HUMAN SKIN EMANATIONS IN THE HOST-SEEKING BEHAVIOUR OF THE MALARIA MOSQUITO *ANOPHELES GAMBIAE*

Aan ons pap en mam

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GENERAL INTRODUCTION

THE ROLE OF MOSQUITOES IN MALARIA TRANSMISSION

Malaria is the most important parasitic infectious human disease and one-third of the world population is under threat of the disease (W.H.O., 1997). The human malaria parasites, *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale*, are transmitted by members of the mosquito genus of *Anopheles* (Diptera: Culicidae). Next to man, other mammals, birds and reptiles suffer from *Plasmodium* spp. infections. Over 100 *Plasmodium* species are described from vertebrates: 4 species occur in humans, 20 species in other primates and another 20 in other mammals, and 40 species in birds and a small number in reptiles. In most cases, the parasites are transmitted by mosquitoes: mammalian malaria is invariably transmitted by anophelines and avian malaria often by culicines (Garnham, 1980). However, lizard malaria has, next to a culicine vector, also a non-mosquito vector, namely sandflies (Diptera: Psychodidae) (Ayala and Lee, 1970).

The occurrence and distribution of the members of the *Plasmodium* genera over possible hosts and vectors depend on various known and unknown factors; specialisation exists at all levels. Each member of the *Plasmodium* genera can only develop in a limited number of insect vector species and the same holds for the development in a vertebrate host. Even differences in parasitic infectivity to vectors between endemic areas have been observed on a large geographic scale (Day *et al.*, 1998). Moreover, from the feeding patterns demonstrated by most vectors (see below) it follows that the parasite is transmitted to only a limited range of potential hosts. Hence, approximately 85 of the over 400 known species and subspecies of *Anopheles* have been conclusively incriminated as vectors of human malaria (Tempelis, 1975; Giles and Warrell, 1993). The ability of the mosquito to transmit human malaria is represented by the vectorial capacity, C, which is the daily rate at which future inoculations arise from a current infective case (Garrett-Jones, 1964; Elliot, 1972). This is defined as:

$$C = m\, a^2\, p^n / - \ln p$$

where m is the number of female *Anopheles* per human individual, a is the daily biting rate of an individual mosquito on humans; p is the daily survival of a female *Anopheles* and n the extrinsic incubation period of the malaria parasite in days. Consequently, the ecology of vectors largely determines the vectorial capacity. Four important factors in the ecology of the vector are recognised: its abundance, its susceptibility for the parasite, its longevity and its contact with man (Charlwood, 1996). The latter factor is the outcome of the host-feeding pattern of the *Anopheles* females. Ecological and behavioural aspects of host-feeding patterns of mosquitoes will be considered in the next section.

HOST-FEEDING HABITS

Hosts of blood feeding mosquitoes can be found in all vertebrate classes. As mentioned above, most vectors have a different feeding habit giving rise to a wide range of feeding patterns: feeding entirely on mammals, readily on birds and mammals, almost entirely on birds, exclusively on amphibians, predominantly on reptiles, one odd mosquito species

(*Uranotaenia lateralis)* feeding exclusively on a single fish species, the mudskipper which rests on top of mud, and finally readily feeding on both poilkilothermic and homeothermic animals vertebrates (Tempelis, 1975). Due to the medical importance of some mosquitoes in transmitting human diseases, this division is generally simplified to just three groups: feeding exclusively on humans or an anthropophilic feeding pattern, feeding on non-human vertebrates or a zoophilic feeding pattern and feeding on both human and non-human vertebrates or an opportunistic feeding pattern. Although, this may be an adequate division for epidemiological and medical studies, it has little biological meaning. It suggests that non-human vertebrates share more characteristics with each other than with man. Scientific arguments for placing man apart from other animals cannot be given. Moreover, the general adoption of this anthropomorphic division often obscures competent research.

According to this division, the Afro-tropical malaria mosquito complex, *Anopheles gambiae sensu lato* embodies one strictly antropophilic sibling species (*An. gambiae sensu stricto),* a zoophilic one (*An. quadriannulatus)* and opportunistic feeders (*An. arabiensis, An. melas, An. merus,* and *An. bwambae*) (Gillies, 1967). Often the question is raised "why" *An. gambiae s.s.* feeds nearly exclusively on humans. This is only a reasonable question when one realises that the same question can be addressed to every other mosquito species with a narrow range of hosts. The generally adopted distinction suggests that zoophilic mosquitoes have a wide range of host species, while this is often not the case e.g. the zoophilic *An. quadriannulatus* feeds exclusively on bovines. The fact, however, that investigators are humans themselves brings about the suggestion that the anthropophilic pattern is more exclusive. The same reasoning is often present in the discussion about the role of carbon dioxide as possible olfactory cue or also called a kairomone (for more details, see section Chemical Ecology) to which mosquitoes respond to find their host. All living vertebrates emit carbon dioxide and it is generally accepted that carbon dioxide is, in concert with other volatile components, a kairomone for virtually all haematophagous insects (Service, 1993; Mboera and Takken, 1997). Nevertheless, the fact that anthropophilic species like *An. gambiae s.s., An. funestus* and *Cx quinquefasciatus* respond poorly to carbon dioxide (Dekker and Takken, 1998a) is frequently explained with the statement that carbon dioxide cannot be a reliable cue for these highly specialised species. This argument is often raised with respect to the profound response of the zoophilic *An. quadriannuluatus* to carbon dioxide (Dekker and Takken, 1998b). Here, again the narrow feeding habit of the latter mosquito is ignored. The fact that the anthropophilic yellow fever mosquito, *Aedes aegypti,* shows a considerable response to carbon dioxide is also disregarded.

Another problem attached to the rather strict division in anthropophilic and zoophilic behaviour is that variation in host preference within sibling species exists. Varying degrees of anthropophily of, for example, *An. gambiae s.s.* are found. While in East Africa this mosquito, indeed, nearly exclusively feeds on humans (Severeni *et al.*, 1990; Petrarca *et al.*, 1991), in West Africa data is present that less than one-third of the *An. gambiae s.s.* caught appeared to have fed on humans (Robert *et al.*, 1991; Charlwood *et al.,* 1998; Diatta *et al.*, 1998). Interestingly, in Senegal, about 60 per cent of *An. arabiensis* and *An. gambiae s.s.* caught had fed on humans and the remainder largely on bovines (Lemasson *et al.,* 1997). In Madagascar, however, *An. arabiensis* feeds for 94% on bovines and the remainder on other non-human vertebrates (Ralisoa Randranasolo and Coluzzi, 1987) while *An. gambiae s.s.* is strictly feeding on man (Lepers *et al.*, 1990). Braack and colleagues (1994) collected *An. arabiensis* by means of human-biting catches in the Kruger National Park of South Africa, an area where human hosts are normally not present. They suggested that the preference for human blood in many areas, therefore, does not imply a dependence on humans for survival. During intensive house spraying campaigns, feeding habits of mosquitoes appear to change. The percentage of malaria mosquitoes feeding on humans decreases through the selection pressure favouring mosquitoes that prefer to bite outdoors, predominantly non-human biting mosquitoes (Garrett-Jones *et al.*, 1980). Another example is given by Gillies (1964) who could induce small changes in feeding preference in two lines of *An. gambiae s.s.* following several generations of laboratory selection for feeding on man or calves. Thus, the degree in which anthropophily is developed may vary in space and time and should, therefore, refer to

a population rather than a species (Garrett-Jones *et al.*, 1980). Host feeding of opportunistic feeders is, at least in some cases, proven to be largely influenced by the availability of hosts that can also change in time and space (Garrett-Jones *et al.*, 1980).

The degree of anthropophily is customarily determined by analysing samples of blood meals of freshly fed mosquitoes collected from trapping devices. The proportion of freshly fed female mosquitoes found to contain human blood is called the human blood index (HBI) (Garrett-Jones, 1964). The HBI is relevant in epidemiological and medical research to estimate the vectorial capacity of the mosquito population (Garrett-Jones, 1964). The HBI should refer to a representative sample of a single species collected in a certain area and period. Aside of the discussion about terminology, the main problem determining the degree of anthropophily with HBI is to obtain a representative and not a biased sample of a population. To analyse blood meals, resting females are aspirated or knocked down by insecticide spraying. Good sampling of possible resting places, indoors and outdoors is crucial. In addition to data on the proportions fed on human in samples collected from different biotopes, information is needed on the relative prevalence of each biotope and average density of blood-fed mosquitoes in each one (Garrett-Jones *et al.*, 1980). Unfortunately, HBI is often based solely on indoor resting females. In a survey in Kenya, 95% of *An. gambiae s.s.* and 63% of *An arabiensis* caught indoors were human fed, while in a cow trap 77% of *An. gambiae s.s.* and 97 % of *An arabiensis* caught were cow fed (Petrarca *et al.*, 1991).

In summary, three major difficulties are recognised in the behavioural division of anthropophily and zoophily. First, the division is largely based on anthropomorphic reasoning that obscures the biological backgrounds. Second, the division of feeding habits used suggests that it is a rigid characteristic of species while the opposite appears to be true. Feeding patterns appear to be the result of innate host preferences and external factors and can vary between places and over time. Third, the degree of anthropophily is often based on biased samples of the population. In conclusion, the established practice to divide mosquito behaviour in anthropophily and zoophily should be approached with great care.

Apart from of the difficulties with the classification, different feeding patterns exist: not all mosquitoes feed on all vertebrates. As stated earlier, feeding patterns of mosquitoes are considered to be the outcome of host preference, physiological state and other factors like host availability and microclimate. About the cause of the feeding preference various theories have been proposed (Van Thiel, 1939). As early as 1932, Roubaud suggested that feeding preference was determined by the morphology of the maxillae of a mosquito. The number of "teeth" on the maxillae, also called maxillary index, was used as measure for the level of anthropophily or zoophily (in Van Thiel, 1939). In the same period, a second group of investigators suggested that mosquitoes choose by means of smell (Grassi, 1902; Hackett and Missori, 1930, in Van Thiel, 1939). Another investigator proposed that feeding preference was the consequence of the microclimate preference of an anopheline population and the microclimate conditions this mosquito finds in human dwellings and stables (Martini and Treuber, 1933, in Van Thiel, 1939). Van Thiel tested all three hypotheses and rejected the first and the third one. By means of behavioural tests he showed that olfactory cues emanating from the host do play a role in host selection, at least of members of the *An. maculipennis*-complex (Van Thiel, 1939). In general, the well-developed olfactory sense of mosquitoes appears to be an important mechanism for exhibiting an innate and hence genetic host preference. Up to now, knowledge of the genetic background of host preference is still limited (Ralisoa Randrianasolo and Coluzzi, 1987). Moreover, basic ecological and behavioural aspects of odour-mediated host selection by malaria vectors and how these affect transmission of malaria are still marginally understood. This while knowledge of vector biology is critical to develop alternative control strategies (Takken and Knols, 1999). The background of odour-mediated host-seeking behaviour of malaria mosquitoes is considered in the following section.

CHEMICAL ECOLOGY

ODOUR-MEDIATED HOST-SEEKING BEHAVIOUR OF MALARIA MOSQUITOES

Already in 1922, Rudolfs recognised the presence of odour-mediated host selection in mosquitoes and, since then, compelling evidence has been provided (reviewed in Takken and Knols, 1999). Next to olfactory signals, other cues may play a role in mosquito host selection like physical cues (warmth and humidity) in close vicinity of the host and visual cues (Takken, 1991). However, since most anopheline mosquitoes and all members of the *An. gambiae s.l.*-complex are nocturnal, visual cues are of minor importance in host selection.

Of the few human breath compounds tested, only acetone and carbon dioxide have shown attractiveness for *An. gambiae* (Takken *et al.*, 1997). It is generally assumed that the man-biting *An. gambiae* is also highly attracted to volatiles emanating from the human skin (Knols and Meijerink, 1997). The first synthetic blend of odours that attracted *An. gambiae* in a bioassay consisted of a mixture of carboxylic acids (Knols *et al.*, 1997). Carboxylic acids are constituents of human skin emanations and are thought to be produced by microbial activity (Nicolaides, 1974). Recently, the first electrophysiological study on the single cell level for *An. gambiae* reported single antennal olfactory neurons sensitive to aliphatic carboxylic acids (Meijerink and Van Loon, 1999). Despite intensive research, attraction of *An. gambiae* to a single component has not been established, yet.

A routine frequently observed in kairomone studies of insects is to monitor various different chemical components and mixtures for electrophysiological and/or behavioural activity of the insect without comprehensive knowledge of the natural occurrence or biological significance of these compounds. For this reason, some background and origin of olfactory stimuli will be described in the following section.

SEMIOCHEMICALS

The olfactory signature of an animal is determined by the total of volatiles emanating from it (Albone, 1984). Some of the volatiles carry information or otherwise mediate interactions between organisms in the shared environment. These so-called semiochemicals operate either between members of the same species or between members of different species, named homeochemicals or pheromones and allelochemicals respectively. The latter comprise chemicals which either favouring the emitting species (allomone) or the receiving species (kairomone) (Albone, 1984; Dicke and Sabelis, 1988). Semiochemicals are found in many of the biological interactions of organisms with members of their own and other species. The total of volatiles emanating from a source makes up its odour. However, odour is a popularly used term for the total of volatiles that we, humans, perceive consciously. Since humans are equipped with a rather poorly developed olfactory system compared to many other organisms, the volatiles that we are aware of are only a fraction of the total of volatiles present. Erroneously, these are frequently regarded as 'the whole story'. An illustrative example is that a literature search on human odour brings forth just reports on malodours arising from feet or arm pits, completely overlooking the olfactory signals given off to others e.g. possible mates (see later), police dogs or haematophagous mosquitoes. Therefore, the term odour will, henceforth, be used in the broad sense incorporating all volatile compounds that are emanating from an organism.

In the last decades, through the improvement of chemical analyses of volatiles emanating from a source or so-called headspace analysis, increasing numbers of semiochemicals have been discovered and identified. Important research fields are found in medical studies, agricultural science and commercial business. An example of the first is the headspace analyses of exhaled air for the diagnosis of diseases (Penn and Potts, 1998). An example on the boundary of both medical and agricultural science is the development of

odour-baited traps for tsetse flies, the vector of sleeping sickness (an important disease of humans and livestock), by the identification of kairomones for this fly (Vale, 1993). Strict agricultural applications are the usage of insect sex pheromones in the control of phytophagous pest insects or the development of a synthetic scent of a male pig for the artificial insemination of sows (Gibbons, 1986). The identity of chemicals that influence human behaviour are of commercial interest, for example, the development of "odour-pillars" in shopping malls to create a comfortable atmosphere or the design of perfumes to affect even human mate-selection (Gibbons, 1986).

In the mosquito-host interaction the scent of the host functions as a kairomone for the mosquito in finding its host for a blood meal. Identification of these kairomones is crucial for the development of odour-baited traps, invaluable for monitoring mosquito populations in the field in service of both biological and medical studies. The fact that many mosquito species can distinguish different host species by their odour implies that host-specific odour blends must exist.

SOURCES OF HOST ODOUR

Olfactory information from the host originates either from the exhaled air or the skin or both. Since some animals spread excretion on themselves, urine and faecal odours will also be briefly mentioned here.

Breath

Vertebrate breath or exhaled air contains plasma-derived volatiles from the lungs and saliva volatiles from the oral cavity together with volatiles from microbial origin, arising from degradation of saliva or food (Albone, 1984). The latter group of volatiles makes up an important part of the exhaled air of ruminants. These animals regurgitate food to the first division of their multi-chambered stomach after being fermented by microorganisms in other stomach divisions. Gas arising from this fermentation contains carbon dioxide, methane nitrogen, oxygen and short-chain fatty acids and is released with belches (Vonk, 1978; Eckert, 1988). However, carbon dioxide is, in general, the main component of vertebrate breath and is released at different rates. For example, humans release about 300 ml per minute, bovines between 1000 and 3500 ml/min and domestic chickens 12-24 ml per kg body weight per minute (Mboera and Takken, 1997). Next to carbon dioxide, more than one hundred other volatile components have been identified from breath (Sastry *et al.*, 1980).

Urine and faeces

Physiological waste products are predominately excreted as urine or faeces. Urine consists principally of a filtrate of blood plasma produced in the kidney. Mammalian urine contains mainly urea, the end product of the nitrogen metabolism, together with electrolytes and organic acids (Zandee and Spaargaren, 1978). Birds and some reptiles and amphibians excrete uric acid and fishes excrete pure ammonia as end product of the nitrogen metabolism. The average pH of human urine is 6.2, while the urine of herbivorous mammals like horses and cows is more alkaline (Albone, 1984). Headspace analyses of human urine demonstrates over 300 identified volatiles including ketones, aldehydes, carboxylic acids phenols and alcohols (Sastry *et al.*, 1980; Albone, 1984). The typical urine-like smell that we normally recognise better than the odour of fresh urine arises after the initially sterile urine is eliminated from the body and readily inoculated with microorganisms that hydrolyse urea into ammonia and androstenol into the offensively odorous androstone (Sastry *et al.*, 1980).

Waste products of the alimentary system are excreted in faeces. Food in most vertebrate alimentary systems is subjected to gastrointestinal digestive secretion and degradative enzymes of the symbiotic intestinal microbes. Since the faecal composition is strongly related to the animal's diet, a wide range of volatiles is found in the headspace of faeces. General faecal odour components are indole and short chain fatty acids (Albone, 1984).

Skin emanations
The skin of animals is both an endogenous and exogenous source of substances including skin secretions and breakdown products of keratin and cell debris and their microbial degradation products. The odour is the result of the interaction between secretions of skin glands and resident bacteria. These together with the hairs provide a perfect place for odour production. Because species-specific olfactory signatures originate from differences between these factors on the skin surface, comparative aspects of mammalian skin will be considered.

SKIN SURFACE EMANATIONS: A COMPARATIVE APPROACH

The mammalian skin that protects an organism against the environment consists of three major layers. The outer layer, or epidermis, contains no blood vessels and receives nutrients by diffusion only from the underlying dermis. Beneath the dermis lays an inner layer of subcutaneous fat tissue. The whole skin protects the organisms against mechanical damage. The structure of the dermis prevents water loss and penetration of foreign microorganisms and substances. The hair and subcutaneous fat provides insulation against cold, and the network of blood vessels in the dermis and the secretion of sweat on the skin surface regulate the body temperature. The secreted emulsion of the epidermis lubricates the skin and has anti-bacterial properties (Sokolov, 1982).

The epidermis that covers the surface body shows a large variation among mammals. The epidermis is most developed in hairless organisms, like humans, or in hairless body parts, like the foot soles of many animals. As mentioned earlier, the epidermis is provided with an emulsion of water and fat. Watery substances are secreted by the sweat glands, the eccrine and the apocrine glands; fats are predominately secreted by the sebaceous glands (Noble and Somerville, 1974). A schematic drawing of the skin surface is given in Figure 1.1. The occurrence and distribution of these glands vary largely between different body parts and different species and consequently also the composition of the epidermal emulsion varies. As a result the composition of the microbial population present on the skin can be largely different. All these factors together determine an animal's body odour.

Sebaceous glands
Sebaceous glands are present in all mammalian skin, except whales and porpoises, and are generally associated with hair follicles. The skin of man, together with that of the lemur (Albone, 1984), is exceptionally rich in sebaceous glands and shows an unequal distribution over the body from 900 glands/cm^2 on the fore head, 100/cm^2 on arms and legs to a total absence of glands on sole and palms. The glands are fully developed after puberty and men have generally larger ones than females. In other mammals the glands are far less numerous but more equally distributed (Sokolov, 1982).

Sebaceous glands are largest and most numerous in mucocutaneous junctions, in interdigital spaces and over the dorsal neck and rump and in some specialised scent glands (Muller, 1995). Studies of the lipids on mammalian skin show that man is unique in its high level of triglycerides, which are principally broken down by *Propionibacterium* spp. and give rise to a high level of free fatty acids. In rabbits and sheep, only low levels of triglycerides and free fatty acids are found, while these compounds are completely absent in the skin lipids of chimpanzees, baboons, hamsters, guinea pigs, dogs, cats and cows (Nicolaides *et al.*, 1968). Skin lipids of non-human mammals contain mainly esters other than triglycerides which are less readily hydrolysed by microbial activity (Albone, 1984). Next to these compounds, squalene is found in high levels in humans only, and sterols and several wax esters are generally found in sebum of mammals. As the lipids are synthesised by the gland itself, the lipid composition does not directly reflect the composition of diet or circulating lipids in blood, except in situations of major deficiencies. Large individual differences are seen in the level of hydrolysis of triglycerides while the other lipid components are rather stable

(Noble and Somerville, 1974). Sebaceous glands provide substances that have anti-bacterial properties, protect against water loss and often play a role in chemical communication between mammals. In humans sebum production is increased during puberty (Blackburn, 1991).

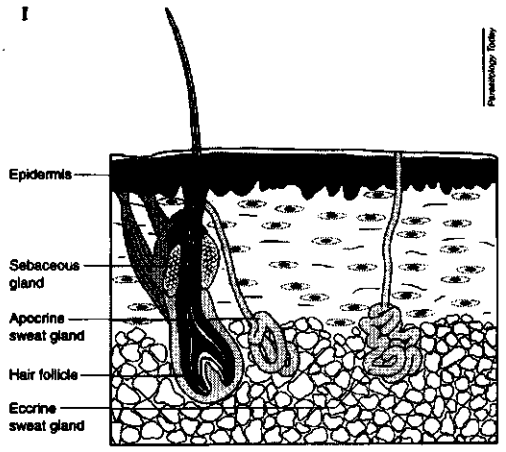

FIGURE 1.1. Schematic drawing of basic hair follicle unit (drawing by P. Kostense and reprinted from Braks et al. *1999a, Infochemicals in mosquito host-selection; human skin microflora and Plasmodium parasites . Parasitology Today. With permission from Elsevier Science.).*

Sweat glands

Sweat glands are present in the skin of mammals only. Despite a debate about the underlying secretory mechanisms, sweat glands are generally divided in eccrine or real sweat glands and apocrine glands (Sokolov, 1982).

Eccrine sweat glands are most numerous and best developed in higher primates. In man, eccrine sweat glands are particularly numerous and are distributed over the whole body surface, especially on palms and soles (140-340 glands/cm ^{2}). Eccrine sweat glands secrete a watery solution directly on the skin surface and are not associated with hair follicles (termed 'atrichial') unlike sebaceous and apocrine glands. Human eccrine sweat is hypotonic to the circulating blood and consists mainly of water (99%), urea which is derived from circulating blood (0.3 g/l), lactic acid which is produced through glycolysis by the gland itself (0.9-3.5 g/l), amino acids and inorganic salts (Albone, 1984). In other primates eccrine glands are less well developed and their distribution is restricted to special sites in other mammals e.g. on footpads and muzzles of Rodentia, Felicidae and Canidae and in the snout of a pig. Feline eccrine sweat contains lactate, glucose and inorganic salts and differs from that of humans in that it is hypertonic, alkaline and more saline (Muller, 1995).

Apocrine glands are found throughout all mammalian haired skin. The glands are located below the sebaceous glands in the hair follicle or pilosebaceous canal and are termed epitrichial. Since sweat is secreted into this canal it is mixed with sebum before it reaches the skin (Jenkinson, 1973). Apocrine glands are largest and most numerous in mucocutaneous junctions, in interdigital spaces and over the dorsal neck and rump of mammals (Muller, 1995). The occurrence of these glands in higher primates is limited and in man restricted to the axillary and genitalian region (Adams, 1980). Although some compounds of apocrine secretion are known (lipids, proteins, ammoniac and reducing sugars), their function is not entirely understood. Apocrine secretion is released under emotional stress (Sastry *et al.*, 1980). The production of apocrine sweat like that of sebum is

under hormonal control and increases during puberty (Blackburn, 1991). The secretion is initially odourless but is modified to a typical smell by coryneform bacteria present in the axillae (Zeng *et al.*, 1992). The secretions of different mammals range from very sparse or oily to that in horse where it is profuse and watery (Jenkinson, 1973). Reports of the composition of sweat of mammals other than human are limited.

Sweat glands are only present in homeothermic animals excluding birds, and not all mammals possess sweat glands. Some mammals like some rodents and the hippopotamus that can create their own microclimate (e.g. by living mainly in holes, wallowing) are lacking sweat glands. So it seems that sweat glands only serve as thermoregulatory organs. However those of pig and marsupials do not respond to heat. Thermoregulation by sweat glands is only seen in primates and ungulates (including bovines and equines). In general, 75 % of the heat loss from the mammalian body is achieved through radiation, conduction and convection, and the rest by evaporation of water from the skin and respiratory passages and excretion of urine and faeces (Muller, 1995). Because canines and felines do not produce a lot of sweat, the heat loss is mainly accomplished through respiratory passages. Cats posses an additional mechanism; they spread watery saliva, which flow is increased by heat, over its coat for the cooling effect by evaporation (Muller, 1995). Next to thermoregulation sweat glands can fulfil other functions like producing skin lubricant, creating antibacterial conditions, excreting waste products and products that are important in mammalian communication (Jenkinson, 1973). According to Jenkinson (1973), sweat glands should therefore be considered basically as protective organs against extreme temperatures, skin damage, bacterial infections, accumulation of waste products and the extinction of species by acting as scent glands. So, in most mammals, the sweat secretions have similar function as sebum.

In humans four different patterns of sweating are recognised, three eccrine and one apocrine based patterns (Fig.1.2). First, sweating upon stimulation of the heat-regulating centre in the hypothalamus is occurring over the whole body. Emotional and mental stimuli induce sweating in restricted areas. Sweating of the palm (and soles) will increase grip in stressful situations. An explanation for emotional sweating in the other regions is not available (Champion, 1970). Gustatory sweating has no obvious physiological function but which occurs commonly upon consuming food.

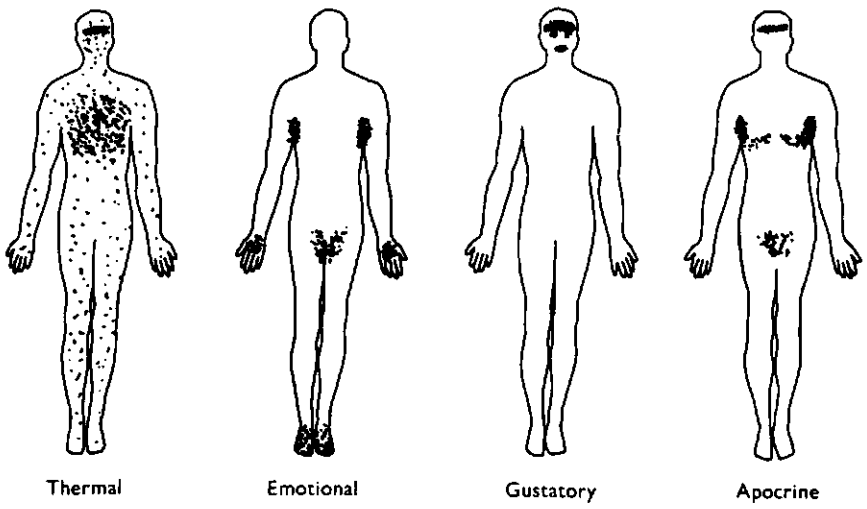

Figure 1.2. Patterns of physiological sweating (After R.H. Champion, 1970, with kind permission from Blackwell Science Publishers, Boston).

The rate and mode of action of sweating differ largely between the different primate and ungulate species. Three different discharge types are recognised (Jenkinson, 1973). First, man together with camel, llama and buffalo and sometimes horse demonstrate a smooth onset of sweating that can be sustained for some time. Cow and eland show a stepwise increase, but this can be also sustained, while sheep, goat, duiker (a small antelope) and gazelle display slow and uncontrolled interrupted discharges of sweat which decrease in strength and magnitude with time.

Hair

Mammals are the only vertebrates possessing hair. The mammalian hair coat insulates, protects the skin against UV light and often fulfils an important signalling function in social interaction (Jenkinson, 1993). A basic hair follicle unit consists of a hair follicle with a hair, an arrector pili muscle, a sebaceous gland and an apocrine gland (Fig. 1.1). Variations in size and distribution of hair exist between different species, and also between body parts within a single species. The presence or absence of hair follicle grouping is a variation upon this basic follicle unit model. Hair follicle grouping, found in cats, dogs, sheep and camels, is the occurrence of grouping of a second type of hair follicle (secondary follicles) together with complete follicles (primary follicles). The secondary follicles are smaller and lacking the arrector pili muscle or sweat gland. The ratio between the two types of hairs determines the appearance of the coat. Man, pig, horse and cow lack such grouping and only possess primary follicles (Jenkinson, 1993). The different appearance of the hair coat of, for example, pig and cow, is due to a large variation in follicle density, respectively 10-20 and 2000 follicles per cm^2.

Three types of hair replacement are recognised, a seasonal, mosaic and wave-like pattern. Wild animals often show a seasonal shedding of the coat to adapt to different ambient temperatures. Humans exhibit a mosaic pattern, where the replacement cycle is not uniform over the body. A wave pattern is found in laboratory rodents whereby the shedding moves gradually from (often) the neck to other parts of the body (Jenkinson, 1993). Clusters of hairs and glands provide a good microclimate and substrate for skin microorganisms. For example, the human axillary region in man carries relatively high densities of microorganisms.

Scent glands

Odours may be produced by skin glands in the general body surface, but often arise from specialised regions in the skin involving high sweat or sebaceous gland density and gland enlargement (Adams, 1980). These scent organs consist often of structures that enhance the storage, modification and release of odorous chemicals. In addition to odours arising from scent organs of the integument, semiochemicals may originate from salivary glands, accessory glands of the eye, vagina or urine, however these will not be discussed here.

The scent organs of small mammals consist primarily of modified sebaceous glands while these organs of larger mammals are often a combination of sebaceous and sweat glands. Specialised sebaceous glands often are equipped with large muscles not only for piloerection but especially to squeeze sebum to the surface when necessary. The most odoriferous regions contain apocrine glands. Eccrine gland specialisation is seen in the plantar surface of mouse foot producing an individual recognition scent. Odour production is most likely to result from incomplete substrate oxidation associated with anaerobic microbial processes as in mammalian cutaneous structures, such as pouches and cavities that restrict oxygen access. General anaerobic odour components are volatile fatty acids, amines and mercaptans (Albone *et al.*, 1977). In human, sweat glands of the palmar and plantar surfaces excrete in stressful and emotional conditions. In females, steroids are secreted which strongly point at a pheromonal function (Adams, 1980).

For many years, the idea that chemical communication within the human race does exist was repudiated by the simple argument that humans do not have the equipment for detecting pheromones. Such an organ has been demonstrated in the tissue between the two

nostrils of the nose of mammals other than humans and is called the vomeronasal organ, or VNO. Despite earlier speculation, conclusive evidence for the presence of a VNO in humans has been provided only a few years ago (Berliner *et al.*, 1996). The astonishing fact that this sixth sense of human was discovered only recently illustrates the underdeveloped consensus of our own behaviour and the underestimation of the importance of olfactory information available in the environment for other organisms.

