbon dioxide concentration as measured in the breath sample, by adding carbon dioxide from a pressurized gas cylinder. 10 ml of distilled water were then added to both bags which were then stored for 24 hrs at 25^0C in order to obtain equal, near-saturated, moisture levels. Both odours were then tested against each other by pumping them (with two S-200 Du Pont constant flow sampler pumps) at 230 ml min^{-1} through teflon tubing into the traps fitted in the windtunnel. The response towards breath was studied for East African *An. gambiae s.s.* only. Similarly, clean moistened air was pumped at 460 ml min^{-1} through an empty vial (control) or vials containing Limburger cheese (30 or 500 mg, for Tanzanian and Liberian mosquitoes respectively) after which the (odorous) air was pumped into the mosquito traps and subsequently entered the flight chamber. Since this cheese contains 44% water (El-erian, 1969) and moisture is reported to influence mosquito behaviour (Clements, 1963; Hocking, 1971; Friend & Smith, 1977; Wright & Kellogg, 1962), we incorporated tests to assess its importance by testing the headspace of the equivalent of water (220 μl) present in 500 mg of cheese. Mosquitoes were released 120 cm downwind from the traps in groups of 50 and given the opportunity to respond to test odours for 20 minutes after which new mosquitoes were introduced. Mosquitoes not leaving the containers were excluded from the analyses. For every test series the odour sources were alternated, in total four times each test day. Catches were compared using Chi-squared tests.

Gas chromatography-Mass spectrometry (GC-MS). Initial GC-MS analyses of Limburger cheese headspace showed that the majority of the volatiles detected were aliphatic fatty acids (A. Cork, *pers. comm.*). It was therefore decided to analyse and compare the acid content of Limburger cheese, human sweat, and foot scrapings. Extracts of acids from Limburger cheese were prepared by diethyl ether/heptane extraction of cheese treated with sodium sulphate (for a detailed description of procedures see chapter 6). Whole human body sweat was collected from a male Burkinabé volunteer and the extracts were prepared from acidified (1N hydrochloric acid) aliquots of sweat using small aliquots of dichloromethane (0.5 ml). The foot scrapings were prepared from detritus collected from under the toe nails of a male Caucasian volunteer and dissolved in dichloromethane. The amounts of detritus collected and solvents used were not quantified. Data for foot odour and the sweat samples represent relative measures of acids only. GC-MS settings were similar to those described in chapter 6.

Results

The results (Table 1A) show that breath-related chemicals do not enhance the attractiveness of carbon dioxide alone for East African *An. gambiae s.s.*. When breath was tested against clean air (Table 1B) we found a slightly significant attraction to one of the subject's breath (PH) only. It seems therefore that the doses of carbon dioxide present in human breath (in all six samples lower than the previously attractive 4.5%) were not attractive to *An. gambiae s.s.*, nor was carbon dioxide at 3.56% when tested against clean air (Table 1C).

***Table 1.** Data from windtunnel bioassay experiments with human breath. Shown are numbers of East African* Anopheles gambiae s.s. *caught in traps baited with human breath against a similar concentration of CO_2 (A), human breath against clean air (B) or CO_2 against clean air (C).*

		Treatments				
A	Person	Breath	CO_2	$[CO_2]$%	N	X^2
	RJ	22	37	3.30	194	3.81ns
	BK	43	47	3.65	196	0.18ns
	PH	36	37	3.49	191	0ns
B	Person	Breath	Clean air	$[CO_2]$%	N	X^2
	RJ	34	32	3.80	191	0.06ns
	BK	36	36	3.28	191	0ns
	PH	50	30	3.54	186	5.00*
C		CO_2	Clean air	$[CO_2]$%	N	X^2
		57	42	3.56	187	2.26ns

ns: not significant ($P > 0.05$); *: $P < 0.05$. N: total number of mosquitoes tested (not all of which reacted to either stimulus).

Traps baited with Limburger cheese caught significantly ($P < 0.001$) higher numbers of both West and East African *An. gambiae s.s.* than control traps (Table 2A). No evidence was found for any influence of moisture on the catches (Table 2B,C), thus confirming the attractiveness of cheese odour.

Table 2. *Data from windtunnel bioassay experiments with Limburger cheese. Shown are numbers of West or East African* Anopheles gambiae s.s. *caught in traps baited with either headspace of (i.e. air blown over) Limburger cheese (30 or 500 mg, see text) or odourless air (A); headspace of 220 µl of water or odourless air (B); headspace of Limburger cheese or headspace of 220 µl of water (C).*

		Treatments			
A	Origin	Cheese	Control	N	X^2
	Liberia	181	75	392	43.9***
	Tanzania	100	31	233	36.3***
B		Water	Control		
	Liberia	92	104	369	0.7ns
C		Cheese	Water		
	Liberia	142	85	383	14.3***

ns: not significant ($P > 0.05$); ***: $P < 0.001$. N: total number of mosquitoes tested (not all of which reacted to either stimulus).

GC-MS analyses of extracts from Limburger cheese, human sweat and foot scrapings are shown in Table 3. Even though the relative composition of the three samples was quite different, it is interesting to note the similarity in aliphatic carboxylic acids present in them. The C_2-C_5 and C_{10}-C_{18} acids dominated in all extracts. A major difference between the cheese and human samples was the absence of aromatic carboxylic acids in the cheese. Ethanoic, propanoic, butanoic, tetradecanoic, hexadecanoic and 9-octadecenoic acids predominated in the cheese extract. Noteworthy is the very high relative contribution of hexadecanoic, octadecanoic and 9-octadecenoic acids in the foot scrapings.

***Table 3.** Carboxylic acids identified by GC-MS in Limburger cheese (relative to 3-methylbutanoic acid and absolute amounts (mg/20 g)) and dichloromethane extracts of acidified human sweat samples and human foot scrapings (toe nail detritus)(relative amounts only).*

	Limburger cheese			
Carboxylic acid	Relative amount[1]	mg/ 20g	Human sweat[1]	Foot scrapings[1]
Ethanoic	883	57.2	413	97
Propanoic	518	33.6	15	443
2-Methylpropanoic	24	1.6	0.2	35
Butanoic	622	40.3	17	446
3-Methylbutanoic	100	6.4	100	100
Pentanoic	1	0.06	2	9
4-methylpentanoic	0	0	1	8
Hexanoic	33	2.1	22	13
Heptanoic	0	0	IS	12
Octanoic	42	2.7	6	25
Nonanoic	0	0	11	31
Decanoic	101	6.5	10	49
Dodecanoic	102	6.6	14	333
Tetradecanoic	247	16.0	166	387
Hexadecanoic	397	25.7	707	1555
9-Hexadecenoic	52	3.3	191	316
Octadecanoic	81	5.2	145	966
9-Octadecenoic	430	27.9	155	3025
9,12-Octadecadienoic	25	1.6	0	315
9,12,15-Octadecatrienoic	28	1.8	0	32
Benzoic	0	0	11	69
Phenylacetic	0	0	63	230
Phenylpropanoic	0	0	13	60

[1] Percentage relative to 3-methylbutanoic acid (100%).

IS = Internal Standard.

Discussion

Our results indicate that, in human breath, volatiles other than carbon dioxide do not play an important role in the host-seeking behaviour of *An. gambiae s.s.*. However, several factors that influenced the experiment need consideration. Collection of breath in Tedlar$^{(r)}$ gas bags was followed by a 24 hr period of storage at 25^0 C. We do not know whether the chemical composition of the samples changed during that period and therefore caused the loss of attraction of mosquitoes to breath. In order to obtain equal relative humidities in the samples, 10 ml of distilled water were added to the bags, and this could also have influenced the composition of the samples. Furthermore, the pumping of odour from the bags into the traps, was followed by dilution with clean air entering the traps. The volume of air passing the ports of the traps was *ca.* 1.6 l sec^{-1} (diameter 5 cm, windspeed 20 cm sec^{-1}). A volume of 230 ml min^{-1} was pumped from the gas bags into this volume before entering the flight chamber. If diffusion of carbon dioxide in this volume would be complete than the resulting carbon dioxide concentration would drop from 3.8% (the highest concentration measured) to 0.0091%, which is 3-4 times lower than the background carbon dioxide concentration (Richards, 1952). At 4.5%, which previously showed a marked attraction of *An. gambiae s.s.* in the same set-up (Knols *et al.* 1994a), this concentration would have dropped to 0.01%, still lower then background levels. This implies that diffusion of carbon dioxide was not complete but rather that 'filaments' or 'pockets' of odour (e.g. Murlis & Jones, 1981), which had a concentration above background level, caused the attraction. However, the diffusion in clean air of carbon dioxide in the gaseous phase does not necessarily reflect the dilution process for all compounds, and this could have caused their concentration to drop below critical levels for initiating behavioural responses. A last point of concern is that even though attraction to breath components was absent, we cannot exclude an interaction between breath odours and other, skin-related, compounds. This has been found to be the case for other Diptera, such as tsetse flies (Willemse & Takken, 1994), but also for several mosquitoes species (Takken & Kline, 1989).

Other studies, however, support our findings. Recently Healy & Copland (1995), while working with the same strain of East African *An. gambiae s.s.* mosquitoes as in this study, arrived at the same conclusion, *i.e.* that chemicals other than carbon dioxide do not enhance the attractiveness of breath. They observed activation and orienting responses in a windtunnel towards carbon dioxide levels *ca.* 0.01% above background. Field studies in Tanzania failed to attract *An. gambiae s.l.* to electric nets baited with human breath, but they were readily caught on nets baited with odour from an occupied bedroom (Knols & Mboera, 1996). Similar results were found when electric nets were baited with carbon dioxide at a human equivalent (300 ml min^{-1}) and when tents were baited with this amount of carbon dioxide they attracted only 9% of *An. gambiae s.l.* compared to the catch of a human odour-baited tent (Mboera *et al.*, 1996). The fact that Gillies & Wilkes (1972) were unable to attract large numbers of *An. gambiae s.l.* (except *An. melas*) to carbon dioxide at an equivalent of two bait calves or the calves themselves further supports the limited role of this compound in the field.

The clear and highly significant responses to Limburger cheese odour in the same experimental conditions are in strong contrast with attraction towards human breath. Volatiles that evaporated from the small quantities of cheese were diluted in double the amount of clean air than that in breath experiments, but nevertheless resulted in marked attraction.

The GC-MS analyses revealed that aliphatic carboxylic acids are present in cheese (see Hanspach, 1981), human sweat (see Nicolaides, 1968, 1974) and foot scrapings (see Kanda *et al.*, 1990), and that aromatic carboxylic acids are absent in the cheese. The low volatility of these aromatic acids and the longer chain aliphatic acids (C_{16} and above) most likely reduces the number of acids that evaporate from cheese/skin and are subsequently used by *An. gambiae s.s.* while host-seeking. Analyses of headspace samples of cheese should confirm this, and are presented in chapter 6.

The ripening of Limburger cheese is effected by coryneform bacteria, in particular strains of *Brevibacterium linens* (Sharpe *et al.*, 1976, El-Erian, 1969) Closely related species, e.g. *Br. epidermidis*, form part of the normal microflora on human feet (Anthony *et al.*, 1992; Pitcher, 1977). Some of these coryneform bacteria are known to produce methanethiol (Anthony *et al.*, 1992; Manning & Moore, 1979), a pungent sulphur compound which is metabolized from L-methionine liberated during proteolytic activity and reported to contribute substantially to both cheese and foot odour (Cogan & Daly, 1987, Anthony *et al.*, 1992, Nicolaides, 1974). It is interesting to note that the presence of high levels of free fatty acids in skin surface lipid is highly characteristic of the human species (Nicolaides, 1968, 1974) and therefore could represent a group of reliable kairomones for this anthropophilic mosquito species.

Although there are obvious similarities in composition between Limburger cheese and foot odour, one should, of course, be extremely cautious in relying on the human nose to identify the compound(s) responsible for the attraction of *An. gambiae s.s.*. Other cheeses produce fatty acids similar to those from Limburger cheese but not necessarily produce 'foot' odour (Hanspach, 1981), for instance domiati and feta type cheeses (Abd El-Salam, 1987). Nevertheless, the anthropomorphic cheese-foot odour analogy formed the basis for this work. It is remarkable that such a distinctly anthropophilic mosquito species is attracted to an odour of non-human origin and this strongly suggests that bacteria are responsible for the production of the 'human specific' odours involved in host-location of this species. In addition, it is now tempting to explain variations in attractiveness of individual human hosts for *Anopheles* mosquitoes on the basis of the metabolic activity of their resident skin flora. Furthermore, since both West and East African *An. gambiae s.s.* responded so well to Limburger cheese, we expect that throughout the Afro-tropical region this species can be attracted by this bait .

Epidemiological studies of many mosquito-borne diseases are seriously hampered by the scarcity of objective sampling methods for mosquito populations. Light traps set beside occupied bed nets provide a reliable method for monitoring populations of *An. gambiae s.s.* (Lines *et al.*, 1991), but are not as attractive as a human host. Ideally, traps baited with a 'standard' host odour, would lure mosquitoes as strongly as a human host and thus provide reliable information on vector population density. One might even foresee the development of odour-baited mosquito traps which might be used *en masse* to reduce the vector population in a village, or in an individual bedroom to divert mosquitoes away from the occupants. Although carbon dioxide is a known attractant it is not convenient for use in African villages either from a gas cylinder or in the form of dry ice. Limburger cheese is the first kairomone ever reported for host-seeking *An. gambiae s.s.* that acts independently of carbon dioxide, and its discovery will assist the development of odour-baited traps for mosquito surveillance and control in sub-Saharan Africa.

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6

Behavioural and electrophysiological responses of female malaria mosquito *Anopheles gambiae s.s.* (Diptera: Culicidae) to Limburger cheese volatiles[1]

ABSTRACT - Limburger cheese, previously shown to attract female *Anopheles gambiae s.s.*, was solvent extracted and chemically fractionated into acid and non-acid fractions. The extracts and aliquots of the headspace odour of the cheese were analysed by gas chromatography and electron impact mass spectrometry. Nineteen saturated and unsaturated aliphatic fatty acids, ranging in carbon chain length from C_2-C_{18}, were detected. The most abundant acids (> 1mg/g) identified in the acid extract were ethanoic, propanoic, butanoic, hexadecanoic and 9-octadecenoic acid. The same compounds were identified in analyses of headspace samples but only trace quantities of the less volatile acids (C_{10}-C_{16}), and no C_{18} acids. Behavioural responses of female *An. gambiae s.s.* towards a range of concentrations of the acid extract (in diethyl ether) were observed in a windtunnel bioassay. The undiluted extract was found to be repellent, but became highly attractive ($P < < 0.001$) at lower doses, and was still significantly attractive ($P < 0.001$) when diluted 10^6x. A synthetic mixture of 12 of the more abundant aliphatic acids identified in the acid extract was found to be significantly attractive ($P < 0.001$) when diluted 10^8x. Electroantennographic (EAG) studies showed significant and reproducible responses to (saturated) Limburger cheese headspace. At doses higher than 0.1%, the synthetic mixture of 12 acids gave significantly higher EAG amplitudes than the solvent control

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(paraffin oil). EAG responses were recorded for C_5 to C_8 acids that were characterised by significant dose-dependencies. Weaker, though significant EAG responses were observed to the less volatile acids (C_9-C_{14}), and only hexadecanoic acid did not elicit a detectable response. The electrophysiological and behavioural response elicited by the fatty acids isolated from Limburger cheese suggests that together they could act as a kairomone for female *An. gambiae s.s.*. The implications of this are discussed together with the occurrence and production of these compounds on human skin.

Introduction

Africa's most important malaria vector, *An. gambiae s.l.* Giles, is thought to consist of a complex of six sibling species with different behavioural traits (White, 1974). They differ in their host preferences from purely zoophilic (*An. quadriannulatus*) through opportunistic (*An. arabiensis*) to highly anthropophilic (*An. gambiae s.s.*), with intermediate levels in different geographical regions (Gillies & Coetzee, 1987). Few studies have been performed on odour-mediated host-seeking behaviour of *An. gambiae s.l.* under field conditions (mainly on *An. melas*) but these indicated that host odours play a major role in this process (Gillies & Wilkes, 1969; 1970; 1974), and influence host-selection (Coluzzi *et al.*, 1979).

Carbon dioxide has long been recognised as a kairomone for many mosquito species (Gillies, 1980; Sutcliffe, 1987; Takken, 1991). In The Gambia, it attracted host-seeking *An. melas* in the field (Gillies & Wilkes, 1969) and studies on *An. gambiae s.s.* and *An. arabiensis* in Burkina Faso gave similar results (C. Costantini, *pers. comm.*). In Tanzania, however, recent field studies have shown a limited response of *An. arabiensis* and *An. gambiae s.s.* to carbon dioxide baited tents and electric nets (Knols & Mboera, 1996; Mboera *et al.*, 1996). We hypothesize that carbon dioxide may well represent a useful kairomone for opportunistic feeders, but that it cannot be a reliable cue for anthropophilic species. In our studies we have therefore focused on identifying attractants other than carbon dioxide for *An. gambiae s.s.*, in particular human-specific kairomones.

Female *An. gambiae s.s.* were found to bite the ankles and feet of a naked, motionless, human volunteer in a series of experiments in which the selection of biting sites by mosquitoes was observed (De Jong & Knols, 1995a). It was concluded that this behaviour was odour-mediated. A windtunnel bioassay for evaluating odours at the behavioural level was subsequently developed (Knols *et al.*, 1994) and used to assess the importance of foot odour in attracting mosquitoes. As part of a screening process Limburger cheese was selected and tested in the bioassay, since it has an odour reminiscent of human foot odour, and found to be highly attractive to *An. gambiae s.s.* in this bioassay (De Jong and Knols, 1995b; Knols and De Jong 1996). The primary odour of this cheese, as perceived by humans, consists of two methyl-branched aliphatic carboxylic acids, 2-methylpropanoic and 3-methylbutanoic acid (Hanspach, 1981). These and other fatty acids are produced through microbial activity during its ripening process (Cogan & Daly, 1987). Similarly, microflora on human skin have been found to produce fatty acids that contribute significantly to the odour associated with feet (Nicolaides, 1974; Kanda *et al.*, 1990).

Electrophysiological techniques have been utilised to identify host-odour attractants for various haematophagous (Hall *et al.*, 1984; Bursell *et al.* 1988) or myiasis causing (Cork, 1994) Diptera. However, very little is known about the sensory physiology of anopheline mosquitoes. For *Aedes aegypti*, the only mosquito species of which olfactory receptors have been characterized to some extent, sensitive neurons for the host-attractant lactic acid were discovered in the grooved peg sensilla (Davis & Sokolove, 1976). Single cell studies on *Ae. aegypti* reported three different types of sensory hairs, including grooved peg sensilla, which responded to fatty acids (Lacher, 1967). Since Limburger cheese is known to have an odour similar to that of human feet, and that the odour consists of aliphatic acids, this study was undertaken to identify the volatile fatty acids present in Limburger cheese and assess their electrophysiological and behavioural significance to the highly anthropophilic *An. gambiae s.s.*, as a first step in the development of an effective odour bait.

Materials and Methods

Chemical procedures

Extraction of acids from Limburger cheese. Limburger cheese (40 g) was ground with anhydrous sodium sulphate (120 g) and sulphuric acid (12 ml, 2.5 M). The resulting mixture was then extracted with diethyl ether/heptane (1:1.3 x 150 ml). Cheese extracts were combined, diluted to 400 ml with diethyl ether/heptane and dried over anhydrous sodium sulphate (20 g)(De Jong & Badings, 1990).

Isolation of free fatty acids. The cheese extract was fractionated into acid and non-acid extracts by passing an aliquot of the cheese extract through a deactivated alumina column (40 g neutral aluminium oxide, deactivated with 4 g water) to remove the fatty acids. The resulting eluant was collected as the non-acid fraction. The alumina column was washed with diethyl ether/heptane (2 x 100 ml), dried (vacuum desiccator, 1 hr) and extracted with methanoic acid in diethyl ether (3% w/w, 4 x 50 ml) to give the acid fraction (De Jong & Badings, 1990).

Headspace samples of Limburger cheese. Limburger cheese (5 g) was placed in a Pyrex conical flask (50 ml) and sealed with a rubber septum (B24). The headspace was allowed to equilibrate for 2 hr at 20^0 C before sampling. Aliquots (1 ml) of the headspace were collected in a gas-tight glass syringe (2 ml, SGE, UK) for GC and GC-MS analysis.

Gas chromatography (GC). GC analyses of Limburger cheese and headspace (1 ml) were performed on a Carlo Erba Mega series 5300 instrument fitted with two Grob split/splitless injectors (250^0 C) and flame ionisation detector (250^0 C). Fused silica columns (25 m x 0.32 mm ID) were used throughout the study, coated with either CP Wax 52CB (chemically-bonded Carbowax 20 M equivalent; Chrompack, The Netherlands). The carrier gas was helium with an inlet pressure of 0.5 kg/cm^2. All injections were made with the split valve closed for 40 sec onto the analytical column held at either 50^0 C or 70^0 for 2 min then temperature programmed at 6^0 C per min to 260^0 C.

Mass spectrometry (MS). Electron impact mass spectra of compounds present in the Limburger cheese extracts and headspace were obtained on a Finnigan MAT Ion Trap Detector (ITD), model 700, under continuous ion monitoring between m/z 40-250 at 220^0 C. Compounds were introduced into the ITD from a Carlo Erba Mega series GC fitted with CP Wax 52CB capillary column run under the same analytical conditions as used for the GC analyses.

Synthetic Limburger cheese acid mixtures. A synthetic acid solution (C_2-

C_{16}) was prepared in diethyl ether based on the relative amounts of each acid observed in the acid extracts of Limburger cheese samples (Table 1). The synthetic chemicals used were purchased from commercial sources and were at least 99% chemically and isomerically pure.

Experimental mosquitoes

The *An. gambiae s.s.* strain used originated from Suakoko, Liberia (courtesy Prof. M. Coluzzi) and was maintained under standard laboratory conditions (27±1°C, 80±5% RH, 12 hr scotophase). Adults were kept in 30 cm square gauze covered cages and fed on 6% glucose. Females were offered blood from a human arm twice weekly for 10 min. Eggs were laid on wet filter paper, emerged in water trays and larvae were fed on Tetramin$^{(r)}$ fishfood. Pupae were collected daily from the trays and allowed to emerge in the adult cages. Experimental females were 4-8 days old and had not received a bloodmeal. For bioassay tests, 200 females were selected randomly from the cages with an aspirator. They were then kept in containers (50 mosquitoes each) until the following morning and offered water-moistened cotton wool only. Females, 4-6 days old, which exhibited an alighting response to a hand of the experimenter were selected from the cages and used for EAG testing.

Windtunnel bioassay

The windtunnel bioassay and general experimental procedures were described by Knols *et al.* (1994). The setup consists of a flight chamber (160 x 60 x 60 cm) in which mosquitoes are released from containers in batches of fifty after which they are given the opportunity to respond to either an experimental or control odour for twenty minutes. These odours are mixed with purified conditioned air (50-70 % RH, 27±2°C) and enter the chamber (20 cm/sec) through two ports (diameter 5 cm, horizontally aligned and 30 cm apart in the upwind side of the tunnel) behind which traps have been mounted. Clean traps, with positions of the odour sources alternated were installed, and fifty additional mosquitoes were added to the chamber after twenty minutes, a procedure repeated four times each test-day. Total catches over the four 20 min test periods were subjected to chi-square analysis to test for attractiveness of the experimental odour.

