The role of skin microbiota in the attractiveness of humans to the malaria mosquito *Anopheles gambiae* Giles

Niels O. Verhulst

Thesis committee

Thesis supervisors
Prof. dr. ir. W. Takken
Personal Chair at the Laboratory of Entomology,
Wageningen University

Prof. dr. M. Dicke
Professor of Entomology,
Wageningen University

Other members:
Prof. dr. J. A. Pickett
Rothamsted Research, Harpenden, UK

Prof. dr. R. W. Sauerwein
Radboud University Nijmegen

Dr. S.E. van Wieren
Wageningen University

Prof. dr. ir. M.H. Zwietering
Wageningen University

This research was conducted under the auspices of the C. T. de Wit Graduate School for Production Ecology and Resource Conservation.

The role of skin microbiota in the attractiveness of humans to the malaria mosquito *Anopheles gambiae* Giles

Niels O. Verhulst

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Thursday 9 December 2010
at 11 a.m. in the Aula

Niels O. Verhulst (2010)
The role of skin microbiota in the attractiveness of humans to the malaria mosquito *Anopheles gambiae* Giles

PhD-thesis, Wageningen University – with references – with summary in Dutch

ISBN 978-90-8585-830-0

ABSTRACT

Malaria is one of the most serious infectious diseases in the world. The African mosquito *Anopheles gambiae sensu stricto* (henceforth termed *An. gambiae*) is highly competent for malaria parasites and preferably feeds on humans inside houses, which make it one of the most effective vectors of the disease. Human body odours are presumably the most important cues that enable *An. gambiae* to find its host. Knowledge on the odours that mediate the host-seeking behaviour of malaria vectors is expected to contribute to novel intervention methods for disease control.

The skin microbiota plays an important role in the production of human body odours and the human microbial and chemical signature displays a qualitative and quantitative correlation. Several studies have indicated a possible role of skin bacteria in the production of volatiles attractive to mosquitoes. The principle goal of this thesis was to understand the role of the human skin microbiota in mosquito-host interactions and to identify which compounds produced by these micro-organisms are involved in the attraction of *An. gambiae* to humans.

Skin bacteria isolated from human feet and grown *in vitro* on agar plates attracted *An. gambiae* and this attraction was affected by incubation time and dilution of the skin microbiota. Semi-field experiments showed similar results and field experiments in Kenya suggested that skin bacterial volatiles also attract other disease vectors. Volatiles produced by five bacterial species common on the human skin showed that volatile blends produced by some species, including *Staphylococcus epidermidis,* were more attractive than blends produced by other species. Volatiles produced by *Pseudomonas aeruginosa* did not affect the behaviour of *An. gambiae*.

Analysis of the volatiles produced by human skin microbiota grown *in vitro* led to the identification of 16 compounds, the majority of which had an effect on *An. gambiae* behaviour. 3-Methyl-1-butanol enhanced the attractiveness of a synthetic blend by a factor of three, and could be used to increase mosquito trap catches for monitoring or vector control purposes. 2-Phenylethanol decreased mosquito catches of a synthetic blend and may act as a spatial repellent.

In order to examine the interaction between the microbiota on the skin and human attractiveness to mosquitoes, skin emanation and skin microbiota samples were taken from 48 individuals. The skin emanations from the individuals varied significantly in attractiveness to *An. gambiae* and several compounds originating from the skin were associated with individuals that were highly attractive or less attractive to mosquitoes. Individuals with a higher abundance of bacteria on their skin were more attractive to *An. gambiae*, whereas individuals with a higher diversity of skin microbiota were less attractive. *Staphylococcus* spp. were associated with individuals that were highly attractive and *Pseudomonas* spp. with individuals that were less attractive to mosquitoes. Human leukocyte antigen (HLA) genes are considered to influence the human body odour profile and HLA profile analysis of the 48 human individuals indicated that these genes may also affect the attractiveness of humans to mosquitoes.

The studies described in this thesis show that volatiles produced by the human skin microbiota play an important role in the host-seeking behaviour of *An. gambiae* and the abundance and composition of the skin microbiota determine an individual's attractiveness to mosquitoes. Optimised blends of the compounds identified can be used in push-pull strategies for the manipulation of mosquitoes, thereby reducing the number of malaria mosquitoes, the human-biting frequency, and the intensity of *Plasmodium* transmission. Research on the role of skin microbiota in the host-seeking behaviour and host preference of biting insects may lead to a better understanding of vector-host interactions and contribute to the fight against vector-borne diseases.

Contents

General introduction

General introduction

01

Niels O. Verhulst

Mosquitoes and malaria transmission

Malaria is one of the most serious infectious diseases in the world. One third of the human population lives in a malaria endemic area and each year around 900.000 people die from the disease (WHO 2009). Most of the victims are children below 5 years of age (Greenwood et al. 2005). In 2008, there were an estimated 243 million cases of malaria worldwide, predominantly (85%) in Africa (WHO 2009).

Plasmodium parasites that cause malaria are transmitted by anopheline mosquitoes. Around 30 of the approximately 450 species of anophelines are major vectors of malaria (White 1982) of which *Anopheles funestus* and the members of the *Anopheles gambiae* Giles complex are most important. Within this complex, *An. gambiae sensu stricto* (henceforth termed *An. gambiae*), is highly anthropophilic, endophilic and endophagic and has a large distribution in Africa (Coetzee et al. 2000) and is, therefore, the most effective vector of human malaria (Takken and Knols 1999).

Mosquito host-seeking behaviour

To acquire their nutrients and to reproduce, mosquitoes use several cues, of which olfactory cues are the most important (Takken and Knols 1999). Semiochemicals are used by female mosquitoes to find sites for oviposition, sugar sources, suitable blood hosts, and possibly, mating partners (Takken and Knols 1999). Other cues can be physical or visual. Physical cues include moisture and temperature, which both increase the response of *Anopheles* mosquitoes to host odours (Takken et al. 1997b, Olanga et al. 2010). The response to visual cues varies markedly between different species. Nocturnal species can only use visual cues to navigate through the landscape. Diurnal species can use visual cues for host seeking and/or oviposition (Allan et al. 1987).

The host-seeking flight of a mosquito can roughly be divided into long-range and short-range attraction. Long distance attraction is mainly determined by olfactory stimuli, whereas attraction from a short distance is mediated by odours and non-olfactory cues, such as heat, body moisture and visual cues (Takken 1991, Takken and Knols 1999, Cardé and Gibson 2010). For *An. gambiae*, odours are probably the most important cues to find its host. *Anopheles gambiae* can distinguish humans from non-hosts by the odours they emit (Pates et al. 2001) and mainly feeds on humans (Costantini et al.

1998, Takken and Knols 1999).

Carbon dioxide (CO_2) is one of the most important olfactory cues for mosquitoes to find their hosts. Several species of mosquitoes have been shown to be attracted to CO_2 (Reeves 1951, Eiras and Jepson 1994, Mboera and Takken 1997), but the role of CO_2 in the behaviour of *An. gambiae* is less clear and studies show variable results (Knols 1996b, Mboera et al. 1997, Takken et al. 1997a, Dekker et al. 2001a). In a laboratory setup CO_2 was a poor kairomone to *An. gambiae* when offered alone, but augmented the attraction to skin emanations (Spitzen et al. 2008). When added to mosquito traps, CO_2 dramatically increases trap catches (Costantini et al. 1996b, Mboera et al. 2000b, Qiu et al. 2007b, Schmied et al. 2008, Jawara et al. 2009). Carbon dioxide alone, however, cannot explain the anthropophilic behaviour of *An. gambiae* as it is also emitted by other non-hosts and therefore other, more human-specific odours, must be involved in the host-seeking process of this mosquito species.

Identification of the volatile organic compounds (VOCs) emitted by humans that attract *An. gambiae* can be difficult because the behavioural effect of the compounds depends largely on the concentration tested. Ammonia, for example, is an important kairomone and attractive to *An. gambiae* (Braks et al. 2001, Smallegange et al. 2005). However, when concentrations of ammonia are too high, it acts as a repellent to *An. gambiae* (Smallegange et al. 2005). VOCs can also have a synergistic effect on mosquito behaviour. Lactic acid plays an important role in the host-seeking behaviour of *Aedes aegypti* L. and can act as a synergist that improves the attraction to human skin emanations (Steib et al. 2001). To *An. gambiae,* lactic acid is only slightly attractive (Braks et al. 2001), however, it shows a synergistic effect in combination with ammonia and carboxylic acids (Smallegange et al. 2005). The combination of ammonia, lactic acid and a mixture of carboxylic acids was significantly more attractive than any of the three components alone (Smallegange et al. 2005).

Differential attractiveness of humans to mosquitoes

Humans are differentially attractive to mosquitoes. Differential attractiveness can be determined by several characteristics. Increased body temperature and moisture both have an effect on human attractiveness to mosquitoes (Smart and Brown 1957, Gilbert et al. 1966, Mukabana et al. 2002). When the body heat component is excluded, however, humans are still differentially attractive

(Qiu et al. 2006, Olanga et al. 2010), suggesting that other factors are also involved.

Other characteristics which have an effect on human attractiveness to mosquitoes are skin colour (Smart and Brown 1957), pregnancy (Lindsay et al. 2000, Ansell et al. 2002, Himeidan et al. 2004) and *Plasmodium* infection (Lacroix et al. 2005, Mukabana et al. 2007), but obviously other physiological factors may also play a role in differential attractiveness among humans. Chemical cues are considered most important as both short- and long-range attractants (Takken 1991, Mboera et al. 1997, Takken and Knols 1999, Olanga et al. 2010) and also affect the differential attractiveness of humans to mosquitoes (Brouwer 1960, Mayer and James 1969, Schreck et al. 1990, Lindsay et al. 1993, Knols et al. 1995, Brady et al. 1997, Bernier et al. 2002, Mukabana et al. 2002, Qiu et al. 2004a, Qiu et al. 2006, Logan et al. 2008). Studying the differential attractiveness of humans to mosquitoes can help to identify VOCs that mediate mosquito host-seeking behaviour (Bernier et al. 2002, Logan et al. 2008).

Human skin microbiota and mosquito host-seeking behaviour

The human skin microbiota plays an important role in the production of human body odours. The intensity of human odour and its chemical composition are correlated with the abundance of certain microorganisms on the human skin (Leyden et al. 1981, Jackman and Noble 1983, Rennie et al. 1990, Rennie et al. 1991, Bernier et al. 2000, Taylor et al. 2003, Ara et al. 2006, Xu et al. 2007). If the attractiveness of humans to mosquitoes is based on the metabolic activity and/or density of the skin microbiota, then this will have a direct impact on the interaction between the mosquito and its host (Knols 1996b, Takken and Knols 1999).

The first evidence for the role of skin microbiota in the production of volatiles attractive to mosquitoes was provided by Schreck and James (1968). A broth culture of *Bacillus cereus* derived from smears from a human arm was attractive to female *Ae. aegypti* and it was suggested that metabolites and/or decomposition derivates produced by the action of micro-organisms on the human skin may contribute to the attractiveness of the skin to mosquitoes.

A study on Limburger cheese volatiles further indicated the role of bacteria in mosquito olfaction (Knols 1996a, Knols and De Jong 1996). Tests

in a dual-choice olfactometer showed that *An. gambiae* was more attracted to traps baited with Limburger cheese than to unbaited traps. *Brevibacterium linens* is involved in the ripening of Limburger cheese and closely related to *Br. epidermidis*, which is a resident of the microbiota on human feet (Anthony et al. 1992, Noble 2004). It is suggested that dairy *Brevibacterium,* like *Br. linens,* may have originated from either human or bovine skin (Jackman 1982). *Brevibacterium epidermidis* could be one of the bacteria on the human skin that are involved in the production of odours attractive to malaria mosquitoes (Knols and De Jong 1996).

Human sweat is attractive to *An. gambiae*, but only after incubation for one or two days at 37ºC (Braks and Takken 1999). When fresh sweat was sterilized through a bacterial filter and incubated for one or two days, there was no longer an effect of the incubation on mosquito attraction and the sample was less attractive than fresh sweat (Braks et al. 2000). During an incubation period, the human skin microbiota converts fresh sweat into aged sweat (Shelley et al. 1953), which is attractive to *An. gambiae* (Braks et al. 2000). This indicates the role of the human skin microbiota in the production of human skin emanations attractive to *An. gambiae*. However, the potential role of skin micro-organisms in the host-seeking behaviour of *An. gambiae* needs further elucidation (Braks et al. 1999, Braks and Takken 1999, Takken and Knols 1999, Braks et al. 2000). The correlation between bacteria present on the human skin and human attractiveness to mosquitoes needs to be investigated and the volatile compounds produced by skin bacteria that attract mosquitoes should be identified. These compounds can then be used for manipulation of the host-seeking behaviour of mosquitoes and thus for mosquito control.

Research objectives

The principle goal of the study described in this thesis was:

Understand the role of the skin microbiota in mosquito-host interactions and identify which compounds produced by these organisms are involved in the attraction of Anopheles gambiae sensu stricto *to humans.*

The role of human skin microbiota was investigated, following four main objectives:

1. *Investigate whether micro-organisms obtained from the human skin produce substances that affect the host-seeking behaviour of* An. gambiae s.s.;
2. *Determine which genera of the human skin microbiota are most important in the production of mosquito behaviour-mediating compounds;*
3. *Investigate whether the differential attractiveness of human individuals to* An. gambiae s.s. *and the skin microbiota composition are correlated;*
4. *Identify compounds produced by microbiota of human skin that mediate mosquito behaviour.*

The outline of this thesis is as follows:

Chapter 2:

Chemical ecology of interactions between human skin microbiota and mosquitoes

The human microbial and chemical signature displays a qualitative and quantitative correlation. Genes may influence the microbiota composition and, thereby, the human odour composition. This chapter gives an overview of the literature on human skin microbiota and how it shapes the human body odour profile. It is discussed how detailed knowledge on the ecology and genetics of human skin microbiota may unravel the evolutionary mechanisms that underlie the interactions between mosquitoes and their hosts.

Chapter 3:

Structural design affects entry response of mosquitoes in olfactometers

Olfactometers have long been used to test the behavioural responses of mosquitoes to odour components and blends. It was tested whether a dual-choice olfactometer equipped with a funnel as trap entry would catch more mosquitoes than when equipped with a baffle. Increasing the total number of mosquitoes trapped can increase the discriminatory power of the system.

Chapter 4:

Cultured skin microbiota attracts malaria mosquitoes

It is hypothesized that host attractiveness and selection of *An. gambiae* is affected by the species composition, density, and metabolic activity of the human skin microbiota. The production and constituency of volatile

compounds by human skin microbiota was examined and the behavioural responses of *An. gambiae* to volatiles from skin microbiota investigated. A synthetic blend, composed of compounds found in the headspace of the skin bacteria, was also tested for its effect on *An. gambiae* host-seeking behaviour.

Chapter 5:

Human skin microbiota and their volatiles as odour baits for the malaria mosquito Anopheles gambiae sensu stricto

The attractiveness of volatiles produced by human skin bacteria to malaria mosquitoes was tested in laboratory, semi-field and field experiments to assess these effects in increasing environmental complexity. A synthetic blend of ten compounds identified in the headspace of skin bacteria (Chapter 4) was also tested for its attractiveness. The results obtained with the skin bacteria and the synthetic blend were evaluated for use in traps to monitor or control mosquitoes.

Chapter 6:

Improvement of a synthetic lure for Anopheles gambiae sensu stricto *using compounds produced by human skin microbiota*

Ten compounds identified in the headspace of skin bacteria (Chapter 4) were tested for their attractiveness to *An. gambiae* in a dual-choice olfactometer and a semi-field bioassay. Compounds may be more attractive in mixtures than when applied alone and, therefore, each compound was added to a blend of three compounds that was already known to be attractive to *An. gambiae*.

Chapter 7:

Differential attractiveness to malaria mosquitoes of volatile blends produced by human skin bacteria

Five commonly occurring species of human skin bacteria were tested for the production of volatiles that attract *An. gambiae* and ranked according to this attractiveness. Bacterial headspace volatiles were identified and synthetic compounds were tested for their attractiveness to *An. gambiae*.

Chapter 8:

Human traits underlying differential attractiveness to malaria mosquitoes

It was hypothesised that the skin bacterial compositions and/or human leukocyte antigen (HLA) profile affect the attractiveness of humans to mosquitoes by influencing the composition of volatiles emanating from human skin. For 48 human individuals the attractiveness to mosquitoes was examined by collecting skin emanations from the foot on glass beads and correlations with their skin emanations, HLA profile and bacterial composition were investigated.

Chapter 9:

General discussion

The results of the research described in this thesis will be discussed in connection to the objectives as specified above. Future research directions will be suggested and the use of skin microbiota for the identification of compounds that influence mosquito behaviour will be discussed.

Human skin microbiota and mosquito interactions

Chemical ecology of interactions between human skin microbiota and mosquitoes

02

Niels O. Verhulst, Willem Takken, Marcel Dicke, Gosse Schraa, Renate C. Smallegange

Microbiota on the human skin plays a major role in body odour production. The human microbial and chemical signature displays a qualitative and quantitative correlation. Genes may influence the chemical signature by shaping the composition of the microbiota. Recent studies on human skin microbiota, using 16S rRNA gene sequencing, found a high inter- and intra-personal variation in bacterial species on the human skin, which is relatively stable over time. Human body odours mediate the attraction of mosquitoes to their blood hosts. Odours produced by skin microbiota are attractive to mosquitoes as shown by *in vitro* studies and variation in bacterial species on the human skin may explain the variation in mosquito attraction between humans. Detailed knowledge on the ecology and genetics of human skin microbiota is needed in order to unravel the evolutionary mechanisms that underlie the interactions between mosquitoes and their hosts.

Published in:
FEMS Microbiology Ecology, 74: 1-9

Introduction

Human-associated microorganisms on the skin have long been studied because of their role as pathogens. For example, *Staphylococcus aureus* and *Streptococcus pyogenes,* which cause skin infections and a variety of other diseases (Bisno and Stevens 1996, Kluytmans et al. 1997), are probably two of the best studied microbial pathogens on the human skin. The role of human skin microorganisms as pathogens and in the defence against diseases has been reviewed extensively by Cogen et al. (2008). Skin-inhabiting bacteria also act as producers of odours which may play a role in the interactions between disease vectors (e.g. mosquitoes, biting midges, biting flies, triatomine bugs, mites, ticks) and their human hosts. Olfaction is the principal sense with which many of these vectors locate their blood hosts, and hence chemical cues affect disease transmission (Takken and Knols 1999, Logan and Birkett 2007). The present paper provides an overview of how skin microorganisms shape the human odour profile and in which way this knowledge may be exploited for novel strategies of vector control and a reduction of vector-borne disease risk. As an example we discuss how skin microbiota affects the interaction between malaria vector mosquitoes and humans, and suggest how this knowledge can be used to identify the microbial volatiles that mediate this behaviour.

Human microbiota

Since molecular tools became available an enormous increase in knowledge on the ecology of the human microbiota has been achieved (Gao et al. 2007, Costello et al. 2009, Grice et al. 2009, Vrieze et al. 2010). The human intestinal microbiota proves to be a complex community of many different species and plays an important role in human health and disease (Egert et al. 2006). Upon disturbance, probiotics can help to restore microbial populations in the intestines and thereby restore the function of the intestinal tract (Ouwehand et al. 2002, Rastall et al. 2005).

In contrast to the intestinal microbiota, until recently only little was known about the microbial community on the human skin. Most of the knowledge gathered was based on research using selective growth media. The application of molecular techniques shows that only a small proportion of the existing microbial community is detected by using these media (Gao et al. 2007, Grice et al. 2008). Recent studies, using molecular techniques, have

shown the complexity of the microbial community on the skin and that humans differ strongly in their skin microbiota profiles (Gao et al. 2007, Fierer et al. 2008, Costello et al. 2009, Grice et al. 2009). This knowledge can help to unravel some of the basic functions of microorganisms on the human skin.

Volatile production by microbiota on the human skin

Human sweat is odourless and only after incubation with bacteria sweat gets its characteristic smell (Shelley et al. 1953). The microbiota of the skin plays an important role in human odour production and the number of certain microorganisms is strongly correlated with the intensity of the odour emitted (Leyden et al. 1981, Jackman and Noble 1983, Rennie et al. 1990, Rennie et al. 1991, Taylor et al. 2003, Ara et al. 2006). The human skin microorganisms are most abundant in the vicinity of skin glands, where they metabolise the skin gland secretions (Figure 2.1).

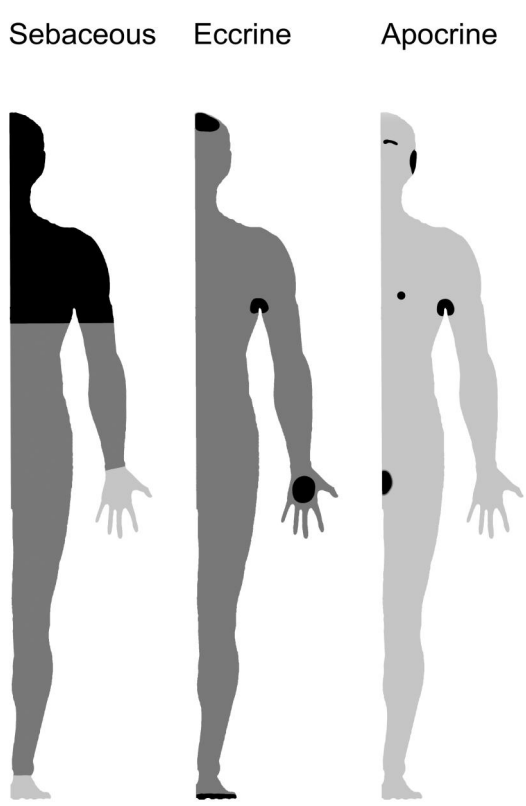


Figure 2.1 Distribution of sebaceous, eccrine and apocrine glands on the human skin surface.

Colours indicate gland densities (light gray = no glands present, dark gray = low densities, black = high densities) (modified after Wilson 2008).

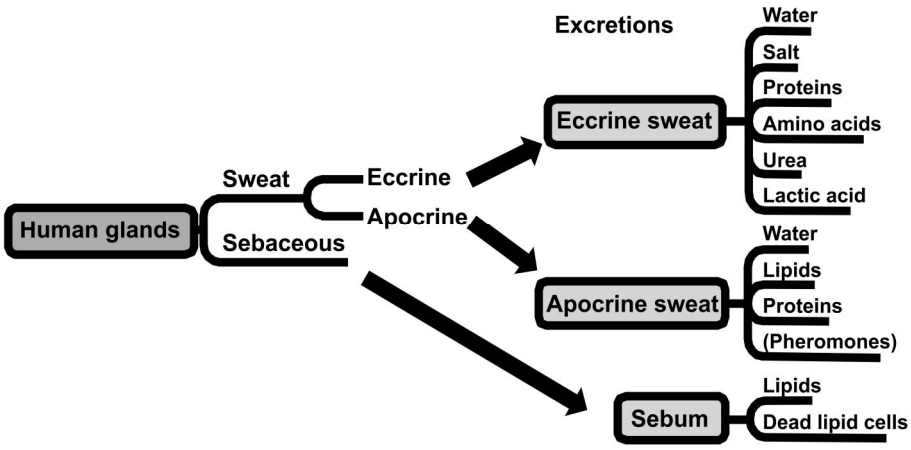


Figure 2.2 Human skin glands and their most important excretions.

(): no convincing evidence

Human skin glands can be divided into sebaceous and sweat glands (Figure 2.2). Sebaceous glands produce sebum, which consists of lipids and dead cells (Figures 2.1 & 2.2) (Stoddart 1990). Sweat glands comprise eccrine and apocrine glands, and produce mainly water (Figures 2.1 & 2.2). Eccrine glands are located all over the body (Figure 2.1) and produce sweat to cool the body by evaporative heat loss when its temperature increases. Apocrine glands are mainly found in the axillary region, and are hypothesized to play a role in human pheromone production (Stoddart 1990). When fresh apocrine secretions are sterilized, they are odourless (Shelley et al. 1953).

To identify which bacteria are responsible for the production of human odour, Leyden et al. (1981) compared the microbiology of the axilla of 229 human subjects with the intensity of their axillary odour. Corynebacteria were found to be responsible for the typical apocrine odour (Leyden et al. 1981). This was confirmed by other studies (Jackman and Noble 1983, Rennie et al. 1991, Taylor et al. 2003), although an association with the total number of aerobic bacteria and the number of micrococci on the skin was also suggested (Taylor et al. 2003).

Most studies have investigated the relation between axillary odour and microbiota composition and therefore focused on apocrine sweat. Feet are a major source of human body odour and contain high numbers of eccrine glands (Figure 2.1). Increased foot odour is associated with higher population

densities of microorganisms with lipase and proteinase activity (Marshall et al. 1987) and/or higher numbers of *Bacillus* species (Ara et al. 2006). The human scalp has a high density of sebaceous glands (Sastry et al. 1980). Sebaceous glands contain numerous propionibacteria that hydrolyze triglycerides into volatile fatty acids (Kearney et al. 1984).

After a correlation between odour production and bacterial composition was recorded, *in vitro* studies further revealed the underlying mechanisms of odour production by specific bacterial species. Gower et al. (1994) showed that 5α-androstenone, which is suggested to contribute to axillary odour in men, is produced by two coryneform bacterial strains when supplied *in vitro* with four non-volatile 16-androstenes. Corynebacteria also play a pivotal role in the generation of volatile fatty acids, which are associated with malodour (James et al. 2004a). When skin lipids are catabolized into long chain fatty acids, it seems that only corynebacteria are capable of transforming these long chain fatty acids into short and medium-chain fatty acids (C2-C11), causing malodour (James et al. 2004a). Brevibacteria and micrococci metabolize these short and medium-chain fatty acids even further (James et al. 2004a). Another example of the specificity of biotransformations by certain bacterial species is the conversion of branched chain amino acids. *Staphylococcus* species, but not *Corynebacterium* species, can convert these amino acids to highly odorous short-chain amino acids (C4-C5) (James et al. 2004a). The above examples illustrate that different species of skin bacteria each have their own specific metabolism and, therefore, generate a characteristic odour profile.

The complete genomes of the skin bacterial species *Bacillus subtilis* (Kunst et al. 1997), *Corynebacterium jeikeium* (Tauch et al. 2005), *Propionibacterium acnes* (Bruggemann et al. 2004) and *Staphylococcus aureus* (Kuroda et al. 2001) have been sequenced. A next step will be to link these sequences to pathways and mechanisms of odour production by these bacteria on the human skin.

Bacterial enzymes involved in odour production, by converting non-volatile compounds into volatile compounds, have been identified and their genes brought to expression in *Escherichia coli* (Natsch et al. 2003, Natsch et al. 2004). Specific odour-producing enzymes can be blocked by inhibitors, which have a higher affinity for the enzyme than the original substrate (Ara et al. 2006).

Another group of components involved in odour production on the

human skin involves odour-binding proteins. In the apocrine glands they transport odour molecules to the skin surface (Spielman et al. 1995, Zeng et al. 1996). When the amount of these Apocrine Secretion Odour-Binding (ASOB) proteins was measured on the skin of individuals of Chinese and non-Chinese descent, a lower amount of protein was detected on the panellists of Chinese ancestry (Jacoby et al. 2004). This may result in lower body odour production.

Research on human body odour is often focused on a specific group of odours or pathways, often related to malodour. Studies that aimed at analyzing all components of human body odour result in long lists of chemicals, often dependent on the methods used for collection and analysis of the body odours. Bernier et al. (1999, 2000) analysed human skin emanations collected on glass beads and reported 346 compounds including carboxylic acids, alcohols, esters, aldehydes, aliphatics, aromatics and ketones. The majority of the intense peaks in the chromatograms were fatty acids and the pattern of these peaks appeared to be similar to that observed from bacteria that convert triglycerides on the skin into these fatty acids (Bernier et al. 2000). The collection and analysis of skin emanations through direct skin contact result in many compounds, often non-volatile (Bernier et al. 2000, Penn et al. 2007). The use of solid phase micro-extraction (SPME) or Dynamic Headspace Sampling (DHS) to analyze compounds released from human skin limits the results to volatile compounds and in these studies often fewer, but also different compounds are detected than in studies analyzing skin emanations through direct skin contact (Curran et al. 2005, Gallagher et al. 2008).

One of the methods commonly used to determine microbial profiles is denaturing gradient gel electrophoresis (DGGE) analysis, which allows for DNA fingerprinting of complex microbial communities. Comparing this microbial profile of the skin with its chemical profile shows a correlation, but only when the subjects strictly followed some basic rules of behaviour, like no deodorant use for 48 hr and wearing a t-shirt provided by the researchers (Xu et al. 2007). The importance of behavioural rules for the volunteers indicates that, in addition to microbial composition, also environmental factors such as grooming habits and diet influence the formation of human body odour (Xu et al. 2007, Havlicek and Lenochova 2008).

Genetic origin of human odour

The human odour profile is, at least partly, genetically based. By smell, humans can match monozygotic twins, but not dizygotic twins, based on their body odours even when the twins are living apart (Wallace 1977, Roberts et al. 2005). Humans are able to discriminate their relatives from non-relatives, based on their odour (Porter et al. 1985, Weisfeld et al. 2003). This kin recognition may indicate a genetic basis for the human odour profile, although the exact mechanism remains unknown (Lenochova and Havlicek 2008).

The volatile compounds released by monozygotic twins can also be matched based on qualitative and quantitative similarities (Sommerville 1994). Recently, Kuhn and Natsch (2009) compared the body odour of monozygotic twins using two-dimensional GC, focusing on volatile carboxylic acids known to be the principal components of body odour, and released by skin bacteria. First, a fresh sweat sample was taken from the axilla of the individuals. Next, to induce production of volatile odours from the fresh sweat sample, a recombinant enzyme from axilla bacteria was used instead of skin bacteria themselves. In this way the influence of personal grooming habits on the bacterial population of the skin was ruled out. The study showed a clear and strong contribution of genetic factors to the relative pattern of volatile fatty acids released and the importance of bacterial enzymes in the production of volatile odorants from fresh sweat samples (Kuhn and Natsch 2009).

In mice, genes of the Major Histocompatibility Complex (MHC) have been shown to influence body odour (Yamazaki et al. 1976, Penn and Potts 1998b) and several studies have tried to link MHC genes to body odour in humans. The genes of the MHC complex encode antigens, which are involved in the immune system. When humans had to judge the body odours coming from worn T-shirts, they preferred body odours from persons with another MHC gene profile (Wedekind and Furi 1997, Wedekind and Penn 2000). This leads to the conclusion that individuals have a distinct body-odour type, which is determined, at least partly, by their inherited MHC alleles.

In 1998, Penn and Potts (1998a) reviewed the available data on the connection between MHC genes and body odour and summarized the possible explanations on how these genes might influence body odour. They combined the hypothesis that MHC genes influence odour by shaping the commensal microbiota (Howard 1977) and the hypothesis that converted MHC molecules transport aromatic molecules (Pearse-Pratt et al. 1992). It was hypothesized that MHC molecules bind allele-specific subsets of peptides,

from which metabolites are volatilized by the activity of the commensal microorganisms (Penn and Potts 1998a). In an experiment to test this hypothesis, the gas chromatography-mass spectrometry (GC-MS) analysis of the axillary odour of 18 volunteers revealed a number of compounds, including 3-methylbutanal. This compound was noted as a potential link to the human leukocyte antigen (HLA) genes (Savelev et al. 2008), which are the genes located in the MHC region of humans, encoding for specific antigens. An *in-vitro* experiment was conducted with axillary bacteria from individuals with high or low abundance of 3-methylbutanal. Media with two different HLA peptides were incubated with the bacteria of individuals with a high or low production of 3-methylbutanal in their armpit. The results showed that different HLA peptides can alter the production of 3-methylbutanal by skin bacteria and that the microbial populations themselves influence the production of 3-methylbutanal (Savelev et al. 2008).

Inter- and intra-personal diversity in skin microbiota

As discussed, odour production on the human skin is in part a function of the composition of the skin microbiota. Because the human odour profile is at least partly genetically based, it can be expected that intrapersonal variation in the microbiota composition over time is lower than interpersonal variation. This has indeed been shown for the composition of faecal microbiota (Holdeman et al. 1976, Eckburg et al. 2005), but studies that investigated intra- and interpersonal variation of skin microorganisms have been rare.

Recently, the human microbial community was studied in more detail, using 16S rRNA gene sequencing. This technique allows for the identification of more bacterial species than is possible with culture-based methods alone (Gao et al. 2007, Grice et al. 2008) and it allows for a better overview of the composition of the human microbial community. On the surface of the average hand palm more than 150 bacterial species were found (Fierer et al. 2008) and 88 on the inner-elbow (Grice et al. 2008). The variation in composition of bacterial communities on the human skin is often high and these communities are more diverse than bacterial communities found in throat, stomach and faecal environments (Fierer et al. 2008, Costello et al. 2009).

In several studies using 16S rRNA gene sequencing, the variation between individuals and the temporal stability of the skin microflora have been investigated with various results. No personal bacterial profile was found when the bacterial variation between the left and right inner elbow of an individual

was compared with the left and right inner elbow of different individuals (Grice et al. 2008) and also no longitudal stability of the microbiota was found when samples from the forearm of four volunteers were taken 8 to 10 months apart (Gao et al. 2007). Other studies, however, showed that the variation between left and right body parts of the same individual was lower than the interpersonal variation (Gao et al. 2007, Fierer et al. 2008, Grice et al. 2009), and sampling multiple skin sites at multiple time points showed that the variation in skin microbiota between individuals was higher than within an individual (Costello et al. 2009, Grice et al. 2009). In addition to this, individuals can be identified by the bacterial traces they leave on objects like a computer keyboard or mouse (Fierer et al. 2010).

These studies, using high throughput molecular techniques, all showed a high bacterial diversity and indicate that the composition of bacterial communities on the human skin depend on skin site characteristics. Detailed studies with multiple sample sites and time points clearly indicate that humans have an individual microbial skin composition that is relatively stable over time (Costello et al. 2009, Grice et al. 2009).

Volatiles of human skin bacteria and vector-host interactions

Several insect families act as vectors of harmful diseases such as malaria, leishmaniasis, river blindness and West Nile virus (Takken and Knols 1999, Resh et al. 2004, Reisen et al. 2005, Kamhawi 2006).These vectors locate their blood hosts, including humans, from a distance using olfactory cues produced by their hosts (Takken and Knols 1999, Logan and Birkett 2007). Recent studies demonstrated the role of skin microbiota in interactions between disease vectors and their blood hosts (Braks and Takken 1999, Verhulst et al. 2009, Ortiz and Molina 2010) and the knowledge on human skin microbiota reviewed in this paper sheds further light on these interactions.

As an example, and because of recent discoveries on its olfactory behaviour, we focus on the malaria vector *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*). This mosquito species is anthropophilic and its host seeking is mainly accomplished by odour-mediated anemotaxis in which human odours provide essential cues (Takken and Knols 1999). Many of the volatiles to which these mosquitoes respond are of bacterial origin. This became evident when washing human feet with a bactericidal soap proved to significantly alter the biting-site selection of *An. gambiae* females on a motionless naked volunteer (de Jong and Knols 1995).

In addition, human eccrine sweat is attractive to *An. gambiae* after incubation with skin bacteria for one or two days (Braks and Takken 1999). Moreover, a recent study showed that the volatiles produced by human skin bacteria *in vitro* are attractive to *An. gambiae* when grown on agar plates (Verhulst et al. 2009). The headspace volatiles from cultures of these bacteria were identified and a synthetic blend was developed that attracted females of this mosquito species (Verhulst et al. 2009). However, it is not clear yet whether bacteria isolated from less-attractive individuals produce these volatiles, whether other substrates can enhance the production of attractive volatiles and whether the production of these attractive volatiles is specific to bacteria species present on human skin only. These important issues deserve being addressed in future studies.

Further research on the role of skin microbiota in the host-seeking behaviour of mosquitoes can possibly reveal the evolutionary mechanisms of vector-host interactions in general. *Rhodnius prolixus* Stål (Hemiptera: Reduviidae: Triatominae), for example, is the vector of Chagas disease and its host-seeking behaviour is influenced by host odours (Guerenstein and Lazzari 2009). Odours produced by bacteria also seem to play an important role for *R. prolixus*, since an anti-bacterial gel reduced the attractiveness of skin washings to this vector (Ortiz and Molina 2010).

The discovery that volatiles produced by human skin microorganisms *in vitro* mediate *An. gambiae* host-seeking behaviour creates new opportunities for the control of this mosquito species. Identifying the volatiles in the headspace of bacterial cultures, or mass production of the bacteria themselves, may lead to the development of new odour-baited trapping systems. African malaria mosquitoes and men have co-evolved for thousands of years, and this process may have led to the development of microorganisms on the human skin that repel mosquitoes. Identification of these repellent microorganisms and the volatiles that they produce can lead to the development of new repellent products. Blockage of odour-producing bacteria or specific odour-producing enzymes in bacteria (Ara et al. 2006) can possibly affect the attractiveness of a person to mosquitoes.

For most vector-borne diseases, the primary factor influencing transmission is human exposure to bites of infected vectors (Téllez 2005). Humans differ in their attractiveness to mosquitoes and these differences remain relatively stable over time (Schreck et al. 1990, Lindsay et al. 1993, Knols et al. 1995, Bernier et al. 2002, Mukabana et al. 2002, Qiu et al. 2004a,

Qiu et al. 2006, Logan et al. 2008). Because the human skin microbiota plays an important role in the production of body odours and is attractive to *An. gambiae* when grown on agar plates, a correlation between skin microbiota composition and a person's attractiveness to mosquitoes can be expected. Within humans this may lead to selection for skin bacteria that are less attractive or even repellent to mosquitoes.

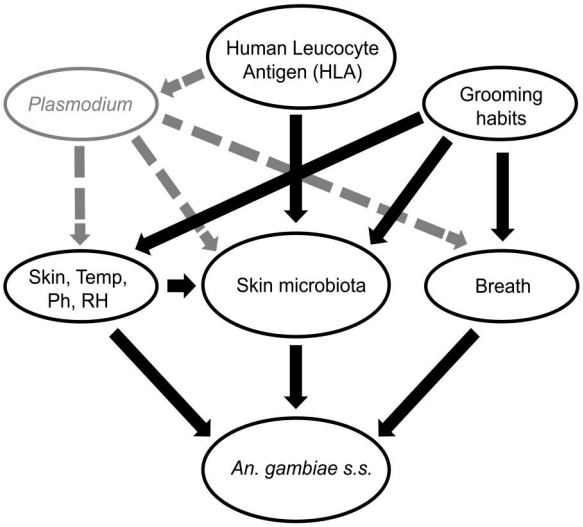


Figure 2.3 Hypothetical model of mosquito-host interactions and the role of skin microbiota (Modified after Knols 1996). *The most important factors involved are shown. Dashed lines indicate the effects when the host is infected with Plasmodium parasites. RH: Relative Humidity.*

Human attractiveness to mosquitoes may change when individuals are infected with *Plasmodium* parasites that cause malaria (Lacroix et al. 2005, Mukabana et al. 2007). Children harbouring *Plasmodium* gametocytes attracted about twice as a many mosquitoes as children without infection or with parasites in the asexual stage (Lacroix et al. 2005). This increased attractiveness of infected children could be explained by an increase in body temperature, increased perspiration or by a change in breath composition. However, these factors are less likely to be involved in the observed change in attractiveness, as the infection was asymptomatic in all of the children involved (Lacroix et al. 2005). It remains to be investigated whether an infection with *Plasmodium* affects the composition and/or number of skin bacteria. If so, it might be an additional explanation for the increase in attractiveness to malaria mosquitoes (Figure 2.3).

Concluding remarks and future directions

The microbiota on the human skin plays a major role in body odour production. Body odour is used by disease vectors to locate their blood hosts and differential attractiveness of humans to mosquitoes may be explained by individual variation in microbial composition on the skin. Differences in observed human attractiveness and a correlation with microbiota composition can be exploited to mediate vector behaviour as a tool for disease control. Bacteria like corynebacteria and brevibacteria can have their own specific metabolism and, therefore, a characteristic odour profile. Recent technological advances revealed pathways and mechanisms of odour production on human skin, and the contribution of specific microorganisms to this odour production. Next, parts of the genome sequences of skin bacteria need to be linked to these pathways. By screening for these specific sequences, the role and significance in odour production of the different bacteria on the human skin can be determined and will reveal their role in the attraction of humans to mosquitoes.