Skin micro-flora

Unlike the high humidity and the abundance of organic matter suggest, the skin is a rather inhospitable substrate for most microorganisms. Moreover, the skin is an efficient protective barrier against microorganisms by maintaining a low pH and excreting antibacterial agents (see above). Only few microbial species can survive. The composition and growth of skin micro-flora depend on skin temperature, humidity, pH, the concentration of inhibitors, and availability of nutrients (Noble, 1993). The distribution of skin microbes is determined by species-specific nutrient demands as well as the presence of cutaneous glands and by physical characteristics of skin sites. Therefore, the composition of the microbial population on the skin differs largely between body area and vertebrate species. Microorganisms of the mammalian skin appear to be distributed as mixed micro-colonies between the layers of the outer stratum corneum: bacteria are often found in close association with yeasts, mainly *Malessezia* species (Lloyd, 1980; Noble, 1993). Bacteria are roughly subdivided in anaerobe (mainly *Propionibacterium* spp.) and aerobe species (Table 1.1). The distributions of yeast, and anaerobe and aerobe bacteria on the skin are given in Figure 1.3. The organisms live in symbiotic groups probably in pockets where the thermal and gaseous microenvironment is favourable and nutrient content of the emulsion is high (Jenkinson, 1993). Many odours emanating from the skin or specialised scent glands are thought to be produced by microbial activity (Albone *et al.*, 1977).

TABLE 1.1[a]. The most important families of the microflora resident on the human skin.

Group	Family	Genus	Example species
Gram-positive cocci	Micrococcaceae	*Micrococcus* *Staphylococcus*	*M. luteus* *S. epidermis*
Diphtheroids-like species	Coryneforms	*Brevibacterium* *Corynebacterium*	*B. epidermis* *C. diphtheria*
	Propionibacteriaceae	*Propionibacterium*	*P. acnes*
Fungi	Yeast	*Malassezia*	*M. furfur*

[a] *modified after Noble, 1993*

The principle groups of aerobe organisms colonising the human skin are coryneforms and staphylococci. Relatively little attention has been paid to the coryneforms from animal skin, which is due to the poor taxonomy of this group. In contrast the taxonomy of staphylococci of human and animal origin has been worked out well, nevertheless mainly with respect to skin infections (Jenkinson, 1990). In man, at least three genera of diphtheroids areregularly present on the human skin, *Brevibacterium, Corynebacterium* and *Propionibacterium. Brevibacterium* spp. have a limited distribution on the human skin and appears only on the toe webs whilst various species are known to be part of the normal flora in cattle. *Brevibacterium linens* is used in dairy industry to enhance flavour and odour in cheeses and may have originated from cattle skin. *Brevibacterium epidermis* is involved in foot infections and causes extensive destruction of the toe web by proteolysis that gives rise to a pungent "cheesy" malodour. *Corynebacterium* spp. is found over the whole human body

surface, but the reports on animals are scarce. Axillary odour has received most attention and consists of three components: the short chain fatty acids providing the 'sweaty' odour, the 'musk' or 'urine-like' odours derived from the 16-androstrenes and the 'unwashed' odour which is derived from isomers of 2-methyl-3-hexenoic acids (Gower *et al.*, 1994). The human coryneforms are able to form the odorous compounds from steroids secreted by the axillary gland that are suggested to be involved in maternal/neonates recognition and may act as human pheromones (Stoddart, 1990). The sweaty acid odour such as isovaleric acid is probably formed by cocci (Lukacs *et al.*, 1991). *Propionibacterium* spp. and to a lesser extent *Corynebacterium* spp. are lipophilic. In humans, *Propionibacterium* spp. are abundant in the highly sebaceous areas like face and shoulders and are the most numerous organisms on the human skin. Members of this genera are rarely reported to be present on animal skin, which may be due to a moderately or poorly developed sebaceous system compared to the human system (Holland, 1993). Various staphylococcal species make part of the normal skin flora of several mammals. Although not in man, many staphylococcal species are involved in animal skin diseases e.g. mastitis in cattle and sheep.

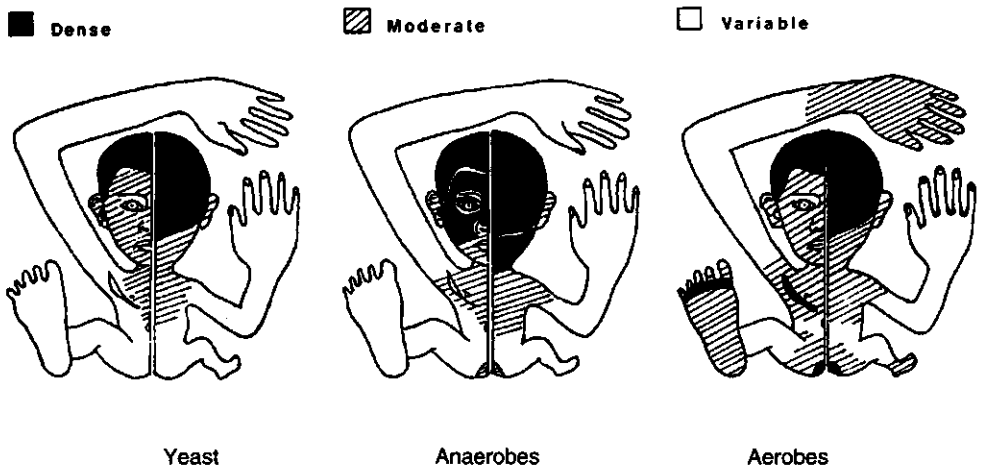

Figure 1.3. Regional and qualitative variation in the cutaneous microflora (Leyden, J.J.; McGinley, K. and Webster, G. 1983, from M.J. Marples, 1965, with kind permission from Thomas Springfield).

As mentioned before, microorganisms play a large role in the production of body odour, either from the normal skin surface or from specialised scent glands. Albone (1984) recognised three possible ecological functions of these odours with respect to fermentation. First, body odour may function in the individual or species recognition in which stability of fermentation product composition with time is important. Second, odour may have a signal function in which a time modulation of the fermentation products is required e.g. reflecting hormonal state. Third, odour may have a defence function, like in the skunk, and a limited variation in fermentation product composition with time is unimportant. This division suggests that microbial-borne volatiles favour only the host of the microorganisms. However, on the contrary, other species often benefit from it. For example, hosts exhibit non-intentionally their presence and identity to haematophagous malaria mosquitoes by their odour. However, odour components responsible for the attraction of the malaria mosquito to their hosts are only being identified at present (Knols, 1996; this thesis). In case microorganisms are involved in the production of these kairomones, these volatiles function as species recognition, the first category in Albone's division, in which stability of the fermentation product composition with time is important. Skin surfaces of all mammals are colonised by a

variety of microorganisms and it is probably that these contribute together with the specific skin substances present to the general body odour which typify many species (Albone, 1984).

AIM AND OUTLINE OF THE THESIS

The aim of the behavioural ecological studies described in this thesis was to elucidate the odour-mediated host-seeking behaviour of the Afrotropical malaria mosquito *An. gambiae s.s.* in relation to human skin emanations. The specific objectives of the work presented in this thesis relate to the source, identification and production of kairomones.

The first section deals with the source of kairomones of *An. gambiae* (Chapters 2-6). In Chapter 2, a field study on the significance of breath and skin emanations in the attraction of *An. gambiae* to humans is described. After recognising that skin emanations play an essential role in this attraction the research was continued in the laboratory to investigate the three research objectives under controlled conditions. In continuation of earlier laboratory studies the role of carboxylic acids was investigated and the results are reported in Chapters 3 and 4. In Chapter 3 replicates of behavioural experiments with carboxylic acid mixtures of a preceding study are described. Chapter 4 describes the role of carboxylic acids in the attractiveness of a sweat sample collected from a human volunteer. On the basis of these data, sweat as a naturally derived host odour stimulus was further investigated. In Chapter 5, it is described that the incubation of freshly collected sweat probably brings about the attraction reported in Chapter 4. In Chapter 6 the significance of sweat in respect to skin washing, another known complex host derived attractant, is investigated.

In the second section the identification of kairomones is described. In Chapter 7 a multidisciplinary approach connecting the behavioural and electrophysiological research is reported. In addition, a method to diminish the variation in the attraction to different sweat samples mentioned in Chapter 5 is described. Both are essential achievements for the ultimate identification of kairomones. In Chapter 8 the role of human sweat components, ammonia and L-lactic acid, in the behaviour of *An. gambiae* is lined out.

The third section describes investigations in the production or biosynthesis of kairomones of *An. gambiae* by incubation of sweat. Experimental results (Chapter 9) and a theoretical discussion (Chapter 10) on the role of skin microflora in the production of kairomones are discussed.

Finally, the results of the three sections are considered in the General Discussion. Further, the outcome of the research is discussed and directions for future studies are proposed.

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SECTION I

SOURCES OF KAIROMONES FOR *AN. GAMBIAE*

HUMAN SKIN AS IMPORTANT SOURCE OF KAIROMONES FOR MAN-BITING *AN. GAMBIAE*: A FIELD STUDY IN TANZANIA

ABSTRACT – A study to sample an outdoor population of *Anopheles gambiae sensu stricto* using odour-baited electric nets was conducted in Kilombero, south-east Tanzania. *An. gambia s.s.* was attracted to human body odour in the presence and absence of breath. The response of the mosquito to body odour with breath was not significantly different to that to body odour alone, indicating an important role of skin emanations in its olfactory-mediated host-finding behaviour. The mosquito showed a poor response to electric nets baited with human skin residues collected on worn clothing. The results suggest that *An. gambiae s.s.* uses highly volatile host odours, other than carbon dioxide, in distinguishing living humans from inanimate materials contaminated with human skin residues. Electric nets are a useful trapping device for testing the attractiveness of odour stimuli in an outdoor situation.

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INTRODUCTION

The host-seeking behaviour of a female mosquito is guided by a range of physical, visual, and olfactory cues (Takken, 1991). The Afro-tropical malaria mosquito *Anopheles gambiae* Giles *sensu stricto* feeds primarily on humans and is highly endophilic and endophagic. It is part of a species complex, whose members differ widely in their host preference (White, 1974). It is thought that for this complex host-odours are the principal cues with which hosts are being found and therefore human odours must contain these cues for *An. gambiae s.s.*.

Carbon dioxide and skin emanations are important olfactory stimuli for mosquitoes in general (Takken, 1991; Mboera and Takken, 1997). Responses of female *An. gambiae* to carbon dioxide have been observed both in the laboratory (De Jong and Knols, 1995b; Healy and Copland, 1995) and in the field (Costantini *et al.*, 1993, 1996; Mboera *et al.*, 1997; Mboera, 1999). Nevertheless, various studies have shown that carbon dioxide is of minor importance for Afro-tropical anthropophilic mosquitoes (Gillies and Wilkes, 1972; Mboera and Takken, 1997; Mboera, 1999), as compared to the zoophilic or opportunistic mosquitoes (Dekker and Takken, 1998a; Knols *et al.*, 1998). Of the components of human skin emanations, *An. gambiae* s.s. has been shown to respond significantly to sweat (Chapter 4) and a mixture of fatty acids (Knols *et al.*, 1997) in the laboratory. Moreover, recent wind-tunnel studies by Knols *et al.* (1997) have shown that the mosquito responds to Limburger cheese volatiles. However, to date reports of the responses of *An. gambiae* s.s. to these stimuli in the field are not available.

Field studies of the odour-guided host-seeking behaviour of malaria mosquitoes in Africa are at least partly hampered by the lack of good sampling techniques to test host odours. Recent studies by Knols *et al.* (1998) and Mboera (1999) have shown that electric nets can be successfully employed in sampling outdoor mosquitoes. Previously, electric nets have been widely used in behavioural studies of tsetse flies (Vale, 1974) and blackflies (Killick- Kendrick *et al.*, 1981). The objective of the present field study was to test the feasibility of sampling outdoor populations of *An. gambiae s.s.* by means of electric nets baited with the complete emanations of a human being. In addition the effect of the removal of breath, the main human carbon dioxide source, from the stimulus was studied. Finally, the attractiveness of human skin residues collected on clothes was assessed.

MATERIALS AND METHODS

Study site. Field experiments were carried out during the long rainy season (April and May 1997) in Njage, 70 km to the west of Ifakara town, Morogoro Region, South-East Tanzania. The village is situated at the southern edge of the Udzungwa mountains, in the Kilombero valley (Charlwood *et al.*, 1995). The experiments were carried out in close vicinity (10 m) to two little houses situated outside the main village and surrounded by large rice fields. There were no other houses within a radius of 50 m. In one of the houses, occupied by one adult man, the density of mosquitoes was monitored daily by CDC miniature light-traps (Model 512 John W. Hock Company, Gainesville, FL).

Experimental design. Odour-baited electric nets, slightly modified after Knols *et al.* (1998), were used as trapping devices in the experiments. The nets consisted of 149 stainless steel wires, which were attached to a metal frame (inner dimensions: 40 x 60 cm) at 4 mm apart. The wires were alternately earthed or charged with 6 kV AC by an inverter transformer oscillator (manufactured by Dr. T. Coates, Bristol, UK) driven by a 12 V car battery. The mosquitoes were electrocuted upon touching the wires and collected in plastic trays, which were filled with soapy water and placed underneath the electric nets. More details of the technical aspects of the electric nets have been described by Knols *et al.* (1998).

Two electric nets were placed 3 m at either side of a small experimental plastic tent (0.65 m high and 1.85 x 1.5 m wide) occupied by one human individual (Fig. 2.1). One of the

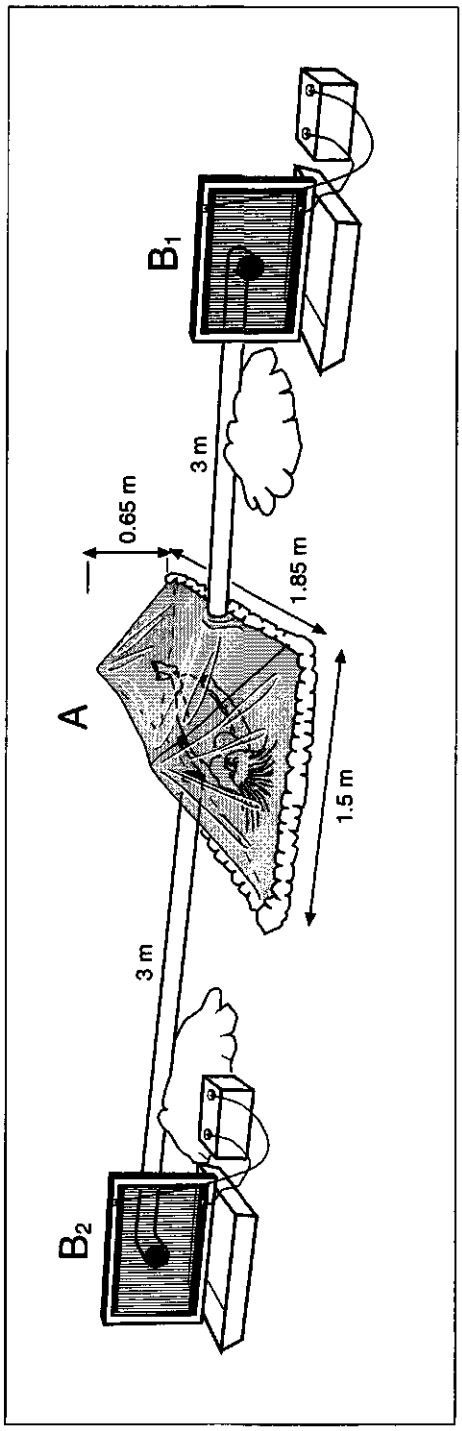

FIGURE 2.1. Schematic drawing of the experimental set up where the odour of a human individual in a small experimental tent (A) was pumped to one of the two electric nets (B_1 and B_2) (drawing by P. Kostense).

electric nets was baited with odours pumped from the individual sleeping under the plastic tent while the unbaited electric net onto which clean air was pumped, was used as a control. Air was led to each electric net through a 3 m long polyvinyl chloride pipe (11 cm internal diameter), by means of a fan (taken from a CDC light-trap, 6V) producing an outlet speed of 1-1.5 m/sec. In this set-up the air of the tent (volume 900 l) was refreshed every 1.5 min.

The outside relative humidity during the experimental nights was above 98% during the experimental nights; the ambient temperature varied between a maximum of 26.0°C at dusk (24.1±1.8°C) and a minimum of 16.3°C at dawn (21.2 ± 2.1°C). There was no measurable difference between the temperature and humidity of the outside air and the air flowing out of the pipe.

Odours. The following three different odour stimuli were tested against clean air (control): (i) Whole body odour (W). The odour was derived from a Caucasian adult female (28 years old) sleeping under the plastic tent. (ii) Whole body odour without breath (W-B). The odour was derived from the same Caucasian adult female sleeping under the plastic tent and breathing through a one-way valve (Harvard-Douglas®) to which a 15 m silicon tubing (9 mm internal diameter) was attached. (iii) Residual body odour (R). No human individual was sleeping in the tent, but her clothes worn for 2 days and bedding materials were present and formed the odour source. Each odour stimulus was tested for four nights from 20.00 to 06.00 hr and equally often on both positions and electric nets. The order of the different odour stimuli was randomised over twelve nights.

At dawn mosquitoes caught on the net and in the water trays were collected and identified morphologically.

Data analysis. Statistical analysis of differences between catches of each stimulus and its control were performed per mosquito species using a *t*-test for paired samples of the log (x+1) transformed data ($P<0.05$). A comparison between the catches of *Anopheles gambiae s.l.* with the different odour stimuli and controls was performed using *t*-test for independent samples with a Bonferroni correction for multiple comparison of α. The catches of other mosquito species were considered inadequate to include in the latter statistical analyses.

RESULTS

During the twelve days of the experiment a total of 592 unfed female mosquitoes were caught on the two electric nets of which *An. gambiae s.l.* formed the main species (67.7%) followed by *Mansonia africana* (11.5%), *An. funestus* (8.0%), *Culex quinquefasciatus* (7.7%), *An. coustani* (4.6%) and *Aedes sudanensis* (0.5%). In a nearby house, an average of 190 mosquitoes/night were collected in CDC light traps which consisted mainly of *An. gambiae s.l.* (83.4%) followed by *An. funestus* (13.9%) and other mosquito species (2.7%).

The total number of female mosquitoes caught and the geometric means (reversed log-transformed means) of the catches per species using different baits are shown in Table 2.1. For *An. gambiae s.l.*, catches with whole body odour (W) and whole body odour without breath (W-B) differed significantly from the control ($P<0.05$). Although the total number of *An. gambiae s.l.* caught with W was larger than with W-B, the difference was not significant ($P = 0.174$). Similarly, no significant difference was found between the catches with residual body odour (R) and the control.

A significant difference was also found between *Ma. africana* catches with whole body odour and its control. For *An. funestus*, a significant difference was observed between catches with whole body odour minus breath and its control. No significant difference was found between an odour-baited electric net and its control for the other species.

TABLE 2.1. The total numbers and geometric means (reversed log transformed mean) ± SD of female mosquitoes of the six different species caught on electric nets with different odour stimuli, Whole body odour (A), Whole body odour minus breath (B) and Residual body odour (C), versus the control electric nets. Means in the same row followed by the different letter are significantly different (t-test for paired samples, P < 0.05). As the standard deviation exceeded the mean, the range of the number of mosquito per night is indicated. The data of An. gambiae s.l. *followed by the different Latin number are significantly different (t-test for independent samples with Bonferroni correction). The data of other species were too small for the latter statistical analysis.*

	A			
Species	Whole body odour		Control	
	N	Mean	N	Mean
An. gambiae s.l.	200	49.1 ± 0.3 a, I	19	2.6 ± 2.2 b, II
An. funestus	22	3.8 ± 1.9 a	1	0-1 b
An coustani	11	1.9 ± 1.4 a	2	0-1 a
Ma. africana	35	7.4 ± 1.0 a	0	0 b
Cx quinquefasciatus	10	2.2 ± 0.6 a	5	1.1 ± 0.7 a
Ae. sudanesis	0	0 a	0	0 a

	B			
Species	Whole body odour minus Breath		Control	
	N	Mean	N	Mean
An. gambiae s.l.	147	34.8 ± 0.5 a, I	13	2.2 ± 1.5 b, II
An. funestus	18	4.3 ± 0.4 a	2	0-1 b
An coustani	8	1.5 ± 1.1 a	1	0-1 a
Ma. africana	26	3.5 ± 2.4 a	3	0-1 a
Cx quinquefasciatus	12	2.7 ± 0.7 a	5	1.2 ± 0.2 a
Ae. sudanesis	1	0-1 a	1	0-1 a

	C			
Species	Residual body odour		Control	
	N	Mean	N	Mean
An. gambiae s.l.	11	1.7 ± 1.5 a, II	3	0-2 a, II
An. funestus	1	0-1 a	3	0-2 a
An coustani	1	0-1 a	4	0-4 a
Ma. africana	2	0-2 a	2	0-1 a
Cx quinquefasciatus	8	0-3 a	6	0-3 a
Ae. sudanesis	0	0 a	1	0-1 a

DISCUSSION

Of the mosquitoes collected *An. gambiae s.l.* dominated both the indoor and outdoor catches in Njage. Previous research showed that the *An. gambiae* complex population of Njage is dominated by high densities of *An. gambiae s.s.* (Mboera, 1999; B.G.J. Knols unpublished data).

Considerable numbers of *An. gambiae* were attracted and caught on the electric nets baited with host odours outdoors. Knols *et al.* (1998) and Mboera (1999) already showed that electric nets are useful tools to test the attractiveness of odours for several mosquitoes species outdoors. The former study using breath-baited electric nets collected mainly zoophilic mosquitoes and only few *An. gambiae*, the latter using carbon dioxide-baited nets,

collected a significant number of *An. gambiae* and *Cx quinquefasciatus*. The present study showed clearly that human odours other than breath play a major role in attracting *An. gambiae* to its main host. Moreover, this study and others in Tanzania (Knols *et al.*, 1998; Mboera, 1999) have confirmed the usefulness of electric nets in sampling an outdoor population of host-seeking *An. gambiae*, against previous argument that the mosquito being highly endophilic and endophagic cannot be caught outdoors.

Role of carbon dioxide. Selective removal of breath, the major human carbon dioxide source, from whole body odour had no significant effect on the overall attractiveness of a human host in our experiments. Costantini *et al.* (1996) observed a significant reduction in the number of *An. gambiae s.s.* caught in an odour-baited entry trap (OBET) by removing breath from whole body odour stimulus. Snow (1970) found similar results in human biting catches and cage catches. Our results show that complete body odour is more attractive than body odour without breath. However, as the differences are not significant, we assume that carbon dioxide is of minor importance in host-seeking behaviour of *An. gambiae s.s.* in East Africa. The disparity may also be explained by the differences in trapping methods used. Electric nets, in contrast to many other sampling devices, intercept mosquitoes during their upwind flight and catch mosquitoes, which fly against the electrocuting wires. We assume that the catches of electric nets are less biased than those obtained with other trapping devices.

Residual body odour. Significant numbers of mosquitoes were caught on electric nets baited with odour taken from a living human than with residual body odour collected on worn clothes. It is of vital interest for host-seeking mosquitoes to be able to discriminate between a living host and human skin residues on non-living materials in the absence of the host. In addition, the preferred host needs to be distinguished from other hosts. In the living host, heat or convention currents have been shown to be an important cue in attracting some species of mosquitoes. The absence of heat in skin residues might also have contributed to the low number of mosquitoes in this case. Nonetheless, Kline (1998) and Mboera (1999) have recently shown that some species of mosquitoes can be collected in a counterflow geometry trap baited with worn clothing. In another experiment in the same study area we have shown that carbon dioxide baited electric nets and counterflow geometry traps have equal sampling efficacy in collecting *An. gambiae* or *Cx quinquefasciatus* (Mboera, 1999). Haddow (1942) found twice as many female *An. gambiae s.l.* in a hut with dirty clothes than in an empty hut by means of resting-catches. No other field reports of attraction of *An. gambiae s.l.* to host odours, other than carbon dioxide, in the absence of the host itself are available. With complete body odour as bait, all possible olfactory host cues (specific and non-specific) are presented and therefore the mosquitoes are attracted to the most highly reliable stimulus. We conclude that complete body odour without breath is as reliable as body odour with breath for *An. gambiae s.s.* These mosquito kairomones must be highly volatile components of host odour, other than carbon dioxide, which provide as reliable 'life signs' for *An. gambiae s.s.* Such components must be produced continuously from a living host, but evaporate quickly from worn clothes.

This phenomenon may explain the discrepancy between laboratory and field findings. Attraction of *An. gambiae s.s.* to human sweat samples (Braks *et al.*, 1997) was established in the laboratory but not in the field (Braks, unpublished data). In the laboratory stimuli need to last only for a few minutes to attract a selected group of receptive female mosquitoes from a limited distance, while in the field stimuli must last for several hours to attract reasonable number of mosquitoes from an open area. It is probable that in the present study important but highly volatile components were lost too rapidly from worn clothes to be effective in the field. The problem of keeping the most volatile stimuli available during a longer experimental period may also have hampered field research in the past. The current studies to identify kairomones for *An. gambiae* on must therefore be complemented with the development of an efficient odour release device and a better mosquito sampling device.

ACKNOWLEDGEMENTS

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CARBOXYLIC ACIDS AS KAIROMONES FOR *AN. GAMBIAE*

ABSTRACT - For the continuation of a preceding behavioural laboratory study on the host-seeking behaviour of *An. gambiae s.s.*, some essential experiments on the role of Limburger cheese and carboxylic acids in this behaviour were repeated. In accordance with previous reports, attraction was established to trapping devices baited with Limburger cheese and mosquitoes were repelled by traps baited with an undiluted natural acid fraction of this cheese in a dual port olfactometer. Unfortunately, attraction to diluted natural and synthetic acid fractions, reported earlier, was not found. A new approach in the search for the identification of important kairomones for *An. gambiae s.s.* is proposed.

INTRODUCTION

The Afro-tropical malaria mosquito *Anopheles gambiae s.s.* demonstrates an anthropophilic host preference or, in other words, feeds predominantly on humans (Garrett-Jones, 1964). This behaviour is mediated by olfactory cues emanating from the human host (for a review see Takken and Knols, 1999).

From preceding research it was concluded that carbon dioxide and other breath volatiles play a limited role in the odour-mediated host-seeking behaviour of *An. gambiae s.s.* and that skin emanations are more important (Knols, 1996). In an experiment on the selection of biting sites, *An. gambiae s.s* preferred to bite on the feet and ankles of a seated naked human volunteer. Washing the feet with antiseptic soap resulted in significant changes in biting preference (De Jong and Knols, 1995a). It was concluded that the selection of biting sites is mediated by odour. Subsequently, through anthropomorphic deduction, the authors selected an odour source of which the scent resembled that of human foot odour, to wit Limburger cheese. In a dual port olfactometer *An. gambiae s.s.* had a high preference for the trapping-devices baited with Limburger cheese to a control (De Jong and Knols, 1995b). Similar responses were also found to the acid fraction of this cheese and to a synthetic mixture of short-chain carboxylic acids present in the cheese and foot odour (Knols *et al.*, 1997). Additionally, significant electrophysiological responses of *An. gambiae s.s.* were observed towards short-chain carboxylic acids (Knols *et al.*, 1997; Meijerink and Van Loon, 1999; Van den Broek and Den Otter, 1999). Reproduction of the behavioural responses to Limburger cheese and the natural and synthetic acid mixture of the cheese was the starting point of the present laboratory study.

MATERIALS AND METHODS

Mosquitoes. The *An. gambiae s.s.* strain used originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome) and was maintained under standard conditions (27 ± 1°C, 80 ± 5% RH, 12 hr scotophase). Adults were kept in 30 cm cubic gauze-covered cages and had access to 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 the larvae were fed on Tetramin fish food. Pupae were collected daily from the trays and were allowed to emerge in the adult cages. In the bioassay 4-8 days old female mosquitoes were used, which had not received a blood meal.

Olfactometer. A dual-port olfactometer (after Knols *et al.*, 1994), was used to study the attractiveness of odour stimuli (Fig. 3.1). The olfactometer consists of a large flight chamber (160x60x60cm) in which mosquitoes are released. Conditioned air (60 ± 5% RH; 27 ± 0.5 °C) enters the flight chamber through two small ports (diameter 5 cm, horizontally aligned, 30 cm apart). The ports of the flight chamber are linked to trapping devices (Knols *et al.*, 1994). These are made of glass containers through which the conditioned air stream enters the flight chamber. The wind speed was fine-regulated to 20 cm/s. Light (6.3 ± 1.4 Lux) was produced by incandescent light bulbs placed on top of the tunnel.

Experimental procedure. Each experimental day consisted of four trial periods for the same odour stimulus. Mosquitoes were tested in the last four hours of the scotophase. In each trial a batch of 50 mosquitoes were simultaneously released from a container placed in the flight chamber at 100 cm downwind from the two ports. Every batch of mosquitoes was allowed to make a choice between the test or control port during 20 minutes. The trap catches were counted afterwards and the distribution between the test and control stimuli was determined. Each trial started with new mosquitoes, clean traps and 100 µl newly applied stimuli. The test stimulus was alternated between the right and left ports every trial to rule out positional effects.

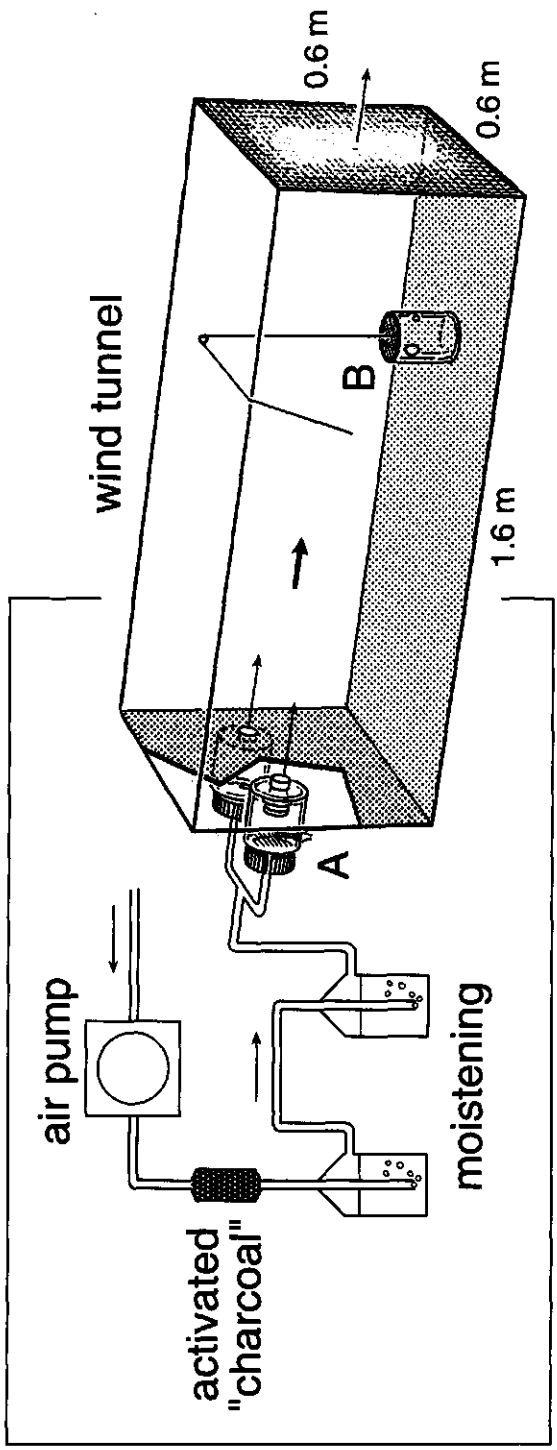

FIGURE 3.1.Diagram of the dual-port olfactometer. A= trapping device, B= release container (drawing by P. Kostense).

Odour stimuli. An acid fraction of Limburger cheese and an artificial mixture of short-chain carboxylic acids from the cheese were used, both originating from the stocks described by Knols *et al.* (1997). For this, the chemical mixtures were diluted with diethyl ether and tested in the dual port olfactometer. The stimuli were applied on clean filter papers on glass slides that were placed in the trapping devices.