Experimental odours (acid extract or the synthetic equivalent as listed in

Table 1) were diluted in diethyl ether to give a concentration range of 10^{-1} to 10^{-9}. 100 μl aliquots of a given concentration was then pipetted onto 5x2 cm Whatman (no. 3) filter paper after which the diethyl ether was allowed to evaporate. Using forceps the filter papers were then placed on glass slides and mounted in the back of the traps. The control odour source consisted of a filter paper on which diethyl ether had been pipetted only. Experimental and control odours were renewed after each of the four tests.

Electrophysiological recording

Experimental setup. Excised head preparations were used, which were taken from cooled (ca. 90 sec in a -18° C freezer) females. The excised head was mounted between two glass electrodes filled with 0.1 M KCl. AgCl-coated Ag-wires were connected to a home-made DC amplifier (amplification 100x), with the recording electrode slid over the tip of the antenna, from which the terminal segment had first been removed; the indifferent electrode was inserted through the foramen magnum of the head. The potential differences between the electrodes were visualized on a paper chart recorder set to a full scale sensitivity of 2 mV. EAG amplitudes were measured manually to an accuracy of 10 μV. A standard off-line method of stimulus delivery was used (Van Loon *et al.*, 1992). An airstream (33 ml sec^{-1}), saturated with water vapour, was led continuously over the preparation.

Limburger cheese headspace. In a preliminary set of experiments, head space odour of Limburger cheese was offered to the preparation. A known amount of cheese (50-100 mg) was placed in a 30 ml glass bottle, which was then capped with an aluminium screwcap incorporating a rubber septum. After 30 min, 1 ml aliquots of head space odour were withdrawn from the bottle with a syringe. This volume was injected manually, in a *ca.* 1 sec pulse, into the continuous airstream over the preparation. As a control, an injection of 1 ml of air drawn from a similar but empty bottle was tested.

Chemicals tested. Aliphatic carboxylic acids were volatilised from an aliquot of paraffin oil (25 μl) that was put on filter paper (30x3 mm) and inserted into a glass Pasteur pipette. Odours were injected into the continuous airstream by inserting the tip of the Pasteur pipette through a small hole in the side of the glass tube (distance of the hole to preparation *ca.* 10 cm). Doses are expressed as % in paraffin oil (v/v; 1% means 225 μg of compound). Fatty acids tested were: 3-methylbutanoic, pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acid. Each compound was tested at five doses in a random order, and no more than three chemicals were tested on individual preparations. This process was replicated for 5-8 individuals per test series. A mixture of these acids in the ratio in which they occur in the headspace of the acid extract (Table 1) was also tested. The maximum EAG depolarisation was calculated for each response and expressed relative to the response elicited by the solvent (paraffin oil). The solvent response was measured at regular intervals during the testing sequence. In some experiments, 2-ethyl hexanoic acid (previously shown to be electrophysiologically-active) was used as a standard. Differences between response amplitudes of test odours and controls as well as for different doses were analyzed using Mann-Whitney U tests.

Results

Chemical analyses

GC and GC-MS analyses of extracts of Limburger cheese showed that the major volatile compounds present in the cheese were saturated and unsaturated aliphatic carboxylic acids ranging in carbon chain length from C_2 to C_{18} (Table 1). In addition two methyl-branched aliphatic carboxylic acids, 2-methylpropanoic and 3-methylbutanoic acid were found. No aromatic carboxylic acids were observed.

GC-MS analyses of the headspace from Limburger cheese confirmed the presence of the aliphatic carboxylic acids, although only trace amounts of the less volatile acids, C_{10} to C_{16} carbon chain length, and no C_{18} acids were observed. The ratio of the more volatile carboxylic acids present in the headspace, C_4 to C_6 carbon chain length, was relatively consistent with that found in the acid extract from the cheese. In addition to the carboxylic acids trace quantities of dimethyldisulphide and dimethyltrisulphide were observed in a 80:1 ratio.

Windtunnel bioassay

The majority (always >90%) of the mosquitoes left the release cage within the experimental period. Since the temperature and humidity of the air

Table 1. Relative (%) and absolute amounts (mg/g) of carboxylic acids identified by GC-MS analyses of the acid extract, headspace and synthetic acid mixture of Limburger cheese.

	Relative amount (%)[a]		Content (mg/g)	
Carboxylic acid	Acid extract	Head-space	Acid extract	Synthetic mixture
Ethanoic	883	68	2.86	2.79
Propanoic	518	170	1.68	1.29
2-Methylpropanoic	24	22	0.08	0.07
Butanoic	622	609	2.00	2.01
3-Methylbutanoic[a]	100	100	0.32	0.27
Pentanoic	1	2	0.003	0.003
Hexanoic	33	23	0.11	0.12
Heptanoic	0	1	0	0
Octanoic	42	8	0.13	0.14
Nonanoic	0	1.5	0	0
Decanoic	101	3.8	0.32	0.39
Dodecanoic	102	4.5	0.33	0.40
Tetradecanoic	247	5.5	0.80	0.80
Hexadecanoic	397	8.3	1.29	1.29
9-Hexadecenoic	52	n/d	0.16	0
Octadecanoic	81	n/d	0.26	0
9-Octadecenoic	430	n/d	1.40	0
9,12-Octadecadienoic	25	n/d	0.08	0
9,12,15-Octadecatrienoic	28	n/d	0.09	0

[a] Percentage relative to 3-methylbutanoic acid (set at 100%); n/d: not detected in headspace analyses.

Table 2. *Results of windtunnel bioassay experiments with the acid extract of Limburger cheese (A) and a synthetic equivalent thereof (B).*

(A)	Acid Extract					Control							
Concentration	1[a]	2	3	4	n[b]	1	2	3	4	n	N[c]	X^2	A/R[d]
undiluted[e]	2	7	4	10	23	1	24	15	41	81	181	32.35***	--
	4	5	3	13	25	2	30	19	63	114	186	56.99***	---
10^{-3}	9	27	29	30	95	9	26	25	21	81	190	1.11ns	0
$5*10^{-4}$	17	34	10	38	99	8	17	19	20	64	195	7.52**	+
10^{-4}	18	21	30	31	100	6	5	9	23	43	185	22.72***	++
	7	17	27	42	93	7	8	18	28	61	198	6.65**	+
10^{-5}	9	16	58	33	116	7	10	14	36	67	191	13.12***	+
10^{-6}	19	30	22	42	113	6	3	3	15	27	198	52.83***	++++
10^{-7}	31	4	28	29	92	6	10	7	20	43	180	17.79***	++
10^{-9}	14	4	14	6	38	2	18	5	10	35	188	0.12ns	0
(B)	Synthetic acid mixture					Control							
10^{-6}	23	10	9	15	57	7	25	20	53	105	197	14.22***	-
10^{-8}	14	27	24	41	106	29	7	25	12	73	190	6.08*	+
	25	34	27	28	114	10	3	13	28	54	191	21.43***	++

[a]: Number indicates test series (20 min. each, 4 per test day).

[b]: n: total catch (used for calculating chi-squared); ns: not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

[c]: N: total number of mosquitoes tested (not all of which reacted to either stimulus).

[d]: A(ttractive)/R(epellent): - = repellent, 0 = neutral, + = attractive.

[e]: composition of sample shown in Table 1.

passing through the ports was normally higher than that of the flight chamber, mosquitoes generally responded to either airstream by flying upwind towards the ports. Hence, warm moistened air, used as a control, was sufficient stimulus to induce trap entries against which the addition of odours was tested. Table 2A shows trap catches for all test series with the acid extract at various concentrations. When offered undiluted (concentrations as in Table 1) the acid extract was highly repellent ($P < 0.001$), the total catches being 3.5 and 4.6 times lower than those in the control. At concentrations of 10^{-3} both treatment and control were equally attractive, and on further dilution the extract became

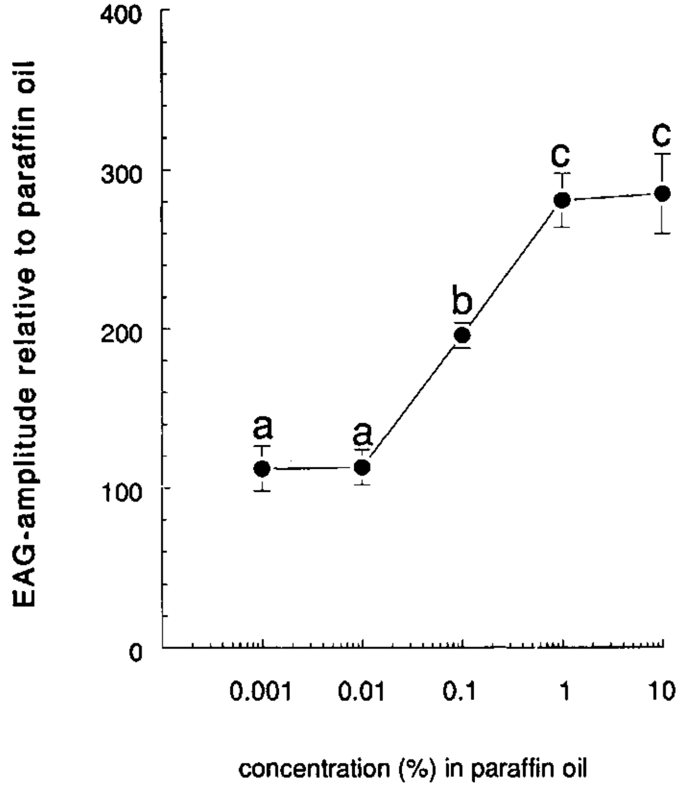

Figure 1. EAG responses relative to the response to paraffin oil (average set at 100%) of An. gambiae s.s. to a mixture of aliphatic carboxylic acids (composition see Table 1) at different doses. Doses marked with 'a' depict responses similar to paraffin oil. Doses without letters in common are significantly different from each other at $p < 0.05$.

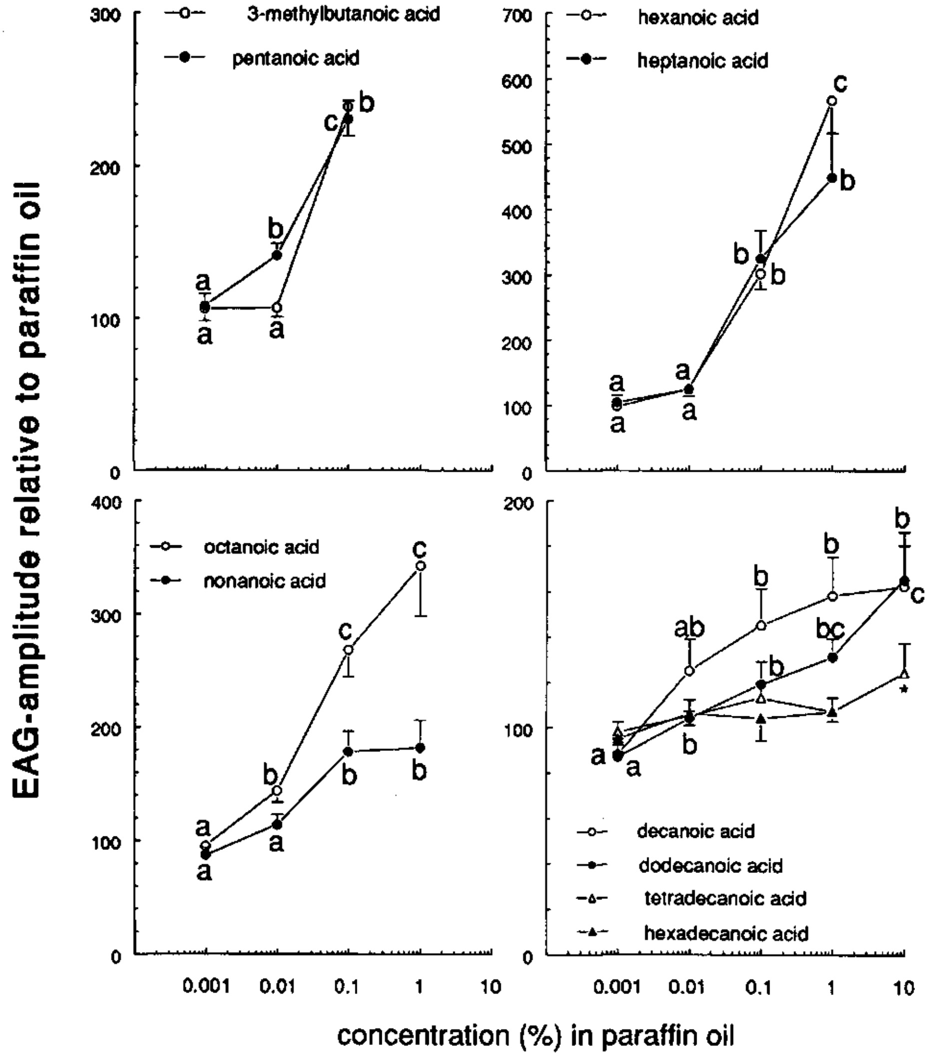

***Figure 2.** EAG responses relative to the response to paraffin oil (average set at 100%) of An. gambiae s.s. to individual aliphatic carboxylic acids at different doses. Doses marked with 'a' depict responses similar to paraffin oil. Doses without letters in common are significantly different from each other at p< 0.05. Tetradecanoic acid gave only a significant response at a dose of 10% (see asterisk). Hexadecanoic acid did not elicit any significant responses. notes: Y-axes have different scales. Letters shown do not indicate dose-response analyses between different compounds.*

significantly more attractive then the control, up to 4.2 times at a concentration of 10^{-6}. Attraction of mosquitoes towards lower concentrations was no longer consistent. Within the range of concentrations when the extract was attractive ($5*10^{-4}$ to 10^{-7}), the extract caught more mosquitoes than the control in 20 out of 24 test series (over 6 experimental days). A synthetic blend of the acids found in the acid extract was found to be repellent at a dilution of 10^{-6}(Table 2B), but was attractive when diluted to 10^{-8}.

EAG recordings

A well-defined and reproducible response was observed to Limburger cheese headspace. The mean response amplitude was 300 μV (SEM 40 μV; n=8). No measurable deflection was seen upon injection of clean air from the control bottle. EAG amplitudes from the synthetic acid mixture differed significantly from the solvent stimulus at doses of 0.1% and above, and were positively correlated with dose (Fig 1).

From Fig. 2 it can be seen that dose dependent EAG-responses were observed for 3-methylbutanoic, pentanoic, hexanoic, heptanoic and octanoic acid, with a threshold of between 0.001 and 0.1% (*i.e. ca.* 0.23 and 22.5 μg of pure compound) with the stimulus delivery method used. The mean absolute response to paraffin oil was 140 μV (n=8, data from one experimental series). Significant but lower amplitude EAG responses were elicited by nonanoic acid (0.01 and 1%), decanoic and dodecanoic acid (at the 0.1% and higher doses), and these increased significantly with dose. Tetradecanoic acid elicited a significant EAG response, but only at the 10% level, and no significant depolarizations were recorded on exposure of the EAG preparation to doses of up to 10% hexadecanoic acid.

Discussion

At present, only a few attractants have been identified for mosquitoes engaged in host-seeking. (+)*L*-lactic acid, isolated from acetone washings of a human arm, was reported by Acree *et al.* (1968) to attract female *Ae. aegypti*. 1-Octen-3-ol, identified as a tsetse fly attractant from cattle odour (Hall *et al.* 1984), was shown to attract several mosquito species, particularly *Ae. taeniorhynchus* (Takken & Kline, 1989). However, both these attractants are ineffective if used in the absence of carbon dioxide, which in itself acts as a kairomone for many different haematophagous Diptera (Sutcliffe, 1987). Schreck *et al.* (1981) isolated material from human hands that was attractive to *Ae. aegypti* in the absence of carbon dioxide but the chemical identity of this attractant has so far not been established (Schreck *et al.*, 1990). Few studies have focused on the identification of host-odour attractants for anopheline mosquitoes. Lysine, cadaverine and estradiol were shown to be attractants for *An. stephensi* (Bos & Laarman, 1975) and (+)*L*-lactic acid for *An. quadrimaculatus* (Price *et al.*, 1979), the latter compound again only showing activity in the presence of carbon dioxide. Limburger cheese odour was the first reported attractant for host-seeking *An. gambiae s.s.*, and acts as a kairomone in the absence of carbon dioxide (De Jong & Knols, 1995b).

The behavioural responses elicited by volatile fatty acids have been evaluated for *Ae. aegypti* and various other Diptera of medical and veterinary importance. Roessler (1961) obtained weak attraction of *Ae. aegypti* to a blend of aliphatic and amino acids, and several carboxylic acids of C_2-C_5 carbon chain length and a substituent at the alpha position were later reported to be attractive for this species (Carlson *et al.*, 1973). Ethanoic acid was found to be repellent to *Glossina morsitans* (Diptera: Glossinidae) (Johns, 1958), but 3-methylbutanoic acid was reported to attract this species (Hughes, 1957). Both *G. morsitans* and *G. pallidipes* were repelled by hexanoic acid (Johns, 1958; Vale, 1980). Swormlure-4, an attractant for the New World screw worm, *Cochliomyia hominivorax* (Diptera: Calliphoridae)(Mackley & Brown, 1984) contains ethanoic, butanoic, pentanoic and benzoic acid. Recently nine other aliphatic acids, identified from larval wound fluids, were found to elicit electrophysiological responses from *C. hominivorax* (Cork, 1994) and in combination with other volatiles identified in the wound fluids were found to attract predominantly *C. hominivorax* in the field (A. Cork & M.J.R. Hall, unpublished data). *Simulium damnosum* (Diptera: Simuliidae) was repelled by methanoic, ethanoic, propanoic and butanoic acid (Thompson, 1976), but *Simulium euryadminiculum* was attracted to ether extracts from the uropygial glands of its host, the common loon, which contain fatty acids (Fallis & Smith, 1964). Ethanoic, propanoic and 2-methylpropanoic acid are produced by bacteria causing summer mastitis in cattle, and serve as allomones to enhance their transmission by *Hydrotaea irritans* (Thomas *et al.*, 1985).

These findings indicate that a broad spectrum of insects associated with vertebrates is repelled/attracted by carboxylic acids which therefore could be expected to play a role in their host-seeking behaviour.

The typical odour of Limburger cheese, is to a large extent caused by short-chain fatty acids. These acids are formed mainly as a result of the lipolytic enzyme action of *Brevibacterium linens* bacteria on glycerides (Cogan & Daly, 1987). Closely related *Coryneform* bacteria occur on the human skin and have similar physiological characteristics (Sharpe *et al.*, 1977). Pitcher (1977, 1978) reported that *Brevibacterium* spp. may be present on human skin, especially in areas such as the toe web, but the taxonomy of this group of bacteria, and its influence on odour production is still under investigation (Anthony *et al.*, 1992). The chemical composition of lipids found on the surface of human skin is usually very similar within and between individuals but the hydrolysis of these triglycerides to free fatty acids was found to vary markedly between individuals (Downing *et al.*, 1969). Differences in the odour composition of individuals is then thought to reflect differences in the relative abundance of the different species of microflora associated with those individuals (Wesley & Musselwhite, 1975, Marshall *et al.*, 1988). Acid extracts of forehead sweat samples taken from five Tanzanian volunteers, of varying relative attractiveness to *An. gambiae*, were found to show large variations (up to 30x) in the amounts of C_2-C_6 acids produced (A. Cork, unpublished data). Thus it is likely that individual humans produce and consequently release different quantities of odorous-compounds, including the carboxylic acids, that could result in the observed differences in attractiveness between individuals to *An. gambiae* (Lindsay, 1993, Knols *et al.*, 1995). Aliphatic acids are produced during the ripening of Limburger cheese, so acid content of the cheese varies with age and storage conditions (Hanspach, 1981, A. Cork, unpublished data). Since the synthetic blend of aliphatic acids tested in the bioassay was inevitably based on an averaged value of acids found in Limburger cheese further work will need to be undertaken to optimise the blend for its attractiveness to *An. gambiae.*

The present study has only considered the aliphatic carboxylic acids present in Limburger cheese. The non-acid fraction is known to contain methanethiol, dimethyldisulphide, dimethyltrisulphide, and 1-octen-3-ol (Larpent, 1989), a known mosquito attractant, and all these compounds are present in human effluvia (Sastry *et al.*, 1980). Microbial breakdown of the amino acids leucine and valine results in the production of butanoic and pentanoic acids and during this process water, carbon dioxide and ammonia are liberated (Hanspach, 1981), of which the former two are known to influence mosquito behaviour. It may therefore be premature to attribute the attractiveness of Limburger cheese to its aliphatic carboxylic acid content alone. Nevertheless, carbon dioxide, ammonia, methanethiol and dimethyldisulphide were all identified in human breath (Krotozynski *et al.*, 1977; Tonzetich, 1977), and breath was shown to have no demonstrable behavioural effect on *An. gambiae s.s.* either in our laboratory bioassay (De Jong & Knols, 1995b), or in the field (Knols & Mboera, 1996).

The determination of free fatty acids in Limburger cheese extract revealed the presence of C_{16} and C_{18} acids which, most probably due to their low volatility, were absent in its headspace. The more volatile but much reduced quantities of ethanoic and propanoic acids observed in the headspace samples relative to those found in the acid extracts probably reflected the relative Pka values of acids with ethanoic and propanoic acids being more firmly bound in the cheese than the less acidic, higher molecular weight, acids. Since the make-up of the synthetic mixture was based on the extract rather than the headspace this could have resulted in higher concentrations of ethanoic and propanoic acid when evaporated from a diethyl ether solution on filter paper in the bioassay experiments. The texture of cheese probably acts as a slow release medium for the acids and the resulting higher doses offered on filter paper could have caused the initial repellency of the undiluted acid extract.

In this study we detected significant EAG responses to all acids tested except hexadecanoic acid. Whereas the threshold of response for the acids was found to increase, as might be expected with increasing carbon chain length, the saturation level of response was found to decrease with increasing carbon chain length (Fig. 2). Furthermore, besides their low volatility, the solvent (paraffin oil) may have influenced the dose arriving at the antenna, which was not compensated for (see Cork, 1994). It is interesting that the EAG responses elicited by the C_6 to C_8 acids at the 0.1% dose were higher than those elicited by the more volatile 3-methylbutanoic and pentanoic acids, suggesting that there are more receptors on the antennae that respond to the former acids. Even though only compounds with carbon chain lengths of C_5 or more were tested in the present study, ethanoic acid was found by De Jong (unpublished data) to elicit significant EAG responses from *An. gambiae s.s.*.

The observation that the maximum EAG-response elicited by the synthetic mixture were lower than those evoked by several of the pure compounds alone may be indicative of an inhibitory interaction at the peripheral sensory level or to a reduction in the release rates of more volatile compounds when offered as a mixture. Our study leads us to conclude that aliphatic carboxylic acids, found in Limburger cheese and ranging in carbon chain length from C_2 to C_{14}, elicit electrophysiological responses and are behaviourally relevant since a synthetic blend of these compounds was shown to elicit a behavioural response from *An. gambiae s.s.* in a laboratory bioassay.