Several studies have indicated a link between HLA genes and the human odour profile (Wedekind and Furi 1997, Wedekind and Penn 2000, Savelev et al. 2008), although the evidence is not fully convincing yet. These HLA studies focused on the axillae as these are suggested to play a role in human pheromone production from the apocrine glands. If HLA genes influence human body odour produced by eccrine glands and not apocrine glands, HLA-determined odours might be easier to detect from other body parts, because apocrine glands are mainly found in the axillae.

Molecular studies have mapped the human microbiota community in detail (Gao et al. 2007, Fierer et al. 2008, Grice et al. 2008, Costello et al. 2009, Grice et al. 2009) and the recently launched Human Microbiome Project (http://www.hmpdacc.org/resource.php) will further characterize the human microbiota. Several studies have examined the stability of the human microbial population over time (Gao et al. 2007, Costello et al. 2009, Grice et al. 2009). The results show that a large number of volunteers and many time points are essential in these studies. To be able to link the human odour profile with its skin microbiota, sequencing the 16S rRNA gene fragments may not give the desired results since these genes are not involved in the metabolism of the bacteria. Sequencing the genome of the skin microbiota and blasting these sequences can indicate which genes involved in certain metabolic pathways are present. Whole transcriptome sequencing involves mRNA, which reflects

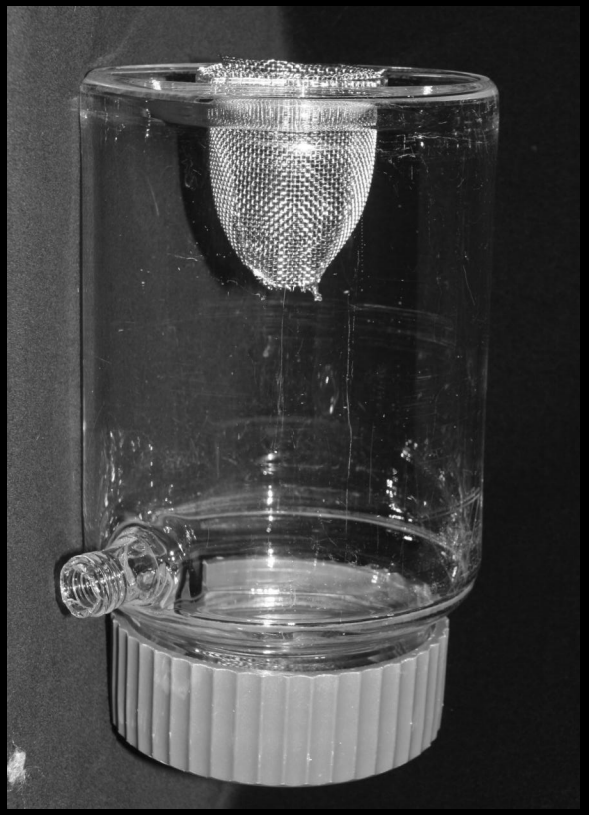
genes that are actively expressed and codes for enzymes that regulate the metabolic activity of the skin microbiota at the time of sampling. At the moment the known metabolic pathways and links to genes are limited and the methods still costly (Zoetendal et al. 2006, Turnbaugh et al. 2007, Vrieze et al. 2010). To reveal a possible relationship between the human genome and the bacterial composition on the skin and the odour profile, blood samples for HLA analysis, detailed mapping of the human skin microbiota and odour samples for GC-MS analysis are required.

More detailed knowledge on how genes, human skin microbiota and human body odour are related can not only help to understand and develop treatment of skin diseases, but possibly also unravel the evolutionary mechanisms behind the host-seeking behaviour of mosquitoes and other blood-feeding insects and lead to novel means of vector-borne disease control.

Acknowledgements

This work was supported in part by a grant from the Foundation for the National Institutes of Health (NIH) through the Grand Challenges in Global Health Initiative (GCGH#121).

Structural design olfactometers



Structural design affects entry response of mosquitoes in olfactometers

03

Niels O. Verhulst, Willem Takken, Renate C. Smallegange

Host-seeking of the malaria mosquito *Anopheles gambiae sensu stricto* is mediated by human odours. Dual-choice olfactometers are used to identify attractive odour components. We examined whether an olfactometer equipped with a funnel as trap entry would catch more mosquitoes than when with a baffle. When tested directly against each other using a human skin extract as stimulus, the funnel caught more mosquitoes in the first of the two experiments performed. In the second experiment, worn socks were used as an attractive odour source instead of human skin washings. This experiment showed that the total response of mosquitoes was significantly higher when funnels were used in both trapping devices than when baffles were used. Increasing the total number of mosquitoes trapped by using a funnel, can make it easier to distinguish which odour is more attractive when odours are tested that only differ little in their attractiveness.

Published in: *Proceedings of the Netherlands Entomological Society meeting* 19: 129-136

Introduction

Odours play an important role in the host seeking of mosquitoes (Takken and Knols 1999). The host-seeking flight of a mosquito can roughly be divided into long range and short range attraction. Long distance attraction is mainly determined by olfactory stimuli, whereas attraction from a short distance is mediated by odours and non-olfactory cues, such as heat, body moisture and visual cues (Gillies and Wilkes 1969, Takken 1991). The malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) (henceforth termed *An. gambiae*), however is nocturnal and therefore visual cues are of minor importance. *An. gambiae* is highly attracted to odours emitted from the human skin (Takken and Knols 1999).

Identifying odours that are used by mosquitoes to locate their host can be important in the development of odour-baited traps. Windtunnels and olfactometers have long been used to test the behavioural response of mosquitoes to odours (Acree et al. 1968, Eiras and Jepson 1991, Geier and Boeckh 1997). In 1994, Knols et al. developed an dual-choice olfactometer to test the behavioural response of *An. gambiae* (Figure 3.1), which was the first effective 2-choice system available for studying the behavioural response of this mosquito species. Since then, numerous experiments have shown the effectiveness of the setup in testing the olfactory response of *An. gambiae* to individual odours (Smallegange et al. 2005), blends of odours (Knols and

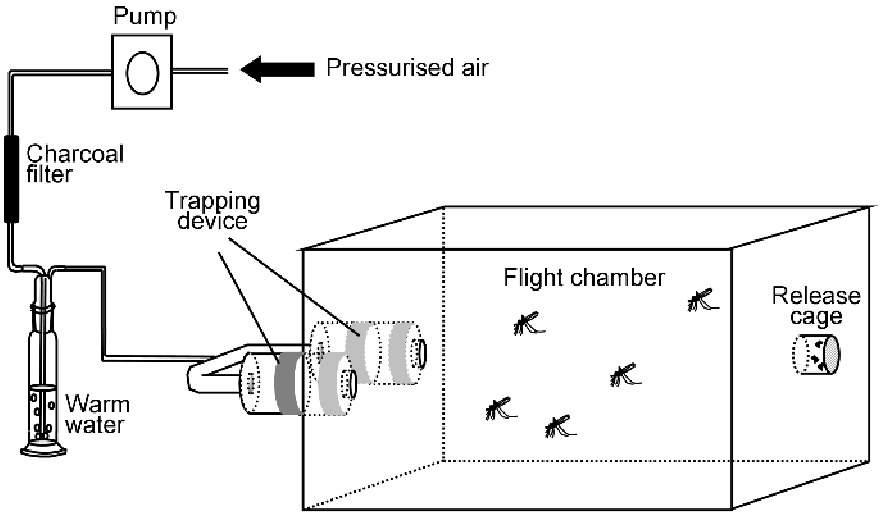


Figure 3.1. A diagram of the dual-choice olfactometer (modified after Pates et al. 2001)

Meijerink 1997, Smallegange et al. 2005) and human skin emanations (Pates 2002, Qiu et al. 2004a, Qiu et al. 2006). However, since 1994 the olfactometer has not been changed in order to increase the effectiveness of the system, or to test why it is effective. Increasing the discriminative power of the system will increase the efficiency of experiments, especially when tested odours differ only slightly in their attractiveness.

It was tested if the use of a funnel instead of a baffle as trap entry in the olfactometer would increase the efficiency of the trapping system. Human skin washing samples and worn socks have shown to be an attractive odour source to *An. gambiae* and were used as a standard odour source (Pates et al. 2001, Pates 2002, Qiu 2005, Agtmaal van 2006).

Materials and Methods

Insects

The *An. gambiae* colony at Wageningen University, The Netherlands, originated from Suakoko, Liberia. The mosquitoes have been cultured in the laboratory since 1988 with blood meals from a human arm twice a week. The adult mosquitoes were maintained in 30 x 30 x 30 cm gauze-covered cages in a climate-controlled room (27 ± 1°C, 80 ± 5% RH, LD 12:12). They had access to a 6% (v/v) glucose solution on filter paper. Eggs were laid on wet filter paper, emerged in tap water in plastic trays and fed daily with Tetramin® baby fish food. Pupae were collected daily and placed in adult cages for emergence.

Olfactometer

A dual-port olfactometer (Figure 3.1) (Knols et al. 1994, Braks and Takken 1999) was used for the experiments. Pressurized air was charcoal filtered, humidified and led through two glass mosquito trapping devices, which were linked to two ports (diameter 5 cm, 30 cm apart). Trapping devices were equipped with either a baffle or a funnel (Figure 3.2), depending on the experiment. The air entered the flight chamber (1.60 x 0.66 x 0.43 m) with a speed of 0.21 ± 0.01 m/s, temperature of 28.5 ± 0.8°C and relative humidity above 80%. The experimental room was maintained at a temperature of 26.6 ± 0.7°C and a relative humidity of 63.6 ± 2.7%.

Experiments were prepared and performed according to the methods described by Smallegange et al. (2005). For each experiment thirty female mosquitoes of 5-8 days old, which had not received a blood meal, were

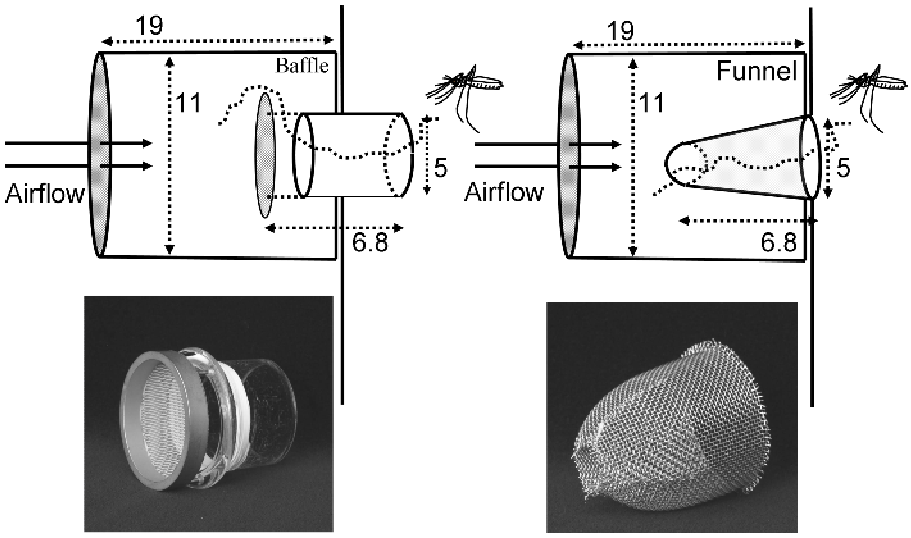


Figure 3.2. Schematic drawing and picture of a trapping device for the olfactometer equipped with a baffle or funnel. *All dimensions are in centimeters.*

selected 14-18 hours before and placed in a cylindrical release cage (d = 8, h = 10 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the dark period, when *An. gambiae* females are known to be more responsive to host-odours (Maxwell et al. 1998, Killeen et al. 2006). In each trial test odours were released in the air stream before a group of mosquitoes was set free from a cage which was placed at the downwind end of the flight chamber, 1.60m from the two ports. Mosquitoes were left in the flight chamber for 15 min. The female mosquitoes that had entered either trapping device were counted at the end of the experiments, after anaesthetization with 100% CO_2. Mosquitoes remaining in the flight chamber were removed with a vacuum cleaner. Each trial started with new mosquitoes, clean trapping devices and new stimuli. Experiments were repeated six times on different days. The sequence of treatments was randomized on the same day and between days. Treatments were alternated between right and left ports in different replicates to rule out any positional effects. Surgical gloves were worn by the experimenter to avoid contamination of the equipment with human volatiles.

Odour sources

Previous research showed that human skin washing with ethanol is a reliable mosquito attractant (Pates 2002). Human skin washing samples were collected, since it has the advantage that it can be used in the behavioural bioassay as a standard attractive odour source. Cotton wool pads with ethanol were used to rub both hands, under the arm pits and feet of a human subject (female, 39 years old) for two minutes. Twenty five cotton wool pads containing human skin emanations were packed in a glass column (1 m long) and eluted with 550 ml absolute ethanol. The ethanol elution was concentrated from 190 ml to 27 ml using a rotating vacuum evaporator, 55°C and 625 mBar. The concentrated skin washing samples were decimally diluted in ethanol and stored at -20 °C until use.

Before each olfactometer experiment 100µl of diluted (1:1000) human skin washing sample was pipetted onto a sand blasted glass slide (10 x 2.5 cm) (Braks and Takken 1999). After evaporation of the ethanol, slides were placed in both trapping devices and mosquitoes released. Previous experiments showed that a dilution of the skin washing sample of 1:1000 was most attractive for *An. gambiae* in the same olfactometer (Agtmaal van 2006).

Worn socks were also used as odour source. Nylon socks (Hema, The Netherlands) were worn for 24 hours by a human volunteer (Male, 28 years old). The volunteer refrained from alcohol, garlic and spicy food for 24 hours. The last shower was without soap and the volunteer did not use any soap, deodorant or perfume when wearing the socks. Socks were stored at -20°C until use.

Experiment 1

It was examined whether using a funnel instead of a baffle would increase the number of mosquitoes caught in the trapping device. The trapping devices in the olfactometer were equipped with either a baffle or funnel at the point of entry. Baffles and funnels were tested directly against each other in the olfactometer to compare trap entry response. Also tests with only baffles or only funnels were done to compare the total trap entry response. Skin washing samples with a dilution of 1:1000 were used as an attractant. As a control, a worn sock was tested against a clean sock in trapping devices with baffles. Clean air experiments were done to determine trap entry response when no odour was present. Experiments were repeated six times on different days. Each trial started with new mosquitoes.

Experiment 2

The experimental setup was equal to that of experiment 1, with the exception that worn socks were used as an attractant instead of skin washing samples. Experiments with baffles and worn socks or funnels and worn socks were performed twice each day. Experiments during which a baffle and a funnel were tested directly against each other, were only performed once a day, as were the control experiments (see experiment 1). Experiments were performed for five days.

Statistics

For each two-choice test in the olfactometer a χ^2-test was used to analyze whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution. A Generalized Linear Model with binomial function (GLM; Genstat for Windows, release 9.2) was used to investigate the effect of treatments on the trap entry response, which is defined as the number of female mosquitoes caught in both trapping devices as the percentage of mosquitoes that flew out of the release cage.

Results

In the first experiment, when a baffle and funnel were tested directly against each other, the trapping device with a funnel caught significantly more mosquitoes than the one with a baffle (χ^2-test, d.f. = 5, $P < 0.001$) (Figure 3.3). A worn sock caught significantly more mosquitoes than a clean sock (χ^2-test, d.f. = 5, $P < 0.001$). The total response was significantly higher when a sock was used compared to the other treatments (GLM, d.f. = 5, $P < 0.05$). No significant effect was found of skin washings, baffles or funnels on the total response (Figure 3.3). The experiments with clean air only showed that the trapping system was symmetrical with a total response of 21-24 %.

In the second experiment, no significant difference between a baffle and a funnel was found when they were tested directly against each other (χ^2-test, d.f. = 4, $P = 0.20$) (Figure 3.4). Again a worn sock caught significantly more mosquitoes than a clean sock (χ^2-test, d.f. = 4, $P < 0.001$). The total response was significantly higher when funnels were used in both trapping devices than when baffles were used (GLM, d.f. = 4, $P < 0.05$) (Figure 3.4).

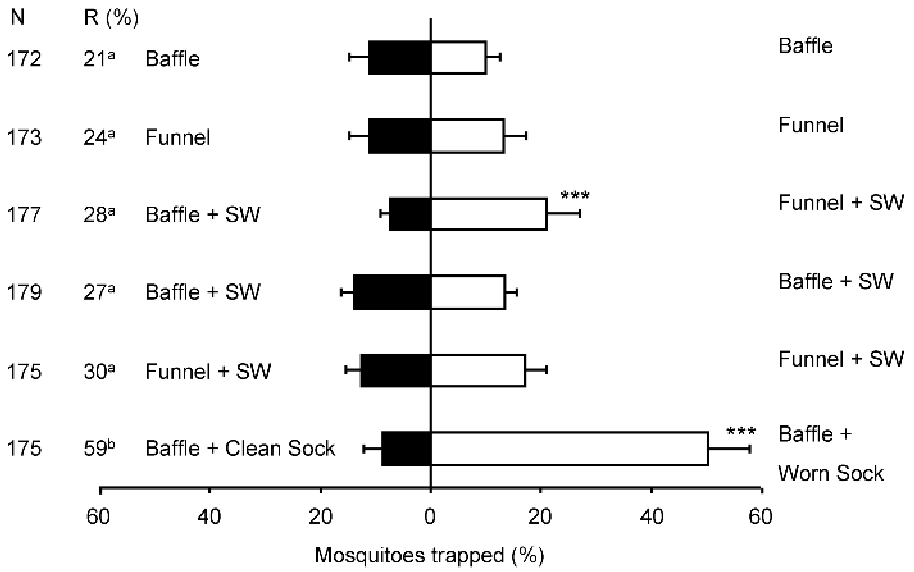


Figure 3.3. Average response of* An. gambiae *when trapping devices were equipped with baffles or funnels.

*Skin washings (SW) or worn socks were used as an attractant. Error bars represent standard errors of the mean; ***: χ^2-test P < 0.001. N = number of mosquitoes released. R = Total response in both trapping devices, data not sharing the same superscript letter differ significantly (GLM, P < 0.05).*

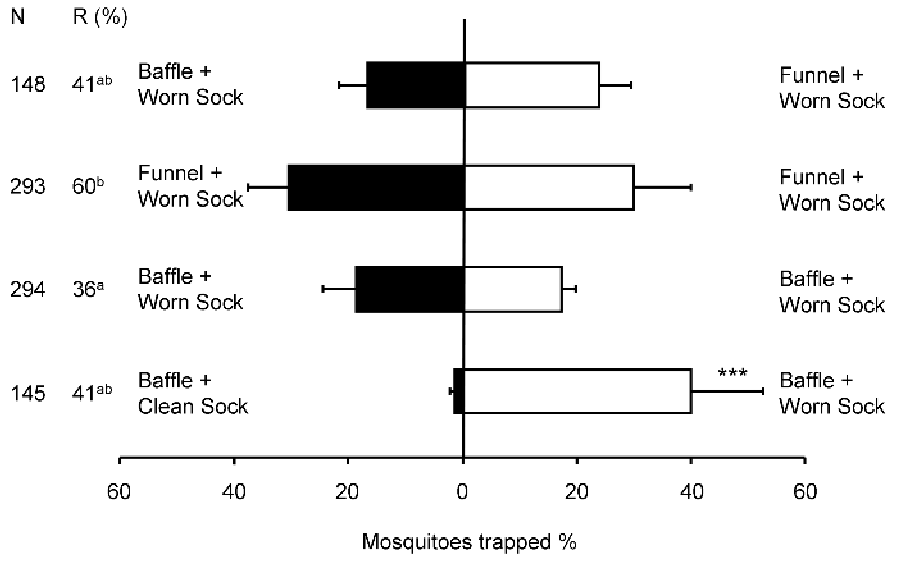


Figure 3.4 Average response of* An. gambiae *when trapping devices were equipped with baffles or funnels.

*Worn socks were used as an attractant. Error bars represent standard errors of the mean; ***: χ^2-test P < 0.001. N = number of mosquitoes released. R = Total response in both trapping devices, data not sharing the same superscript letter differ significantly (GLM, P<0.05).*

Discussion

When baffles and funnels were tested directly against each other, the two experiments showed conflicting results. In the first experiment, the trapping device with a funnel caught significantly more mosquitoes than the trapping device equipped with a baffle. In the second experiment no significant difference was found between the two types of trap entrance. The only difference between the experiments was the use of skin washings in the first experiment and worn socks in the second experiment. Worn socks were highly attractive, skin washings were not. Maybe this shows the discriminative power of a funnel when an odour is only slightly attractive. Previous experiments have shown that skin washing samples can be attractive to *An. gambiae* (Pates 2002, Agtmaal van 2006), whereas our results did not show any attractiveness of the samples. Although van Agtmaal (2006) showed that a dilution of 1:1000 of the original sample showed maximum attractiveness, storage at -20ºC might have had a negative influence on the concentration or composition of the original sample. Further research should indicate what affected the skin washing samples and what made them scarcely attractive to *An. gambiae*.

Because the first experiment showed that skin washing samples were not attractive, worn socks were used in the second experiment to increase the total number of mosquitoes caught. In this second experiment, the total number of mosquitoes caught when using a funnel was significantly higher than when using a baffle. Thus, trapping devices equipped with a funnel-like entrance are more efficient than traps equipped with a baffle. When odours are tested which only differ little in their attractiveness, increasing the total number of mosquitoes trapped by using a baffle can make it easier to distinguish which odour is most attractive. Another advantage of the funnel is that it is easier to fit in the tunnel and less breakable than the glass baffle. Research on odours that influence the host seeking behaviour of mosquitoes should lead to a trap which can be used to trap and/or monitor mosquitoes in the field. When developing traps, our results indicate that funnels might be better to use than baffles to trap *An. gambiae*.

Acknowledgements

We are grateful to F. K. M. van Aggelen, A. J. Gidding, L. Koopman for assistance with mosquito rearing and Yu Tong Qiu for providing skin washing samples. This project was funded by the Bill & Melinda Gates Foundation (GCGH#121).

Cultured skin microbiota attracts mosquitoes

Cultured skin microbiota attracts malaria mosquitoes

04

Niels O Verhulst, Hans Beijleveld, Bart GJ Knols, Willem Takken, Gosse Schraa, Harro J Bouwmeester, Renate C Smallegange

Host-seeking of the African malaria mosquito, *Anopheles gambiae sensu stricto*, is guided by human odours. The precise nature of the odours, and the composition of attractive blends of volatiles, remains largely unknown. Skin microbiota plays an important role in the production of human body odours. It is hypothesized that host attractiveness and selection of *An. gambiae* is affected by the species composition, density, and metabolic activity of the skin microbiota. A study is presented in which the production and constituency of volatile organic compounds (VOCs) by human skin microbiota is examined and the behavioural responses of *An. gambiae* to VOCs from skin microbiota are investigated.

Blood agar plates incubated with skin microbiota from human feet or with a reference strain of *Staphylococcus epidermidis* were tested for their attractiveness to *An. gambiae* in olfactometer bioassays and indoor trapping experiments. Entrained air collected from blood agar plates incubated with natural skin microbiota or with *S. epidermidis* were analysed using GC-MS. A synthetic blend of the compounds identified was tested for its attractiveness to *An. gambiae*. Behavioural data were analysed by a χ^2-test and GLM. GC-MS results were analysed by fitting an exponential regression line to test the effect of the concentration of bacteria.

More *An. gambiae* were caught with blood agar plates incubated with skin bacteria than with sterile blood agar plates, with a significant effect of incubation time and dilution of the skin microbiota. When bacteria from the feet of four other volunteers were tested, similar effects were found. Fourteen putative attractants were found in the headspace of the skin bacteria. A synthetic blend of 10 of these was attractive to *An. gambiae*.

The discovery that volatiles produced by human skin microorganisms *in vitro* mediate *An. gambiae* host-seeking behaviour creates new opportunities for the development of odour-baited trapping systems. Additionally, identification of bacterial volatiles provides a new method to develop synthetic blends, attractive to *An. gambiae* and possibly other anthropophilic disease vectors.

Published in: *Malaria Journal* 8: 302.

Introduction

The African malaria mosquito *Anopheles gambiae sensu stricto* (hereafter referred to as *An. gambiae*) preferably feeds on human beings inside houses and is therefore one of the most effective vectors of *Plasmodium* malaria parasites (Takken and Knols 1999). Although visual and physical cues play a role in the host-seeking behaviour of *An. gambiae*, host-seeking is mainly accomplished by odour-mediated anemotaxis in which volatile organic compounds (VOCs) of human origin provide essential cues (Takken and Knols 1999). Humans are differentially attractive to mosquitoes because of the odours they emit (Kelly 2001, Qiu et al. 2006, Logan et al. 2008). The skin microbiota plays an important role in the production of human body odours (Leyden et al. 1981) and without bacteria human sweat is odourless (Shelley et al. 1953). Many volatile compounds seem to be widespread among bacteria, although others are strain-specific. Some strains can produce up to 60 different volatile compounds (Kai et al. 2009). Differences in foot odour production can be explained by micro-floral differences between humans (Marshall et al. 1988) and a recent study by Xu et al. (2007) provides more evidence that there is a connection between the microbial composition on human skin and chemical signature of humans. If host-selection by *An. gambiae* is based on the species composition, metabolic activity and/or density of the skin microbiota, then this will bear a direct impact on the number of bites received per person and the resulting risk of infection (Takken and Knols 1999). The non-random nature of host selection remains poorly understood, yet has an important impact on exposure to disease (Smith et al. 2006).

A study on Limburger cheese volatiles revealed the putative role of bacteria in mosquito olfaction (Knols and De Jong 1996, Knols et al. 1997). Knols et al. (1997) suggested that bacteria involved in the ripening of Limburger cheese may have originated from human skin and hence that these bacteria are responsible for the production of 'human-specific' VOCs that mediate the host-seeking process of malaria mosquitoes. Washing the feet with a bactericidal soap significantly altered the selection of biting sites of *An. gambiae* on a motionless naked volunteer (Knols and Meijerink 1997). In addition, human eccrine sweat is attractive to *An. gambiae*, but only after incubation for one or two days (Braks and Takken 1999). Microorganisms on the skin are responsible for the conversion of fresh sweat into sweat attractive to *An. gambiae* (Shelley et al. 1953, Braks and Takken 1999).

The human odour profile consists of more than 350 compounds (Bernier et al. 2000, Meijerink et al. 2000). It was examined whether human skin microbiota produces attractive VOCs (kairomones) (Dicke and Sabelis 1988) for *An. gambiae* when cultured *in vitro* and whether analysis of entrained odours collected from these microorganisms can lead to a synthetic blend attractive to *An. gambiae*. In this paper an attractant is defined as a compound or blend of compounds, which causes insects to make oriented movements towards its source (Dethier et al. 1960).

Methods

Insects

The *An. gambiae* s.s. culture originated from Suakoko, Liberia (courtesy Prof. M. Coluzzi). Mosquitoes have been cultured in the laboratory since 1988 and received blood meals from a human arm twice a week. Adults were maintained in 30-cm cubic gauze-covered cages in a climate-controlled chamber (27 ± 1° C, 80 ± 5% RH, LD 12:12). They had access to a 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper and placed in tap water in plastic trays and fed daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed in 30-cm cubic cages for emergence.

Skin microbiota sampling

Skin microbiota samples were taken from a human foot, because there is evidence that this body part produces VOCs that influence the selection of biting sites by *An. gambiae* (de Jong and Knols 1995). Each volunteer (all healthy males, Caucasian, aged 23, 25, 28, 29, and 31 years) was asked not to shower, drink alcohol and eat spicy food 24 hours before the experiment and not to use soap during the last shower. Volunteers were provided a nylon sock, which had to be worn 24 hours before the experiment. Samples were taken from the foot of each volunteer by using a sampling ring and washing buffer as described by Taylor et al. (2003). A sterile Teflon sampling ring (internal diameter 2.9 cm) was placed in the centre of the underside of the foot, and 0.75 mL of full-strength wash fluid (75 mM sodium phosphate buffer (pH 7.9) + 0.1% (v/v) Triton X-100, Merck, The Netherlands) was added. The surface of the skin, within the ring, was gently scrubbed with a sterile glass stick for 1 min and the fluid was pipetted in a 2 ml sample tube (Eppendorf®). Immediately thereafter the process was repeated at the same site, and the two samples were pooled and diluted 5x in half strength wash fluid (Taylor et al.

2003). Diluted microbiota samples (100 µl) were spread on Colombia (sheep) blood agar plates (Tritium, The Netherlands; http://www.tritium-microbiologie.nl/) before use in the behavioural experiments.

The number of colony-forming units (cfu) in the samples taken from the human feet was determined using selective plates for the five microbiota genera most abundant on human skin. According to the method described by Taylor et al. (2003), media were selective for staphylococci, aerobic corynebacteria, micrococci, propionibacteria and *Pityrosporum* species (Tritium, The Netherlands).

Olfactometer bioassays

A dual-port olfactometer (Figure 4.1) (Knols et al. 1994, Braks and Takken 1999) was used to evaluate host-seeking responses of female mosquitoes to VOCs produced by microbiota from the human skin. Pressurized air was charcoal-filtered, humidified, and passed through two glass mosquito trapping devices equipped with baffles, which were linked to both ports (diameter 5 cm, 30 cm apart) of the olfactometer. The air entered the flight chamber (1.60 x 0.60 x 0.60 m) with a speed of 22 ± 1 cm/s, temperature of 28.3 ± 0.5°C, and relative humidity above 80%. The experimental room was maintained at a temperature of 26.7 ± 0.8°C and a relative humidity of 64.5 ± 3.5%.

Experiments were prepared and performed according to the methods described by Smallegange et al. (2005). For each test 30 (mated) female mosquitoes of 5-8 d old, which had never received a blood meal, were selected 14-18 h before the experiment and placed in a cylindrical release cage (d = 8, h = 10 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the scotophase, when *An. gambiae* females are known to be highly responsive to host odours (Maxwell et al. 1998, Killeen et al. 2006). In each trial, test odours were released in the air stream before a group of mosquitoes was set free from a cage which was placed at the downwind end of the flight chamber, 1.60 m from the two ports. Mosquitoes were left in the flight chamber for 15 min. Specimens that entered each of the two trapping devices were counted at the end of the experiments. Mosquitoes remaining in the flight chamber were removed with a vacuum cleaner. Each trial started with a fresh batch of mosquitoes, clean trapping devices, and new stimuli. A randomized complete block design was used which included five test treatments and one control treatment (so six treatments total) over six days. For each treatment, 30 female mosquitoes

were released in the olfactometer. The sequence of test odours was randomized on the same day and between days. Test stimuli were alternated between right and left ports in different replicates to rule out any positional effects. Surgical gloves were worn by the researcher at all times to avoid contamination of equipment with human volatiles.

Excised blocks of blood agar (1.5 x 1.5 x 0.3 cm) with or without microbiota were placed on a glass slide (1.5 x 1.5 cm) and then heated on a brass block (Figure 4.1). One blood agar block was placed in each trapping device. The temperature of each block (34.0 ± 0.2°C) was regulated with a universal thermostat with external sensor (UT 100, Conrad electronic, The Netherlands).

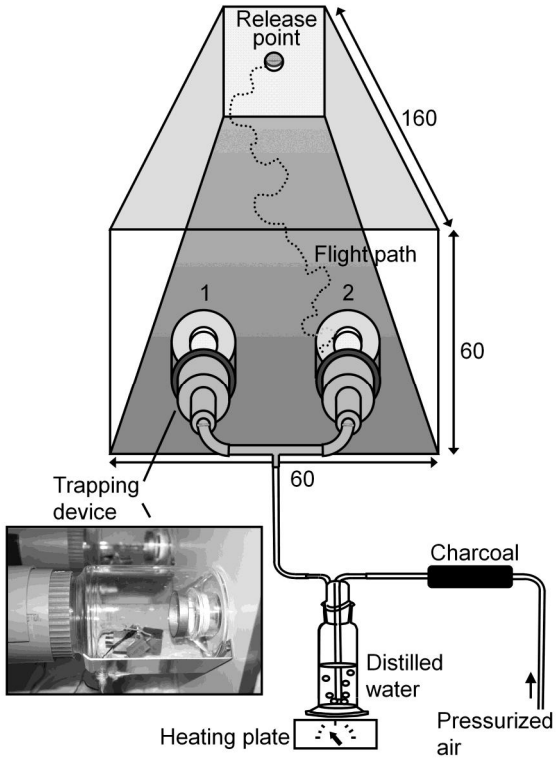


Figure 4.1. Dual-choice olfactometer with two CO_2 measurement positions (1, 2) (Modified after Knols et al. 1994, Braks and Takken 1999). The photograph shows a trapping device and an excised block of blood agar with microbiota on a heated brass block, regulated at 34 ± 0.2°C by a universal thermostat with external sensor. All dimensions are in cm.

In the first series of experiments diluted microbiota samples (100 µl) of one of the volunteers (28 years old) were spread on Colombia (sheep) blood agar plates (Tritium, The Netherlands) and incubated for 0, 6, 12, 24, 36, 72 hours at skin temperature (34°C) (Healy and Copland 2000) before testing. Small blocks (1.5 x 1.5 x 0.3 cm) of blood agar incubated with skin microbiota were tested against small blocks of sterile blood agar.

In the second series of experiments all samples, originating from one of the volunteers (28 years old), were incubated for 12 h, after it had been decimally diluted (1:1; 1:10; 1:100; 1:1,000 or 1:10,000). Sterile control blood agar plates were incubated together with the plates with the skin microbiota. Excised blocks of blood agar incubated with skin microbiota were tested against blocks of sterile blood agar.

For a third series of experiments a bacterial sample was taken as described above from five volunteers (male, Caucasian) to test whether the main result obtained by using bacteria of the volunteer in the first series of experiments could be repeated with samples from other volunteers. The volunteer from the first series of experiments was one of the five volunteers in this experiment (# 4). Bacterial samples of each volunteer were diluted to a concentration of $2.63 \cdot 10^2$ cfu per cm^2 (similar as found in the first series of experiments), spread on blood agar plates and incubated for 12 hours (based on the result of the first series of experiments). Blocks of blood agar with microbiota from the volunteers were tested against a control of sterile blood agar in the olfactometer.

To verify that *An. gambiae* is attracted to VOCs released by microorganisms common on human skin (Noble 2004) , an olfactometer experiment was conducted with a reference strain of *Staphylococcus epidermidis* (DSMZ 11047). Small blocks (1.5 x 1.5 x 0.3 cm) of blood agar grown with *S. epidermidis* at a concentration of $2.63 \cdot 10^2$ cfu per cm^2 for 12 hours were tested against a control of sterile blood agar in the olfactometer.

In the first series a negative control of blood agar with microbiota that was not incubated was randomized with the treatments. In the second and third series two excised blocks of blood agar (1.5 x 1.5 x 0.3 cm) without microorganisms were tested against each other and randomized with the treatments as a control.

Experiments in which only clean moist air was released from both ports of the olfactometer were conducted to test the symmetry of the trapping system. Six tests were performed on one day which showed that the system was symmetrical (χ^2-test, d.f. = 1, P = 1.00, trap entry response 14.5%).

Experimental room trapping

Two Mosquito Magnet-X (MM-X) (American Biophysics Corporation, USA) (Kline 1999) traps were placed in a large netting cage of 233 x 250 x 330 cm (Howitec Netting BV, Bolsward, The Netherlands), inside a climate controlled

room (T = 25 ± 0.5°C, RH = 72 ± 4 %). The traps were placed at 2 m distance from each other. Blood agar plates with a mix of skin bacteria ($2.63 \cdot 10^2$ cfu per cm^2) were incubated for 12 hours at 34°C and tested against incubated sterile blood agar plates. Before the experiment, blood agar with or without skin microbiota was cut into two pieces, and placed inside a metal holder (11.5 x 5 x 1 cm). These holders (Figure 4.2) were then placed in the air outlet of a MM-X trap.

For each test 50 (mated) female mosquitoes, five to eight days old, which had never received a blood meal, were selected 14-18 h prior to the experiment and placed in a cylindrical release cage (d = 8, h = 17.5 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the scotophase. The mosquitoes were released from the release cage in the centre of the large cage. After 4 h, the MM-X traps were closed and transferred to a freezer to kill the mosquitoes. Experiments were repeated for six days, altering the side of each treatment every day. Surgical gloves were worn to avoid contamination of equipment with human volatiles.

On six mornings, experiments with unbaited traps in the MM-X setup were done to test the symmetry of the trapping system. Sterile blood agar was tested against sterile agar without blood to test the effect of the blood in the agar. As a control, sterile blood agar (1.5 x 1.5 x 0.3 cm) was tested against sterile blood agar.

A blend of ten volatile compounds originating from incubated skin bacterial samples and identified in the first part of the study by GC-MS (see below) (1-butanol; 2,3-butanedione; 2-methyl-1-butanol; 2-methylbutanal; 2-methylbutanoic acid; 3-hydroxy-2-butanone; 3-methyl-1-butanol; 3-methylbutanal; 3-methylbutanoic acid; 2-phenylethanol) were tested in the MM-X setup. Only compounds that were found to be significantly more abundant in the bacterial samples than in the control of sterile agar were tested (Table 4.1), except for 2-hydroxy-3-pentanone, which is not commercially available, and the three compounds that could not be identified.

One hundred µL of each compound (Fluka, Sigma, ≥95%; Table 4.1), either pure or diluted in distilled water, was dispensed from sealed sachets (25 x 25 mm) of Low Density PolyEthylene (LDPE; Audion Elektro, The Netherlands). The thickness of the polyethylene material was varied to adjust release rates for each compound (Torr et al. 1997) (Table 4.1). LDPE sachets were suspended from a hook and placed inside the black tube of the MM-X trap (Figure 4.2). The control consisted of an equal number of sachets with the

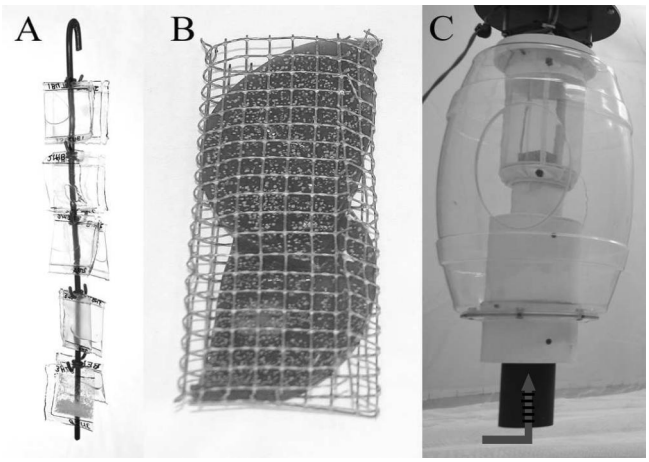


Figure 4.2. Odour release methods in MM-X traps. *(A) Hook with 10 LDPE sachets for release in MM-X traps; (B) metal holder with blood agar with bacteria (11.5 x 5 x 1 cm) for adjustment in inner tube; (C) MM-X trap, arrow indicates the position of LDPE sachets (A) and agar samples (B).*

Table 4.1. Compounds present in the odour blend which was tested in experimental room trapping experiment.

Compound (dilutions in H_2O)	Release rates µg/h	Supplier	Purity	LDPE thickness
1-butanol	168	Sigma	>99%	0.10 mm
2,3-butanedione (1:1000)	48	Fluka	>99%	0.10 mm
2-methyl-1-butanol	545	Sigma	>99%	0.03 mm
2-methylbutanal (1:1000)	31	Sigma	95%	0.10 mm
2-methylbutanoic acid (1:1000)	39	Sigma	98%	0.10 mm
3-hydroxy-2-butanone(solid)	32	Sigma	≥97%	0.03 mm
3-methyl-1-butanol	431	Fluka	≥99.8%	0.10 mm
3-methylbutanal (1:1000)	29	Fluka	≥98%	0.10 mm
3-methylbutanoic acid (1:1000)	34	Sigma	99%	0.10 mm
2-Phenylethanol	261	Fluka	≥99%	0.05 mm
distilled water 0.10 mm	31			0.10 mm
distilled water 0.05 mm	131			0.05 mm
distilled water 0.03 mm	186			0.03 mm

Each compound was released from a separate LDPE sachet. The thickness of the sachets determined the release rates (µg/h), which was measured by weighting the LDPE sachets before and after the experiment.

same size and thickness as the sachets containing experimental compounds, but filled with distilled water only. Release rates (µg/h) were measured by weighing the sachets before and after the experiments (Torr et al. 1997).