Data analysis. The total number of mosquitoes caught in the treatment-trapping device in four replicates was compared with the total number in the control trapping device using Chi-squared tests.

RESULTS AND DISCUSSION

Significantly more mosquitoes were caught in the trapping devices baited with Limburger cheese than in the control ($P< 0.05$) (Table 3.1). This is in agreement with data reported in a preceding study by De Jong and Knols (1995b). They demonstrated, for the first time, that *An. gambiae s.s.* could be attracted to a blend of non-human derived volatiles. This finding is encouraging to achieve the original goal of the research namely to develop odour-baited traps for monitoring mosquito populations in the field.

TABLE 3.1. Responses of An. gambiae s.s. *to Limburger cheese, a range of dilutions of the acid fraction of Limburger cheese and an artificial mixture of carboxylic acids (N=4).*

Odour source	Stimuli		Response [a]		Chi square [b]
	Treatment	Control	Treat.	Control	
Limburger cheese	100 gram cheese	blank	58	12	* a
	undiluted	Diethyl ether	18	77	* r
	undiluted	Diethyl ether	40	101	* r
Natural acid	undiluted	Diethyl ether	44	100	* r
fraction of	10^2 times diluted	Diethyl ether	73	93	ns
Limburger	10^3 times diluted	Diethyl ether	70	89	ns
cheese	10^4 times diluted	Diethyl ether	75	79	ns
	10^5 times diluted	Diethyl ether	55	64	ns
	10^6 times diluted	Diethyl ether	65	82	ns
	10^6 times diluted	Diethyl ether	91	67	ns
	10^6 times diluted	Diethyl ether	51	43	ns
	10^1 times diluted	Diethyl ether	50	62	ns
Artificial	10^2 times diluted	Diethyl ether	52	65	ns
mixture of	10^3 times diluted	Diethyl ether	71	68	ns
carboxylic	10^5 times diluted	Diethyl ether	50	54	ns
acids	10^6 times diluted	Diethyl ether	91	67	ns
	10^7 times diluted	Diethyl ether	53	62	ns

[a] *The response is given as the total number of mosquitoes caught in either the treatment or control trapping device.*

[b] *Significant differences (*: $P \leq 0.05$, r: repellent, a: attractive) or no significant differences (ns: $P>0.05$) found between the total number of mosquitoes caught in the treatment and control trapping device (Chi-squared test)*

Similar to the data reported by Knols and colleagues (1997), significantly fewer mosquitoes were caught in the trapping devices baited with undiluted natural acid extract of the cheese than in the control traps. This purely odour-mediated choice confirms previous reports that *An. gambiae s.s.* perceives carboxylic acids (Knols *et al.*, 1997; Meijerink and

Van Loon, 1999; Van den Broek and Den Otter, 1999). Carboxylic acids are frequently reported to be present in human emanations (Marples *et al.*, 1970; Nicolaides, 1974; Sastry *et al.*, 1980; Albone, 1984; Korting *et al.*, 1988; Stoddart, 1990; Noble, 1993). In a previous study, *An. gambiae s.s.* was significantly attracted to trapping devices baited with 10^4, 10^5, 10^6 and 10^7 times diluted natural acid cheese extract and to a 10^8 diluted synthetic acid mixture (Knols *et al.*, 1997). Attraction of the yellow fever mosquito, *Aedes aegypti,* to carboxylic acids has also been reported (Carlson *et al.*, 1972; Müller, 1968). However, in the present study, neither diluted solutions of the acid fraction of the cheese nor the artificial mixture of short-chain carboxylic acids attracted significantly more *An. gambiae s.s.* than the control ($P \geq 0.05$). Since the attractiveness of Limburger cheese could be verified in the present study, the latter outcome was highly unexpected. The same stock of both natural and synthetic acid mixture had been used for preparing the stimuli as in the preceding study (Knols *et al.*, 1997). A possible explanation for this result is that the composition of the stock solutions has changed over time due to oxidation of components or evaporation of important volatiles. However, chemical analyses of the stock solutions at the time of the present study did not reveal any difference with the initial composition (A. Cork, pers. commun.). Possibly, the 'resolution' of the dual port olfactometer bioassay may have decreased since the previous study, in a way that only strong stimuli like the whole blend of Limburger cheese or undiluted acid extract could induce a distinct behavioural choice of the mosquitoes. Nevertheless, my attempt to reinforce the results obtained with carboxylic acids in the preceding study was not succesfull. Although *An. gambiae s.s.* was again attracted to Limburger cheese, this was not further explored. Because skin emanations other than foot odour had not been investigated, it was decided to study more naturally derived host odour sources to continue the investigation in the identification of natural kairomones for host-seeking *An. gambiae s.s.*. The results of behavioural ecological research on the source, identification and production of kairomones for *An. gambiae s.s.* is reported in the following chapters

THE ATTRACTION OF *AN. GAMBIAE* TO HUMAN SWEAT

ABSTRACT - Behavioural responses of the malaria mosquito *Anopheles gambiae sensu stricto* to human sweat were studied in a dual-port olfactometer. The role of fatty acids present in human sweat was investigated by manipulating the release of acidic compounds in the headspace of human sweat by changing the pH of the sweat samples. The number of mosquitoes attracted to pure human sweat (pH 7-8) was significantly higher than that attracted to an equivalent amount of distilled water. After acidification of sweat to pH 1.5 the attractiveness of human sweat was lost. The alkalisation to pH 8-9 did not affect the attractiveness of human sweat. The results suggest that in addition to fatty acids other chemicals present in sweat may play a role in odour-guided host-seeking of malaria mosquitoes.

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INTRODUCTION

Female malaria mosquitoes (Diptera: Culicidae) need a blood meal for egg production and are guided to their host by odour, in addition to physical and visual factors (Takken, 1991). Many studies have examined human body odours for their attractiveness to mosquitoes, in particular the yellow fever mosquito *Aedes aegypti*. Air led over human body parts was found attractive for many mosquito species (*Ae. aegypti*: Mayer and James, 1969; Von Rahm, 1956; Skinner *et al.*, 1965; Thompson and Brown, 1955; *Ae. aegypti, Anopheles quadrimaculatus*: Willis, 1948; *An. arabiensis, Culex quinquefasciatus*: Omer, 1979; *An. atroparvus*: Laarman, 1955). Although human sweat has long been suspected of being attractive to mosquitoes, early investigations (Howlett, 1910; Rudolfs, 1922) found little evidence for this supposition (Thompson and Brown, 1955). However, later experiments showed that sweat can be attractive for *Ae. aegypti* (Parker, 1948; Willis, 1948; Brown *et al.*, 1951; Thompson and Brown, 1955; Roessler, 1961; Maibach *et al.*, 1966; Müller, 1968). Despite the long history of research into odour-guided host-seeking by mosquitoes little is known, in general, about the identity of the attractive components. Carbon dioxide is an attractant to many mosquito species, however, it appears to be of minor importance for the anthropophilic *An. gambiae sensu stricto* (Mboera and Takken, 1997). In addition to carbon dioxide, 1-octen-3-ol and lactic acid are reported as kairomones in mosquitoes other than *An. gambiae s.s.* (Acree *et al.*, 1968; Kline, 1994; Geier, 1995). Knols and De Jong (1996) showed that *An. gambiae s.s.* is attracted to Limburger cheese, the odour of which is (anthropomorphically speaking) reminiscent to odour of human feet. It was also shown that *An. gambiae s.s.* was attracted to a natural and synthetic blend of fatty acids present in this cheese (Knols *et al.*, 1997). Fatty acids are abundantly present in volatile emanations of both Limburger cheese and human feet (A. Cork, unpublished data) and we set out to investigate the role of these acids in the host-seeking behaviour of *An. gambiae s.s.*. To this end we studied the responses of *An. gambiae s.s.* to natural (complex) odour sources like sweat. To our knowledge studies on the role of human sweat in the behaviour of *An. gambiae s.s.* has not been reported.

In the following, results are given of behavioural responses of the malaria mosquito *An. gambiae s.s.* to human sweat, studied in a dual-port olfactometer. The role of fatty acids present in human sweat was investigated by manipulating the release of acidic compounds in the headspace of human sweat by changing the pH of the sweat samples (Müller, 1968).

MATERIAL AND METHODS

Mosquitoes. The *An. gambiae s.s.* strain used originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome) and was maintained under standard conditions (27 ± 1°C, 80 ± 5% RH, 12 hr scotophase). Adults were kept in 30 cm cubic gauze-covered cages and had access to 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 the larvae were fed on Tetramin fish food. Pupae were collected daily from the trays and were allowed to emerge in the adult cages. In the bio-assay 4-8 days old female mosquitoes were used, which had not received a bloodmeal.

Olfactometer. A dual-port olfactometer slightly modified after Knols *et al.* (1994) was used to study the attractiveness of odour stimuli (Chapter 3). The olfactometer consists of a large flight chamber (160 x 60 x 60cm) in which mosquitoes are released. Conditioned air (60 ± 5% RH; 27 ± 0.5 °C) enters the flight chamber through two small ports (diameter 5 cm, horizontally aligned, 30 cm apart). The ports of the flight chamber are linked to trapping devices (Knols *et al.*, 1994). These are made of glass containers through which the conditioned airstream enters the flight chamber. The windspeed was fine-regulated to 20 cm/s. Light (6.3 ± 1.4 Lux) was produced by incandescent light bulbs placed on top of the tunnel.

Experimental procedure. Each test day consisted of four trial periods for the same odour stimulus. Each stimulus trial was replicated on three different test days (n=12). Mosquitoes were tested in the last four hours of the scotophase. In each trial a batch of 50 mosquitoes were simultaneously released from a container placed in the flight chamber at 100 cm downwind from the two ports. Every batch of mosquitoes was allowed to make a choice between the test or control port during 20 minutes. The trap catches were counted afterwards and the distribution between the test and control stimuli was determined. Each trial started with new mosquitoes, clean traps and newly applied stimuli. The test stimulus was alternated between the right and left port every trial to rule out positional effects.

Odour stimuli. Human sweat samples were collected from the forehead and trunk regions of an adult volunteer in Burkina Faso. The volunteer was asked not to wash for at least 24 h prior of sweat collection. Placing the volunteer in an enclosed vehicle after vigorous exercise stimulated sweating. Sweat was collected with glass Pasteur pipettes and stored in glass bottles (c. 50 ml) at -20°C before shipment to Europe. Part of the sweat sample was analysed using GC-MS procedures, which have been described elsewhere (Cork and Park, 1996).

Three different odour stimuli were used. First the natural untreated sweat was tested. Secondly the sweat sample was acidified to pH 1.5 (as measured with Indicator Paper "Merck") by adding 20 µl 5% hydro-chloric acid to 0.5 ml pure sweat. For the third test series the sweat was alkalised to pH 8-9 by adding 20 µl 5% sodiumcarbonate to 0.5 ml sweat.
In each trial, 0.1 ml sweat (pure, acidified or alkalised sweat) was applied on a Whatman (no. 3) filter paper and was tested directly against the control, which consisted of an equivalent amount of water on filter paper. Filter papers with sweat or water were placed inside the glass traps, from where the odorous air was led into the flight chamber.

Data analysis. The attractiveness of a stimulus in each trial is defined as the percentage of mosquitoes that where caught in the stimulus port relative to the total amount of mosquitoes caught. The arcsine-square root transformed data were tested with a paired t-test for significant differences ($P<0.05$) between the control and test stimuli (Sokal and Rohlf, 1995).

RESULTS

The data of each separate trial are presented in Table 4.1. The natural sweat and the alkalised sweat were found to be significantly more attractive than the control ($P< 0.01$). The attractiveness of natural sweat appeared to be lost by acidification. The mosquitoes seemed to be slightly repelled by the acidified sweat, but the differences were not significant.

DISCUSSION AND CONCLUSION

The results indicate that, in our bioassay, *An. gambiae s.s.* is attracted to human sweat collected from the forehead and trunk. Although sweat can be attractive for *Ae. aegypti* (a.o. Maibach *et al.*, 1966), its attractiveness appears to depend strongly on the location of the body where the sample is collected. It was shown that *Ae. aegypti* is attracted to armpit sweat (Parker, 1948; Brown *et al.*, 1951; Thompson and Brown, 1955; Müller, 1968), but not to forehead sweat (Thompson and Brown, 1955) or leg and trunk sweat (Müller, 1968). However, Eiras and Jepson (1991) could not establish attraction to trunk sweat, while later they showed that trunk sweat in combination with convection current is more attractive than convection current alone (Eiras and Jepson, 1994). Possible reasons for the contradictory findings about the attractiveness of sweat may be found in the different methods used to collect and store the sweat samples and to determine its attractiveness. As Parker (1948)

already mentioned, the comparison of 'attractiveness' from different reports is difficult because it is not clear whether they refer to approach, alightment or probing. In the present study we consider only those mosquitoes that entered the ports during upwind flight and, therefore, clearly were attracted to the odour stimulus from a distance. Alighting and probing behaviour were not recorded.

*TABLE 4.1. The number of mosquitoes responding to natural, acidified and alkalised sweat in comparison to the response to destilled water (control), ** indicates a significant difference from 50% (P < 0.01)*

	TREATMENTS					
	Series 1		Series 2		Series 3	
Trial number	Natural sweat	Control	Acidified sweat	Control	Alkalised sweat	Control
1	19	16	15	17	26	10
2	22	16	14	31	30	13
3	28	16	21	17	31	6
4	26	12	18	21	13	5
5	20	11	10	13	17	11
6	28	17	8	9	23	6
7	29	14	9	16	25	18
8	28	13	10	17	8	5
9	7	3	9	7	12	6
10	13	9	6	9	19	7
11	28	8	10	12	13	7
12	20	17	8	8		
Sum	268	152	138	177	217	94
Mean	22.3	12.7	11.5	14.8	18.1	7.8
Percentage	64.0	36.0 **	44.6	55.4	69.3	30.7 **
SD	6.9	6.9	7.6	7.6	7.9	7.9

The results with the chemically treated sweat samples fully support the findings of Thompson and Brown (1955), who showed that the attractiveness of human sweat (collected from the armpit) for *Ae. aegypti* was not affected by alkalisation with sodium carbonate and that it was also lost by acidification of the pure sweat sample with hydrosulphate. Even a significant repellent action of the acidified sweat was observed (Thompson and Brown, 1955). They concluded that the attractiveness shown by armpit sweat is decreased by the release of volatile acids present and normally held by esterification, whereas the release of any volatile bases present has essentially no effect on the attractiveness. In contrast Müller (1968) found that attractive arm sweat lost its attractiveness for *Ae. aegypti* after alkalisation. He also found that mosquitoes were attracted to single organic acids like formic, acetic, propanoic and lactic acid. The latter also lost its attractiveness at alkaline pH. The loss of attractiveness of alkalised sweat was suggested to be due to the absence of the attractive acidic components in the headspace of sweat, because these compounds are kept in the solution. The fact that in our experiments the alkalised sweat is still attractive suggests that also non-acidic compounds can attract malaria mosquitoes. The slightly repellent action of acidified sweat may be due to the high concentration of acidic compounds in the headspace. Knols *et al.* (1997) also found repellence at high concentrations of the fatty acid mixture, of which dilutions were highly attractive.

Considering the results of the present study, the data from Knols *et al.* (1997) and other published data, we conclude that in addition to fatty acids, non-acidic compounds may play a role in odour-guided host seeking of *An. gambiae s.s.*. The nature of these

compounds is not yet known. Replicates with sweat from other individuals are necessary to be able to generalise the results found.

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5

INCUBATED HUMAN SWEAT BUT NOT FRESH SWEAT ATTRACTS *AN. GAMBIAE*

ABSTRACT - A dual-port olfactometer was used to quantify behavioural responses of the malaria mosquito *Anopheles gambiae s.s.* to volatiles emitted by human sweat samples collected from 3 human volunteers. Significant attraction ($P< 0.05$) was found to sweat of two volunteers after two days and to one of the volunteers' sweat after one day of incubation at 37°C. There was no attraction found to any of the fresh sweat samples. The pH of the sweat, that became attractive after 1 or 2 day(s), had changed from acidic (5.5-5.75) to alkaline (8.25-8.75), while the sweat of the third volunteer (pH 7) did not show a change in pH during incubation. Microorganisms, present in all fresh sweat samples, showed a distinct growth in the incubated sweat samples. The concentration of lactic acid decreased on average with 23 % in two days of incubation. The role of the pH, skin microflora and lactic acid in the differential attractiveness of human sweat samples is discussed.

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INTRODUCTION

Females of the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* seek humans during the night and rely mainly on olfactory cues, so called kairomones, to find their host (Takken, 1991; Knols, 1996). Despite intensive research the identity of these kairomones is not known. Carbon dioxide present in mammalian breath has been shown to be an attractant for many mosquito species, however it appears to be of minor importance for the anthropophilic *An. gambiae s.s.* (Mboera and Takken, 1997). It is widely assumed that important kairomones for *An. gambiae s.s.* emanate from the human skin (Knols, 1996). In addition to carbon dioxide, only lactic acid and 1-octen-3-ol have repeatedly been reported as kairomones for mosquito species other than *An. gambiae s.s.* (Acree *et al.*, 1968, Kline *et al.*, 1990; Geier *et al.*, 1996).

Although sweat has long been suspected as a kairomone source for *An. gambiae*, evidence was not present until Braks *et al.* (1997, Chapter 4) found attraction to sweat samples, collected from volunteers from Burkina Faso (Cork and Park, 1996), using a two-choice olfactometer. Their attempt to repeat the experiment with freshly collected sweat samples from other volunteers failed; no attraction was found (Braks, unpublished data). The latter sweat samples were acidic (pH 5.5), which is in accordance with the literature (Noble and Somerville, 1974), however the sweat sample from Burkina Faso had an alkaline pH of 8. To our knowledge alkaline sweat is only described as the result of microbial decomposition of sweat components into ammonia during incubation (Bergeim and Cornbleet, 1943; Müller, 1968; Weiner and Hellmann, 1960). Moreover, studies on the responses of *Aedes aegypti* to pure sweat samples, collected without the interference of solvents or absorbents, are not clear (Skinner *et al.*, 1965; Müller, 1968; Eiras and Jepson, 1991, 1994).

An. gambiae s.s. has also been shown to be attracted to the volatiles of Limburger cheese, which, to a human, are reminiscent of human foot odour (De Jong and Knols, 1995b), and to a synthetic blend of fatty acids occurring both in this cheese and in human foot odour (Knols *et al.*, 1997). These fatty acids are produced during ripening of the cheese by the inoculated bacteria (Cogan and Daly, 1987). Some fatty acids in foot odour have also been shown to be of bacterial origin (Noble and Somerville, 1974). However, Braks *et al.* (1997) showed that in addition to fatty acids other chemicals must play a role in odour-guided host-seeking of malaria mosquitoes as sweat samples kept their attractiveness while the fatty acids in the headspace were artificially minimised (Braks *et al.*, 1997).

The present study was initiated on the elucidation of the ambiguous responses of mosquitoes to sweat samples. Therefore, the effect of the incubation of sweat samples on their attractiveness for *An. gambiae s.s.* is investigated, together with the variance between samples of different human individuals. Here, the behavioural responses of *An. gambiae s.s.* to freshly collected sweat and to sweat, which had been incubated for one and two day(s) of three different human volunteers are reported. The role of pH, lactic acid concentration and bacterial growth in the differential attractiveness of sweat samples is discussed.

MATERIAL AND METHODS

Mosquitoes. The *An. gambiae s.s.* strain used originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome) and was maintained at 27 ± 1°C, 80 ± 5% RH and a photo/scotophase of 12:12. Adults were kept in 30 cm cubic gauze-covered cages and had access to 6% (v/v) glucose solution. Females were offered blood from a human arm twice a week for 10 min. Eggs were laid on wet filter paper, hatched in trays of water and the larvae were fed Tetramin fish food. Pupae were collected daily from the trays and were allowed to emerge in the adult cages. In the bioassay 4 to 8-day-old female mosquitoes were used, which had not received a blood meal. One day before the experiments females were randomly selected from the adult cages in batches of 30, and put in small release containers. They had access to cotton wool soaked with tap water only.

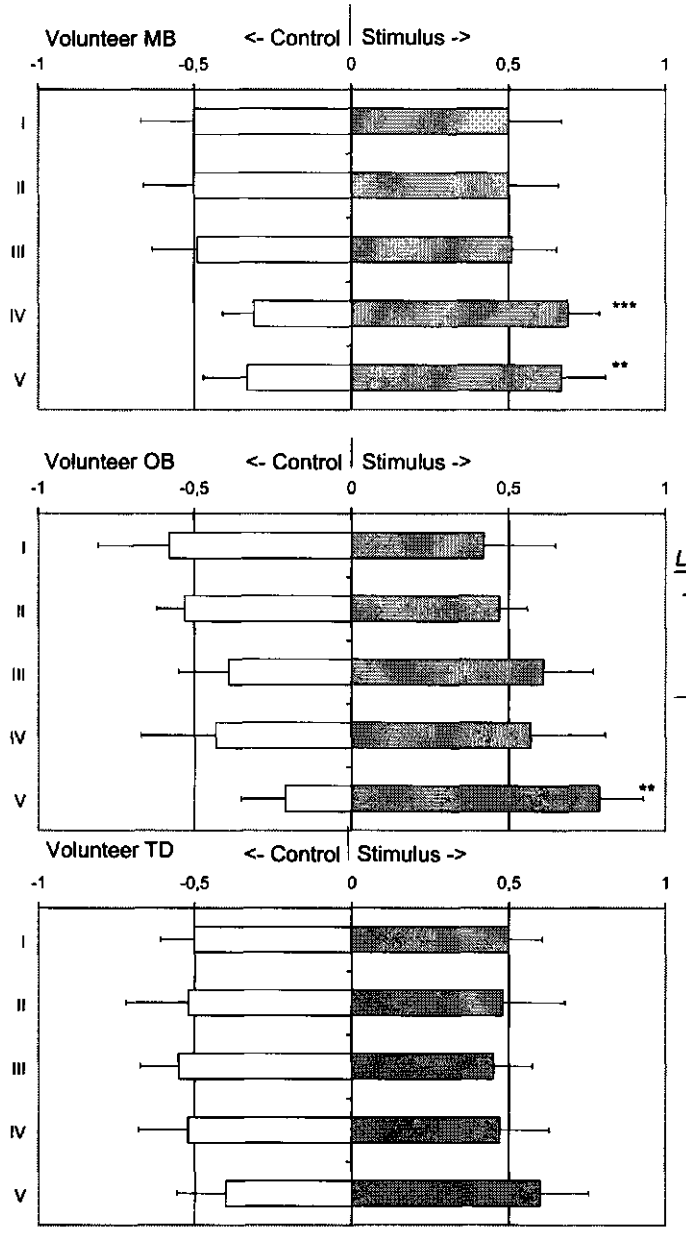
Olfactometer. A dual-port olfactometer, modified from Knols *et al.* (1994), was used to study the attractiveness of sweat samples. The olfactometer consisted of a flight chamber (160 x 60 x 60 cm) in which mosquitoes were released. Air, obtained from a pressurised air source, acquired room temperature when passing through 10 m long silicon tubes (7 mm diameter) in the room. Subsequently, the air was purified by means of activated charcoal and humidified by passing it through two bottles filled with demineralised water. This conditioned air (60 ± 5% RH; 27 ± 0.5 °C) entered the flight chamber (55 ± 5% RH; 27 ± 0.5 °C) through two small ports (diameter 5 cm, horizontally aligned, 30 cm apart). The ports of the flight chamber were linked to trapping devices (for details see Knols *et al.*, 1994). These were made of glass containers through which the conditioned air entered the flight chamber. The odour source, applied on the rough surface of a sand blasted glass slide (5x2 cm), was placed in the glass container and odorous air was passed on to the flight chamber. The wind speed was 20 cm/s. Light (6.3 ± 1.4 Lux) was produced by 9 incandescent light bulbs suspended over the roof of the tunnel. In each test a batch of 30 mosquitoes was released from a container placed in the flight chamber, 100 cm downwind from the two ports. The trap catches were counted and the distribution between the test and control stimuli was determined. The mosquitoes that not had chosen one of the two ports were removed from the flight chamber after each test. Each subsequent test started with new mosquitoes, clean traps and newly applied odour stimuli.

Odour stimuli. Sweat droplets were collected from the foreheads of three Caucasian human volunteers, one female (MB, 27 yr.) and two males (OB, 25 yr. and TD, 28 yr.) with sterile glass Pasteur pipettes and put in glass vials (2 ml). From each volunteer 1.5 ml sweat was collected twice on different days. Sweat production was stimulated by physical exercise in a warm humid room (30°C, 90 % RH). Directly after collection, the sweat sample (1.5 ml) was divided in three sub samples of 0.5 ml each. One sub-sample was directly stored at -5 °C. The other sub-samples were incubated under aerobic conditions at 37 °C for one or two day(s), after which they were also stored at -5 °C. Afterwards the pH (Indicator Paper, Merck) and lactic acid concentration (Lactic Diagnostic Kit Sigma, NR 735) of each sample was determined.

The presence and growth of micro-organism was indicated by the number of colonies on agar plates (Iso Sensi Test, Biotrading) which were inoculated with 50 µl of 10%, 1%, and 0.1 % diluted sweat samples. Control plates inoculated with 50 µl sterile distilled water. The agar plates were incubated for 1 day at 37 °C under aerobic conditions.

Experimental procedures. For testing of sweat, 0.1 ml sweat (fresh, 1 day or 2 days incubated) was applied directly onto the glass slide and tested directly against the control, which consisted of an equivalent amount of distilled water. Control tests to assess the response of the mosquitoes to clean air over untreated slides or with distilled water only were also performed. Glass slides were placed inside the glass traps through which air was led into the flight chamber.Behavioural responses of mosquitoes were tested during the last four hours of the dark period. Each experiment consisted of five 15-min tests examining the three differently treated sweat sub samples, which originated from one collection day of one volunteer, and the two control trials. From each collection from each volunteer this set of five tests was replicated on four days. The odour stimuli were tested the same number of times from the right and the left port to rule out positional effects and the order of the different tests were randomised per experimental day.

Statistical analysis. For each 15 min test, the numbers of mosquitoes caught in the test and control port were calculated as proportions of the total number of mosquitoes caught. Subsequently, an arcsine square root transformation for normalisation was applied on the proportions. A Student-t-test was performed to examine differences between test and control odours. The sweat samples were collected from three different volunteers and the data were analysed separately for each volunteer (Sokal and Rohlf, 1995).



LEGEND FIG. 5.1:

Trial	Control	Stimulus
I	clean air	clean air
II	water	water
III	water	fresh sweat
IV	water	1 day old sweat
V	water	2 day old sweat

*FIGURE. 5.1. Attractiveness expressed as the mean proportion of mosquitoes that was caught in the test port (positive proportion) and control (negative proportion) relative to the total number caught with the sweat samples of each volunteer in the eight replicates. The horizontal lines represent the standard errors. The total number of mosquitoes caught in eight replicates are shown in Table 5.1. Asterisks mark significant differences found between the test and control stimulus (Student t-test, * P<0.05, ** P< 0.01, ***P< 0.001)*

RESULTS

Bioassay. No significant differences were found between the fresh sweat samples and the control for any of the volunteers. For volunteer MB, the sweat samples that had been incubated for one and two day(s) were significantly more attractive than the control ($P<0.05$). For volunteer OB, a significant attraction was found for the sweat samples that had been incubated for two days ($P< 0.01$), but not for those after one day of incubation. For volunteer TD, none of the sweat stimuli tested gave rise to a significant difference in attraction. The control tests showed that *An. gambiae* entered the trap in response to clean or slightly humidified air currents, but that no differences were found between the ports (Fig.5.1).

Measurements. Table 5.1 shows the pH and lactic acid concentration of the different sweat samples and the numbers of skin microorganisms present in fresh sweat samples. The pH of the sweat samples of volunteers MB and OB showed a shift from acidic (pH 5.5-5.75) when fresh to alkaline (7.75-8.75) after one or two day(s) of incubation. The pH of the two fresh sweat collections of TD were not acidic but neutral and the pH did not change to alkaline during incubation. The fresh sweat samples of all volunteers contained substantial amounts of lactic acid (1.59-3.83 mg/ml), which decreased on average to 77.2 ± 12.5 % in two days of incubation. Microorganisms were abundant in all fresh sweat samples and no colonies were found on the control plates. The numbers of skin microorganisms present in sweat samples incubated for one or two day(s) could not be determined, as the agar plates of all dilutions were completely overgrown, indicating that exponential growth of microorganisms had occurred.

DISCUSSION

Several methods have been developed for collecting human emanations for testing their attractiveness for mosquitoes (Parker, 1948; Thompson and Brown, 1955; Roessler, 1961; Skinner *et al.*, 1965; Bar-Zeev *et al.*, 1977; Schreck *et al.*, 1981; Geier *et al.*, 1996). Although sweat has long been suspected as a kairomone source, pure sweat droplets are rarely tested (Skinner *et al.*, 1965; Müller, 1968; Eiras and Jepson, 1991 and 1994) and, until recently, only for *Ae. aegypti.*

Surprisingly, no attraction was found to any of the fresh sweat samples of the different volunteers which seems to contradict earlier reports of behavioural responses of *An. gambiae* s.s. to sweat samples (Braks *et al.*, 1997). However, the results show that sweat can become attractive after incubation at body temperature for one or two day(s). We suggest that the sweat sample used by Braks *et al.* (1997) may have undergone alterations between collection and testing, which resemble the incubation process. Our assumption is supported by the fact that the latter sweat sample had an alkaline pH of 8 like our incubated sweat, instead of a normal slightly acidic pH (Noble and Somerville, 1974). Bergeim and Cornbleet (1943) showed that incubated sweat becomes alkaline due to ammonia formation by bacteria from the urea present. In addition they found that the lactic acid concentration of sweat decreased during incubation, due to bacterial action. The acidity of freshly secreted sweat is due to the production and secretion of lactic acid by the eccrine sweat glands (Thurmon and Ottenstein, 1952). All our sweat samples showed a decrease in lactic acid concentration after two days of incubation. Müller (1968) also reported a pH shift from acidic to alkaline in incubated sweat samples. However, he found that *Ae. aegypti*, was attracted to freshly collected sweat from the fore arm and that the attractiveness was lost after incubation. This loss in attractiveness was suggested to be due the absence of lactic acid and other acidic components in the headspace of alkaline sweat (Thompson and Brown, 1955; Müller, 1968). Lactic acid has repeatedly been shown to be an important kairomone for *Ae. aegypti* (Acree *et al.*, 1968; Müller, 1968; Geier *et al.*, 1996). Since we only found attraction to alkaline sweat and because the artificial alkalisation to pH 10 did not affect the

TABLE 5.1. Total number of mosquitoes caught in eight replicates.

Volunteer	MB		OB		TD	
Trial[a]	Control	Stimulus	Control	Stimulus	Control	Stimulus
I	72	77	56	32	57	59
II	56	56	55	30	61	61
III	76	67	52	78	75	57
IV	44	94	51	79	76	72
V	47	95	26	78	61	88

[a] *For trial codes see legend of Figure 5.1.*

TABLE 5.2. Values of pH and lactic acid concentration (mg/ml) of fresh and one or two day(s) incubated (37°C) sweat samples of three volunteers (MB, OB and TD), collected on two different days (1 and 2)[a].