Human sebum consists of up to 60% of triglycerides (Nicolaides, 1974). The breakdown of these triglycerids to free glycerol by the skin microflora results in the production of free fatty acids of which a third is made up of tetradecanoic, hexadecanoic, octadecanoic, 9-octadecenoic and 9,12-octadecadienoic acids. In addition some 200 different fatty acids make up the remaining two-thirds, the majority occurring in trace amounts only (review by Stoddart, 1990). The presence of free fatty acids on human skin makes man unique among other animals whose skin surface lipids consist largely of mono- and diester waxes, sterol esters and free sterol. Nicolaides *et al.* (1968) compared the skin surface lipid composition of man with several domestic animals such as cow, pig, goat, sheep, dog, cat and chicken and concluded that only man produces a surface lipid which consists mainly of triglycerides and their breakdown products, *i.e.* di- and monoglycerides and free fatty acids. Therefore fatty acids may provide a group of semiochemicals which can be used by *An. gambiae s.s.* to effectively distinguish between its preferred host (man) and the (domesticated) animals surrounding him.

We conclude that a) C_2-C_{14} aliphatic fatty acids elicit electrophysiological and behavioural responses in *An. gambiae s.s*, b) these compounds originate from human skin and are produced by microorganisms through decomposition of triglycerides, c) this feature is unique to human skin and therefore may present a reliable cue for anthropophilic *An. gambiae s.s.*.

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Part III:

Host attractiveness and odour-mediated host-seeking behaviour of mosquitoes: a field approach

7

Differential attractiveness of isolated humans to mosquitoes in Tanzania[1]

ABSTRACT - Individually occupied tents, modified for sampling mosquitoes, were used to assess the attractiveness of three humans for *Anopheles gambiae* Giles *s.l.*, *An. funestus* Giles, *Culex quinquefasciatus* Say and *Mansonia* spp. in North-East Tanzania. Significant ($P < 0.025$) differences in attractiveness were observed for the first three species, whereas the latter species did not show a preference for any of the three persons. Because of the objective sampling method used, our results form the first conclusive evidence that *isolated* people vary in their attractiveness to mosquitoes. This variation in attractiveness of humans is discussed within the framework of host-seeking by mosquitoes.

Introduction

Of the many factors influencing host-selection by mosquitoes (Burkot, 1988), those causing preferential feeding on certain humans are still poorly understood. Adults have been reported to be more attractive than children for *An. albimanus* (Muirhead-Thomson, 1951), *An. gambiae s.l.* (Thomas, 1951; Port *et al.*, 1980; Carnevale *et al.*, 1978) and *An. farauti* (Spencer, 1967), whereas men are more attractive to *Aedes aegypti* than women (Gilbert *et al.*, 1966; Rahm, 1956). There appears to be a direct relationship between the number of bites received and the size of the host (Port *et al.*, 1980) and in addition to this body odours (Brouwer, 1960; Schreck, 1981, 1990) seem to influence the choice by mosquitoes for a particular individual upon encountering a group of hosts. There is also evidence for species-specific attractiveness of humans (Curtis *et al.*,

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1987), and it was recently shown that during a series of night biting catches test person A attracted large numbers of *An. funestus* and few *An. squamosus* whereas the reverse was found for person B (Knols *et al.*, 1995).

Lindsay *et al.* (1993) studied the attractiveness of Gambian men sleeping individually in experimental huts, and found some individuals more attractive for *An. gambiae s.l.* than others. These huts, however, were only 12 m apart, and interactions between odours from different huts with their occupants can not be excluded, since this distance falls well within the range of attraction of the mosquito species studied (Gillies & Wilkes, 1969). Also it was impossible to assign huts to individual subjects, which hindered objective analysis since it is known that odour 'residues' remain present in huts after having been occupied for some time. Haddow (1942) showed that the presence of worn clothing in empty huts attracted significantly higher numbers of *An. gambiae s.l.* and *An. funestus* than huts without clothing and that huts were frequented for some days by host-seeking mosquitoes after having been vacated. It is therefore important to eliminate the interaction caused by the test person's odours and odour residues present of previous occupants when assessing an individual's attractiveness to mosquitoes.

In the present study we modified tents according to a design by Sharpe *et al.* (in press) and used them to assess whether isolated human subjects differ in their attractiveness to some important disease transmitting mosquitoes in Tanzania.

Materials and Methods

The experiment was conducted during the long rainy season (April-June) at Kisiwani, Tanga region, NE-Tanzania. The site consists of open maize farmland with partial coconut, mango and citrus tree cover.

The tents (Fig. 1) consisted of light-grey PVC with two exit-traps (Muirhead-Thomson, 1948). Mosquitoes could enter through entrance slits, cut just underneath the roof on both sides of the tent, which overhung (20 cm) and prevented outdoor light being visible from inside the tent.

A tent, camping bed and (unimpregnated) rectangular bed net was assigned to each of three test persons (male Tanzanians, aged 21, 22 and 24 years). Every night the subjects entered their bed nets at 21.30 hrs until the following morning 07.00 hrs. The exit-traps were then emptied and the interior of the

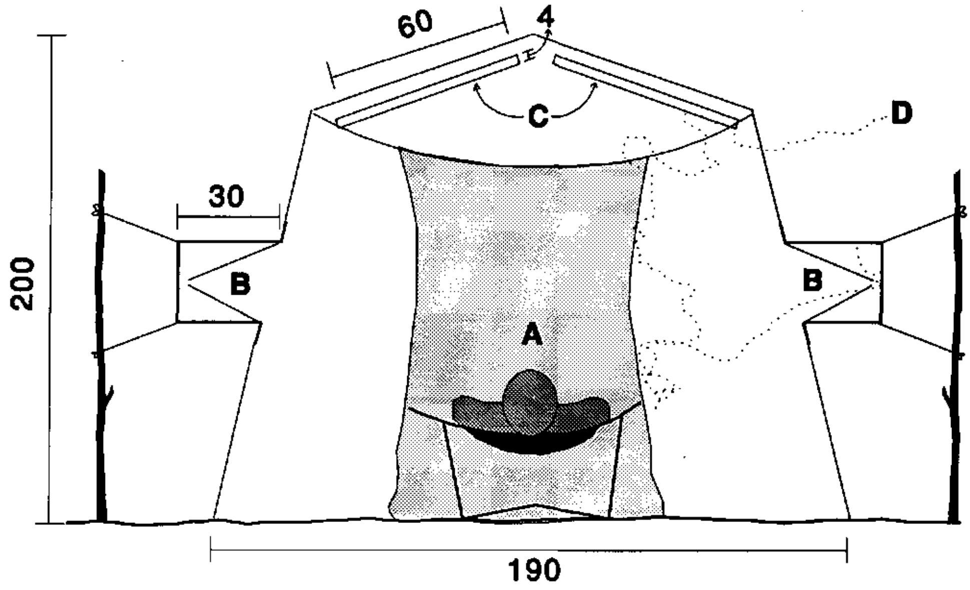

Figure 1. The experimental tent (dimensions in cm), modified for sampling mosquitoes. A: Test person on camp bed under mosquito net; B: Muirhead-Thomson exit trap; C: Mosquito entry slit; D: Mosquito flight path.

tent searched for mosquitoes for a fixed 10 min period. The occupants with their tents, beds and bed nets were then moved between sites for a total of nine test days (randomized block design with three replicates). The current set-up excluded variation caused by possible odour residues in the tent, the remaining variation, being site, day and person. All catches were transformed to $\log(x+1)$ and after a satisfactory check for normality of the distribution subjected to 3x3 Latin square analysis of variance (Snedecor & Cochran, 1989). A F-test significant at $p<0.05$ was followed by a LSD-test to test for differences of attractiveness between persons.

Table 1. ANOVA tables and mean trap catches per person/night for Anopheles gambiae s.l. *(A),* An. funestus *(B),* Culex quinquefasciatus *(C) and* Mansonia *spp. (D).*

(A)	Source	df	MS	F	Person	n	Mean	Index
	Day	8	0.153	3.602 ns	1	45	4.294	1.000a
	Site	2	0.033	0.781 ns	2	48	4.151	0.967a
	Person	2	0.463	10.883 ***	3	13	1.113	0.260b
	Error	14	0.042					

(B)	Source	df	MS	F	Person	n	Mean	Index
	Day	8	0.161	4.000 *	1	27	1.702	1.000a
	Site	2	0.071	1.774 ns	2	14	1.119	0.657a
	Person	2	0.325	8.100 ***	3	2	0.167	0.098b
	Error	14	0.040					

(C)	Source	df	MS	F	Person	n	Mean	Index
	Day	8	0.076	1.109 ns	1	108	10.537	1.000a
	Site	2	0.024	0.346 ns	2	155	14.613	1.387a
	Person	2	0.436	6.341 **	3	53	4.805	0.456b
	Error	14	0.069					

(D)	Source	df	MS	F	Person	n	Mean	Index
	Day	8	0.085	1.716 ns	1	26	1.948	1.000a
	Site	2	0.151	3.050 ns	2	21	2.093	1.075a
	Person	2	0.005	0.105 ns	3	19	1.770	0.909a
	Error	14	0.050					

n: total number caught; ns: not significant, *: $p<0.05$, **: $p<0.025$, ***: $p<0.005$; Indices not followed by the same letter are significantly different at $p<0.05$.

Results and Discussion

The exit-trap catches are shown in Table 1. Despite the relatively low mosquito densities during the study period significant differences caused by person variation were found for *An. gambiae s.l.*, *An. funestus* and *Cx. quinquefasciatus* but not for *Mansonia* spp. For these first three species it was found that person 3 was significantly less attractive than person 1 and 2 who did not differ in their attractiveness. Since there was no active involvement of the test persons in catching the mosquitoes, and each person occupied his own tent it can be concluded that the only causal factor was differential responses of host-seeking mosquitoes towards the test person's body odours. Human body odours are known to be distinctive and specific (Stoddart, 1990) and varying compositions of this odour complex might determine whether or not a mosquito initiates a search for a host upon detecting host odour. Though there is ample evidence that mosquitoes select a particular host while given a choice it is therefore interesting that *isolated* individuals also differ in their attractiveness. Not only does this influence effective epidemiological modelling, in which it is generally assumed that the number of bites received by humans is equal and constant, but also on ecological studies such as those comparing the efficacy of CDC-light traps with human biting catches (Lines *et al.*, 1991, Githeko *et al.*, 1994).

More than 90% of the *An. gambiae sensu lato* population in the study area consists of *An. gambiae sensu stricto* (Mnzava & Kilama, 1986). Therefore all three mosquito species that discriminated between the hosts are strongly endophilic, and obtain the majority of their bloodmeals from man (Garrett-Jones *et al.*, 1980). *Mansonia* spp., on the other hand will also feed outdoors and are much more liberal in the range of hosts fed upon. It is expected that mosquitoes with a broad host range respond to commonly present odours such as carbon dioxide, and recent laboratory experiments, on the selection of biting sites by mosquitoes on humans, have provided strong evidence for this (De Jong & Knols 1995a; Knols *et al.*, 1994b). Since the individuals involved in the experiment were roughly the same size, weight and age it can be expected that the amounts of carbon dioxide expired were about the same, which might have caused no difference in the number of *Mansonia* mosquitoes attracted. Consequently it can be concluded that the three anthropophilic species did not base their host discrimination on carbon dioxide but must have selected hosts on a particular blend of human-specific odours. Recent laboratory experiments with a windtunnel bioassay (Knols *et al.*, 1994a) suggest that the human skin microflora might be responsible for producing the compound(s) attracting *An.*

gambiae s.s. (De Jong & Knols, 1995b). It is common to find varying densities of different species of bacteria on the human skin (Wesley & Musselwhite, 1975) and this results in varying amounts of volatiles produced. Therefore it could well be that host discrimination by the mosquito species observed in this study was based on differential responses to volatile substances of bacterial origin and further research in the field of skin microbiology might therefore resolve the long-standing question as to why some people are more attractive to mosquitoes than others.

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8

Species-specific attraction of *Anopheles* mosquitoes (Diptera: Culicidae) to different humans in South-East Tanzania[1]

ABSTRACT - Differential attractiveness of humans to mosquitoes has frequently been reported. From these studies it appears that humans not only differ in terms of numbers of mosquitoes attracted but also that certain mosquito *species* prefer individuals within a group. This species-specific attraction was studied for six people doing human biting catches (HBC) alone or in groups (2 or 3 persons) for 5 consecutive nights. A negative correlation between group size and mean individual catch was observed. Mean individual catches showed significant differences in attractiveness to *Anopheles funestus* Giles and *An. squamosus* Theobald but not to *An. gambiae s.l.* Giles and *An. ziemanni* Grünberg. The catches of the former two species were inversely correlated. Our results provide new evidence for mosquito-species specific host-seeking strategies possibly mediated by different host odours or blends thereof.

Introduction

The variation in attractiveness of humans to mosquitoes has been the subject of many studies. Maibach *et al.* (1966) examined the attractiveness of 838 adult men to *Aedes aegypti* and found one man clearly less attractive than others. In olfactometer studies Brouwer (1960) demonstrated that differences in attractiveness of test persons to *An. stephensi* were based on body odours. Lindsay *et al.* (1993) studied attractiveness of adult men to *An. gambiae s.l.* in

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the field. The observed variation in attractiveness was reported to be consistent over a period of at least two years. Adults have been shown to be more attractive than children for *An. albimanus* (Muirhead-Thomson, 1951), *An. gambiae s.l.* (Carnevale *et al.*, 1978) and *An. farauti* (Spencer, 1967). Sex-specific attractiveness to *Aedes aegypti* was reported by Rahm (1956), who, in laboratory studies, found men consistently more attractive than women. A person's blood group was once thought to influence attractiveness to *An. gambiae* (Wood *et al.*, 1972) but this was later refuted by Thornton *et al.* (1976). A direct relationship between the number of bites received and size of the host for *An. gambiae* was demonstrated by Port *et al.* (1980).

The above mentioned studies provide sufficient evidence that mosquitoes, upon encountering a group of hosts, perceive small differences in their attractiveness and select particular individuals for taking a bloodmeal. In addition, recent field studies in Tanzania (Knols *et al.*, 1995, see chapter 7) have demonstrated that *isolated* people also vary in their attractiveness to important disease transmitting mosquitoes.

The human biting catch (HBC), whereby mosquitoes are collected while feeding on exposed limbs of collectors, is still widely used to estimate population densities of disease vectors. Burkot (1988), however, has shown that non-homogenous mosquito biting rates can have a serious impact on the epidemiology of disease and models to estimate transmission rates, which work on the assumption that equal numbers of bites are received by all individuals in a population, should therefore be interpreted with care.

Curtis *et al.* (1987) first found evidence that individuals can be attractive to particular mosquito *species*. They found that one collector was much more attractive to *An. coustani* and *Mansonia* spp. than three others but less attractive to *Culex quinquefasciatus*. This phenomenon might complicate the understanding of an individual's attractiveness to mosquitoes even further and we therefore decided to study mosquito catches of six humans in an attempt to collect further proof for the existence of mosquito-species specific attractiveness of humans. In the present work we compared the attractiveness of six individuals for several *Anopheles* mosquito species. Group-size effects were also studied.

Materials and Methods

The experiment was carried out near Namawala village, Kilombero district, South-east Tanzania, during the long rainy season (May), when mosquito densities are highest. The area consists of open woodland interspersed with food crops, mainly maize and rice.

Six Tanzanian adults (5 men, 1 woman, aged 18-35 yrs), seated outdoors on chairs, conducted the biting catches by exposing their lower limbs and feet to mosquito bites. Flashlights were used to locate landing and probing mosquitoes, which were then caught using a suction tube. All mosquitoes were stored in paper cups and taken to the laboratory for identification the following day. In this way biting catches were executed for five consecutive nights between 23.00 and 02.00 hrs. Every half hour the collectors would change positions (ca. 50 m apart) according to a randomized experimental design. In this way, each night, every person would catch mosquitoes for 1 half-hour period alone, for 1 hour in the presence of another collector, and 1.5 hrs with two other collectors. Thus the majority of the catches occurred in the presence of other humans. All collectors were under medical supervision and had access to curative antimalarial drugs.

Data were analysed using a one-way analysis of variance for differences in catch per species between the collectors. Species-specific attractiveness was calculated using a general analysis of variance of $\log(x+1)$ transformed data after a satisfactory check for normality of the distribution.

Results

Table 1 shows the mean 3-hr catches for the six collectors over the five night period for the four predominant *Anopheles* species. These species comprised 92% of the total catch ($n=3569$), the other species being (totals caught between brackets) *An. pharoensis* Theobald (1), *An. wellcomi* Theobald (76), *An. seydeli* Edwards (3), *An. rivulorum* Leeson (37), *Aedes* spp. (7) and *Culex* spp. (155). The variation in total catch for the six collectors was not significant. No differences in the number of *An. gambiae s.l.* and *An. ziemanni* caught by the collectors was found. However these differences were significant for *An. funestus* and *An. squamosus*. Besides this difference in attractiveness of the collectors to these two species it is interesting to note the inverse correlation of their catches, i.e. the lowest *An. funestus* catch (by collector Ka) corresponded with the highest *An. squamosus* catch while the reverse was found for collector An. A full analysis of variance (Table 2) indeed shows that there is a highly significant (P<0.001) collector*species interaction, applying to collectors Ka and An.

The analysis of group size versus mean catch per collector is shown in Figure 1. This indicates a significant decrease in catch when collectors were grouped as compared to their mean individual catches. As expected, the absolute number of mosquitoes caught increased with biomass from 892 (1 person) to 1262 (two persons) to 1415 (three persons).

Table 1. *Mean catch per 3-hr sampling period and total number of mosquitoes caught per species/collector for four Anopheles spp. by six collectors over a five night period. Mean catches under which letters are not similar are significantly different (P< 0.05). Highlighted data depict observed species-specific attractiveness of collectors 2 and 3.*

Species (*Anopheles*)	Collector Ra	 Ka	 An	 Si	 Ad	 Ni	Total (species)
An. gambiae s.l.	29.0	34.4	55.4	41.4	31.2	31.2	1113
An. funestus	32.6 ab	9.0 c	40.4 a	37.0 ab	26.2 ab	21.4 bc	833
An. squamosus	23.8 b	47.4 a	14.0 b	22.8 b	28.8 ab	27.2 ab	820
An. ziemanni	18.8	18.0	23.2	10.8	19.8	14.2	524
Total (Collector)	521	544	665	560	530	470	3290

Discussion

Of the many factors that seem to be influencing host selection by *Anopheles* mosquitoes few, if any, are fully understood. The wealth of literature on the subject does not provide means to assess a person's attractiveness *a priori* simply because the exact nature of it is still unknown. The present study has demonstrated that different people can attract different *Anopheles* species,

***Table** 2. ANOVA table for* An. funestus *and* An. squamosus *catches (ns: not significant; *: $P<0.05$; ***: $P<0.001$)*

Source	df	SS	MS	F
Day	4	0.688	0.172	3.60*
Collector	5	0.322	0.081	1.34ns
Species	1	0.003	0.003	0.06ns
Day*Collector	20	0.659	0.033	3.44*
Day*Species	4	1.349	0.337	1.41ns
Collector*Species	5	2.690	0.538	11.59***
Coll.*Day*Species	20	0.957	0.048	
Total	59	6.668		

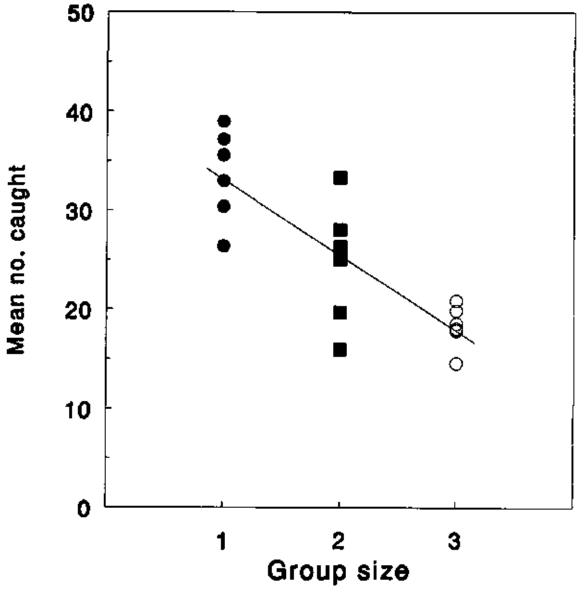

***Figure** 1. Correlation between mean catch per collector ($n=6$) and group size ($Y=40.8-7.6X$, $r^2=0.82$, $P<0.001$)*

and that groups of collectors have lower mean catches than individual collectors.

The reported species-specific attractiveness of *An. funestus* and *An. squamosus* for collectors Ka and An could be caused by a variety of factors. First, though not very likely, it is possible that their sensitivity to bites of both species differs, resulting in different catch compositions even though equal numbers of both species were attracted. Secondly, due to the experimental set-up, it is not known whether major differences in catch composition occurred while collecting in groups or individually. It is likely that in the presence of other collectors the catch composition changes, but since the majority of the collections was done in groups (in fact 50% of the total experimental period collectors Ka and An were catching mosquitoes at the same sites) the observed differences seem to be independent of group size. Another possibility is the existence of olfactory based differences in host-seeking behaviour of both *Anopheles* spp. If so, than this would imply use of different host-emitted volatiles while locating a host or use of similar volatiles but different preferences for blends of these. *An. funestus* is strongly anthropophilic whereas *An. squamosus* has a more zoophilic nature (Gillies & De Meillon, 1968), which might explain their different uses of host-derived olfactory cues.

Even though no differences in catch for *An. gambiae s.l.* was observed it should be noted that since catches were performed outdoors they may well represent mixed catches of *An. gambiae sensu stricto* and *An. arabiensis*, known to occur sympatrically in the area. Again, the former species represents a strongly anthropophilic one whereas the latter, in East Africa, is a more catholic feeder. Since we did not discriminate between the two members of this species complex we can not yet rule out the existence of species-specific attractiveness of the collectors to them. Lastly, *An. ziemanni* is a truly opportunistic species and since it might therefore use common mammalian odours, such as carbon dioxide, to locate its host, this might explain the absence of a preference for a certain individual, since the amounts given of by adults are roughly the same.

The observed decrease in mean catch per individual with group size is in concordance with the findings of Haddow (1942), who studied mosquito numbers in huts occupied by 1, 5, 10 or 15 persons. Mean catches dropped dramatically between 1 and 5 occupants but stabilized above 10. This implies that both in and outdoors the mean number of mosquitoes per person, recruited from within the active range of host odours, is negatively correlated with body mass.

The results of our work show that the use of the human biting catch for studying mosquito populations is by no means a reliable method due to intrinsic differences of attractiveness of collectors to mosquitoes in terms of numbers but also in terms of *species*. Furthermore it is shown that the sensitivity of the human biting catch, particularly when dealing with low mosquito densities, increases when collectors sample the population individually instead of in groups.