Volatile entrainment and GC-MS analysis

Volatiles were entrained using purge and trap (Figure 4.3) on Tenax-TA 20/35 (Alltech), from blood agar plates with different concentrations of human foot bacteria (n = 2 for each concentration). In addition, headspace samples of *S. epidermidis* on agar plates at a concentration of $2.63 \cdot 10^2$ cfu per cm^2 were taken (n = 4). Control samples consisted of sterile blood agar plates with wash buffer without bacteria added to it. Plates that had been incubated for 12 hours at 34 °C were placed in a cuvette. To reduce background volatiles, air was sucked into the cuvette through a standard glass cartridge containing 100 mg Tenax-TA (Figure 4.3). Headspace volatiles were entrained at a flow rate of 100 ml/min for two hours on a second cartridge containing 100 mg Tenax-TA connected to the outlet of the cuvette. The whole setup was placed in an incubator at 34°C to allow growth of the bacteria at skin temperature. Tenax-TA cartridges were conditioned before the experiments by heating for 1 hour at 320°C under a flow of He (60 mL/min).

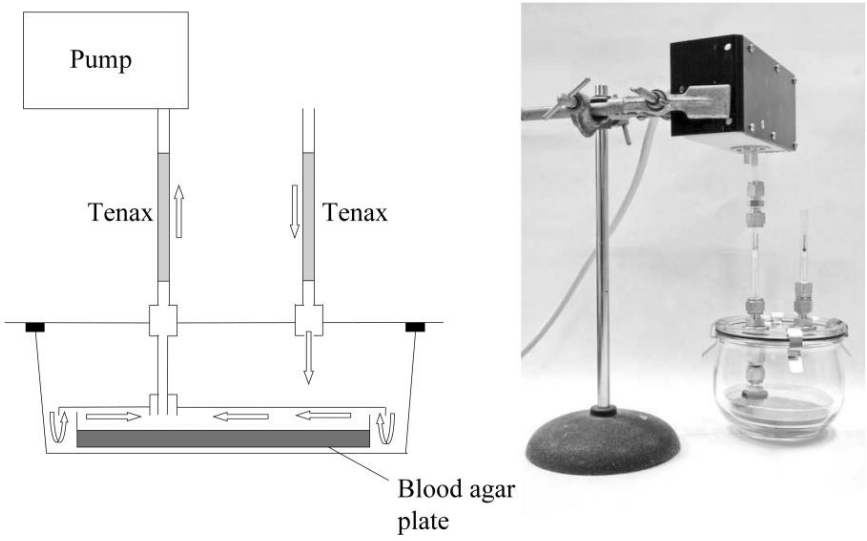


Figure 4.3. Headspace sampling method of skin bacteria.

Schematic representation and photograph of the volatile sampling method used to collect the headspace of blood agar plates incubated with or without skin microbiota. Arrows indicate the direction of the airflow.

Samples were analysed by thermal desorption from the cartridge onto a cold trap and subsequent thermal desorption for introduction into the Trace GC Ultra coupled to a Trace DSQ (both from Thermo Scientific, The Netherlands). The thermal desorption programme consisted of a 3 min He dry purge and 1 min He prepurge both at 30°C. Cartridge desorption was performed at 250°C for 3 min and the volatiles were focused on a general purpose hydrophobic cold trap (Markes) at 0°C. Injection onto the analytical column was achieved by heating of the cold trap at the maximum speed (>60° C/s) to 250°C and splitting of the carrier gas (He) resulting in an injection of 1/6 of the total volatile amount. The transfer line between the cold trap and the GC was kept at 160°C.

A 30 m x 0.25 mm ID x 1.0 µm Rtx-5 MS with He carrier gas (1.0 mL/ min) was used. The GC oven temperature programme was: 3 min at 45°C, ramping of 8°C/min to 280°C and a 2 minute hold at 280°C. The transfer line between the GC and MS was kept at 275°C. Mass-spectra were recorded by electron impact ionization at 70 eV, scanning in positive mode from 35-300 *m/ z* with a scan speed of five scans/s and an ion source temperature of 250°C. The filament was switched off from 13.6-13.8 min because of a high background peak.

Peak analysis was performed using Xcalibur software and peak deconvolution by AMDIS, (http://chemdata.nist.gov/mass-spc/amdis/). The obtained spectra were compared to the NIST-library. Calculated and reported retention indices and injection of authentic synthetic reference compounds (Table 4.1) provided additional information for identification.

Carbon dioxide measurements

Carbon dioxide (CO_2) is assumed to play an important role in mosquito host-seeking behaviour (Mboera and Takken 1997). Therefore, CO_2 levels in the olfactometer were measured on two days. Simultaneously, *An. gambiae* females were released to investigate whether their response to skin microbiota is (partly) due to CO_2 emission from the skin microbiota. The concentration of CO_2 inside the olfactometer was measured at two different positions (Figure 4.1) using a Xentra 4100 CO_2 analyzer (Servomex, The Netherlands), at intervals of 3 min, according to the method described by Spitzen et al. (2008). Carbon dioxide concentrations were measured over a range of 0 - 1030 ppm with an accuracy of 0.1 ppm. The data were downloaded to a PC using Das Wizard© 2.0 software (Measurement Computing Corporation, USA).

For this purpose, blood agar plates with a concentration of skin microorganisms of $2.63 \cdot 10^2$ cfu per cm^2, incubated for 12 h at 34°C, were prepared, and blocks of 1.5 x 1.5 x 0.3 cm, heated on a brass block (34°C), were tested in the olfactometer.

Statistics

For each two-choice test in the olfactometer and MM-X setup a χ^2-test was used to analyze whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution ($P < 0.05$). A Generalized Linear Model (GLM, $P < 0.05$; Genstat for Windows, release 9.2) with binomial function, linked in logit, dispersion estimated, was used to investigate the effect of treatments on the trap entry response in the olfactometer experiments. The trap entry response is defined as the number of female mosquitoes caught in both trapping devices as the percentage of mosquitoes that flew out of the release cage (Qiu et al. 2006).

Differences in CO_2 concentrations at the different time intervals and between blood agar with or without skin microbiota were tested using a t-test for each time-interval ($P < 0.05$; Genstat for Windows, release 9.2).

The abundance of compounds in the chromatograms of the GC-MS analysis were fitted to an exponential regression line (Genstat for Windows, release 9.2) to test whether the concentration of microbiota present on blood agar plates had a significant effect on the abundance of each compound in the headspace samples of these plates ($P < 0.01$).

Differences between the abundance of compounds in the chromatograms of *S. epidermidis* and sterile blood agar plate headspace samples were tested using ANOVA. When a treatment effect was found (ANOVA, $P < 0.05$) a t-test was used to compare pairwise differences of the mean (Genstat for Windows, release 9.2).

Results

Olfactometer bioassays

Traps with sterile blood agar caught significantly more mosquitoes than traps with clean moist air (χ^2-test, d.f. = 1, $P < 0.001$). Blood agar on which skin microbiota were growing (initial concentration $2.63 \cdot 10^2$ cfu per cm^2), however, caught significantly more mosquitoes than sterile blood agar after 12, 24, 36

and 72 h of incubation (Figure 4.4A; χ^2-test, d.f. = 1, $P < 0.01$). The trap entry response, expressed as the number of female mosquitoes caught in both trapping devices divided by the number of mosquitoes that flew out of the release cage, was significantly higher during the tests with microbiota incubated for 12 h than with the other treatments (GLM, d.f. = 5, $P < 0.05$), except for the samples that had been incubated for 6 h (Figure 4.4A).

Based on colony counts on blood agar plates, the bacterial density on the sole of the volunteer's foot was estimated to be $1.90 \cdot 10^5$ cfu per cm^2. Selective plates showed that staphylococci were most abundant ($1.86 \cdot 10^5$ cfu per cm^2); corynebacteria ($5.22 \cdot 10^4$ cfu per cm^2) and propionibacteria ($4.54 \cdot 10^3$ cfu per cm^2) were present in lower numbers. Micrococci and *Pityrosporum* were not found during any of the experiments.

In the second series of experiments the 1:10 dilution was chosen such that the concentration of bacteria before incubation was the same as in the first experiment ($2.63 \cdot 10^2$ cfu per cm^2), and higher and lower concentrations could be tested (1:1; 1:100; 1:1,000 and 1:10,000). The total bacterial density on the sole of the foot was estimated to be $1.14 \cdot 10^7$ cfu per cm^2, the

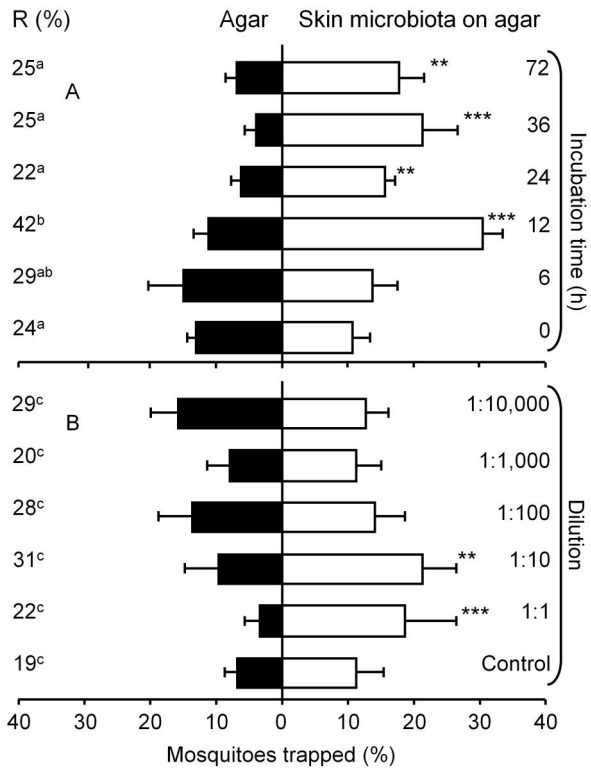


Figure 4.4. Mean response of An. gambiae to skin microbiota in an olfactometer. *Microbiota were grown on blood agar and tested at different times of incubation (A) and in different dilutions of the most attractive treatment (12 h incubation) (B). Six times 30 mosquitoes were released per treatment. Error bars represent standard errors of the mean; ***: χ^2-test $P<0.001$; **: χ^2-test $P<0.01$. R = The trap entry response expressed as the number of female mosquitoes caught in both trapping devices divided by the number of mosquitoes that flew out of the release cage. Data followed by different letters differ significantly at $P<0.05$ (GLM).*

staphylococci density $9.27 \cdot 10^6$, corynebacteria $2.16 \cdot 10^6$ and propionibacteria $5.73 \cdot 10^5$ cfu per cm^2.Traps with blood agar with skin microbiota dilutions of 1:1 or 1:10 caught significantly more *An. gambiae* than traps with sterile blood agar (Figure 4.4B; χ^2-test, d.f. = 1, $P < 0.01$). The trap entry response was not significantly different between treatments (Figure 4.4B; GLM, d.f. = 5, $P > 0.05$). The control experiments with sterile blood agar on both sides showed no positional bias (Figure 4.4B; χ^2-test, d.f. = 1, $P = 0.157$).

In the third series of experiments the blood agar with bacteria from each volunteer ($2.63 \cdot 10^2$ cfu per cm^2, 12h incubation) caught significantly more *An. gambiae* than the sterile blood agar (χ^2-test, d.f. = 1, $P < 0.05$).

Carbon dioxide levels measured at the outlet of both ports of the olfactometer were equal at both ports of the olfactometer when incubated (12 h) blood agar with microbiota was tested against sterile blood agar (Table 4.2; t-test, d.f. = 1, $P > 0.05$).

Volatile entrainment and GC-MS analysis

Regression analysis of the results of the volatile entrainment of headspace odours from the blood agar with diluted microbiota, originating from a human foot, and from the sterile (control) blood agar revealed 14 compounds that were more abundant when bacterial concentrations were higher (Table 4.3, Figure 4.5; exponential regression, d.f. = 7, $P < 0.01$). The 1:1 concentration was not included in the analysis because the abundance of compounds in the chromatograms showed a clear decline at this concentration (Figure 4.5), probably because of bacterial overgrowth on the plate.

Table 4.2. Mean carbon dioxide concentrations during behavioural experiments in the olfactometer

	Mean CO_2 concentration (ppm) ± SE		
Time (min)	**Sterile blood agar**	**Skin microbiota on blood agar**	**P-value**
1-3	439.90 ± 1.63	441.18 ± 1.72	0.58
4-6	438.02 ± 1.58	438.59 ± 1.63	0.80
7-9	435.20 ± 1.52	435.60 ± 1.58	0.85
10-12	432.74 ± 1.48	434.09 ± 1.52	0.52
13-15	430.71 ± 1.43	432.17 ± 1.47	0.48

Carbon dioxide concentrations (ppm ± SE) were measured in front of the trapping device (Figure 4.1) with skin microbiota on blood agar and in front of the trapping device with sterile blood agar. Time indicates minutes after release of mosquitoes and start of each experiment.

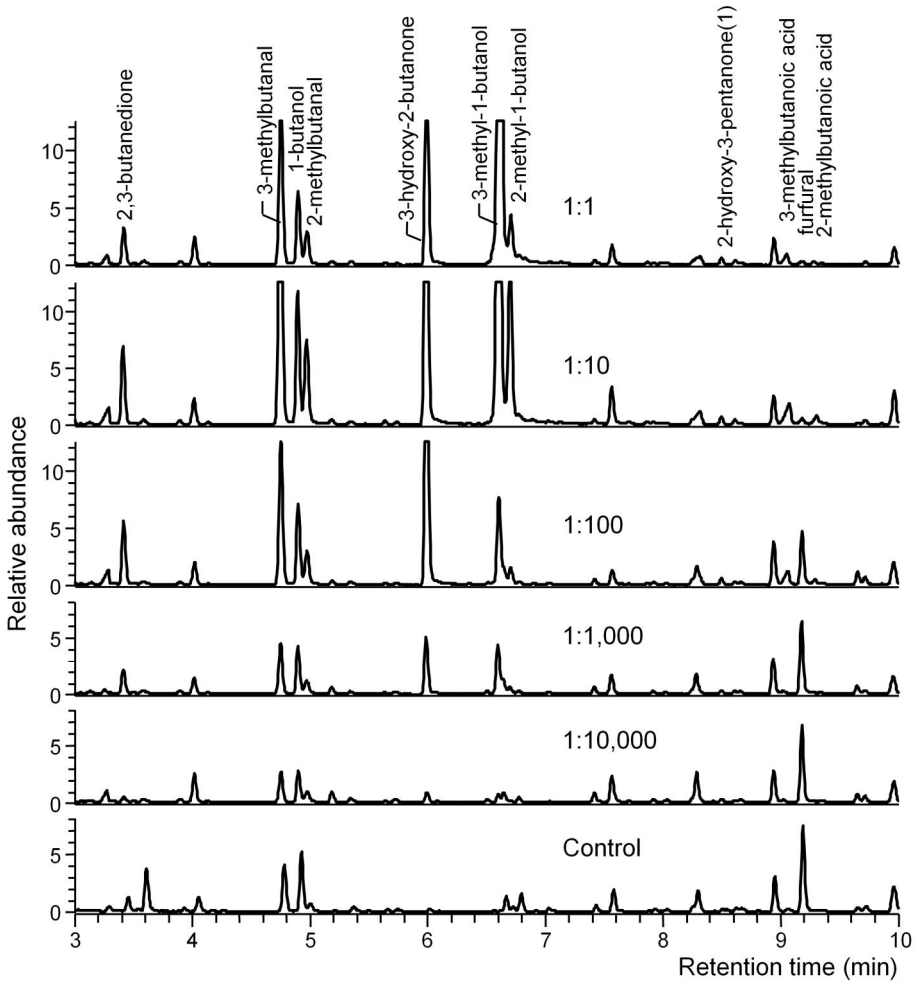


Figure 4.5. GC-MS chromatogram of headspace samples of skin microbiota.

Part of GC-MS chromatograms of headspace samples of blood agar plates with different dilutions of skin microbiota and a blood agar plate without skin microbiota showing 11 compounds that were present in significantly higher or lower amounts in the control (Table 4.3). All compounds were identified on the basis of matching spectra (NIST library), and verified by injection of a standard (except 2-hydroxy-3-pentanone).

A significant reduction of furfural was found when microbiota concentrations increased (Table 4.3, Figure 4.5; $P < 0.001$).

Staphylococcus epidermidis

Blocks (1.5 x 1.5 x 0.3 cm) of blood agar with a reference strain of *S. epidermidis* caught significantly more *An. gambiae* than sterile blocks of blood agar (Figure 4.6; χ^2-test, d.f. = 1, $P < 0.001$). Headspace analysis revealed five compounds that were present in the headspace of *S. epidermidis* samples, but were absent or present in significantly lower quantities in the control of blocks of sterile agar (Figure 4.6, 7; ANOVA, d.f. = 1, $P < 0.05$). These five compounds were also found in the headspace of the microbiota collected from the human foot. Two other compounds, furfural and hexanal, were found in reduced quantities in the headspace of the *S. epidermidis* samples compared to the control (Figure 4.6 and 4.7; $P < 0.05$).

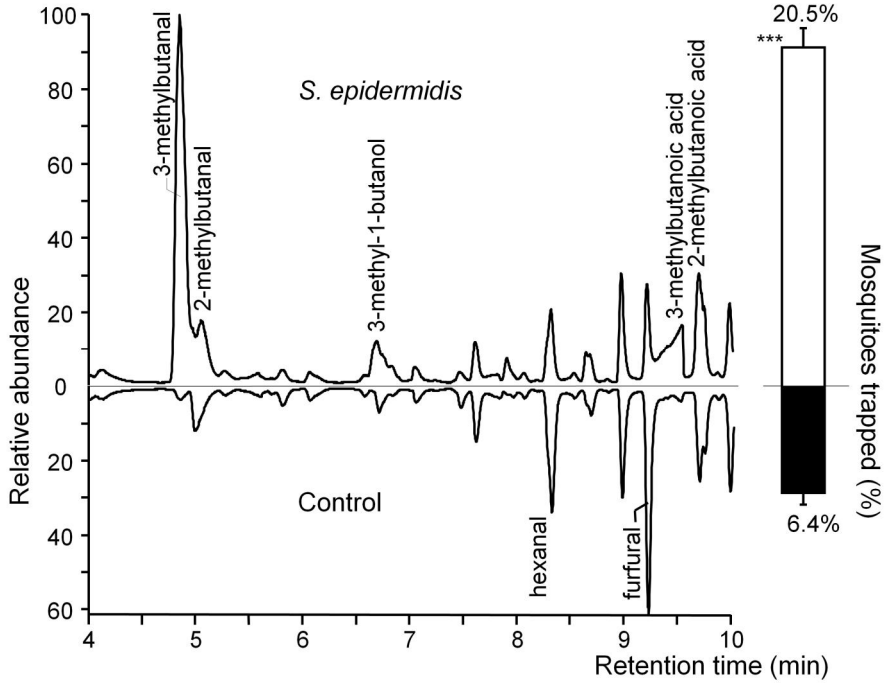


Figure 4.6. GC-MS chromatogram of the headspace of S. epidermidis and the behavioural response of An. gambiae.

*Part of a GC-MS chromatogram of the headspace of a blood agar plate with S. epidermidis and a blood agar plate without skin microbiota indicating the compounds that were present in significantly different amounts (Figure 4.7). Compound names are indicated when present in significantly higher or lower amount in the treatment compared to the control. Bars represent the average response of released An. gambiae in a dual-port olfactometer to both odour sources. Error bars represent standard errors of the mean; ***: χ^2-test $P<0.001$.*

Table 4.3 Bacterial headspace compounds.

Compounds found in lower amounts in diluted samples and control	RT (min)	R^2 (%)	P-value	Exponential regression parameters A + B*(R**X)			Studies on human odour
				A	B	R	
1-butanol[1]	4.89	83.2	<0.001	$2.21*10^{7}$	$-1.79*10^{7}$	$3.09*10^{-16}$	2,3
2,3-butanedione[1]	3.42	78.1	0.002	$2.34*10^{7}$	$-1.99*10^{7}$	$3.77*10^{-246}$	
2-hydroxy-3-pentanone	8.49	94.1	<0.001	$1.39*10^{6}$	$-1.02*10^{6}$	$8.54*10^{-26}$	
2-methyl-1-butanol[1]	6.70	97.1	<0.001	-	$5.05*10^{6}$	$2.89*10^{4}$	2
2-methylbutanal[1]	4.97	98.7	<0.001	$1.11*10^{7}$	$-1.04*10^{7}$	$1.32*10^{-13}$	4.5
2-methylbutanoic acid[1]	9.30	96.8	<0.001	$1.52*10^{6}$	$-1.41*10^{6}$	$8.56*10^{-22}$	2
3-hydroxy-2-butanone[1]	5.99	95.0	<0.001	$8.51*10^{7}$	$-8.24*10^{7}$	$2.31*10^{-34}$	2
3-methyl-1-butanol[1]	6.61	97.0	<0.001	-	$5.22*10^{7}$	$4.25*10^{3}$	2
3-methylbutanal[1]	4.75	98.1	<0.001	$2.74*10^{7}$	$-2.51*10^{7}$	$7.89*10^{-15}$	5
3-methylbutanoic acid[1]	9.07	92.9	<0.001	$5.42*10^{6}$	$-4.69*10^{6}$	$6.63*10^{-28}$	2,3
2-phenylethanol[1]	15.70	86.0	<0.001	$1.14*10^{7}$	$-7.12*10^{6}$	$1.01*10^{-23}$	
unknown 1	16.48	94.7	<0.001	$6.28*10^{5}$	$-6.12*10^{5}$	$5.72*10^{-18}$	
unknown 2	20.22	96.5	<0.001	$7.41*10^{5}$	$-7.04*10^{5}$	$5.50*10^{-14}$	
unknown 3	23.51	94.1	<0.001	$1.93*10^{6}$	$-1.96*10^{6}$	$4.80*10^{-48}$	
Compounds found in higher amounts in diluted samples and control							
furfural	9.18	82.8	<0.001	$1.03*10^{6}$	$1.34*10^{7}$	$1.49*10^{-30}$	

Compounds detected in the headspace of the skin microbiota samples, but not present or in significantly higher or lower amounts in the diluted samples and control (exponential regression, P<0.01). Increase in the abundance of compounds is explained by the parameters in the exponential regression model (A + B (R**X); X=concentration of bacteria). Numbers in last column refer to studies in which compounds were reported previously. All compounds were identified on the basis of matching spectra (NIST library), and verified by injection of standard (except 2-hydroxy-3-pentanone). [1]Compounds tested in MM-X traps. RT=retention time. R^2=coefficient of determination.*

[2](Meijerink et al. 2000), [3](Gallagher et al. 2008), [4](Bernier et al. 2000), [5](Curran et al. 2005)

Experimental room trapping

To test the attractiveness of the volatiles produced by foot microbiota to *An. gambiae* on a larger scale, skin microbiota samples on blood agar ($2.63 \cdot 10^2$ cfu per cm^2) were tested against sterile blood agar in a dual-choice test using MM-X traps. A MM-X trap baited with skin microbiota caught significantly more mosquitoes (Figure 4.8; χ^2-test, d.f. = 1, P <0.001) than a MM-X trap baited with sterile blood agar only. The MM-X traps together caught on average 66% of the mosquitoes released.

Sterile blood agar tested in the MM-X setup against sterile agar without blood showed no significant differences in mosquito numbers caught (Figure 4.8; χ^2-test, d.f. = 1, P = 0.74). On average, 52.5% of the mosquitoes

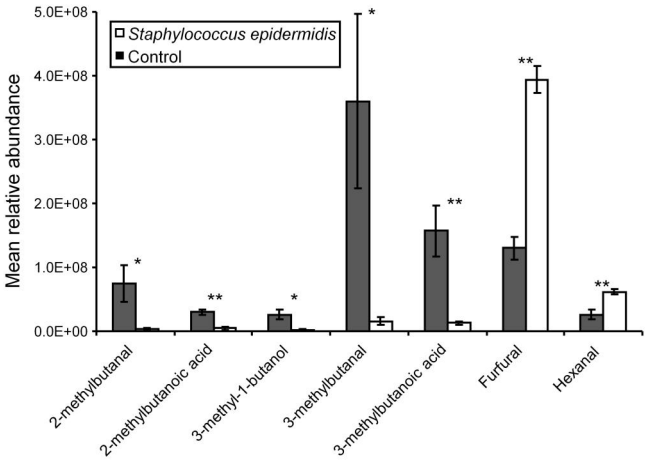


Figure 4.7. Mean relative abundance of headspace compounds emitted by* S. epidermidis *on blood agar. *Compounds were present in significantly different amounts in the headspace of blood agar plates with* Staphylococcus epidermidis *than in that of blood agar plates without* S. epidermidis *(Control). Error bars represent standard errors of the mean; **: χ^2-test P<0.01; *: χ^2-test P<0.05.*

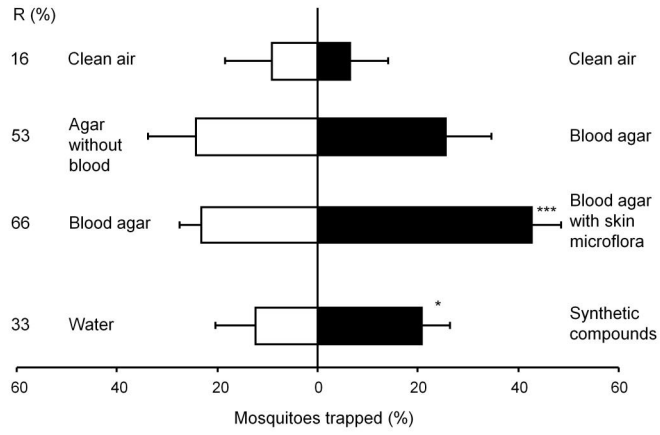


Figure 4.8. Mean response of* An. gambiae *in the experimental room trapping experiments. *Error bars represent standard errors of the mean; ***: χ^2-test P< 0.001; *: χ^2-test P< 0.05. R = Trap entry response (%).*

that left the release cage were caught. Two unbaited MM-X traps caught equal numbers of mosquitoes showing that the system was symmetrical (Figure 4.8; χ^2-test, d.f. = 1, P = 0.24). Together the unbaited traps caught on average 16% of the released mosquitoes.

Synthetic blend

The MM-X trap containing the ten synthetic odours caught significantly more mosquitoes than the control trap (Figure 4.8; χ^2-test, d.f. = 1, P = 0.011). Thirty-three percent of the mosquitoes that left the release cage were caught by the two traps (Figure 4.8).

Discussion

Good understanding of olfactory-mediated behaviour is crucial for the development of mosquito control strategies using host-derived semiochemicals (Logan and Birkett 2007). Through the analysis of organic volatiles produced by microbiota isolated from the human skin, compounds were identified that affect the host-seeking behaviour of *An. gambiae*. This finding underlines the important role of microbiology in the elucidation of mosquito-host interactions. Using the compounds identified a synthetic mixture attractive to this mosquito species was developed.

Although blood agar alone was attractive to the mosquitoes, the microbiota mixture used in this study caught significantly more mosquitoes than sterile blood agar, and it is therefore concluded that the volatiles produced by the microbiota themselves are chiefly responsible for the observed effects. The blood agar used in these experiments is a medium rich in organic substances and many of the volatiles identified in the headspace analysis of the blood agar plates incubated with bacteria were also found in the sterile blood agar plates (Figures 4.5-4.7). This could explain why blood agar itself was attractive to the mosquitoes. However, the abundance and composition of volatiles emanated by incubated blood agar plates was much different from those of sterile blood agar plates, causing the mosquito's choice for the former. Volatiles associated with blood can be attractive to mosquitoes (Allan et al. 2006). The indoor trapping experiments with agar with and without blood however, showed that blood itself was not a source of attractiveness for the mosquitoes (Figure 4.8).

The results obtained with skin microbiota are likely representative for

humans in general, as the skin microbiota from five men caused attractiveness to *An. gambiae*. To determine the possible correlation between skin microbiota composition and the attractiveness of humans to malaria mosquitoes, the attractiveness of the volunteers needs to be investigated. A research like this would require a higher number of volunteers (Qiu et al. 2006).

Classification of the microbiota in the foot samples used in the first and second olfactometer experiments showed that staphylococci were most abundant and corynebacteria occurred in low numbers only. Micrococci and *Pityrosporum* were not found on the foot of the volunteer, although these are reportedly present on the feet of 30-58% of healthy humans (Marshall et al. 1987, Noble 2004). *Staphylococcus* species have been reported to produce 3-methylbutanoic acid (James et al. 2004a), a compound that was also detected in the headspace analysis in this study and is associated with foot malodour (Ara et al. 2006). Corynebacteria and propionibacteria are capable of catabolizing skin lipids to Long Chain Fatty Acids (LCFAs; C14-C30) and LCFAs to Volatile Fatty Acids (VFAs; C2-C12) (Gower et al. 1994, James et al. 2004b), which were also present in our headspace samples (2-methylbutanoic acid, 3-methylbutanoic acid). Previous studies have shown that aliphatic carboxylic acids play a role in the host-seeking behaviour of *An. gambiae* (Knols et al. 1997, Qiu 2005, Smallegange et al. 2005).

The human odour profile consists of more than 350 compounds (Bernier et al. 2000, Meijerink et al. 2000). Eight of the 14 putative attractive compounds found in the present study have been reported previously from studies on human odour or human sweat (Bernier et al. 1999, Bernier et al. 2000, Meijerink et al. 2000, Curran et al. 2005, Gallagher et al. 2008) (Table 4.3), which links our results on odour production by *in vitro* cultured skin microbiota to these previous studies.

With the new approach presented here it is possible to identify compounds that affect the host-seeking behaviour of *An. gambiae*. Although there is no information on the correlation between the release rate of the compounds present in the synthetic odour blend and the actual concentration of the odorant chemicals in the headspace of the microbiota, the behavioural response to a blend of 10 compounds that were abundantly present among the bacteria-derived VOCs suggests that this strategy of kairomone identification is an effective means of kairomone discovery. Testing the blend of ten compounds in indoor trapping experiments represents an intermediate research step between laboratory-based olfactometer studies and (semi-)field

studies, as the MM-X traps are currently also used in semi-field and field experiments in Africa (Njiru et al. 2006, Qiu et al. 2007b, Jawara et al. 2009).

Although the synthetic blend was more attractive than the control, the trap catches were lower than when skin microbiota or agar alone, were tested. Both quantity and quality of constituents present in synthetic blends are known to have an effect on trapping efficacy (Vale and Hall 1985, Knols et al. 1997, Bosch et al. 2000, Smallegange et al. 2005). As the release rates of the chemicals from the sachets depended on the volatility of the compounds and the size and thickness of the LDPE sachets (Torr et al. 1997) (Table 4.2), we expect that a stronger positive effect of the blend can be achieved by influencing the release rates of the individual components in the blend by variation of these characteristics. In addition, some compounds that were present in the blend may have had a negative effect on the attractiveness, depending on concentration, and other compounds that may increase the attractiveness of the blend may be missing (Smallegange et al. 2009).

Inhibition of the metabolism of certain skin bacteria may reduce a person's attractiveness to malaria mosquitoes. Indeed, some compounds like citral, citronellal and geraniol block foot odour-producing enzymes in bacteria (Ara et al. 2006, Debboun et al. 2007). This knowledge can lead to the development of a new class of odour-masking or inhibitory compounds, which can be exploited in the protection from mosquito bites, aiming at compounds that reduce the production of attractive volatiles on the human skin.

Skin microorganisms are known to determine the human odour profile (Taylor et al. 2003) and with the results presented here it is plausible that the composition of the skin microbiota determines an individual's attractiveness to malaria mosquitoes. The discovery that human skin microorganisms mediate malaria mosquito behaviour provides new opportunities for the control of this disease, for example biotechnological approaches using bacteria for mass production of mosquito attractants or modification of the composition of the microbial flora on the human skin to reduce attractiveness.

Conclusions

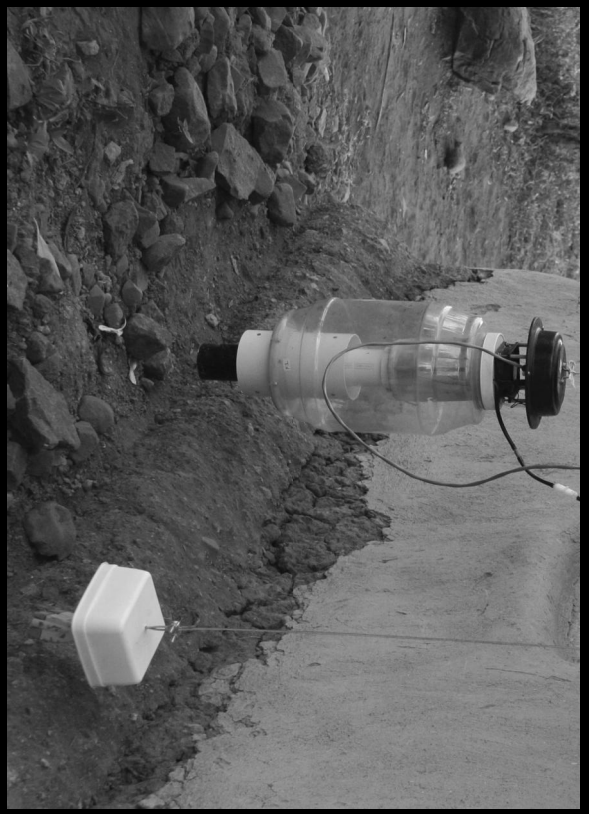
Skin microorganisms attract malaria mosquitoes when grown on blood agar in both olfactometer and indoor trapping experiments. A study with five volunteers showed that this effect is probably representative for humans in general. Analysis of the headspace of the skin bacteria in a dilution series resulted in fourteen putative kairomones. A synthetic blend of 10 of these was

attractive to *An. gambiae*. This approach to identify semiochemicals could potentially be a novel means of vector-borne disease control through the deployment of semiochemical-baited trapping systems (Kline 2007, Logan and Birkett 2007). Further knowledge of the effect of skin bacteria on mosquito attraction could support the development of new repellents by blocking skin bacteria or the mass production of skin bacteria as mosquito attractant.

Acknowledgements

We would like to thank all volunteers for their participation and are grateful to F. K. M. van Aggelen, A. J. Gidding and L. Koopman for assistance with mosquito rearing. We thank Prof. Dr. M. Dicke, Dr. J. J. A. van Loon and Dr. Y. T. Qiu for commenting on an earlier version of this manuscript, R. de Vrijer-Albers for assistance with the microbiology work, Dr. F. C. Griepink for providing LDPE material and Dr. H. M. Smid for making the photographs. This study was funded by a grant from the Foundation for the National Institutes of Health (NIH) through the Grand Challenges in Global Health Initiative (GCGH#121). BGJK is supported by a VIDI grant (#864.03.004) from the Netherlands Organization for Scientific Research (NWO).

Skin microbiota and their volatiles as odor baits



Skin microbiota and their volatiles as odour baits for the malaria mosquito *Anopheles gambiae*

05

Niels O. Verhulst, Wolfgang R. Mukabana, Willem Takken and Renate C. Smallegange

Host seeking by the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) is mainly guided by human odours. The skin microbiota plays an important role in the production of these human body odours and skin bacteria grown on agar plates attract *An. gambiae s.s.* in the laboratory. In the present study the attractiveness of volatiles produced by human skin bacteria to *An. gambiae s.s.* was tested in laboratory, semi-field and field experiments to assess these effects in increasing environmental complexity. A synthetic blend of ten compounds identified in the headspace of skin bacteria was also tested for its attractiveness. Carbon dioxide significantly increased mosquito catches of traps baited with microbial volatiles in the semi-field experiments and was therefore added to the field traps. Traps baited with skin bacteria caught significantly more *An. gambiae s.s.* than control traps, both in the laboratory and semi-field experiments. Traps baited with the synthetic blend caught more mosquitoes than control traps in the laboratory experiments, but not in the semi-field experiments. Although bacterial volatiles increased mosquito catches in the field study, affecting several mosquito vector species, these effects were not significant for *An. gambiae* s.l. It is concluded that volatiles from skin bacteria affect mosquito behaviour under laboratory and semi-field conditions, and after fine tuning, have potential to be developed as odour baits for mosquitoes.

Submitted

Introduction

Anopheles gambiae Giles *sensu stricto* (hereafter referred to as *An. gambiae*) is one of the most important malaria vectors in Africa. This mosquito species is considered to be highly anthropophilic and volatiles of human origin provide essential cues in its host-seeking behaviour (Takken and Knols 1999). Human body odours account for most of the attractiveness of humans to *An. gambiae* (Costantini et al. 1996a, Costantini et al. 1998), whereas heat and moisture play a minor role (Olanga et al. 2010).

Carbon dioxide (CO_2), a major component of breath, is an important olfactory cue that acts as a mosquito activator (Gillies 1980, Takken 1991, Mboera and Takken 1997, Spitzen et al. 2008). Semi-field and field studies have shown that CO_2 increases trap catches of several species of mosquitoes, including *An. gambiae* (Costantini et al. 1996a, Qiu et al. 2007b, Schmied et al. 2008, Jawara et al. 2009), although high concentrations CO_2 can inhibit the response of mosquitoes to host odours (Lefèvre et al. 2009). Other breath components do not increase the attraction of *An. gambiae* to humans (Braks 1999, Mukabana et al. 2004).

Skin microbiota plays an important role in the production of human body odours (Leyden et al. 1981, Stoddart 1990) and without bacteria human sweat is odourless (Shelley et al. 1953). The bacterial composition of human skin can be correlated with body (mal)odour and its chemical profile (Rennie et al. 1991, Taylor et al. 2003, Ara et al. 2006, Xu et al. 2007). Several groups of bacteria have been identified to play specific roles in converting non-volatile compounds on the human skin into volatile compounds (James et al. 2004b, James et al. 2004a).

Washing the feet of a volunteer with a bactericidal soap significantly altered the selection of biting sites of *An. gambiae* (de Jong and Knols 1995), and although human sweat is attractive to *An. gambiae*, this was only found after incubation of sweat with skin bacteria (Braks and Takken 1999). Interestingly, skin bacteria that are cultured on blood agar are attractive to *An. gambiae* in olfactometer experiments (Verhulst et al. 2009). Attractiveness was found to depend on the incubation time and the concentration of the bacteria. In the headspace of skin bacteria growing on blood agar fourteen putative attractants have been identified. A synthetic blend of 10 of these was attractive to *An. gambiae* in laboratory experiments (Verhulst et al. 2009). Eight of these compounds have been identified before in studies on human odours (Bernier et al. 1999, Bernier et al. 2000, Meijerink et al. 2000, Gallagher et al. 2008).

Insecticide treated nets (ITNs) and artemisinin-based therapies have contributed considerably to the reduction in malaria cases in the past decade. However, mosquito resistance to pyrethroids used in ITNs and parasite resistance to antimalarial medicines are major threats for malaria control (N'Guessan et al. 2007, WHO 2009). Vector-control strategies have historically been shown to be of vital importance and should be considered to play a prominent part in measures to reduce malaria incidence (Takken and Knols 2009). Host-derived attractants could play an important role in these vector-control strategies and may be used for monitoring vector densities or in push-pull strategies (Agelopoulos et al. 1999, Cook et al. 2007, Logan et al. 2008, Takken and Knols 2009, Okumu et al. 2010c).

Mosquito Magnet-X (MM-X) traps are commonly used in field studies to test attractants (Kline 2007, Qiu et al. 2007b, Jawara et al. 2009, Okumu et al. 2010c). The purpose of the current study was to examine whether volatiles released by human skin bacteria grown on agar, and a blend of 10 compounds identified in an earlier study (Verhulst et al. 2009) may be used as odour baits in MM-X traps. This was studied in three setups of increasing environmental complexity, i.e. in laboratory, semi-field and field experiments.