		Volunteers					
	Collections	MB		OB		TD	
		1	2	1	2	1	2
pH	Fresh sweat	5.5	5.5	5.75	5.75	7.0	7.0
	1 day incubated sweat	8.25	8.5	7.75	8.25	7.0	7.25
	2 days incubated sweat	8.75	8.75	8.25	8.75	7.5	7.5
Lactic acid [b]	Fresh sweat	2.00	2.07	2.37	2.82	2.74	3.83
	1 day incubated sweat	1.83 (91.5%)	1.75 (84.5%)	1.94 (81.8%)	3.17 (112.0%)	2.37 (86.5%)	3.58 (93.5%)
	2 days incubated sweat	1.59 (79.55)	1.77 (85.5%)	1.73 (73.0%)	2.44 (86.5%)	1.48 (54.0%)	3.25 (84.9%)
Microorganisms [c]	Fresh sweat	1.7×10^4	0.9×10^4	3.0×10^4	1.0×10^4	1.6×10^7	1.7×10^4

[a] *.The number of microorganisms in fresh sweat samples are indicated in the last line*
[b] *.Concentration lactic acid [mg/ ml]. % = (concentration lactic acid in incubated sweat sample / concentration lactic acid in fresh sweat sample) x 100%*
[c] *.Colony forming units [CFU/ ml]*

attractiveness of human sweat (Braks *et al.*, 1997), we doubt that lactic acid is very important in the attraction of *An. gambiae s.s.*.

Although attraction has been observed to alkaline sweat samples only, not all alkaline sweat samples have been found attractive. Most likely other chemical changes than the observed shift in pH occur during incubation and the resulting increased bacterial activity. Nevertheless, growth of microflora has also been observed in unattractive incubated sweat samples of one volunteer (TD) while there was no change in pH. The results suggest that the presence and growth of skin flora, pH and individual differences in sweat composition together give rise to differential responses to sweat samples between volunteers. These findings may explain the variability in attractiveness of humans (Muirhead- Thompson, 1951; Brouwer, 1960; Maibach *et al.*, 1966; Lindsay *et al.*, 1993; Knols *et al.*, 1995;).

Although consensus is reached that the typical odour of the armpit is the product of bacterial decomposition of odourless apocrine sweat (Stoddart, 1990), little is known about the role of the skin flora in the production of other human odours from eccrine sweat and sebum. Khan *et al.* (1969) suggested that increased bacterial decomposition of sweat might cause an increased attraction of mosquitoes to a human arm while sweating, but conclusive evidence was lacking. The present study provides strong indications that the skin flora plays a role in the production of volatiles. We hypothesise that a continuous bacterial action on secretions on the human skin results in volatiles which function as kairomones for mosquitoes.

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BEHAVIOURAL RESPONSES OF *CULEX QUINQUEFASCIATUS* AND *AN. GAMBIAE* TO SKIN WASHINGS

ABSTRACT - Behavioural responses of *Culex quinquefasciatus* and *Anopheles gambiae sensu stricto* to human odour, represented by skin rubbings and skin washings, to lactic acid and carbon dioxide as well as a possible synergistic action of carbon dioxide were investigated in this study. Mosquitoes were tested in batches and individually in a small dual port olfactometer.

Batches of *Cx quinquefasciatus* showed a high preference for the skin rubbing. At 600 PPM, there was no response to carbon dioxide, but mosquitoes were repelled at a concentration of 2000 PPM. There was no synergistic action of carbon dioxide in combination with other host odours. Lactic acid was attractive but only in high doses of 5 and 31 mg on filter paper. Ethanol washings of hands and feet seemed to be more attractive than the control, however not significantly. Significant preference for the skin washing was observed when tested against 0.31 mg lactic acid, indicating that lactic acid is not the only attractive component on the skin. In the experiments testing individual mosquitoes, significant attraction was found to a second skin washing made from hands and feet and a skin washing made from the back. No preference was observed between these two sources of skin washings. A freeze-dried sample of skin washing was less attractive than the untreated washing, suggesting that important volatiles were lost during the freeze-drying and reconstitution of the sample.

The Afrotropical malaria mosquito, *An. gambiae s.s.*, showed a high preference for the ethanol washings of hands and feet compared to a control.

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INTRODUCTION

The mosquito *Cx quinquefasciatus* Say (Diptera: Culicidae), one of the most important vectors of bancroftian filariasis (Subra, 1981; Mboera, 1999), is widely distributed in the tropical and subtropical areas of the world. Its local distribution is strongly associated with human settlements as it feeds primarily on man and prefers open drains and latrines as oviposition sites. *Cx quinquefasciatus* has been proven to be difficult to control because of its relatively high tolerance to insecticides causing large problems in many tropical cities. Therefore, the need to perfect alternative mosquito control strategies has become urgent (Kline, 1994). For the study of vector biology and epidemiology of vector-borne diseases, and for developing control methods, good sampling tools are necessary.

Studies on mosquito behaviour have shown that chemical cues or kairomones play an important role in attracting female mosquitoes to their host (Takken and Knols, 1999), indicating that odour-baited traps may provide a tool for monitoring mosquito populations. The history of research on host-seeking behaviour of the anthropophilic yellow fever mosquito, *Aedes aegypti,* is long. So far, only a few attractive components of human odour have been identified for this species: carbon dioxide and lactic acid (Geier, 1995). Contradictory statements about the role of carbon dioxide for *Cx quinquefasciatus* are available; some authors found carbon dioxide attractive (Omer, 1979; Reisen and Pfunter, 1987; Costantini *et al.*, 1996; Dekker and Takken, 1998b) others found it repellent or inactive (Harden and Poolson, 1969; Service, 1993; Mboera *et al.*, 1998). Little is known about the role of lactic acid in the behaviour of *Cx quinquefasciatus*.

In this study the behavioural responses of *Cx quinquefasciatus* to lactic acid and carbon dioxide as well as a possible synergistic action of these components were investigated. In order to compare the attractiveness of lactic acid and carbon dioxide to that of natural human host odours, a skin rubbing on filter paper and ethanol washings of hands and feet (Geier, 1995) were tested as well. Comparisons were also made between the attractiveness of such washings and a washing derived from the human back. In addition, behavioural responses of Afrotropical malaria mosquito, *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*), to ethanol washings of hand and feet were determined.

MATERIAL AND METHODS

Insectary and dual port olfactometer. The behavioural responses of two mosquito species, *Cx quinquefasciatus* Say and *An. gambiae,* to olfactory stimuli have been investigated. The *Cx quinquefasciatus* strain originated from Colombo, Sri Lanka (courtesy C.F. Curtis) and the *An. gambiae* strain from Suakoko, Liberia (by courtesy of Prof. M. Coluzzi, Rome). The mosquitoes were kept in a climate-controlled room (28 ± 1°C, 80 ± 5 % RH, LD 12:12) and tested the last 4 hours of the dark period (for details see Mboera *et al.*, 1998; Chapter 3).

Behavioural responses of *Cx quinquefasciatus* to olfactory stimuli were determined in a small dual port olfactometer (16 x 16 x 126.5 cm) consisting of a flight chamber and two trapping compartments (40 cm length) (modified after Mboera *et al.*, 1998). Air used was pumped from outside by an electric pump, filtered by glass wool and active charcoal, heated by a water bath (50°C) and humidified by leading it through demineralised water. Subsequently, the air stream was split and led through the two trapping compartments into the flight chamber via two ports (diameter 5 cm, 2.5 cm, modified after Knols *et al.*, 1994). Mosquitoes were released at the downwind side of the flight chamber. Stimuli were presented in the right or left trapping compartment or in both.

Behavioural responses of *An. gambiae* were determined in a large dual-port olfactometer, consisting of a Perspex flight chamber (160 x 66 x 43 cm) (modified after Braks and Takken, 1999), was used to study the attractiveness of candidate stimuli. Charcoal filtered, humidified (65 ± 5% relative humidity), warm air (27 ± 0.5 °C) was led via a Perspex mosquito trapping device, which was linked to two ports (diameter 4 cm, 28 cm apart), into

the flight chamber with a speed of 20 cm/ sec. Dim light (1 lux) was produced by one incandescent light bulb (75 watt) and was filtered and scattered through a screen of yellow cloth hanging 1 m above the flight chamber.

Experimental set up. Cx quinquefasciatus was tested in batches (series 1-3) and individually (series 4 and 5). At the start of the first series, batches of around twenty female mosquitoes were selected by luring them to a human hand from the rearing cages into small release cages. All stimuli, except carbon dioxide, were presented on filter papers (Whatman, no. 4), which were placed in vertical position in metal paper clips. 100 µl of the skin washings and lactic acid were applied and placed in the trapping compartments after the solvent (ethanol) was allowed to evaporate. After application of the stimuli the release cage was placed at the downwind side of the tunnel and after 5 minutes the mosquitoes were released. When no mosquito was flying in the flight chamber anymore (4.5 min ± 2.1), the mosquitoes in the left or right compartment, flight chamber and release cage were counted and removed. Series 1 and 2 consisted of 6 stimuli in 10 replicates and series 3 consisted of 5 stimuli in 8 replicates (Table 6.1). To avoid contamination of any surface with skin residues, the researcher wore surgical gloves at all times.

In the second set of experiments, individual *Cx quinquefasciatus* were released from the test cages and given 3 min to make a choice and subsequently removed from the set-up. For each mosquito new stimuli were applied. To minimise the amount of skin washing used another application method was practised here. The skin washings (10 µl) were placed in glass capillaries (6.5 cm, 3 mm Ø), which were surrounded by a heated brass block (60 °C) (modified after Geier, 1995). After evaporation of the solvent, air branched off from the main stream was blown through introducing the stimuli into the trapping compartments. Two resistances and 6V adapters brought about heating of the brass block. Series 4 and 5 consisted of three stimuli which order was randomised each morning. During each experimental day ten mosquitoes were tested per stimulus which position was alternated between the right and the left compartment each time five mosquitoes had been tested (Table 6.2).

An. gambiae was exposed to skin washings in batches of 30 mosquitoes (series 6-9). 200 µl of the skin washings were applied on sand blasted glass slides (no filter paper) and placed in the trapping compartments after the solvent (ethanol) had been allowed to evaporate. A control glass slide with an equivalent amount of ethanol was placed in the opposite trapping device. During a 15-min trial, 30 mosquitoes were released from a release cage at the downwind end of the flight chamber and allowed to choose between one of the two trapping devices. Stimuli were alternated between the trapping devices with each trial. Each trial started with fresh mosquitoes, clean trapping devices and freshly applied odour stimuli. Each stimulus trial was replicated 6 times (Table 6.3).

Odour stimuli and tests. In the first set of experiments batches of *Cx quinquefasciatus* were exposed to skin rubbings, skin washings, lactic acid and carbon dioxide (Table 6.1). A skin rubbing (**'SR'**) was made by rubbing a filter paper (Whatman®) over the skin of forearm and hand of the experimenter for 10 seconds and was tested directly. The control consisted of a clean filter paper (**'C'**). Skin washings (**'SW'**) were made after Geier (1995). Hands, forearms, feet and ankles of 60 people (23-60 yr., 40 males and 20 females) were rubbed with cotton wool, drenched in ethanol, for 5 min. The cotton wool samples were left to dry overnight at room temperature, packed in a glass tube (150 x 2.8 cm) and the skin residues were extracted with ethanol for six hours at 1.5 ml/min. Subsequently, the washing was concentrated to 50 ml using a vacuum excitator, kept at -20°C for an hour and centrifuged (3000G, 5 min). The supernatant was used as skin washing stimulus (**'$SW_{60}H1$'**). Half of the washing (22.5 ml) was concentrated a second time to 10 ml (**'$SW_{60}H2$'**). The solid fraction and L-lactic acid concentration (Lactate Diagnostic Kit, Sigma) were determined (Table 6.4). The behavioural response was also tested to 5 mg of L-lactic acid on a filter paper (**'L_5'**, 50 mg L-lactic acid/ ml ethanol, Sigma®) and to a concentration array around 31 mg on a filter paper (0.31, 3.1, 31 and 310 mg/filter paper, '$L_{0.031}$-L_{31}'), which is the amount of L-lactic acid

present in 100 µl $SE_{60}H2$ (Table 6.4). Carbon dioxide (4.5 % CO_2 in synthetic air) was tested at concentrations 600 ('**CO_{600}**') and 2000 PPM ('**CO_{2000}**') in the air stream. Control of the tests with skin washing or lactic acid consisted of filter paper with an equivalent ethanol ('**CE**').

For the second set of experiments, testing individual *Cx quinquefasciatus* (Table 6.2), again a skin washing was made by rubbing hands, forearms, feet and ankles of 50 people other than rubbed for $SW_{60}H$ (18-50 yr., 33 males and 17 females) ('**$SW_{50}H$**'). Of these people the back was rubbed in a similar way and the samples of cotton wool were extracted ('**$SW_{50}B$**') separately from the rubbing of the extremities. The treatments of the cotton wool samples were similar to the samples $SW_{60}H1$ and $SW_{60}H2$. The attractiveness of both washings was tested against a control, an equivalent ethanol (**E**), and against each other (series 4). The solid fraction was determined by freeze-drying (Table 6.4). For this, 1 ml extract with 0.75 ml demineralised water was put at -80 °C for 4 hours where after it was put in a freeze drier. The freeze-dried sample of skin washing of the extremities was reconstituted with 1 ml ethanol ('$SW_{50}H$*fd*') and the effect of the treatment on the attractiveness was tested in series 5.

For the third set of experiments, behavioural responses of batches of *An. gambiae* to $SW_{60}H2$ and $SW_{50}H$ against a control (**E**) were determined (Table 6.3).

TABLE 6.1. Results of experiments testing behavioural responses of batches of Cx quinquefasciatus *to carbon dioxide and L-lactic acid separately or in combination with skin rubbings, skin washings made of hands and feet of 60 human volunteers.*

Stimuli [a]		Response				
Test	Control	Test	Control	Attractiveness	t-test [c]	n [d]
		Series 1				
SR	C	156	28	82 ± 6	*	10
L_5	CE	152	37	79 ± 6	*	10
CO_{2000}	-	95	132	42 ± 6		10
$SW_{60}H1$	CE	112	68	59 ± 8		10
$SW_{60}H1+CO_{2000}$	CE	86	127	40 ± 5		10
L_5+CO_{2000}	CE	95	118	39 ± 6		10
		Series 2				
$L_{0.031}$	CE	48	82	44 ±10		10
$L_{0.31}$	CE	72	72	46 ± 10		10
$L_{3.1}$	CE	66	66	48 ± 8		10
L_{31}	CE	118	24	79 ± 5	*	10
$SW_{60}H2$	CE	68	61	54 ± 11		10
$SW_{60}H2$	$L_{0.31}$	131	38	78 ± 7	*	10
		Series 3				
CO_{600}	C	86	64	61 ± 7		8
$SW_{60}H2$	CE	89	38	63 ± 9		8
$SW_{60}H2+$ CO_{600}	CE	100	67	64 ± 6		8
SR	CE	112	22	83 ± 7	*	8
SR+ CO_{600}	CE	139	15	89 ± 6	*	8

[a] *For abbreviations used in the first and second column see Material and Methods*

[b] *The percentage of mosquitoes that flew into the test compartment of the total of mosquitoes that flew in the test and control compartment. Averages of the individual tests are presented (in % ± standard error).*

[c] * *significantly different from 50% (paired two sample for means t-test for significant differences, P < 0.05)*

[d] *number of replicates.*

Data analysis. The attractiveness of a stimulus in the experiments in which batches of mosquitoes, either *Cx quinquefasciatus* or *An. gambiae* were tested, is defined as the percentage of mosquitoes trapped in the test compartment of the total number of mosquitoes that responded by flying into any compartment. To normalise the non-parametric data the percentages of the individual experiments are transformed by an arcsine-square root transformation (Sokal and Rohlf, 1995). The transformed data were tested with a paired-two-sample-for-means t-test for significant differences from 50%.

In the experiment testing *Cx quinquefasciatus* individually, the total number of mosquitoes caught in the test-compartment was compared with those entering the control compartment, using Chi-squared tests.

RESULTS

Results of the experiment testing batches of *Cx quinquefasciatus* are shown in Table 6.1. In series 1, the skin rubbing and the lactic acid were significantly attractive ($P<0.05$). The most diluted skin washing appears to be attractive, although this was not significant. The highest concentration of carbon dioxide was repellent as more females flew into the control compartment than in the compartment releasing carbon dioxide. This repellence seems to overrule the attractiveness of lactic acid and the skin washing. In series 2, the high dose of lactic acid was significantly attractive. Lower concentrations were indifferent. Significant preference for the skin washing was observed when tested against lactic acid (0.31 mg), however not when tested to a control. In series 3, significant attraction was found to the skin rubbing tested alone and in combination with a low concentration of carbon dioxide.

Results of series 4 (Table 6.2) with individual mosquitoes show that skin washings made of human extremities and back were both significantly more attractive than the control and indifferent when tested against each other. In series 5, the reconstituted freeze-dried skin washing of the extremities was significantly more attractive than the control and the same held again for the untreated skin washing. When the two skin washings were tested against each other, the untreated skin washing was significantly preferred ($P<0.05$), suggesting that important volatiles were lost during the freeze drying and reconstitution of the sample.

TABLE 6.2. Results of experiments testing behavioural responses of individual Cx quinquefasciatus *to skin washings made of hands and feet, and of the back of 50 human volunteers, and reconstituted freeze dried skin washing of hands and feet.*

Stimuli [a]		Response				
Test	Control	Test	Control	χ^2-test [b]	N [c]	N_{active} [d]
		Series 4				
$SW_{50}H$	E	29	5	*	76	34
$SW_{50}B$	E	25	2	*	82	27
$SW_{50}B$	$SW_{50}H$	18	12	ns	64	30
		Series 5				
$SW_{50}H$	E	14	0	*	66	14
$SW_{50}Hfd$	E	7	1	*	62	8
$SW_{50}Hfd$	$SW_{50}H$	3	18	*	72	21

[a] *For abbreviations used in the first and second column see Material and Methods*
[b] **: significant difference in χ^2-test ($P < 0.05$), ns: not significant in χ^2-test ($P > 0.05$)*
[c] *Number of individual mosquitoes tested*
[d] *Total number of mosquitoes in left and right trapping compartment*

Results of the experiment with *An. gambiae* are shown in Table 6.3. Significantly more mosquitoes were caught in the trapping devices baited with ethanol skin washings of hands and feet than in the control ($P<0.05$).

TABLE 6.3. Results of experiments testing behavioural responses of batches of An. gambiae *to skin washings made of hands and feet of 50 or 60 human volunteers.*

Stimuli [a]		Response				
Test	Control	Test	Control	Attractiveness	t-test [c]	n [d]
		Series 6				
$SW_{60}H2$	E	82	19	81 ± 3	*	6
		Series 7				
$SW_{50}H$	E	83	34	69 ± 13	*	6

[a] *For abbreviations used in the first and second column see Material and Methods*
[b] *The percentage of mosquitoes that flew into the test compartment of the total of mosquitoes that flew in the test and control compartment. Averages of the individual tests are presented (in % ± standard error).*
[c] *significantly different from 50% (paired two sample for means t-test for significant differences, P < 0.05)*
[d] *number of replicates.*

TABLE 6.4. Description of the different skin washings used (for abbreviations used see Material and Methods).

Skin Extract	Skin site	Number of volunteers	Number of rubbings/ ml	Lactic acid mg/ml	Solid fraction mg/ml	Stimulus size ml	Release method
$SW_{60}H1$	Hands+ feet	60	1.2	1.6	56.7	0.1	Filter paper
$SW_{60}H2$	Hands+ feet	60	3.0	3.1	25.2	0.1	Filter paper
$SW_{50}H$	Hands+ feet	50	0.9	0.8	20.5	0.01	Heated capillary
$SW_{50}H$*fd*	Hands+ feet	50	-	-	20.5	0.01	Heated capillary
$SW_{50}B$	Back	50	1.0	0.5	22.5	0.01	Heated capillary
$SW_{50}B$*fd*	Back	50	-	-	22.5	0.01	Heated capillary

fd= freeze dried skin washing reconstituted with 1 ml ethanol

DISCUSSION

There was no attractive or synergistic effect of carbon dioxide, confirming previous investigations with *Cx quinquefasciatus* in a similar set up (Mboera *et al.*, 1998). Omer (1979) found in a wind tunnel study carbon dioxide attractive for *Cx quinquefasciatus*, but only when the carbon dioxide was applied in pulses (20 s on / 20 s off). In field studies, more *Cx quinquefasciatus* were collected in carbon dioxide baited traps than in unbaited traps (Reisen and Pfuntner, 1987; Costantini *et al.*, 1996). However, others have failed to show attraction to carbon dioxide in the field (Harden and Poolson, 1969). Recent field data showed that carbon dioxide accounted for only about 25% of the attraction of *Cx quinquefasciatus* to a human host (Mboera and Takken, 1999) indicating that other host odours are more important. A high concentration of carbon dioxide counteracts the attraction

of skin rubbing or lactic acid, which finding supports the hypothesis of Service (1993) that carbon dioxide repels at higher concentrations. Many authors report the combined action of carbon dioxide and chemicals present on the human skin inducing or increasing the attractiveness of those chemicals for *Ae. aegypti* (Roessler, 1961; Acree *et al.*, 1968; Mayer and James, 1969; Smith *et al.*, 1970; Carlson *et al.*, 1973; Geier, 1995). Omer (1979) showed similar effects for *Cx quinquefasciatus*. The contradiction found between our results and those of Omer (1979) might be due to differences between populations of *Cx quinquefasciatus* or the bioassay used.

The high preference for skin rubbings from the forearm indicates that attractive volatiles are present on the skin. Geier (1995) showed that ethanol skin washings of human hands and feet were attractive to *Ae. aegypti*. In test series 1 and 3 the skin washings on filter paper were slightly attractive, but in the second not. Skin washings of extremities and back tested in heated capillaries were attractive, which is in line with results of Geier using the same release method. In a pilot experiment releasing the skin washings of 60 people in heated capillaries, significant attraction to these washings was found ($P<0.05$) (72% ± 8, unpublished data). The relatively low attractiveness of the skin washing on filter paper compared to the data of Geier (1995) was probably due to a low dose, 5 times lower than that used by Geier or may have been caused by a difference in response to human skin emanations between *Ae. aegypti* and *Cx quinquefasciatus*. As *An. gambiae* showed a clear preference for trapping devices containing the skin washings, the concept of species-specific responses is supported.

The fact that L-lactic acid is dominantly present in eccrine sweat on human skin suggests that this chemical could play a role in the attractiveness of the skin rubbing (Woodroffe and Shaw, 1974). Eccrine sweat glands are spread over the whole body; in high densities on the soles of the feet, hands and head, in average densities on the limbs and in low densities on the back and the face (Marples, 1969). Acree *et al.* (1968) identified L-lactic acid as the major attractive component for *Ae. aegypti* in an acetone skin washing. In test series 1 and 2, attraction to L-lactic acid at relatively high doses (5.0 and 31.0 mg on filter paper) was found while there was no attraction to lower dosages in test series 2. Possibly these dosages were too low to act as an attractant. In the literature, many examples are given of the reverse, *Ae. aegypti* being attracted to low doses of lactic acid, but repelled by higher doses in the range over which attraction was found here (Müller, 1968; Smith *et al.*, 1970). The fact that the mosquitoes showed a high preference for the skin washing over the lactic acid shows that L-lactic acid is not the only attractive component on the skin. This is confirmed in the literature (Smith *et al.*, 1970; Geier, 1995).

The experiments, testing individual mosquitoes, show that the skin washings made of hands and feet, and back are both attractive for *Cx quinquefasciatus*. De Jong and Knols (1996) found a significant biting preference for body parts with low densities of eccrine sweat glands. However, since other parameters associated with these sites could not be ruled out, a causal correlation between gland density and biting preference has not been demonstrated. In our study, the skin washings of hands and feet, and back made of as many as 50 volunteers did not differ in dry weight and lactic acid concentration and were also equally attractive. Mboera *et al.* (1998) already showed in a similar set up that *Cx quinquefasciatus* was significantly attracted to worn socks. These findings suggest that, despite differences in gland densities between hands and feet on the one hand, and the back on the other hand, the composition of the washings did not differ dramatically. In addition, the essential volatiles used by mosquitoes to recognise humans are probably not limited to emanations from specific sites but might be general characteristics of human skin. It is concluded that *Cx quinquefasciatus* and *An. gambiae* are attracted to emanations from human skin, and that lactic acid is one of the components that cause the attraction of the former species. In the laboratory set-up carbon dioxide does not affect the host-seeking response of *Cx quinquefasciatus*.

As the skin washing was made according to the method used by Geier (1995), the different skin washings can be considered as analogous odour stimuli. Thus, for the first time, attraction to volatiles from a single host odour source in the absence of carbon dioxide

has been demonstrated in three of the most medically important mosquito species with distinct differences in ecology and behaviour (Mboera, 1999; Takken and Knols, 1999)

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SECTION II

IDENTIFICATION OF KAIROMONES FOR *AN. GAMBIAE*

BEHAVIOURAL AND ELECTROANTENNOGRAM (EAG) RESPONSES OF *AN. GAMBIAE* TO POOLED HUMAN SWEAT

ABSTRACT - Behavioural and electroantennogram (EAG) responses of female *Anopheles gambiae sensu stricto* mosquitoes to pooled samples of human sweat, either fresh or incubated at 37 °C for two days, were analysed. No responses were obtained to fresh sweat while the incubated sweat produced behavioural as well as EAG responses. The sweat samples of all volunteers, that had been analysed individually, showed a similar trend in change of pH, osmolality and microbial density during incubation. The role of differences between olfactory signatures of human individuals in the search for kairomones for the *An. gambiae* is discussed.

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INTRODUCTION

Human skin emanations play an important role in the host-seeking behaviour of the man-biting malaria mosquito *Anopheles gambiae s.s.* (henceforth termed *An. gambiae*) (Takken and Knols, 1999). Earlier studies showed that behaviourally attractive components are present in human sweat (Chapter 4 and 5). Behavioural experiments conducted with sweat showed that alkalisation of the attractive sweat, which results in a decreased release of acidic compounds, had no effect on the attractiveness of the sweat (Braks *et al.*, 1997). This finding suggests that, in addition to carboxylic acids, other sweat-borne compounds play a role in the host seeking behaviour of *An. gambiae.* However, when repeating this study with freshly collected sweat from other volunteers, it appeared to be not attractive at all (Braks and Takken, 1999). In the past, conflicting results have been reported concerning the attraction of *Aedes aegypti* mosquitoes to complex odour mixtures of human origin as well (Willis, 1947; Parker, 1948; Brown *et al.*, 1951; Thompson and Brown, 1955; Müller, 1968; Price *et al.*, 1979; Eiras and Jepson, 1991 and 1994). The first indication that the differential attractiveness of sweat samples for *An. gambiae* might be due to differences in chemical composition of the sweat samples came from pH measurements (Braks and Takken, 1999). Attractive, incubated sweat appeared to have an alkaline pH of 8, in contrast to fresh and un-attractive sweat that had an acidic pH of around 5.5 (Noble and Somerville, 1974; Braks *et al.*, 1997; Braks and Takken, 1999). Surprisingly, freshly collected sweat from the individuals tested was not attractive for *An. gambiae*, while incubation of the sweat resulted in a strong attraction. Incubation of the sweat was accompanied by a distinct growth of microorganisms, suggesting that the change in pH as well as the attractiveness of the incubated sweat might be the result of chemicals produced by these bacteria. Unfortunately, determining the density of microorganisms in the sweat after incubation failed due to unexpectedly strong microbial growth in the different sweat samples (Braks and Takken, 1999).

The present study served two goals. First, the ranges of three characteristics of sweat samples questioned in Braks and Takken (1999) were explored. Second, volatiles that are produced by any human host were studies while ignoring possible differences between individual sweat samples. Hence, sweat of fourteen human volunteers was collected. For the first objective, the course of the pH, the osmolality (a measure of soluble particles dissolved) and microbial density of the different sweat samples over time were determined. For the second objective, we decided to pool the sweat samples to minimise volunteer-specific effects. Behavioural and antennal olfactory responses of female *An. gambiae* mosquitoes to fresh and incubated pooled sweat were determined.

MATERIALS AND METHODS

Insects. An. gambiae originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome). Mosquitoes were reared at 27 °C, 80% R.H. and 12:12 L:D photoperiod. Adults were held in gauze cages (30 x 30 x 30 cm). They had access to a 6% glucose solution and were given the opportunity to feed on a human arm twice a week. Eggs were laid on wet filter paper and transferred to water trays. Larvae were fed Tetramin fish food. For the behavioural and EAG studies 5 – 8 days old non-blood fed female mosquitoes were used.

Sweat collection. The collection of sweat from volunteers engaged in physical exercise by cycling on a hometrainer took place in a humidified (70% RH) room at 30 °C. Fourteen Caucasian volunteers, nine males and five females with ages ranging from twenty-one to fifty-two, were asked each to collect 3 of ml sweat from the forehead. Volunteers had not used soap or any other cosmetic products 24 hours prior to the sweat collection. From each individual 1.5 ml sweat was directly placed in a freezer (-5 °C), while the other 1.5 ml was incubated for 1 to 2 days under aerobic conditions at 37 °C and subsequently stored in a freezer. Both sweat samples will further be referred to as 'fresh sweat' and 'incubated sweat' respectively. A pooled sweat sample (pool A) of the individual sweat samples was obtained

by pooling 1.2 ml sweat from thirteen of the individuals and 0.8 ml from one individual; this was done both for the fresh and for the incubated sweat. A second sweat sample (pool B) was obtained from three male Caucasian volunteers, with ages ranging from thirty-three to fifty-one, each of whom collected 6 ml sweat from the forehead. Equal volumes of their sweat samples were pooled. The pH of all the sweat samples was determined before and after incubation (Indicator Paper, Merck).

Analysis of sweat. The pH (Indicator Paper, Merck) and osmolality (Digital micro-osmometer type 3, Vogel) of the fourteen sub-samples of fresh and incubated sweat pool A were determined. Osmolality, by definition, is an expression of the total of solute particles dissolved in one kilogram of solvent without regard for particle size, density, configuration or electrical charge. Osmolality is expressed as mOs/kg, where Os is defined as the concentration, expressed on a molal basis of the osmotically active particles in true solution e.g. 1000 mmol (1 mol) NaCl in 1 kg water = 2000 mOsm/kg (under ideal conditions) (prescription of Digital micro-osmometer type 3,Vogel). Further, the presence and growth of microorganisms were indicated by the number of colony forming units (CFU) per ml sweat. For this, a-selective agar plates ((Iso Sensi Test, CM471, Oxoid Ltd. UK) were inoculated with 45 µl of 10 to 10^{7} times (v/v) diluted fresh or incubated sweat samples of pool A. Control plates were inoculated with 45 µl of sterile distilled water. After the agar plates had been left to incubate for one day at 37 °C under aerobic conditions, the CFU were counted. The growth rate, µ [day^{-1}], was calculated as follows:

$$\mu = \frac{\ln(N_2) - \ln(N_0)}{\ln(2)}$$

where N_0 and N_2 are the numbers of CFU per ml sweat on day 0 and day 2 of the incubation, respectively. The L-lactic acid concentration of fresh and incubated sweat samples of pool A and B was also determined (Lactic Diagnostic Kit Sigma, NR 735).

Behavioural assay. A dual-port olfactometer, consisting of a Perspex flight chamber (1.6 x 0.66 x 0.43 m) (modified after Braks and Takken, 1999) was used to study the attractiveness of fresh and incubated pooled sweat. Charcoal filtered, humidified (65 ± 5% RH) air with a temperature of 27 ± 0.5 °C was led, via Perspex trapping devices, through two ports (diameter 0.04 m, 0.28 m apart) into the flight chamber with a speed of 20 cm/ sec. Light (1 lux) was produced by one incandescent light bulb (75 W) and was filtered and scattered by a piece of yellow cloth hanging 1 m above the flight chamber.