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9

Electric nets for studying odour-mediated host-seeking behaviour of mosquitoes (Diptera: Culicidae)[1]

ABSTRACT - The feasibility of using odour-baited electric nets for sampling host-seeking mosquitoes was studied in Tanzania. Three designs, with operating principles similar to those used for tsetse flies, were evaluated: Small square nets (15x17cm), with electrocuting wires 2.5 mm apart and charged with 2 kV, larger rectangular nets (28x40cm) with wires at 4 mm and charged with 6kV, and a large cylindrical net (diameter 60 cm, height 40cm), with wires 4 mm apart and charged with 25 kV. The small nets were baited with human breath and the rectangular/cylindrical nets with carbon dioxide at a human equivalent (300 ml min^{-1}). All designs proved efficient in sampling mosquitoes and a representative sample of the species present in the area was obtained. Both odour baits attracted mainly zoophilic mosquitoes and the very low catches of *Anopheles gambiae s.l.* and *An. funestus* (3% and 8.5% of the total catch for human breath and carbon dioxide respectively) indicate that these odours alone are unsuitable attractants for these anthropophilic species in the field. The carbon dioxide baited cylindrical net was used to assess the direction of approach to an odour bait, and confirmed that mosquitoes engage in positive anemotaxis upon detecting a host. Direct observations on the behaviour of mosquitoes around a carbon dioxide baited rectangular net gave a sampling efficiency of 42.8%.

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Introduction

Electric nets, initially developed as sampling tools for tsetse flies by Vale (1974), have played a paramount role in studying the behavioural ecology of these insects in the field. They have been used to examine the attractiveness of host odours (Vale *et al.*, 1986) and synthetic kairomones (Vale & Hall, 1985), analyse tsetse behaviour around hosts and in odour plumes (Torr, 1988), estimate proportions of flies feeding on hosts (Vale, 1977) and for testing sampling efficiencies of numerous trap and target designs (e.g. Vale, 1982). All these studies have contributed to the development of economic and environmentally safe baits (Vale, 1993), now widely used to reduce the trypanosomiasis risk in East and Southern Africa (e.g. Willemse, 1991).

The tools available for studying outdoor host-seeking behaviour of mosquitoes are few, and they all operate on the principle of an active air current sucking insects into collecting bags/cages (many examples to be found in Service (1993)), or have an active odour-laden air current pumped out of traps guiding mosquitoes towards them (Costantini *et al.*, 1993). These methods have two major disadvantages. Firstly it is not known what proportion of approaching mosquitoes enter the trap upon arrival at its entrance and secondly, being non-directional sampling devices, they cannot be used to study mosquito flight behaviour.

Gillies *et al.* (1978) and Gillies & Wilkes (1981) adopted Vale's technology to study flight behaviour of mosquitoes in relation to prevailing wind directions and the influence of wind on the flight speed of West African mosquito species. Their electric nets were modified by reducing the distance between wires from 8 (for tsetse flies, see Vale (1974)) to 5 mm and they were charged with a lower voltage output (2 kV). In France, Killick-Kendrick *et al.* (1986) successfully sampled sandflies (Diptera: Psychodidae), which are considerably smaller than mosquitoes, with human odour-baited electric nets. Although these studies have clearly demonstrated the potential of using electric nets for sampling mosquitoes, they have never been used to assess the attractiveness of odour baits.

We are currently in the process of screening human odours for behavioural activity in the laboratory (Knols *et al.*, 1994a; De Jong & Knols, 1995b) in order to develop odour-baited traps for the African malaria vector *An. gambiae s.s.*. Evaluation of candidate odours under field conditions depends heavily on the availability of sampling devices which can be used outdoors. We therefore studied the feasibility of using electric nets, baited with human breath or carbon dioxide, for this purpose.

Materials and Methods

Study area. The experiments were done near Namawala village, Kilombero district, South-East Tanzania, towards the end of the long rainy season (March-June). Rice is the main crop in the area, and rice fields provide ideal breeding sites for a wide variety of mosquito species. Further details of the ecological setting and demography of the area can be found in Smith *et al.* (1993) and Charlwood *et al.* (1995). During the experiments density and composition of the mosquito population were monitored regularly with miniature CDC light traps (John W. Hock co., FL, USA) set beside occupied bed nets (Lines *et al.*, 1991), in a house near the experimental sites.

Electric nets. Three types of electric nets were used in the experiments. Small square nets (15x17cm, fig. 1a) consisted of a grid of 70 stainless steel wires (diameter 0.2mm), attached to Fabory(r) zinc plated draw springs (0.5x3.5x20mm) on one side (conductors) and nylon loops (Damyl(r) fishing line) on the opposite side (insulators). Alternating wires (at 2.5mm) were charged with 2 kV AC or earthed respectively. The power source was an inverter transformer oscillator (hereafter 'spark box', manufactured by Dr. T. Coates, Bristol, UK) driven by a 12 V (50 Ah) car battery. The nets were surrounded by a metal frame (30x30cm) covered with white mosquito netting. When operating sparks were continuously visible. Mosquitoes alighting on or colliding with the grid were stunned or killed and collected in a plastic transparent tray (45x45cm) underneath the net, filled with water to which a natural soap or washing powder was added. A plastic polythene sheet was suspended over the nets so that they could be operated during light rains.

The larger rectangular nets (28x40cm, fig 1b) were manufactured with the same materials as the small nets but the 70 wires were set at 4mm distance and a 6 kV AC spark box was used.

The large cylindrical net (diameter 60 cm, height 40cm, for top view see fig 2) consisted of 467 wires set at 4mm, and a 25 kV AC spark box was used.

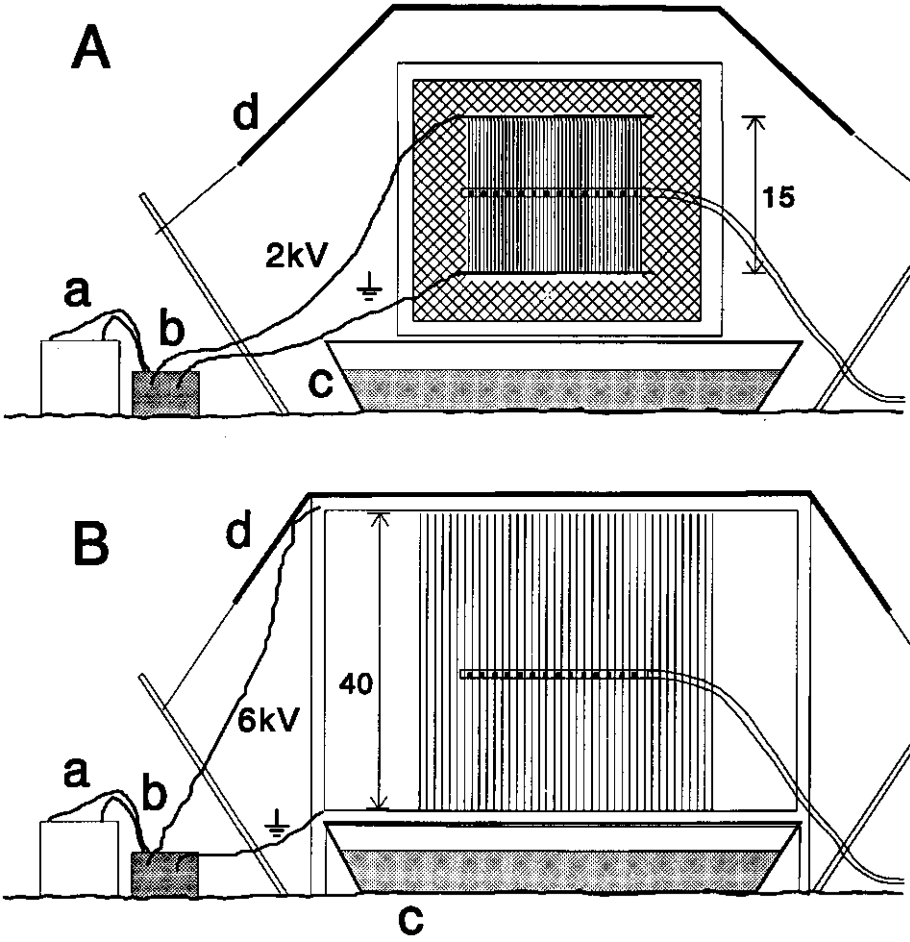

Figure 1. Details of the electric nets (dimensions in cm). (A) small electric net, (B) large rectangular net. Odour dispensers are fixed horizontally across the grids. a= 12 V car battery, b= spark box, c= collecting tray with water, d=polythene cover sheet.

The top and bottom of the net was covered by a plastic sheet so that odour could only pass through the wires. Four trays, each covering an arc of 90° of the net, were used to collect mosquitoes. The rectangular and cylindrical nets were not spontaneously discharging ('sparking') while operating.

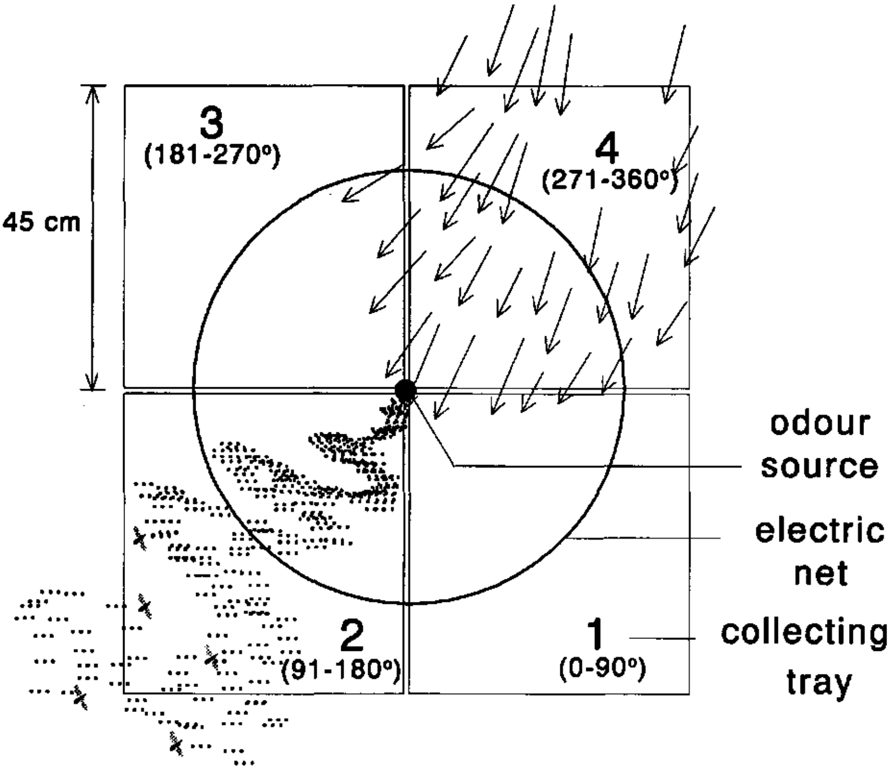

Figure 2. *Top view of a cylindrical electric net with collecting trays (1-4) placed underneath. Arrows indicate wind direction with resulting (imaginary) CO_2 odour plume (more details see text).*

Odours. Human breath was led to a small electric net by using a Harvard-Douglas⁽ʳ⁾ one-way breathing valve to which 12 m of silicon tubing (9mm inner diameter) was attached. The person from which the breath was obtained (BGJK) was sitting indoors, but was inhaling air through silicon tubing from outside the building. A nose-clip was used to prevent accidental exhaling of breath and avoid inhaling of other body odours. In this way only (outdoor) air that had passed the lungs was led to the net. A 15 cm long plastic transparent tube with three 1 mm holes every 10 mm was attached to the end of the silicon tubing. This tube was fixed to one side of the net, touched the wire grid and provided a down-wind carried front of breath odour. The rectangular/cylindrical nets were baited with 300 ml min^{-1} of carbon dioxide, this being the equivalent expired in breath by an adult human at rest. This was pumped from a pressurized gas cylinder through silicon tubing (inner diameter 5 mm), passed a flow-meter, and was dispensed through a similar tube as used for dispensing breath.

Experimental protocols. The small electric nets were used to test the attractiveness of human breath. Two similar nets at 5 m distance were used, one baited with breath odour, the other net serving as a control. Experiments were conducted between 20.30 and 23.30 hrs local time. Every half hour the nets were switched off and mosquitoes collected from the trays and wire grids after which the odour source was moved to the control net. After collecting the mosquitoes the nets were not operated for 15 min to prevent killing mosquitoes that were attracted by the person collecting the mosquitoes. Half hour catches were stored in 96% ethanol and identified the following day.

The setup for the carbon dioxide baited rectangular nets was the same as above except that experiments were run between 22.30 and 04.30 hrs., the tube for dispensing carbon dioxide was 25 cm long, and catches were collected only once at the end of the experiment. The attractiveness of the small and rectangular nets, both baited with carbon dioxide, was compared during one night (22.00-04.30 hrs).

The cylindrical net was baited with carbon dioxide through a similar tube as the rectangular nets, but this dispenser was fixed vertically in the centre of the net. The experiment was run for five days between 22.00-02.00 hrs and catches from the four trays, each covering a quarter of the net's surface, were collected at hourly intervals. Previous experiments showed that unbaited nets do not catch mosquitoes, and we therefore assumed that electrocuted specimens had responded to the odour bait. A Gill$^{(r)}$ 3-axis standard ultrasonic anemometer, of which the centre of the transducers was aligned with the centre of the net, was connected to a laptop computer and placed at 1 m from the net. The apparatus measured horizontal/vertical windspeeds (in cm ±3%) and the wind direction (±3 degrees, for horizontal windspeeds >5 cm sec^{-1}) every second. The net and anemometer were placed in a wide open field with short grass.

Direct observations on mosquito behaviour around a rectangular net baited with carbon dioxide were done for 9 nights by both authors while seated in a pit covered by a tent. A small window was fixed perpendicular to the net at 5 cm. Human odour was pumped away from the tent by two fans connected to lay-flat tubing. A torch (3V) was placed underneath the transparent collecting tray, shining upwards, so that mosquitoes approaching the net could be seen in the dark. The efficiency (in %) of the net was defined as the number of mosquitoes electrocuted divided by the total number observed while approaching the net. This was determined for three spark box voltage outputs: 2 and 6 kV (non-sparking nets) and 25 kV (continuously sparking net).

Data analysis. Since most experiments were carried out during the latter part of the rainy season, when mosquito numbers were low, data could not be analysed per species and therefore catches were pooled per treatment. Effects of human breath and CO_2 on mosquito attraction were calculated after normalizing catches using $\log(x+1)$ transformation, by subjecting the data to 2x2 Latin square analysis of variance. Catches from the CO_2 baited cylindrical net (catch/tray/hour) were converted into percentages. Anemometer data were converted likewise to give percentages of wind/tray/hour. Pooled data per 4-hr period (1 night) were then subjected to a Friedman two-way analysis of variance to analyse the effect of wind direction on the approach direction of mosquitoes. Data on the catching efficiency of different spark boxes were converted into proportions, variances were normalized using the arcsine-square root transformation and then subjected to analysis of variance.

Results

Table 1 shows the results obtained with carbon dioxide or human breath baited electric nets. Both carbon dioxide ($F=45.2$, $p<0.001$) and human breath ($F=162.0$, $p<0.001$) caught significantly more mosquitoes than the control nets. Even though both nets produced continuous sparks, which were clearly visible in the dark, these apparently did not influence the attraction of mosquitoes. Electrocuted mosquitoes were easily identifiable and never burned which allowed them to be dissected. Only one female was bloodfed and no half-gravid or gravid specimens were caught, indicating that the odours attracted host-seeking females. Some male *Mansonia africana* were caught, which are known to respond to host odours since mating takes place near the host (McIver *et al.*,

Table 1. *Numbers of mosquitoes caught with different odour baits and electric net designs. (A): CO_2 (300 ml min^{-1}) baited large rectangular nets (6kV, wires at 4 mm), (B): Human breath baited small square nets (2 kV, wires at 2.5 mm), and (C) comparison of both types of nets, baited with CO_2 (300 ml min^{-1}). CDC light trap catches were collected during the same periods the electric nets were run.*

	(A)			(B)			(C)		
Mosquito species	CO_2	Control	CDC Trap	Breath	Control	CDC Trap	CO_2 (Small net)	CO_2 (Large net)	CDC Trap
Anopheles squamosus	15	0	0	36*	1	0	8	3	0
Anopheles pharoensis	0	0	0	4	0	0	0	0	0
Anopheles coustani	6	0	0	15	0	9	2	13	0
Anopheles gambiae s.l.	8	0	13	2	0	47	4	8	4
Anopheles funestus	3	0	58	1	0	35	1	5	10
Anopheles ziemanni	4	0	0	0	0	0	0	0	0
Anopheles wellcomi	2	0	0	0	0	0	0	0	0
Mansonia africana	76**	3	26	22	0	49	25	59	1
Mansonia uniformis	1	0	0	4	1	4	0	1	0
Culex spp.	12	1	84	14	1	39	7	41	6
Aedes sudanensis	2	1	0	1	0	2	0	1	0
Total	129	5	181	99	3	185	47	131	21
Average per hour	5.4	0.2	7.5	16.5	0.5	30.8	5.5	15.4	2.5

* Includes 1 bloodfed specimen; ** Includes 6 male specimens

Table 2. *Numbers of mosquitoes caught with the cylindrical electric net baited with CO_2 (300 ml min^{-1}). Catches show the totals caught per hour (over the 5-night experimental period) for the four collecting trays (see fig. 2).*

	Tray 1 (0-90°)				Tray 2 (91-180°)				Tray 3 (181-270°)				Tray 4 (271-360°)			
Mosquito species	1[a]	2[b]	3[c]	4[d]	1	2	3	4	1	2	3	4	1	2	3	4
Anopheles squamosus	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
Anopheles coustani	3	3	2	6	4	4	8	10	1	3	6	4	1	1	1	3
Anopheles gambiae s.l.	2	1	2	2	4	0	1	3	2	1	1	1	0	1	2	0
Anopheles funestus	1	1	1	0	2	0	3	2	1	1	2	3	0	0	1	0
Anopheles wellcomi	0	0	0	1	0	1	1	2	0	1	0	0	0	0	0	0
Mansonia africana	7	5	6	7	4	8	2	6	3	5	3	7	1	5	5	6
Culex spp.	1	3	3	4	1	2	4	3	1	1	2	3	1	2	4	1
Aedes sudanensis	0	0	0	1	1	0	1	0	0	0	0	2	0	0	0	0
Total	14	13	14	21	16	16	20	26	8	12	14	21	3	9	13	10

a: 22.00-23.00 hrs; b: 23.00-24.00 hrs; c: 24.00-01.00 hrs; d: 01.00-02.00 hrs.

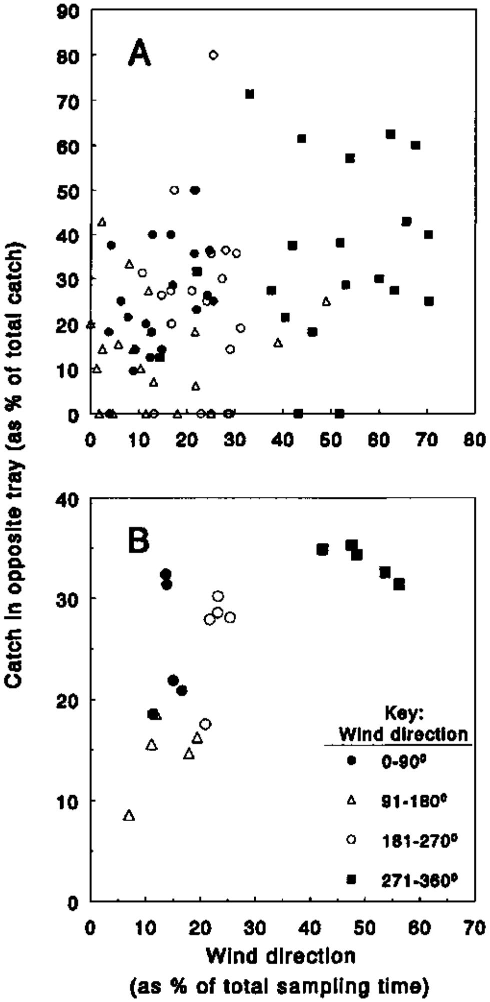

Figure 3. Relationship between the wind direction (as % of total sampling time) per quarter (see symbols) of the cylindrical electric net versus the mosquito catch (n=230) in the opposite quarter (as % of total catch). A: per tray/hour (5 nights x 4 trays x 4 hours); B: per tray/night (5 nights x 4 trays).

1980). Three zoophilic species, *Anopheles squamosus*, *An. coustani*, and *Mansonia africana* (Gillies & De Meillon, 1968), accounted for 75 and 74% of the human breath and CO_2 catches respectively. The indoor operated CDC light traps caught considerable numbers of *An. funestus* and *An. gambiae s.l.*, indicating that these anthropophilic species were present but not attracted to carbon dioxide or human breath in the outdoor setup. When compared for one night, the carbon dioxide baited large rectangular net was 2.8 times more effective than the small net and caught 6.2 times more mosquitoes than the CDC trap. The electric nets sampled 12 mosquito species whereas the indoor-operated CDC traps sampled 8 species (both including 2 unidentified *Culex* spp.). This difference was probably caused by the exophilic/exophagic behaviour of some species. Human biting catches during the experimental period did not detect additional mosquito species, which shows that the electric nets sampled all species present in the area.

Catches from the carbon dioxide baited cylindrical net are presented in Table 2. Tray 2 (n=78) caught significantly more mosquitoes than tray 4 (n=35; F=11.68, p=0.0085). Catches were also significantly higher during the last hour of the experiment (n=78) then in the first hour (n=41; F=9.4, p=0.025). Average horizontal windspeeds were generally lower than 50 cm sec^{-1}, and the wind mainly came from one direction during most of the observation period (each night *ca.* 50% of the time between 271-360°) which resulted in an odour plume 'leaving' the net between 90-180° (see fig. 2). The relationship between the percentage of time the wind entered a quarter of the net versus the catch in the *opposite* quarter is shown in fig 3. It is obvious that the more the wind originated between 271-360° (tray 4), the higher the catches were in the opposite tray (2), when shown per hour (fig 3a), but particulary when shown per night (fig 3b). The opposite is true for the tray with the lowest catches, where the wind originated from. This indicates that mosquitoes arrived at the downwind side of the odour source, implying that the perception of carbon dioxide was followed by positive anemotactic responses.

Direct observations of mosquitoes flying around the large rectangular net showed that, unlike tsetse flies, mosquitoes approached the net from the downwind side in a slow flying manner, and sometimes even landed on the frame or the underside of the roof suspended over it. On three occasions, when the net was charged with 2 kV, mosquitoes contacted the grid but were not electrocuted. With the 6 or 25 kV spark boxes this was not seen, which makes the overall number of collisions followed by escapes extremely low (<2%), particularly when compared with the much higher figure for tsetse flies (*ca.*

Table 3. *Catching efficiencies of a CO_2 (300 ml min^{-1}) baited rectangular electric net when powered by three different spark box capacities.*

	Sparkbox capacity (kV AC)								
	2	6				25			
Date	26/6	21/6	24/6	25/6	28/6	22/6	23/6	29/6	1/7
Hrs observed	2.75	2	2	2	2	1.5	3	1	1
No. approached	27	13	23	36	50	22	53	16	13
No. caught	12	5	4	20	22	6	31	1	6
% efficiency	44.4	38.5	17.4	55.6	44.0	27.3	58.5	6.3	46.2
Mosquito species									
Anopheles squamosus	2	1	0	0	0	0	3	0	1
Anopheles coustani	3	2	0	2	3	1	4	0	1
Anopheles gambiae s.l.	1	0	0	0	0	0	0	0	0
Anopheles funestus	1	0	1	2	7	1	1	0	1
Anopheles wellcomi	1	0	0	4	2	0	3	0	0
Mansonia africana	3	2	2	9	10	4	19	1	3
Mansonia uniformis	0	0	0	1	0	0	1	0	0
Culex spp.	1	0	1	2	0	0	0	0	0

30%, Griffiths & Brady, 1994). It is not clear, however, to what extent the torch-light influenced mosquito behaviour. There was no significant effect of the three different spark box voltage outputs on the trapping efficiency of the net ($F=0.1$, $p>0.05$) indicating that a continuous standing electrical charge across the net (with 2 or 6 kV spark boxes) does not affect mosquito behaviour. The average sampling efficiency for the three output voltages was 42.8%, which is similar to what has been reported for tsetse flies (45%, see Packer and Brady (1990)).