Materials and methods

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* colony used for the laboratory experiments originated from Suakoko, Liberia. Mosquitoes were cultured in the Laboratory of Entomology of Wageningen University, The Netherlands, since 1988 and received blood meals from a human arm twice a week. Adult mosquitoes were maintained in 30-cm cubic gauze-covered cages in climate-controlled chambers (27 ± 1°C, 80 ± 5% RH, LD 12:12). They had access to a 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper and placed in tap water in plastic trays and fed daily on Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed in 30-cm cubic cages for emergence.

The *An. gambiae* Giles *sensu stricto* colony at the Thomas Odhiambo campus of the International Centre of Insect Physiology and Ecology (ICIPE), Nyanza Province, western Kenya, originated from Mbita Point and has been cultured since 2001. Mosquitoes were fed three times a week on a human arm and larvae were reared in filtered water from Lake Victoria. Other rearing procedures were similar to those used for rearing mosquitoes in Wageningen.

Skin microbiota

Skin microbiota samples were taken from the foot of a human volunteer (male Caucasian, 29 yrs old) by using a sampling ring and washing buffer as described by Taylor et al. (2003). A sterile Teflon sampling ring (internal diameter 2.9 cm) was placed in the centre of the underside of the foot, after which 0.75 mL of full-strength wash fluid (75 mM sodium phosphate buffer (pH 7.9) + 0.1% (v/v) Triton X-100, Merck, The Netherlands) was added. The surface of the skin, within the ring, was gently scrubbed with a sterile glass stick for 1 min and the fluid was pipetted in a 2 ml sample tube (Eppendorf, Germany). The process was repeated at the same site immediately thereafter and the two samples were pooled (Taylor et al. 2003).

Microbiota samples (100 µl) were decimally diluted in half-strength wash fluid, spread on Colombia blood (sheep) agar plates (Tritium, The Netherlands) and incubated for 24 h at 34°C to determine the concentration of microbiota in the original sample. The original sample was then diluted in half-strength wash fluid to a concentration of 263 colony forming units (cfu) per cm^2 on the blood agar plates and incubated for 12 h at 34°C to obtain plates with bacterial counts similar to the plates that have been proven to be attractive to *An. gambiae* in previous experiments (Verhulst et al. 2009). As a control, 100 µl of half-strength wash fluid (Taylor et al. 2003) was spread on a blood agar plate and incubated for 12 h at 34°C.

Before each experiment blood agar incubated with or without skin microbiota was cut into two pieces, and placed inside a metal holder (11.5 x 5 x 1 cm). These holders were then placed in the odour outlet of an MM-X trap (Verhulst et al. 2009).

Synthetic odours

A synthetic blend of 10 compounds, present in the headspace of incubated skin bacterial samples and identified in a previous study (Table 5.1) (Verhulst et al. 2009), was tested for its attractiveness as an odour bait. One hundred µl of each compound (Fluka, Sigma, ³95%; Table 5.1), either pure or diluted in distilled water, was dispensed from sealed sachets (25 x 25 mm) of Low Density PolyEthylene (LDPE; Audion Elektro, The Netherlands). Each sachet contained one compound. Concentrations of the diluted samples were increased 10 times compared to a previous study (Verhulst et al. 2009), as in semi-field and field experiments wind can dilute the odours released. The thickness of the polyethylene material was varied to adjust release rates for

Table 5.1. Compounds present in the synthetic odour blend and used to bait MM-X traps in laboratory and semi-field experiments.

Compound (dilutions in H_2O)	LDPE thickness (mm)	Release rates laboratory (µg/h ± SE)	Release rates semi-field (µg/h ± SE)	T-test (P-value)
1-butanol	0.10	150 ± 22	101 ± 35	0.21
2,3-butanedione (1:100)	0.10	21 ± 19	41 ± 6	0.84
2-methyl-1-butanol	0.03	468 ± 51	1260 ± 594	0.20
2-methylbutanal (1:100)	0.10	18 ± 15	30 ± 11	0.70
2-methylbutanoic acid (1:100)	0.10	25 ± 12	36 ± 33	0.85
3-hydroxy-2-butanone (solid)	0.03	250 ± 34	242 ± 64	0.64
3-methyl-1-butanol	0.10	68 ± 17	71 ± 32	0.72
3-methylbutanal (1:100)	0.10	21 ± 6	24 ± 19	0.98
3-methylbutanoic acid (1:100)	0.10	18 ± 13	31 ± 18	0.79
2-phenylethanol	0.05	189 ± 24	244 ± 54	0.50
distilled water	0.10	7 ± 13	21 ± 11	0.43
distilled water	0.05	46 ± 25	61 ± 30	0.95
distilled water	0.03	64 ± 26	76 ± 36	0.76

Each compound was released from a separate LDPE sachet. The thickness (mm) of the sachets determined the release rates (µg/h ± SE; N = 6). The result for the t-test to compare release rates between laboratory and semi-field rates are given (P-value).

each compound (Table 5.1) (Torr et al. 1997). LDPE sachets were suspended from a hook and placed inside the odour outlet of a MM-X trap (Verhulst et al. 2009). The control consisted of an equal number of LDPE sachets with the same size and thickness as the sachets containing experimental compounds, but filled with distilled water only. Release rates (µg/h) were measured by weighing (laboratory, Mettler AC100; semi-field, Sartorius A200S; accuracy both 0.1 mg) the sachets before and after the experiments (Torr et al. 1997).

Previous studies (Njiru et al. 2006, Schmied et al. 2008, Spitzen et al. 2008, Jawara et al. 2009) have shown that carbon dioxide increases trap catches substantially and, therefore, CO_2 was added to the odour sources in the semi-field and field experiments. Carbon dioxide (≥99.9%) was provided from pressurized cylinders (Carbacid Investments Ltd., Kenya) through silicone tubing (Ø 7 mm; Rubber B.V., The Netherlands) at a rate of 250 ml/ min, regulated by a flow meter (Sho-Rate; Brooks Instruments, The Netherlands) in the semi-field experiments and regulated by an orifice (American Biophysics Corporation, USA) in the field experiments. The pre-installed plug on the MM-X trap was used to release the gas directly into the odour outlet. Worn nylon socks were used as a positive control in the field experiments as they are known to attract *An. gambiae* in laboratory experiments (Dekker et al. 2001b, Spitzen et al. 2008, Smallegange et al. 2010, Qiu et al. in press) and to induce a synergistic effect in combination with carbon dioxide in semi-field (Njiru et al. 2006, Schmied et al. 2008) and field (Kline 1998, Jawara et al. 2009, Okumu et al. 2010c) setups. A nylon sock (40 Den, 100% polyamide, Hema, The Netherlands,) was worn for 12 h by a volunteer (male Caucasian, 29 yrs old) and stored in a freezer at – 20°C until use. Socks were placed in the odour outlet of the MM-X trap and held in position by a small metal wire.

Temperature and humidity

In each experiment (laboratory, semi-field and field), a data logger recorded temperature and humidity every ten minutes (TinyTag Ultra, model TGU-1500, INTAB Benelux, The Netherlands).

Laboratory experiments

Laboratory experiments were conducted at the Laboratory of Entomology, Wageningen University, The Netherlands. Two MM-X traps (American Biophysics Corporation, USA; Kline,1999) were placed in a large netting cage measuring 233 × 250 × 330 cm (Figure 5.1; Howitec Netting BV, The Netherlands), inside a climate controlled room (T = 24.5 ± 0.4 ºC, RH = 89.3 ± 6.8%) (Verhulst et al. 2009, Smallegange and Takken 2010). The traps were placed at 2 m distance from each other, 15 cm from the ground (Schmied et al. 2008, Jawara et al. 2009).

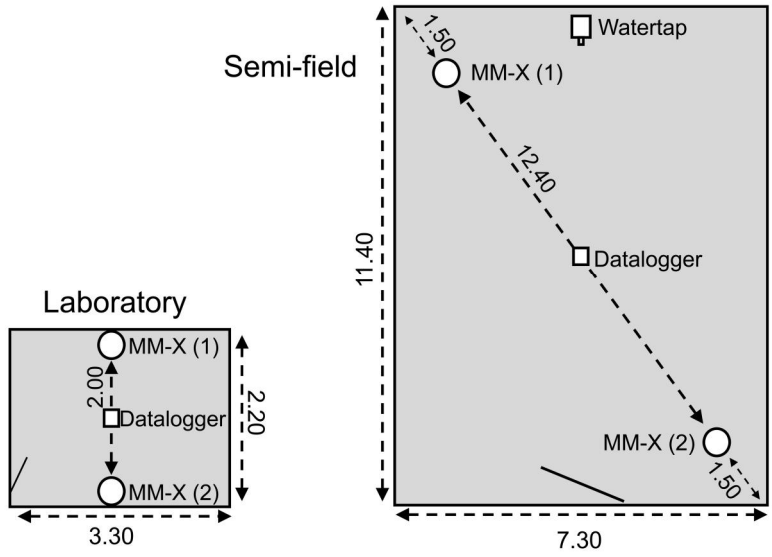


Figure 5.1. Top view schematic drawing of laboratory and semi-field setups.
Distances in meters. Mosquitoes were released from the centre, next to the data logger.

For each test 50 female mosquitoes, 5-8 d old, which had been given a mating chance and had never received a blood meal, were selected 14-18 h prior to the experiment (Smallegange et al. 2009) and placed in a cylindrical release cage (d = 8, h = 17.5 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the scotophase. The mosquitoes were released from the release cage in the centre of the netting cage. After 4 h, the MM-X traps were closed and transferred to a freezer to kill the mosquitoes. Experiments were repeated for six days, alternating the positions of treatment and control every day. Surgical gloves were worn during handling traps and release cages, to avoid contamination of

equipment with human volatiles.

Initially, experiments with unbaited traps were done to test the symmetry of the system, followed by experiments in which blood agar with skin microbiota was tested against sterile blood agar. The results of these experiments have been published before (Verhulst et al. 2009), and are included to be able to compare these results directly with the results of the synthetic compounds and the semi-field and field results. In addition, synthetic compounds were released from LDPE sachets with different thicknesses (Table 5.1) and tested against a control of sachets filled with water.

Semi-field experiments

Semi-field experiments were conducted at the Thomas Odhiambo campus of the International Centre of Insect Physiology and Ecology (ICIPE), Nyanza Province, western Kenya. Experiments were conducted in a greenhouse with a glass-panelled roof and gauze-covered side walls as described by Njiru et al. (2006). Inside, sand covered the floor and a large mosquito-netting cage (11 × 7 × 2.5 m; mesh width 3 mm) was suspended from the ceiling to the floor. Two MM-X traps, were placed in the greenhouse at 12.40 m from each other at 1.50 m from the corners (Figure 5.1), with the odour outlet positioned 15 cm above ground level (Schmied et al. 2008, Jawara et al. 2009).

For each test 200 female mosquitoes, 3–5 d old, which had been given a mating chance and had never received a blood meal, were selected six h before the experiments (Njiru et al. 2006). Mosquitoes were placed in a 1 L (d = 11-13 cm, h = 15 cm) cup, covered by mosquito netting, and were offered water-moistened cotton wool only. Every test night, 200 experimental mosquitoes were released from the centre of the greenhouse at 9.30 pm. At 6.30 am the following morning traps were collected, placed in the freezer and mosquitoes counted. Every afternoon the mosquitoes remaining in the greenhouse were captured and the sand in the greenhouse moistened to prevent dust and to lower temperature. Experiments were repeated for six nights, alternating the position of treatment and control every night. Surgical gloves were worn to avoid contamination of equipment with human volatiles.

Experiments with two unbaited traps were done to test the symmetry of the system and the trap entry response without an odour bait. Agar with skin microbiota was tested against sterile agar. To determine the effect of CO_2 on trap catches, CO_2 was added to the skin microbiota in another experiment and tested against sterile agar with CO_2. The synthetic odours were only tested in

combination with CO_2, as preliminary experiments showed that traps baited with synthetic odour alone caught very few mosquitoes (data not shown; on average 4 mosquitoes per trap per night).

Field experiments

The field experiments were performed in Lwanda (00º28.'S, 034º17'E), in the basin of Lake Victoria, Nyanza Province, western Kenya at an altitude of 1169 m above sea level. Lwanda is a rural village with a variety of mosquito breeding habitats (Minakawa et al. 1999). A previous study by Mathenge et al. (2004) showed that *Anopheles gambiae* s.l. and *An. funestus* Giles were the main species complexes present. The area has a main rainy season from March to May, and a short rainy season from October to December (Seyoum et al. 2003). Experiments were conducted in December 2008 and in July 2009.

Four mud-walled houses with thatched roofs, at a distance of 65 to 340 m from each other, were selected for the experiments based on several criteria (household, location cooking spot, roof construction, vegetation around the house and all houses had a insecticide treated bednet (ITN)). One MM-X trap was placed outside every house, under the eave, near a window, with the odour outlet positioned at 15 cm above ground level (Schmied et al. 2008, Jawara et al. 2009). Traps operated on a 12V car battery. Unperfumed Vaseline® petroleum jelly was put around the suspension cable, electrical cables and tubing to prevent ants from reaching the mosquitoes caught in the trap. Surgical gloves were worn to avoid contamination of equipment with human volatiles.

Each trap was provided with CO_2 at a rate of 250 ml/min from pressurized cylinders. In addition, the traps were baited with the following odour sources: agar with or without skin bacteria, a worn sock as a positive control (Njiru et al. 2006).

The effects of the treatments were tested following a 4 by 4 Latin square design, during two series of eight nights (December 2008 and July 2009). Each night, traps were run from 06.30 pm to 06.30 am the next morning, when traps were collected, placed in the freezer (to kill the surviving insects) and mosquitoes counted.

The female mosquitoes caught in each trap were morphologically identified to genus and counted. Anophelines were identified to species. Female *An. gambiae* s.l. mosquitoes were placed in a 2-ml Eppendorf tube with dry silica gel and a piece of cotton wool. These mosquitoes were transported to the

Laboratory of Entomology, Wageningen University, for species identification. A subsample of 92 randomly selected *An. gambiae s.l.* was identified by polymerase chain reaction (PCR) (Scott et al. 1993).

Data analysis

For each two-choice test in the laboratory and semi-field setup a χ^2-test was used to analyze whether the total (i.e. sum of all replicates) number of mosquitoes that was caught in one of the MM-X traps and the total number that was caught in the other MM-X trap differed from a 1:1 distribution ($P < 0.05$).

Differences in release rates from LDPE sachets between laboratory and semi-field were tested using a t-test ($P < 0.05$; Genstat for Windows, release 9.2).

A Generalized Linear Model with Poisson distribution, linked in logarithm, dispersion estimated, was used to investigate the effect of treatments on the trap catches in the field experiments (GLM, $P < 0.05$; Genstat for Windows, release 9.2). The effect of house and night on mosquito catches were tested and fitted as parameters in the model.

Results

Laboratory trapping

When no odour bait was present 16% of the mosquitoes were caught by the two traps without a preference for either of the two traps (Figure 5.2: χ^2-test, df = 1, $P = 0.69$) (Verhulst et al. 2009). From this we concluded that the experimental setup was symmetrical. Traps baited with incubated skin microbiota on agar caught significantly more mosquitoes than traps baited with sterile agar (Figure 5.2: χ^2-test, df = 1, $P < 0.001$) (Verhulst et al. 2009). The traps baited with the synthetic odour blend attracted significantly more mosquitoes than the control traps baited with water alone (Figure 5.2: χ^2-test, df = 1, $P = 0.002$).

Release rates of the synthetic compounds from the LDPE sachets were comparable to the release rates measured in previous experiments (Table 5.1) (Verhulst et al. 2009), except for the release rates of 3-hydroxy-2-butanone which was considerably higher and 3-methyl-1-butanol which was considerably lower than in previous experiments.

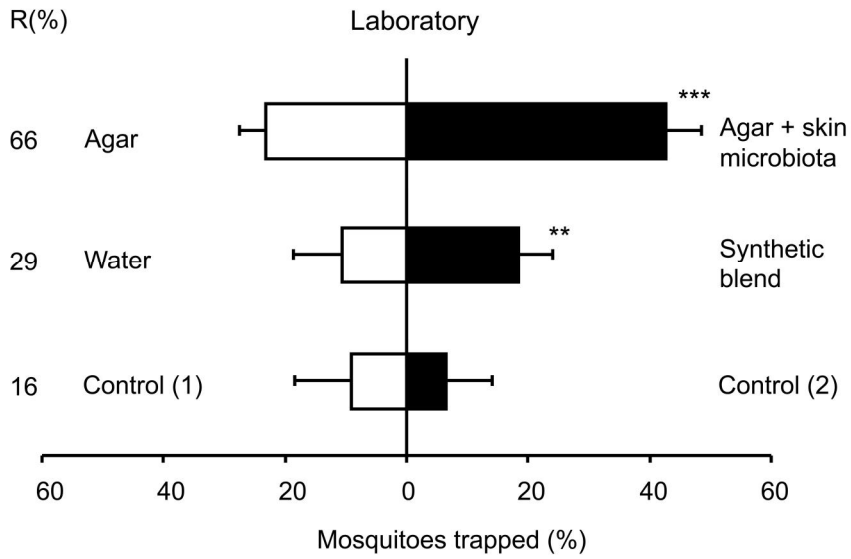


Figure 5.2. Mean response of* An. gambiae *in laboratory experiments.

*Error bars represent standard errors of the mean; ***: χ^2-test P<0.001; **: χ^2-test P<0.01. N = 300 R= The trap entry response expressed as the number of female mosquitoes caught in MM-X traps divided by the number of mosquitoes that flew out of the release cage (%). Control: unbaited MM-X trap. Skin bacteria and control experiment results were published before in Verhulst et al. (2009).*

Semi-field experiments

During the nights of the semi-field experiments (December 2008), the average temperature was 22.2 ± 1.1 ºC and the average humidity 82.4 ± 4.9%. Control experiments with two unbaited traps showed that the experimental setup was symmetrical (Figure 5.3, χ^2-test, df = 1, P = 0.64). The two traps together caught on average 5.0% of the mosquitoes released.

Similar to the results of the laboratory experiments, traps baited with bacteria on agar caught more mosquitoes than traps with sterile agar alone (Figure 5.3, χ^2-test, df = 1, P < 0.001). When CO_2 was added, the percentage of mosquitoes caught increased from 25 to 72. Traps baited with bacteria and CO_2 caught more mosquitoes than traps baited with CO_2 only (Figure 5.3, χ^2-test, df = 1, P < 0.001). The blend of synthetic compounds tested in combination with CO_2, showed no effect of the blend on the number of mosquitoes caught (Figure 5.3, χ^2-test, df = 1, P = 0.86).

The release rates of compounds from LDPE sachets was not

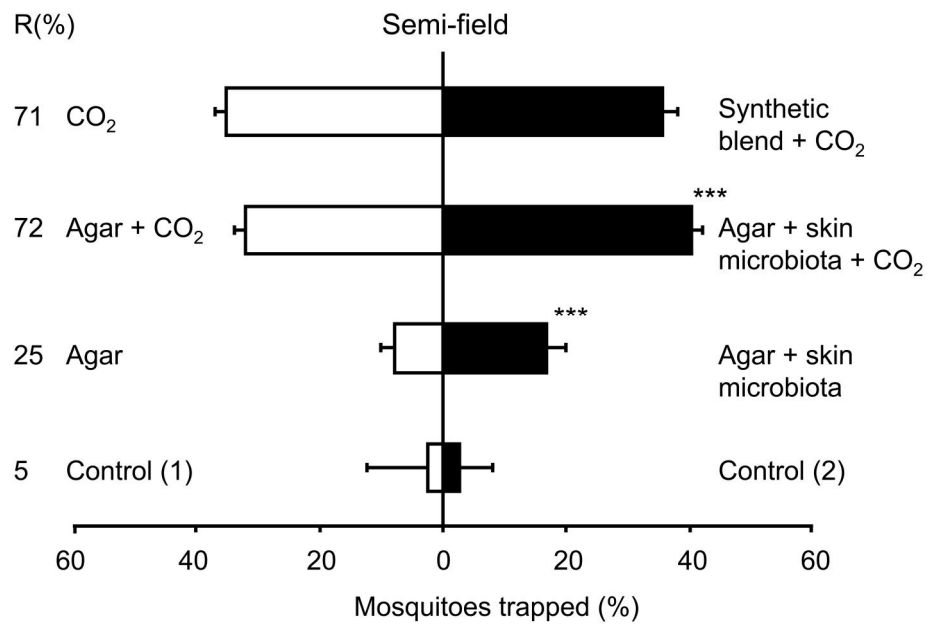


Figure 5.3. Mean response of* An. gambiae *in semi-field experiments.

*Error bars represent standard errors of the mean; ***: χ2-test P<0.001; N = 1200 R= The trap entry response expressed as the number of female mosquitoes caught in MM-X traps divided by the number of mosquitoes that flew out of the release cage (%). Control: unbaited MM-X trap.*

significantly different between the laboratory and semi-field experiments (Table 5.1, T-test, $P > 0.05$). The release rate of 2-methyl-1-butanol was high in the first two nights of the semi-field experiments compared to the laboratory experiments, resulting in a high mean release rate and standard error (Table 5.1). After two nights the sachet was replaced, resulting in a release rate during the subsequent experimental nights comparable to the release rates of 2-methyl-1-butanol during laboratory experiments.

Field experiments

The average temperature measured during the nights of the first series of field experiments in November/ December 2008 was 22.7 ± 1.5 ºC and the average humidity 81.0 ± 5.4 %. During the second series of experiments in July 2009 the average night temperature was 20.9 ± 3.1 ºC and the average humidity 73.0 ± 11.7 %.

In total 2,594 mosquitoes were caught, of which 1176 (45%) were

Culex spp., 798 (31%) *Mansonia* spp., 336 (13%) *An. gambiae* s.l., and 169 (7%) *Aedes* spp. Other anopheline specimens (115; 4%) were mainly *An. coustani* Laveran. Results of the PCR analysis of a random subsample showed that of the *An. gambiae* s.l. complex 1% was *An. gambiae s.s*, 90% *An. arabiensis* and 9% of the samples could not be identified.

House ($P < 0.001$) and night ($P < 0.05$) had a significant effect on the trap catches of most mosquito species (Table 5.2) and were therefore included in the GLM. The trap near house 2 caught significantly fewer mosquitoes than the other traps ($P < 0.05$). Treatment had a significant effect on the number of *Mansonia* spp. and the number of *Aedes* spp. caught (Table 5.2; $P < 0.05$). The effect of treatment on the number of *An. gambiae* s.l. caught was not significant (Table 5.2; $P = 0.08$). The effect of traps with skin bacteria combined with CO_2 on *An. gambiae* s.l. catches compared to CO_2 alone, was almost significant ($P = 0.07$). Significantly more *Mansonia* spp. mosquitoes were caught in MM-X traps baited with skin bacteria + CO_2 or a worn sock + CO_2 than in traps baited with CO_2 alone ($P = 0.03$ and $P = 0.001$, respectively). Only few *Aedes* spp. were collected. Traps baited with agar + CO_2 or a worn sock + CO_2, however, caught significantly more *Aedes* spp. mosquitoes than traps baited with CO_2 alone ($P = 0.02$ and $P = 0.005$ respectively).

Discussion

Volatiles released by human skin microbiota were attractive to *An. gambiae s.s.* in both laboratory and semi-field experiments (Figures 5.2 & 5.3). In the semi-field experiments, the effect of skin microbiota was enhanced by the addition of carbon dioxide (Figure 5.3). The results of the field experiments suggest an effect of skin bacteria volatiles on trap catches of *An. gambiae* s.l. under natural conditions.

Results obtained in the laboratory were, to a large extent, confirmed by the results obtained with skin bacteria in the semi-field. The synthetic blend, consisting of ten compounds identified in the headspace of human skin bacteria, was attractive in the laboratory and not under semi-field conditions (Figure 5.2 & 5.3). It is likely that concentrations of both the volatiles in the synthetic blend and in the natural blend emitted by the skin bacteria should be higher in semi-field respectively field experiments than in laboratory experiments to obtain attraction of these blends of semiochemicals under natural conditions. Further experiments should confirm this. The effect of the

Table 5.2. Mosquito catch in field experiments.

	House	Night	Treatment	n	Treatments			
					CO_2	Agar + CO_2	Bacteria agar + CO_2	Worn Sock + CO_2
Anopheles gambiae s.l.	<0.001	0.013	0.084	16	3.63 ± 1.01^{a}	4.19 ± 1.25^{ab}	6.00 ± 1.54^{ab}	7.19 ± 1.82^{b}
Culex spp.	<0.001	0.002	0.660	16	15.88 ± 4.03^{a}	20.81 ± 6.19^{a}	16.25 ± 4.63^{a}	20.56 ± 5.26^{a}
Mansonia spp.	<0.001	0.053	0.003	16	8.25 ± 1.53^{a}	10.31 ± 1.97^{ab}	14.25 ± 2.84^{bc}	17.06 ± 3.40^{c}
Aedes spp.	<0.001	0.001	0.020	16	1.25 ± 0.54^{a}	3.31 ± 1.12^{b}	2.38 ± 0.55^{ab}	3.63 ± 0.71^{b}

The effect of house, night and treatment (GLM, $y = house*x_1 + night*x_2 + treatment*x_3$, P-values given) on the mean number of mosquitoes caught per night in field experiments in Kenya (± standard error). Mean mosquito numbers followed by different letters in the same row are significantly different at $P < 0.05$ (GLM).

synthetic blend may be further improved by testing the biologically active components present in the blend by coupled Gas Chromatography - ElectroAntennoGram (GC-EAG) (Qiu et al. 2004b) through either a sequential procedure starting with a weakly attractive mixture and adding the components one by one (Okumu et al. 2010c) or a substraction procedure starting with the ten compound blend (Smallegange et al. 2009). This will elucidate the individual effect of each compound, which may result in the development of a more effective synthetic blend consisting of less than ten compounds.

The addition of CO_2 to the traps in the current semi-field study increased the total number of mosquitoes trapped almost threefold (Figure 5.2), which emphasizes its important role in mosquito host-seeking behaviour. The increase in mosquito catches by addition of CO_2 has also been observed in similar semi-field experiments in Tanzania (Schmied et al. 2008) and for this reason CO_2 has been added to odour-baited traps in various studies for increased responsiveness of *An. gambiae* s.l. (Jawara et al. 2009, Okumu et al. 2010c).

As the volatiles from the natural mixture of human skin bacteria were attractive to *An. gambiae s.s.* in the laboratory and semi-field experiments, testing of different species of skin bacteria may lead to the identification of bacterial species more attractive than a natural mixture of bacteria and to the identification of new attractants. Synthetic compounds attractive to *An. gambiae s.s.* can be identified by gas chromatography - mass spectrometry (GC-MS) analysis of headspaces collected from bacteria (Verhulst et al. 2009), but it should be taken into account that concentration is known to be an important factor in the development of an attractive blend (Vale and Hall 1985, Smallegange et al. 2005, Smallegange and Takken 2010).

The results from the field study may have been confounded by the relatively low densities of anopheline mosquitoes, both in December 2008 and in July 2009, probably because of low rainfall during the study periods. In a previous study the abundance of *Anopheles* mosquitoes was much higher and more than 50% of the *An. gambiae* s.l. were *An. gambiae s.s.* (Mathenge et al. 2004). In addition, a study performed in a nearby area in Kenya and still running at the time of the field experiments described in the current paper suggests that the area-wide coverage of insecticide-treated bed nets caused a population decline in *An. gambiae s.s.*, the target species in the current study, and a proportionate increase in *An. arabiensis* (Bayoh et al. 2010).

Anopheles arabiensis was the main *An. gambiae* s.l. sibling species

caught in the field traps and, interestingly, the current results suggest that this mosquito species may be attracted to traps baited with human skin bacteria. *Anopheles arabiensis* is one of the main malaria vectors in Africa (Gillies and Coetzee 1987, Coetzee et al. 2000) and has an opportunistic host preference in contrast to *An. gambiae s.s.*, which is considered to be more anthropophilic (White 1974, Githeko et al. 1994, Githeko et al. 1996). More *Mansonia* spp. were caught in traps baited with both skin bacteria and CO_2 than in traps baited with CO_2 alone. *Mansonia* spp. are important nuisance mosquitoes due to aggressive biting and have been associated with West Nile virus and Rift Valley Fever transmission (Fontenille et al. 1998, Diallo et al. 2005). Future studies should reveal whether some mosquito species are more attracted to volatiles produced by skin microbiota than others and if these volatiles play a role in the host preference of mosquitoes.

From the laboratory and semi-field results it was clear that human skin bacterial volatiles attract *An. gambiae s.s.* when applied in mosquito traps. Encouraging results were obtained in the field study, suggesting that several disease vectors may be lured into mosquito traps by human skin bacterial volatiles. More knowledge on the role of skin bacteria in mosquito-host interactions could help to improve bacterial baits or baits composed of synthetic bacterial metabolites. These baits may play a role in future vector-control strategies for monitoring vector densities or in push-pull strategies.

Acknowledgements

We would like to thank Tony Njeru for assisting with semi-field and field experiments. We acknowledge Frans van Aggelen, David Alila, André Gidding, Elizabeth Masinde, David Owaga, Dennis Veldhuizen, and Léon Westerd for rearing mosquitoes and Phoebe Mdadi, Mike Okal and Evelyn Odinga for organizing materials and for their help with semi-field experiments. Our thanks also go to the inhabitants of Lwanda for allowing us to work in their village, and Woody Foster and Tom Guda for kindly making available MM-X traps. The generous provision of additional traps by Woodstream USA is greatfully acknowledged. This study was funded by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health Initiative (GCGH#121). This study was approved by the joint Kenyatta National Hospital/University of Nairobi ethical review committee (protocol approval number P102/7/2004 amended in 2008).

Improvement synthetic lures using bacterial compounds

Improvement of a synthetic lure for *Anopheles gambiae sensu stricto* using compounds produced by human skin bacteria

06

Niels O. Verhulst, Phoebe A. Mbadi, Gabriella Bukovinszkiné Kiss, Wolfgang R. Mukabana, Joop J.A. van Loon, Willem Takken, Renate C. Smallegange

Anopheles gambiae sensu stricto is considered to be highly anthropophilic and volatiles of human origin provide essential cues during its host-seeking behaviour. A blend of three human-derived volatiles, ammonia, lactic acid and tetradecanoic acid, attracts *An. gambiae*. Bacteria isolated from the human skin produce volatiles that attract *An. gambiae*. The purpose of the current study was to test the effect of ten compounds present in the headspace of human bacteria on the host-seeking process of *An. gambiae*. The effect of each of the ten compounds on the attractiveness of a basic blend of ammonia, lactic and tetradecanoic acid to *An. gambiae* was examined.

The host-seeking response of *An. gambiae* was evaluated in a laboratory setup using a dual-port olfactometer and in a semi-field facility in Kenya using MM-X traps. Odorants were released from LDPE sachets and placed inside the olfactometer as well as in the MM-X traps. Carbon dioxide was added in the semi-field experiments, provided from pressurized cylinders or fermenting yeast.

The olfactometer and semi-field setup allowed for high-throughput testing of the compounds in blends and in multiple concentrations. Compounds with an attractive or inhibitory effect were identified in both bioassays. 3-Methyl-1-butanol was the best attractant in both setups and increased the attractiveness of the basic blend up to three times. 2-Phenylethanol reduced the attractiveness of the basic blend in both bioassays by more than 50%.

Identification of volatiles released by human skin bacteria led to the discovery of compounds that have an impact on the host-seeking behaviour of *An. gambiae*. 3-Methyl-1-butanol may be used to increase mosquito trap catches, whereas 2-phenylethanol has potential as a spatial repellent. These two compounds could be applied in push-pull strategies to reduce mosquito numbers in malaria endemic areas.

Submitted

Introduction

Host-seeking mosquitoes are mainly guided by chemical cues released by their blood hosts (Takken and Knols 1999, Smallegange and Takken 2010). Some of these cues have already been identified for the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) and include ammonia, lactic acid and carboxylic acids (Knols et al. 1997, Braks et al. 2001, Smallegange et al. 2005, Smallegange et al. 2009, Okumu et al. 2010c), which are released from the human skin. These compounds are more attractive in mixtures than when applied alone (Smallegange et al. 2005, Okumu et al. 2010c). Lactic acid, for example, is only slightly attractive on its own (Braks et al. 2001), but when combined with ammonia and carboxylic acids, this combination shows a synergistic effect (Smallegange et al. 2005).

Blends of ammonia, lactic acid and carboxylic acids have been shown to be attractive in the laboratory (Smallegange et al. 2005, Smallegange et al. 2009), and in semi-field and field setups when carbon dioxide (CO_2) was added (Qiu et al. 2007b, Okumu et al. 2010c). However, a blend of ammonia, lactic acid and carboxylic acids is still less effective than humans odours, when compared at close distance (Okumu et al. 2010c, Smallegange et al. 2010) and its attractive effect can probably be improved by the addition of compounds other than aliphatic carboxylic acids (Smallegange et al. 2009).

Another chemical cue that plays an important role in mosquito host-seeking behaviour, including that of *An. gambiae,* is CO_2 (Gillies 1980, Mboera and Takken 1997). In the field, trap catches increase when CO_2 is added to an odour blend (Costantini et al. 1996b, Mboera et al. 2000a, Qiu et al. 2007b, Schmied et al. 2008, Jawara et al. 2009).

Humans are differentially attractive to mosquitoes (Schreck et al. 1990, Knols et al. 1995, Bernier et al. 2002, Mukabana et al. 2002, Qiu et al. 2006, Logan et al. 2008) and focusing on these differences can reveal new compounds that mediate the mosquito host-seeking process (Bernier et al. 2002, Logan et al. 2008). Analyses of human skin emanations, however, often result in hundreds of compounds (Bernier et al. 2000, Penn et al. 2007), which makes identification of active compounds laborious, as each compound potentially may contribute to the overall attraction of the emanations. Recently it was shown that volatiles released by human foot bacteria grown *in vitro* attract *An. gambiae* (Verhulst et al. 2009). Chemical analysis of the headspace collected from the cultures of these skin bacteria narrowed down the number of putative attractants to fourteen. A synthetic blend consisting of ten of these

was attractive to *An. gambiae* (Verhulst et al. 2009), although not as attractive as the volatiles released by the skin bacteria themselves (Verhulst et al. 2009). In addition to this, when tested in semi-field experiments in Kenya, traps baited with CO_2 and the blend of compounds did not catch more mosquitoes than the control traps baited with CO_2 (Chapter 5). Possibly the concentrations of the chemicals tested were too low to attract the mosquitoes from a distance or some of the compounds in the blend acted as inhibitors, masking the attractive effect of the other components in the blend.

The purpose of the current study was to test the effect of each of the ten compounds present in the headspace of human foot bacteria on the host-seeking process of *An. gambiae*. Compounds may be more attractive in mixtures than when applied alone (Geier et al. 1999, Smallegange et al. 2005) and therefore the effect of each of the ten selected compounds on the attractiveness of a blend of ammonia, lactic acid and tetradecanoic acid (Smallegange et al. 2009) to *An. gambiae* was examined. Experiments were performed in an olfactometer and in a semi-field setup to compare results obtained under laboratory and semi-field conditions.

Methods

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* colony used for the laboratory experiments originated from Suakoko, Liberia. Mosquitoes were cultured in the Laboratory of Entomology of Wageningen University, The Netherlands, since 1988 and received blood meals from a human arm twice a week. Adult mosquitoes were maintained in 30-cm cubic gauze-covered cages in climate-controlled chambers (27 ± 1°C, 80 ± 5% RH, LD 12:12). They had access to a 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper and placed in tap water in plastic trays. Larvae were fed daily on Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed in 30-cm cubic cages for emergence.

The *Anopheles gambiae* Giles *sensu stricto* colony at the Thomas Odhiambo campus of the International Centre of Insect Physiology and Ecology (ICIPE), Nyanza Province, western Kenya, were used for the semi-field assays. The colony originated from Mbita Point and has been cultured since 2001. Mosquitoes were fed three times a week on a human arm and larvae were reared in filtered water from Lake Victoria. They had access to a 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper

and placed in tap water in plastic trays. Larvae were fed daily on Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed in 30-cm cubic cages for emergence.

Compounds

The ten compounds identified in previous experiments (Verhulst et al. 2009), were dispensed from sealed sachets (25 x 25 mm) of Low Density PolyEthylene sheet (LDPE; Audion Elektro, The Netherlands) (Torr et al. 1997). Each sachet contained 100 µL of each of the ten diluted or undiluted compounds. In the laboratory experiments, compounds were tested in sachets of 0.20 mm sheet thickness and diluted in paraffin oil (Merck, Germany) (1:100; 1:1,000 or 1:10,000). In the semi-field experiments, compounds were tested undiluted in sachets of 0.20, 0.10 or 0.03 mm sheet thickness. The compounds 1-butanol, 2-methyl-1-butanol, 2-methylbutanal, 2-methylbutanoic acid, 3-hydroxy-2-butanone and 3-methylbutanoic acid were purchased from Sigma (Germany) and 2,3-butanedione, 3-methyl-1-butanol, 3-methylbutanol, 3-methylbutanal and 2-phenylethanol from Fluka (Germany). All compounds had purity levels between 95 and 99.8%.

For each of the ten compounds it was tested whether it increased or reduced mosquito catches of a basic blend of ammonia, L-(+)-lactic acid (henceforth termed lactic acid) and tetradecanoic acid (Smallegange et al. 2009) upon addition to the latter blend. Ammonia (100 µl of a 25% solution in water; analytical grade, Merck) and tetradecanoic acid (50 mg, >99%, Sigma) were released from separate LDPE sachets of 0.03 mm sheet thickness, while lactic acid (100 µl of a 88–92% aqueous solution, Riedel-de Haën) was released from a third LDPE sachet of 0.05 mm sheet thickness.

Carbon dioxide was added in the semi-field experiments as it has been shown to increase trap catches of *An. gambiae* under semi-field conditions (Njiru et al. 2006, Schmied et al. 2008),. Four compounds (2-methyl-1-butanol, 2-methylbutanal, 2-methylbutanoic acid and 3-methylbutanoic acid) were tested together with carbon dioxide (≥ 99.9%) provided from pressurized cylinders (Carbacid Investments Ltd., Kenya) (Table 6.1) through silicone tubing (Ø 7 mm; Rubber B.V., The Netherlands) connected to the Luer connection at the underside of the trap's top lid. The carbon dioxide was released at a rate of 500 ml/min regulated by a flow meter (Sho-Rate; Brooks Instruments, The Netherlands). The other six compounds (1-butanol, 2,3-butanedione, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 3-

Table 6.1 Response of **Anopheles gambiae** *in an olfactometer to compounds identified in bacterial headspace samples.*

Compound	Dilution	N	Treatment	Control	χ2-test (P-value)	Effect
1-butanol	1:100	167	34	39	0.56	
	1:1,000	160	23	38	0.05	
	1:10,000	163	32	17	0.03	+
2,3-butanedione	1:100	166	25	42	0.04	-
	1:1,000	166	25	39	0.08	
	1:10,000	169	44	28	0.06	
2-methyl-1-butanol	1:100	165	22	25	0.66	
	1:1,000	164	24	45	0.01	-
	1:10,000	168	38	22	0.04	+
2-methylbutanal	1:100	174	33	49	0.08	
	1:1,000	172	13	22	0.13	
	1:10,000	171	31	18	0.06	
2-methylbutanoic acid	1:100	167	49	25	0.01	+
	1:1,000	171	49	41	0.40	
	1:10,000	166	38	43	0.58	
3-hydroxy-2-butanone	1:100	168	21	29	0.26	
	1:1,000	170	29	36	0.39	
	1:10,000	170	36	17	0.01	+
3-methyl-1-butanol	1:100	163	29	31	0.80	
	1:1,000	158	20	28	0.25	
	1:10,000	157	41	25	0.048	+

See next page

Table 6.1 continued

Compound	Dilution	N	Treatment	Control	χ2-test (P-value)	Effect
3-methylbutanal	1:100	170	34	18	0.03	+
	1:1,000	168	24	21	0.65	
	1:10,000	172	16	16	1.00	
3-methylbutanoic acid	1:100	163	22	25	0.66	
	1:1,000	161	33	24	0.23	
	1:10,000	161	30	18	0.08	
2-phenylethanol	1:100	162	24	30	0.41	
	1:1,000	167	15	40	<0.001	-
	1:10,000	155	11	25	0.02	-

The ten test compounds were applied in three dilutions (1:100; 1:1,000; and 1:10,000) in LDPE sachets. The effect of the compounds on mosquito behaviour was examined by adding them individually to the attractive basic blend (treatment) and to test this combination against the basic blend (control). N = number of mosquitoes released. The effect of the compound tested on the 'attractiveness' of the basic blend is indicated: + = significant increase of mosquito catches compared to the control, - = significant reduction of mosquito catches compared to the control.

methylbutanal and 2-phenylethanol) were tested together with yeast-produced CO_2 (Table 6.1) as described by Smallegange et al. (in press). Carbon dioxide was produced by mixing 17.5 g of dry yeast (Angel Instant Dry Yeast Co. Ltd., China), 250 g sugar (Sony Sugar, South Nyanza sugar Co. Ltd., Kenya) and 2.0 L tap water in a plastic bottle of 3 L, which results in a release rate of approximately 135 ml/min. Mixing took place 30 minutes before mosquitoes were released, at ambient temperature, until the dry yeast was dissolved.