The behavioural responses to fresh and incubated sweat samples of pool A and B were tested. For each test 50 µl sweat was applied on a sand-blasted glass slide (5 x 2 cm), which was placed in the left or right Perspex trapping device and was tested to an equivalent amount of distilled water. Mosquitoes were released from a container at the rear end of the flight chamber. During each 15 min-test, 30 mosquitoes had the opportunity to choose between one of the ports. Each stimulus was tested six times against its control. Stimuli were alternated between the right and left port with each test. Behavioural tests were performed during the last four hours of the dark period.

Electrophysiology. Female mosquitoes were lured by a human hand, caught in an aspirator, cooled in a freezer (- 5 °C) for ± 1.5 min., and mounted on a small Perspex holder (Lacher, 1971). Wings were glued with Perfax glue (Henkel, The Netherlands). Legs were removed, and one of the antennae was carefully fastened with the proximal segments to double-sided sticky tape (Sellotape, H.P.N., The Netherlands), after which the tip of the distal segment of the antenna was removed. The other antenna was fastened over the entire length to the double- sided sticky tape to enhance the stability of the preparation. Electroantennograms were recorded with AgCl-coated Ag-electrodes. Glass electrodes were filled with a saline solution containing 9 g NaCl, 2 g KCl, 1.34 g $CaCl_2.2H_2O$ and 5 g PVP per litre H_2O. The tip

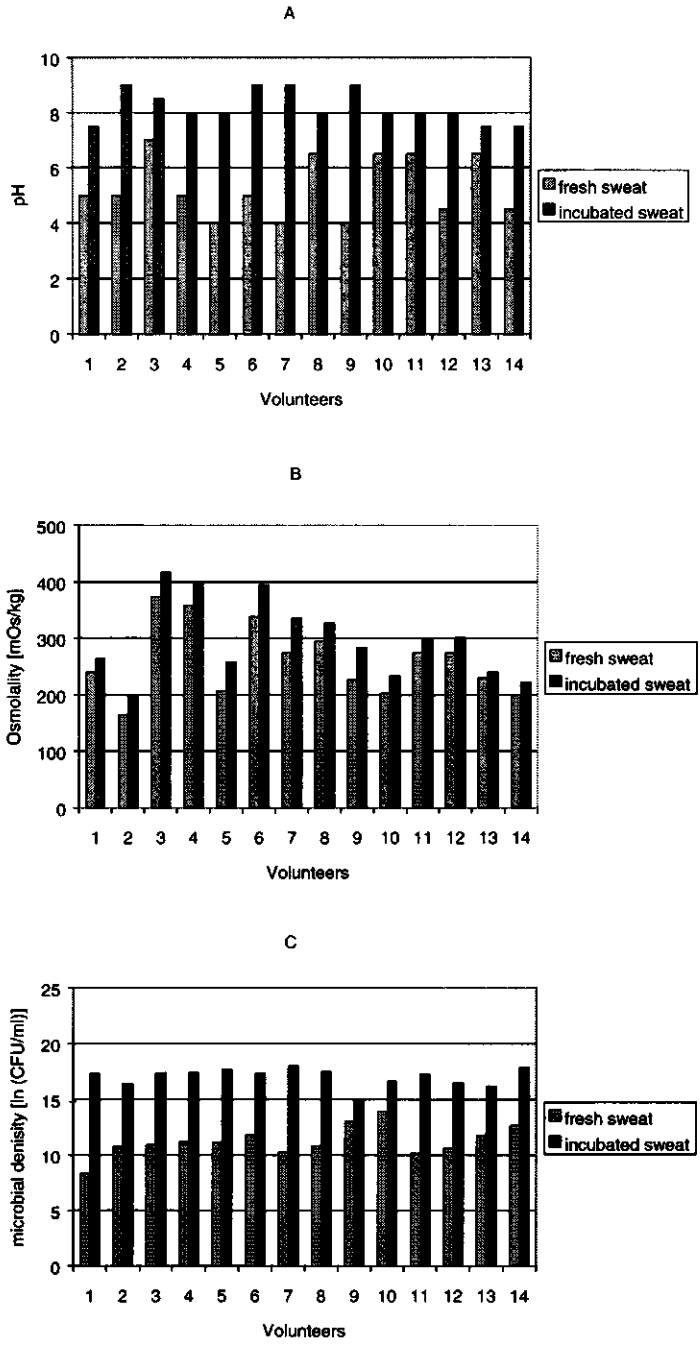

FIGURE 7.1. Data on the pH (a), osmolality (b) and microbial density (natural logarithm of CFU/ml) (c) of the separate sweat samples from the 14 volunteers of pool A.

of the glass electrodes was removed and the recording electrode was placed over the cut end of the antenna. The indifferent electrode was inserted into the eye of the mosquito. A moistened, charcoal filtered, continuous air stream (14.5 ml/ sec) was led through a glass tube (i.d. 1 cm) ending 0.5 cm from the preparation holder. Stimuli were prepared by applying 25 µl of the sweat, its headspace on a 2.4 cm^2 filter paper (Schleicher & Schuell, Dassel, Germany). Filter papers were placed in a 150 mm glass Pasteur pipette that was sealed with para-film at both ends. Stimulus puffs (volume 1.6 ml) lasted for 0.5 sec and were injected into the air stream, at a location 10 cm from the outlet of the tube, by using a stimulus controller (Syntech, Hilversum, The Netherlands).

Signals were amplified 10x and directly imported via an IDAC interface box and A/D converter (Syntech, Hilversum, The Netherlands) into an Intel Pentium-based personal computer. Recordings were analysed by means of EAG software version 2.6 (Syntech, Hilversum, The Netherlands).

To normalise the electroantennogram responses, EAG values were corrected for the response to the control, an equivalent amount of water. Corrected values were expressed as percent response to the standard 1-octen-3-ol, which was a 1% solution (25 µl) in diethylether (v/v). Each preparation was stimulated with the standard to allow comparison of relative effectiveness of a stimulus among individuals. EAG responses did not decrease during the life span of a preparation, most likely because living mosquitoes were used. Nevertheless, for the EAG experiments performed in response to the identified components, the standard was applied after every two stimulations.

RESULTS

Analyses of sweat. The pH, osmolality and microbial density increased in each of the sweat sub-samples of pool A. The range of pH, osmolality and microbial density of the different sweat samples are shown in Figure 7.1. On average the pH of sweat samples changed from 5.3 ± 1.1 when fresh to 8.2 ± 0.6 when incubated. The osmolality of the fresh sweat samples of 261.3 ± 63.2 mOs/kg increased during incubation to 298.2 ± 68.7 mOs/kg. The growth rate of the microbes over the two incubation days was 8.3 ± 2.7. The pooled fresh samples of pools A and B contained 3.0 and 2.4 mmol/ml L-lactic acid, respectively, and the pooled incubated sweat samples of pools A and B 2.9 and 1.7 mmol/ml, respectively.

Behavioural responses to fresh and incubated sweat. The trapping devices baited with incubated sweat samples of both pool A and B caught a significantly larger number of mosquitoes than the control trap (Table 7.1). The catches with both the fresh samples were not significantly different from the control.

EAG recordings from fresh and incubated sweat. Electroantennogram responses to the different sweat samples were corrected for the response to the control and compared. Mean responses to the incubated sweat samples of pool A (- 0.24 mV ± 0.02; N = 35) as well as of pool B (- 0.21 mV ± 0.03; N = 11) were significantly larger (pool A: $P < 0.001$; pool B: $P < 0.01$; Wilcoxon's matched pair signed rank test) than those obtained from the fresh sweat samples of pool A (0.00 mV ± 0.01; N = 35) and B (0.01 mV ± 0.02; N = 11). No significant differences were observed between the incubated sweat samples from pool A and B ($P = 0.2$; Mann-Whitney U test). The mean pH value for the fresh and incubated sweat from pool A was 5 and 8 respectively and for the fresh and incubated sweat samples from pool B these values were 7 and 9. Mean normalised EAG values corrected for their response to the control and expressed as percent response to the standard 1-octen-3-ol are shown in Figure 7.2.

TABLE 7.1. Data from the dual-port olfactometer experiments with sweat samples collected from 14 volunteers (Pool A) and three volunteers (Pool B). Shown are the numbers of An. gambiae s.s. *caught in trapping devices baited with fresh sweat against the control (an equivalent amount of distilled water) and incubated sweat against the control.*

Odour source	Stimuli		Response [a]		Chi-square	N [c]		
	Treatment	Control	Treat.	Control		T	C	=
Pool A	Fresh sweat	Water	24	32	ns	2	4	0
	Incubated sweat	Water	85	16	***	6	0	0
Pool B	Fresh sweat	Water	21	19	ns	2	2	2
	Incubated sweat	Water	43	8	***	6	0	0

[a] *The response is given as the total number of mosquitoes caught in either the treatment or control trapping device.*

[b] *Significant differences (*: P≤ 0.05, **: P≤ 0.01 or ***: P≤ 0.001) or no significant differences (ns: P>0.05) found between the total number of mosquitoes caught in the treatment and control trapping device (Chi-squared test)*

[c] *N= number of replicates. The distribution of the preferences for either the treatment (T), the control (C) or no preference (=) in the individual replicates are also shown.*

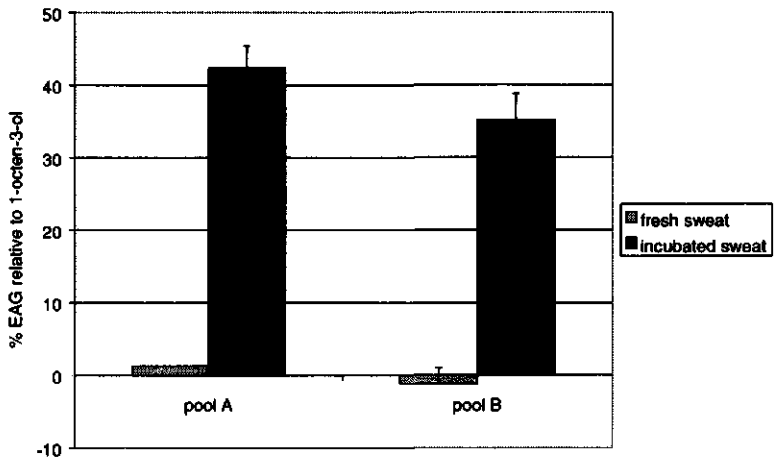

FIGURE 7.2. Normalised mean EAGs expressed as percent response to the standard 1-octen-3-ol (25 µl of 1% solution in diethylether (v/v)) from An. gambiae *females in response to fresh and 42-52 hours incubated sweat samples from a pooled sample of 14 (pool A) and a pooled sample of 3 (pool B) volunteers respectively. Markers above and below bars represent standard error of the mean (SEM).*

DISCUSSION

The behavioural bioassay showed that sweat incubated for two days was attractive to *An. gambiae* females in contrast to the fresh sweat, which showed no significant attractiveness. In addition to Braks and Takken (1999) who found that sweat obtained from two out of three individuals became attractive after an incubation period of two days, we found that pooled sweat from a group of 14 as well as from a group of 3 volunteers gave similar degrees of attractiveness after incubation. This suggests that *An. gambiae* responded to volatiles that are generally present in incubated human sweat, while the absolute composition of the sweat samples collected by the different volunteers varied largely (Figure 7.1; Noble and Somerville, 1974). General trends could also be observed, despite these differences: in each of the sub-samples of pool A, the pH, osmolality and microbial density increased during incubation. The abundant growth of microflora (Figure 7.1) and the fact that the sweat elicits a behavioural and olfactory response only after incubation, strongly suggests that attractants are produced by microbial activity. The change in pH is reported to be due to the production of ammonia from nitrogen-components of sweat and the reduction of L-lactic acid to the utilisation of this component by skin microorganisms during incubation (Bergeim and Corntbleed, 1943; Smith, 1971). Hence, skin microorganisms are presumed to break sweat-borne compounds down into smaller ones and, probably, this is represented in the general increase of the osmomality. Field experiments conducted by Haddow (1942) showed that unwashed children are more attractive to *An. gambiae* than washed children and corroborate indications that the skin microflora might play a role in the production of kairomones.

In agreement with the behavioural data, incubated sweat but not fresh sweat induced EAG responses. The lack of EAG reactions with fresh sweat indicates that the nature or quantity of volatiles emanating from fresh sweat was inadequate to produce measurable EAG-responses. Comparison of fresh and incubated sweat can lead to exclusion of many volatiles that are potentially responsible for the attraction. Electrophysiological responses and chemical analyses of the headspace of both the fresh and incubated sweat samples used in this study are reported elsewhere (Meijerink *et al.*, accepted). As the sweat was collected from the natural host of *An. gambiae*, it is conceivable that attractive volatiles arising from the sweat might have ecological meaning.

Individual humans are differently attractive to mosquitoes (Carnevale *et al.*, 1978; Port *et al.*, 1980; Curtis *et al.*, 1987; Lindsay *et al.*, 1993; Brady *et al.* 1997) and that some of this variation can be explained by the composition of their odour profile (Knols *et al.*, 1995). However, *An. gambiae* demonstrates a high preference for any human individual to any other mammalian species, implying that host-specific odour blends must exist. As no essential differences were found between the behavioural and electrophysiological responses to sweat samples composed of a collection from either three or fourteen volunteers, it is suggested that volatiles generally emanating from human sweat samples are involved.

Compounds showing kairomonal activity in a field situation can be applied in studies of the population dynamics and used in mosquito density monitoring programs thereby serving as an alternative for human biting catches. Eventually application of an optimised bait might lead to a reduction in malaria mosquito populations which in combination with other malaria control measures might contribute to malaria control.

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ROLE OF HUMAN SWEAT COMPONENTS, AMMONIA AND LACTIC ACID, IN THE BEHAVIOUR OF *AN. GAMBIAE*

ABSTRACT - In a study of the role of human sweat in the behaviour of the anthropophilic malaria mosquito *Anopheles gambiae s.s.*, it was found that freshly collected eccrine sweat was attractive but that incubated sweat was significantly more attractive than fresh sweat. The behavioural response to L-lactic acid and ammonia, the main constituents of sweat, was investigated. L-lactic acid was attractive at one concentration only (1.11 µmol/100 µl) and removal of the L-lactic acid from the sweat by enzymatic decomposition did not affect the attractiveness of sweat. Ammonia present in the sweat caused attraction over a range of 1.34 x 10^{-2} - 1.34 mmol on glass slides and at 10.3 - 103 PPM in an air stream. It is concluded that a) human sweat contains kairomones for host-seeking *An. gambiae*, b) ammonia is an important kairomone for this mosquito and c) that L-lactic acid does not appear to play an important role in the attraction of *An. gambiae* to sweat.

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INTRODUCTION

Vertebrate hosts reveal their presence and identity to blood feeding mosquitoes mostly by odour. The olfactory cues or kairomones are contained in the expired air and/or skin emanations. Host-seeking *Anopheles gambiae* Giles *sensu stricto* (henceforth termed *An. gambiae*), being highly anthropophilic, orient mainly to human skin emanations (Mboera and Takken, 1997; Costantini *et al.*, 1998; Takken and Knols, 1999). The volatiles emanating from the skin originate either from the secretions of skin glands or the skin microorganisms or both. Gland secretions can be divided into water-soluble products, mainly from eccrine sweat glands, and fat-soluble products from the sebaceous and apocrine glands. Recently, Braks *et al.* (1997) reported upwind responses of *An. gambiae* to human sweat collected from volunteers performing physical exercise in a warm humid room. Sweat samples that had been incubated for two days at 37°C were attractive while freshly collected sweat samples were not (Braks and Takken, 1999). The volatiles responsible for the attraction were probably produced by skin microorganisms during incubation in the sweat volume (Braks *et al.*, 1999a).

Thermally induced sweat consists principally of eccrine excretion with L-lactic acid, ammonia, urea and electrolytes as the main components (Noble and Somerville, 1974). L-lactic acid has been shown to play an important role in the host-seeking behaviour of the yellow fever mosquito *Aedes aegypti* L. (Acree *et al.*, 1968; Geier *et al.*, 1996), but behavioural responses of *An. gambiae* or other mosquito species to L-lactic acid have not been reported. Ammonia has occasionally been mentioned in association with host-seeking *Aedes*-mosquitoes (Rudolfs, 1922; Müller, 1968), but not with *An. gambiae*, and there is no recent report on the attraction of *Aedes* spp. to ammonia. The other main constituents of eccrine sweat are not volatile.

In this study, we investigated the general features of sweat samples of different human individuals to study the role of the constituent volatiles responsible for the attraction of *An. gambiae* to sweat (Braks *et al.*, 1997; Meijerink *et al.*, accepted). For this, sweat collections of fifteen volunteers were pooled before testing to minimise volunteer-specific effects as reported by Braks and Takken (1999). The attractiveness of the sweat samples over time was determined to assess the volatility level of components responsible for the attraction. The significance of the behavioural responses to incubated sweat was investigated by testing it to two other host-derived stimuli, skin washings (Braks *et al.*, 1999b, Chapter 6) and a human hand *in vivo*. The role of two main eccrine sweat components, L-lactic acid and ammonia, in the attraction of *An. gambiae* to sweat was investigated by determining the time course in their concentration in the sweat during incubation, the dose-response relationships and the effect of the selective removal of L-lactic acid from sweat on its attractiveness.

MATERIALS AND METHODS

Insects. The *An. gambiae* strain used originated from Suakoko, Liberia (by courtesy of Prof. M. Coluzzi, Rome) and was maintained under standard insectary conditions (27 ± 1°C, 80 ± 5% relative humidity, photo/scotophase of 12:12 LD). Adults were kept in 30-cm cubic gauze-covered cages and had access to 6% glucose solution. Females were offered blood from a human arm for 10 min twice a week. Eggs were laid on wet filter paper, hatched in water trays and the larvae were fed on Tetramin® fish food. Pupae were collected daily from the trays and were allowed to emerge in the adult cages. For the bioassay 4 to 8-day-old female mosquitoes, which had not received a blood meal, were used. Behavioural experiments were performed during the last four hours of the dark period.

Olfactometer. A dual-port olfactometer, consisting of a Perspex flight chamber (1.60 X 0.66 X 0.43 m) (modified after Brals and Takken, 1999), was used to study the attractiveness of

possible stimuli. Charcoal filtered, humidified (65 ± 5% relative humidity), warm air (27 ± 0.5 °C) was led via a Perspex mosquito trapping device, which was linked to two ports (diameter 4 cm, 28 cm apart), into the flight chamber with a speed of 20 cm/ sec. Dim light (1 lux) was produced by one incandescent light bulb (75 watt) and was filtered and scattered through a screen of yellow cloth hanging 1 m above the flight chamber.

Collection and treatment of sweat samples. Sweat droplets were collected from the foreheads of fifteen Caucasian human volunteers (eleven males and four females with ages ranging from 25 to 52 years) with sterile glass Pasteur pipettes and put in 5 ml glass vials. From each volunteer 3 ml of sweat was collected. Sweat production was stimulated by physical exercise on a hometrainer in a warm humid room (30°C, 90 % relative humidity). Immediately after collection, 1.5 ml sweat was directly stored at -20 °C and the other half was incubated under aerobic conditions at 37 °C for two days, after which it was also stored at -20 °C. The pH of the fresh sweat samples collected by the different volunteers ranged from 4.4 to 7.4 (5.81 ± 0.96) and the pH of the incubated sweat ranged from 6.3 to 9.0 (8.8 ± 0.76). A pooled sample was composed of sub-samples collected by the different volunteers (0.6 ml sweat per volunteer). The pH (micro pH-electrode, Inlab 423, Mettler), L-lactic acid concentration (Lactic Diagnostic Kit Sigma, NR 735), urea and ammonia concentration (Indophenol method according to Berthelot; Gips and Wibbens-Alberts, 1968) of the sweat were determined.

Selective removal of L-lactic acid from the sweat was achieved by adding 50 µl lactate-oxidase (from *Pediococcus spp.*, 34 U/mg, Sigma, 50 U/ 0.1 ml buffer) and 100 µl catalase (from bovine liver, 3100 U/mg, Sigma, 50 mg/ ml buffer) to 1 ml sweat (after Geier, 1995). The buffer (pH 6.4) contained 0.136 gr $KHPO_3$, 0.278 ml 1N NaOH per 100 ml distilled water. After an incubation period of two hours at 37°C, the L-lactic acid of the incubated sweat was oxidised (confirmed with Lactic Diagnostic Kit). To oxidise the remaining L-lactic acid of the fresh sweat, an extra 20 µl lactate-oxidase and 40 µl catalase was added. After another half-hour incubation period all lactic acid was oxidised. The control samples (*sham*) consisted of sweat or distilled water that were treated similarly, only omitting the lactate-oxidase from the buffer.

Collection of skin washings. A skin washing that previously has been proven to be attractive for *An. gambiae* (Chapter 6) was made by rubbing forearms, feet and ankles of 60 volunteers (23-60 yr., 40 males: 20 females) for 5 min with cotton wool, drenched in 96% ethanol. The cotton wool samples were left to dry overnight at room temperature and subsequently packed in a glass tube (150 x 2.8 cm). The skin residues were extracted with ethanol at 1.5 ml/min for six hours. The washing was concentrated to 50 ml using a vacuum excitator, kept at -20 °C for an hour and centrifuged (3000G, 5 min). Half of the supernatant (22.5 ml) was concentrated a second time to 10 ml and was used as skin washing (for details see Braks *et al.*, 1999b).

Preparation of chemicals. 1 g L-lactic acid (L-lactic acid sodium salt, Fluka) was dissolved in 10 ml ethanol (>98% pure, Merck) and concentrations from to 0.001 to 100 mg/ml (1.11×10^{-5} -1.11 M) were tested in the olfactometer. The aqueous ammonia solution was purchased from Merck (min. 25 %, 1l = 0.91 kg) and diluted further with distilled water. The ammonia concentration of the stock solution was 13.4 M (227.8 g/ l) (Indophenol method, Berthelot Gips and Wibbens-Alberts, 1968). A concentration array from 1.34×10^{-4} to 13.4 M ammonia solution applied on a glass slide was tested against distilled water. In contrast to L-lactic acid, ammonia is highly volatile and it was, therefore, also tested in gaseous form from gas sampling bags to sustain a constant stimulus concentration. For this, 250 µl of respectively 25%, 2.5 % and 0.25% ammonia solution were put in a 80 l Teflon gas sampling bag (SKC Menro Int. BV.). Subsequently, the bags were filled with 80 l humidified warm air from the air supply of the olfactometer (65 ± 5% relative humidity, 27 ± 0.5 °C) at least 15 hours prior to the experiment to allow the solution to evaporate. This procedure resulted in ammonia

concentrations of 1.03, 10.3 and 103 PPM in the bags. The control stimuli consisted of an equivalent amount of air taken from another gas sample bag filled with air and 250 µl distilled water.

Behavioural assay. The stimuli tested comprised of sweat, a skin washing, L-lactic acid and ammonia. In each trial within an experiment, except the one testing a human hand *in vivo* or ammonia from gas sampling bags, a standard volume of a stimulus was applied on a sandblasted glass slide (5 x 2 cm) that was placed in the left or right Perspex trapping device. A control glass slide with an equivalent amount of sweat or solvent was placed in the opposite trapping device. The standard stimulus volume for sweat was 50 µl and for L-lactic acid or ammonia solution 100 µl, and 200 µl for skin washing. To test the attractiveness of a human hand *in vivo*, the experimenter put her hand through a slot of the trapping device into the air stream. From the gas sampling bag, odorous air was pumped at 0.25 l /min, (air pump, MG-4, AMETEK) through Teflon tubes (diameter 7 mm) into the trapping device where it merged with the main air stream (23.5 l/min).

During a 15-min trial, 30 mosquitoes were released from a container at the downwind end of the flight chamber and were allowed to choose between one of the two trapping devices. Stimuli were alternated between the trapping devices with each trial. Each subsequent trial started with fresh mosquitoes, clean trapping devices and freshly applied odour stimuli except for one experiment testing the attractiveness of sweat over time.

Experimental set-up. Three different sets of experiments were performed:

i. Responses to fresh and incubated sweat and attractiveness of sweat over time. The responses of *An. gambiae* to fresh and incubated sweat were determined by testing them against an equivalent amount of distilled water or each other (Exp. #1). Subsequently the preference for either incubated sweat or skin washing against each other was investigated. A similar experiment was done to examine the preference for either incubated sweat or a human hand *in vivo* by testing them against each other (Exp. #2 and #3). In addition the attractiveness of sweat over time was assessed in dual choice tests with fresh and incubated sweat while using the same glass slides during six subsequent trials with a 5 min. interval (Exp. #4).

ii. Responses to L-lactic acid. First, a concentration series of L-lactic acid in ethanol (1.11×10^{-3} - 1.11×10^{2} M) was tested against ethanol (Exp. #5). Subsequently, the effect of the selective removal of L-lactic acid on the attractiveness of sweat was investigated by testing lactate-oxidase-treated sweat samples (Exp. #6 and #7) against *sham*-sweat samples and *sham*-water.

iii. Responses to ammonia. In these experiments, a concentration series of ammonia was tested either as an aqueous solution applied on glass slides ($13.4 - 1.34 \times 10^{-4}$ M) (Exp. #8) or in gaseous form (1.0 - 103.0 PPM) at a rate of 250 ml/min from a gas sampling bag (0.084 -8.4 µmol/min) (Exp. #9).

Data analysis. The total number of mosquitoes caught in the treatment-trapping device in six replicates was compared with the total number in the control trapping device using Chi-squared tests.

RESULTS

Chemical composition of pooled sweat. Pooling of the sweat samples resulted in a single sample of fresh sweat with pH 5.3 and a single sample of incubated sweat with pH 8.5. The L-lactic acid and urea concentration of the sweat decreased during the incubation by 31.1% and 21.4 %, respectively. In contrast, the ammonia concentration of sweat increased more than 7.5 times (785.1%) in the same period (Table 8.1).

TABLE 8.1. Chemical composition of pooled sweat from 15 volunteers.

Parameter	Fresh sweat	Incubated sweat
pH	5.3	8.5
L-lactic acid (mM)	32.2	22.2
Urea (mM)	19.6	15.3
Ammonia (mM)	6.3	49.4

Responses to fresh and incubated sweat and the attractiveness of sweat over time. Significantly more mosquitoes were caught in the trapping devices baited with fresh (P= 0.004) or incubated sweat than in the control ($P<<$ 0.001). When tested against each other, the mosquitoes responded more to incubated sweat than to fresh sweat ($P<<$ 0.001) (Table 8.2). Significantly more mosquitoes were caught in the trapping device baited with incubated sweat than the one baited with skin washing ($P<<$ 0.001). A human hand attracted significantly more mosquitoes than incubated sweat ($P<<$ 0.001). When the attractiveness of sweat over time was studied, the preference for incubated sweat existed only during the first 20 min. and was lost in the subsequent time intervals. In the fourth time interval, 60-75 min. after introduction in the olfactometer, a preference for the fresh sweat to the incubated sweat was found (P=0.0067). There was no significant difference between the number of mosquitoes caught in the two trapping devices in the other time intervals ($P\geq$0.05) (Figure 8.1).

TABLE 8.2. Responses of An. gambiae *to sweat (Exp. # 1, # 2 and # 3)*

	Stimuli		Response[a]		Chi-	N[c]		
Exp.	Treatment	Control	Treat.	Control	square	+	-	n
1	Fresh sweat	Water	32	13	**	6	0	0
	Incubated sweat	Water	93	6	***	6	0	0
	Incubated sweat	Fresh sweat	81	15	***	6	0	0
2	Incubated sweat + ethanol	Skin washing 1 + water	48	19	**	5	0	1
3	Hand	Incubated sweat	92	35	*	6	0	0

[a] *The response is given as the total number of mosquitoes caught in either the treatment or control trapping device.*

[b] *Significant differences (*: P≤ 0.05, **: P≤ 0.01 or ***: P≤ 0.001) or no significant differences (ns: P>0.05) found between the total number of mosquitoes caught in the treatment and control trapping device (Chi-squared test)*

[c] *N= number of replicates. The distribution of the preferences for either the treatment (+), the control (-) or neutral (n) in the individual replicates are also shown.*

Responses to L-lactic acid. The trapping device baited with 1.11 µmol L-lactic acid caught significantly more mosquitoes than the control (with an equivalent amount of ethanol) ($P<<$ 0.001). The number of mosquitoes caught in the traps baited with any of the other L-lactic acid concentrations did not differ significantly from the control catches ($P\geq$0.05) (Table 8.3, Exp. #5). Fresh sweat treated with lactate oxidase that selectively decomposed L-lactic acid and sham-treated fresh sweat were significantly more attractive than the water controls (resp. P= 0.024 and $P<<$ 0.001) and when tested against each other did not differ from each other in attractiveness ($P\geq$ 0.05) (Table 8.3, Exp. #6). Incubated sweat treated with lactate oxidase and sham-treated incubated sweat were also significantly more attractive than the

control ($P<< 0.001$) and no difference was found between the two differently treated incubated sweat samples when tested against each other ($P \geq 0.05$) (Table 8.3, Exp. #7).

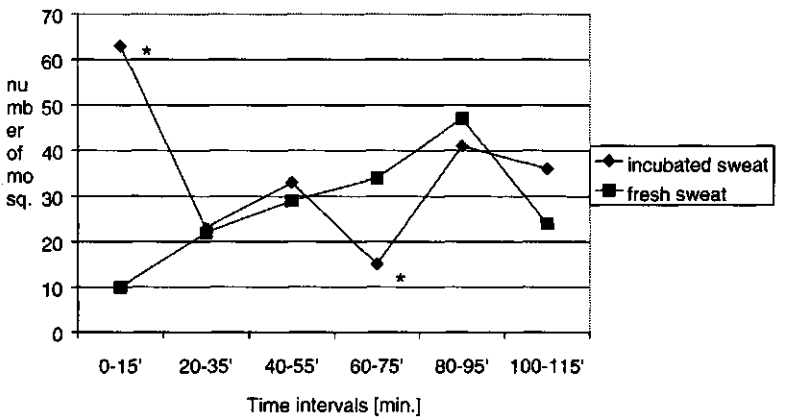

FIGURE 8.1. Responses of An. gambiae to fresh and incubated sweat over time (Asterisks indicate significant differences P< 0.05).

TABLE 8.3. Responses of An. gambiae *to L–lactic acid (Exp. #5) and sweat samples treated with lactate-oxidase (Exp. #6 and #7).*

Exp	Stimuli		Response[a]		Chi-	N[c]		
	Treatment	Control	Treat.	Control	square	+	-	n
5	$1.11x10^{-3}$ µmol LA[d]	Ethanol	33	39	ns	3	3	0
	$1.11x10^{-2}$ µmol LA	Ethanol	31	31	ns	4	2	0
	$1.11x10^{-1}$ µmol LA	Ethanol	32	28	ns	2	2	2
	1.11 µmol LA	Ethanol	48	14	***	5	0	1
	$1.11x10^{-2}$ mmol LA	Ethanol	28	40	ns	3	3	0
	$1.11x10^{-1}$ mmol LA	Ethanol	39	25	ns	3	3	0
6	Fresh sweat + LO[e]	Water *sham*	41	23	*	5	0	1
	Fresh sweat *sham*	Water *sham*	53	18	***	6	0	0
	Fresh sweat + LO	Fresh sweat *sham*	41	47	Ns	1	4	1
7	Incubated sweat + LO	Water *sham*	69	16	***	6	0	0
	Incubated sweat *sham*	Water *sham*	60	14	***	5	1	0
	Incubated sweat + LO	Incubated sweat *sham*	65	49	ns	5	1	0

a/ b /c : see description under Table 8.2.
d LA= L-lactic acid
e LO= lactate oxidase

Responses to ammonia. Ammonia applied on glass slides in quantities of 1.34×10^{-2} - 1.34 mmol attracted significantly more mosquitoes to the trapping device than the control ($P<<$ 0.001) (Table 8.4, Exp. #8). Also the odorous air from the gas sampling bags containing

103.0 or 10.3-PPM ammonia attracted significantly more mosquitoes to the traps than the control airflow control ($P<< 0.001$). The catches with 1.34 -1.34x10 $^{-2}$ µmol ammonia or an odorous air from the gas sampling bags containing 1.03-PMM did not differ from the control ($P \geq 0.05$) (Table 8.4, Exp. #9).