Discussion

The electric nets currently used in tsetse research have an overall sampling efficiency of 40-50% (Griffiths & Brady, 1994). Undersampling is mainly caused by a) the high proportion of flies bouncing off the grid, apparently unscathed and b) active avoidance behaviour of flies 'seeing' the net. The low flight speed at which mosquitoes collided with the nets made contact sufficiently long to stun or kill them. Since an unbaited net caught extremely low numbers, it is likely that randomly flying mosquitoes were aware of it and actively avoided colliding with the grid. Dispensing of odours at the grid, however, caused mosquitoes to stay near the net which increased their chance of being electrocuted. Even though our nets showed a relatively low catching efficiency when baited with carbon dioxide, it is likely that different configurations (distance between electrocuting wires, spark box capacity and discharge frequencies, size and shape of the net, more potent odour baits) can increase this figure. Also, since individual mosquitoes could have been making repeated approaches it is likely that our efficiency estimates were biased in a conservative manner. At present, the large rectangular net (frame size 60 x 40 cm) charged with 6 kV and wires placed at 4 mm distance (fig 1b) seems to be the best design at hand. Gillies *et al.* (1978) used 0.15 mm steel wires spaced at 5 mm intervals, and reported that approximately 24% of the mosquitoes passed through the grids without being stunned or killed, the overall efficiency being 75-80%. However, with wires at 4 mm intervals we never observed mosquitoes passing through the grid but still found a much lower efficiency which indicates that additional factors, yet unknown, influence the catching ability of the device. Infrared light-sensitive video systems can aid a further understanding of behaviour of mosquitoes around the nets. It is for instance of utmost importance to know whether landing responses can be induced, maybe with the addition of a visual stimulus ('target'), since this will open up avenues to develop odour-baited insecticide impregnated gadgets to control mosquitoes.

Human breath and carbon dioxide at a human equivalent mainly attracted zoophilic mosquitoes, which is in concordance with our findings that mosquitoes with a broad host spectrum respond well to carbon dioxide (Knols *et al.*, 1994b; De Jong & Knols, 1995). *Anopheles gambiae s.l.* and *An. funestus*, both highly anthropophilic (White, 1974) were not attracted to human breath or carbon dioxide which supports laboratory results (De Jong & Knols 1995b, Healy & Copland, 1995). In additional experiments (unpublished) we have shown that these species did not actively avoid the electric nets, but were readily caught on nets baited with human odour from a house (through lay-flat tubing). In West Africa Gillies & Wilkes (1972) and Costantini (pers. comm.) caught good numbers of *An. gambiae s.l.* with carbon dioxide and this seems to indicate differential responses to this compound across the African continent. Carbon dioxide has an activating effect on East African *An. gambiae s.s.* (Healy & Copland, 1995) and attracts mosquitoes in a windtunnel setup (Knols *et al.* 1994a) but its role as a kairomone in the open field remains unclear. However, as suggested by Gillies (1980), it is possible that carbon dioxide synergizes the effect of other host odours under field conditions (e.g. 1-octen-3-ol, see Kline and Takken, (1990)).

To our knowledge this study was the first to use cylindrical nets to study odour-mediated mosquito flight behaviour. The low flight speed of mosquitoes made it possible to assess approach directions to an odour source even when the diameter of the net was only 60 cm. Apparently optomotor-steered positive anemotaxis was used to locate the carbon dioxide source, a flight mechanism commonly used by insects to detect a source of odour (e.g. Murlis *et al.*, 1992). These results confirm earlier observations by Gillies and Wilkes (1969; 1972) who used ramp traps in a similar way to study orientation behaviour of mosquitoes towards calves and carbon dioxide.

To date, the knowledge on how mosquitoes orient themselves at night while searching for a host is still very limited, which is mainly caused by the absence of suitable sampling devices. The search for attractants which can be used to lure mosquitoes requires methods that can be operated in the absence of man whereas the study of odour-mediated flight behaviour can only be meaningful if sucking/blowing air currents (from traps) are absent. Electric nets meet these requirements and the work presented in this paper shows that they can be used in much the same way as in tsetse fly research.

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10

The response of *Anopheles gambiae s.l.* and *An. funestus* (Diptera: Culicidae) to tents baited with human odour or carbon dioxide in Tanzania[1]

ABSTRACT - Field studies on the response of *Anopheles gambiae s.l.* Giles and *Anopheles funestus* Giles to tents baited with human odour or carbon dioxide were conducted in South-East Tanzania. Two exit traps and a CDC miniature light trap set beside a bed net were used to sample mosquitoes that entered the tent. Human odour, pumped from an underground pit into a bed net attracted a similar number of mosquitoes as a bed net occupied by a man. Significantly fewer mosquitoes were caught in a tent into which carbon dioxide (300 ml min^{-1}) was pumped than in a human odour baited tent (9 and 27% for *An. gambiae s.l.* and *An. funestus* respectively). A five-fold increase of the carbon dioxide concentration (to 1500 ml min^{-1}) did not increase the catches of *An. gambiae s.l.* whereas those of *An. funestus* rose to 69% of the catches by man. Species identifications of *An. gambiae s.l.* catches showed that *An. arabiensis* prevailed and that the proportions of *An. arabiensis/An. gambiae s.s.* did not differ between treatments. It is concluded that in the indoor situation described, human odour other than carbon dioxide is the principle cue to which these malaria vectors are attracted and that the physical presence of a host and carbon dioxide, when used as a kairomone on its own, account for only a minor part of the overall attractiveness of man, particularly for *An. gambiae s.l.*.

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Introduction

Effective transmission of mosquito-borne diseases requires at least two successful contacts between female mosquitoes and their hosts. These contacts become established whenever a mosquito, by means of a complex behaviour, locates the host by responding to chemical, physical and visual cues produced by it (Clements, 1963, Takken, 1991). Methods aimed at interrupting this transmission necessitate a detailed knowledge of the factors governing this process. The relative importance of these factors varies between mosquito species and depends mainly on the degree of host-preference. The yellow fever mosquito, *Aedes aegypti*, is a diurnal anthropophilic species (Gillett, 1972a), and even though vision plays an important role during host-seeking (Gillett, 1972b), its specialism to feed on man requires olfactory responses to human-specific odours. *Eretmapodites chrysogaster*, a diurnal opportunistic species (Haddow, 1956), does not actively search for hosts but waits until a host enters its field of vision, after which olfactory cues guide its final approach. In the former case olfaction dominates the host-seeking process whereas in the latter case vision is the major cue used.

The extent to which each of these factors, or a combination thereof, influence the host-seeking behaviour of nocturnal *Anopheles* species remains largely unknown. By placing a cow in an underground pit, Vale (1974) separated visual and olfactory cues and demonstrated the importance of host odours in the host-seeking behaviour of tsetse flies (Diptera: Glossinidae). A similar experiment, to assess the attractiveness of human odour to mosquitoes in the absence of visual and physical cues has never been undertaken. The purpose of our study was to determine the response of the two most important African malaria vectors, *An. gambiae s.l.* and *An. funestus* to human odour or carbon dioxide when pumped from a pit and released inside tents which were modified for sampling mosquitoes. Specifically we sought to determine a) the relative attractiveness to mosquitoes of human odour in the physical presence or absence of man and, 2) the responses to different levels of carbon dioxide released from the tents.

Materials and Methods

Study area. The experiments were carried out at Kikulukutu village near

Namawala, Kilombero district, South-East Tanzania, during the long rainy season (March-June), when mosquito densities are highest. Kikulukutu borders an extensive rice-growing area which provides ideal breeding sites for mosquitoes. The ecological setting and demography of the area have been described by Smith *et al.* (1993) and Charlwood *et al.* (1995).

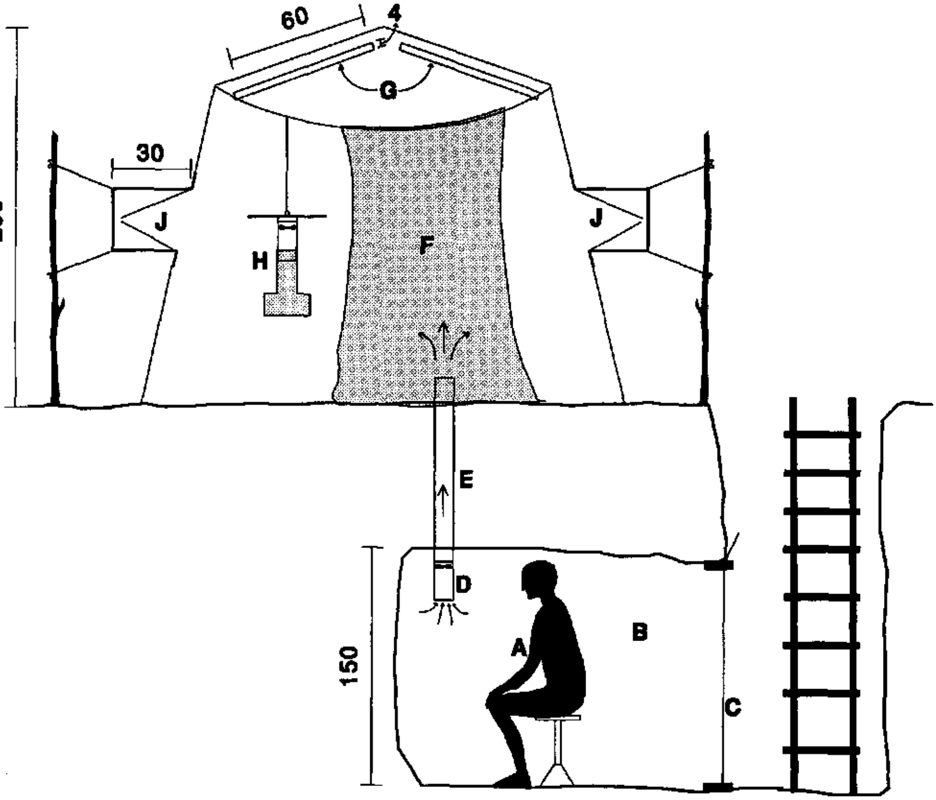

Figure 1. *The experimental setup (dimensions in cm). The test person (A) was seated in an underground pit (B), sealed by a polythene sheet (C). His odour was pumped by a fan (D) through a PVC tube (E) into a bed net (F). Mosquitoes entered through entry-slits (G) and were caught by a CDC light trap (H) or window exit traps (J).*

Experimental setup. The tents (Fig. 1) used in the experiments have been described by Knols *et al.* (1995). They were floorless, had a sloping roof 2m high at the apex, with a ground floor area of 1.9x1.9m, and were made of light-grey polyvinylchloride (PVC). Mosquitoes could enter through slits just beneath the roof on both sides of the tent, which overhung (20 cm) and prevented direct outside light from entering the tent. Two exit traps (Muirhead-Thomson, 1948) were fitted to the walls of the tents. Inside, an unimpregnated rectangular bed net was hung, next to which a standard miniature CDC light trap (John W. Hock Co., FL, USA) was operated, the shield of the trap being suspended 1 m above ground level close to the net (Lines *et al.*, 1991).

Underneath each tent a pit was dug, large enough to contain a test person. A 1m long PVC pipe (diameter 11 cm), to which the fan of a CDC light trap was fixed, protruded 15 cm above ground level in the tent and was used to pump odours (at *ca.* 1.5 m sec^{-1}) from the pit into the bed net. The pits could be closed with a polythene sheet, pinned onto a wooden door frame. The distance between the tents was 30 m, and they were situated *ca.* 50 m from the nearest village house.

Experimental protocols. The first experiment investigated the feasibility of catching mosquitoes with only human odour being present in the tent. Odour from a 29 year old male Tanzanian volunteer was pumped from the pit between 23.00-03.00 hrs during four nights. 'Odour' from an unoccupied pit was pumped into a second tent which served as a control. Each night the test person would alternate between the pits. This would not only exclude day or site effects but would also show whether pits could become contaminated by odour residues which would temporarily make them unsuitable for use as a control treatment. The fans in the pits were left running from 03.00 hrs until dawn as a precautionary measure against this.

The second experiment compared the attractiveness of a man seated inside a bed net versus a bed net baited with human odour pumped from a pit. Two Tanzanian test persons, 29 and 35 years of age, alternated between the two positions (bed net or pit) and the two sites for a total of 16 nights (between 23.00-06.00 hrs).

A third series of experiments compared the attractiveness of a man inside a bed net versus a bed net baited with carbon dioxide. The gas was dispensed from a pressurized gas cylinder through 5 mm silicon tubing, passed a flow-meter, and was then led into the tent under the bed net. The outlet of the tubing was fixed over a CDC fan at ground level to increase the dispersal of the gas. Similar fans were installed under the test person and in the control tent. Two doses of carbon dioxide were tested, 300 ml min^{-1} and 1500 ml min^{-1}, being the equivalent expired in breath by one or five adults respectively. The first dose was tested for 3 nights between 23.00-03.00 hrs, the second dose for 6 nights between 24.00-02.00 hrs. A third tent with neither a human bait nor carbon dioxide was used as a control.

In all experiments a tent, bed net and traps were assigned to the respective treatments and moved daily between sites to avoid contamination with odours.

The proportions in which *An. arabiensis* and *An. gambiae s.s.* occurred in catches with human odour and carbon dioxide (300 ml min^{-1}) were assessed by identifying specimens with the polymerase chain reaction (Scott *et al.*, 1993).

Data collection and analysis. Exit traps were emptied, and CDC light trap catches retrieved from the tents at 06.00 hrs every morning. To prevent daytime resting mosquitoes from being caught the following night the interior of the tents was thoroughly searched for mosquitoes which were then removed from it. Catches from light traps and exit traps were summed, transformed to $\log(n+1)$ and after a satisfactory check for normality of the distribution subjected to Latin square analyses of variance (Snedecor & Cochran, 1989). An F-test significant at $p<0.05$ was followed by a Least Significant Different test to sort out differences between treatment means.

Results

Table 1 shows that human odour, pumped from a pit, attracted significantly more mosquitoes of both species than 'odour' from a control pit. Within the four-day experimental period no effect of odour residues on the catch in the control treatment could be noticed, these being 0,4,0,5 for *An. gambiae s.l.* and 0,1,1,3 for *An. funestus.*

When the catches of mosquitoes collected from a man-baited tent were compared with those from a human odour-baited tent (Table 2) it was found that human odour was the single most important factor determining the size

Table 1. *ANOVA tables, log-transformed mean catches/day and indices of comparison for human odours pumped into a tent from an underground pit versus odours pumped from un unoccupied pit (control).*

Anopheles	Source	df	MS	F-value	Odour	n	Mean	Index	Aa/Ag	pcr
gambiae s.l.	Day	3	0.020	0.63ns	human	106	21.90	1a	0.84	69
	Site	1	0.760	24.39*	control	9	1.34	0.061b	0.89	9
	Odour	1	1.963	62.97**						
	Error	2	0.031							
funestus	Day	3	0.074	3.16ns	human	34	7.32	1a		
	Site	1	0.004	0.17ns	control	5	1.38	0.188b		
	Odour	1	0.592	25.43*						
	Error	2	0.023							

df= degrees of freedom; MS= Mean squares; *: $p<0.05$; **: $p<0.025$; ns=not significant
n: number of mosquitoes caught; indices not followed by the same letter are significantly different at $p<0.05$.
Aa/Ag: proportion of *An. arabiensis/An. gambiae s.s.*; pcr: number identified.

Table 2. *ANOVA tables, log-transformed mean catches/day and indices of comparison for human odours pumped into a tent from an underground pit versus a man occupied tent.*

Anopheles	Source	df	MS	F-value	Human inside:	n	Mean	Index
gambiae s.l.	Block	3	0.044	0.37ns	Tent	45	2.14	1a
	Site	1	0.002	0.01ns	Pit	68	3.01	1.405a
	Position	1	0.089	0.75ns				
	Person	1	0.007	0.06ns				
	Error	25	0.119					
funestus	Block	3	0.047	0.59ns	Tent	204	10.94	1a
	Site	1	0.014	0.18ns	Pit	161	8.35	0.764a
	Position	1	0.089	1.13ns				
	Person	1	0.013	0.17ns				
	Error	25	0.079					

df= degrees of freedom; MS= Mean squares; ns=not significant
n: number of mosquitoes caught; indices not followed by the same letter are significantly different at $p < 0.05$.

Table 3. *ANOVA tables, log-transformed mean catches/day and indices of comparison for a human occupied tent versus a 300 or 1500 ml min^{-1} CO$_2$ baited tent.*

Anopheles	Source	df	MS	F-value	Odour	n	Mean	Index	Aa/Ag	pcr
gambiae s.l.	Day	2	0.043	2.16ns	Human	300	99.20	1a	0.78	156
	Site	2	0.039	1.95ns	CO$_2$ (300 ml/min)	32	8.80	0.088b	0.87	31
	Odour	2	2.192	108.90*	Control	3	1.00	0.010c	0.50	2
	Error	2	0.020							
funestus	Day	2	0.001	1.52ns	Human	56	17.70	1a		
	Site	2	0.098	108.19*	CO$_2$ (300 ml/min)	16	4.80	0.274b		
	Odour	2	1.035	1139.46**	Control	1	0.26	0.015c		
	Error	2	0.000						Aa/Ag	pcr
gambiae s.l.	Day	5	0.106	1.83ns	Human	126	18.37	1a	0.64	106
	Site	2	0.040	0.69ns	CO$_2$ (1500 ml/min)	13	1.46	0.080b	0.55	11
	Odour	2	2.295	39.47**	Control	2	0.26	0.014c	1.00	1
	Error	8	0.058							
funestus	Day	5	0.082	1.15ns	Human	67	9.51	1a		
	Site	2	0.070	0.97ns	CO$_2$ (1500 ml/min)	51	6.59	0.693a		
	Odour	2	1.476	20.67**	Control	1	0.26	0.027b		
	Error	8	0.071							

df= degrees of freedom; MS= Mean squares; *: $p<0.01$; **: $p<0.001$; ns=not significant; n: number of mosquitoes caught; indices not followed by the same letter are significantly different at $p<0.05$. Aa/Ag= proportion of *An. arabiensis*/*An. gambiae s.s.*; pcr= number identified.

of catches since the physical presence of man in addition to his odour did not increase catches significantly. None of the other main effects were significant, and the two volunteers were equally attractive to both mosquito species.

When tents were baited with the equivalent of carbon dioxide produced by an adult human catches were only 9 and 27% (for *An. gambiae s.l.* and *An. funestus* respectively) of the catch obtained from a tent occupied by a man, in both cases a significant reduction (Table 3). However, carbon dioxide caught significantly more mosquitoes than unbaited tents. A five-fold increase in the amount of carbon dioxide released in the tent did not affect *An. gambiae s.l.* catches, but increased the *An. funestus* catch to 69% of that by man, which made both treatments equally attractive for this species. Species identifications showed that the majority of *An. gambiae s.l.* were *An. arabiensis*. The relative proportions of *An. arabiensis*/*An. gambiae s.s.* in human odour and carbon dioxide samples were not significantly different from each other (X^2 test).

Discussion

By pumping human odour from an underground pit into a tent we excluded the influence of visual cues, convection currents and radiant body heat, the remaining stimuli being moist air, body odours and carbon dioxide. An immediate consequence of pumping odours is that they become diluted with air. Some 855 litres min^{-1} of odour-laden air were pumped from the pit into the tent, which meant that the entire volume of the tent (*ca* 7 m^3) was replaced every 8 min. The 300 ml min^{-1} of carbon dioxide expired by the test person (4.5% in exhaled breath) was diluted in this volume and mixing by the fan and diffusion inside the tent could have reduced the concentration to a minimum of 0.035%, similar to background levels (0.03-0.04%, Richards, 1952). The active range of attraction of this carbon dioxide when leaving the tent must therefore have been small, probably less than a metre (Gillies, 1980). The attraction of both mosquito species can then only be explained as orientation from a distance to body odours other than carbon dioxide. It is concluded that body odour guided the mosquitoes to the immediate vicinity of the tent, where, prior to entering it, they experienced increased carbon dioxide and moisture levels near the entry slits. This is similar to what was found for *An. melas* (a member of the *An. gambiae* complex) in an outdoor situation in The Gambia, where the active range of attraction was much larger than what could be expected on the basis of carbon dioxide expired by the calves used in that study (Gillies and Wilkes, 1969; 1970).

Within the experimental periods we could not find an effect of odour residues, left behind by the test persons in the pits, on mosquito catches. This effect, however, has been described for both species by Haddow (1942), who found host-seeking mosquitoes visiting houses for a period after they had been vacated. Similarly, in Burkina Faso, Costantini (*pers. comm.*) found new houses to be much less attractive than old structures even though both were occupied by a similar number of people and were constructed of similar materials. How long it takes before the effect becomes apparent, and how long it takes to cease remains unclear but taking such effects into account is of utmost importance in research involving experimental units (huts, tents) occupied by man.

After the mosquitoes entered the tent, the physical presence or absence of a human (in the bed net) did not affect the total number caught. This indicates that even if visual and physical properties of the host are presented to the mosquito, albeit from within the bed net, chemical cues still dominate the host-seeking process at this stage of final approach, *ca.* 2 m from the host. Although it has long been thought that body odours only play a role at distances from the host where physical and visual cues are not detectable (Laarman, 1955; Clements 1963, Gillies & Wilkes, 1972) the present results show that they continue to play a role even in the immediate vicinity of the host. This supports laboratory studies in which it was demonstrated that even the selection of biting sites on a human host by *An. gambiae s.s.* was odour-mediated (De Jong & Knols, 1995).

When the attractiveness of carbon dioxide was compared with that of a man, an important difference was the fact that no dilution of odours took place inside the tent. The fans, installed in the bed nets, increased the circulation of odour but did not influence the total volume of odorous air in the tent, which probably resulted in a more passive diffusion of odours through the entry slits. Consequently a gradual built-up in the carbon dioxide concentration occurred in the tents during the experimental period, and the concentration near the entrance slits must therefore have been higher than in the other experiments. Nevertheless, the equivalent of carbon dioxide produced by an adult human attracted significantly fewer mosquitoes of both species than a test person, though for both species the catch was significantly higher than that of the control tent. The large reduction in catch compared to a human baited tent could have been caused by the smaller range of attraction of carbon dioxide. However, a five-fold increase in carbon dioxide concentration should then have extended this range and attract more mosquitoes, which for *An. gambiae s.l.* was clearly not the case. In contrast, *An. funestus* catches rose to the extent that they were no longer different to those from the test person. For *An. gambiae s.l.* this could have meant that the low dose of carbon dioxide already induced the maximum response, which therefore could not be increased by a higher dose. For *An. funestus* the responses were clearly enhanced by the higher dose. However, several additional factors could have caused the increase of catches for this species. Firstly, in East Africa, *An. funestus* belongs to a species complex (Gillies & Coetzee, 1987), and it is possible that zoophilic members of this complex, such as *An. rivulorum*, represented a larger proportion of the catch when higher doses were used. Both species occur sympatrically in this region, but their relative contributions to the catches could not be ascertained due to difficulties of identifying *An. rivulorum* from CDC light trap catches. Secondly, even if similar numbers were attracted to the tents by both doses, the higher dose could have enhanced the slit-entry response, resulting in higher catches. Furthermore, even if the number that entered the tents was similar, it is not known what proportion escaped through the entry slits. The higher dose of carbon dioxide might have stimulated mosquitoes to remain around the bed net longer which therefore increased their chance of being caught. Whether differential responses towards varying doses of carbon dioxide took place at the physiological or behavioural level therefore remains unclear.