The bottles were connected to the MM-X traps (American Biophysics Corp., USA) (Kline 1999) using the original MM-X tubing (micron filter and orifice removed) and the Luer connection at the underside of the trap's top lid. The connections were sealed by Teflon tape to prevent leakage of carbon dioxide.

Olfactometer experiments

A three layer dual-port olfactometer (Posey et al. 1998) was used to evaluate host-seeking responses of female mosquitoes to the ten compounds identified in a previous study (Verhulst et al. 2009). Pressurized air was charcoal-filtered, humidified, and passed through two poly-methyl-methyl-acrylaat (PMMA) mosquito trapping devices equipped with funnels (Verhulst et al. 2008), which were linked to both ports (diameter 5 cm, 25 cm apart) of the olfactometer. The air entered the flight chamber (1.50 x 0.50 x 0.50 m) at a speed of 0.21 ± 0.02 m/s. Temperature and humidity were recorded using data loggers (MSR145S, MSR Electronics GmBH, Switzerland). Temperature of the air entering the two trapping devices was 27.0 ± 1.2°C, and relative humidity was above 80%. The air temperature in the flight chamber was 25.8 ± 0.7°C and relative humidity was 77.3 ± 8.6 %. The experimental room was maintained at a temperature of 25.8 ± 0.8°C and a relative humidity of 64.5 ± 5.1%.

Experiments were prepared and performed according to the methods described by Smallegange et al. (2005). For each test 30 female mosquitoes of 5-8 d old, which had been provided a mating opportunity and not been offered a blood meal, were selected 14-18 h before the experiment and placed in a cylindrical release cage (d = 8 cm, h = 10 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the scotophase, when *An. gambiae* females are known to be highly responsive to host odours (Maxwell et al. 1998, Killeen et al. 2006).

The compounds tested were diluted 1:100; 1:1,000 or 1:10,000 in paraffin oil. Each diluted solution of the volatile compounds was contained in a LDPE sachet and placed in the trapping device together with the three sachets containing the basic blend (ammonia, lactic acid, tetradecanoic acid). The control trap was baited with the sachets making up the basic blend and one LDPE sachet containing 100 µl paraffin oil. All sachets were suspended by a hook as described before (Verhulst et al. 2009). Clean air was tested against clean air, to test the symmetry of the system and to determine the mosquito response when no odour stimulus was present. The attractiveness of the basic blend was established by testing the three LDPE sachets containing ammonia, lactic acid and tetradecanoic acid against three sachets of the same size and sheet thickness, one empty and two filled with distilled water.

In each trial, test odours were released in the air stream before a group of mosquitoes was set free from a cage which was placed at the downwind end of the flight chamber, 1.50 m from the two ports. After 15

minutes specimens that entered each of the two trapping devices were counted after anaesthesia with CO_2. Each trial started with a fresh batch of mosquitoes, clean trapping devices, and new stimuli. Surgical gloves were worn by the researcher at all times to avoid contamination of equipment with human volatiles.

The sequence of test odours was randomized on the same day, between days and between the three layers of the olfactometers. Each treatment was repeated six times and test stimuli were alternated between right and left ports in different replicates to rule out any positional effects.

Semi-field experiments

Semi-field experiments were conducted at the Thomas Odhiambo campus of ICIPE, Kenya. Experiments were conducted as described before (2006), (Chapter 5) in a greenhouse with a glass-panelled roof and gauze-covered side walls. Inside, sand covered the floor and a large mosquito-netting cage (11 x 7 x 2.5 m; mesh width 3 mm) was suspended from the ceiling to the floor. Four MM-X traps (American Biophysics Corp., USA) (Kline 1999) were placed in the corners of the greenhouse (Figure 6.1), with the odour outlet positioned 15 cm above ground level (Schmied et al. 2008, Jawara et al. 2009). MM-X traps were used because they have a high discriminatory power (Schmied et al. 2008).

For each test 200 female mosquitoes, 3-6 d old that had been held together with male mosquitoes to allow mating and that had not received a blood meal, were selected 8 h before the experiments. Mosquitoes were placed in a 1 L (d = 11-13 cm, h = 15cm) cup, covered by mosquito netting, and were offered water-moistened cotton wool only. Every test night, the mosquitoes were released from the centre of the greenhouse at 8.00 pm. At

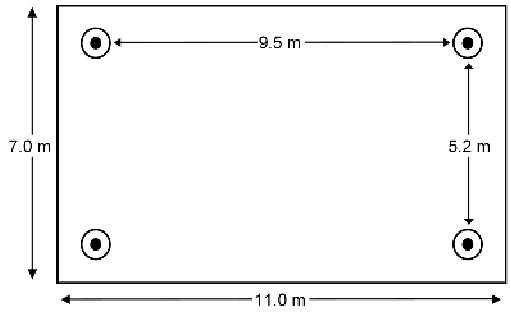


Figure 6.1 Schematic drawing of semi-field setup (top view).
The rectangle represents the outline of the screened cage. Mosquitoes were released from the centre. Circles indicate the positions of the four MM-X traps.

6.30 am the following morning traps were collected and placed in a freezer, after which mosquitoes were counted. Every afternoon the mosquitoes remaining in the greenhouse were captured and the sand in the greenhouse moistened to prevent dust formation and to lower the temperature. Surgical gloves were worn by the researcher to avoid contamination of equipment with human volatiles.

Each of the four traps in the greenhouse was provided with CO_2 (either from a cylinder or yeast-produced) and the basic blend released from three LDPE sachets containing either ammonia, lactic acid or tetradecanoic acid (see above). Three traps were provided with LDPE sachets containing the compound to be tested, each in a sachet of different thickness to test various release rates. Sachets were used with a sheet thickness of either 0.03 mm, 0.10 mm or 0.20 mm and release rates were measured by weighing (laboratory: AC100, Mettler, Germany; semi-field, A200S Sartorius, Germany; accuracy both 0.1 mg) the sachets before and after the experiments (Torr et al. 1997). LDPE sachets were suspended by hooks in the black tube of the MM-X trap (Verhulst et al. 2009). Treatments and traps were randomized over the four positions to complete 3 series of a 4 x 4 Latin square in 12 nights.

A data logger (TinyTag Ultra, model TGU-1500, INTAB Benelux, The Netherlands) recorded temperature and humidity during the experiments in the middle of the screenhouse, every ten minutes. During the nights of the semi-field experiments (March – September 2009), the average temperature was 23.6 ± 2.6 ºC and the average relative humidity 71.5 ± 18.4%.

Statistics

To test whether the release rates of each compound varied exponentially with LDPE sheet thickness as described by Torr et al. (1997), the release rates of each compound and the LDPE thickness were fitted to an exponential regression line (Genstat, release 12.1).

For each two-choice test in the olfactometer a χ^2-test was used to analyze whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution ($P < 0.05$).

A Generalized Linear Model (GLM, Binomial, logit link function, dispersion estimated) was used to investigate the relative attractiveness of each combination of odours tested in the traps in the semi-field experiments,

expressed as the number of mosquitoes caught in one of the traps divided by the total number of mosquitoes trapped in all four traps during each experimental night (Qiu et al. 2004a, Qiu et al. 2006). Two-sided t-probabilities were calculated to test pairwise differences between proportions. Effects were considered to be significant at $P < 0.05$. For each series of 12 nights in which a compound was tested, the effect of CO_2 source, trap location, temperature and humidity on mosquito catches was tested and fitted as parameters in the GLM model when significant. Another GLM (Binomial, logit link function, dispersion estimated) was used to test the effect of CO_2 source (cylinder or yeast-produced) on catches of traps baited with the basic blend alone.

Results

Olfactometer experiments

The results of the olfactometer experiments in which no odour stimuli were placed in either of the trapping devices showed that the olfactometer was symmetrical in all three layers (χ^2-test, d.f. = 1, $P > 0.05$). Trapping devices baited with the basic blend caught significantly more mosquitoes than trapping devices baited with LDPE sachets with water alone (χ^2-test, d.f. = 1, $P < 0.001$). These results are in accordance with previous experiments (Smallegange et al. 2009).

Of the ten compounds tested, five compounds (1-butanol, 2-methylbutanoic acid, 3-hydroxy-2-butanone, 3-methyl-1-butanol and 3-methylbutanal) increased the number of mosquitoes caught in the trapping devices baited with the basic blend compared to the trapping devices baited with the basic blend alone (Table 6.1; χ^2-test, d.f. = 1, $P < 0.05$). This depended, however, on the concentration tested (Table 6.1). Three compounds (2-methyl-1-butanol, 2,3-butanedione and 2-phenylethanol) caused fewer mosquitoes to enter the trapping devices compared to the trapping devices baited with the basic blend alone, dependent on the concentration tested (Table 6.1; χ^2-test, d.f. = 1, $P < 0.05$). 2-Phenylethanol was the only compound that reduced the number of mosquitoes caught in the trapping devices with the basic blend at the two lowest concentrations tested (Table 6.1; χ^2-test, d.f. = 1, 1:1,000 $P < 0.001$ and 1:10,000 $P = 0.02$). A concentration of 1:1,000 of 2-methyl-1-butanol reduced the number of mosquitoes caught in the trapping devices baited with the basic blend (χ^2-test, d.f. = 1, $P < 0.05$), whereas a concentration of 1:10,000 of this compound led to an increased number of mosquitoes caught (Table 6.1).

Semi-field experiments

An exponential correlation was found between LDPE thickness and release rates of six of the ten compounds tested (Table 6.2). The CO_2 source used (pure CO_2 from a pressurized cylinder or CO_2 produced by fermenting yeast) had no significant effect on trap catches (GLM, d.f. = 1, P = 0.96). The spatial position of the trap in the greenhouse had a significant effect on the trap catches of all compounds tested (Table 6.3; GLM, d.f. = 3, P < 0.05), except in the case of 2-methyl-1-butanol (GLM, d.f. = 3, P = 0.54), and was therefore included in the GLM model. Treatment had a significant effect on trap catches with 2,3-butanedione, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 3-methylbutanoic acid or 2-phenylethanol (Table 6.3; GLM, d.f. = 3, P < 0.05).

Traps to which either 2,3-butanedione, 3-methyl-1-butanol or 3-methylbutanoic acid was added caught significantly more mosquitoes than traps baited with the basic blend and CO_2 alone, depending on the sachet sheet thickness used to release the test compound from (Table 6.3; GLM, d.f. = 3, P < 0.05). Traps baited with either 2-methylbutanal, 3-hydroxy-2-butanone, 3-methylbutanoic acid or 2-phenylethanol and the basic blend and

Table 6.2 Correlation between LDPE thickness and release rate of bacterial volatiles as determined by weight loss of the LDPE sachets.

			Exponential regression parameters $A + B*(R^X)$		
	R^2 (%)	P-value	A	B	R
1-butanol	12.8	0.06	0.00036	0.0200	5.22E-18
2,3-butanedione	64.3	<0.001	0.00387	0.0975	5.28E-13
2-methyl-1-butanol	–[1]	0.46	-0.00042	0.0052	1.49E-12
2-methylbutanal	31.7	<0.01	0.00709	0.0595	6.96E-13
2-methylbutanoic acid	2.6	0.26	0.01020	9.585E-19	6.275E+79
3-hydroxy-2-butanone	58.7	<0.001	0.000242	0.0122	1.27E-19
3-methyl-1-butanol	61.7	<0.001	0.0003722	8.561	2.307-125
3-methylbutanal	33.8	<0.001	0.00745	0.0495	2.88E-11
3-methylbutanoic acid	–[1]	0.85	0.01007	3.244E-19	5.125E+79
2-phenylethanol	35.3	0.001	0.001333	3.12E-19	6.80E+80

The release rate of each compound (Y, g/night) was fitted by an exponential regression model ($A + B*(R^X)$; X = LDPE thickness, mm) (Torr et al. 1997). R^2 = coefficient of determination. [1]Residual variance exceeds variance of response variate.

Table 6.3 Mean trap catches of **Anopheles gambiae** *in a semi-field setup to bacterial volatiles.*

			BB	0.03		0.10		0.20	
Compound	**Trap location**	**Treatment**	**Mean ± SE**	**Mean ± SE**	**Effect**	**Mean ± SE**	**Effect**	**Mean ± SE**	**Effect**
1-butanol	<0.001	0.10	38.4 ± 6.7	47.0 ± 9.5		37.4 ± 6.7		28.6 ± 4.9	
2,3-butanedione	<0.001	0.02	29.0 ± 4.4	29.5 ± 6.3		55.2 ± 14.4	+	30.0 ± 7.2	
2-methyl-1-butanol	0.54	0.20	24.9 ± 4.3	23.2 ± 2.8		22.6 ± 3.5		33.4 ± 6.3	
2-methylbutanal	0.04	0.07	29.6 ± 4.7	18.4 ± 3.3	-	30.1 ± 5.2		23.3 ± 3.9	
2-methylbutanoic acid	0.001	0.93	19.6 ± 3.1	19.0 ± 4.6		18.0 ± 3.9		20.1 ± 4.3	
3-hydroxy-2-butanone	<0.001	<0.001	44.0 ± 9.3	52.8 ± 6.4		33.3 ± 5.8	-	27.8 ± 4.3	-
3-methyl-1-butanol	<0.001	<0.001	19.1 ± 3.3	29.1 ± 6.2		61.3 ± 9.6	+	29.8 ± 6.4	+
3-methylbutanal	0.007	0.670	25.8 ± 7.2	22.3 ± 3.6		24.4 ± 7.1		18.9 ± 2.3	
3-methylbutanoic acid	<0.001	<0.001	32.4 ± 4.1	11.9 ± 0.8	-	58.0 ± 5.8	+	13.0 ± 1.9	-
2-phenylethanol	0.002	0.002	47.3 ± 8.1	23.3 ± 3.1	-	26.0 ± 4.1	-	26.7 ± 5.0	-

All traps were provided with CO_2 and the basic blend (BB). To three traps a LDPE sachet of different thickness (0.03 mm, 0.10 mm or 0.20 mm) was added containing one of the 10 test compounds The effect of trap location and treatment (GLM, y = locationx_1 + treatment*x_2, P-values given) on the mean number of mosquitoes caught per night (± standard error) is given. The effect of the compound tested on the 'attractiveness' of the BB is indicated: + = significant increase of mosquito catches compared to the BB, - = significant reduction of mosquito catches compared to the BB (GLM, P < 0.05).*

CO_2 caught significantly fewer mosquitoes than traps with the basic blend and CO_2, depending on the sachet sheet thickness used to apply the test compound (Table 6.3; GLM, d.f. = 3, $P > 0.05$). 2-Phenylethanol was the only compound that reduced trap catches independent of the LDPE thickness tested, compared to the number of mosquitoes caught by traps to which no 2-phenylethanol was added (Table 6.3; GLM, d.f. = 3, $P < 0.05$).

Discussion

The identification of volatiles produced by human skin bacteria narrows down the number of putative mosquito attractants emitted by humans (Verhulst et al. 2009). The three-layer olfactometer and semi-field system allowed for high-throughput testing of these volatiles. Many of the compounds identified in the headspace of the human foot bacteria, caused a behavioural response of *An. gambiae* in both olfactometer and semi-field experiments, with an attractive or repellent effect, dependent on the compound and concentration tested.

Eight out of the ten compounds tested in the olfactometer had a significant effect on the number of mosquitoes caught when compared with the numbers attracted to the basic blend only. Six of these significantly increased the number of mosquitoes caught with the basic blend when tested at a specific concentration. In the semi-field system, six out of the ten compounds tested had a significant effect on the trap catches when combined with CO_2 and the basic blend and tested against CO_2 and the basic blend alone. At certain concentrations, three of these compounds increased the ´attractiveness´ of the basic blend combined with CO_2.

Both olfactometer and semi-field results indicate that the concentration at which each compound is tested has an important effect on the response of *An. gambiae*. This has also been observed in previous experiments with *An. gambiae* in laboratory and (semi-)field experiments (Braks et al. 2001, Smallegange et al. 2005, Smallegange et al. 2009, Okumu et al. 2010c, Smallegange and Takken 2010, Qiu et al. in press). As has been mentioned previously (Smallegange et al. 2009), it is difficult to estimate and compare the concentrations of the volatiles in the odour blends tested as encountered by the mosquitoes in the two different bioassays. The different results obtained with 2,3-butanedione, 3-hydroxy-2-butanone and 3-methylbutanal in the two bioassays show this clearly. This underlines the importance of control over the concentration tested and testing more than three concentrations in future experiments is likely to provide a better understanding of the effect of each

compound (Smallegange et al. 2005).

Of four compounds (1-butanol, 2-methyl-1-butanol, 2-methylbutanoic acid and 3-methylbutanoic acid) the release rates from the LDPE sachets in the semi-field experiments was very low or the variation too high to find a significant correlation with LDPE sheet thickness (Table 6.2), even though we aimed to standardize the release rate of each of the candidate compounds by using LDPE material (Torr et al. 1997). The use of LDPE tubes may reduce variation in the release rate, depending on the compound tested (Torr et al. 1997), while the use of nylon strips or open glass vials (Okumu et al. 2010a) could increase the release of compounds.

2-Methylbutanal reduced the attractiveness of the basic blend in the semi-field system at the highest concentration tested. In the olfactometer, the highest concentration also reduced the number of mosquitoes caught, although the effect was marginally significant (Table 6.1, P = 0.08). 2-Phenylethanol clearly inhibited mosquito attraction exerted by the basic blend as its addition reduced the number of mosquitoes caught by more than 50% in both bioassays. Future experiments with 2-phenylethanol may elucidate its potential as a spatial repellent for personal or household protection.

3-Methyl-1-butanol increased the number of mosquitoes caught with the basic blend in both experimental setups when tested at the lowest concentrations. In the semi-field, traps baited with 3-methyl-1-butanol applied in a LDPE sachet of 0.01 mm sheet thickness together with the basic blend and CO_2 caught three times more mosquitoes than traps baited with the basic blend and CO_2 alone. A field study has shown that synthetic odour blends can compete with natural host odour when placed in separate huts (Okumu et al. 2010c) and the results obtained with the combination of 3-methyl-1-butanol, the basic blend and CO_2 are encouraging as a novel attractant for future use in malaria vector monitoring or reduction.

In the semi-field experiments, carbon dioxide was added from two different sources: either from a pressurized cylinder containing pure CO_2 or from a bottle containing fermenting yeast. In a previous study, traps baited with yeast-produced CO_2 caught more mosquitoes than traps baited with CO_2 supplied from cylinders (Smallegange et al. in press). In the current study, however, no difference was found in trap catches between the cylinder and yeast-produced CO_2 when added to the basic blend, although concentrations were different from the previous study. For field application the use of yeast-produced CO_2 is preferable as it has several advantages above CO_2 from

cylinders: it is cheaper, easier to handle and easier to obtain (Smallegange et al. in press).

Conclusions

To date only a limited number of compounds that have an impact on the host-seeking behaviour of *An. gambiae* have been identified (Smallegange and Takken 2010). The identification of volatiles released by human skin bacteria resulted in a selection of ten compounds of which eight had an effect on host-seeking behaviour in the laboratory and six in the semi-field system. Two compounds showed a similar result in both bioassays and in multiple concentrations. 2-Phenylethanol reduced, whereas 3-methyl-1-butanol increased the attractiveness of the basic blend in both setups.

Carbon dioxide produced by yeast-sugar solutions applied from bottles, together with ammonia, lactic acid, tetradecanoic acid and 3-methyl-1-butanol applied in LDPE sachets can be an easy to use, cost-effective combination for monitoring and possible reduction of *An. gambiae* populations. In so-called push-pull systems (Agelopoulos et al. 1999, Cook et al. 2007, Logan and Birkett 2007, Logan et al. 2008, Jawara et al. 2009, Okumu et al. 2010c), the synthetic odour blend including 3-methyl-1-butanol may be used to 'pull' mosquitoes into traps. 2-Phenylethanol is a candidate compound to be added as a spatial repellent to 'push' mosquitoes away from human dwellings.

Acknowledgements

We would like to thank Frans van Aggelen, David Alila, André Gidding, Elizabeth Masinde, David Owaga, Dennis Veldhuizen, and Léon Westerd for rearing mosquitoes and, Mike Okal and Evelyn Olanga for their help with semi-field experiments. This study was funded by a grant from the Foundation for the National Institutes of Health (FNIH) through the Grand Challenges in Global Health Initiative (GCGH#121).

Differential attractiveness of skin bacteria to mosquitoes

Differential attractiveness to malaria mosquitoes of volatile blends produced by human skin bacteria

07

Niels O. Verhulst, Rob Andriessen, Ulrike Groenhagen, Gabriella Bukovinszkiné Kiss, Stefan Schulz, Willem Takken, Joop J.A. van Loon, Gosse Schraa, Renate C. Smallegange

The malaria mosquito *Anopheles gambiae sensu stricto* is mainly guided by human odour components to find its blood host. Skin bacteria play an important role in the production of human body odour and attract *An. gambiae* when grown *in vitro*. The role of single skin bacterial species in the production of volatiles that mediate the host-seeking behaviour of mosquitoes has remained largely unknown and is subject of the present study. Headspace samples were taken to identify volatiles that mediate this behaviour and can be used as potential attractant or repellent.

Five commonly occurring species of skin bacteria were tested in an olfactometer for the production of volatiles that attract *An. gambiae*. Odour blends produced by some bacterial species were more attractive than blends produced by other species. In contrast to odours from the other bacterial species tested, odours produced by *Pseudomonas aeruginosa* were not attractive to *An. gambiae*. Headspace analysis of bacterial volatiles in combination with behavioural assays led to the identification of six compounds that elicited a behavioural effect in *An. gambiae*.

Our results provide, to our knowledge, the first evidence for a role of selected bacterial species, common on the human skin, in determining the attractiveness of humans to malaria mosquitoes. This information will be used in the further development of a blend of semiochemicals for the manipulation of mosquito behaviour.

Submitted

Introduction

Blood-feeding mosquitoes are guided by odours during host finding (Takken and Knols 1999). The African malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) (henceforth termed *An. gambiae*) is one of the most important malaria vectors because of its preference to feed on humans (Takken and Knols 1999). Odour contributes to the differential attractiveness of humans to anthropophilic mosquitoes and these differences remain relatively stable over time (Knols et al. 1995, Bernier et al. 2002, Mukabana et al. 2002, Qiu et al. 2006, Logan et al. 2008).

Several volatile compounds that mediate host-seeking behaviour in mosquitoes have already been identified and tested in laboratory, semi-field or field studies. Carbon dioxide (CO_2), for example, plays an important role, presumably for mosquito activation and attraction over longer distances (Gillies 1980, Takken 1991, Spitzen et al. 2008). Ammonia, lactic acid and carboxylic acids are released from human skin and were attractive in laboratory, semi-field and field experiments when offered in a blend (Smallegange et al. 2005, Smallegange et al. 2009, Okumu et al. 2010c). However, the factors determining the quantity of compounds and composition of the odour blend released from the human skin that mediate mosquito behaviour have received little attention.

Bacteria play an important role in the production of human odours (Verhulst et al. 2010). Freshly secreted human sweat is odourless (Shelley et al. 1953) and only limited attractive to *An. gambiae* compared to sweat incubated with skin bacteria (Braks and Takken 1999). In addition, there is a strong correlation between human body odour and the species composition of skin bacteria (Rennie et al. 1990, Rennie et al. 1991, Taylor et al. 2003, Ara et al. 2006). Recent laboratory, semi-field, and field studies have shown that volatiles from bacteria obtained from human skin and grown *in vitro* on agar attract *An. gambiae* (Verhulst et al. 2009). The identification of the volatiles present in the headspace of natural human skin bacterial cultures led to the development of a synthetic blend consisting of ten compounds that was attractive to *An. gambiae* (Verhulst et al. 2009).

Volatiles produced by bacteria are often widespread among bacterial taxa, but some transformations of organic compounds lead to the production of volatiles that are species- specific (Schulz and Dickschat 2007). *Bacillus subtilis* has been associated with foot odour and corynebacteria species play a role in the synthesis of androsterone sulphate, which leads to a mixture of 16-

androstenes, producing a typical axillary smell (Gower et al. 1994). Staphylococci and corynebacteria play a role in the formation of volatile fatty acids (James et al. 2004b, James et al. 2004a), which have a distinct sweaty odour. Of these two bacterial genera, only *Staphylococcus* species can convert branched-chain amino acids to highly odorous short-chain volatile fatty acids (James et al. 2004a), that also play an important role in the host-seeking behaviour of *An. gambiae* (Knols et al. 1997, Smallegange et al. 2005, Smallegange et al. 2009).

Recent technological advances allow for high throughput 16s rDNA sequencing of the microbial community found on the human skin (Turnbaugh et al. 2007, Costello et al. 2009, Grice et al. 2009, Fierer et al. 2010). Studies using this technique revealed that the distribution of bacterial species on the human skin depends on local skin site characteristics (Costello et al. 2009, Grice et al. 2009) and that human individuals each have a unique composition of microbiota on their skin (Fierer et al. 2010). This composition remains relatively stable over time (Costello et al. 2009). Many different species of bacteria have been identified, some of which had not previously been associated with human skin. Pseudomonads, for instance, are extremely versatile organisms, which are primarily found in soil, water, decomposing organic materials, and also found in the intestinal flora. They are not commonly associated with human skin (Bojar and Holland 2002, Grice et al. 2008). *Pseudomonas aeruginosa* is the best known *Pseudomonas* species on the human skin, often found as a secondary invader of wounds and regarded as a transient species (Bojar and Holland 2002). A molecular study of the skin microbiota on the inner elbow, however, showed that *Pseudomonas* species were commonly present (Grice et al. 2008).

Volatiles produced by a mixture of human skin bacteria grown on agar attract *An. gambiae* (Verhulst et al. 2009). However, the role of single skin bacterial species in the production of volatiles that mediate the host-seeking behaviour of mosquitoes has remained largely unknown. In the present study the relative attractiveness of five common skin bacterial species to *An. gambiae* was investigated. The species *Bacillus subtilis, Brevibacterium epidermidis, Corynebacterium minutissimum, Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were chosen, because they are commonly found on the human skin and are associated with differences in body odour production (Kloos and Musselwhite 1975, McGinley et al. 1978, Anthony et al. 1992, Noble 2004, Ara et al. 2006, Grice et al. 2008). Headspace samples of

batch cultures of each bacterial species were taken to identify potential attractive or repellent compounds.

Results

Response of *An. gambiae* to volatiles from different bacterial species

A three layer dual-port olfactometer (Figure 7.1) (Posey et al. 1998) was used to evaluate host-seeking responses of female mosquitoes to volatile organic compounds (VOCs) produced by the bacterial species. The experiments with two unbaited traps showed no positional bias (χ^2-test, d.f. = 1, P = 0.48). Trapping devices baited with standard medium caught similar numbers of mosquitoes as unbaited trapping devices (χ^2-test, d.f. = 1, P = 0.56).

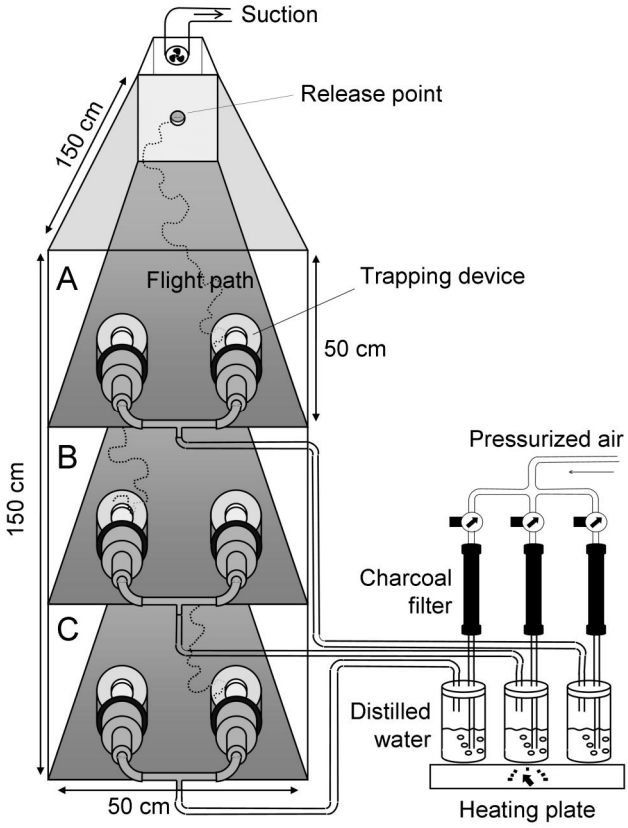


Figure 7.1. Schematic drawing of the three-layer dual-choice olfactometer used to examine the response of* Anopheles gambiae *to volatile organic compounds (VOCs).

Bacterial broths of *B. subtilis, Brev. epidermidis, C. minutissimum, P. aeruginosa* and *S. epidermidis* (Table 7.1) were tested at the time of exponential growth and in the stationary phase, when the number of bacteria in the broth stayed constant (Figure 7.2). No preference of *An. gambiae* was found for trapping devices baited with bacteria at the time of exponential growth compared to trapping devices baited with standard medium alone (Figure 7.3; χ^2-test, d.f. = 1, P > 0.05), except for trapping devices baited with

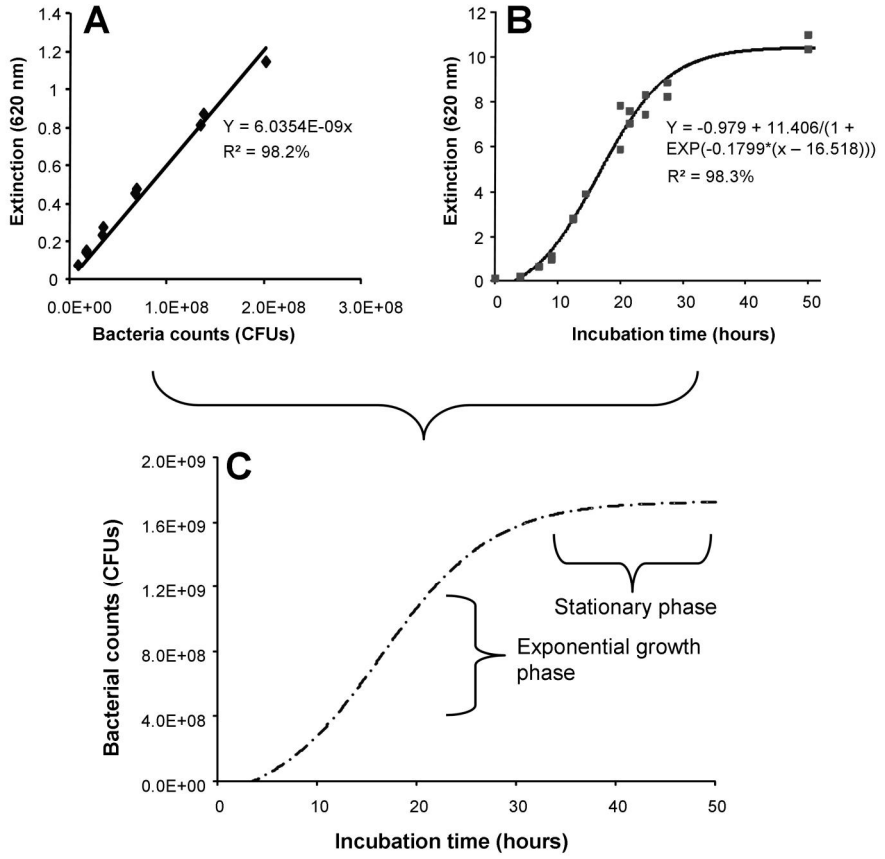


Figure 7.2. Combining spectrophotometer extinction values and bacterial numbers to plot a growth curve.

Corynebacterium minutissimum *is shown as an example. A: Correlation between the number of bacteria (determined by colony forming units, CFU) in standard medium and the optical density (extinction) of the medium in a spectrophotometer. B: Growth over time of* C. minutissimum *measured as optical density (extinction) and fitted by a logistic S-shaped curve (Genstat, release 12.1). C: Combining data from graphs A and B in a growth curve represented by the number of C. minutissimum (CFU) in standard medium over time.*

Table 7.1. Bacterial species used for experiments and headspace analyses.

Species	Origin
Staphylococcus epidermidis	DSMZ, Braunschweig, Germany, Nr. 20044
Corynebacterium minutissimum	DSMZ, Braunschweig, Germany, Nr. 20651
Brevibacterium epidermidis	DSMZ, Braunschweig, Germany, Nr. 20660
Pseudomonas aeruginosa	Laboratory of Microbiology, Wageningen University, The Netherlands, Isolate P8, Delft, 1957
Bacillus subtilis	Laboratory of Microbiology, Wageningen University, The Netherlands, Isolate B28, Marburg strain, J.W. Woldendorp. Delft, 1961

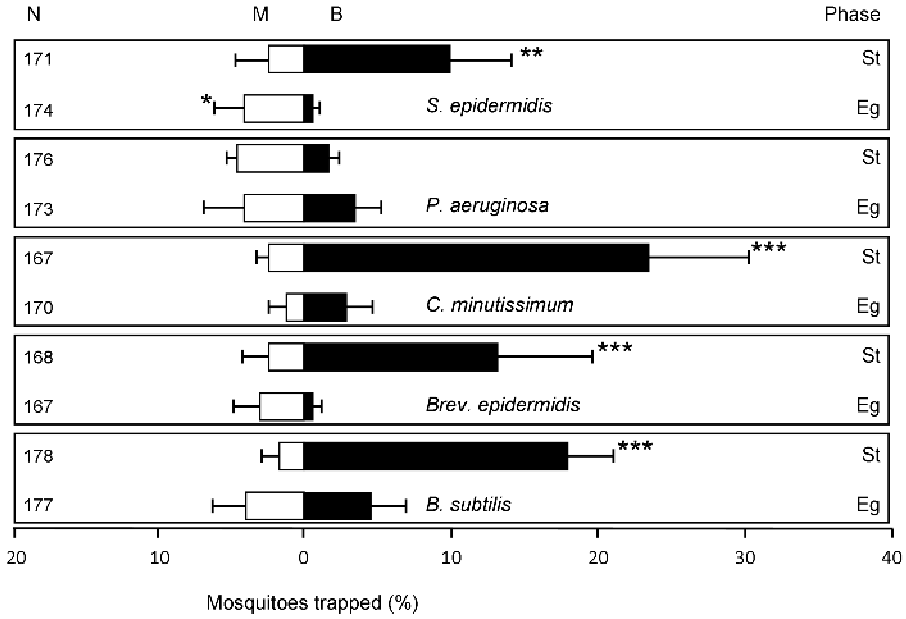


Figure 7.3. Response of An. gambiae to VOCs released from broths of five species of skin bacteria.

*Bacterial species were tested at the time of exponential growth (Eg) and during the stationary phase (St). The percentage of mosquitoes caught in the trapping device baited with the bacterial broth (B) and in the trapping device baited with medium alone (M) are given. N = number of mosquitoes released. Error bars represent standard errors of the mean; ***: χ^2-test P < 0.001; **: χ^2-test P < 0.01: *: χ^2-test P < 0.05.*

S. epidermidis which caught significantly fewer mosquitoes than medium alone (Figure 7.3; χ^2-test, d.f. = 1, P = 0.034). Trapping devices with bacteria in the stationary phase caught significantly more mosquitoes than the trapping devices with clean medium for all species of bacteria (χ^2-test, d.f. = 1, P ≤ 0.005), except for *P. aeruginosa* (Figure 7.3; χ^2-test, d.f. = 1, P = 0.13).

Ranking different species of bacteria

In order to compare the response of *An. gambiae* to the different bacterial broths in the same series of experiments, broths of *B. subtilis, Brev. epidermidis, C. minutissimum,* and *S. epidermidis* in their stationary phase were tested against ammonia, which is a known attractant for *An. gambiae* (Braks et al. 2001, Smallegange et al. 2005). Olfactometer trapping devices baited with volatiles of *S. epidermidis, B. subtilis* and *Brev. epidermidis* caught fewer mosquitoes than trapping devices baited with ammonia, and thus, the relative attractiveness of these species was lower than 0.5 (Figure 7.4). The volatiles of *C. minutissimum* lured more mosquitoes into trapping devices than ammonia (Figure 7.4). Comparing the four species, the relative attractiveness of *C. minutissimum* was significantly higher than that of *S. epidermidis* and *B. subtilis* (Figure 7.4; GLM, d.f. = 3, P = 0.014 respectively P = 0.042).

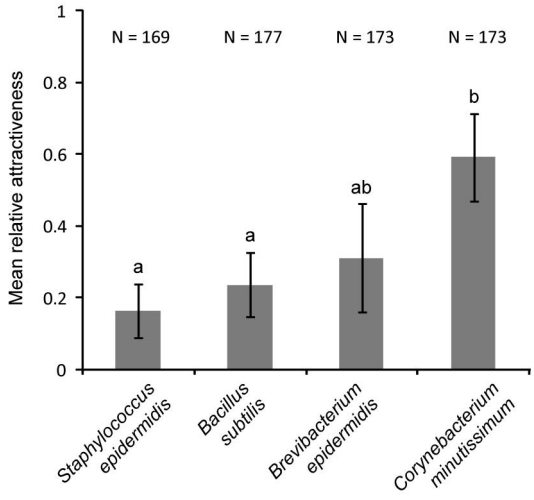


Figure 7.4. Ranking different species of skin bacteria for attractiveness to An. gambiae.

Results of dual-choice olfactometer experiments in which VOCs of different skin bacterial species in liquid medium were tested for their attractiveness to An. gambiae against ammonia released from an LDPE sachet. Bars show the mean relative attractiveness (i.e. the number of mosquitoes entering the trapping device baited with bacteria as a proportion of the total number entering either this trapping device or the trapping device baited with ammonia). N = total number of mosquitoes released. Error bars represent standard errors of the mean. Means not sharing the same superscript letter differ significantly at P < 0.05 (GLM).

The relative attractiveness of *Brev. epidermidis* was equal to the relative attractiveness of the other bacterial species (GLM, d.f. = 4, P > 0.05). No effects of bacterial density, temperature, humidity, air pressure, flight chamber, day and time of testing on the relative attractiveness were found (GLM, P > 0.05) and these factors were, therefore, not included in the GLM model.

Headspace analysis

More than 50 compounds were identified in the headspace of the standard medium. When the bacterial species were individually added to the medium and incubated, in total more than 150 volatile compounds were distinguished (Supplemental Table S7). As the bacterial broths of *B. subtilis, Brev. epidermidis, C. minutissimum* and *S. epidermidis* were attractive only in the stationary phase, we compared the headspaces of the stationary phase with those of the exponential growth phase. This comparison yielded ten headspace compounds (Table 7.2A, Supp. Table S7) that were more abundant in broths in the stationary phase than in broths in the exponential growth phase. Comparing the headspace results of the *P. aeruginosa* broths in the stationary phase with the broths of the other bacterial species in the stationary phase resulted in four headspace compounds that were less abundant and one compound that was more abundant in *P. aeruginosa* broths than in broths of the other species (Table 7.2B, Supp. Table S7). Butyl 2-methylbutanoate, pentathiane and 2-pentadecanone yielded from both comparisons. Butyl butyrate was the only compound found in higher amounts in the *P. aeruginosa* broth than in the broths of the other bacteria, except for the *S. epidermidis* broths (Table 7.2B, Supp. Table S7).