TABLE 8.4. Responses of An. gambiae *to ammonia from glass slides (Exp. #8) or gas sampling bags (Exp.#9).*

Exp	Stimuli		Response [a]		Chi-	N [c]		
	Treatment	Control	Treat.	Control	square	+	-	n
8	1.34x10 $^{-2}$ µmol NH_3	water	31	22	ns	4	1	1
	1.34x10 $^{-1}$ µmol NH_3	water	35	24	ns	3	2	1
	1.34 µmol NH_3	water	35	33	ns	2	1	3
	1.34x10 $^{-2}$ mmol NH_3	water	69	15	***	6	0	0
	1.34x10 $^{-1}$ mmol NH_3	water	63	21	***	6	0	0
	1.34 mmol NH_3	water	48	17	***	5	0	1
9	1.03 PPM NH_3	clean air	26	21	ns	5	0	1
	10.3 PPM NH_3	clean air	70	26	***	5	1	0
	103 PPM NH_3	clean air	60	22	***	4	2	0

[a]/[b] f : see description under Table 8.2.
[d] NH_3 = ammonia

DISCUSSION

Responses to fresh and incubated sweat and the attractiveness of sweat over time. An. gambiae was attracted to eccrine sweat pooled from 15 individuals and, on all occasions, incubated sweat was preferred to fresh sweat when tested simultaneously. In a separate study we have observed behavioural and electrophysiological responses of *An. gambiae* to pooled incubated sweat (Meijerink *et al.*, accepted) and it appears that incubated sweat is an attractive stimulus for this mosquito in the laboratory. This positive effect of incubation is probably due to the action of skin microorganisms present in aqueous skin collections (Braks and Takken, 1999). However, in contrast to our previous findings (Braks and Takken, 1999; Meijerink *et al.*, accepted), in the present study fresh sweat was preferred to the control (water). This result suggests that kairomones to which *An. gambiae* responds were also present in fresh sweat but that the quantity or quality of the attractive volatiles was enhanced during incubation. Skin microorganisms are presumed to break down sweat-borne compounds into smaller, more volatile components (Braks *et al.*, 1999a). This together with the fact that the preference of the mosquitoes for the incubated sweat was lost within 20 minutes after exposure in the olfactometer suggests that the components responsible for the preference of *An gambiae* for incubated sweat to fresh sweat are highly volatile. In addition, it was found that *An. gambiae* preferred incubated sweat (50 µl) to a skin washing (200 µl). This skin washing had previously been found highly attractive for *An. gambiae* (Chapter 6). Similar results have been reported for *Culex quinquefasciatus* (Chapter 6, Braks *et al.*, 1999b). Geier and colleagues (1996) already demonstrated that skin washings were highly attractive for *Ae. aegypti*. Here, we show that, at least for *An. gambiae*, incubated sweat is a more potent stimulus than skin washings. As Geier demonstrated that skin washings were equally attractive for *Ae. aegypti* as a human hand when tested simultaneously in a y-tube olfactometer, we expected that incubated sweat for *An. gambiae* would be, at least, as attractive as a human hand. However, a human hand appeared to be more attractive than incubated sweat (Exp #3). The contradiction between the present findings with *An. gambiae* and those of Geier with *Ae. aegypti* may be due either to the different mosquito species tested or the different bioassays used. In the current set up of our olfactometer, an alteration

of humidity or temperature of the air stream by introducing a human hand in the air stream cannot be ruled out. Therefore, the observed preference of *An. gambiae* for a human hand may have resulted from factors other than olfactory cues.

Role of L-lactic acid. The sweat samples contained L-lactic acid within the range of 4.0-40 mM as reported by Noble and Somerville (1974). The observed reduction of L-lactic acid during incubation was probably due to the utilisation of this component by skin microorganisms (Bergeim and Corntbleed, 1943; Smith, 1971). Only one out of the six concentrations of L-lactic acid tested was attractive for *An. gambiae*, namely 1.11 µmol/100µl. This quantity at the source was close to that of the L-lactic acid present in the incubated sweat applied (1.1 µmol/50µl). Attraction to a broader range of L-lactic acid concentrations (2.2-222.2 mM) has been shown for *Ae. aegypti* (Geier *et al.*, 1996). The latter authors showed that the attractiveness of skin washings to *Ae. aegypti* was completely lost after the selective removal of L-lactic acid. However, skin washings containing L-lactic acid induced a higher response than L-lactic acid alone indicating a strong synergistic effect of L-lactic acid with other components present in the skin washings (Geier *et al.*, 1996). In contrast, the selective removal of lactic acid from the sweat in our study did not affect its attractiveness, indicating that L-lactic acid does not play an important role in the attraction of *An. gambiae* to sweat. Such a limited role had previously been suggested following artificial alkalisation to pH 10, when volatilisation of L-lactic acid is reduced (Braks *et al.*, 1997). The differences between these studies with *Ae. aegypti* and *An. gambiae* indicate that the L-lactic acid is a stronger stimulus for *Ae. aegypti* or that the responses to sweat and skin washings are not based on the same set of volatiles. The latter is likely, considering the different procedures followed for the collection of the odour samples and the differences in attractiveness of the stimuli over time. The skin washings were made by rubbing hands and feet with cotton wool soaked in ethanol which were left to dry for a day at room temperature. The skin washings proved to be attractive for *Ae. aegypti* for more than two months after application on filter paper, at room temperature (Geier, 1995). This suggests that the attraction to skin washings was not based on highly volatile components. In contrast, the sweat in the present study was placed in airtight vials immediately after collection and stored at –20°C when fresh or incubated for two days at 37°C. This procedure prevented the loss of (highly) volatile components either present in the sweat or produced during incubation.

Role of ammonia. To our knowledge, this is the first report showing that *An. gambiae* responds to ammonia. Our investigation of ammonia was initiated mainly after recognising that the pH of the sweat samples increased during incubation (Braks and Takken, 1999). This was probably due to the production of ammonia from urea and amino acids by microorganisms present (Bergeim and Corntbleed, 1943; Müller, 1968). The concept of a rise in pH, observed in the sweat of all volunteers during incubation (see Material and Methods), accompanied by an increase of the ammonia concentration and a decrease of the urea concentration is confirmed in this study. Since more ammonia was formed than could be explained by the reduction of urea, it is likely that other nitrogen-components, such as amino acids, present in sweat (Gitlitz *et al.*, 1974) must have been deaminated. From the results of the experiments with different concentrations of ammonia, we deduct that the behavioural threshold of ammonia must lay between 1.34 and 13.4 µmol/100µl. The quantity of ammonia in the incubated sweat at the source, 2.46 µmol, lays within this transition interval; the ammonia in the fresh sweat stimulus, 0.315 µmol, lays below this interval. The results from tests in which ammonia was released from the gas sampling bags demonstrated a clear response of *An. gambiae* to a low but constant flow, ranging from 0.84-8.4 µmol/min. These odorous air flows of ammonia were even further diluted (~ 100 times) by the main stream of clean air. Considering these responses to ammonia together with the differences in ammonia concentration between fresh and incubated sweat and the low attractiveness of sweat over time, we suggest that ammonia contributes to the differential attractiveness of fresh and incubated sweat. The responses to fresh sweat, which contains only a low quantity of ammonia, indicate that additional components must also play a role in the attraction of *An.*

gambiae to sweat. An electrophysiological study into other potential kairomones detected in sweat is reported elsewhere (Meijerink *et al.*, submitted).

Rudolfs (1922) recognised ammonia together with carbon dioxide as the ultimate decomposing products of the human body and reported positive behavioural responses of *Ae. sollicitans* and *Ae. cantator* to these compounds. Müller (1968) found that ammonia was not attractive for *Ae. aegypti* but that it increased the activity of this mosquito. A similar response was found with trunk sweat that had been incubated for 24 hours. He suggested that the latter attraction was caused by the large quantity of ammonia present in the incubated sweat. Ammonia responses associated with host-location have also been reported from other haematophagous arthropods, e.g. ticks (El-Zaidy, 1958; Haggart and Davis, 1980; Sonenshine *et al.*, 1986) and horse flies (Hribar *et al.*, 1992). In addition, sensitivity to ammonia has been reported for insects such as haematophagous Triatomidae (Taneja and Guerin, 1995, 1997) and body louse (Mumcuolglu *et al.*, 1986) where ammonia functions as an aggregation pheromone derived from the faeces of con-specifics, and for fruit flies (Mazor *et al.*, 1987) where it is an oviposition cue.

Ammonia is a breakdown product in the protein metabolism of animals and is excreted as urea in the urine of all mammals. Low levels of ammonia in plasma are already toxic and therefore normally prevented. Ammonia levels in plasma may increase during heavy exercise (Czarnowski and Górski, 1991). Recent research showed that relatively large quantity of ammonia are lost with sweat during heavy exercise (3.36 ± 2.08 mmol/ 30 min), probably preventing excessive increase of ammonia levels in the blood (Czarnowski and Górski, 1991). At rest, the loss of ammonia through sweating is probably limited as the concentration of ammonia in blood is extremely low (~35 µmol/l, Czarnowski and Górski, 1991) and the sweat rate is low (~0.5 l sweat per 24 hours under basic metabolic conditions, Walter, 1972). Nonetheless, the latter brings about an accumulation of non-volatile sweat components like urea on the skin (Jenkinson, 1980). Urea together with other nitrogen-rich compounds are utilised by skin microorganisms resulting in the emission of ammonia as ultimate breakdown product (Bergeim and Corntbleed, 1943). The emanation rate of ammonia from the skin either of physiological or bacterial origin or both is not known.

The main function of eccrine sweating is to regulate body temperature. This form of thermoregulation is nearly exclusively found in humans and to a lesser extent in anthropoid primates. In ungulates, apocrine glands that are found on their general body surface (Folk and Semken, 1991) fulfil this function of eccrine glands, which they lack. Humans have apocrine glands only in restricted areas as the armpit and groin (Stoddart, 1990). The composition of apocrine sweat differs largely from eccrine sweat; apocrine sweat is a milky viscid excretion containing mainly proteins and reduced sugars and eccrine sweat is a watery solution of mainly lactic acid, electrolytes, urea and ammonia. Urea is found only in small quantities on the skin of cattle (Jenkinson *et al.*, 1974). However, all mammals excrete ammonia as urea in urine and, since many animals cover themselves with their excreta, ammonia is strongly associated with animal odours. Although ammonia is not restricted to human emanations, the level of ammonia emission might be human-specific. Haggart and Davis (1980) suggested that the two types of ammonia sensitive neurons found in ticks encode for different levels of ammonia in the environment, one encoding low ammonia levels as those produced from sweat and the other for encoding high levels of ammonia produced from urine and feces along an animal trail. Recently, electrophysiological studies showed that neurons on the antennae of *An. gambiae* that responded to incubated sweat (Meijerink *et al.*, accepted) also are sensitive to ammonia (Meijerink *et al.*, submitted).

We conclude that ammonia is an important kairomone for host-seeking *An. gambiae*. L-lactic acid does not appear to play an important role in the attraction of *An. gambiae* to sweat.

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SECTION III

PRODUCTION OF KAIROMONES FOR *AN. GAMBIAE*

SKIN MICROFLORA AS PRODUCERS OF KAIROMONES FOR *AN. GAMBIAE*

ABSTRACT - Behavioural responses of the malaria mosquito *Anopheles gambiae s.s.* to volatiles released from pooled and individual sweat samples collected from human volunteers were quantified in a dual-port olfactometer. Both fresh and incubated sweat were attractive to *An. gambiae*, but incubated sweat was significantly more selected when tested against each other. The enhancement of the attractiveness of the sweat following incubation appeared to be due to bacterial growth during incubation. The pH value of a sweat sample did not affect behavioural responses to sweat. The role of specific bacterial groups and substrates in odour-mediated host-seeking behaviour is discussed.

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INTRODUCTION

Night-active blood-feeding mosquitoes locate and identify their vertebrate hosts mostly by odour. Volatiles emanating from the human skin are important orientation cues for the host-seeking *Anopheles gambiae* Giles sensu stricto (henceforth termed *An. gambiae*) which exhibits a high preference for human hosts (Mboera and Takken, 1997; Costantini *et al.*, 1998; Takken and Knols, 1999) above other mammals. Skin emanations predominantly originate from the secretions of skin glands and the action of skin microorganisms upon these secretions (Braks *et al.*, 1999a). Gland secretions can be divided into water-soluble components, mainly from eccrine sweat glands, and fat-soluble products from the sebaceous and apocrine glands. Within the skin microorganisms, two major groups of bacteria are recognised, Gram-positive cocci and diphtheriod-like organisms. Members of the former can be recovered from nearly all body sites with *Staphylococcus epidermis* predominating. At least three genera of diphtheriods are regularly present on the human skin; *Brevibacterium* spp., *Corynebacterium* spp. and *Propionibacterium* spp.. *Brevibacterium* spp. has a limited distribution and appear mainly on the toewebs. *Corynebacterium* spp. is found over the entire skin surface. The *Propionibacterium* spp. is associated with sebum rich areas, especially the face and scalp (Holland, 1993).

Although human sweat has long been suspected to be a kairomone source for *An. gambiae*, evidence was not presented until Braks *et al.* (1997) found attraction to sweat samples. Indications for microbial involvement in the production of kairomones were recently demonstrated by showing that incubation of freshly collected sweat enhances its attractiveness for *An. gambiae* (Braks and Takken, 1999; Meijerink *et al.*, accepted; Chapter 8). Incubation of sweat was accompanied by a distinct change of its pH value, from acidic to alkaline (Braks and Takken, 1999). The pH-shift might have changed the volatilisation of components already present in the sweat and affecting its attractiveness.

The specific questions that are being addressed here are as follows. Is there an effect of pH shift *per se* on the attractiveness of sweat to *An. gambiae* and what is the effect of incubation of sweat in the absence of microorganisms? Which bacteria from the skin are present in sweat and what is their development during incubation? The time course of the concentration of three major sweat constituents, L-lactic acid, urea and ammonia of sweat samples, before and after incubation, is also examined.

MATERIALS AND METHODS

Insects. The *An. gambiae* strain used originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome) and was maintained under standard conditions (27 ± 1°C, 80 ± 5% relative humidity, photo/scotophase of 12L:12 D). Adults were kept in 30-cm cubic gauze-covered cages and had access to 6% (v/v) glucose solution. Females were offered blood from a human arm twice a week for 10 min. Eggs were laid on wet filter paper, emerged in water trays and the larvae were fed on Tetramin fish food. Pupae were collected daily from the trays and were allowed to emerge in the adult cages. In the bioassay 4- to 8-day-old female mosquitoes were used, which had not received a blood meal.

Olfactometer. A dual-port olfactometer, consisting of a Perspex flight chamber (1.60 X 0.66 X 0.43 m) (Chapter 3) was used to study the attractiveness of sweat samples. Charcoal-filtered, humidified (65 ± 5% RH), warm air (27 ± 0.5 °C) was led via a Perspex trapping device, which was linked to two ports (diameter 4 cm, 28 cm apart), into the flight chamber with a speed of 20 cm/ sec. Light (1 lux) was produced by one light bulb (75 W) and was filtered by a piece of yellow cloth hanging 1 m above the flight chamber.

Collection and treatment of sweat samples. To test the effect of changes in pH value and sterilisation on attractiveness, pooled sweat collected from eight volunteers was used (Investigation I). To examine the presence and development of microorganisms and their

effects, sweat samples were collected from three volunteers and analysed separately (Investigation II).

Investigation I: Droplets of sweat were collected from the foreheads of eight Caucasian human volunteers (five males and three females with ages ranging from 25 to 41 years) with sterile glass Pasteur pipettes and put in glass vials (5 ml). From each volunteer 3 ml of sweat was collected. Sweat production was stimulated by physical exercise in a warm humid room (30°C, 90 % relative humidity). Immediately after collection, each sweat sample was divided in two sub-samples of 1.5 ml. One sub-sample was directly stored at -20 °C and the other was incubated under aerobic conditions at 37 °C for two days, after which it was also stored at -20 °C. The pH value of sweat of each volunteer was determined seperately with a micro pH-electrode (Inlab 423, Mettler) and was 5.6 ± 1.0 when fresh and 8.5 ± 0.3 after incubation.

A pooled fresh sweat sample was composed of the sub-samples collected from eight volunteers (1.0 ml per volunteer). Two-third of the pooled fresh sweat was sterilised using a bacterial filter (Millipore, Millex GS, pore size 0.22 µm). Half of the sterilised pooled fresh sweat was directly stored at -20 °C; the other sterile half was incubated under aerobic conditions at 37 °C for two days, after which it was also stored at -20 °C. Subsequently, the pH of the sweat volume was determined. Combining the sub-samples of the different volunteers made a pooled incubated sweat sample. Half of the pooled incubated sweat was also sterilised using a similar bacterial filter, after which it was stored at -20 °C.

The pH value of 0.8 ml of pooled fresh sweat (pH 4.9) was artificially increased by adding small amounts (1-2µl) of a base (total of 13 µl, 0.1 N NaOH) until the value of the pooled incubated sweat (pH 8.4) was reached. Likewise, 0.8 ml of pooled incubated sweat (pH 8.4) was artificially acidified (16 µl, 0,5 % HCL) until the pH value of the pooled fresh sweat (pH 4.9) was reached. During these treatments the pH value of the sweat was constantly monitored with the micro pH-electrode.

Investigation II. Sweat droplets were collected from the foreheads of three Caucasian human volunteers, two males (A and B) and one female (C) with ages of 34, 51 and 28 respectively, as described above. From each volunteer, 3 ml of sweat was collected twice on different days. Immediately after collection, each sweat sample was divided in two sub-samples of 1.5 ml. One sub-sample was directly stored at -20 °C and the other was incubated under aerobic conditions at 37 °C for two days, after which it was also stored at -20 °C. L-lactic acid concentration (Lactic Diagnostic Kit Sigma, NR 735) and urea and ammonia concentration (indophenol method according to Berthelot; Gips and Wibbens-Alberts, 1968) of the sweat were determined.

The presence, identity and growth of microorganisms were indicated by the number of colony forming units (CFU) on selective agar plates which were inoculated with 30 µl of 10 to 10^7 times diluted sweat samples (v/v) (fresh or incubated for one or two days). Three different kinds of selective agar media were used: Aerobic Coryneform Agar with Phosphomycin (ACAP) which allows growth of bacteria commonly found on the skin (predominantly aerobic coryneforms and micrococci) but is inhibitory to staphylococci; Staphylococcal Selective Agar (SSA) which is inhibitory to bacteria other than staphylococci; and Tween Reinforced Clostridial Agar (TRCA) which is selective for isolation of propionibacteria (recipes of media kindly provided by Prof. K.T. Holland, Department of Microbiology, University of Leeds, UK). The SSA and ACAP plates were incubated for two days at 37 °C under aerobic conditions and the TRCA plates were incubated for seven days at 37 °C under anaerobic conditions in jars applied with Anaerocult A. Control plates of each agar medium were inoculated with 30 µl of sterile distilled water and incubated. At the end of the incubation periods the CFU were counted. The growth rates, µ [day^{-1}], of the members of the different bacterial groups were calculated as follows:

$$\mu = \frac{\ln(N_t) - \ln(N_{t-1})}{\ln(2)}$$

where N_t (N_{t-1}) is the number of CFU per ml sweat on day 1 or day 2 (on day 0 or day 1 respectively) of the incubation [CFU/ml].

Behavioural assay. Behavioural experiments were performed during the last four hours of the dark period. In each trial of the different experiments, 50 µl of sweat was applied on a sand-blasted glass slide (5 x 2 cm) which was placed in the left or right Perspex trapping device and, in the opposite trapping device, a glass slide with an equivalent amount of distilled water or another sweat sample was placed as a control treatment. Mosquitoes were released from a container at the downwind end of the flight chamber. During each test 30 mosquitoes had the opportunity to choose between one of the ports for a time period of 15 min. Stimuli were alternated between the right and left trapping devices with each trial. The different tests with pooled sweat were replicated eight times and with individual sweat four times. The specific experiments were as follows.

Investigation I. Four different experiments were done with pooled sweat. In the first experiment the attractiveness of five differently treated pooled sweat samples was determined by testing them each against a control (an equivalent amount of distilled water). The five treatments were *i.* pooled fresh sweat, *ii.* sweat collected, incubated for two days and subsequently pooled, *iii.* sterilised pooled fresh sweat, *iv.* sweat collected, pooled, sterilised and subsequently incubated and *v.* sweat collected, incubated, pooled and subsequently sterilised. In the second experiment, the preference of *An. gambiae* for either fresh or incubated sweat was determined by testing them against each other. In the third experiment the role of bacteria in the production of kairomones was investigated by testing sweat that had been sterilised when fresh and subsequently incubated against non-sterilised fresh sweat. To check for a possible negative effect of the procedure *per se* on the attractiveness of sweat, sterilised fresh sweat was also tested against non-sterilised fresh sweat. In the fourth experiment the effect of the artificial modification of the pH value of sweat on the response of the mosquitoes was investigated by testing alkalinised fresh sweat and acidified incubated sweat against their origins.

Investigation II. In two separate experiments the preference of the mosquitoes for either fresh or incubated sweat was tested. In each experiment six trials were performed testing one of the two collections (fresh against incubated sweat) of each volunteer twice.

Data analysis. In the first investigation (Exp. #1-4), the total number of mosquitoes caught in the treatment-trapping device in eight replicates was compared with the total number in the control trapping device using Chi-squared tests. In the second investigation (Exp #5-6) the total number of mosquitoes caught in the trapping device with incubated sweat in four replicates was compared with the total number in the trapping device with fresh sweat (Chi-squared tests).

RESULTS

Investigation I. Significantly more mosquitoes ($P< 0.001$) entered trapping devices baited with the differently treated pooled sweat samples than the control (Table 9.1, Exp. #1). When tested against each other, more mosquitoes responded to incubated than to fresh sweat ($P<< 0.001$) (Table 9.1, Exp. #2). Significantly fewer mosquitoes responded to sweat that had been sterilised and subsequently incubated than to fresh sweat ($P=0.046$) and no difference was found between the numbers responding to sterilised fresh sweat and fresh sweat ($P= 0.577$) (Table 9.1, Exp. #3). When tested against each other, the response to fresh sweat of which the pH was artificially modified to 8.4 did not differ from that to untreated fresh sweat ($P=0.816$). Similarly, the pH-modified incubated sweat was not different in attractiveness from the non-modified incubated sweat ($P= 0.108$) (Table 9.1, Exp. #4).

The pH value of the incubated sweat in the absence of bacteria (sweat collected, pooled, sterilised and subsequently incubated) was acidic (pH 5.0).

TABLE 9.1. Results of behavioural experiments testing the effect of pH value and sterilisation on the attractiveness of An. gambiae *to sweat (N=8).*

Exp	Stimuli		Response [a]		Chi-	N [c]		
	Treatment	Control	Treat.	Control	square	+	-	n
1	Fresh sweat	Water	86	19	***	8	0	0
	Incubated sweat	Water	108	14	***	8	0	0
	Sterilised fresh sweat	Water	49	16	***	7	0	1
	Incubated sterilised sweat	Water	63	16	***	6	1	1
	Sterilised incubated sweat	Water	105	5	***	8	0	0
2	Incubated sweat	Fresh sweat	63	12	***	7	0	1
3	Incubated sterilised sweat	Fresh sweat	12	24	*	1	4	3
	Sterilised fresh sweat	Fresh sweat	13	16	ns	3	3	2
4	Alkalised fresh sweat	Fresh sweat	36	38	ns	3	3	2
	Acidified incubated sweat	Incubated sweat	36	51	ns	2	4	2

[a] *The response is the total number of mosquitoes caught in either the test or control trapping device.*

[b] *Significant differences (*: P< 0.05, **: P< 0.01 or ***: P< 0.001) or no significant differences (ns: P≥0.05) found between the total number of mosquitoes caught in the test and control strapping device (Chi-squared test)*

[c] *N= number of replicates. The distribution of the preferences for either the test (T), the control (C) or no preference (=) in the individual replicates are also shown.*

[d] *fresh sweat = pooled fresh sweat; incubated sweat = sweat collected, incubated for two days and subsequently pooled; sterilised fresh sweat = sterilised pooled fresh sweat; incubated sterilised sweat = sweat collected, pooled, sterilised and subsequently incubated; sterilised incubated sweat = sweat collected, incubated, pooled and subsequently sterilised.*

Investigation II. On all occasions, viable cell counts (CFU) were formed on the three different selective agar media inoculated with a dilution of defined volumes of fresh sweat or sweat incubated at 37°C for one and two days (Table 9.2). The results indicated that both aerobe coryneforms and staphylococci grew within the first day of incubation (from day 0 to day 1) but not on the second incubation day (from day 1 to day 2) for the replicate experiments. However this was not the case for propionibacteria, where only one of the samples supported growth and the mean of the data indicated no growth. Data on the growth rate of CFU on ACAP and SSA-agar media pointed at a distinct growth on the first day of incubation (from day 0 to day 1) but not on the second incubation day (from day 1 to day 2). No mean growth was found in the CFU on TRCA, neither during the first nor during the second day of the incubation (Fig. 9.1).

In all but one behavioural experiment significantly more mosquitoes were caught in the trapping devices baited with incubated sweat than in the devices with fresh sweat ($P< 0.05$). Although the results from tests with the fresh and incubated sweat of the first collection of volunteer A showed a similar trend, the difference was not significant ($P= 0.057$) (Table 9.3).

The time course of the pH value and concentration of L-lactic acid, urea and ammonia showed similar trends in each of the sweat collections (Table 9.4). The pH value changed from either slightly alkaline (C) or acidic (A and B) to alkaline pH (>8) following incubation of

two days at 37 °C. After the incubation period, the L-lactic acid and urea concentration of the sweat decreased to, on average, 82 ± 9 % and 69 ± 11 % of the concentration in fresh sweat respectively. In contrast, the ammonia concentration of all sweat samples increased, on average, more than 18.5 ± 13 times in the same period.

TABLE 9.2. Natural logarithm of the viable cell count (CFU) per ml sweat collected from three volunteers (A, B and C) on two different occasions (I and II).

Agar media[a]	Sweat sample					
	A					
	Collection I			Collection II		
	Day 0	Day 1	Day 2	Day 0	Day 1	Day 2
ACAP	6.72	14.76	10.06	5.45	8.29	12.02
SSA	8.64	15.47	15.71	7.29	16.27	17.83
TRCA	11.44	8.11	8.11	11.69	9.03	3.51
	B					
	Collection I			Collection II		
	Day 0	Day 1	Day 2	Day 0	Day 1	Day 2
ACAP	9.44	9.32	8.85	9.68	13.83	12.72
SSA	13.98	22.31	15.46	14.92	14.60	15.54
TRCA	10.19	9.57	8.81	13.60	13.63	16.48
	C					
	Collection I			Collection II		
	Day 0	Day 1	Day 2	Day 0	Day 1	Day 2
ACAP	11.70	16.36	14.51	13.82	16.36	17.50
SSA	13.66	18.51	15.62	13.94	19.22	17.98
TRCA	9.24	17.73	20.50	13.11	14.47	16.12

[a] *ACAP= Aerobic Coryneforms Agar with Phosphomycine; SSA= Staphylococcal Selective Agar; TRCA= Tween Reinforced Clostridial Agar*

TABLE 9.3. Results of behavioural experiments testing the preference of An. gambiae *for fresh or incubated sweat collected from three volunteers (A, B and C) on two different occasions (I and II).*

Experiment	Stimuli		Response[a]		Chi square[b]	N[c]		
	Incubated	Fresh	Incubated	Fresh	*P*	I	F	=
5	A I	A I	26	14	ns	4	0	0
6	A II	A II	38	13	***	4	0	0
5	B I	B I	30	11	**	4	0	0
6	B II	B II	20	8	*	4	0	0
5	C I	C I	27	13	*	4	0	0
6	C II	C II	34	7	***	4	0	0

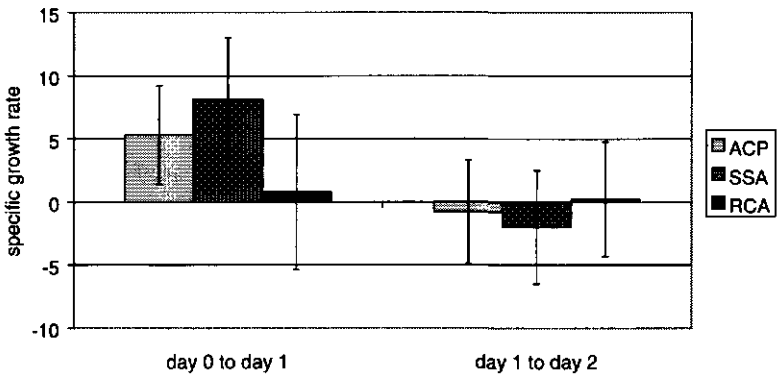
[a] *The response is the total number of mosquitoes caught in either the trapping device baited with incubated or fresh sweat.*
[b] *See legend under Table 9.1.*
[c] *N= number of replicates. The distribution of the preferences for either incubated sweat (I), fresh sweat (F) or no preference (=) in the individual replicates are also shown.*

TABLE 9.4. pH value and concentration of L-lactic acid, urea and ammonia of fresh and incubated sweat collected from three volunteers (A, B and C) on two different occasions (I and II).

		pH		L-lactic acid (mM)		Urea (mM)		Ammonia (mM)	
Volunt.	Coll	Day 0	Day 2	Day 0	Day 2	Day 0	Day 2	Day 0	Day 2
A	I	5.2	8.8	35.5	31.0	34.7	21.1	9.4	62.7
A	II	4.9	8.7	48.8	42.1	46.4	27.0	12.7	92.6
B	I	5.5	8.9	37.7	30.0	40.2	24.2	4.3	62.9
B	II	5.2	8.8	51.0	46.6	59.4	44.9	0.8	32.8
C	I	7.4	8.6	35.5	28.8	25.9	22.1	0.7	17.4
C	II	7.6	8.3	25.5	16.6	22.9	16.8	2.7	45.2
r ± std [a]				0.82± 0.09		0.69± 0.11		18.53± 12.88	

[a] $r=\Sigma(mM\ compound_{incubated}/\ mM\ compound_{fresh})/n$

r < 1: reduction after incubation



r > 1: rise during incubation,

FIGURE 9.1. Mean growth rates of the different bacterial groups. Error bars represent standard deviation.