Although carbon dioxide is considered to be a universal attractant for blood-feeding Diptera, our observations have indicated that the degree of attraction of these anthropophilic mosquito species to this compound is rather limited. In a separate field study by Knols and Mboera (1996) it was shown that both *Anopheles* species were not attracted to human breath or the equivalent of carbon dioxide therein in an outdoor situation. It is likely that these anthropophilic mosquitoes show less dependency on carbon dioxide in their host-seeking behaviour since this compound provides no information on the identity of the host upwind of them. It is somewhat surprising that the more zoophilic *An. arabiensis*, which dominated in the catches, did not show a higher response to carbon dioxide but apparently relied more heavily on other odours to locate the source. *An. gambiae s.s.* is activated by (Healy & Copland, 1995) and attracted to carbon dioxide in the laboratory (Knols *et al.*, 1994), and its role as a kairomone has clearly been established. Similar results have been reported for *An. arabiensis* (Omer, 1979). In the field, however, the biological significance of carbon dioxide might only be expressed in the interaction with other odours, perhaps in a synergistic manner, in the proximity of the host. Currently the relative attractiveness of a test person from which the breath has been removed or the carbon dioxide has been filtered is being assessed as a next step to unravel the role of this kairomone in the host-seeking behaviour of these important malaria vectors.

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11

General discussion and conclusions

The lack in understanding of mosquito-host interactions has led to generalizations based on findings for mosquito species with major differences in their behavioural ecology. For instance, the variability in host range and circadian activity are believed to be important determinants influencing the perception of and behavioural responses to host-related cues. It seems obvious, therefore, that diurnal, exophagic, exophilic and zoophilic mosquitoes will exploit the available cues in a different way than nocturnal, endophagic, endophilic and anthropophilic species. The vast majority of research on mosquito host-seeking behaviour has focused on the diurnal anthropophilic *Ae. aegypti*. This species, however, shows relatively high levels of plasticity in these characteristics (Mukwaya, 1976; 1977), which may not be surprising considering its widespread distribution throughout the tropics.

Studies on the host-seeking behaviour of the members of the *An. gambiae* complex have been comparatively few and have been summarised in chapter 1. These have revealed that even for individual sibling species the plasticity in characteristics such as host range and in/outdoor feeding can be substantial in different areas of its distribution range. Moreover, even within single localities sibling populations have been found to differ in behavioural characteristics such as house-entry behaviour (Coluzzi *et al.*, 1979). These populations show differences in their genetic make-up and may well be undergoing a process of becoming genetically isolated, and their divergence may lead to variations in host-seeking strategies.

In the early stages of *Anopheles* research it was realised that the extrapolation of findings for other mosquito species to that of *An. gambiae s.s.* would be difficult, if not impossible. No references to laboratory studies on odour-mediated host-seeking behaviour of *An. gambiae s.s.* were available, and field studies reported primarily on *An. gambiae s.l.*. The majority of field studies on sibling species within the complex involved the West African *An. melas* (Gillies & Wilkes, 1969; 1970; 1972).

The importance of chemical cues in host-seeking by *Anopheles gambiae s.s.*

The identification and synthesis of behaviourally active human odours for *An. gambiae s.s.*, necessitated clarification of the relative importance of chemical cues in the host-seeking process of this species. This question was addressed in Burkina Faso with the use of a so-called OBET trap (Odour-Baited-Entry-Trap)(Costantini *et al.*, 1993; 1996) and in Tanzania with tents modified for sampling endophilic/endophagic mosquitoes (chapter 7) and electric nets (chapter 9). In both countries, albeit with different sampling techniques, it was observed that *An. gambiae s.s.* and *An. arabiensis* significantly preferred human odour above an equivalent of carbon dioxide produced by man. Also, in both situations, it was observed that an increase of the carbon dioxide concentration to levels five times higher than that produced by a single man, did not affect catch levels. However, the proportions of *An. gambiae s.s.* caught with carbon dioxide relative to the numbers caught with human odour differed markedly between the two countries. In Burkina Faso, carbon dioxide attracted *ca.* 50% as many *An. gambiae s.s.* compared to human odour, whereas in Tanzania this was only 9%. This 5.6-fold difference could have been caused by differences in trapping techniques, but it may well be that differential responses to carbon dioxide occur in both populations. Nevertheless, the real importance of these findings was the fact that, in particular in the Tanzanian situation, human odour other than carbon dioxide plays a major role in the attraction of *An. gambiae s.s.* to its host. Since in both situations man was not physically present, but only odour was presented to the mosquitoes, these experiments were very much similar to the initial trials showing the importance of olfactory cues in host-seeking by tsetse flies (Vale, 1974). Further evidence for the limited role of carbon dioxide in host-seeking by this mosquito was provided by results from experiments in which electric nets were baited with human breath (including carbon dioxide) or carbon dioxide only (chapter 9). Human breath or an equivalent of carbon dioxide therein attracted large numbers of zoophilic mosquito species, but both highly anthropophilic species (*An. gambiae s.s.* and *An. funestus*) were not attracted to these odours. In contrast, an (outdoor) electric net baited with human odour (through lay-flat tubing) from a bedroom occupied by four people attracted both these species (Knols & Mboera, unpublished data). In Burkina Faso it was also shown that human odour minus breath/head odour attracted 60% as many *An. gambiae s.l.* as whole human odour (C. Costantini, *pers. comm.*). These results demonstrated that carbon dioxide is not a prerequisite for attracting *An. gambiae s.s.*. Similar results were obtained by Snow (1970) who observed that even though a carbon dioxide absorbing apparatus reduced the overall number of *An. gambiae s.l.* attracted, it did not entirely prevent mosquitoes locating and biting the host. Results by Healy & Copland (1995) support the limited role of breath volatiles (other than carbon dioxide) for East African *An. gambiae s.s.*, similar to what was reported by De Jong & Knols (chapter 5) while working with the same strain of mosquito species.

Notwithstanding the fact that carbon dioxide alone evokes clear behavioural responses in *An. gambiae s.s.* (chapter 4) it is concluded that it is only partially responsible for the overall attractiveness of man. It not being a cue signifying the identity of a potential blood host, these findings support our initial hypothesis (based on the reliability/detectability hypothesis by Vet & Dicke (1992)) that human-specific odours must play a more prominent role in host-seeking by this mosquito species than carbon dioxide. Moreover, the results indicate that it may well be possible to attract *An. gambiae s.s.* in the absence of carbon dioxide, which is an important prerequisite for the development of odour-baited traps for use under field conditions.

Selection of biting sites by mosquitoes on man: a new approach to kairomone identification

The first part of this thesis described observations on the selection of biting sites by five different mosquito species on the same human host. The original idea to study this phenomenon stemmed from reported preferred landing and feeding sites by mosquitoes as well as other Diptera of medical and veterinary importance. Haddow (1945) found that irrespective of position a preference for biting the face of naked humans existed for *Ae. simpsoni*. Gillett (1971) reported biting by *Sabethes belisarioi* to occur exclusively on the nose of man. Personal observations on *An. gambiae s.l.* in Africa and on colonized specimens of this species showed that a preference for biting of the lower extremities (foot region) exists. A similar preference for biting the foot region was also found for the highly anthropophilic *An. farauti* in Papua New Guinea (Charlwood, 1984). The laboratory studies reported in chapters 2 and 3 not only revealed that four out of five species showed a preference for biting a specific part of the body but it was shown for the first time that odours from these regions play a role in this process. It was demonstrated that foot odour plays a role in the selection of biting sites by *An. gambiae s.s.* and breath influences this process for *An. atroparvus* and *An. albimanus*. Furthermore it should be noted that the preferred region of biting by *An. albimanus* (on the face) was predicted *a priori*, after examining the broadness of its host range (chapter 3). Thus, the levels of anthropophily/zoophily may be reflected in the selection of biting sites by different mosquito species. Both *An. atroparvus* and *An. albimanus* are opportunistic and their preference for biting the face reflected their responses to exhaled air (and most likely its carbon dioxide content). Carbon dioxide is produced in relatively high quantities by all vertebrate hosts and thus provides both a reliable and detectable source for opportunistic mosquito species. Accordingly, it was assumed that *An. gambiae s.s.* responded to human-specific components of foot odour.

It would thus be of interest to establish whether the selection of biting sites by the different members of the *An. gambiae* complex differs. Preliminary results for *An. arabiensis* show a similar pattern as that for *An. gambiae s.s.* but the plasticity in host-preference by this sibling species may well have influenced its selectivity. Populations of *An. arabiensis* in the field (South Africa) also bit the feet and ankles (Braack *et al.*, 1994). If, however, the strictly zoophilic *An. quadriannulatus* shows a similar preference for biting the face as *An. atroparvus* and *An. albimanus* then this methodology will indeed provide a tool for examining levels of anthropophily/zoophily. Such a tool could then be applied in genetic studies in which the aim is to drive genes coding for zoophily into anthropophilic *An. gambiae s.s.* populations using transposable elements. Alternatively, successive back-crossing of female *An. gambiae s.s.* with *An. quadriannulatus* males may ultimately render the former species more liberal in its host-selection (C.F. Curtis, *pers. comm.*). The successes of these technologies could then be examined in the laboratory by observing the gradual change from 'foot' biting to 'face' biting of *An. gambiae s.s.*.

Additional experiments on the selection of biting sites by *An. gambiae s.s.* have shown that not only foot odour but also convection currents influence this process. When the test person was laying, hence reducing convection currents to a minimum, biting occurred randomly over the body. When the subject was standing, and only one foot was washed with bactericidal soap (see chapter 2), a significant preference was found for biting the unwashed foot (De

Jong & Knols, unpublished data). Positioning of the host thus seems to influence the selection of biting sites. Similar findings were reported for *Cx. quinquefasciatus* (Self *et al.*, 1969) and *Er. chrysogaster* (Haddow, 1956) observing biting below knee level while seated and random biting in a laying position. Although it can be concluded that observations on the biting behaviour of haematophagous Diptera may reveal the origin of chemical cues that play a role in host-seeking other factors may affect preferential biting as well. Even though Vale (1974) observed *G. m. morsitans* biting on the head and eye region of warthog, and suggested that odour from this region could be a causal factor, it was later shown that fly density was a major determinant. High density levels evoked increased levels of defensive behaviour of the warthog, *i.e.* grooming and tail whisking, which diverted the flies from biting the body to the head (Torr, 1994). Other preferences for biting the heads of hosts have been found for tabanids (*Chrysops* spp.)(Magnarelli & Anderson, 1980), sandflies (in Lane, 1993), and blackflies (Duke & Beesley, 1958). Parasite-induced preferences for specific biting sites, thus increasing the transmission of the pathogen, have been reported for microfilariae that cluster at certain sites under the skin dermis (P. Mellor, *pers. comm.*) and for *Leishmania* parasites, in which sandflies fed predominantly on cutaneous lesions (Coleman & Edman, 1988). Visual responses to hosts, host-hairiness, thickness of the skin, length of the proboscis and combinations of several of these factors have also been found to cause unequal distributions of biting by insects on hosts (Knols & De Jong, *in prep.*).

Odour-mediated host-seeking behaviour and kairomone identification: a laboratory approach

The second part of this thesis focused on the development of a laboratory bioassay and the subsequent search for human odour attractive to *An. gambiae s.s.* using this bioassay. Windtunnel studies on this species originally examined the flight responses of individual females to odour complexes such as those collected from a human arm or human breath (e.g. Takken & Knols, 1990). These were recorded using infra-red sensitive video equipment. Even though these emanations elicited responses of *An. gambiae s.s.*, data analyses were often laborious and responses were never studied simultaneously with control odours. This hindered objective analysis of results because of daily variation in response levels.

The development of the bioassay as described in chapter 4 resulted in a system which is almost similar to the conventional dual-port bioassay developed for studying olfactory responses of *Ae. aegypti* (Schreck *et al.*, 1967). Both systems are designed to study responses of groups of females to test odour(s) which can be directly compared to control odour(s). Initial responses to conditioned air entering the flight chamber from both ports showed the absence of preference for any of them, thus demonstrating the symmetry of the setup. The marked variation in catches could initially not be attributed to any environmental factor, but has since been shown to depend heavily on the relative humidity of the air entering the chamber, and most likely the difference in RH between the air in the flight chamber versus that entering it (H. Otten, unpublished data). Initial studies showed clear responses to carbon dioxide (4.5%), but these decreased when RH values of the test odour were lower than that of the flight chamber.

Responses towards human breath of three volunteers did not indicate an influence of odour other than carbon dioxide on mosquitoes in this set-up. Since then these findings have been confirmed by Healy & Copland (1995), who studied responses of individual female *An. gambiae s.s.* towards human breath in a windtunnel. Their studies were done with *An. gambiae s.s.* from the same locality in Tanzania and upwind flight responses were attributed to the activating effect of carbon dioxide only. An important consideration should be that possible interactions between breath odours and other body odours can not be ruled out. The absence of clear responses towards human breath supports the findings from the experiments on the selection of biting sites reported in the previous chapters. In these experiments *An. gambiae s.s.* was observed flying through the cone of exhaled breath without any noticeable change in behaviour, much in contrast with the behaviour observed for *An. atroparvus.* Laarman (1955) found marked responses towards human breath in windtunnel studies with this latter species.

Headspace samples of small quantities of Limburger cheese elicited clear responses by *An. gambiae s.s.*, as reported in chapters 6 and 7. Preceding studies on the effect of human foot odour in a smaller windtunnel showed clear responses of *An. gambiae s.s.* to so-called 'anklets' worn around the ankles of a volunteer. The absorption of water in the control anklet was compensated for and revealed that foot odour was responsible for the observed attraction. The significance of these findings was not only that foot odour attracted this mosquito, but that this effect could be observed in the absence of carbon dioxide (A. Roosen, unpublished data). Similar results have been obtained with extracts from diethyl ether washings of human feet (T. Healy, *pers. comm.*).

The main advantage of the fact that *An. gambiae s.s.* showed clear responses to Limburger cheese volatiles has been the drastic reduction in the broadness of the spectrum of compounds present in human foot odour responsible for this effect. Headspace analyses of Limburger cheese identified C_2-C_{18} carboxylic acids as the main constituents and the similarity in the acid content of toe scrapings and Limburger cheese volatiles is clearly present. Fatty acids are, however, produced over the entire skin surface (Stoddart, 1990) and it may well be that a specific blend of volatile acids emanating from human feet causes the observed behavioural responses. Some preliminary experiments with only the acids of higher (C_2-C_6) or lower volatility (C_8-C_{16}) did not result in similar responses as the entire spectrum of acids found in the cheese's headspace, which indicates that the behavioural effect only occurs in the presence of compounds from both groups. Recent experiments with *Ae. aegypti* have shown that besides the known attractant L-lactic acid (Acree *et al.*, 1968) two additional fractions from skin washing are necessary to elicit the same level of attractiveness as an entire skin extract (Geier & Sass, 1996). These findings indicate that behavioural responses may occur to a limited number of compounds (or fractions of skin extracts), but that similar levels of attractiveness as whole skin extracts may only be obtained if the full scala of behaviourally active compounds are present ('pattern recognition', Pappenberger & Boeckh, 1996).

A further interesting aspect of the attraction of *An. gambiae s.s.* to Limburger cheese is the fact that its odour originates from the metabolic activity of its surface microflora, which consists of a single species namely *Brevibacterium linens*. Closely related coryneform bacteria (e.g. *Brevibacterium epidermidis*) are found on human feet and have been reported to contribute to foot odour (Anthony *et al.*, 1992; Pitcher, 1977). It should be realised that other cheeses also produce fatty acids, but do not have these species of bacteria in common. Despite the similarity in bacteria species of cheese and feet it can therefore not be concluded that this micro-organism is solely responsible for the production of (a group of) kairomones for *An. gambiae s.s.*. On the human skin other bacteria, such as *Micrococcus* and *Staphylococcus* spp., have been reported to produce fatty acids as well, for instance in the axilla (Rennie *et al.*, 1991). In preliminary experiments in which we isolated *S. epidermidis* from human feet we observed that it produced 2-methylpropanoic and 3-methylbutanoic acids while growing on a blood agar medium. Though we have not (yet) been able to attract *An. gambiae s.s.* to volatiles directly produced by bacteria on artificial media, these preliminary results suggest that the skin microflora (at least partially) influences the olfactory mediated part of the host-seeking process (see below).

Similar phenomena, i.e. the attraction of insects to odours produced by microorganisms on their host, have been observed. New world screwworm, *Cochliomyia homnivorax*, is attracted to fatty acids produced by bacteria resident in wound fluid of hosts (Cork, 1994). *Hydrotaea irritans* is attracted to ethanoic, propanoic and 2-methylpropanoic acid, produced by bacteria causing summer mastitis in cattle (Thomas *et al.*, 1985).

Though fatty acids may only be part of the blend of human odour attracting *An. gambiae s.s.*, it is interesting to note that large variations exist in the production of these compounds on the skin (e.g. Downing *et al.*, 1969). The skin surface lipid composition of different individuals is normally very constant with respect to squalene, sterol and wax esters, but the resident microflora determines the breakdown levels of triglycerides to free glycerol during which free fatty acids are liberated. Thus it is tempting to explain the variations in attractiveness of individuals to *An. gambiae s.s.* on the basis of the metabolic activity of their resident skin microflora. The source of triglycerides is sebum, which is excreted by the sebaceous glands, distributed over the entire skin surface. Sebum production increases during sexual maturity, and the reported preference of *An. gambiae s.s.* for biting adults over children may, at least in part, be based on large differences in fatty acid production. If the human skin microflora is indeed responsible for the attraction of *An. gambiae s.s.* to man, which the attraction to volatiles of bacterial origin of Limburger cheese suggests, then this merits the inclusion of a new field of research within the context of this work namely skin microbiology. The normal human skin microflora consists of several species in varying densities depending on such factors as skin pH, skin humidity etc. Each species of bacteria has its own ecological niche which may be altered by favouring conditions for other species. The application of systemic pro-biotica, a relatively new field in skin microbiology (Tannock, 1994), may thus favour conditions on the human skin for species of the microflora which do not produce kairomones that attract *An. gambiae s.s.*, altering the blend of kairomones (thus interfering with 'pattern recognition') or even decrease populations of kairomone producing bacteria.

Host attractiveness and odour-mediated host-seeking behaviour of mosquitoes: a field approach

The latter part of this thesis described the results of field experiments in Tanzania. In conjunction with the suggestion that the human skin microflora is (in part) responsible for the attraction of *An. gambiae s.s.* it was assessed whether isolated individuals showed differences in attractiveness to mosquitoes. Using an objective means to catch mosquitoes attracted to three individuals it was shown that three anthropophilic species (*An. gambiae s.l.*, *An. funestus* and *Culex quinquefasciatus*) were attracted to one person in higher numbers than to the other two volunteers. *Mansonia* spp., which are more opportunistic, did not show a preference. Even though differences in attractiveness to malaria vectors were reported in the past (e.g. Lindsay, 1993), this experiment demonstrated for the first time that isolated individuals also vary in their attractiveness. Secondly it was observed that whenever a group of people is exposed to mosquitoes not only the numbers caught vary, but that the relative contribution of different species to the catches varies as well. This seems to imply that different mosquito species either use different kairomones or respond to different 'patterns' of the same compounds. A practical application of this work is that mosquito sampling using the human biting catch (HBC) is more effective when catchers are active individually instead of in groups.

Two new methods were developed for studying odour-mediated host-seeking behaviour of mosquitoes in the field. Tents were modified for sampling endophagic mosquitoes by constructing artificial eaves for their entry and instalment of exit- and CDC traps inside the tent. Several of these tents were used in latin square designs, and a major advantage was the fact that they could be moved between sites and be assigned to single treatments, thus preventing contamination with different odours. It could be demonstrated that only human odour is responsible for differences in attractiveness of humans to mosquitoes (chapter 7), that the physical presence of man does not affect the number of mosquitoes attracted when compared with human odour only, and that carbon dioxide accounts for only 9% of the overall attractiveness of man (chapter 10).

Electric nets are extensively used for studying host-seeking behaviour of tsetse flies. Although attempts were undertaken to use similar nets in the USA (S. Torr, *pers. comm.*) for sampling mosquitoes, these failed to do so. However, modification of the commonly used nets, by altering the distance between electrocuting wires, different charge voltages etc., resulted in systems which are equally effective in sampling mosquitoes than those used for tsetse flies (chapter 9). Furthermore, these studies showed that under natural conditions mosquitoes engage in positive anemotaxis upon perceiving host odour (carbon dioxide). The electric nets were able to attract and catch several mosquito species present in the area, and thus provide a new tool for objective sampling of mosquito populations and studying the attractiveness of laboratory identified kairomones under field conditions.

The attractiveness of laboratory identified kairomones was tested in Tanzania, during two rainy seasons when densities of malaria vectors are highest. Originally, unlighted standard CDC light traps were baited with Limburger cheese, much in the same way these traps are baited with 1-octen-3-ol (Takken & Kline, 1989). Unbaited traps rarely catch *An. gambiae s.l.* in an outdoor situation. The maximum catch obtained with 0.5 gr of cheese was six *An. gambiae s.l.* on a single night. Cheese extracts and fatty acid mixtures never showed any effect on catches compared to controls. Direct observations (at dusk) showed that the air current produced by light traps prevented many mosquitoes from approaching the vicinity of the trap, and it may well be that *An. gambiae s.l.* were attracted to the odours in higher numbers but were simply not caught. Alternatively it may well be that response levels to the odours similar to those in the laboratory can only be reproduced in the field in the presence of other yet unidentified compounds. Windtunnel tests always brought females in the direct vicinity of the odour source (ca. 1 m), and these odours may not be behaviourally active at greater distances. The laboratory observations that the RH of the air into which the odours are pumped influences response levels could mean that moistened air in addition to odour might also be important in the field. The composition of the odour presented to the mosquitoes in the laboratory was fairly constant due to renewal of the sample for every test (lasting 20 min). However, it may be that the release of odours (due to differences in volatility) in the field was different because of longer testing intervals. Lastly, the tests with odours always occurred in the vicinity of occupied houses, since a) this is where highest densities of vectors occur, and b) this is where interception of host-seeking females should occur with odour-baited trapping devices. However, it can be expected that the attractiveness of a limited number of human odours is outweighed by the full blend of human compounds from nearby huts (normally occupied by several individuals). This 'competition' effect may thus interfere with experiments assessing the responses of mosquitoes towards artificial baits.

Conclusions

With respect to the specific research goals (in bold face) the following conclusions can be drawn:

1. to investigate the importance of chemical cues in the host-seeking behaviour of *An. gambiae s.s.*.