The headspace samples of the individual bacterial species, *C. minutissimum, B. subtilis* and *Brev. epidermidis* contained many sulfur-containing compounds. *Staphylococcus epidermidis* headspace samples contained higher amounts of acetoin and 3-methyl-1-butanol compared to the headspace samples of the other bacterial species. The latter compound has been identified before in the headspace of *S. epidermidis* and other skin bacteria grown *in vitro* after isolation from human feet (Verhulst et al. 2009).

Response of *An. gambiae* to volatiles identified in bacterial headspace

The addition of L-(+)-lactic acid (henceforth termed lactic acid) and carboxylic acids to ammonia had increased trap catches of *An. gambiae* in previous

***Table 7.2. Relative abundance of compounds that were potentially attractive or repellent to* An. gambiae.**

A

Compounds	RI	*Bac* Eg	*Bac* St	*Bre* Eg	*Bre* St	*Cor* Eg	*Cor* St	*Sta* Eg	*Sta* St	*Pse* Eg	*Pse* St
Dimethyldisulfide	n.d. [1]		++		++				+++		+++
Butyl acetate	826	+++	++++	++	++++		++++		++++		+++
Butyl isobutyrate	958		++		++		++				+
Butyl 2-methylbutanoate	1042		++		++		+++		++		
Dimethyltetra-sulfide	1208	++	++++		++++		++++		+++		++++
Pentathiane	1437		++		++		++				
Dimethylpenta-sulfide	1455		++		++		++		++		++
2-Pentadecanone	1695		++		++		++		+		
Hexathiepane	1707		++		++		++		+		+
Octasulfur	2025		++++		++++		++++		++	+	+++

B

Compounds	RI	*Bac*	*Bre*	*Cor*	*Sta*	*Pse*
Butyl butyrate	997				++	++
Butyl 2-methylbutanoate	1042	++	++	+++	++	
Pentathiane	1437	++	++	++		
2-Pentadecanone	1695	++	++	++	+	

Compounds were selected from the volatiles detected in the headspace of broths of five different bacterial species. The selection criteria are described in the Material and Methods section. ***A****: Exponential growth phase (Eg) compared with stationary phase (St) of* B. subtilis *(Bac),* Brev. epidermidis *(Bre),* C. minutissimum *(Cor) and* S. epidermidis *(Sta). The abundance of* P. aeruginosa *(Pse) is given as a reference;* ***B:*** *Stationary phase of* P. aeruginosa *(Pse) compared with the stationary phase of the other four bacterial species. Abundance based on GC-MS analysis: + 0 – 0.5%; ++ 0.5 – 10%; +++ 10 – 30%; ++++ 30 – 100%, relative to the largest peak in the total ion chromatogram. For the full list of compounds identified, see* Supplemental Table S7. *[1]not determined, [2]not commercially available.*

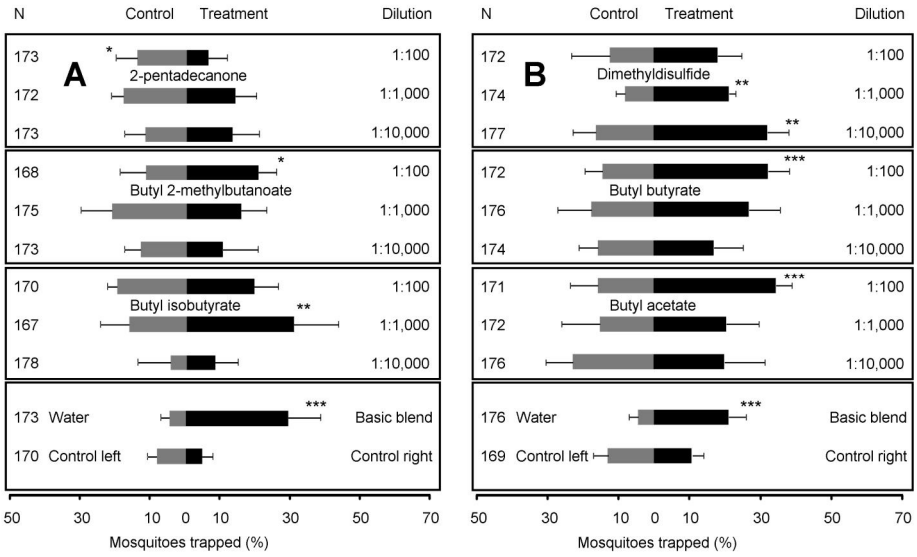


Figure 7.5. Responses of* An. gambiae *to individual compounds added to a basic blend in an olfactometer.

*Compounds were added in three concentrations (dilution 1:100, 1:1,000 or 1:10,000) to the basic blend (ammonia, lactic acid, tetradecanoic acid) (basic blend + compound = Treatment) and tested against the basic blend (Control) alone in two series (A and B). N = number of mosquitoes released. Error bars represent standard errors of the mean; ***: χ^2-test P < 0.001; **: χ^2-test P < 0.01: *: χ^2-test P < 0.05.*

experiments (Smallegange et al. 2005, Smallegange et al. 2009). Therefore, it was tested whether any of the above six compounds identified from the bacterial broth headspace samples could increase trap catches when added to a basic blend of ammonia, lactic acid and tetradecanoic acid (Smallegange et al. 2009).

Trapping devices baited with a basic blend of ammonia, lactic acid, and tetradecanoic acid in LDPE sachets caught significantly more mosquitoes than trapping devices baited with LDPE sachets containing water (Figure 7.5A,B; χ^2-test, d.f. = 1, P < 0.001), which is in accordance with the results of previous experiments (Smallegange et al. 2009).

Six of the 11 compounds selected for testing (Table 7.2) were commercially available and were examined for any impact on the response to the basic blend. Trapping devices baited with the basic blend and butyl 2-methylbutanoate, butyl butyrate or butyl acetate at the 1:100 dilution caught significantly more mosquitoes than trapping devices baited with the basic

blend alone (Figure 7.5A, B, χ^2-test, d.f. = 1, P = 0.043, P < 0.001 and P < 0.001, respectively). By contrast, at the highest concentration tested (1:100), 2-pentadecanone decreased the attractiveness of the basic blend significantly (χ^2-test, d.f. = 1, P = 0.020). Butyl isobutyrate at a dilution of 1:1,000 added to the basic blend caused a significantly increased response (χ^2-test, d.f. = 1, P = 0.007). When dimethyldisulfide was added at the two lowest concentrations to the basic blend, trapping devices baited with this 4-compound-blend caught significantly more mosquitoes than trapping devices baited with the basic blend alone (Figure 7.5B, χ^2-test, d.f. = 1, P = 0.001, and P = 0.003, respectively).

Discussion

Four of the five bacterial species grown *in vitro* in liquid medium, produced volatiles that were attractive to the malaria mosquito *An. gambiae* at the concentrations tested (Figure 7.3). The ranking of these four bacterial species shows that some species commonly found on the human skin produce volatile blends that are more attractive to *An. gambiae* than volatile blends of other bacterial species. This shows that not all micro-organisms present on the human skin contribute equally to the attractiveness conferred to mosquitoes. *Corynebacterium minutissimum* ranked significantly higher than *B. subtilis* and *S. epidermidis* in the second series of experiments (Figure 7.4). Volatiles of *P. aeruginosa* were not attractive, and may even attenuate the effect of the volatiles from the other bacterial species.

The difference between the response of *An. gambiae* to volatiles released by skin bacteria in their exponential growth phase and stationary phase could in part be explained by the higher number of bacteria in the stationary phase. However, the headspace analyses show clearly that some compounds were present in the headspace samples of the bacterial broths in the stationary phase, but were not detected in samples of the bacterial broths in the exponential growth phase (Table 7.2A, Supp. Table S7). This indicates qualitative differences in volatile production between the two growth phases.

The outermost layer of the human skin, the stratum corneum, is predominantly an oxic environment. However, certain micro-environments of the skin, such as glands and hair follicles, contain reduced levels of oxygen or no oxygen at all. Under these micro-aerophilic or anoxic conditions only bacteria with fermentative metabolic characteristics will be active (Wilson 2008). During fermentation processes, organic compounds are converted into

many different smaller compounds, often of a volatile nature (Wilson 2008). *Staphylococcus epidermidis,* for example, metabolizes glycerol and lactic acid, forming large amounts of acetic and propionic acid as fermentation products (James et al. 2004a). Fermentation reactions, which occurred during the stationary phase of our experiments, could explain the high diversity of compounds found in the headspace of broths at this stage. Four of our bacterial species are known to express fermentation reactions in the absence of oxygen (Welsch and Thibaut 1948, James et al. 2004b, James et al. 2004a, Romero-Garcia et al. 2009). Interestingly, *P. aeruginosa* grows best in the presence of oxygen or nitrate and has only limited fermentative capacities (Eschbach et al. 2004, Schreiber et al. 2006). To test whether fermentation in the absence of oxygen plays a role in the production of volatiles attractive to *An. gambiae*, the bacterial strains should be grown under anoxic conditions and tested for attractiveness to *An. gambiae*.

All five bacterial species could be grown in the same liquid medium and although this is probably not the most suitable medium for all species tested, it allowed for a comparison of the volatile blends produced against the same background. Trapping the volatiles from the headspace samples of bacteria using the CLSA technique resulted in more than 150 volatiles of which 11 were considered for further testing for their effect on *An. gambiae's* host-seeking behaviour (Table 7.2A,B; Supp. Table S7). Dimethyltetrasulfide, pentathiane, dimethylpentasulfide, hexathiepane and octasulfur were not commercially available. It will be interesting to synthesize these compounds and test them for their attractiveness to *An. gambiae*. All of the remaining six compounds induced a behavioural response of *An. gambiae* in the olfactometer when tested in combination with the basic blend that consisted of ammonia, lactic acid and tetradecanoic acid (Figure 7.4A,B). And, therefore, these six compounds are candidates to be studied in future experiments under African semi-field or field conditions.

Based on the headspace composition it was not expected that butyl butyrate would increase the attractiveness of the basic blend to *An. gambiae*, since this compound was present in higher quantities in the headspace of *P. aeruginosa* broths than in that of broths containing *B. subtilis, Brev. epidermidis* or *C. minutissimum*. An explanation may be that the concentration of butyl butyrate tested in the olfactometer was different from the concentration released by *P. aeruginosa* broths, or other compounds present in the headspace samples of *P. aeruginosa* broths may have influenced the

behavioural response of *An. gambiae*.

Dimethyldisulfide is the most interesting compound to test in semi-field and field experiments to evaluate as a mosquito bait under natural African conditions, because it was the only compound that increased the number of *An. gambiae* caught when added to the basic blend at the lowest concentration (Figure 7.5B). Dimethyldisulfide is a known bacterial volatile (Stotzky et al. 1976, Tomita et al. 1987), also found in human skin emanations (Bernier et al. 2000) and has been shown to be attractive on its own and in blends with lactic acid and acetone in dual-port olfactometer experiments to the yellow fever mosquito *Aedes aegypti* L. (Bernier et al. 2003, Allan et al. 2006, Bernier et al. 2007).

We have shown previously that skin bacteria play an important role in the host-seeking behaviour of *An. gambiae* (Verhulst et al. 2009). To find their host, *An. gambiae* females use host-specific cues in addition to CO_2 and physical cues like heat and moisture (Takken and Knols 1999). As different bacterial species produce volatile blends of highly different composition it can be hypothesized that *An. gambiae* females are guided by volatiles released by skin bacteria that are specific for the human skin. The results from the present study support this hypothesis. *Pseudomonas aeruginosa* is present on the human skin (Morrison and Wenzel 1984, Grice et al. 2008), but is also very common in the environment (Feltman et al. 2001) and, therefore, not human-specific. This may explain why *An. gambiae* was not attracted to the volatile odour blend produced by this bacterial species (Figure 7.3). *Corynebacterium minutissimum* is strongly associated with human skin (Sarkany et al. 1962, Collins and Jones 1983, Wauters et al. 1998), and to our knowledge not commonly found on other substrates. It was also the most attractive bacterium in our study (Figure 7.4). In addition, corynebacteria and brevibacteria are present in high numbers on the human foot (Wilson 2008), which is the body part most attractive to *An. gambiae* (de Jong and Knols 1995).

Skin micro-organisms are known to determine the human odour profile (Rennie et al. 1990, Rennie et al. 1991, Taylor et al. 2003, Ara et al. 2006). In a previous study, using solid agar based medium (Verhulst et al. 2009), a mixture of human skin bacteria from the foot was found to produce compounds attractive to *An. gambiae*. In the present study it was shown that not all bacterial species produce blends of volatile organic compounds that attract *An. gambiae* and that some bacterial species produce odour blends that are more attractive than others. The GC-MS analyses of headspace volatiles led to the

identification of compounds that, depending on the concentration tested, either increased or decreased the attractiveness of a basic blend. Attractive synthetic odour blends are of potential use in semiochemical-baited trapping systems for monitoring or controlling malaria vectors (Kline 2007, Logan and Birkett 2007, Smallegange et al. 2010). The present study provides further evidence that the composition of skin microbiota determines the attractiveness of humans to malaria mosquitoes. More detailed knowledge on the relationship between human skin microbiota and human body odour will contribute to our understanding of the evolutionary mechanisms underlying the anthropophilic host-seeking behaviour of mosquitoes and other blood-feeding insects and the development of novel means of vector-borne disease control (Verhulst et al. 2010).

Materials and Methods

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* culture originated from Suakoko, Liberia and has been cultured in the laboratory in Wageningen since 1988. Mosquitoes received blood meals on a human arm twice a week. During the present study, the *An. gambiae* strain was switched to membrane feeding, using human blood obtained from the blood bank (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands). Blood was offered through Parafilm® using a Hemotec® PS5 (Discovery Workshops, UK) feeder at 38ºC. During feeding, a sock releasing human odour was wrapped around the membrane and 5% CO_2 (human equivalent, (Gillies 1980)) was added next to the membrane at 250 ml/min. Olfactometer experiments showed no behavioural differences between mosquitoes reared on a human arm and mosquitoes reared on a membrane (G. Bukovinszkiné Kiss, unpublished data). Mosquitoes reared on membranes were used during the olfactometer experiments testing synthetic compounds.

Adult mosquitoes were maintained in 30-cm cubic gauze-covered cages in a climate-controlled chamber (27 ± 1°C, 80 ± 5% RH, LD 12:12). They had access to a 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper and placed in tap water in plastic trays. Larvae were fed daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed in 30-cm cubic cages for eclosion.

Bacteria

Five bacterial species (*Bacillus subtilis, Brevibacterium epidermidis, Corynebacterium minutissimum, Pseudomonas aeruginosa, Staphylococcus epidermidis*) commonly found on human skin were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) or from the Laboratory of Microbiology (Wageningen University and Research Centre, Wageningen, The Netherlands) (Table 7.1). All species were initially cultivated in species-specific media to enable exponential growth (Table 7.3). Subsequently, each species was incubated in a standard liquid medium (Table 7.4) to exclude the possible effect of different media on volatile production and thereby mosquito responses. Bacteria were incubated in tubes containing 5 ml standard medium at 34°C shaking at 225 rpm (Innova TM 4000, New Brunswick Scientific Co.). After incubation, bacteria were stored in glycerol stocks containing 300 µl glycerol and 700 µl medium at -80°C.

To assess the number of bacteria tested in each experiment, reference graphs were created to determine the correlation between the number of colony-forming units (CFUs) in the standard medium and the optical

Table 7.3. Medium ingredients used for initial growth of each bacterial species (DSMZ, Germany).

Bacterial species	Ingredients	Amount	Supplier
B. subtilis, P. aeruginosa	Proteose peptone	5.0 g	Fluka
	Meat extract	3.0 g	Fluka
	Distilled water	1000 ml	
Brev. epidermidis, S. epidermidis	Pancreatic digest of casein (Peptone C)	10.0 g	Difco
	Yeast extract	5.0 g	Difco
	Glucose	5.0 g	Merck
	Sodium chloride (≥ 99.5%)	5.0 g	Merck
	Distilled water	1000 ml	
C. minutissimum	Infusion from heart muscle	18.5 g	Fluka
	Glucose	5.0 g	Merck
	Distilled water	1000 ml	

Table 7.4. Ingredients of standard liquid medium used for growth of the five bacterial species.

Ingredient	Amount	Supplier
Infusion from heart muscle	2.0 g	Fluka
Pancreatic digest of casein (Peptone C)	13.0 g	Difco
Yeast extract	5.0 g	Difco
Sodium chloride (≥ 99.5%)	5.0 g	Merck
Distilled water	1000 ml	

density of the medium, measured by a spectrophotometer (SmartSpec^{tm} 3000, Bio-Rad). First, the optical density of at least six samples of 1 ml during growth of the bacteria was measured at 620 nm. Bacterial concentrations resulting in an extinction value measured with the spectrophotometer above 1 were diluted with standard medium, because the spectrophotometer is not accurate at optical densities above this value.

Secondly, for quantification of the bacterial population, each sample that had been measured in the spectrophotometer was diluted decimally, spread on species-specific agar (Table 7.3) plates and incubated at 34°C. CFUs were counted on dilution plates (between 25 and 250 colonies per plate), 3-6 days after spread plating. Count data were converted to number of bacteria per millilitre of liquid medium (determined by number of CFUs). The correlation between optical density and number of bacteria was fitted with a linear regression line, which was used as a reference line for calculation of the bacterial numbers tested in each experiment (Microsoft Excel; Table 7.5; example for *C. minutissimum* given in Figure 7.2A).

Table 7.5. Correlation between the number of CFUs in liquid medium and the extinction coefficient.

Bacterial species	Linear equation	R^2 (%)
B. subtilis	Y = 1.452E-09X	96.0
Brev. epidermidis	Y = 2.349E-09X	99.2
C. minutissimum	Y = 6.035E-09X	98.2
P. aeruginosa	Y = 9.883E-10X	98.6
S. epidermidis	Y = 3.877E-09X	91.8

Extinction was measured in a spectrophotometer at 620 nm. Y = Extinction coefficient. X = Number of CFUs. R^2 = coefficient of determination.

Table 7.6. Exponential growth rate and fitted equation of the bacterial species in liquid medium.

Bacterial species	Growth optimum (h)	Logistic growth equation	R^2 (%)
B. subtilis	15.3	Y= -0.044 + 4.840/(1 + EXP(-0.374*(T – 15.27)))	96.1
Brev. epidermidis	8.7	Y= -0.0794 + 11.262/(1 + EXP(-0.341*(T – 8.717)))	97.8
C. minutissimum	16.5	Y= -0.979 + 11.406/(1 + EXP(-0.1799*(T – 16.518)))	98.3
P. aeruginosa	13.0	Y= -0.092 + 5.151/(1 + EXP(-0.320*(T – 13.02)))	89.7
S. epidermidis	10.9	Y= -0.223 + 8.310/(1 + EXP(-0.470*(T – 10.900)))	94.7

Y = Extinction coefficient. T = Time (hours). R^2 = coefficient of determination.

Next, for each bacterial species the optical density of the broth was measured at regular intervals to determine the growth curve. The growth curve of bacteria in a batch culture follows an S-curve (Zwietering et al. 1990). In the exponential growth phase, which follows upon a lag phase, the bacterial growth rate will reach its maximum. This stage is followed by the stationary phase, in which the growth rate slows down due to nutrient depletion and/or accumulation of toxic products, and in which the maximum number of bacteria is reached. The exponential growth and stationary phase of each bacterial species were determined by fitting the observed optical density values to a logistic S-shaped curve (Genstat, release 12.1; Table 7.6; Figure 7.2B). For visualization, the growth curve describing the number of bacteria over time was plotted by integrating the reference line and fitted S-shaped curve (Figure 7.2C, 7.6).

Olfactometer bioassays

A three layer dual-port olfactometer (Figure 7.1, Tupola, The Netherlands) (Posey et al. 1998) was used to evaluate host-seeking responses of female mosquitoes to volatile organic compounds (VOCs) produced by the bacterial species. Pressurized air was charcoal-filtered, humidified, and passed through two poly-methyl-methyl-acrylaat (PMMA) mosquito trapping devices equipped with funnels (Verhulst et al. 2008), which were linked to both ports (diameter 5 cm, 25 cm apart) of the olfactometer. The air entered the flight chamber (polycarbonate,1.50 x 0.50 x 0.50 m) at a rate of 0.21 ± 0.02 m/s. Temperature, humidity and air pressure were recorded using dataloggers (MSR145S, MSR Electronics GmBHm, Switzerland). The air entered the two

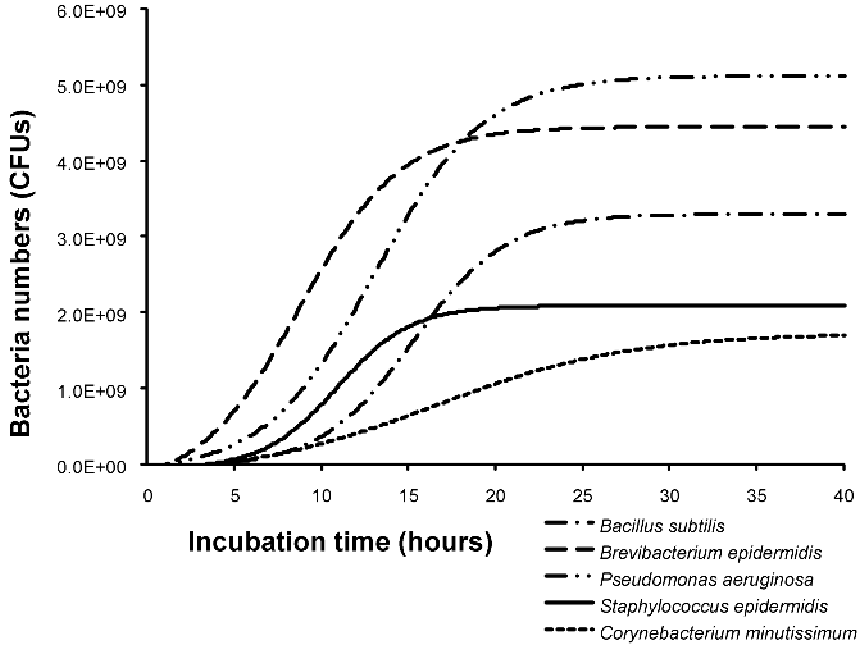


Figure 7.6. Growth curve of the five bacterial species in standard liquid medium.
Bacterial numbers were determined by counting colony forming units (CFUs).

trapping devices at a temperature of 27.0 ± 1.0°C, and had a relative humidity above 80%. The air temperature in the flight chamber was 26.3 ± 0.9°C and its relative humidity was 66.0 ± 8.2%. The experimental room was maintained at a temperature of 26.3 ± 1.0°C and a relative humidity of 59.6 ± 8.3%.

Experiments were prepared and performed according to the methods described by Smallegange et al. (2005). For each test 30 (mated) female mosquitoes of 5-8 d old, which had never been offered a blood meal, were selected 14-18 h before the experiment and placed in a cylindrical release cage (d = 8 cm, h = 10 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the scotophase, when *An. gambiae* females are known to be highly responsive to host odours (Maxwell et al. 1998, Killeen et al. 2006).

In each trial, test odours were released in the air stream before a group of mosquitoes was set free from a cage which was placed at the downwind end of the flight chamber, 1.50 m from the two ports. Mosquitoes were left in the flight chamber for 15 min. Specimens that entered each of the two trapping devices were counted at the end of the experiments after

anesthesia with CO_2. Mosquitoes remaining in the flight chamber were removed with a vacuum cleaner. Each trial started with a fresh batch of mosquitoes, clean trapping devices, and new stimuli.

The sequence of test odours was randomized on the same day, between days and between the three layers of the olfactometers. Each treatment was repeated six times and test stimuli were alternated between right and left ports between different replicates to rule out any positional effects. Surgical gloves were worn by the researcher at all times to avoid contamination of equipment with human volatiles.

Response of *An. gambiae* to volatiles from different species of bacteria

The volatiles of a broth of each bacterial species (Table 7.2) in liquid medium (Table 7.4) were tested for their effect on *An. gambiae*. Broths were tested at the time of exponential growth and in the stationary phase, when the number of bacteria in the broth stayed constant. Broths were incubated on the day before the experiments and kept at 4ºC overnight. Before the experiments, broths were incubated one more hour at 34°C, 225 rpm.

Five minutes before each experiment, 100 µl of the bacterial broth was spread on a sandblasted glass slide (75 x 25 mm) and positioned in the middle of one of the trapping devices of the olfactometer. Clean standard medium (100 µl) was used as a control and placed on a sandblasted glass slide in the other trapping device. The response of *An. gambiae* to the medium itself was tested in a separate experiment. One hundred µl of medium on a sand-blasted glass slide was placed inside one of the trapping devices in the olfactometer and tested against an unbaited trapping device. Two unbaited traps were offered to test the response of the mosquitoes when no odour was present and to test the symmetry of the system.

Ranking different species of bacteria based on mosquito response

In order to compare the responses of *An. gambiae* to the different bacterial broths, broths of *B. subtilis, Brev. epidermidis, C. minutissimum,* and *S. epidermidis* were tested against ammonia, which is a known attractant for *An. gambiae* (Braks et al. 2001, Smallegange et al. 2005). Because all bacterial species were tested in the same randomized series of experiments and against a standard of ammonia, it was possible to rank the bacteria according to their relative attractiveness (Qiu et al. 2006).

A volume of 100 µl of the bacterial broths on a sand blasted glass slide was tested against ammonia (100µl, 25% in water; analytical grade, Merck) released from Low Density PolyEthylene (LDPE; Audion Elektro, The Netherlands) sachets (25 x 25 mm, 0.20 mm thick) (Torr et al. 1997). LDPE sachets loaded with ammonia were suspended from a hook inside the olfactometer trapping device (Figure 7.7).

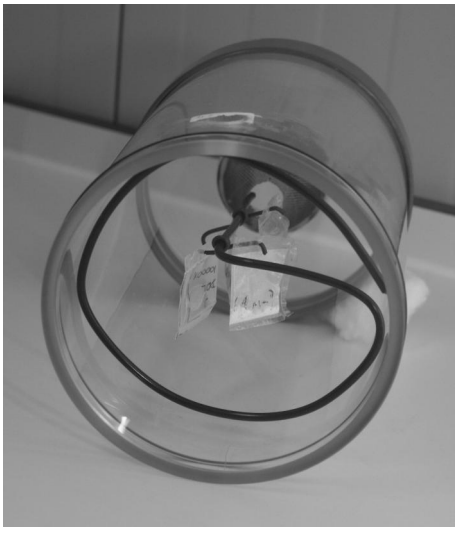


Figure 7.7. LDPE sachets used to test synthetic compounds.

Sachets were suspended from a hook inside an olfactometer trapping device. Gauze cover of trapping device not shown.

Headspace volatile analysis

Bacterial volatiles were trapped on active charcoal using a Closed-Loop Stripping Apparatus (CLSA) (Boland et al. 1984, Dickschat et al. 2005). In this system, air is continuously pumped (MB-21E, Senior Flextronics, USA) through a closed system over the bacterial broth present in a closed 250 ml Erlenmeyer flask and through an activated charcoal filter (5 mg, Chrom Tech, Germany), in which the volatiles were absorbed.

The Erlenmeyer flask was first rinsed with acetone (Technical grade, CVH Chemie-Vertrieb GmbH & Co, Germany), next with distilled water and autoclaved before use. The flask was filled with 50 ml standard medium (Table S7.4) and 200 µl bacterial suspension from a glycerol stock was added. Next, the broth was placed in an incubator at 34°C. After 3 h the broth was placed for 3 h (exponential growth phase) or 20 h (stationary phase) at 34°C in the CLSA setup. The airstream in the CLSA setup was directed towards the surface of the incubation mixture, leading to constant mixing of the culture.

Trapped volatiles were extracted from the charcoal filter by rinsing the filter three times with 10-15 µl dichloromethane (≥99.8%, Merck, Germany). Before use, the active charcoal filter had been rinsed with pentane (≥99%, Sigma-Aldrich, Germany), methanol (≥99.8%, Merck, Germany), and dichloromethane (≥99.8%, Merck, Germany).

Extracts were analyzed by gas chromatography – mass spectroscopy (GC-MS, GC 7890A/MSD 5975C, Agilent Technologies, USA). The GC-MS system was equipped with a split/splitless injector and an MS fused silica capillary column HP-5 MS (30 m, 0.25 mm internal diameter, 0.25 microns phase thickness, Agilent Technologies, USA) with He as the carrier gas (1.2 ml/min). The GC oven temperature was kept at 50°C for 5 min, followed by raising the temperature with 5°C/min to 320°C. Mass-spectra were recorded by electron impact ionization at 70 eV.

Compounds were identified by comparison of their mass spectra and gas chromatographic retention indices with those of authentic reference compounds and use of commercial mass spectral libraries (Wiley 7, NIST 08). Quantification of abundance was done in a semi-quantitative way by assigning each component to one of four classes dependent on the relative abundance in the total ion chromatogram 0 – 0.5%, 0.5 – 10%, 10 – 30%, 30 – 100%, relative to the total ion count of the largest peak in the chromatogram.

Two criteria were used for the selection of headspace compounds that were potentially attractive or repellent to *An. gambiae*. 1) The abundance of compounds in the stationary phase of broths of *B. subtilis, Brev. epidermidis, C. minutissimum* and *S. epidermidis* that were attractive to *An. gambiae* was compared with the abundance of compounds in the exponential growth phase of these broths, which were unattractive to *An. gambiae*. Compounds with a higher overall abundance in the stationary phase or a higher overall abundance in the exponential growth phase were selected. 2) The abundance of compounds in the stationary phase of broths of *B. subtilis, Brev. epidermidis, C. minutissimum* and *S. epidermidis*, which were attractive to *An. gambiae,* were compared with the abundance of compounds in the stationary phase of *P. aeruginosa* broths. The latter did not attract *An. gambiae*. Compounds with a higher overall abundance in *P. aeruginosa* broths or a higher overall abundance in the broths of the other bacterial species were selected.

Response of *An. gambiae* to volatiles identified in bacterial headspace samples

Six volatile compounds originating from bacterial broths and identified by GC-MS (butyl acetate, butyl isobutyrate, butyl butyrate, butyl 2-methylbutanoate, dimethyldisulfide and 2-pentadecanone) were commercially available and tested in the olfactometer. All compounds were purchased from Sigma Aldrich (Germany) with purity levels ranging between 97 and 99%. Each compound was tested in three dilutions in paraffin oil (Merck, 1:100; 1:1,000 and 1:10,000) and released from LDPE sachets (25 x 25 mm, 0.20 mm thick) (Torr et al. 1997). Each sachet contained 100 µl of the dilution tested.

It was tested whether any of the above six compounds identified from the bacterial broth headspace samples could increase trap catches when added to a basic blend of ammonia, L-(+)-lactic acid and tetradecanoic acid (Smallegange et al. 2009). Ammonia (25% in water; analytical grade, Merck) and tetradecanoic acid (>99%, Sigma) were released from LDPE sachets 0.03 mm thick, while lactic acid (88–92% aqueous solution, Riedel-de Haën) was released from LDPE sachets of 0.05 mm thick. Sachets were 25 x 25 mm in size and contained either 100 µl ammonia solution, 100 µl lactic acid solution, or 50 mg tetradecanoic acid.

To test whether the six selected compounds identified from bacterial broth headspace samples could increase trap catches of *An. gambiae* when added to the basic blend, each dilution of the compounds was offered in an olfactometer trapping device together with the basic blend and tested against a control of a trapping device that contained only the basic blend and an LDPE sachet with paraffin oil. The six compounds were tested in two series (A and B) of three compounds. Each dilution of the compounds was tested six times and the position of the two odour sources was alternated between right and left ports among the replicate experiments. To test the symmetry of the system, two unbaited traps were offered. In addition, the basic blend was tested against LDPE sachets of the same size and thickness filled with distilled water to show that this blend is attractive.

Statistics

The spectrophotometer extinction values measured at different bacterial densities were fitted to a linear regression line (Microsoft Excel). To plot growth curves of each bacterial species, the extinction values measured at different incubation times during growth of each bacterial species were fitted to

a logistic S-shaped curve (Genstat, release 12.1).

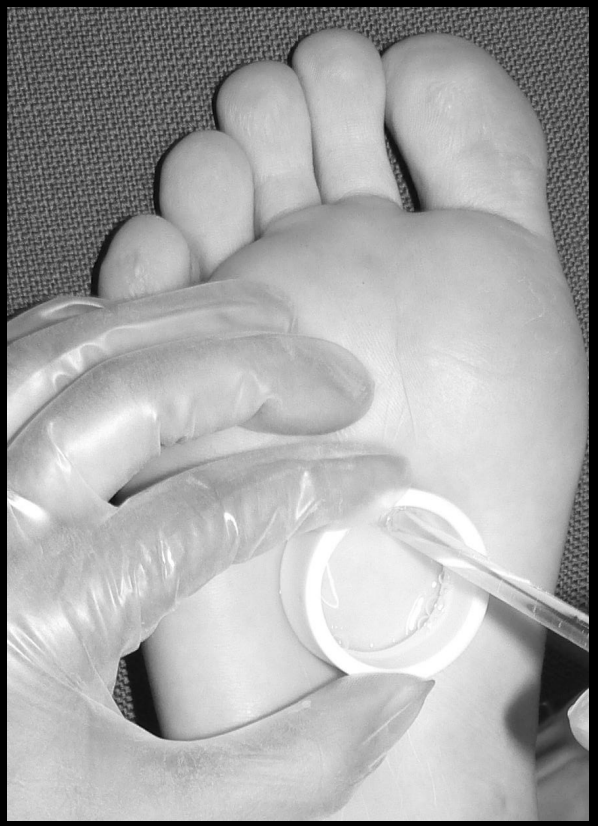
For the two-choice test in the olfactometer a χ^2-test was used to analyze whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution ($P < 0.05$).

The relative attractiveness of each bacterial species was tested with a Generalized Linear Model (GLM; Genstat, release 12.1; Binomial, linked in logit, dispersion estimated). The relative attractiveness is expressed as the number of mosquitoes caught in the trapping device baited with the bacterial odour divided by the total number of mosquitoes trapped in both trapping devices (bacteria trap + ammonia trap) during each experiment (Qiu et al. 2004a, Qiu et al. 2006). Two-sided t-probabilities were calculated to test pairwise differences between means. Effects were considered to be significant at $P < 0.05$. For each series of experiments the effects of bacterial density, temperature and humidity of the flight chamber, air pressure, flight chamber (Figure 7.1: A, B, C), day and time of testing on the relative attractiveness were tested and fitted as parameters in the GLM model when significant.

Acknowledgements

We would like to thank F. K. M. van Aggelen, A. J. Gidding, D. van Veldhuizen and L. Westerd for mosquito rearing. We thank Dr. F. C. Griepink for providing LDPE material, T. Nawrath for establishing headspace sampling procedures and Prof. M. Dicke for constructive comments on this manuscript.

Traits underlying differential attractiveness



Traits underlying the differential attractiveness of humans to mosquitoes

08

Niels O. Verhulst, Hans Beijleveld, Yu Tong Qiu, Chris Maliepaard, Dan Knights, Stefan Schulz, Donna Berg-Lyons, Chris Lauber, Willem Verduyn, Gerrit W. Haasnoot, Roland Mumm, Rob Knight, Marcel Dicke, Willem Takken, Joop J.A. van Loon, Harro Bouwmeester, Frans H.J. Claas, Renate C. Smallegange

Chemical cues are considered to be the most important cues for mosquitoes to find their hosts and humans can be ranked for attractiveness to mosquitoes based on the chemical cues they emit. Human leukocyte antigens (HLA) genes are considered to influence human body odour and may therefore affect human attractiveness to mosquitoes. Another trait affecting human attractiveness to mosquitoes is the composition of the human skin microbiota. A correlation exists between the composition of human skin microbiota and human body odour. In the present study the possible effects of HLA profile and skin microbiota composition on the attractiveness of humans to the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* were examined.

Skin emanations were collected from the feet of 48 individuals and tested for attractiveness to the malaria mosquito *An. gambiae*. The chemical composition of these emanations was determined by gas chromatography – mass spectroscopy (GC-MS) and the response of the mosquito antennal olfactory system determined by gas chromatography – electroantennographic detection (GC-EAD). Blood samples were taken for HLA analysis and skin bacterial samples collected to determine the composition of skin microbiota by plate counts and 16S rRNA sequencing.

The skin emanations from individuals differed significantly in attractiveness to *An. gambiae*. HLA profiling suggested that HLA gene Cw*07 affects the attractiveness of humans to mosquitoes. Individuals with a high abundance of bacteria on their skin were significantly more attractive to *An. gambiae* then others, whereas individuals with a higher diversity of the skin microbiota were less attractive to *An. gambiae*. *Staphylococcus* spp. were associated with individuals that were significantly more attractive to mosquitoes then others and *Pseudomonas* spp. with individuals that were significantly less attractive. GC-MS and GC-EAD analysis revealed several compounds that were associated with individuals who expressed differential attractiveness to mosquitoes.

It is concluded that skin microbiota plays an important role in the production of body odours that influence the attractiveness of humans to mosquitoes. The compounds identified may be used for disruption of mosquito behaviour in malaria interventions.

Introduction

Female mosquitoes use physical and chemical cues to find their hosts. Physical cues include heat, moisture and vision and play a role during orientation and landing (Khan et al. 1968, Allan et al. 1987, Cardé and Gibson 2010). Chemical cues, however, are considered most important as both short and long range attractants, especially for nocturnal mosquito species (Takken 1991, Mboera and Takken 1997, Takken and Knols 1999, Olanga et al. 2010), and play a role in the differential attractiveness of humans to mosquitoes as has been shown in many studies (Brouwer 1960, Mayer and James 1969, Schreck et al. 1990, Lindsay et al. 1993, Knols et al. 1995, Brady et al. 1997, Bernier et al. 2002, Mukabana et al. 2002, Qiu et al. 2006, Logan et al. 2008). Humans can be ranked for attractiveness to mosquitoes by testing the emanations from their total body (Lindsay et al. 1993, Knols et al. 1995, Mukabana et al. 2002) or by testing emanations from a part of the body (Smart and Brown 1957, Mayer and James 1969, Logan et al. 2008). To eliminate the effect of physical parameters like skin temperature or humidity, skin emanations can also be collected on glass beads which can be tested for attractiveness to mosquitoes (Schreck et al. 1990, Bernier et al. 1999, Qiu et al. 2006).

Several factors have been shown to influence the differential attractiveness of humans to mosquitoes. The landing response of mosquitoes is higher with increased skin temperature or a drier skin (Smart and Brown 1957, Gilbert et al. 1966). Human body mass or surface area and mosquito catch are positively correlated (Spencer 1967, Port et al. 1980) and this might explain the preference of mosquitoes for adults over children (Muirhead-Thomson 1951, Spencer 1967). There is at least one report of a correlation between gender and attractiveness to mosquitoes (Muirhead-Thomson 1951), although more recent studies did not confirm this (Carnevale et al. 1978, Kirk et al. 2000, Qiu et al. 2006, Logan et al. 2008). A genetic background for human attractiveness to mosquitoes was investigated among 197 monozygotic and 326 dizygotic twin pairs (Kirk et al. 2000). The study indicated that a strong genetic influence on frequency of being bitten by mosquitoes exists. The volatile blends released by twins can be matched by human sniffers (Wallace 1977, Roberts et al. 2005) and gas-chromatography (GC) analysis (Sommerville 1994, Kuhn and Natsch 2009). The genetic background of human body odour is determined, at least partly, by the human leukocyte antigen (HLA) genes of the major histocompatibility complex (MHC)

(Wedekind and Furi 1997, Penn and Potts 1998a, Wedekind and Penn 2000, Verhulst et al. 2010) that serve functions in the human immune system.

The differential attractiveness of humans determined by the attractiveness of their skin emanations remains relatively stable over time (Qiu et al. 2006). This, together with the genetic background of the human odour profile (Sommerville 1994, Roberts et al. 2005, Kuhn and Natsch 2009) and of the number of mosquito bites received by humans (Kirk et al. 2000), supports the hypothesis that genetic factors, in particular HLA genes, are involved in determining differential attractiveness of humans to mosquitoes, and HLA genes are suspected to underlie this effect (Logan 2008, Verhulst et al. 2010).