DISCUSSION

Effect of incubation, sterilisation and pH value on the attractiveness of sweat. All pooled sweat samples were more attractive than water and, therefore, contained components attractive for *An. gambiae*. The responses to incubated sweat, however, seemed to be stronger than to fresh sweat. Statistical support for this observation was demonstrated in the second and third experiment: mosquitoes selected the incubated sweat significantly more than fresh sweat, as reported earlier (Chapters 7 and 8; Meijerink *et al.*, accepted). It was also found that the incubation of sweat in the absence of bacteria did not enhance its attractiveness, but even diminished it: sterilised fresh sweat was preferred to sweat that was collected, pooled, sterilised and subsequently incubated. The decrease in attraction might be caused by processes like oxidation or evaporation of volatiles during the incubation procedure. The incubation of sweat, after removal of bacteria, was not accompanied by a shift in pH value, which suggests that the shift in pH value of untreated sweat was the result of bacterial action upon sweat components. Artificial modification of the pH of fresh and

incubated sweat did not affect the attractiveness of sweat (Table 9.1, Exp. #4). In an earlier report (Chapter 5) alkalisation of sweat (pH 7-8) to pH 8-9 did not affect the attractiveness of human sweat, while acidification to pH 1.5 caused a slightly repellent response. The latter was probably caused by the release of a high concentration of acidic compounds through the rather strong acidification (Braks *et al.*, 1997) as also reported for Aedes aegypti by Thompson and Brown (1955). In contrast Müller (1968) found that attractive sweat from a human arm lost its attractiveness for *Ae. aegypti* after alkalisation and explained this finding by the loss of lactic acid in the headspace. Lactic acid, however, appears to have a limited role in the attraction of *An. gambiae* to sweat (Chapter 8). We conclude that the enhancement of the attractiveness of sweat after incubation is due to microbial activity rather than to other chemical or physical processes occurring during incubation.

Presence and development of microorganisms and the attractiveness of sweat. All fresh sweat samples collected by the three different volunteers contained bacteria from all three bacterial groups. The absolute number and development of organisms of the three main bacterial groups differed largely between days of incubation, sweat collections and volunteers (Table 9.2). The growth rate, however, showed a distinct growth of the coryneform and staphylococcal species during the first day of incubation. No mean growth was found for these two bacterial groups on the second day of incubation and no significant growth at all was found for the anaerobe diphtheroid bacteria. The latter result was expected, as these predominantly lipophilic organisms would not flourish under lipid-poor environment; thermally induced sweat is stated to consist principally of an aqueous eccrine excretion, which does not contain lipids (Noble and Somerville, 1974). In addition, growth of anaerobe microbes was also not expected as the sweat was incubated under aerobic conditions. However, contrary to our expectation, a distinct growth of diphtheroid bacteria was seen in some of the sweat samples. This indicates either that the availability of lipids was not limited or that an anaerobic microclimate is present in sweat or that the growing bacteria were not strictly anaerobe. In another study, Meijerink *et al.* (accepted) showed that the headspace of thermally induced sweat does, indeed, contains components derived from the sebum, next to eccrine components. Growth of microorganisms in human sweat samples during incubation was already suggested previously, although not shown by Bergeim and Corntbleed (1943). From the data of this first effort to examine the bacterial population using a rather crude categorisation into three common bacterial groups, we were able to show that bacteria belonging to a wide range of groups are present in fresh sweat and that general bacterial growth occurs during incubation.

Data from the behavioural assay with sweat from three volunteers showed that all incubated sweat samples induced similar responses of the mosquitoes when tested against fresh sweat: incubated sweat was preferred to fresh sweat. These results confirm the outcome of the experiments with pooled sweat (see above). Considering this, together with the observed variation in bacterial populations and proliferation, we suggest that the enhancement of the attractiveness of sweat results from a-specific bacterial growth producing attractive volatiles for *An. gambiae.* However, the possibility that the attraction might have been caused by the action of a single bacteria species while the effect of other bacterial development is negligible cannot be ruled out. The identity of the volatile component(s) responsible for the attraction is not known. From our chemical analysis of the sweat volumes we consider ammonia as one of the potential candidates as it is nearly absent in sweat when fresh and the concentration has largely increased after incubation. Behavioural and electrophysiological responses to ammonia and other sweat components are reported elsewhere (Chapter 8; Meijerink *et al.*, submitted). L-lactic acid and urea decrease in all sweat samples after incubation independent of specific bacterial development.

General discussion. We conclude that, through the action of bacteria, volatile components, which are attractive for *An. gambiae,* are released from the sweat. To our knowledge, only Schreck and James (1968) investigated the role of bacterial volatiles in host-seeking

behaviour of mosquitoes and found behavioural preference of *Ae. aegypti* for air led through broth cultures of transient skin bacteria, *Bacillus cereus*, compared to control air. At first sight the attraction to the action of bacterial species not belonging to the resident skin microflora was peculiar since this could never have been responsible for the attractiveness of the human skin. However, considering our data, we suggest that the production of volatiles might be a general process rather than a process specific to a limited range of bacterial species. Many odours emanating from the mammalian skin or its specialised scent glands are thought to be produced by microbial activity (Albone *et al.*, 1977). Other examples of insects responding to bacterial-produced volatiles emanating from the mammalian skin have been reported (for a review see Braks *et al.*, 1999a). Species-specific odours emanating from mammals are probably the result of the substrate and the action of a range of skin bacterial species together. Therefore, a skin-borne odour may be a highly available and reliable kairomone for host-specific mosquitoes that rely on olfactory cues rather than visual cues for the localisation of a blood meal.

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10

HUMAN SKIN MICROFLORA AND THE MOSQUITO-HOST-PARASITE INTERACTION

ABSTRACT - The interaction between the African malaria vector *Anopheles gambiae* and its human host has traditionally been viewed within a bitrophic context considering the human and the mosquito. Recently, the influence of the *Plasmodium* parasite on the interaction has been recognised as it affects the physiology and/or behaviour of humans and mosquitoes. However, studies on odour-mediated host-seeking behaviour of *An. gambiae* and other Diptera have provided evidence that a fourth group of organisms should be taken into consideration. Human skin microflora play a role in the production of odorous compounds that may function as kairomones for mosquitoes. Here, the role of human microflora is introduced into the process of odour-mediated host selection and the interaction in a multi-partite context is reviewed to identify research avenues that will enhance our limited knowledge of this aspect of malaria transmission

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INTRODUCTION

Every two or three days, the female malaria mosquito, *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae), seeks a human host from which it obtains a blood meal for egg development. While host seeking, it exploits olfactory information derived from volatiles emanating from its human host (Takken and Knols, 1999). The identification of individual kairomones is hindered by the complexity of whole human odour, some 300-400 compounds have been described (Bernier *et al.*, 1999). Carbon dioxide (CO_2), a major constituent of exhaled breath and a kairomone for many mosquito species, appears to be of minor importance for host-recognition by *An. gambiae* (Mboera and Takken, 1997). In a field study in Tanzania, Mboera *et al.* (1997) found that tents baited with CO_2 caught only 9% of the number of mosquitoes caught by a tent baited with whole human odour. Considering the highly anthropophilic behaviour of this species, it is not surprising that CO_2 plays only a limited role as it is not a species-specific compound, but merely reveals the presence of a potential host to the mosquito. Other, more human-specific volatiles that originate from human skin are believed to serve as reliable kairomones for *An. gambiae*. Recently, in the laboratory, Braks *et al.* (1997) demonstrated behavioural preferences of this mosquito towards human sweat. There is evidence that individual humans are differentially attractive to mosquitoes and that some of this variation can be explained by the composition of their odour profile (Knols *et al.*, 1995; Brady *et al.*, 1997). Furthermore, field studies have shown that humans may receive more landings and bites from certain mosquito species than from others (Knols *et al.*, 1995), which supports the hypothesis that specific olfactory signals are used for host-selection.

The volatiles emanating from the skin originate from either the secretions of skin glands or the skin microflora or both. Gland secretions can be divided into water-soluble products, mainly from eccrine sweat glands, and fat-soluble products from the sebaceous and apocrine glands. Lactic acid is the only major volatile component of eccrine secretions. Despite its low volatility, L (+)-lactic acid has been shown to play an important role in the host-seeking behaviour of *Aedes aegypti* (Diptera: Culicidae) (Geier *et al.*, 1996). Sebum, an oily secretion of the sebaceous glands, contains free long-chain fatty acids with low volatility that contribute partially to the human odour. More important are the microbial breakdown products of these different gland secretions, which are responsible for the production of a distinctive human olfactory signature. Direct microbial involvement in the production of kairomones was recently demonstrated by showing that incubation of sweat enhances its attractiveness (Braks and Takken, 1999; Chapter 9). Understanding the role and nature of skin microorganisms in the production of 'human' odours may facilitate kairomone identification and development of traps (Vale, 1993) or anti-mosquito products (repellents). It may also contribute to understanding patterns in mosquito responses to different humans.

SEMIOCHEMICALS OF MICROBIAL ORIGIN

Various examples of bacterial involvement in arthropod-vertebrate interactions via the production of olfactory products are known. The adult female of the New World screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae), feeds and oviposits on animal wounds and is an important agent of myiasis; olfactometer tests showed that these flies can orient to volatiles produced by a bacterium, *Providencia rettgeri*, present in the wounds associated with myiasis (Hammack *et al.*, 1987). The sheep blow fly, *Lucilia cuprina* (Diptera: Calliphoridae), moves and probes in response to volatile kairomones produced by the bacterium *Pseudomonas aeruginosa*, the causative agent of dermatitis associated with fleece rot lesions (Morris *et al.*, 1997). *Hydrotaea irritans* (Diptera: Muscidae) feeds on protein-rich secretions found on the facial surface, belly and teats of its host and is the primary vector of bacteria that cause summer mastitis of cattle (Chirico *et al.*, 1997). Volatiles associated with mastitis secretions, which are produced by *Peptococcus indolicus* (Thomas *et al.*, 1985), increase the probability of the fly finding the infected feeding site. In tsetse fly

(Diptera: Glossinidae) control, good performance of traps baited with buffalo urine appeared to be due to the gradual production of phenolic compounds by the bacterium *Aerococcus viridans* (Okech and Hassanali, 1990). The haematophagous blackfly, *Simulium euryadminiculum* (Diptera: Simuliidae), and the tick, *Ixodes (Afrixodes) matopi* (Acarina: Ixodidae), respond to the odour of scent organs of vertebrates (Fallis and Smith, 1964; Spickett *et al.,* 1981), which are cutaneous structures that enhance the storage, modification and release of volatile chemicals mainly produced by microbes. Microbial decomposition of nitrogen-containing compounds such as urea and amino acids give rise to the highly volatile component ammonia to which many haematophagous arthropods respond (Chapter 8; Taneja and Guerin, 1997)

These examples clearly demonstrate that microorganisms, residing on vertebrate skin, play an important role in the production of 'host' volatiles that mediate behavioural responses between arthropods and their vertebrate hosts.

HUMAN SKIN MICROFLORA

Human skin is inhospitable to most microorganisms; thus only limited species can survive. The composition and growth of skin microflora depend on skin temperature, humidity, pH, the concentration of inhibitors, and availability of nutrients (Holland, 1993). Nutrients are derived mainly from eccrine and sebaceous gland secretions. The distribution of skin microbes is determined by species-specific nutrient demands as well as the presence of cutaneous glands and by physical characteristics of skin sites. All these factors may give rise to large intra and inter-individual differences in composition of the residential skin microflora (Korting *et al.,* 1988). Human skin microflora can be divided roughly into three groups: Gram-positive cocci, Diphtheroid-like organisms, and fungi (see Chapter 1, Table 1.1). Gram-positive cocci can be recovered from nearly all body sites, with *Staphylococcus epidermis* predominant. At least three genera of diphtheroids are common. *Brevibacterium* spp. have a limited distribution and appear mainly on the toe webs. *Corynebacterium* spp are found over the entire skin surface and, together with various micrococcal species, in high densities in the armpit. The anaerobic *Propionibacterium* spp. together with the fungus *Malassezia furfur* are abundant in the areas with high densities of sebaceous glands such as the face and scalp (Noble, 1993). *Propionibacterium* spp, *Malassezia furfur* and to a lesser extent *Corynebacterium* spp. are lipophilic (Korting *et al.,* 1988).

Differential attractiveness of humans to *An. gambiae* has frequently been reported. Adults receive more bites than children do and there is a direct relationship between the size of the host and number of bites (Knols *et al.*, 1995; Brady *et al.,* 1997). However, as the selection of biting sites by *An. gambiae* is influenced by body odours in the proximity of the host (De Jong and Knols, 1995a), this preference for biting adults may also be based on olfactory signals. Ontogenetic development of skin glands and other skin features give rise to age-related differences in the skin microflora and, therefore, possibly also in odour profile. As *Plasmodium* gametocyte prevalence appears to be higher in children than in adults (Githeko *et al.*, 1994), mosquitoes could reduce the risk of acquiring an infection by responding to kairomones produced by adults. Such 'avoidance' behaviour may actually increase a female's fitness, as *Plasmodium* infections generally affect this in a negative manner (Hurd *et al.,* 1995).

VOLATILES FROM SKIN MICROFLORA

Complete oxidation of nutrients by skin microflora would result primarily in the production of water and CO_2. In general, however, incomplete oxidation produces other, small, volatile breakdown and excretory molecules. The origin of these volatiles is known only in limited cases. Humans are unique in having a high level of triglycerides (Stoddart, 1990), which are broken down principally by *Propionibacterium* spp and which give rise to a large number of

both long-chain and short-chain free fatty acids (Noble, 1993). *Corynebacterium* spp are responsible for the modification of the initially odourless apocrine secretions, including androsterone sulphate and dehydroepiandosterone, into the typical axillary smell of 5-androst-16-en-3-one and short chain fatty acids (Gower *et al.*, 1994). *Brevibacterium epidermis* is responsible for production of methanethiol and iso-valeric acid, components of pungent foot odour (Marshall *et al.*, 1988) and *An. gambiae* responds positively to volatiles produced by a related species *Brevibacterium linens* (De Jong and Knols, 1995b). Limburger cheese, the odour of which is similar to that produced by feet, obtains its flavour by microbial action of *B. linens* and raises by 2-3 times the number of mosquitoes in olfactometer traps. Similar responses were also found to the acid fraction of this cheese and an artificial mixture of short-chain fatty acids occurring in the cheese and foot odour (Knols *et al.*, 1997). Additionally, significant electrophysiological responses of *An. gambiae s.s.* were observed towards short-chain fatty acids (Meijerink and Van Loon, 1999). Similarly *Ae. aegypti* was attracted by air led through broth cultures of transient skin bacteria, *Bacillus cereus* (Schreck and James, 1968)

PLASMODIUM AND VECTOR-HOST INTERACTIONS

The transmission of parasites by haematophagous vectors depends largely on the behaviour and physiology of the vector and vertebrate host. Therefore, parasites that modify important features of their arthropod vectors and vertebrate hosts may be at a selective advantage relative to those strains of parasites that do not. Aspects of the feeding behaviour of the vector, including host preference, probing time, biting persistence and rate, have a large effect on parasite transmission. Although, there are many published examples of behavioural and physiological changes in animal behaviour induced by parasites (Moore, 1993), examples within the *Plasmodium-An. gambiae*-human interaction are only starting to be documented (Koella and Packer, 1996; Koella *et al.*, 1998). In the following section, we review some studies of similar interactions illustrating possible parasite-induced effects in the former, with emphasis on odour-mediated host selection.

Host selection by mosquitoes is mediated at the proximal level by receptors sensitive to general and/or specific host cues, thus it is conceivable that parasites may modify the vectors' receptor sensitivity, specificity or both to alter the nature of the mosquito response. Conversely, parasite infection may change the nature of volatiles produced by the vertebrate to increase its attractiveness to host-seeking arthropods (P. Taylor, *pers. commun.*). Body odour has been shown to be indicative of many diseases, including the mosquito-borne yellow fever (Lidell, 1976). Penn and Potts (1998) suggest that parasitic infections can change an individual's odour by changing the composition of residential skin microbes and by affecting the immunological and endocrine system. In many vertebrates, parasitic infections induce an increased body temperature and thus increased respiration and CO_2 output. *Culex* mosquitoes prefer to bite lambs infected with Rift Valley Fever virus compared with uninfected controls, probably as a result of this mechanism. The sandfly *Lutzomyia longipalpis* (Diptera: Phlebotominae) shows selective feeding on lesions of *Leishmania*-infected mice, thus enhancing transmission (Coleman and Edman, 1988). Parasite effects on olfaction of vectors have often been proposed (Moore, 1993), but only observed in *Ae. sierrensis* infected with a ciliophoran parasite, which does not infect vertebrates (Egerter and Anderson, 1985). In that study, infected mosquitoes were less responsive to human odour than uninfected mosquitoes. It is possible that decreased probability of blood-feeding-associated mortality enhances survival of the mosquito host and consequently of the parasite. Another example of parasitic modification of the behaviour of a haematophagous vector has been observed for lizard malaria, *Plasmodium mexicanum*, transmitted by the sandfly *Lutzomyia* spp. (Diptera: Phlebotominae). Infected sandflies prefer higher environmental temperatures than uninfected flies. This change in temperature preference may represent an adaptive manipulation of the sandfly by *P. mexicanum* to increase the

chance that the parasite completes development before the vector takes its last blood meal (Schall, 1996).

Once a vertebrate blood source has been located, parasites may still exert an effect on the feeding behaviour of the vector to enhance transmission. The outcome of the interaction between mosquito and vertebrate host is determined by the efficiency of blood location and uptake by the mosquito and the degree of behavioural tolerance of the host to mosquito attack. Decreased probing efficiency due to malaria infection is associated with the presence of sporozoites in the salivary glands and a concomitant reduction in salivary apyrase activity in *Ae. aegypti* (Rossignol *et al.*, 1984) *Plasmodium falciparum*-infected *An. gambiae* show a correlated reduction in blood location efficiency although the levels of apyrase for *Anopheles* mosquitoes naturally infected with appropriate species of *Plasmodium* have not been determined (Wekesa *et al.*, 1992). Ultimately, this pathology increases the number of partial blood meals a mosquito must take in order to be fed fully (Moore, 1993). *Aedes* mosquitoes infected with La Cross virus tend to probe more and engorge less than uninfected mosquitoes. Other examples of similar effects are abound (Moore, 1993).

Defensive behaviour of vertebrates, a major determinant of mosquito feeding success, may be reduced in ill hosts, either because they are less perceptive to nuisance caused by blood-feeding arthropods or because they are incapable of responding (Moore, 1993). Day *et al.* (1983) showed that *Ae. aegypti* engorged almost exclusively on mice infected with malaria parasites when the gametocytes were most infective, but this laboratory model was based on a highly artificial system; in fact natural rodent malaria infections tend to be chronic and do not induce clinical illness in thicket rats. This preference was apparently due to a decrease in defensive behaviour (Landau and Chabaud, 1994). In another study, although no difference in attraction of tsetse flies to uninfected cattle and cattle infected with *Trypanosoma congolense* was observed, feeding success on infected cattle was 75% greater than on uninfected animals (Baylis and Nambiro, 1993). It is difficult to verify this phenomenon for human hosts of malaria because clinical episodes often do not coincide with the presence of gametocytes in the peripheral bloodstream. However, metabolic effects that are relevant to mosquito choice or close attraction may manifest several days later when gametocytes are present. No reports on the relative attractiveness of infected or non-infected humans to malaria mosquitoes are available. In addition to reduced defensive capacity of the vertebrate, a *Trypanosoma* spp. infection induces vasodilatation and reduces the haemostasis of the blood so as to increase feeding efficiency of the tsetse fly. A similar effect on feeding efficiency has been postulated based on the change in blood hematocrit associated with malaria infection in vertebrates (Kingsolver, 1987).

Parasite effects on interactions between haematophagous vectors and their vertebrate hosts appear to be a general feature of these interactions rather than a peculiarity. Additional research is required to fill gaps in our knowledge of parasite-associated changes in the mosquito-human interaction.

CONCLUDING REMARKS

By reviewing the mosquito-host interaction in a broad context we have shown that this may affect our current understanding of malaria transmission. This and other arthropod/vertebrate relationships have traditionally been considered as bitrophic interactions, but are now found to be more complex. A multidisciplinary approach that integrates parasitology, medical entomology and skin microbiology may thus significantly enhance our understanding of these complex biological phenomena. More specifically, the mosquitoes that transmit human malaria and their human hosts may be significantly affected by other organisms. Human skin microflora may affect the attractiveness of human hosts for mosquitoes and consequently, the strength of the mosquito-host interaction. Evidence is mounting that malaria parasites play a strong role in regulating the feeding behaviour of their anopheline hosts (Koella *et al.*, 1998) with the consequence that transmission, a prerequisite for parasite reproduction, is enhanced. Perhaps it is time to begin to look at the responsiveness of infected and

uninfected anopheline mosquitoes to volatiles produced by skin microflora to see if this aspect of the interaction between mosquitoes and man can also be exploited by malaria parasites. The introduction of non-random host choice into existing epidemiological models has already been shown to have important quantitative and qualitative effects (Kingsolver, 1987). Investigations into the factors underlying the intra- and interspecific differences in host choice are necessary for a better understanding of the micro-epidemiology of malaria. The identification of volatiles responsible for host attraction and their microbial origin may lead to the development of new methods to interrupt host-vector contact. A more rational approach to repellent development will be possible once kairomones and the producing bacteria have been identified. Such kairomones may also be used in conjunction with traps to monitor mosquito populations. The co-evolution between the four groups of organisms involved in malaria transmission, their web of interactions and reproductive strategies should form the basis for future studies on malaria transmission, and may reveal a wealth of insight into the intricate life-cycle of this disease.

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11

GENERAL DISCUSSION AND CONCLUSIONS

In this thesis the results of behavioural investigations into the source, identity and production of kairomones in the odour-mediated host-seeking behaviour of the Afrotropical malaria mosquito *Anopheles gambiae* Giles *sensu stricto* are reported. To colleagues, the most remarkable conclusion drawn from this thesis is probably the fact that I did not study the effect of carbon dioxide on *An. gambiae* in the laboratory. This while carbon dioxide, a volatile compound emitted with exhaled air of all vertebrates, is considered a general kairomone in the host-location behaviour of most haematophagous insects. As for many other mosquito species, responses to carbon dioxide have been reported for *An. gambiae* (Costantini *et al.*, 1996; Knols, 1996; Mboera, 1999). However, the role of carbon dioxide in the host-seeking behaviour of *An. gambiae* appears to be rather limited in comparison to body odours (Chapter 2; Knols, 1996; Mboera and Takken 1997). In the field, traditionally, the role of carbon dioxide is determined by comparing the number of mosquitoes caught in traps baited with carbon dioxide with those baited with the complete host odour. From the ratio of the catches the relative contribution of carbon dioxide to the total attraction is determined (Costantini *et al.*, 1996; Mboera *et al.*, 1997; Mboera and Takken, 1999). However, this approach should be questioned as maximal attraction is not a simple summation of the partial attraction of the individual components as is illustrated in the following two examples. First, *Ae. aegypti* is highly attracted to ethanol washings made from the skin. The attraction to the skin washing is lost after selective removal of lactic acid, while this mosquito is only slightly attracted to lactic acid alone (Geier, 1995). Such synergistic action of lactic acid hampers the calculation of a relative contribution of this component to the total attraction. Second, in my field work, *An. gambiae* was equally attracted to electric nets baited with human body odour in the presence and absence of breath and consequently of carbon dioxide (Chapter 2). This while in another experiment run simultaneously and only 2 km away, electric nets baited with carbon dioxide attracted significantly more mosquitoes than the unbaited nets (Mboera, 1999). Thus, for another reason than synergism, the relative contribution of an attractive component, here carbon dioxide, cannot be determined easily. Nevertheless, from the field study we learnt that it was possible to attract *An. gambiae* in the absence of carbon dioxide. For a long time carbon dioxide was considered to be an essential element of an odour-bait in the field. Since the application of carbon dioxide in the field is rather laborious, this result was very promising for the improvement of odour-baited traps with other volatiles. For this reason, it was decided to focus on the source, identification and production of kairomones emanating from the human skin.

The most substantial achievement of the present investigation is the entrapment of 'a human in a bottle'. In other words, the 'development' of an olfactory stimulus that is not artificial but rather true to nature (*a*), is inducing stable distinct behavioural and electrophysiological responses of *An. gambiae* despite storage (*b*), and can be subjected to chemical analyses (*c*). Sweat samples, collected from human volunteers and subsequently incubated, fulfilled all these conditions essential for understanding the source and identity of mosquito kairomones and even their production.

SOURCE OF KAIROMONES FOR *AN. GAMBIAE*

Evidence was provided that the human skin is the major source of important olfactory cues for host-seeking *An. gambiae* (Chapter 2) and that sweat collected from the skin contains at

least some attractive volatiles (Chapter 4). As reported earlier, sweat has long been suspected as a source of kairomones for *An. gambiae*, however, conclusive evidence was not provided until now. The sweat samples used in this investigation consisted principally of secretions of the eccrine sweat glands, but not exclusively. As sweat was collected as droplets from the skin of human volunteers and not directly from the eccrine glands, the presence of substances from the epidermis and the sebaceous glands could not be excluded. In addition, sebum was to be expected to occur in the samples as the collection site, the human forehead, holds high densities of sebaceous glands. Unfortunately, time was lacking to quantify the lipid-content of the sweat samples. Nevertheless, chemical analysis of headspace of the sweat samples showed the presence of volatiles originating from the sebum, like fatty acids and fatty acid ethyl esters acids (Cork and Park, 1996; Meijerink *et al.*, accepted). In comparison with other mammals, the human skin surface contains high levels of both long-chain and short-chain fatty acids (Chapter 1; Nicolaides, 1974). Over 200 acids acids have been described and are believed to contribute to our distinct olfactory signature (Stoddart, 1990). Especially, the 'sweaty' odour sensed by humans is ascribed to short chain fatty acids such as isovaleric acid (Lukacs *et al.*, 1991). Knols and colleagues (1997) demonstrated attraction of *An. gambiae* to Limburger cheese and to a natural and synthetic acid extract of this cheese. For the first time attraction was established to a limited number of identified volatiles other than carbon dioxide. Conceivably, short chain fatty acids play a role in odour-guided host-seeking of *An. gambiae*. As free fatty acids are derived from the sebum, the sebaceous glands should be assigned as the source for kairomones within the skin. However, fatty acids appear not to bring about the attraction to sweat (Chapter 4) and therefore other chemicals attractive to *An. gambiae* must be present in sweat, comprising eccrine and sebaceous secretions.

All sweat tested in this study originated from the forehead of human volunteers. This body site was initially chosen for the ease of the collections; the forehead holds higher densities of both eccrine and sebaceous glands compared to other body sites. Attraction to skin emanations from other body parts *in vivo* has certainly been reported in many behavioural studies including the present one (Takken and Knols, 1999). *Cx quinquefasciatus* is equally attracted to ethanol washings of feet and hands as to such washing of the back of volunteers. Due to lack of time, the skin washings of the back have not been tested for *An. gambiae*. The feet of humans are reported as a source of important volatiles in the orientation of *An. gambiae* (Knols, 1996). This suggestion arose partly from the experiment that mosquitoes preferred to bite on the ankles and feet of human volunteers and by washing of the feet this preference was diverted to a random biting pattern (De Jong and Knols, 1995a). However, a human volunteer laying full length on the floor was bitten randomly over all body parts (Dekker *et al.*, 1998). Putting the feet up showed that the mosquitoes preferred again the lowest part of the human body and nearly no bites were received on the feet. These data suggest that factors other than olfactory cues largely determine the selection of biting sites. I hypothesise that *An. gambiae* responds to human skin emanations, in general, and displays a rather large tolerance to variation in quality and quantity of the composition. Due to species-specific physiology and anatomy the variation of the odour composition of skin emanation within a species is always smaller than between species. For this reason mosquitoes can probably distinguish between possible host species by their species-characteristic odour composition. Conceivably, some compounds are essential and probably always present in skin emanations. The identification and production of these components were the principal aims of this study.

IDENTIFICATION OF KAIROMONES FOR *AN. GAMBIAE*

The present investigation provided indications that volatiles emanating from sweat other than short chain fatty acids (Chapter 4) are potential kairomones in the odour-guided host-seeking behaviour of *An. gambiae*. The results of the field study (Chapter 2) suggest that reliable olfactory host signals for *An. gambiae* comprise highly volatile components that continuously

emanate from the human skin (but evaporate quickly from worn clothes). This concept is strengthened by the results obtained in Chapter 8. First, the fact that the preference of the mosquitoes for the incubated sweat was lost within 20 minutes after exposure in the olfactometer suggests that the components responsible for the preference of *An. gambiae* for incubated sweat to fresh sweat are highly volatile. Second, *An. gambiae* preferred incubated sweat to skin washings. Although incubated sweat and the ethanol washings both are attractive stimuli for *An. gambiae* that originate from the skin, the stimuli are different in composition. Due to the preparation process, skin washings lack highly volatile compounds while such compounds are preserved in incubated sweat (Chapter 6). Maybe for this reason, incubated sweat is a more potent stimulus for *An. gambiae* than skin washings. And third, attraction of *An. gambiae* to a single highly volatile sweat component, ammonia, has been established under laboratory conditions. The latter is one of the most interesting findings of the present study, and is strengthened by electrophysiological responses recorded by Meijerink *et al.* (submitted).

Early in the 20th century, ammonia had been described as a possible kairomone for *Aedes* species (Rudolfs, 1922), but it has been forgotten since then. This is partly due to the fact that ammonia is often absent in headspace analyses as ammonia has already evaporated from the source ahead of analyses or cannot be detected as it is masked by the solution peak of the gaschromatographs. Ammonia is the ultimate product of decomposing proteins or other nitrogen molecules, generally occurring in nature. Consequently, arthropods make use of this compound to find conspecifics, food or hosts (for references see Chapter 8). Recognition of the fact that many arthropods are sensitive to ammonia has actually led to the suggestion that the attraction of *An. gambiae* to Limburger cheese (De Jong and Knols, 1995b; Chapter 3) was possibly based on fatty acids and ammonia emanating from it (M. van Helden, pers. commun.). At that time, behavioural or electrophysiological responses of *An. gambiae* to incubated sweat or ammonia itself had not yet been considered. Ultimately, the present investigation on the role of ammonia was initiated after recognising that the enhancement of the attractiveness of sweat during incubation was accompanied by a distinct increase in the ammonia concentration of sweat by bacterial activity (Chapter 8). The finding of the present study that *An. gambiae* is indeed attracted to ammonia restores interest in the initial suggestion. Although ammonia has never been described in headspace analyses of Limburger cheese, its presence in addition to short chain fatty acids is most likely. The characteristic odour of Limburger cheese is caused by compounds that are the result of bacterial activity on the milk proteins (Engels, 1997; Cogan and Daly, 1987).

Ammonia is a general product of the nitrogen metabolism of the human body and, therefore, is not only present in skin emanations but also in breath, next to urine (Robin *et al.*, 1959). Ammonia concentration of exhaled air ranges from 0.1 to 10 PPM or 0.45-2.36 µmol/min in rest and during exercise respectively. Expired ammonia originates from the plasma by diffusion through the alveolars and mucus of dead space and saliva (>10mmol/l) (Ament *et al.*, 1999). As with breath, the ammonia in sweat increases exponentially, preventing excessive toxic increase of ammonia (from contracting muscles) in the plasma during exercise (Czarnowski and Górski, 1991). Although ammonia present in skin emanations originates also from the plasma, it predominantly arises from the hydrolysis of sweat urea by bacteria (Huizinga *et al.*, 1994). Unfortunately, the amount of ammonia that actually emanates from the human skin has never been quantified. In a laboratory set up, the threshold for the attraction of *An. gambiae* to ammonia lays between 1-10 PPM in sampling bags (0.08-0.8 µmol NH_3/min) (Chapter 8). Expired air was not attractive for *An. gambiae* in the laboratory (De Jong and Knols, 1995b) and appeared not to play a significant role in the attraction of this mosquito to a host in the field (Costantini *et al.*, 1996; Chapter 2). This while the rate of ammonia from a person at rest (~0.45 µmol/min) falls within the threshold interval determined (0.08-0.8 µmol NH_3/min). As the concentration in human breath appears to be too low to induce a behavioural response in *An. gambiae*, the actual threshold must be close to the upper limit of the threshold interval.