- Carbon dioxide at a human equivalent caught 9% of the number caught with man. A five-fold increase in the carbon dioxide concentration did not alter this figure (chapter 10).
- Human breath did not elicit behavioural responses of *An. gambiae s.s.* in the laboratory bioassay, nor in the field (chapter 5 & 9).
- Carbon dioxide at 4.5% was highly attractive in the laboratory bioassay (chapter 4).
- Though carbon dioxide has been shown to elicit responses of *An. gambiae s.s.* in the laboratory, its role as a kairomone under field conditions remains unclear (chapter 9,10). Human odour (other than carbon dioxide) plays a prominent role in the attraction of this species to a host (chapter 10).

2. to develop tools for studying these chemical cues, in particular carbon dioxide and human odours, both under laboratory and field conditions.

- A laboratory bioassay for rapid screening of the 'attractiveness' of odours was developed (chapter 4).
- Tents were modified for sampling mosquitoes, by installing window exit traps and constructing artificial eaves (chapter 7). Pits

were used to examine the effect of human odour in the physical absence of man (chapter 10).

- Electric nets were developed and proved to be an efficient tool for studying odour-mediated behaviour of mosquitoes in the field. Not only do these provide a new method for screening the effect of odours on the behaviour of mosquitoes in the field, but they have also proven to be useful in studying flight behaviour of mosquitoes (chapter 9).

3. to identify odour complexes/individual kairomones that influence the host-seeking behaviour of *An. gambiae s.s.*.

- Carbon dioxide (4.5 % in clean air) was attractive to *An. gambiae s.s.* in the laboratory (chapter 4, see **1.**)
- Human breath odour other than carbon dioxide, of three volunteers, was not attractive to *An. gambiae s.s.* (chapter 5).
- Foot odour was incriminated as playing a role in the selection of biting sites on the human body (chapter 2).
- Limburger cheese odour proved to be highly attractive to *An. gambiae s.s.* in the windtunnel setup (chapter 5).
- Evidence was obtained that kairomones are (at least in part) produced by the skin microflora.
- The acid extract of Limburger cheese was found to elicit behavioural responses in the windtunnel, even at very low concentrations. A synthetic blend of 12 fatty acids identified from Limburger cheese headspace was also found to be significantly attractive (chapter 6).
- Electroantennographic studies showed significant and reproducible responses to Limburger cheese headspace, the synthetic mixture of 12 fatty acids and several individually tested fatty acids (chapter 6).
- The synthetic blend of fatty acids described in this thesis is the first group of (host-seeking) kairomones identified for *An. gambiae s.s.*. Even though clear behavioural responses to this blend were observed it is not yet clear whether other (human) odours affect its behaviour in addition to this blend.

4. to conduct studies on odour-mediated host-seeking behaviour of *An.gambiae s.l.* in order to determine the potential for using the laboratory-identified kairomones as attractants under field conditions

- Sampling devices were developed for testing behavioural responses of *An. gambiae s.l.* and other mosquito species to human odour, Limburger cheese and fatty acids in the field (chapter 6,9 & 10).
- Even though carbon dioxide elicited clear behavioural responses of *An. gambiae s.s.* in the laboratory (chapter 4) no such effect was found in the field. Human breath did not give higher catches than control treatments, both in the laboratory and field (chapter 9).
- Limited attraction of *An. gambiae s.s.* was found to Limburger cheese odour, but no such effect was found for blends of fatty acids.

Odour-mediated host-seeking behaviour of *An. gambiae s.s.* in relation to its human host: a case for adding the skin microflora and *Plasmodium* parasites as interactants.

The interaction between *An. gambiae s.s.* and its human host has been shown to depend on a wide variety of factors both on the side of the host and the vector as shown in figure 1. This interaction is normally described within a bitrophic context, i.e. the two sole organisms influencing the interaction are man and mosquito. The factors influencing the host-seeking process and the various phases thereof have been described in chapter 1.

Studies on odour-mediated host-seeking behaviour of *An. gambiae s.s.*, presented in this thesis, have confirmed the existence of some of these factors (some under field conditions) but in addition have shown the overall interaction to be more complex as indicated below:

- It was demonstrated that in a windtunnel setup human breath did not attract more *An. gambiae s.s.* than the carbon dioxide content of that breath. Neither did variations in carbon dioxide production by volunteers affect catches in this setup (chapter 6). *An. gambiae s.l.* was not attracted to human breath (chapter 5, chapter 10).
- In Tanzania it was shown that differences in attractiveness (i.e. the

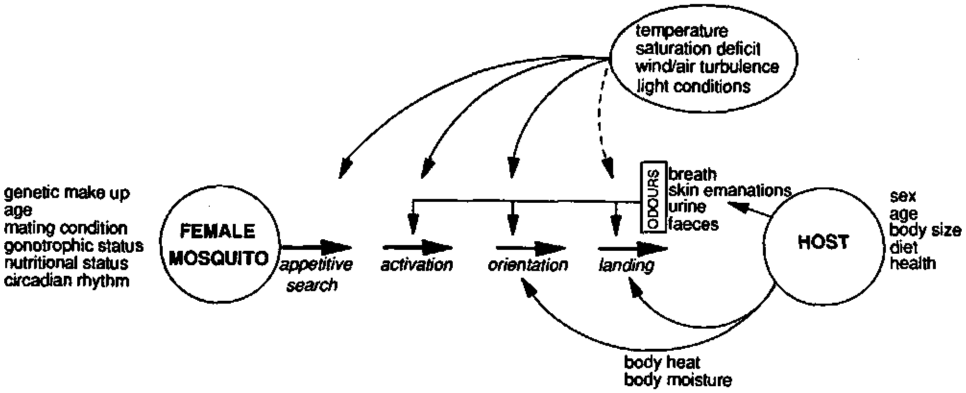


Figure 1. Mosquito-host interactions and the factors governing this within a bitrophic context (modified after Takken, 1996).

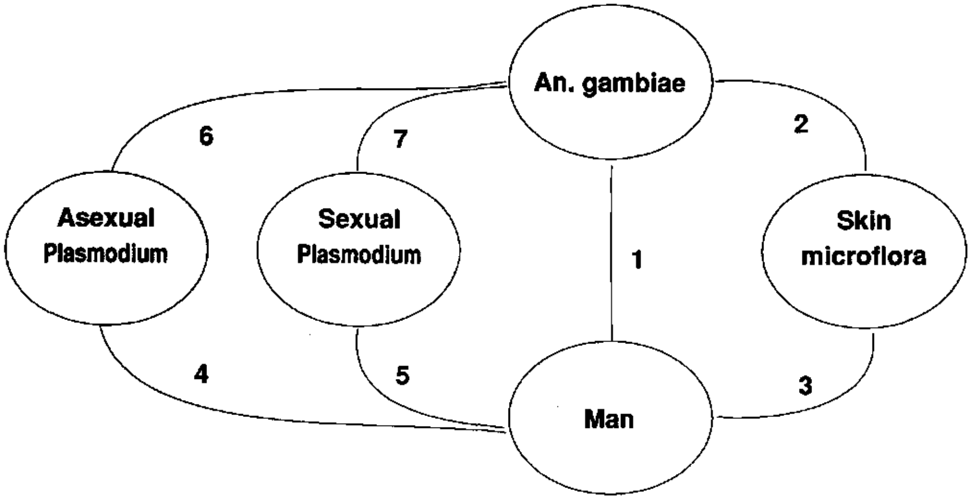
number of mosquitoes caught with catching devices other than the human landing catch) of isolated individuals varied (chapter 8). It was also observed that different mosquito species prefer different individuals in a group (chapter 9). The role of carbon dioxide at a human equivalent was shown to be limited when compared to the complete set of human effluvia (chapter 11).

- Skin-related odours have been demonstrated to influence the host-seeking process. For instance, the selection of biting sites on a human host was influenced by foot odour (chapter 3).
- The odour of Limburger cheese was shown to elicit behavioural responses of *An. gambiae s.s.* (chapter 6). The specific odour of this cheese resembles human foot odour, and is produced by bacteria (*Brevibacterium linens*) during its ripening process. A synthetic blend of volatile carboxylic acids identified from the headspace and extracts of this cheese have been shown to attract *An. gambiae s.s.* in a windtunnel setup, even at very low concentrations (10^{-8}) (chapter 7). These compounds are also found on human skin and are liberated during the breakdown of triglycerides to free glycerol by the skin microflora.

These results suggest that the human skin microflora not only influences the composition of the odour profile of different humans, but that kairomones involved in the odour-mediated stages of the host-seeking process by *An. gambiae s.s.* are (at least in part) of bacterial origin. A similar interaction exists between Dipteran larvae and parasitic wasps that use allelochemicals, produced by microbes on the feeding substrate of the larva, to locate it. Despite the involvement of a third organism (the microbe) this latter interaction is classified by Dicke & Sabelis (1988) as a bitrophic interaction due to the strong biological relationship between microbe and microbivore. Rather than considering the microbial origin of the infochemical they view the interaction in an ecological context, in which only the larva and larval parasite are the interactants, hence classifying the infochemicals as kairomones.

It can be argued that the microflora on the human skin substrate plays a similar role in mosquito-host interactions, and that the *origin* of the infochemicals is of minor importance. However, if intra-specific host-selection is based on the metabolic activity and/or density of the skin microflora then this bears a direct impact on the ecology of the mosquito-host interaction (i.e. the number of bites per person and the resulting chance of infection with *Plasmodium* parasites). Secondly, it was argued in chapter 6 that fatty acids are fairly unique to the human skin and their microbial origin may therefore also be the basis for inter-specific host-selection (i.e. the anthropophily of *An. gambiae s.s.*). Thirdly, the fact that *An. gambiae s.s.* can be attracted to volatiles produced by bacteria on *different* substrates (skin, cheese, milk (?)) renders the substrate less important in mosquito-host interactions than the bacteria on it, underlining its influence on the mosquito-host interaction. Since the skin microflora not only produces kairomones but may directly influence the ecology of the mosquito-host interaction in terms of intra- and inter-specific host-selection this justifies its recognition as a separate entity (Figure 2).

The infochemical-based interactions between these three groups of organisms (man-skin microflora-mosquito) may in turn be affected by a fourth organism namely the *Plasmodium* malaria parasite. As discussed in chapter 1, it has been suggested that the sexual stages of *Plasmodium* might affect the composition of the human effluvia, or even be responsible for the production of a so-called 'gametocyte pheromone' (Burkot, 1988). According to the terminology by Dicke & Sabelis (1988) such an infochemical should be termed an allomone in (*Plasmodium*-mosquito interaction), it being adaptively favourable to the parasite but not to the mosquito. Due to the parasite's negative effect on mosquito biology it can not be classified as a kairomone



1. Kairomones of non-bacterial origin (e.g. carbon dioxide).
2. Kairomones of bacterial origin (e.g. fatty acids).
3. Conditions of man affecting the skin microflora (pH of the skin, temperature and humidity of the skin, disease (other than malaria)), thus influencing interaction 2.
4. Effect of clinical malaria (circulation of large numbers of asexual parasites in the blood) on interaction 3 (exude salts and water through sweating, increased skin temperature, which may alter the skin microflora), in turn influencing interaction 2.
5. Effect of sexual parasite stages on man; possible existence of a gametocyte produced allomone, influencing interaction 1.
6. Effect of asexual parasites on mosquito behaviour; sporozoites induce increased probing behaviour with possible visits to more than one host (repeated orientation to hosts (interactions 1 & 2).
7. Effect of sexual parasites on mosquito behaviour (induced dispersal/migration?).

***Figure* 2.** *Mosquito-host interactions (numbered) within a multipartite context, in which the skin microflora and* Plasmodium *parasite are considered separate entities.*

within the human-mosquito interaction, since it would be adaptively favourable for a mosquito not to respond to this infochemical. Also, such an infochemical would be detrimental to man, since it might attract mosquitoes that cause new, more severe infections. Malaria parasites may thus produce an infochemical affecting the mosquito-host interaction, with negative effects for both the interactants.

An additional effect of the presence of the malaria parasite in man may be its influence on the human skin microflora. Episodes of malaria alter the environment of the skin, through a raised body temperature, increased exudation of salts and water through intense sweating. Thus the composition and density of the microflora may be affected and consequently the production of odours influencing host-seeking by *An. gambiae s.s.*. Though speculative, it may well be that the onset of gametocyte production, which normally occurs several days after an episode (and thus the alteration of the skin microflora) coincides with optimal production (either qualitative or quantitative) of kairomones by the host, ultimately increasing the transmission potential for *Plasmodium* parasites. The important effects of the presence of *Plasmodium* parasites on man, mosquito, and possibly man's microflora merits its recognition as a fourth entity influencing the interaction between man and mosquito (Figure 2).

Some of the interactions between the four organisms, however, are not based on infochemicals, but nevertheless influence each other. Within the human host, for instance, the occurrence of high densities of asexual *Plasmodium* parasites suppress the formation of sexual stages, and thus the possible production of allomones. The presence of sporozoites in the salivary glands of the mosquito affect its probing behaviour which may result in frequenting more than one host prior to full engorgement. Whether dispersal/migration of mosquitoes is affected by the uptake of sexual blood stages, or the presence of sporozoites in the salivary glands, is unknown but would enhance the transmission c.q. spread of *Plasmodium* to possible new hosts. Lastly, the presence of *Plasmodium* inside a human host may, after a malaria episode, alter the microflora on the skin. Even though these interactions between different organisms are not based on infochemical use, they ultimately affect the odour-mediated interaction between man and *An. gambiae s.s.*. It is concluded that the study of odour-mediated host-seeking behaviour of *An. gambiae s.s.* will benefit from analyses within a multipartite context, rather than the conventional bitrophic (man-mosquito) context.

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Summary

Odour-mediated host-seeking behaviour of the Afro-tropical malaria vector *Anopheles gambiae* Giles

Malaria remains the single most important parasitic disease of man in tropical regions of the world. It is estimated that 40% of the world's population, in 102 countries, is at risk from the disease. Some 100-200 million cases occur annually worldwide, of which 90 million in Africa, with 1-2 million deaths.

Efforts to control malaria by chemoprophylactic and/or curative drugs are seriously jeopardized due to widespread parasite resistance, and *Anopheles* mosquito vectors are rapidly developing physiological resistance against insecticides commonly used for indoor spraying or impregnation of bed nets. Trials with the malaria vaccine SPf66 have shown a 31% morbidity reduction in Tanzania but hardly had an effect on disease in The Gambia. The deteriorating malaria situation in the world thus urges the development of new control methods which need to be environmentally safe, socio-economically acceptable, and applicable for use within primary health care systems.

The African mosquito *Anopheles gambiae sensu stricto* Giles, is a prime vector of malaria due to its distinct preference for biting man (anthropophily). It generally feeds indoors (endophagy) between midnight and dawn and rests indoors after feeding for the first 1-2 days of its gonotrophic cycle (endophily). High survival rates and (seasonal) occurrence in high densities further increase its vectorial capacity. DDT resistance has been reported in several countries and other control measures such as environmental management are not possible due to its breeding in a wide variety of (sunlit) water bodies. Exactly how a female *An. gambiae s.s.* exploits human-related cues to orient itself while locating a host at night remains largely unknown. Field studies on closely related sibling species have shown that olfactory cues evoke behavioural responses from mosquitoes over considerable distances, but the identity of these kairomones has not been established. Furthering the knowledge of this process might ultimately result in the development of new control strategies analogous to those for tsetse flies. The host-seeking behaviour of tsetse flies has received considerable interest over the last two decades and has resulted in bait systems now widely applied for monitoring and control of

trypanosomiasis in several African countries.

The goal of the work presented in this thesis was to gain more insight in the chemical ecology of *An. gambiae s.s.* and the resulting interactions with its human host. This encompassed identification of kairomones and the development of tools to study their effect on mosquito behaviour in the laboratory and field. The work was carried out in collaboration with research groups in the United Kingdom, Italy and Tanzania within a European Community funded project titled: 'Behavioural studies on malaria vectors'.

Haematophagy, disease transmission, and factors affecting host-seeking by mosquitoes *(Chapter 1)*

The two possible evolutionary routes leading to haematophagy are described, these being the prolonged association with vertebrates and morphological pre-adaptation for piercing the skin surface. Three associations, depending on the time spent on the host, exist between vertebrates and haematophagous Diptera and pathogen transmission is suggested to benefit most in association with temporary ectoparasites. The host-seeking process, defined as any kind of behaviour that increases the chance to encounter a suitable host followed by blood-feeding, is categorised into two classes, namely 'appetitive search' behaviour prior to contacting host-related cues and activation/orientation upon perceiving these. Three classes of cues produced by hosts are defined namely olfactory, physical and visual cues. The relative importance of cues belonging to different classes depends on the behavioural ecology of the insect, and is affected by factors such as circadian rhythm and host range. Carbon dioxide, expired by all vertebrates, has been incriminated to affect the host-seeking process of all Dipteran blood-feeders. It is thought that this chemical dominated the olfactory-mediated part of host-seeking during the evolution of haematophagy. Specialism towards feeding on specific hosts required olfactory responses to odours other than carbon dioxide typifying the identity of those hosts. To date, only few of these compounds have been identified and for mosquitoes none of these are used on a large scale in the field.

An. gambiae s.s. belongs to a complex of six sibling species with large variations in host-specificity and feeding habits. Host-specificity is genetically determined and differential responses to olfactory cues from different hosts have been observed in the field. Although carbon dioxide has been found to elicit responses from the various siblings, this chemical obviously can not be responsible for these differential responses. Odours other than carbon dioxide must influence this host-selection process and identification of kairomones for the highly anthropophilic *An. gambiae s.s.* is addressed in subsequent chapters of this thesis.

Selection of biting sites by mosquitoes on man: a new approach to kairomone identification *(Chapters 2 & 3)*

A preliminary study on the selection of biting sites by haematophagous Diptera showed that only few species display a random biting pattern on their hosts. The vast majority preferentially bites specific parts of the body, and these preferences have been attributed to factors intrinsic to the behaviour of the insect (e.g. visual responses to extremities of hosts) or to the behaviour of the host (e.g. defensive behaviour). By studying the selection of biting sites by five mosquito species on the same volunteer under identical experimental circumstances it was observed that a) different species prefer different parts of the body, b) that for at least three of these species this process was influenced by odour emanating from the preferred biting region and c) that these preferences might not only reveal the origin of chemical cues involved in host-seeking, but that the biting pattern can provide information on the range of hosts fed upon. *An. gambiae s.s.* showed a strong preference for biting the legs and foot region (76% of all bites) and alteration of the odour from this region, by washing the feet with a bactericidal soap, significantly altered this preference (to 36%). Likewise, the significant preference of *An. atroparvus* and *An. albimanus* for biting the face (50 and 49% of all bites respectively) was influenced by breath since the pattern of biting changed dramatically after breath was removed from the host (to 19 and 20% respectively).

Studying the selection of biting sites by mosquitoes was proven to be a new method to reveal the origin of olfactory cues influencing their host-seeking behaviour.

Odour-mediated host-seeking behaviour and kairomone identification: a laboratory approach *(Chapter 4,5 & 6)*

At the onset of the present study it was realised that a reliable laboratory bioassay was essential to facilitate rapid screening of behavioural effects of (human) odours on *An. gambiae s.s.*. The then existing windtunnel, which was constructed to study responses of individual females, was accordingly modified and resulted in a system closely resembling conventional dual-port olfactometers used for screening behavioural effects of odours on the yellow fever mosquito *Aedes aegypti*. Using this system, test odours were classified as 'attractive' whenever trap catches were significantly higher (using chi-squared) then those of control traps. Carbon dioxide at a human equivalent (4.5% in clean air) caught 4 times more *An. gambiae s.s.* than control traps baited with clean air only.

Results from chapters 2 & 3 showed that foot odour played a role in the selection of biting sites for *An. gambiae s.s.* and breath influenced this process for *An. atroparvus*/*An. albimanus*. Since carbon dioxide at a human equivalent was clearly 'attractive' it was assessed whether whole human breath was similarly attractive. It was shown that human breath (of three volunteers) was not attractive when tested against the carbon dioxide concentration (3.30-3.65%) of that breath only. When breath of the same volunteers (with carbon dioxide concentrations between 3.28-3.80%) was tested against clean air this was also not attractive, nor was carbon dioxide at a concentration of 3.56%. The difference in response to this latter concentration of carbon dioxide and the highly 'attractive' 4.5% concentration was attributed to the dilution of the gas to levels which did no longer result in catches significantly different from those in the control traps.

The headspace of Limburger cheese, to the human nose reminiscent of foot odour, was significantly attractive to *An. gambiae s.s.* from East and West Africa. GC and GC-MS analyses of the acid fractions of Limburger cheese, human sweat and toe scrapings showed strong similarities in their aliphatic fatty acid content and composition. The fact that *An. gambiae s.s.* responded to odours of bacterial origin (produced by *Brevibacterium linens* on the cheese) strongly suggests that the human skin microflora is (at least in part) responsible for the production of kairomones for this species. Nineteen saturated and unsaturated fatty acids, ranging in carbon chain length from C_2-C_{18} were identified in the acid fraction of the cheese, whereas twelve of these were present in its headspace. In the windtunnel bioassay, the undiluted acid extract was repellent, but became significantly attractive at concentrations of $5*10^{-4}$ to

10^{-7}. A synthetic blend of the twelve acids identified in the headspace was significantly attractive at a concentration of 10^{-8}. Electroantennographic studies showed significant and reproducible responses to (saturated) cheese headspace, the synthetic mixture, and all individual acids but hexadecanoic acid. These fatty acids form the first group of kairomones identified for *An. gambiae s.s.*.

Host attractiveness and odour-mediated host-seeking behaviour of mosquitoes: a field approach *(Chapter 7,8,9 & 10)*

The study of the effects of kairomones on mosquito behaviour in Tanzania was initially hampered by the absence of sampling devices. Standard CDC light traps, commonly used for sampling malaria vectors indoors, were not applicable for outdoor use. Tents ('mobile huts') were modified for sampling mosquitoes by fitting exit traps and constructing artificial eaves for mosquito entry. These tents could be positioned at various sites, in randomized block designs, and could be washed which make them ideal for studying the attractiveness of different odours. Initially it was assessed whether three isolated individuals varied in their attractiveness to *An. gambiae s.l.*, *An. funestus*, *Culex quinquefasciatus* and *Mansonia* spp., which for all but the latter species was the case. These differences were attributed to the odour of the volunteers since this was the only variation factor not eliminated in the experimental setup. Also, this experiment showed for the first time that *isolated* individuals vary in their attractiveness to mosquitoes.

In addition to these results it was found that different mosquito species show preferences for different individuals within a group. Catches of *An. funestus* and *An. squamosus* were inversely correlated for two individuals of the group. Furthermore, a negative correlation between group size and individual catch was observed, which suggests that mosquito sampling is more efficient if individuals are not grouped.

The tents were also used to assess the relative importance of the physical presence of man versus his odour only in the number of mosquitoes attracted (and caught). This was done by positioning a man inside a bed net or underground in a pit from which odours were pumped into the bed net. Both treatments caught equal numbers of *An. gambiae s.l.* and *An. funestus*, which indicates that human odour is the main factor in attracting these species to a bait inside a house. When carbon dioxide (at a human equivalent) was released inside a bed net the catches were 9 and 27% of those by man for *An. gambiae s.l.* and *An. funestus* respectively. A five-fold increase in carbon dioxide dose increased the catch of the latter species to 69% but those of *An. gambiae s.l.* remained the same. The relative contribution of carbon dioxide to the overall attractiveness of man to *An. gambiae s.l.* was thus limited and underlined the importance of other human odours in host-seeking by this species.