How genes might influence body odour was summarised by Penn and Potts (1998a). They hypothesised that MHC molecules bind allele-specific subsets of peptides, which are volatilised by the activity of the commensal microorganisms. This hypothesis was confirmed at least partly by an *in vitro* experiment in which HLA peptides determined the production of 3-methylbutanal by skin bacteria (Savelev et al. 2008).

The skin microbiota plays a crucial role in the production of human body odour. Without skin bacteria human sweat is odourless (Shelley et al. 1953) and there is a strong correlation between human body odour and the presence of certain microorganisms (Leyden et al. 1981, Rennie et al. 1990, Rennie et al. 1991, Taylor et al. 2003, Ara et al. 2006). Detailed skin microbial profiles, as assessed through denaturing gradient gel electrophoresis (DGGE) analysis can be linked with the chemical odour profile of the human skin (Xu et al. 2007).

Several studies have indicated a possible role of skin bacteria in the production of volatiles attractive to mosquitoes. Washing the feet with a bactericidal soap significantly altered the selection of biting sites of the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (hereafter referred to as *An. gambiae*) on a sitting naked volunteer (de Jong and Knols 1995). Human eccrine sweat was found attractive to *An. gambiae*, but only after incubation with skin bacteria for one or two days (Braks and Takken 1999). Recently, it was shown that volatiles produced by human skin bacteria *in vitro* are attractive to *An. gambiae* when grown on agar plates and tested in an olfactometer or mosquito traps (Verhulst et al. 2009) (Chapter 5 & 7). When five bacterial species commonly found on human skin were grown *in vitro* and tested for the attractiveness of their headspace volatile blend to *An. gambiae*, some species were more attractive than others. One species, *Pseudomonas*

aeruginosa, was not attractive to *An. gambiae*. Headspace analysis of skin bacteria grown *in vitro* resulted in sixteen compounds that influenced mosquito behaviour when applied in different blends (Verhulst et al. 2009) (Chapter 6 & 7).

In the present study the traits underlying the differential attractiveness of humans to the malaria mosquito *An. gambiae* were examined. It is hypothesised that the skin microbiota composition and/or the HLA profile influence the blend of volatiles released from the human skin and thereby affects the attractiveness of humans to mosquitoes (Verhulst et al. 2010). Skin emanations were collected on glass beads from 48 individuals (Schreck et al. 1990, Bernier et al. 1999, Qiu et al. 2006) and tested for their attractiveness to *An. gambiae* in an olfactometer. Correlations between the attractiveness of these individuals and the chemical profile of their skin emanations, HLA profile and bacterial composition were examined.

Materials and methods

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* colony originated from Suakoko, Liberia. Mosquitoes have been cultured in the laboratory since 1988 and received blood meals from a human arm twice a week. Adults were maintained in 30-cm cubic gauze-covered cages in a climate-controlled chamber (27 ± 1° C, RH 80 ± 5%, LD 12:12). They had access to a 6% (w/v) glucose solution on filter paper. Eggs were laid on wet filter paper and placed in tap water in plastic trays and larvae were fed daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed in 30-cm cubic cages for emergence.

Volunteers

The attractiveness of 48 adult males aged between 20 and 64 years to *An. gambiae* was examined. Forty-six men were Caucasian, one man was Asian and one Hispanic. All volunteers were non-smokers, free from chronic illnesses and not using any medication on a regular basis. Twenty-four hours before each experiment the volunteers were requested to refrain from drinking alcohol (Shirai et al. 2002, Lefèvre et al. 2010), eating garlic, onions or spicy food, not to take a shower, not to use perfumed cosmetics and to wear socks provided by the research team (100% polyamide, 40 denier, Hans Textiel, The

Netherlands). Before use, socks were washed twice with 70% ethanol and dried in a ventilated oven at 80°C for at least one h. Volunteers were requested not to use soap during their last shower before the experiment.

Olfactometer bioassay

A dual-choice olfactometer was used to determine the attractiveness of the volunteers to female *An. gambiae* according to the methods described by Qiu et al. (2006). Skin emanations from each individual were collected by rubbing six glass beads (15 mm in diameter, contained in a Teflon holder, Figure 8.1A) for ten min against the underside of the left foot.

The dual-port olfactometer (Pates et al. 2001), consisting of a glass flight chamber of 1.60 × 0.66 × 0.43 m, was used to study the behavioural response of female mosquitoes to the odour stimuli collected on glass beads. Pressurised charcoal-filtered air was led through two Perspex mosquito trapping devices, which were linked to two ports (diameter 4 cm, 28 cm apart), into the flight chamber at a speed of 0.21 ± 0.01 m/s. Dim light of approximately 1 Lux was provided. The experimental room was maintained at a temperature of 27.9 ± 0.7 °C and a relative humidity of 62.3 ± 5.8 %. The temperature inside the flight chamber was 27.9 ± 1.7 °C and the humidity 69.0 ± 4.6 %. The humidity of the air led into the trapping devices was maintained above 80 % and its temperature was 28.0 ± 1.5 °C.

One day before the experiments, 250 µL of a solution of 2.5 % ammonia in water (Merck, Amsterdam, The Netherlands) was injected into an 80 L Teflon air sample bag (SKC Gulf Coast Inc., Houston, TX, USA). Next, the bag was filled with 60 L humidified and charcoal-filtered pressurised air. The air sample bag was left horizontal overnight to allow the ammonia solution to evaporate. This procedure resulted in an ammonia concentration of 136 ppm in the bag (Smallegange et al. 2005). At this chosen concentration ammonia elicited moderate attraction to female *An. gambiae* according to previous dose – response experiments (Smallegange et al. 2005). Another 80 L air sample bag filled with 250 µL distilled water and 60 L air was prepared in a similar way to be used as the control stimulus. During the experiments, the air was pumped at 0.23 L/min (air pump model 224-PCXR4; SKC Gulf Coast Inc., USA) from the air sample bags through Teflon tubes (diameter 7 mm, Rubber BV, The Netherlands) into the trapping devices, where it merged with the main air stream of 23.5 L/min (Smallegange et al. 2005).

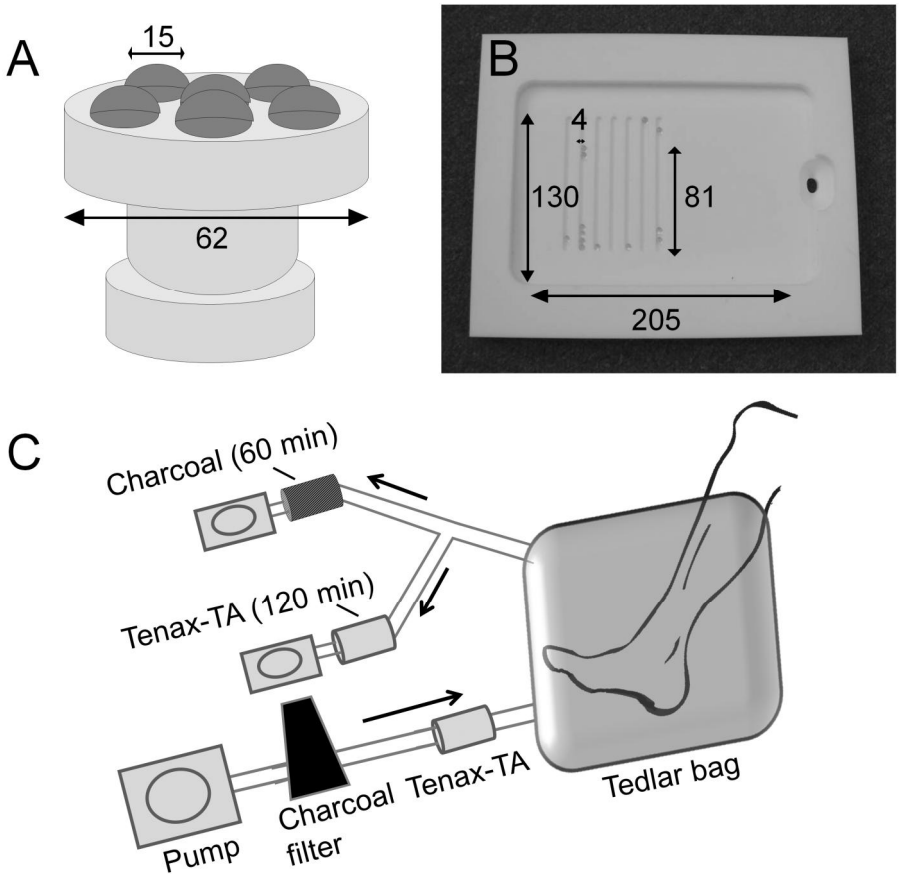


Figure 8.1. Methods for collecting skin emanations of human feet.

Distances in millimetres. A) Teflon holder with six glass beads for collecting skin emanations to be used for mosquito attractiveness tests in the olfactometer. B) Teflon tray for sampling skin emanations for gas chromatography – mass spectroscopy (GC-MS) analysis. The foot was rubbed over 100 small glass beads (a few are shown on the photo). C) Headspace collection of volatiles for GC-MS and gas chromatography – electroantennographic detection (GC-EAD) analysis. Filtered air was pumped into a 25 L Tedlar bag. Air was sucked through two cartridges with adsorbent (sampling time between brackets), which were used for further GC-EAD and GC-MS analysis. Arrows indicate airflow.

In order to test the attractiveness of the volunteers individually, the glass beads that had been in contact with the feet of each individual were placed in one of the trapping devices, into which clean air from an air sample bag was pumped. Gaseous ammonia was pumped into the other trapping device, which contained six clean glass beads.

For each test 30 (mated) female mosquitoes of 5-8 d old, which had not been given the chance to take a blood meal, were selected 14-18 h before the experiment and placed in a cylindrical release cage (d = 8, h = 10 cm) with access to tap water from damp cotton wool. The experiments were performed during the last 4 h of the scotophase, when *An. gambiae* females are known to be highly responsive to host odours (Maxwell et al. 1998, Killeen et al. 2006). In each trial, mosquitoes were released simultaneously from a cage placed at the downwind end of the flight chamber, 1.60 m from the two ports. They were allowed to fly upwind and enter one of the trapping devices. After 15 minutes, the number of mosquitoes trapped in each trapping device was counted.

Odour samples of each volunteer were taken and tested for their attractiveness six times in total; twice in a row on three different mornings (Qiu et al. 2006). Test stimuli were alternated between left and right ports to rule out any positional effects. In addition, experiments with ammonia against clean beads and experiments without any odour source were performed. Each experiment started with new mosquitoes, clean trapping devices and freshly handled glass beads. To avoid contamination of the equipment with human volatiles, surgical gloves were worn during all experiments.

After use, the trapping devices were washed in a dish washer at 45 °C with biological soap (Sonnett tabs, Sonnet OHG, Germany). The glass beads were cleaned by rinsing in a solution of 10% Helmanex® II cleaning concentrate (Hellma GmbG & Co KG, Germany) in water, subsequently in distilled water, and finally in ethanol (99.8 % purity; Merck, Germany). The rinsed beads were dried in an oven at 200 °C for at least one h. Between experiments, the Teflon holder was cleaned with 70 % ethanol and quick-dried with a heat gun (Ferm B.V., The Netherlands).

Analysis of human skin emanations

Emanations on glass beads

Glass beads have the advantage that emanations collected on them can be removed by thermodesorption for analysis by gas chromatography – mass spectroscopy (GC-MS), avoiding the need of solvent extraction (Schreck et al. 1990, Bernier et al. 1999, Qiu et al. 2006). Therefore, directly after two subsequent olfactometer experiments, skin emanations from the left foot of each volunteer were collected on small glass beads (4 mm in diameter, Witeg, Germany).

For this purpose, one hundred beads were divided over seven ridges in

a Teflon tray (Figure 8.1B). Volunteers were asked to rub the sole of their left foot (just behind the toes) over the beads for 10 min. The beads coated with human skin emanations were then transferred to five steel cartridges. The cartridges were placed in an autosampler (Ultra 50:50 TD, Markes International Ltd, UK). Emanations on the beads were analyzed by using thermodesorption followed by GC-MS. The system consisted of a thermal desorption autosampler, an electrically-cooled trap for focusing (Unity, Markes International Ltd, UK) and a flow controller (Air server, Markes International Ltd, UK) for thermal desorption injection into a Trace GC Ultra (Thermo Scientific, USA) coupled to a quadruple mass detector (DSQ, Thermo Scientific, USA).

The cartridges were dry purged for one minute with helium (5.0 grade) at 30 °C to remove residual oxygen. Cartridges were desorbed at 150 °C for 10 min and the volatiles were focused on a cold trap (general purpose hydrophobic, Markes, UK) at -10 °C. Analytes were transferred to the analytical column by heating the cold trap to 250 °C for 3 minutes and splitting of the helium carrier gas resulting in an injection of 1/6 of the total amount. The transfer line between the cold trap and the GC was kept at 160 °C.

A DB5 GC column (RTX-5ms, 30mx0.25mmID, 1.0-µm film thickness; Restek, USA) with helium as carrier gas at a constant flow rate of 1.0 mL/min was used. The GC temperature was programmed for 3 minutes at 45 °C followed by a ramp of 8 °C/min to 280 °C and a 2 minute hold at 280 °C. The transfer line between the GC and MS was kept at 275 °C. Mass-spectra were recorded by electron impact ionization at 70 eV, scanning in positive mode from 35-300 *m/z* with a scan speed of 5 scans/s and an ion source temperature of 250 °C.

After analysis, the cartridges were conditioned at 320 °C for 1 h (TC-20, Markes, UK) with a flow of 60 mL/min. The Teflon tray was cleaned between experiments with 70 % ethanol (Merck, Germany) and quick-dried with a heat gun (Ferm B.V., The Netherlands).

Headspace sampling

Foot volatiles were collected by placing the left foot and lower leg from each volunteer in a 25 L Tedlar air sampling bag (SKC, Gulf Coast Inc. Houston, USA), which had been cleaned by heating in an oven at 110 ºC while charcoal filtered air (1 L/min) was pumped through the bag for 16 h. One of the corners of the bag was cut open so that one foot of the first volunteer of each

experimental day could be put in the bag until the bag reached approximately 10 cm below the knee, where it was sealed with tape around the bag, in such way that the tape did not touch the leg (Figure 8.1C).

Room air was filtered through active charcoal (Darco Granular, 4-12 mesh particles size, Sigma, Germany) and 350 mg clean Tenax-TA 20/35 (Alltech, The Netherlands) and pumped (KNF, Laboport, USA) into the bag at a flow rate of 250 mL/min. At the outlet of the bag a tube with a T-piece was connected to split the airflow. To each tube end, an adsorbent cartridge was connected to collect the headspace volatiles. The cartridges (Markes, UK) were then connected to a small air sampler pump (PAS-500, Spectrex, USA), which sucked the air through the cartridge at a rate of 100 mL/min (Figure 8.1C). One of the two cartridges was made of stainless steel and loaded with 200 mg clean Tenax-TA 20/35 and volatiles were trapped for the full two h sampling period. The other cartridge was made of glass and loaded with 5 mg of active charcoal (CLSA-Filter, Daumazan sur Arize, France) and volatiles were trapped for 60 min during the last hour of sampling (Figure 8.1C).

The 200 mg Tenax-TA adsorbent in the stainless steel cartridge was eluted with 4 mL diethyl ether (spectrophotometric grade, ≥ 99.9 %, Sigma-Aldrich). The elution was subsequently evaporated under a constant flow of pure nitrogen gas at room temperature to concentrate the solution to approximately 150 µl. Pooled samples were made representing headspace samples of individuals which were classified as High Attractive or Low Attractive (HA and LA; see statistics) to mosquitoes based on an their relative attractiveness. Ten µl of the headspace samples of seven individuals with a high relative attractiveness to mosquitoes (HA7) were pooled and ten µl of the headspace samples of seven individuals with significantly lower (LA7) mosquito attractiveness were pooled. The remaining of these 14 samples and the other 34 samples were pooled (Pooled diethyl Ether samples, PE48). The glass cartridge with active charcoal was eluted with 50 µl of dichloromethane (≥ 99.9 %, Aldrich) and the elutions from all 48 headspace samples were pooled (Pooled dichloroMethane samples PM48). All four elutions of the headspace samples were used for gas chromatography – electroantennographic detection (GC-EAD) analysis (see below).

GC-EAD

In the GC-EAD setup, three antennae, of 5-8 d old *An. gambiae s.s.* females from which the top segment had been removed, were mounted in series (Park

and Baker 2002). The cervix of the first mosquito head was connected to the indifferent electrode, the excised tip of one antenna of the first head and the cervix of the second head were both attached to a glass capillary filled with Ringer solution. Similarly, the second and the third antennae were connected and the tip of the third antenna was attached to the recording electrode. Both the indifferent electrode and the recording electrode, glass capillary electrodes filled with insect Ringer and an AgCl-coated silver wire (diameter 0.5 mm), were inserted into it the capillaries (Qiu et al. 2004a). The indifferent electrode was grounded and the recording electrode was connected to a DC preamplifier (10X; Syntech, The Netherlands). A moistened, charcoal-filtered, continuous air stream (600 mL/ min) was led through a metal tube, coated inside with Teflon (10 mm in diameter), ending 0.5 cm from the preparation. Each of the pooled samples described above was tested on evoking a GC-EAD response in 12-15 antennae preparations.

A GC (series 8000, Carlo-Erba, Italy) was equipped with a cold-on-column injector connected with a 1 m guard column (fused silica with intermediate polarity), which was then connected to a DB5 column (RTX-5ms, 30mx0.25mmID, 1.0-µm film thickness; Restek, USA) (Smid et al. 2002, Qiu et al. 2004c). Helium carrier gas with a flow rate of 1.3 mL/min was mixed with a 30 mL/min make-up helium flow and the mixture split to the flame ionization detector (FID) and mounted antennae with a ratio of 1:1 (v/v). The flow leading to the antennae went through a 1.5 m heated transfer line (0.32 mm diameter, non-polar fused silica, Interscience, The Netherlands). The transfer line was kept at 225 °C and the outgoing flow was mixed with the charcoal-filtered, humidified air 17 cm before the outlet to the antennae. The GC column temperature was programmed as follows: 20 s secondary cooling at 45 ºC, 1 min at 45 ºC, followed by a ramp of 10 ºC/min to 275 ºC and a 5 minute hold at 275 ºC. The FID temperature was set at 280 ºC.

To identify the compounds that elicited GC-EAD responses, extracts were analyzed by gas chromatography – mass spectroscopy (GC-MS, GC 7890A/MSD 5975C, Agilent Technologies, USA). The GC-MS system was equipped with a split/splitless injector and an MS fused silica capillary column HP-5 MS (30 m, 0.25 mm internal diameter, 0.25 microns phase thickness, Agilent Technologies, USA) with He as the carrier gas (1.2 ml/min). The GC oven temperature was kept at 50°C for 5 min, followed by raising the temperature with 5°C/min to 320°C. Mass-spectra were recorded by electron impact ionization at 70 eV.

Compounds were identified by comparison of their mass spectra and gas chromatographic retention indices with those of authentic reference compounds and use of commercial mass spectral libraries (Wiley 7, NIST 08). GC peaks that were synchronised with an EAD response were matched with the GC-MS identification by the Kovats Retention Index (KRI).

Identification and abundance of skin emanations

The chromatograms obtained from the skin emanations collected on glass beads of individuals that significantly differed in attractiveness (HA and LA, see statistics) were screened for the presence of 33 selected compounds (Table 8.1). The selection of the 33 compounds was based on previous studies or on the GC-EAD results obtained during the present study. The following selection criteria were used: compounds were either 1. Present in the headspace extracts of skin emanations of HA and LA individuals that elicited electrophysiological responses in antenna preparations (GC-EAD), 2. Identified in the headspace of skin bacteria attractive to *An. gambiae* (Verhulst et al. 2009), 3. Identified as attractive to *An. gambiae* in previous experiments (Smallegange et al. 2009), or 4. Compounds produced in greater quantities by less attractive individuals in an experiment in which individuals were ranked for attractiveness to the yellow fever mosquito *Aedes aegypti* L. (Logan et al. 2008).

For each compound, a commercially available authentic reference was injected to determine the retention time on the GC with the applied settings and GC column used for analysis of the emanations on glass beads (see above). Next, the chromatograms showing skin emanations were analyzed for selected components with a processing setup in Xcalibur (Version 2.07, Thermo Scientific, USA). For each component, expected retention time, characteristic mass (Table 8.1), and integration settings were inserted in a processing setup. Next, a sequence of the chromatograms from the skin emanations of the individuals that significantly differed in attractiveness (HA and LA) was batch processed with the processing setup. Identified peaks were evaluated in the Quan browser and identifications and peak areas manually adjusted were necessary. The abundances of each compound in the chromatograms determined by integration of the peak area was exported to Excel for further statistical analysis.

Table 8.1 Characteristics of compounds screened for in the chromatograms of the skin emanations collected from the foot.

Compound	RT	CAS Nr.	Origin	Mass
2,3-butanedione	3.55	431-03-8	(Verhulst et al. 2009)	Not detected
3-methylbutanal	4.83	590-86-3	(Verhulst et al. 2009)	58
1-butanol	4.90	71-36-3	(Verhulst et al. 2009)	Not detected
2-methylbutanal	5.10	96-17-3	(Verhulst et al. 2009)	57
3-hydroxy-2-butanone	6.00	513-86-0	(Verhulst et al. 2009)	88
3-methyl-1-butanol	6.70	123-51-3	(Verhulst et al. 2009)	Not detected
2-methyl-1-butanol	6.70	137-32-6	(Verhulst et al. 2009)	Not detected
2-hexanone	8.16	591-78-6	GC-EAD	Not detected
3-methylbutanoic acid	9.07	503-74-2	(Verhulst et al. 2009) / GC-EAD	60
dimethylsulfoxide	9.21	67-68-5	GC-EAD	Not detected
2-methylbutanoic acid	9.36	116-53-0	(Verhulst et al. 2009)	74
E-2-hexenol	10.07	928-95-0	GC-EAD	Not detected
heptanal	10.80	111-71-7	GC-EAD	70
lactic acid	10.80	79-33-4	(Smallegange et al. 2009)	45
α-pinene	11.95	80-56-8	GC-EAD	Not detected
1-butoxy-2-propanol	12.08	5131-66-8	GC-EAD	Not detected
6-methyl-5-hepten-2-one	12.77	409-02-9	(Logan et al. 2008)/ GC-EAD	108
β-pinene	12.90	18172-67-3	GC-EAD	Not detected
octanal	13.10	124-13-0	(Logan et al. 2008)	84
2-ethyl-1-hexanol	13.65	104-76-7	GC-EAD	83
limonene	13.86	138-68-3	GC-EAD	93
acetophenone	14.74	98-86-2	GC-EAD	105
nonanal	15.15	124-19-6	(Logan et al. 2008)/ GC-EAD	98
2-phenylethanol	15.65	60-12-8	(Verhulst et al. 2009)	91
benzyl acetate	17.07	140-11-4	GC-EAD	Not detected
decanal	17.30	112-31-2	(Logan et al. 2008)/ GC-EAD	112
sulfolane	17.77	26-33-0	GC-EAD	Not detected
methylsalicylate	17.82	119-36-8	GC-EAD	Not detected
geranylacetone	21.72	689-67-8	(Logan et al. 2008)	151
tetradecanoic acid	26.40		(Smallegange et al. 2009)	185

=> See next page

Table 8.1 continued

Compound	RT	CAS Nr.	Origin	Mass
2-hydroxy-3-pentanone	Not injected	5704-20-1	(Verhulst et al. 2009)	Not detected
2,3-heptanedione	Not injected	600-14-6	GC-EAD	Not detected
3-methylnonane	Not injected	5911-04-6	GC-EAD	Not detected
trimeprazine	Not detected	84-96-8	GC-EAD	Not detected

Synthetic references were injected to determine the retention time (RT) for each compound. Column 'Origin' refers to the criteria based on which each compound was selected. When compounds were detected in the chromatograms of the skin emanations, their abundance in the samples was determined by selecting a characteristic mass. Three compounds were not commercially available and therefore not injected. One synthetic reference compound (trimeprazine) was injected, but not found in the reference chromatograms.

Skin bacterial diversity

The skin bacterial diversity of the feet of the individuals was determined by selective and non-selective plate counts and 16S rRNA sequencing. The results from the selective and non-selective plate counts would give a first indication whether the skin microbiota on the human skin affects the attractiveness of human skin emanations to *An. gambiae s.s.* The 16S rRNA genes contain hypervariable regions and sequencing this region provides a more detailed species-specific signature of the microbiota on the human foot.

Sample collection

On each experimental day, a bacterial sample was taken from the sole of the left foot of each individual subsequent to determining this individual's attractiveness to mosquitoes during two successive olfactometer experiments and collection of skin emanations using the small glass beads as described above. Bacterial samples were taken by using a sampling ring and washing buffer as described before (Taylor et al. 2003, Verhulst et al. 2009). A sterile Teflon sampling ring (internal diameter 2.9 cm) was placed in the centre of the sole of the foot, and 0.75 mL of full-strength wash fluid (75 mM sodium phosphate buffer (pH = 7.9) + 0.1 % (v/v) Triton X-100, Merck, The Netherlands) was added. The surface of the skin, within the ring, was gently scrubbed with a sterile glass stick for 1 min and the fluid was pipetted into a 2 mL sample tube (Eppendorf, Germany). Immediately thereafter the process

was repeated at the same site, and the two samples were pooled. Seven hundred µL of the pooled sample was added to 300 µl glycerol (87 %, Merck, Germany) and stored at -80 °C for later identification by 16S rRNA sequencing. The remainder of the sample was used for plate counts on selective and non-selective media.

Selective and non-selective plate counts

Within three h after the bacterial sample collection, each sample was decimally diluted, spread on Colombia (sheep) blood agar plates (Tritium, The Netherlands) and incubated at skin temperature (34 °C) to determine bacterial densities by counting colony forming units (CFU). A range of selective media was used to determine the diversity of the human skin microbiota samples according to the method described by Taylor et al. (2003). Media were either selective for staphylococci, aerobic corynebacteria, micrococci or propionibacteria species (Tritium, The Netherlands).

DNA extraction

To collect DNA for subsequent molecular analysis, microbial DNA was extracted from 103 bacterial samples using the Mo-Bio Power Soil kit (MO BIO Laboratories, Inc., USA). Briefly, 250 µL of thoroughly mixed bacterial sample in glycerol was transferred into a bead tube containing the kit solution C1 and processed according to Costello et al. (2009), or into a well of a 96-well extraction plate containing C1 and processed according to the manufacturer's instructions. An additional 28 samples were extracted using the FastPrep DNA soil kit (MP Biomedicals, USA). Extracted samples were stored at -20 °C until needed for the polymerase chain reaction (PCR).

PCR amplification of the V2 region of bacterial 16S rRNA genes.

For each sample, 16S rRNA genes were amplified before sequencing, according to the methods described by Costello et al. (2009). The PCR primer set used was optimised for phylogenetic analysis of pyrosequencing reads (Liu et al. 2007, Fierer et al. 2008). The forward primer (5'-GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3') contained the 454 Life Sciences primer B sequence, the broadly conserved bacterial primer 27F, and a two-base linker sequence ('TC'). The reverse primer (5'-GCCTCCCTCGCGCCATCAGNNNNNNNNNNNNCATGCTGCCTCCCGTAG

GAGT -3') contained the 454 Life Sciences primer A sequence, a unique 12-nt error-correcting Golay barcode used to tag each PCR product (NNNNNNNNNNNN) (Fierer et al. 2008), the broad-range bacterial primer 338R, and a 'CA' linker sequence inserted between the barcode and the rRNA primer. PCR reactions were carried out in triplicate in 25 µl reactions with 2.0 µl (30µM) of each 6 µM forward and reverse primers, 1 3 µl template DNA, and 1X of either Platinum PCR SuperMix (Invitrogen, USA) or HotMasterMix, 1 µl of 25 mM Magnesium solution (5 PRIME, USA), and 10 µl PCR Water (MO BIO Laboratories, Inc., USA). The PCR program consisted of initial denaturation at 94°C for 3 minutes followed by 35 cycles at 94°C for 45 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 90 seconds, with a final extension of 10 minutes at 72°C (Costello et al. 2009). Replicate amplicons were pooled and visualised on 1.0 % agarose gels using SYBR Safe DNA gel stain in 0.5X TBE (Invitrogen, USA). Amplicons were cleaned using the UltraClean-htp 96-well PCR Clean-up kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's instructions.

Amplicon quantification, pooling, and pyrosequencing

Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, USA) as described by Costello et al. (2009). Following quantification, cleaned amplicons were combined in equimolar ratios into a single tube. The final pool of DNA was precipitated on ice for 45 minutes following addition of 5 M NaCl (0.2 M final concentration) and 2 volumes of ice-cold 100 % ethanol (Costello et al. 2009). The precipitated DNA was centrifuged at 7,800x g for 40 minutes at 4 °C, and the resulting pellet was washed with an equal volume of ice-cold 70 % ethanol and centrifuged again at 7,800x g for 20 minutes at 4°C. The supernatant was removed, the pellet air dried for 10 min and resuspended in 100 µL of nuclease-free water (MO BIO Laboratories, Inc., USA) (Costello et al. 2009). The ratio of absorptions at 260 nm vs 280 nm to assess concentrations of the pooled DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher, USA). Pyrosequencing was carried out using 5PRIME MasterMix (5PRIME, USA) on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, USA).

Post-processing of DNA sequences

All post-processing of the pyrosequencing output was performed with the

QIIME software package (Caporaso et al. 2010). First appropriate denoising of the 454 pyrosequencing output was performed using the PyroNoise algorithm (Quince et al. 2009). Then the UCLUST software (http://www.drive5.com/usearch/usearch.pdf) was used to pick clusters of operational taxonomic units (OTUs) at the 97 % similarity level. The Ribosomal Database Project (RDP) classifier software (Wang et al. 2007), with the default training taxonomy, assigned taxonomic labels to the resulting OTUs. Finally, the OTUs were placed in a *de novo* phylogenetic tree with FastTree 2 (Price et al. 2010).

HLA analysis

From each volunteer five to 10 mL of blood was collected for DNA isolation in EDTA tubes (Vacutainer, BD, USA) to prevent clotting. Genomic DNA was isolated using a commercial semi-automated beads-based assay (Chemagen, Germany).

HLA-A,-B and Cw typing was done with the reverse line hybridisation strip assay (RELI™ SSO, Invitrogen, Washington DC, USA). HLA-DRB and DQB typing was performed with a reversed approach of the PCR/SSOP technique described previously. (Verduyn et al. 1993) Briefly; using Biotin-labelled generic primers the polymorphic regions of the HLA genes were amplified by PCR. After amplification the PCR fragments were hybridised under critically conditions to HLA specific probes. Signals to discriminate for positive and negative probe reaction were achieved by adding Horseradish peroxdase streptavidin followed by a luminogen (Amersham ECL Kit, GE Healthcare Biosciences Pittsburgh USA).

With computer assisted analysis software (SCORE, Nellcor, USA) the probe hybridization patterns were interpreted to HLA types. (Helmberg et al. 1998)

Statistical Analysis

Olfactometer data

For each volunteer, the fraction of mosquitoes trapped in the trapping device from which the headspace of the handled beads was released compared to the number trapped in the trapping device from which ammonia-containing air was released were analysed with a generalised linear model (GLM; Binomial, logit link function; dispersion estimated, Genstat, Release 12.1; (Qiu et al. 2006)). This method was also used to analyse the results of the control

experiments without any odour sources) in the olfactometer and of the experiments in which ammonia was tested against clean beads.

Another GLM (Binomial, logit link function, dispersion estimated) was used to investigate differences between individuals in their relative attractiveness, expressed as the fraction of mosquitoes caught in the trapping device baited with the glass beads releasing odour of the individual under test divided by the total number of mosquitoes trapped in the two trapping devices together (Qiu et al. 2006). Two-sided t-probabilities were calculated to test pairwise differences between means. Individuals were classified into three groups according to their relative attractiveness. Individuals were classified as highly attractive (HA) when their relative attractiveness was significantly higher than the group of low attractive individuals (LA). The relative attractiveness of the remaining third group was not significantly different from the other two groups. Effects were considered to be significant at $P < 0.05$.

Abundance of compounds in skin emanations

The abundances of the skin emanation components identified in the chromatograms of the HA and LA individuals were analysed using projection to latent structures – Partial Least Squares Discriminant Analysis (PLS-DA) using the software programme SIMCA-P 12.0 (Umetrics, Umeå, Sweden) (Eriksson et al. 2006, Bruinsma et al. 2010). The objective of PLS-DA is to find a model that separates classes of observations on their X-variables. PLS-DA is a supervised technique, so class memberships of the observations need to be predefined (Eriksson et al. 2006). Therefore, the response was a vector y containing the values 1 and 0 as dummy variables for the LA and HA individuals respectively. The X-matrix contained the integrated peak areas, which were log transformed, centred and scaled to unit variance. The number of significant PLS components was determined by cross-validation (Eriksson et al. 2006).

Skin bacterial diversity

The effect of average bacterial densities, determined by the logarithm of the counts of CFUs on the selective and non-selective plates, on the relative attractiveness of the individuals was analysed using a GLM (Binomial, logit link function; dispersion estimated, Genstat).

The 16S rRNA sequence results were used to determine the diversity of skin bacterial communities of the individuals. A phylogenetic diversity (PD)

test (Faith 1992), was used to compare the branch length of the parts of the phylogenetic tree covered by the samples. To control for sequencing effort, multiple rarefaction analyses (Gotelli and Colwell 2001) were performed on all samples at various simulated sequencing depths (1,000 rarefaction samples at each sequencing depth from 500 to 3,000 at intervals of 500). A t-test was used (R programming environment, http://www.R-project.org) to test for a significant difference in microbial diversity between the LA and HA group at a simulated depth of 500, 1000 and 1500 sequences. No statistics were performed for the diversity at sequence depths above 1500 because the samples of some individuals did not contain more than 1500 sequences.

Based on previous experiments (Chapter 7), there was reason to believe that the relative abundance of the following five bacterial species might be significantly related to the attractiveness of the host to *An. gambiae* mosquitoes: *Bacillus subtilis, Brevibacterium epidermidis, Corynebacterium minutissimum, Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. The taxonomic assignments from the RDP classifier had only genus-level classifications for most OTUs, so OTUs were first grouped at the genus level according to their assigned lineages. Next, an ANOVA (R programming environment, http://www.R-project.org) test was performed to determine whether the observed variance in the relative abundances of the five genera was partitioned according to the LA and HA group. These results were corrected for multiple comparisons using the false discovery rate (FDR) correction (Benjamini and Hochberg 1995). The data were also controlled for sequencing effort. As with the diversity analysis described above, 1000 stochastic rarefactions of the data were used at a simulated sequencing depth of 1500; the P-values obtained by performing ANOVA on each of those rarefied OTU-tables were averaged.

An unguided search for any bacterial taxa significantly different in occurrence between the HA and LA group was also performed. To avoid making an excessively large number of statistical tests, only genera that were present in at least 20 samples were considered. Results were FDR corrected.

To test for a possible correlation between the bacterial diversity as determined by 16s rRNA sequencing and the abundance of the selected compounds (Table 8.1) as determined by the peak areas, a distance matrix was used for each dataset: an unweighted Unifrac distance matrix for the 16s rRNA data and a Spearman distance matrix for the compounds' abundances. Next, these matrices were compared in three steps (Qiimi software, (Caporaso

et al. 2010)): 1. Principal coordinates analysis (PCoA) applied to each distance matrix. 2. Procrustes analysis (Sibson 1978) performed on each pair of PCoA matrices to rotate the PCoA scores in one matrix to be as close as possible (i.e. minimize sum of squared distances, SSD) to those in the other matrix, while preserving the relative distances within each matrix. 3. Performed 1000 Monte-Carlo simulations with scrambled sample IDs to estimate the distribution of SSD values under the null hypothesis that the two PCoA matrices are not related, and counted the proportion of "null" SSD scores that were better than (or less than) the SSD from step 2. This is a permutation test (Dwass 1957) of the P-value of our real SSD from step 2.

HLA analysis

A two-tailed Fisher's Exact test was used to test for possible correlations between individuals classified as HA or LA and their HLA profiles. P-values were corrected for multiple comparisons conform the Bonferroni method. Odds ratios were calculated according to Woolf Haldane's test (Svejgaard and Ryder 1994).

To test for possible correlations between HLA genes and the peak areas of selected compounds in the gas chromatograms of the skin emanation samples (Table 8.1), random forest regression was performed (Breiman 2001). A random forest regression was done with 500 regression trees and 22 variables tried at each split and performed with the Random Forest package (Liaw and Wiener 2002) in the R programming environment (http://www.R-project.org).

Results

Olfactometer bioassay

The experiments without any odour source showed that there was no positional bias in the olfactometer (GLM, $P = 0.78$). Trapping devices baited with gaseous ammonia (136 ppm) attracted significantly more mosquitoes than trapping devices with clean beads (GLM, $P = 0.04$), which was in accordance with previous experiments (Smallegange et al. 2005, Qiu et al. 2006). Trapping devices baited with skin emanations on glass beads from thirteen individuals caught significantly more mosquitoes than trapping devices baited with ammonia and clean beads (GLM, $P < 0.05$: volunteer # 1, 2, 13, 30, 34, 38, 40, 46, 47, 48, 51, 58, 59, Figure 8.2). The skin emanations of the 35

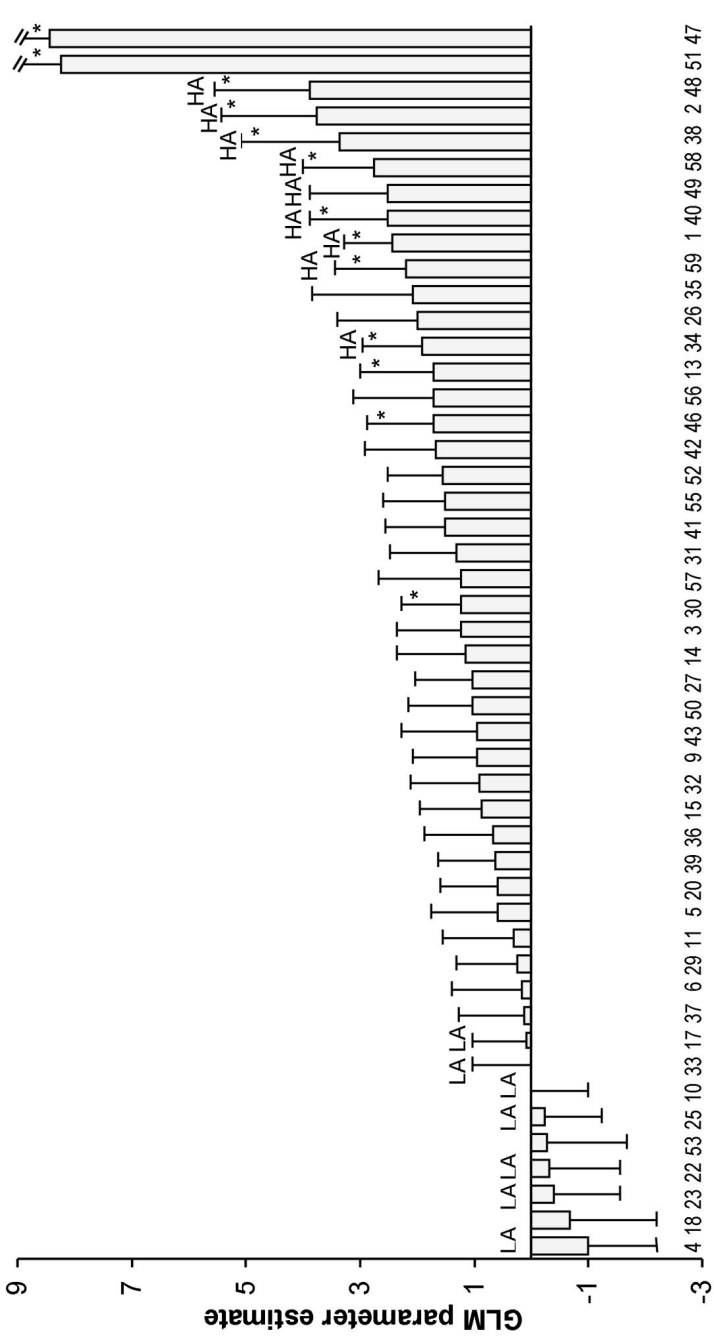


Figure 8.2. Results of olfactometer bioassays in which glass beads carrying human skin emanations from 48 individuals were tested against gaseous ammonia.