In addition to incubated sweat, *An. gambiae* is also attracted to fresh sweat and skin washings (Chapter 6 and 8). As ammonia is nearly absent in these stimuli, attractive volatiles

other than ammonia must be present. L-lactic acid was considered as a potential candidate for another attractant for *An. gambiae* for the following two reasons. First, L-lactic acid is an essential component of eccrine sweat regulating the acidity of the skin (Noble and Somerville, 1974) and, consequently, it is present in the sweat collections and skin washings tested. Second, for a long time, L-lactic acid has been known as an important attractant for host seeking *Ae. aegypti* (Acree *et al.*, 1968). However, unexpectedly the role of L-lactic acid in the attraction of *An. gambiae* to sweat appears to be rather limited. Selective removal of L-lactic acid from sweat did not affect the behavioural responses of *An. gambiae* to sweat (Chapter 8). This while attraction of *Ae. aegypti* to skin washing was completely lost by the selective removal of this compound from skin washings (Geier, 1995). As this experiment has not been done with *An. gambiae* the role of L-lactic acid in the attraction of this mosquito to skin washing is not clear. However, there are two explanations possible. First, L-lactic acid does not play any role in the attraction of *An. gambiae*, and the mosquito's responses to skin washings will not be affected by its selective removal. Second, L-lactic acid does play a role in the attraction to skin washings, but not to sweat. As already suggested in Chapter 8 the responses to sweat and skin washings are not based on the same set of volatiles. Volatiles responsible for the attraction of *An. gambiae* to sweat other than ammonia have not been behaviourally tested, although electrophysiological responses to a number of sweat volatiles were described recently (Meijerink *et al.*, submitted).

Differential attractiveness between human individuals for a mosquito species is well documented and this is, at least partly, due to the differences in olfactory signals given of by the individuals (Knols *et al.*, 1995; Brady *et al.*, 1997). This phenomenon has led to a widespread and initially obvious suggestion to approach the identification of possible kairomones for *An. gambiae* by comparing the headspaces of differently attractive individuals. However, the complexity and variation of the host odour composition prevent a clear outcome by headspace comparison. In addition, differential attractiveness only exists in a choice situation (more hosts available) while a hungry mosquito will bite any person in a no-choice situation. Differential attractiveness might be based on marginal differences that are extremely difficult to resolve. For the identification of important volatiles, we need to focus first on the characteristics of human odour in comparison with other mammals.

PRODUCTION OF KAIROMONES FOR *AN. GAMBIAE*

The incubation of the human sweat samples consistently enhanced the attractiveness of sweat (Chapter 5, 7 and 8) and this was proven to be due to the growth of bacteria during incubation (Chapter 9). As sweat is an olfactory stimulus that is not artificial but rather true to nature, strong indications are provided for the suggestion by Knols (1996) that microbial activity is involved in the production of attractive volatiles for *An. gambiae*. With sweat as a 'human in bottle', a start has been made for investigations in the production of volatiles *in vitro*. This multidisciplinary approach is new and of great value to the understanding of the attraction of mosquitoes to human. As mentioned in Chapter 10, understanding the role and nature of skin microorganisms in the production of human odours may facilitate kairomone identification and development of traps (Vale, 1993) or mosquito repellents.

As incubated sweat is attractive while fresh sweat is not (Chapter 5) or less attractive (Chapter 7, 8 and 9), one could think that attraction of mosquitoes can be avoided by washing oneself each day. However, this idea is not valid for the following reason. In our experiments, sweat production was stimulated for the collection of large quantities. These collections consist predominantly of freshly secreted eccrine sweat, the components of which have not been subjected to bacterial degradation yet. In two days of incubation, a substantial number of volatile components are produced that are attractive to mosquitoes. However, a continuous bacterial action on secretions is apparent on the human skin *in vivo*. Hands that just have been washed are indeed less attractive to mosquitoes (W. Takken, pers. commun.), but washing is only effective in removing the volatile components from the skin for a short period.

As the microflora is not removed by washing, the metabolic conversion of skin secretions continues.

FUTURE RESEARCH

To the layman, the most remarkable conclusion drawn from this thesis will probably not concern the results reported here, but the limited knowledge regarding how mosquitoes find us. When I just started my literature search preceding the experimental study I was left with the same feeling. It has been known since 1898 that malaria is transmitted by mosquitoes (Desowitz, 1991) and since the 1920's, compelling evidence has been provided that nocturnal malaria mosquitoes especially rely highly on olfactory cues to find a suitable host for taking a blood meal. And still..... the identity of the kairomones emanating from the host is not known. Nevertheless, now at the end of this PhD-study, explanations for this hiatus appear to be abundant.

Contrary to expectation, substantial information about human body odour is not available. This is not because such research is not carried out, but because it is actually 'big business'. The unravelling of the human body odour is of major concern to cosmetic and perfume industries and military forces. For this reason many data on human odour are not accessible. Furthermore, most investigations deal predominantly with malodours arising from axillae and feet or, more recently, human pheromones (Chapter 1). Information regarding the 'normal' human odour or in other words all of the volatiles emanating from a human body is sparse. As humans have only a limited olfactory capacity, investigators have to rely on detecting devises other than their own nose for discovering the wealth of olfactory information available for a mosquito. The identification of kairomones for mosquitoes requires a multi-disciplinary approach, incorporating behavioural, electrophyiological, and chemical studies together with field trials. Behavioural and electrophysiological responses of mosquitoes to olfactory stimuli have been generally explored under controlled conditions in the laboratory. The diurnal yellow fever mosquito, *Ae. aegypti*, a cosmopolitan species has long been the primary subject of such laboratory studies (Clements, 1999). This was at least partly due to the fact that American investigators had been especially interested in this mosquito because many of their cities, including Washington D.C., had been under threat of yellow fever for several decades. Advantageously, many studies on other mosquito species could base their investigations on the information already derived from the studies with *Ae. aegypti*. However, since different mosquito species have often a completely different behavioural ecology, the results obtained from those investigations appeared to be only partly applicable to other mosquito species. Comprehensive research on the odour-mediated host-seeking behaviour of *An. gambiae* was started only recently (Knols, 1996; Healy and Copland, 1995). The delicate nature of the antennae of the mosquito has long hindered electrophysiological research. Already for decades, electroantennal responses to olfactory stimuli, predominantly (sex) pheromones, are reported for insects other than mosquitoes, especially for Lepidopteran species. Most identifications of semiochemicals for insects have been accomplished by direct linkage of electrophysiological responses to identified chemicals from a headspace, called coupled EAG-GS/MS (Electroantennogram-Gaschromatography/ Mass-spectrogram). As mentioned in Chapter 1, semiochemicals operate either between members of the same species or between members of different species, named homeochemicals or pheromones and allelochemicals respectively. The latter comprise chemicals that either favour the emitting species (allomone) or the receiving species (kairomone). Pheromones are, in principal, advantageous for both emitter and receiver. As a consequence, the nature of the composition, release and perception of pheromones usually differs essentially from that of kairomones and consequently so does the nature of the identification process. In general, pheromones are composed of a rather stable and limited number of highly specialised compounds that are perceived by the antennae of the receiver, which is covered with many receptors tuned to these compounds. Although the nature of the kairomones for mosquitoes is still not understood, we formulate

the following hypothesis. Host-seeking mosquitoes orient to volatiles that are released by a potential host. Unlike the composition of pheromones, the composition of the volatiles emitted by a mammalian host is rather variable and complex and mosquitoes need to be able to find their host despite this variation. Again unlike the perception of pheromones, the antennae of the mosquito appear to be sensitive to a broad range of host odours. The complexity of host odours together with the rather unspecific receptors complicates the identification of kairomones.

In recent years, much progress has been made in electrophysiological studies on *An. gambiae s.l.* (Meijerink, 1999; Van den Broek and Den Otter, 1999). The development of coupled EAG-GS/MS for mosquitoes is still in progress. This application will be advantageous especially for the identification of the kairomones from the broad spectrum of volatiles emanating from host odour. In summary, the recent progress in behavioural, electrophysiological and chemical studies restores optimism in the identification of kairomones for *An. gambiae* in the near future. The significance of the identified kairomones needs to be evaluated in field trials. The ultimate goal in the identification of these kairomones is to develop effective and objective odour-baited traps that can be used to monitor mosquito populations in the field (see below). An additional goal is to answer the fundamental but at the same time public question mentioned at the beginning of this section: How do mosquitoes find 'us'? And this brings us to the problem of mosquito control.

During the decades following the discovery of Ross, much attention was paid to the eradication of the 'bug' transmitting the disease with the use of insecticides rather than gathering ecological information about this vector. This was partly due to the difficulties associated with studying a small, nocturnal insect in the field. At first sight this may seem a trivial argument, but it has hindered research ['nobody will argue that information could have been gathered more easily if an elephant had been the vector']. Furthermore, since the beginning of the century the interest in medical research has been growing exponentially and malaria studies have focused mainly on the *Plasmodium* parasite causing the disease. This has led to the development of a range of anti-malarial drugs, mainly synthesised derivatives of the natural anti-malarial drug quinine. Unfortunately, despite the early optimism for the eradication of the disease by insecticides or anti-malarial drugs, more people are dying from malaria now than ever before. The limited knowledge regarding the behaviour and ecology of the vector is responsible for part of the disappointing results of control campaigns (Box 1). On the one hand information about the dynamics and behaviour of a mosquito population is essential for the development and implementation of control measures, adjusted to each specific region. On the other hand, such information is necessary to evaluate the effects of control measures. Nevertheless, the importance of 'back to basics' research is still not generally recognised. There is still debate how the mosquitoes meet and mate, and why and how mosquitoes distinguish suitable hosts from the wide range of vertebrates. We do not know why one species of mosquito dominates in one region and another 'similar' region is lacking mosquitoes. We are ignorant how far the mosquito flies to take a blood meal and from whom. We have no idea of the background in the variance of feeding preferences. We lack insight if the malaria parasite plays a role in the mosquito-host interaction. Such basic information about the behavioural ecology of the vector is essential to the understanding of malaria epidemics.

The above questions underline our lack of knowledge about essential aspects of mosquito bionomics that must be addressed before adequate malaria control campaigns can be implemented. The results of this thesis contribute to a better understanding of the odours that we produce and that mosquitoes respond to. Future studies should aim to complete the identification of the full pocket of odourant stimuli and assess their effects in the field.

BOX 1. SHORT OVERVIEW OF VARIOUS MALARIA CONTROL MEASURES

Insecticides - In the beginning of the century, scientists attempted to eradicate malaria by killing all vectors. The first approach was to kill the larval stage of the mosquito by adding larvicides, like Paris green, to breeding sites. Later, in the 1930's, the adult mosquito was attacked by spraying houses indoors with insecticides. Initially, short lived pyrethrum was used but it was readily replaced in the 1940s by insecticides that remain active over extended periods of time like DDT (dichlorodiphenyl trichlorethylene) (Desowitz, 1991). DDT treatments have led to an overall eradication of malaria from temperate regions of the world such as Europe and control of malaria mosquito populations in the tropical regions. Insecticides are still widely used in house spraying, larval control, impregnating bed nets or other means of personal protection. However, the development of physiological and behavioural resistance to DTT and other insecticides has hindered global eradication. Physiological resistance involves reduced sensitivity of the mosquito to the insecticide and examples of behavioural resistance include avoidance behaviour of the mosquito, such as increased exophily (Roberts and Andre, 1994).

Bed nets - A more basic measure against malaria is the use of bed nets, either impregnated with insecticides or not. This simple method prevents the mosquito from biting an individual and therefore is effective against transmission. The main problem with bed nets is that they only protect you when they do not have holes and when you are sleeping under them. In addition, as with all insecticides, it is only a matter of time until behavioural and physiological resistance of the mosquito to the insecticide on the bed nets develops.

Repellents – Some households cannot afford bed nets and may use other control measures such as burning coils that repel approaching mosquitoes. Coils often contain insecticide components which repel but do not kill mosquitoes. Other repellent substances such as deet (N,N-diethyltoluamide) may be used on skin or clothes and sometimes bed nets (Chou *et al.*, 1997). Other effective repellents include plant derived repellents such as neem oil from seeds of *Azadirachta indica* A Juss (Meliaceae) (Sharma and Ansari, 1994) and quwenling produced from lemon eucalyptus oil.

Anti-malarial drugs - The medical revolution of the 20th century brought new optimism for the control of malaria. An anti-malarial alkaloid from the bark of a tree, *Cinchona ledgeriana*, was already known for centuries by the inhabitants of the Andean region in South America to be an efficient medicine to combat high fevers of the malaria disease. Quinine was introduced into Europe by the 1630s. Not until 1920 could quinine be manufactured to produce a chemically pure preparation of predictable activity. However, the drug, chloroquine, was only available on the market from the early 50s. The introduction of this rather cheap and effective drug against malaria made the labour-intensive house spraying campaigns redundant. Although it had been very effective in the beginning, drug resistance already occurred in the first decade after introduction (Desowitz, 1991). Currently, chloroquine resistance is found all over the world. Similar trends are being seen in nearly all quinine-derived drugs and can be expected to occur against other anti-malarial drugs.

Vaccines - The development of an effective vaccine to protect non-immune individuals has been a goal of many studies for a long time. Initially optimism for the development of a malaria vaccine was based on the fact that individuals who are continually exposed to infection eventually develop some level of immunity. However, despite all efforts, an effective vaccine is not yet available. Research is mainly hampered by the complexity of the parasite life-cycle. The different stages of the parasite in the human host are morphologically distinct and, consequently, the immunity is stage-specific (Holder, 1999). Recently, progress has been made in developing a transmission-blocking vaccine. This vaccine works for the sexual stages of the parasite; gametocytes that a mosquito picks up from a vaccinated individual are unable to mate and produce sporozoites in the mosquito, and consequently further transmission is blocked. The vaccinated individuals themselves are not protected against the malaria disease. This altruistic vaccine is an illustrative example of the difficulties encountered in the development of a 'traditional' vaccine.

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SUMMARY

Malaria is an infectious disease caused by a parasite (*Plasmodium* spp.) that is transmitted between human individuals by mosquitoes, belonging to the order of insects, Diptera, family of Culicidae (mosquitoes) and genus of *Anopheles* (malaria mosquitoes). Mosquitoes feed on humans (and other animals) because they need blood for their reproduction. Like most other haematophagous insects, only the female mosquitoes bite and use the protein-rich blood meal for egg development. Whilst feeding on a person infected with malaria, the mosquito can be pick up the parasites from the blood stream. After a developmental period in the mosquito, the parasites can be transmitted to another person when the mosquito takes a next blood meal. Thus, malaria transmission depends largely on the characteristics of the mosquito population. Knowledge about the ecology, behaviour, infection level and size of the mosquito population is essential for the development, implementation and evaluation of control programs. Development of an adequate trapping device for monitoring the mosquito population is of high priority for ecological and epidemiological studies.

Malaria is one of the most important human parasitic infectious diseases and one third of the world population is under threat of the disease. Most victims are found in the sub-Saharan countries of Africa. The Afrotropical malaria mosquito, *Anopheles gambiae sensu stricto*, is the most important vector since it strongly prefers to feed on humans. Like most anopheline species, *An. gambiae s.s.* is nocturnal and its host-seeking behaviour is mainly odour-mediated. Consequently, odour-baited traps are considered as possible monitoring devices. However, despite the important role of this mosquito in malaria transmission, knowledge regarding host odour components (or kairomones) that bring about the attraction to humans is limited. For the development of odour-baited traps, attractive host odours need to be identified. In this thesis a behavioural ecological investigation to the source, identification and production of kairomones for *An. gambiae* (henceforth simply termed 'malaria mosquitoes') is described.

THE SOURCE OF KAIROMONES FOR MALARIA MOSQUITOES

Since the beginning of the century it has been recognised that malaria mosquitoes utilise host odours in their host-seeking behaviour. The source of these olfactory stimuli is expired air, the skin or both. Carbon dioxide present in expired air is an important kairomone for many haematophagous insects. For this reason carbon dioxide is often used in odour-baited traps. From field research (Chapter 2) we learnt that malaria mosquitoes can find their host in the absence of breath, and, thus, the presence of carbon dioxide is not compulsory for finding a host. This suggests that volatiles from the skin of the host also play a role in the attraction of malaria mosquitoes. The addition of skin volatiles to a carbon dioxide baited trap will probably bring about higher trap catches. Moreover, for logistic reasons, an odour-baited trap without presence of carbon dioxide is preferable. Carbon dioxide is highly volatile and can be delivered only by gas cylinders or dry ice (= frozen carbon dioxide), which is impractical in the African field situation. The composition of body odour is complex: more than 300 components have been identified. However, a synthetic blend of the complete human odour has not yet been synthesised. For this reason, the identification of some important components that attract malaria mosquitoes was initiated. A prerequisite for the identification was the entrapment of natural skin emanations separate from the skin. Sweat appeared to be an attractive complex olfactory stimulus since it is not artificial but rather true to nature (Chapter 4 and 5) in the bioassays in the laboratory and it forms the 'heart' of the thesis.

THE IDENTIFICATION OF KAIROMONES FOR MALARIA MOSQUITOES

Sweat was collected from the foreheads of a number of volunteers, who performed exercises on a hometrainer in a warm and humid room. The behavioural response of the malaria mosquitoes to this fresh sweat was rather variable; they were attracted to some fresh sweat samples (Chapter 8 and 9) but not to others (Chapter 5 and 7). However, the response of the mosquitoes to sweat that had been incubated for two days at body temperature was stable, and all incubated sweat samples were attractive to the mosquitoes. It appeared that the incubation released volatile components that were attractive to mosquitoes. Sweat is basically a watery solution of lactic acid, urea and ammonia. After incubation the lactic acid and urea concentration had decreased and the ammonia concentration showed a distinct increase (Chapter 8 and 9). For this reason ammonia was tested in the bioassay. For the first time, malaria mosquitoes were attracted to a single component other than carbon dioxide, namely ammonia. Lactic acid is an essential kairomone for another mosquito species, the yellow fever mosquito *Aedes aegypti*. However, the selective removal of lactic acid from the sweat did not affect the reaction of malaria mosquitoes. Therefore, we conclude that lactic acid is not an essential component of attractive odour blends for malaria mosquitoes. Urea was not tested, as it is not volatile. The fact that attraction was sometimes found to the fresh sweat with a rather low concentration of ammonia indicates that components other than ammonia also play a role in the host-seeking behaviour of malaria mosquitoes. The identity of these components needs further exploration.

THE PRODUCTION OF KAIROMONES FOR MALARIA MOSQUITOES

The skin of humans (and other animals) forms a good habitat for some microorganisms (bacteria and fungi), together called the skin microflora. During the collection of sweat samples, microorganisms are taken up with the sweat. An exponential growth of microorganisms in the sweat samples is found during incubation (Chapter 4, 6 and 7). Sweat constituents are broken down into more volatile components by the growing microorganismal population and this appears to bring about the enhancement of the attractiveness of sweat to malaria mosquitoes (Chapter 9). Such processes also probably play a role in the production of kairomones on the skin. However, this needs further exploration.

CONCLUSIONS

Kairomones for malaria mosquitoes originate from the human skin, in addition to carbon dioxide from exhaled air. Microorganisms of the skin flora play an important role in the production of kairomones for malaria mosquitoes *An. gambiae s.s.*. Ammonia is one of the components responsible for the attraction of malaria mosquitoes to sweat.

SAMENVATTING

De humane infectieziekte malaria wordt veroorzaakt door een parasiet (*Plasmodium* spp.) die tussen mensen wordt overgedragen door malariamuggen, behorende tot de insectenorde Diptera ('tweevleugeligen'), de familie der Culicidae ('steekmuggen') en het geslacht der anophelines ('malariamuggen'). Steekmuggen steken mensen (en andere dieren) omdat ze bloed nodig hebben voor de voortplanting. Evenals bij de meeste andere bloedzuigende insecten steken alleen de vrouwelijke malariamuggen en gebruiken zij de eiwitrijke bloedmaaltijd voor de ontwikkeling van hun eitjes. Wanneer een malariamug een met malaria geïnfecteerd persoon steekt kunnen parasieten tegelijk met het bloed door de mug worden opgenomen. Nadat de malariaparasieten een ontwikkeling hebben ondergaan in de mug, kunnen ze tijdens een volgende 'bloedmaaltijd' van de mug weer worden overgedragen op een andere persoon. De overdracht van malaria wordt dus in hoge mate bepaald door de eigenschappen van de malariamuggen. Voor de ontwikkeling, realisatie en evaluatie van malariabeheersingsprogramma's is kennis over de ecologie, het gedrag, de infectiegraad en de grootte van de malariamuggenpopulatie noodzakelijk. Een adequate vangmethode voor het bemonsteren van de muggenpopulatie is daarom onontbeerlijk

Malaria is de een van de belangrijkste parasitaire infectieziekte bij de mens en eenderde van de wereldbevolking leeft in een risicogebied. De meeste slachtoffers vallen in de gebieden in Afrika ten zuiden van de Sahara. De Afrikaanse malariamug, *Anopheles gambiae sensu stricto,* is de belangrijkste vector omdat deze mug mensen als bloeddonor (oftewel als gastheer) verkiest boven andere zoogdieren. Evenals de meeste andere malariamuggensoorten is *An. gambiae s.s.* 's nachts actief en oriënteert zich voornamelijk op geuren om haar gastheer te vinden. Aangezien de muggen zich voornamelijk oriënteren op geuren, ligt het ontwikkelen van geurvallen voor het bemonsteren en bestuderen van muggenpopulaties voor de hand. Echter, ondanks de belangrijke rol die deze mug speelt in de overdracht van malaria is er weinig bekend over de componenten van deze gastheergeuren, ook wel kairomonen genoemd, die de aantrekking van *An. gambiae* tot mensen tot stand brengen. Voor de ontwikkeling van een zogenaamde 'mierenlokdoos' voor muggen is de identificatie van belangrijke gastheergeuren essentieel. In dit proefschrift is een gedragsecologisch onderzoek verricht naar de bron, identiteit en productie van kairomonen voor *An. gambiae s.s.* (vanaf hier afgekort als 'malariamuggen').

DE BRON VAN KAIROMONEN VOOR MALARIAMUGGEN

Sinds het begin van deze eeuw is bekend dat malariamuggen gebruik maken van geuren om een mens te vinden. De bron van deze olfactorische informatie is de adem of de huid van de gastheer of de combinatie van beide. Veel bloedzuigende insecten oriënteren zich op de koolstofdioxide aanwezig in uitgeademde lucht van de potentiële gastheer. Om deze reden is koolstofdioxide ook een gangbaar bestanddeel van geurvallen. Echter uit het veldonderzoek (Hoofdstuk 2) is gebleken dat malariamuggen een gastheer ook kunnen vinden bij afwezigheid van adem en dus bij afwezigheid van koolstofdioxide. Dit wijst erop dat vluchtige verbindingen die afkomstig zijn van de huid ook een rol spelen in de aantrekking van malariamuggen tot mensen. Het is waarschijnlijk dat het aantal malariamuggen dat aangetrokken wordt door de val zal toenemen door de toevoeging van huidgeurstoffen aan geurvallen. Uit praktisch oogpunt is het zelfs wenselijk om een geurval te kunnen ontwikkelen zonder koolstofdioxide. Koolstofdioxide is zeer vluchtig en kan alleen met behulp van gascilinders of droogijs (= bevroren koolstofdioxide) aangeboden worden. Dit is zeer onpractisch in het veld (lees: het Afrikaanse platteland) waar de muggenpopulatie uiteindelijk bemonsterd zal moeten worden. De samenstelling van lichaamsgeur is zeer complex en er

zijn al meer dan 300 chemische componenten zijn reeds geïdentificeerd. Een "synthetische formule" van de complete mensengeur is echter nog niet op de markt. Om deze reden is er onderzoek gestart om enkele belangrijke componenten van de lichaamsgeur te identificeren waardoor de mug wordt aangetrokken. Een belangrijke voorwaarde voor de identificatie is dat de complexe natuurlijke geur 'opgevangen' kan worden en afzonderlijk van de gastheer getest kan worden. Uit het huidige onderzoek (hoofdstuk 4 en 5) is gebleken dat menselijk zweet aantrekkelijk is voor de malariamuggen en het is daarom nader onderzocht.

DE IDENTIFICATIE VAN KAIROMONEN VOOR MALARIAMUGGEN

Zweetmonsters zijn verzameld van het voorhoofd van een groot aantal vrijwilligers. Het zweten werd gestimuleerd door oefeningen te verrichten op een hometrainer in een warme, vochtige kamer. De gedragsreactie van de malariamuggen op dit vers verzamelde zweet was nogal wisselend. Sommige verse zweetmonster waren attractief (Hoofdstuk 8 en 9) maar andere niet (Hoofdstuk 5 en 7). De reacties van de muggen op zweet, nadat het gedurende twee dagen bij lichaamstemperatuur was bewaard, waren stabiel: de muggen werden aangetrokken tot elk geïncubeerd zweetmonster. Uit dit onderzoek bleek dat er tijdens de incubatie stoffen gevormd worden die voor malariamuggen aantrekkelijk zijn. Zweet bestaat hoofdzakelijk uit een waterige oplossing van melkzuur, ureum en ammonia. Na de incubatie was de hoeveelheid ureum en melkzuur afgenomen terwijl de hoeveelheid ammonia sterk was toegenomen (Hoofdstuk 8 en 9). Daarom is de gedragsreactie op ammonia alleen getest en bleek dat malariamuggen worden aangetrokken door ammonia. Het is de eerste keer dat malaria muggen worden aangetrokken door een enkele chemische component anders dan koolstofdioxide. Melkzuur is ook alleen getest, daar is echter geen duidelijke reactie op gevonden. Melkzuur is een belangrijke component binnen het gastheerzoekgedrag van een andere muggensoort, de gelekoortsmug *Aedes aegypti*. Bij de selectieve verwijdering van melkzuur uit het zweet bleef de reactie van malariamuggen hetzelfde. Hierdoor lijkt het erop dat melkzuur geen essentieel bestanddeel is van een attractief geurmengsel voor deze mug. Ureum is niet getest aangezien het geen vluchtige component is. Het feit dat er ook aantrekking is gevonden op vers zweet met een zeer lage concentratie van ammonia duidt erop dat er naast ammonia andere componenten zijn waarop malariamuggen reageren. De identificatie van deze andere componenten zal echter nog plaats moeten vinden.

DE PRODUCTIE VAN KAIROMONEN

De huid van mensen (en andere dieren) vormt een heel geschikt leefklimaat voor sommige micro-organismen (bacteriën en schimmels), die samen de huidflora genoemd wordt. Micro-organismen werden meegenomen met het zweet tijdens het verzamelen en blijken zich exponentieel te vermeerderen in het zweet tijdens de incubatie (Hoofdstuk 4, 6 en 7). Tijdens dit proces worden zweetcomponenten afgebroken in vluchtige stoffen en dat heeft de verhoogde aantrekking van malariamuggen tot zweet veroorzaakt (Hoofdstuk 9). Waarschijnlijk spelen dergelijke processen ook een rol in de productie van vluchtige stoffen op de huid en zijn de micro-organismen op de huid verantwoordelijk voor de productie van geurstoffen die door muggen gebruikt worden bij de lokalisatie van hun gastheer. Dit aspect dient nog verder te worden onderzocht.

CONCLUSIE

Uit dit promotieonderzoek is gebleken dat, belangrijke, kairomonen voor malariamuggen van de menselijke huid afkomstig zijn, naast een mogelijke rol van koolstofdioxide uit adem. Micro-organismen van de huidflora spelen een belangrijke rol in de productie van de

kairomonen voor malariamuggen, *An. gambiae s.s.*. Ammonia is één van de componenten die verantwoordelijk zijn voor de aantrekking van malariamuggen tot menselijk zweet.

Braks, M.A.H, Meijerink, J. and Takken, W. (*submitted)* The role of human sweat components, ammonia and L-lactic acid, in the behaviour of the anthropophilic malaria mosquito, *Anopheles gambiae* (Culicidae; Diptera). *Journal of Comparative Physiology A.*

Meijerink, J.; Braks, M.A.H. and Van Loon, J.J.A. (*submitted*) Olfactory receptors on the antennae of the malaria mosquito *An. gambiae* are sensitive to ammonia and other sweat-borne components. *Journal of Comparative Physiology A.*

Mboera, L.E.G.; Knols, B.G.J.; Braks, M.A.H. and Takken, W. (*submitted*). Comparison of four trapping systems baited with carbon dioxide in sampling of an outdoor mosquito population in Tanzania. *Medical and Veterinary Entomology.*

Meijerink, J.; Braks, M.A.H.; Brack, A.; Adam, W.; Posthumus, M.; Van Beek, T. and Van Loon, J.J.A. (*accepted)* Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *Journal of Chemical Ecology.*

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Op 16 augustus 1969 werd ik, Marieta Braks, geboren in het Noord-Limburgse dorp Middelaar. In 1981, ging ik naar het Elshofcollege in Nijmegen waar ik in 1987 het diploma Atheneum B behaalde. Vervolgens vertrok ik naar Wageningen om te gaan studeren aan de Landbouw Hogeschool. Na het behalen van de propaedeuse Humane Voeding in 1988, besloot ik om Biologie te gaan studeren aan de Universiteit van Utrecht. In 1994 behaalde ik mijn doctoraalexamen met als hoofdrichting Neuroethologie (UU) en met een buitenlandse onderzoeksonderwerp Mariene Biologie (RUG) in Corsica, Frankrijk.

In 1995 werd ik aangenomen als Onderzoeker in Opleiding bij de vakgroep Entomologie aan de Landbouw Universiteit Wageningen binnen het onderzoeksprogramma naar de rol van gastheergeuren bij het gastheerzoekgedrag van malariamuggen. Deze positie sloot precies aan bij mijn vooropleiding, mijn fascinatie voor de tropen en mijn voorkeur om niet met gewervelde proefdieren te hoeven experimenteren, maar met toch-al-irritante muggen. In 1997 heb ik binnen dit project voor drie maanden onderzoek verricht in het veld in Tanzania. Dit, tezamen met het onderzoek in Wageningen, resulteerde in dit proefschrift.

On 16 August 1969, I, Marieta Braks, was born in the southern Dutch village, Middelaar. In 1981, I started my secondary education at the 'Elshofcollege' in Nijmegen and concluded it in 1987. I left for Wageningen to study at the 'Landbouw Hogeschool'. However after conferring the certificate upon the propaedeutical year of Human Nutrition in 1988, I decided to start the Biology curriculum at the Utrecht University. My specialisation during this M.Sc. study was Neuro-Ethology with an extra research subject in Marine Biology performed on Corsica, France. In 1994 I graduated.

In 1995, I started a Ph.D. study at the Department of Entomology of the Wageningen Agricultural University within a research project 'Odour-guided host-finding by haematophagous mosquitoes'. This research matched my foregoing education, my interest for the tropics and my preference to execute experiments with annoying 'bugs' rather than vertebrates. In 1997, I spent three months in Tanzania to perform field assays. The laboratory and field research resulted in this thesis.