Electric nets (a grid of wires electrocuting insects when they contact them) have played a paramount role in the study of tsetse fly behaviour, and it was studied whether similar sampling systems were applicable for outdoor use, and more specifically to study responses to odours and analyse flight behaviour. Various designs were tested, and when baited with carbon dioxide, sampled all mosquito species present in the study area. Furthermore it was shown that primarily zoophilic mosquito species were attracted to human breath or carbon dioxide, but that the anthropophilic species (*An. gambiae s.l.* and *An. funestus*) hardly responded to these baits. Whole human odour baited nets caught higher numbers of these latter species, thus confirming the results from the tent experiments. A large cylindrical net was used to assess the flight direction of mosquitoes upon contacting carbon dioxide and showed that they engage in positive anemotaxis. The overall efficiency (number caught/number approached) of a rectangular electric net (28 x 40 cm, wires at 4 mm), when baited with carbon dioxide, was 42%. This work showed that electric nets can be used for outdoor sampling of mosquitoes, for analysing responses towards laboratory identified kairomones, and for the analysis of mosquito flight behaviour.

Odour-mediated host-seeking behaviour of *An. gambiae s.s.* in relation to its human host: a case for adding the skin microflora and *Plasmodium* parasites as interactants *(Chapter 11)*

The general discussion reviews the results of the research in a broader context. The preceding chapters suggested that olfactory cues influencing the host-seeking behaviour of *An. gambiae s.s.* originate from the human skin. Their production is (at least partially) the result of the metabolic activity of the skin microflora. It is argued that the influence of the skin microflora on the interaction between man and mosquito is of such importance that this justifies the recognition of the skin microflora as a separate entity. In addition to this it is hypothesized that the influence of the *Plasmodium* malaria parasite may affect the production of infochemicals mediating the interaction between man and mosquito, or, alternatively, is responsible for the production of these allomones. It is concluded that the study of odour-mediated host-seeking behaviour of *An. gambiae s.s.* will benefit from analysing the interactions between man and mosquito within a multipartite, rather than the conventional bitrophic (man-mosquito) context.

Samenvatting

De invloed van geurstoffen op het gastheerzoekgedrag van de Afrikaanse malariamug *Anopheles gambiae* Giles

Malaria is de meest belangrijke parasitaire infektieziekte in de tropen. Veertig procent van de wereldbevolking, in 102 landen, loopt het risiko op besmetting. Jaarlijks raken 100 tot 200 miljoen mensen geinfekteerd, waarvan 90 miljoen in Afrika, met 1 tot 2 miljoen slachtoffers.

De direkte bestrijding van de malariaparasiet met behulp van preventieve dan wel curatieve medicijnen wordt tegenwoordig sterk bemoeilijkt door het op steeds grotere schaal optreden van resistentie tegen deze middelen. Daarnaast blijken gangbare insekticiden voor huisbespuitingen of impregnatie van klamboes steeds minder effektief vanwege fysiologische resistentie bij *Anopheles* muggen, verantwoordelijk voor de overdracht van de ziekte. Proefnemingen met het recentelijk ontwikkeld malariavaccin SPf66 in Tanzania resulteerde in een morbiditeitsreduktie van 31%, doch het vaccin bleek veel minder effektief in soortgelijke proeven in Gambia. Daar malaria een steeds belangrijker gezondheidsprobleem dreigt te worden, en conventionele bestrijdingsmethoden steeds minder effektief zijn, is het van uitermate groot belang om nieuwe, milieuvriendelijke en socio-economisch acceptabele, methoden te ontwikkelen die binnen primaire gezondheidstelsels op het Afrikaanse platteland kunnen worden ingezet.

De Afrikaanse mug *Anopheles gambiae sensu stricto* Giles, is, gezien haar sterke voorkeur voor het steken van de mens, een van de belangrijkste malariavektoren ter wereld. In het algemeen steekt de mug binnenshuis tussen middernacht en zonsopkomst waarna zij 1 tot 2 dagen binnenshuis verblijft gedurende de eiontwikkeling. De relatief lange levensduur van deze soort, en haar voorkomen in relatief hoge dichtheden tijdens de regens verhogen haar zogenaamde vektoriele capaciteit. In verscheidene landen is de mug resistent tegen het insekticide DDT, en andere bestrijdingsmethoden, zoals het droogleggen van broedplaatsen zijn niet mogelijk daar deze soort haar eieren afzet in een grote verscheidenheid aan (zonbeschenen) typen stilstaand water.

Er bestaat onduidelijkheid over hoe *An. gambiae s.s.* haar gastheer lokaliseert en hoe ze daarbij de door de gastheer afgegeven signalen gebruikt. Veldstudies in West Afrika hebben aangetoond dat nauw verwante soorten aangetrokken worden door geuren afgegeven door de gastheer en dat deze muggen aktiveren en aantrekken over afstanden van ongeveer 80 meter. De identifikatie van deze geurstoffen zou een belangrijke bijdrage kunnen leveren aan het ontwikkelen van nieuwe bestrijdingsmethoden. Gedetailleerde studies naar het gastheerzoekgedrag van tsetsevliegen sinds de zeventiger jaren hebben inmiddels geresulteerd in de ontwikkeling van zeer efficiënte vangmethoden die nu in verscheidene Afrikaanse landen worden toegepast om de overdracht van slaapziekte te bestrijden.

Het onderzoek gepresenteerd in dit proefschrift had tot doel inzicht te verwerven in de interaktie tussen *An. gambiae s.s.* en haar menselijke gastheer, met name de rol van geurstoffen binnen deze interaktie. De identifikatie van deze geurstoffen en de invloed daarvan op het gedrag van deze muggesoort werd bestudeerd in het laboratorium en de Afrikaanse veldsituatie. Het onderzoek werd uitgevoerd in samenwerking met onderzoeksgroepen in Engeland, Italië en Tanzania en werd gefinancierd door de Europese Unie binnen het projekt 'Behavioural studies on malaria vectors'.

Bloed als bron van voedsel en reproduktie, de overdracht van ziekten, en faktoren die het gastheerzoekgedrag van muggen beinvloeden *(Hoofdstuk 1)*

Twee mogelijke manieren waarop het bloedzuiggedrag van insekten is ontstaan worden gepresenteerd. De nauwe relatie tussen insekten levend van dierlijke produkten (veren, haren, uitwerpselen etc.) zou uiteindelijk leiden tot het leven op dieren en het exploiteren van bloed als efficiënte eiwitbron. Daarnaast zou de ontwikkeling van zuigende monddelen, aangepast voor het penetreren van harde planteweefsels, vervolgens het penetreren van de dierlijke huid hebben vergemakkelijkt. Drie samenlevingsvormen tussen bloedzuigende insekten en hun gastheren, afhankelijk van de hoeveelheid tijd doorgebracht op de gastheer, worden erkend. De overdracht van ziekten is het meest efficiënt door insekten die slechts korte tijd op hun gastheer verblijven. Gastheerzoekgedrag, gedefiniëerd als ieder type van gedrag dat de kans vergroot op het aantreffen van een gastheer gevolgd door het nemen van een bloedmaaltijd, wordt opgesplitst in twee categoriëen, namelijk 'appetatief' zoekgedrag en aktivatie/oriëntatie respectievelijk voor- en nadat gastheergeuren zijn waargenomen door de mug. Gastheren geven drie klassen van signalen af namelijk olfactorische (chemische), fysische en visuele. Het afzonderlijke belang van deze drie klassen binnen het gastheerzoekgedrag is afhankelijk van de gedragsoecologie van het insekt met name faktoren als circadiane aktiviteit en gastheervoorkeur. Kooldioxide, hetgeen geproduceerd wordt door alle vertebraten, speelt een rol binnen het gastheerzoekgedrag van alle bloedzuigende insekten. Waarschijnlijk heeft deze verbinding een belangrijke rol gespeeld binnen de ontwikkeling van het gastheerzoekgedrag van bloedzuigende insekten. Specialisatie voor het nemen van bloedmaaltijden op een gelimiteerd aantal gastheersoorten ging vervolgens gepaard met het reageren op gastheerspecifieke geurstoffen. Tot op heden zijn slechts enkele van deze gastheerspecifieke geurstoffen bekend, en is er nauwelijks sprake van toepassing van deze stoffen voor het vangen van muggen in de veldsituatie.

An. gambiae s.s. behoort tot een soortcomplex van zes nauw verwante soorten, waarbinnen grote verschillen bestaan voor wat betreft gastheervoorkeur en gedragsoecologie. Gastheervoorkeuren zijn genetisch vastgelegd en bepalen de respons ten aanzien van gastheergeurstoffen in het veld. Daar kooldioxide door alle potentiele gastheren wordt geproduceerd kan deze verbinding niet bepalend zijn voor deze verschillen in gedrag. De identifikatie van (mens-specifieke) gastheergeurstoffen die het gedrag van *An. gambiae s.s.* beinvloeden wordt beschreven in de volgende hoofdstukken.

Het steekgedrag van muggen op de mens: Een nieuwe methode ter identifikatie van geurcomplexen die het gastheerzoekgedrag beinvloeden

(Hoofdstuk 2 & 3)

Een inleidende literatuurstudie naar het steekgedrag van bloedzuigende insekten op hun gastheren liet zien dat slechts weinig insekten over het hele lichaam van de gastheer steken. De meerderheid heeft een voorkeur voor het steken op bepaalde plekken, hetgeen wordt toegeschreven aan het gedrag van het insekt (bijvoorbeeld visuele responsen ten opzichte van uitstekende delen van de gastheer (oren, poten etc.)) of het gedrag van de gastheer (afweerreacties die insekten 'dwingen' op ander delen te steken). Studies naar het steekgedrag van vijf verschillende soorten muggen onder identieke experimentele omstandigheden op dezelfde menselijke gastheer lieten zien dat: a) verschillende soorten een voorkeur hebben voor verschillende delen van het lichaam, b) dat voor minstens drie van deze soorten de voorkeur (gedeeltelijk) bepaald werd door geuren afkomstig van dat lichaamsdeel, en c) dat dit steekgedrag niet alleen informatie verschaft omtrent de geuren die een rol spelen bij het steekgedrag, maar dat een indruk verkregen kan worden omtrent de gastheerspecificiteit van de muggesoort. *An. gambiae s.s.* vertoonde een sterke voorkeur voor het steken op de onderbenen, enkels en voeten (76% van alle steken) en het wassen van de voeten met een medicinale zeep deed deze voorkeur signifikant afnemen (tot 36%). *An. atroparvus* en *An. albimanus* vertoonden een sterke voorkeur voor het steken op het gelaat (50 en 49% van alle steken respektievelijk) en dit gedrag werd beinvloed door uitgeademde lucht, daar de afvoer van deze lucht resulteerde in een signifikante afname van het aantal steken (tot 19 en 20% respektievelijk).

De analyse van het steekgedrag van muggen op de mens kan aldus informatie verschaffen omtrent de origine van geurstoffen die het gastheerzoekgedrag bepalen.

Het gastheerzoekgedrag en identifikatie van kairomonen: De aanpak in het laboratorium *(Hoofdstuk 4,5 & 6)*

Aan het begin van het huidige onderzoek werd al snel duidelijk dat een snelle toetsmethode ter analyse van het effekt van (menselijke) geurstoffen op het gedrag van *An. gambiae s.s.* van groot belang zou zijn. Het tot die tijd gebruikte systeem analyseerde het vlieggedrag van individuele muggen, en werd vervolgens omgebouwd tot een simpel twee-keuze systeem waarbij de responsen van groepen muggen kunnen worden bekeken, vergelijkbaar met conventionele keuze-systemen voor *Aedes aegypti*. Geteste geuren werden geclassificeerd als 'attraktief' wanneer vangsten daarmee signifikant hoger waren dan vangsten voor de kontrole geur. Kooldioxide, in menselijke concentratie (4.5% in schone lucht) was zeer attraktief en gaf vangsten vier maal hoger dan de controle.

De resultaten van hoofdstuk 2 en 3 lieten zien dat voetengeur een rol speelt bij het steekgedrag van *An. gambiae s.s.* en dat uitgeademde lucht dit gedrag beinvloedt voor *An. atroparvus*/*An. albimanus*. Gezien de attraktiviteit van kooldioxide werd vervolgens bepaald of andere verbindingen in adem een additionele rol spelen bij de aantrekking van deze soort. Adem van drie vrijwilligers was niet aantrekkelijker in vergelijking tot eenzelfde concentratie van kooldioxide (3.30-3.65%) in die adem. Wanneer adem (met kooldioxide concentraties variërend tussen 3.28 en 3.80%) werd getest tegen een controle van schone lucht bleek ook deze niet aantrekkelijk. Ook gaf een concentratie van 3.56% kooldioxide geen signifikant hogere vangsten dan een controle van schone lucht. Het verschil in respons ten opzichte van deze concentratie in vergelijking met de sterk attraktieve concentratie van 4.5% werd toegeschreven aan de verdunning die optreedt bij het pompen van deze geur in het valsysteem, resulterend in vangsten niet signifikant afwijkend van controlevangsten.

De vluchtige verbindingen afgegeven door Limburgse kaas, voor de mens sterk gelijkend op voetengeur, werd zeer aantrekkelijk bevonden voor *An. gambiae s.s.* uit zowel Oost- als West Afrika. GC en GC-MS analyses lieten een sterke overeenkomst zien in vetzuur compositie tussen menselijk zweet, Limburgse kaas en teenschraapsels. De aantrekking van *An. gambiae s.s.* door geuren van bacteriële oorsprong (geproduceerd door *Brevibacterium linens* waarnee Limburgse kaas wordt geinoculeerd) geeft aan dat de menselijke huidflora (op zijn minst ten dele) verantwoordelijk is voor de produktie van attraktieve stoffen voor deze muggesoort. Negentien verzadigde en onverzadigde vetzuren, varierend in ketenlengte van C_2 tot C_{18} werden aangetroffen in de zuurfractie van de kaas, waarvan twaalf in de afgegeven geur. Het onverdunde zuurextract van de kaas bleek signifikant afstotend te zijn in windtunnelproeven, doch werd signifikant attraktief na verdunning tot een concentratie van $5*10^{-4}$ tot 10^{-7}. Een synthetisch mengsel van de twaalf vetzuren geindentificeerd in de kaasgeur was signifikant attraktief bij een verdunning tot 10^{-8}. Verzadigde kaaslucht, het synthetisch mengsel en alle individuele vetzuren (behalve palmitinezuur) gaven signifikante en reproduceerbare responsen in electroantennogram proeven. Deze vetzuren zijn de eerste groep verbindingen waarvan is aangetoond dat ze het gedrag van *An. gambiae s.s.* beinvloeden.

Gastheer attraktiviteit en gastheerzoekgedrag van muggen: De aanpak in de Afrikaanse veldsituatie *(Hoofdstuk 7,8,9 & 10)*

Experimenten met geurstoffen in het veld werden oorspronkelijk bemoeilijkt door de afwezigheid van effektieve bemonsteringsmethoden. Standaard CDC lichtvallen, die algemeen worden ingezet voor het bemonsteren van malariamuggen binnenshuis, bleken niet effektief voor gebruik in het open veld. Tenten ('mobiele hutten') werden vervolgens omgebouwd voor het vangen van muggen met behulp van zogenaamde 'exit-traps', en openingen onder het tentdak werden gecreëerd waardoor muggen de tent konden binnenkomen. Het voordeel van de tenten was dat deze op verscheidene plekken geplaatst konden worden, hetgeen analyse van resultaten met behulp van Latijnse vierkanten mogelijk maakte, en ze bleken wasbaar, waardoor het testen van verschillende geuren zonder contaminatie mogelijk was. De tenten werden in eerste instantie gebruikt om verschillen in attraktiviteit te bestuderen tussen geïsoleerde gastheren voor *An. gambiae s.l.*, *An. funestus*, *Culex quinquefasciatus* en *Mansonia* spp., hetgeen alleen voor de laatste soort(en) niet het geval was. Deze verschillen in attraktiviteit werden toegeschreven aan verschillen in gastheergeuren daar dit de enige variabele faktor was in de proefopzet. Daarnaast bleek dit experiment voor het eerst aan te tonen dat geïsoleerde individuen verschillen in hun attraktiviteit voor muggen.

Tevens werd aangetoond dat verschillende muggesoorten een voorkeur hebben voor verschillende individuen binnen een groep. Zo bleken de vangsten van *An. funestus* en *An. squamosus* omgekeerd evenredig gecorreleerd. Een negatieve correlatie tussen de grootte van de groep en individuele vangst gaf aan dat het bemonsteren van muggen efficiënter is wanneer individuen geïsoleerd vangsten verzamelen.

De tenten werden vervolgens gebruikt om te bestuderen of de attraktiviteit van een mens (in een tent) verschilt van enkel de menselijke geur (gepompt uit een ondergrondse tunnel) in een tent. Beide behandelingen vingen gelijke aantallen van *An. gambiae s.l.* en *An. funestus*, hetgeen aangeeft dat de menselijke geur de belangrijkste faktor is tijdens het lokaliseren van een gastheer binnenshuis. Wanneer kooldioxide (in menselijk equivalent) werd vergeleken met menselijke geur, opnieuw in tenten, bleek dit slechts 9% van het aantal muggen gevangen met mensengeur op te leveren voor *An. gambiae s.l.* en 27% voor *An. funestus*. Een vijf maal hogere dosis aan kooldioxide gaf geen verandering in het aantal *An. gambiae s.l.*, doch deed de *An. funestus* vangsten toenemen to 69% van de vangsten met mensengeur. Deze experimenten lieten zien dat kooldioxide slechts in beperkte mate bepalend is voor de menslijke attraktiviteit en dat andere menselijke geuren een belangrijkere rol spelen binnen het zoekgedrag van *An. gambiae s.l.*.

De toepassing van electrische vangnetten binnen het onderzoek naar het gastheerzoekgedrag van tsetsevliegen heeft een enorme bijdrage geleverd aan opheldering van dit process. Het werd daarom onderzocht of soortgelijke systemen van toepassing zouden kunnen zijn binnen het onderzoek naar muggegedrag in het open veld ten aanzien van gastheergeuren. Verschillende typen netten werden getest en en bleken (na de nodige aanpassingen) in staat tot het bemonsteren van muggen. Wanneer kooldioxide werd gebruikt als lokstof werden alle in het gebied aanwezige soorten in meerdere of mindere mate bemonsterd. Juist zoöphiele soorten werden in grote getalen aangetrokken door zowel uitgeademde lucht als kooldioxide, terwijl de mens-specifieke (*An. gambiae s.l.* en *An. funestus*) soorten vrijwel afwezig waren in de vangsten. Deze laatse twee soorten werden wel aangetrokken tot elektrische netten voorzien van menselijke geuren, hetgeen de resultaten van de tent-experimenten ondersteunt. Een cylindrisch net werd gebruikt om de aanvliegrichting ten aanzien van geurstoffen te bepalen en liet voor het eerst (in afwezigheid van kunstmatige luchtstromen) zien dat muggen op geurstoffen reageren door middel van positieve anemotaxis. De efficiëntie (aantal muggen geëlectrokuteerd/aantal muggen aangetrokken) van een rechthoekig net (28 x 40 cm, draden op 4 mm afstand) voorzien van kooldioxide, was 42%. Dit werk liet voor het eerst zien dat elektrische netten gebruikt kunnen worden in het open veld voor het bepalen van de attraktiviteit geurstoffen en voor bestudering van vlieggedrag van muggen.

De menselijke geur en haar invloed op mug-gastheer interakties: de erkenning van het belang van de huidflora en de malariaparasiet op deze interakties *(Hoofdstuk 11)*

In hoofdstuk 11 worden de resultaten uit voorgaande hoofdstukken in een bredere kontext geplaatst. Het is gebleken dat geurstoffen afkomstig van de menselijke huid het gastheerzoekgedrag van *An. gambiae s.s.* beinvloeden. Deze stoffen worden (ten dele) geproduceerd door micro-organismen. Gezien het belang van de huidflora op de interaktie tussen mens en mug, binnen de oecologische kontext, wordt voorgesteld de huidflora te erkennen als een aparte entiteit. Daarnaast is de aanwezigheid van de malariaparasiet in de mug en menselijke gastheer waarschijnlijk van invloed op de interactie. Zo zou de parasiet de gastheer kunnen aanzetten tot het produceren van stoffen die de attraktiviteit van het individu verhogen, of zou de parasiet zelf zo'n stof (allomoon) kunnen produceren. Het lijkt daarom gerechtvaardigd ook de malariaparasiet als een aparte entiteit te erkennen. De studie naar de door gastheergeuren beinvloedde interactie tussen de mens en *An. gambiae s.s.* zal aldus gebaat zijn bij een analyse binnen een multipartite in plaats van een conventionele bitrofische (mens-mug) kontext.

Curriculum vitae

Op 6 September 1965 werd ik, Bart Geert Jan Knols, geboren te Meerssen, Zuid Limburg. Zeventien jaar later, in Augustus 1983, behaalde ik het VWO diploma aan de Scholengemeenschap 'Stella Maris' te Meerssen, waarna ik besloot Biologie te gaan studeren aan de Landbouwuniversiteit te Wageningen. Al snel werd daar mijn interesse gewekt voor werk in de tropen. In 1986/1987 verbleef ik één jaar in Kenya aan het International Centre for Insect Physiology and Ecology, alwaar ik in een Maasai gebied werkte aan de oecologie en bestrijding van tsetse vliegen. In de verdere doctoraal-fase verdiepte ik mij in de populatiedynamika van *Biomphalaria* slakken, verantwoordelijk voor de transmissie van bilharzia, aan de Rijksuniversiteit Leiden. Vervolgens besteedde ik een half jaar aan een literatuuronderzoek over de oecologie van Indonesische malariamuggen (i.s.m het ILRI in Wageningen). Tenslotte, vanaf 1989, bestudeerde ik voor een half jaar het vlieggedrag van de Afrikaanse malariamug *Anopheles gambiae* t.a.v. menselijke geuren in een kleine windtunnel aan de vakgroep Entomologie. Op 1 September 1989 werd het doctoraal diploma Biologie met predikaat *cum laude* behaald.

Hierna trad ik voor 2.5 jaar in dienst bij het consultancy bureau RDP Livestock Services en reisde af naar Zambia alwaar ik tot Februari 1992 werkzaam was als 'tsetse research biologist' in het tsetse control project Senanga West, in Western Province, Zambia.

Op 6 April 1992 trad ik voor drie jaar in dienst bij de vakgroep Entomologie als toegevoegd onderzoeker in het door de Europese Unie gefinancierde projekt 'Behavioural studies on malaria vectors', hetgeen tenslotte leidde tot dit proefschrift. Bijna een jaar van het onderzoek werd uitgevoerd in Tanzania i.s.m. het National Institute for Medical Research.

Recentelijk heb ik aan de Universiteit van Massachusetts (Amherst, USA) gewerkt aan het vlieggedrag van motten in windtunnels (middels een beurs van de CIBA foundation) en vanaf 1 Maart 1996 zal ik opnieuw aan het gedrag van malariamuggen werken in Oost- en Zuidelijk Afrika (middels een beurs van de Niels Stensen stichting).