*Bars show the parameter estimate results form the GLM (Binomial, logit link function, dispersion estimated) used to investigate the relative attractiveness to An. gambiae of each individual. Two groups of individuals could be distinguished that differed significantly in attractiveness (HA = High Attractive, LA = Low Attractive; t-test, P < 0.05). Error bars represent the standard error of the mean from six replications. Standard errors for the relative attractiveness of individual 51 and 47 were too large to display. *Trapping devices baited with glass beads carrying skin emanations from this individual caught significantly more mosquitoes than trapping devices baited with ammonia and clean beads.*

remaining individuals caught similar numbers of mosquitoes as the trapping devices releasing ammonia.

There were significant differences in relative attractiveness between the individuals (GLM, $P < 0.01$). The trapping devices baited with skin emanations of nine individuals catching high numbers of mosquitoes (HA group, volunteer # 1, 2, 34, 38, 40, 48, 49, 58, 59) were significantly more attractive than trapping devices baited with skin emanations of seven individuals catching lower numbers of mosquitoes (LA group, volunteer # 4, 10, 17, 22, 23, 25, 33; t-test, $P < 0.05$, Figure 8.2). The age of the individual or time of testing on each experimental day did not have a significant effect on the relative attractiveness (GLM, $P = 0.41$ and $P = 0.08$ respectively).

GC-EAD

The GC-EAD analysis yielded 28 GC peaks that reproducibly coincided with antennal responses in more than 40 % of the preparations. The 28 compounds were tentatively identified by GC-MS analysis (Table 8.2). Eighteen compounds eliciting EAD responses were identified from sample PE48, two from PM48, 12 from HA7 and seven from LA7. Several components occurred in several samples, mostly in both PE48 and HA7 or LA7 (Table 8.2). The 28 compounds that elicited EAD activities belonged to eight different chemical groups including alcohols, aldehydes, alkanes, aromatics, carboxylic acid, esters, heterocyclics, sulphur-containing compounds and terpenoids.

More than 90 % of the antennal preparations exposed to sample PE48 elicited EAD responses to butyl acetate, and at least 80 % of the preparations elicited EAD responses to 1-butoxy-2-propanol, decanal, heptanal, a-pinene and trimeprazine present in the PE48 sample (Table 8.2). 80 % of the preparations elicited an EAD response to 2-hexanol present in the PE48 sample. More than 70 % of the preparations exposed to components occurring in HA7 elicited EAD responses to heptanal, 2,3-heptanedione, 2-hexanone, *E*-2-hexenol, 3-methylbutanoic acid and trimeprazine (Table 8.2). The highest responses to compounds contained in sample LA7 were found in response to acetophenone (69%) and 6-methyl-5-hepten-2-one (60 %; Table 8.2).

GC-MS

Of the 33 compounds selected, based on GC-EAD results or previous studies (see Materials and Methods), 17 could be identified in the chromatograms of the skin emanations of the 48 individuals (Table 8.1). PLS-DA could not

Table 8.2. Identification of compounds eliciting antennal responses (GC-EAD) in headspace samples of human feet.

Tentative identification	KRI	Preparations with EAD response (%)	Sample*
3-hydroxy-2-butanol	759	53	PE48
toluene	778	60	PE48
butyl acetate	793	93	PE48
2-hexanone	808	80	HA7
2-hexanol	814	80	PM48
2,3-heptanedione	849	75	HA7
3-methylbutanoic acid	851	75, 56	HA7, LA7
E-2-hexenol	885	60, 75, 44	PE48, HA7, LA7
heptanal	904	87, 75	PE48, HA7
2,5-dimethylpyrazine	912	60	PE48
dimethylsulfoxide	921	60	LA7
a-pinene	932	80, 50	PE48, LA7
1-butoxy-2-propanol	941	87, 67	PE48, HA7
b-pinene	974	73, 67	PE48, HA7
6-methyl-5-hepten-2-one	991	62	LA7
trimeprazine	1000	80, 71	PE48, HA7
2-acetylthiazol	1016	67	PE48
limonene	1027	43	HA7
2-ethyl-1-hexanol	1029	54	LA7
3-methylnonane	1052	73, 64	PE48, HA7
acetophenone	1063	69	LA7
1-methyl-1-phenylethanol	1086	53	PE48
undecane	1099	67	PE48
nonanal	1104	67	PE48
benzyl acetate	1175	55	PM48
naphthalene	1179	53	PE48
methylsalicylate	1192	67, 50	PE48, HA7
decanal	1202	80, 64	PE48, HA7

Compounds that elicited an EAD response in more than 40% of the antennal preparations (N = 12-15) are shown. KRI = Kovats Retention Index. PE48: pooled sample of foot headspace from 48 individuals trapped on Tenax and eluted with diethyl ether; PM48: pooled sample of foot headspace from 48 individuals trapped on active charcoal and eluted with dichloromethane; HA7: pooled samples of foot headspace from seven individuals with high attractiveness; LA7: pooled samples of foot headspace from seven individuals with low attractiveness.*

significantly differentiate the LA and HA group based on the peak areas of the selected compounds. To get an indication which compounds associated best with the LA or HA group, the first two PLS components were calculated (R^2X = 0.457, R^2Y = 0.614, Q^2 = -0.195; Figure 8.3 and 8.4). The score plot shows that volunteer #2 was an outlier compared with the other individuals in the HA or LA group (Figure 8.3). The loading plot shows that the LA group correlates best with 2-phenylethanol and limonene (Figure 8.4). No compounds were closely correlated with the HA group.

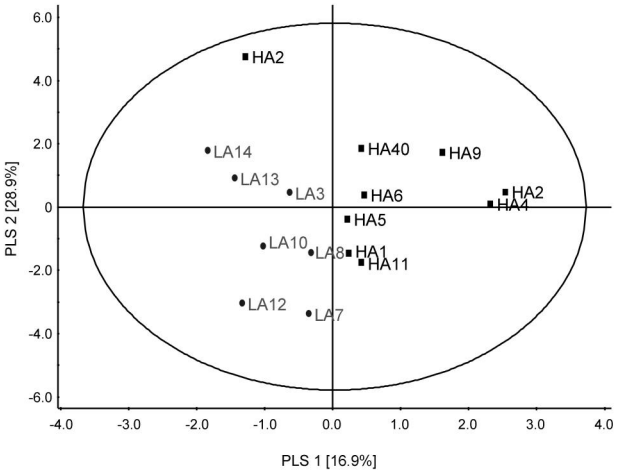


Figure 8.3. Multivariate data analysis of the volatile pattern of skin emanations of LA (Low Attractive) and HA (High Attractive) individuals.

Score plot of projection to latent structures-discriminant analysis (PLS-DA). Percentage variation explained for each PLS axis is given in parentheses. The ellipse defines the Hotelling's T^2 confidence region (95%).

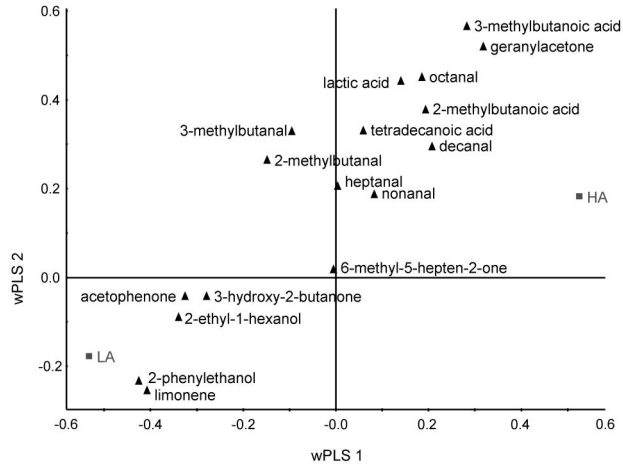


Figure 8.4. Multivariate data analysis of the volatile pattern of skin emanations of LA (Low Attractive) and HA (High Attractive) individuals.

Loading plot of projection to latent structures-discriminant analysis (PLS-DA) as based on the amounts (log) of 17 volatile compounds from the skin emanations of LA and HA individuals.

Skin bacterial diversity

Selective and non-selective plate counts

On average, $5.8 \cdot 10^5$ bacteria were present per cm^2 on the sole of a human foot as determined by counting CFUs on blood agar plates. The average number of *Staphylococcus* spp. was $6.9 \cdot 10^5$ per cm^2 foot, *Corynebacteria* spp. $2.2 \cdot 10^5$ per cm^2, *Micrococcus* spp. $1.1 \cdot 10^4$ per cm^2 and *Propionibacteria* spp. $1.3 \cdot 10^4$ per cm^2 as determined by counting CFUs on selective plates. Of all samples, 98.7% contained *Staphylococcus* spp., 92.9% of the samples *Corynebacteria* spp., 89.6% *Micrococcus* spp. and 85.7% *Propionibacteria* spp.

The average number (log) of bacteria present per cm^2 on the sole of a human foot as determined by counting CFUs on blood agar plates had a significant effect on the relative attractiveness of the individuals to *An. gambiae* (Figure 8.5, GLM, P = 0.003). The abundance of *Staphylococci* spp. was also positively correlated with the relative attractiveness of the individuals to *An. gambiae* (Figure 8.6, GLM, P = 0.01). There was a high correlation between the number of bacteria determined by CFU counts on blood agar plates and *Staphylococcus* spp. selective plates, probably because many of the bacterial colonies found on the blood agar plates were *Staphylococci* spp. The number of *Corynebacteria* spp., *Micrococcus* spp. and *Propionobacteria* spp. did not show a correlation with the relative attractiveness of the

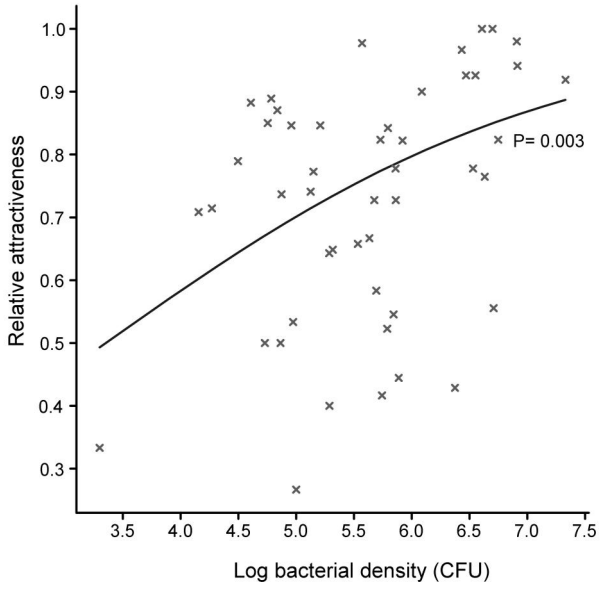


Figure 8.5. Correlation between the number of bacteria (log), determined by counts of colony forming units (CFUs) on non-selective plates and the relative attractiveness of the individuals.

The relative attractiveness is expressed as the number of mosquitoes caught in the trapping device releasing the odour of the individual under test divided by the total number of mosquitoes trapped in both trapping devices during each experiment (Qiu et al. 2004a).

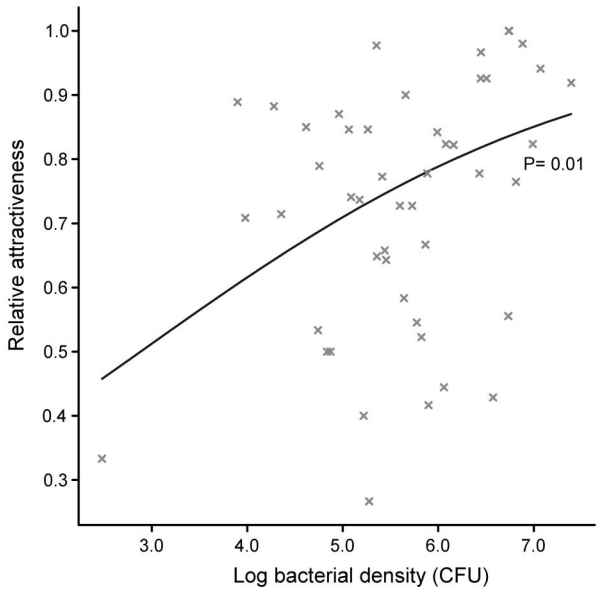


Figure 8.6. Correlation between the number of Staphylococcus spp. bacteria (log), determined by counts of colony forming units (CFUs) on* Staphylococcus *spp. selective plates and the relative attractiveness of the individuals.

The relative attractiveness is expressed as the number of mosquitoes caught in the trapping device releasing the odour of the individual under test divided by the total number of mosquitoes trapped in both trapping devices during each experiment (Qiu et al. 2004a)

individuals (GLM, P = 0.085, P = 0.28 and P = 0.41, respectively).

Individuals #4 and #18 were outliers and leverage points in the analyses. The level of significance changed when these individuals were omitted from the analyses; the results, also then, remained significant.

16S rRNA sequencing

The phylogenetic diversity scores were significantly different between the HA individuals and the LA individuals. The Phylogenetic Diversity (PD) scores of communities on the LA individuals were significantly higher than those on the HA hosts at a simulated depth of 500, 1000 and 1500 sequences (Figure 8.7, t-test, P = 0.041, P = 0.022 and P = 0.028, respectively). At a sequence depth of 1500 the PD diversity scores of bacterial communities on the LA individuals were 37% higher than the PD diversity scores of bacterial communities on the HA individuals.

The relative abundances of OTUs classified within the *Staphylococcus* and *Pseudomonas* genus were significantly different between HA and LA individuals (ANOVA, P < 0.006 and P = 0.04, respectively). The abundance of *Staphylococcus* spp. was 4.17 times higher in the HA group than in the LA group and the abundance of *Pseudomonas* spp. 2.88 times higher in the LA group than in the HA group. *Brevibacterium* spp. and *Corynebacterium* spp. were not found to be significantly different between the two groups (ANOVA, P

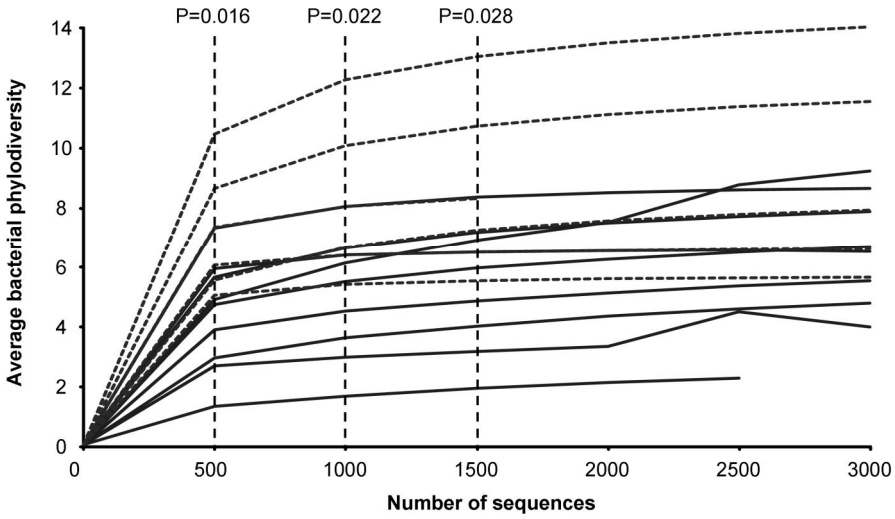


Figure 8.7. Rarefaction curves showing bacterial diversity on foot surfaces from LA (Low Attractive) and HA (High Attractive) individuals.

Phylogenetic diversity was estimated by measuring the average total branch length per sample after a specified number of individual sequences have been observed (Faith 1992). P-value for the difference in diversity score between LA and HA individuals is given at three sampling depths (not calculated for higher number of sequences, because the samples of some individuals did not contain more than 1500 sequences).

= 0.56 and P = 0.20, respectively). The abundance of *Bacillus* spp. was not tested because *Bacillus* spp. were present in only a limited number of samples.

In subsequent tests, which were controlled for sequencing effort, these results were maintained. As with the diversity analysis described above, 1000 stochastic rarefactions of the data were used at a simulated sequencing depth of 1500 and P-values averaged obtained by performing ANOVA on each of those rarefied OTU tables. On average, differences in abundances in *Staphylococcus* spp. and *Pseudomonas* spp. remained significant after rarefaction ($P < 0.05$).

In addition, an unguided search was performed for any taxa that were significantly different between the HA and LA groups. To avoid making an excessively large number of statistical tests, only genera were considered that were present in at least 20 samples. The relative abundance of 13 taxonomic groups at genus level was significantly higher in the LA than in the HA groups, and of one taxonomic group significantly higher in the HA group than in the LA

group. However, none of the P-values remained significant after FDR correction for multiple testing and the 13 taxonomic groups identified should only be suggestive for further research (Table 8.3).

Comparing the bacterial diversity and the abundance of the selected volatile compounds (Table 8.1), as determined by the peak areas in the chromatograms, did not result in any significant correlations (non-parametric estimate, P = 0.255). Of the 1000 scrambled Monte-Carlo simulations to estimate the distribution of SSD values, 225 were better than the SSD values obtained from the Procrustes analysis performed on each pair of PCoA matrices.

HLA

When the HLA profiles of the HA and LA group were compared, HLA antigen Cw*07 occurred significantly more often in the HA group than in the LA group (Fisher's Exact test, P = 0.0073). After correction for testing multiple alleles this correlation was only marginally significant (Bonferroni corrected P - value, P = 0.064).

Testing the correlation between HLA profiles and peak areas of compounds in the chromatograms of the skin emanations with the random forest method only showed a few compounds whose presence could be explained by the HLA profile, but all with very low explained variance.

Discussion

In this study, it was demonstrated that skin emanations collected from the feet of individuals exhibit significant variation in attractiveness to *An. gambiae*. This has been shown before for the skin emanations from human hands (Qiu et al. 2006). The present study focussed on the attractiveness of human foot emanations to *An. gambiae* as it was previously found that volatile organic compounds (VOCs) from human feet influence the selection of biting sites by *An. gambiae* (de Jong and Knols 1995). In the current study there was a significant correlation between the composition and abundance of the microbiota on the feet and the degree of attractiveness of the foot emanations of the individuals to *An. gambiae*. HLA profile analysis indicated one HLA gene that may influence this attractiveness of humans to *An. gambiae*.

MHC genes influence the body odour of mice (Yamazaki et al. 1976, Penn and Potts 1998b) and several studies have suggested a relation between human body odour and the HLA genes located in the MHC region of

Table 8.3. Relative abundance of taxonomic bacterial groups at genus level that differed significantly (before FDR correction) between the HA (High Attractive) and LA (Low Attractive) individuals.

Order/ Family/ Genus	HA	LA	P-value before FDR correction	P-value after FDR correction
Bacillales/ Staphylococcaceae/ Staphylococcus	$5.54*10^{-1}$	$1.33*10^{-1}$	0.006	0.76
Clostridiales/ Ruminococcaceae/ Other	$3.16*10^{-4}$	$5.01*10^{-3}$	0.007	0.45
Burkholderiales/ Comamonadaceae/ Variovorax	$1.50*10^{-4}$	$3.89*10^{-3}$	0.010	0.42
Clostridiales/ Lachnospiraceae/ Lachnospiraceae Incertae Sedis	$8.96*10^{-4}$	$1.47*10^{-2}$	0.011	0.34
Thermoanaerobacterales/ Incertae Sedis III/ Caldicellulosiruptor	$3.75*10^{-4}$	$4.38*10^{-3}$	0.019	0.45
Clostridiales/ Lachnospiraceae/ Other	$1.33*10^{-3}$	$2.43*10^{-2}$	0.019	0.39
Bacteroidales/ Rikenellaceae/ Alistipes	$6.09*10^{-4}$	$8.90*10^{-3}$	0.021	0.37
Clostridiales/ Ruminococcaceae/ Faecalibacterium	$1.78*10^{-3}$	$2.03*10^{-2}$	0.022	0.33
Clostridiales/ Lachnospiraceae/ Roseburia	$1.00*10^{-3}$	$1.09*10^{-2}$	0.023	0.31
Clostridiales/ Peptostreptococcaceae/ Peptostreptococcaceae Incertae Sedis	$2.23*10^{-4}$	$2.90*10^{-3}$	0.035	0.43
Pseudomonadales/ Pseudomonadaceae/ Pseudomonas	$1.66*10^{-3}$	$4.78*10^{-3}$	0.037	0.40
Clostridiales/ Lachnospiraceae/ Dorea	$7.68*10^{-4}$	$9.72*10^{-3}$	0.039	0.39
Bacteroidales/ Bacteroidaceae/ Bacteroides	$4.03*10^{-3}$	$3.87*10^{-2}$	0.041	0.38
Flavobacteriales/ Flavobacteriaceae/ Flavobacterium	$3.48*10^{-5}$	$1.27*10^{-3}$	0.044	0.38

Mean relative abundances of the HA and LA group are given.

humans (Wedekind and Furi 1997, Wedekind and Penn 2000). The present study provides evidence for a positive correlation between the presence of HLA gene Cw*07 and the attractiveness of human skin emanations to mosquitoes, although the effect was marginally significant after correction for multiple comparisons (Bonferroni-corrected P = 0.064). HLA-Cw genes play a role in the modulation of natural killer (NK) cell alloreactivity (Colonna et al. 1993), that constitutes the first line of innate immunity. If HLA gene Cw*07 influences a person's attractiveness to *An. gambiae*, then this will have an impact on the number of bites received and therefore, possibly, the intensity of *Plasmodium* transmission. Future field studies are necessary to assess whether the frequency of HLA gene Cw*07 is significantly lower in malaria endemic areas and whether individuals carrying this gene are bitten more often. A targeted study, however, would involve fewer individuals if two groups would be selected, one group consisting of individuals with and the other group of persons without HLA-Cw*07.

The abundances of the selected compounds for GC-MS analysis did not correlate with the HLA profiles or skin bacterial composition of the individuals. Based on the finding in this study that the presence of HLA gene Cw*07 and skin bacterial composition influence human attractiveness to mosquitoes, it was hypothesised that a link with VOC composition and/or abundance would exist. However, of only 17 compounds the abundance was determined in the skin emanation profiles. The other compounds could not be identified, possibly because of the different sampling methods used for the GC-MS and GC-EAD analysis. More than 300 compounds can be identified in human skin emanations collected on glass beads (Bernier et al. 1999, Bernier et al. 2000) and it is plausible that other compounds than the 17 quantified in the current study correlate with HLA profile or skin bacteria composition. Automatic alignment and comparison of chromatograms would be essential for the analysis of more than 300 compounds in the chromatograms. This requires chromatograms with low background noise and samples obtained in a relatively short period to prevent large shifts in retention time. The latter, however, can not be realized in mosquito behavioural studies with large groups of individuals.

Several studies have indicated a possible role of skin bacteria in the production of volatiles that mediate the differential attractiveness of humans to mosquitoes (de Jong and Knols 1995, Braks and Takken 1999, Verhulst et al. 2009, Verhulst et al. 2010). The present study shows that both abundance and

composition of the human skin microbiota have an effect on the attractiveness of individuals to mosquitoes. The abundance of *Staphylococcus* spp. on the feet as determined by both selective plate counts and 16S rRNA sequence analysis was positively correlated with the attractiveness of the individuals (Figure 8.6). This corroborates previous findings that volatiles released by *in vitro* grown *Staphylococcus epidermidis* were attractive to *An. gambiae* females (Verhulst et al. 2009) (Chapter 7). The abundance of *Pseudomonas* spp. on the feet was negatively correlated with the attractiveness of the individuals as determined by 16S rRNA sequence analysis. This is in accordance with *in vitro* experiments showing that *Pseudomonas aeruginosa* does not produce VOCs attractive to *An. gambiae*, in contrast to VOCs produced by four other bacterial species, all commonly found on human skin that did attract *An. gambiae* (Chapter 7). The results from the present study indicate that *Pseudomonas* species produce compounds that repel *An. gambiae* or odours that attenuate the effect of the attractive volatiles emanating from the human skin.

16S rRNA analysis of the skin bacterial profiles revealed that LA individuals had a significantly higher bacterial diversity on their feet than HA individuals (Figure 8.7). Possibly a heterogeneous microbiota includes more bacteria that produce volatiles that attenuate the attractiveness of LA individuals to mosquitoes and may explain the interference effect described for *Ae. aegypti*. higher levels of specific compounds were found to be responsible for decreased attractiveness of individuals to *Ae. aegypti*. Being less attractive to mosquitoes can be caused by an 'in-built defence system' of naturally produced compounds that interfere with the way mosquitoes find their human hosts (Logan 2008, Logan et al. 2008).

The current study shows that the skin microbiota plays an important role in this 'in-built defence system' and may therefore have an impact on *Plasmodium* transmission (Braks et al. 1999, Verhulst et al. 2010). Individuals with a higher microbial diversity are less attractive to mosquitoes, are therefore bitten less and have a lower chance to become infected with *Plasmodium* and consequently a higher survival probability. The fact that increased diversity has a positive effect on health is not new. Genetic heterozygosity of an individual, for example, enhances resistance to infectious diseases (Penn et al. 2002, McClelland et al. 2003) and a reduced diversity of faecal microbiota is associated with Crohn's disease (Manichanh et al. 2006). The hypothesis that a higher skin bacterial diversity can have a positive effect on human health by

reducing the risk of *Plasmodium* infection should be confirmed by field studies aimed at monitoring skin bacterial diversities and *Plasmodium* transmission in a malaria endemic area.

The screening for compounds in the chromatograms obtained from the feet emanations of individuals that differed in attractiveness (HA and LA) did not result in compounds that were significantly correlated to either group. However, calculating the first two components in the PLS-DA analysis indicated that two compounds (2-phenylethanol and limonene) were more abundant in the samples taken from LA individuals (Figure 8.4). Limonene has insecticidal properties (Kassir et al. 1989, Ibrahim et al. 2001), is a common constituent of essential oils that repel mosquitoes (Omolo et al. 2004, Debboun et al. 2007) and inhibits volatile production by human skin microbiota (Ara et al. 2006). 2-Phenylethanol was previously identified in the headspace of skin bacteria (Verhulst et al. 2009) and inhibited the attraction of *An. gambiae* to a blend consisting of ammonia, lactic acid and tetradecanoic acid (Chapter 5). Together with the results obtained in this study, this suggests that 2-phenylethanol can lower a person's attractiveness and may be used as a mosquito repellent.

The PLS-DA analysis partly matched the GC-EAD results. 2-Ethyl-1-hexanol and acetophenone in LA samples elicited an EAD response, which is in correspondence with the results obtained by the PLS-DA that associated these two compounds with LA volunteers (Table 8.2, Figure 8.4). Heptanal and decanal in HA samples elicited an EAD response (Table 8.2) and were associated with HA volunteers in the PLS-DA (Figure 8.4). Limonene in HA samples however elicited an EAD response (Table 8.2) but was associated with LA volunteers in the PLS-DA (Figure 8.4).

Another compound that elicited EAD responses and has been tested in previous studies examining its effect on the response of host-seeking mosquitoes is 6-methyl-5-hepten-2-one. This compound was found in LA samples and elicited EAD responses. It has an inhibitory effect on the attractiveness of a blend consisting of ammonia and lactic acid to *An. gambiae* females (Qiu et al. in press). GC-EAD analysis on the antennae of *Ae. aegypti* (Logan et al. 2008) revealed five compounds, among which 6-methyl-5-hepten-2-one, that were produced in greater quantities by less attractive individuals and that reduced the upwind flight of *Ae. aegypti* females (Table 8.1). Four of these five compounds elicited EAD responses in the present study (Table 8.2). Future olfactometer experiments are needed to reveal

whether the compounds identified in the GC-EAD analysis that have not been tested previously in behavioural bioassays play a role in the host-seeking process of *An. gambiae*.

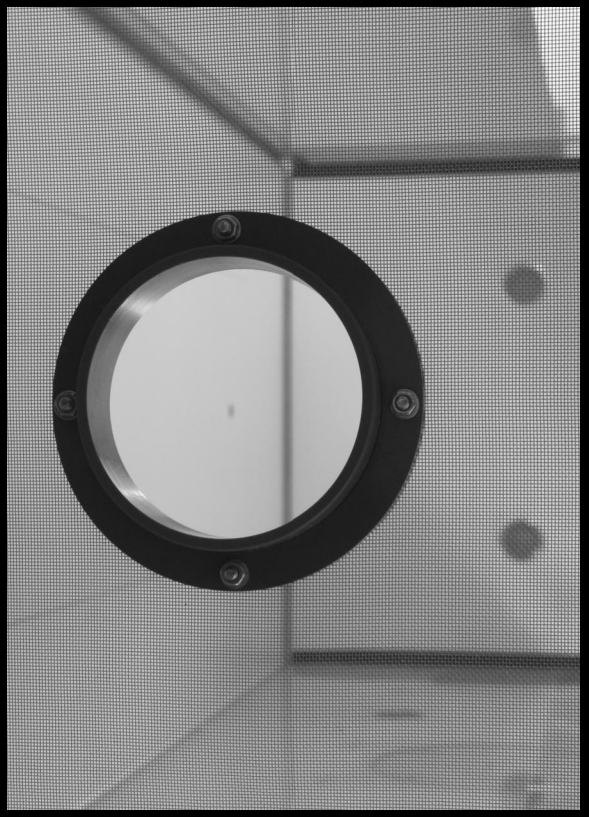
The results presented in the current study provide a better understanding of the traits affecting the production of volatiles released from the human skin that determine a person's attractiveness to mosquitoes. HLA profiling suggested that HLA composition influences the attractiveness of humans to mosquitoes. Both the abundance and composition of the skin microbiota influence the attractiveness of humans to *An. gambiae*. The number of bacteria present on the skin and the degree of attractiveness to the malaria mosquito *An. gambiae* were positively correlated, whereas a highly diverse skin microbiota reduces an individual's attractiveness and may therefore reduce the risk of getting bitten and contracting malaria. *Staphylococcus* spp. were associated with individuals that were highly attractive and *Pseudomonas* spp. with individuals that were less attractive to mosquitoes. The results suggest that a person's attractiveness to mosquitoes can be modified by manipulation of the composition of skin microbiota, for example with probiotics. Several VOCs were associated with persons that were highly attractive and others with persons that were less attractive to mosquitoes in both PLS-DA and GC-EAD analysis. Compounds and bacterial species associated with less attractive individuals are of interest for the development of repellents. Compounds and bacterial species associated with individuals that are highly attractive may contribute to the development of attractants to apply in lure and kill strategies or to be used to monitor malaria mosquito populations.

Acknowledgements

We would like to thank all volunteers for their participation and are grateful to F. K. M. van Aggelen, A. J. Gidding and L. Koopman for mosquito rearing. We acknowledge G. Leenderts for the construction of the devices for sampling skin emanations, H. Heilig for his help with DNA extraction and S. Nakielny for the initial analysis of the sequencing data. We would like to thank M. Ratering, M. van Hasselt and A. Godkewitsch for taking blood samples and providing materials.

This study was approved by the Dutch medical ethical review committee (METC, Project number ABR NL16928.081.07 amended in 2007) and funded by a grant from the Foundation for the National Institutes of Health

Discussion



General discussion

09

Niels O. Verhulst,

The principle goal of this thesis was to:
Understand the role of the skin microbiota in mosquito-host interactions and identify which compounds produced by these organisms are involved in the attraction of Anopheles gambiae sensu stricto *to humans.*

Four main objectives were formulated in the introduction of this thesis **(Chapter 1)** and these will be discussed one by one in the current chapter to identify whether the objective has been achieved and to place the results in a broader scientific and social context. At the end of this chapter, future research opportunities and possible applications based on the findings of this thesis will be discussed.

Investigate whether micro-organisms obtained from the human skin produce substances that affect the host-seeking behaviour of *Anopheles gambiae sensu stricto.*
Volatiles produced by human skin bacteria, that were grown on agar or liquid medium, attracted *Anopheles gambiae sensu stricto* (hereafter referred to as *An. gambiae*) females in an olfactometer and to mosquito (MM-X) traps **(Chapters 4, 5, 7)**. The volatiles released by skin bacteria grown on agar attracted significantly more *An. gambiae* in laboratory and semi-field experiments than volatiles released by agar without skin bacteria. It is concluded that micro-organisms from the human skin produce volatiles (e.g. organic compounds) that affect the host-seeking behaviour of *An. gambiae*.

Also other disease vectors are attracted to the volatiles emitted by bacteria. Broth cultures of *Bacillus cereus* derived from smears from a human arm are attractive to the yellow fever mosquito, *Aedes aegypti* L. (Schreck and James 1968) and an anti-bacterial gel reduced the attractiveness of human skin washings to the kissing bug *Rhodnius prolixus* Stål (Hemiptera: Triatominae), a vector of Chagas disease (Ortiz and Molina 2010). Field experiments in Kenya, suggested that skin bacterial volatiles attract not only *An. gambiae s.s.* but also *An. arabiensis* and *Mansonia* species **(Chapter 5)**.

At the moment, tools for monitoring host-seeking mosquitoes are limited and often depend on traps baited with carbon dioxide (Kline 2007, Qiu et al. 2007a). The field trial described in **Chapter 5** provided the first evidence that bacterial volatiles from human skin in combination with carbon dioxide may be used for the monitoring of host-seeking mosquitoes. However, the agar used in the trial as a substrate for the bacteria to grow on, dried out within

a couple of hours and the volatiles may no longer have been available. Use of an improved substrate as well as a different method of application will probably increase mosquito catches. After improvement of the substrate, preferably containing locally available and cheap ingredients, skin bacteria provide a cheap and easy-to-use bait for field monitoring of African malaria mosquitoes. In addition, identification of the volatiles produced by skin bacteria led to the identification of several compounds that improved the attractiveness of a basic blend to mosquitoes **(Chapters 4, 6 & 7)**. Optimising these blends could result in odour baits that are more attractive than baits depending on natural skin bacterial mixtures. Depending on the delivery method used, the release rate of the consituents of these synthetic blends will most likely be more consistent over time than that of the skin bacterial volatiles (Torr et al. 1997).

Determine which genera of the human skin microbiota are most important in the production of mosquito behaviour-mediating compounds.

Experiments with *Staphylococcus epidermidis* cultured on agar plates or in liquid medium **(Chapter 4 & 7)** showed that volatiles emitted by individual bacterial species common on the human skin are attractive to *An. gambiae*. The attractiveness of *Staphylococcus* spp. volatiles to *An. gambiae* was confirmed in an experiment with 48 volunteers, showing that the abundance of *Staphylococcus* spp. on the human skin was positively correlated with individuals that are highly attractive to *An. gambiae* **(Chapter 8)**.

Not all bacteria species commonly present on the human skin produce volatiles that attract *An. gambiae* and some bacterial species are more attractive than others **(Chapter 7)**. *Pseudomonas aeruginosa* was not attractive to *An. gambiae* and the relative attractiveness of *Corynebacterium minutissimum* was significantly higher than that of *S. epidermidis* and *Bacillus subtilis*. From these results it cannot be concluded whether the observed effects are species-specific or account for whole genera, or even families of bacteria. Often the metabolic capacities of species within a genus are relatively similar (Noble 2004). However, *S. epidermidis* and *S. hominis*, for example, are two closely related skin bacteria species but their metabolic capabilities and volatile production are different (Noble 2004). Testing of multiple bacterial species within one genus or family should indicate whether the production of volatiles attractive to mosquitoes is species-specific.

Volatiles produced by *P. aeruginosa* were not attractive to *An. gambiae*

when grown *in vitro* **(Chapter 7)** and the abundance of *Pseudomonas* spp. on the human skin was positively correlated with individuals that are less attractive to *An. gambiae* **(Chapter 8)**. This suggests that *P. aeruginosa* produces compounds that attenuate or mask the attractiveness of other volatiles or that *P. aeruginosa* produces volatiles that repel *An. gambiae*. Future experiments may elucidate the effect of *Pseudomonas* spp. volatiles on host-seeking mosquitoes. Tests with *P. aeruginosa* alone will indicate whether this bacterial species produces masking and/or repellent compounds. An olfactometer, however, is not suitable for testing repellents and therefore another bioassay should be used (Dogan and Rossignol 1999). If volatiles released by *Pseudomonas* spp. are not repellent, but do inhibit the attractiveness of a worn sock or an attractive synthetic blend, there is probably an attenuating or masking effect of *Pseudomonas* spp. volatiles.

African malaria mosquitoes and mankind have co-evolved for thousands of years, and this process may have led to the development of microorganisms on the human skin that repel mosquitoes (Verhulst et al. 2010) or attenuate or mask the attractiveness of humans, as suggested above for *Pseudomonas* spp. On the other hand, *An. gambiae* may have specialised on volatiles produced by species like *Staphylococcus* spp. to find its host.

Investigate whether the differential attractiveness of human individuals to *An. gambiae sensu stricto* and the skin microbiota composition are correlated.

The results presented in **Chapter 8** showed that both the composition and abundance of the skin microbiota had an effect on the differential attractiveness of human skin emanations to *An. gambiae*. Human individuals that were less attractive to *An. gambiae* had a lower overall abundance of bacteria on the skin, but a higher bacterial diversity.

Comparing the composition of skin microbiota and the attractiveness of the volunteers **(Chapter 8)** resulted in a few bacterial genera that could be associated with individuals that were highly attractive (*Staphylococcus* spp.) or less attractive (*Pseudomonas* spp.) to *An. gambiae*. This was a targeted approach based on previous results **(Chapter 7)**. The untargeted approach did not result in any significant correlations between bacterial genera present on human feet and human attractiveness to mosquitoes **(Chapter 8).** The variation in microbiota on human skin is often high (Fierer et al. 2008, Costello et al. 2009) and increasing the sample size of persons participating in a similar

study may lead to the identification of additional genera associated with highly attractive or less attractive volunteers.

The composition of bacteria on the skin of individuals tested for attractiveness to mosquitoes was determined by 16S rRNA sequencing **(Chapter 8)**. Although this 16S region is commonly used to determine phylogenetic distances, the genes in this region are not involved in the metabolism of the bacteria. Sequencing the genome of the skin microbiota and blasting these sequences will indicate which genes, involved in certain metabolic pathways, are present. Whole transcriptome sequencing reflects genes that code for enzymes that regulate the metabolic activity of the skin microbiota at the time of sampling. Although the number of metabolic pathways that have been unravelled is still limited and the methods for whole transcriptome sequencing still costly (Zoetendal et al. 2006, Turnbaugh et al. 2007, Vrieze et al. 2010), there is much progression in this field and sequencing techniques are rapidly becoming cheaper. Elucidation of the relation between metabolic activity of bacteria and attractiveness of individuals to mosquitoes will not only further reveal the role of human skin microbiota in mosquito attraction, but will also reveal the underlying mechanisms leading to the production of the volatiles that affect that attractiveness **(Chapter 2)**.

The experiment with 48 individuals, described in **Chapter 8**, suggested that the human leucocyte antigens (HLA) play a role in the underlying mechanisms leading to the production of volatiles that mediate the attractiveness to mosquitoes. HLA gene Cw*07 was correlated with the attractiveness of human skin emanations to mosquitoes, although the effect was marginally significant after correction for multiple comparisons. As mentioned in **Chapter 2**, HLA genes were proven to affect the composition of human body odour, and the present study suggests that at least one of these genes may affect the degree to which humans are attractive to *An. gambiae*. To obtain a definitive answer on the question whether HLA genes affect the attractiveness of humans to mosquitoes, the size of the population studied should be larger, or the study should be targeted at HLA-Cw*07 (i.e., compare a group consisting of individuals with to another group of persons without HLA-Cw*07).

Identify compounds produced by microbiota of human skin that mediate mosquito behaviour.

In **Chapter 4**, fourteen putative attractants were found in the headspace of skin bacteria obtained from the sole of a human foot and grown *in vitro*. A synthetic blend of ten of these was attractive to *An. gambiae* in olfactometer and indoor trapping experiments **(Chapters 4 & 5)**. Testing these ten compounds one by one in combination with a basic blend, consisting of ammonia, lactic acid and tetradecanoic acid, resulted in the identification of compounds that increased or decreased the attractiveness of the basic blend **(Chapter 6)**, depending on the concentration of the test compound. When the headspaces of five species of skin bacteria, with differential attractiveness to *An. gambiae*, were compared, additional compounds were found that were differentially emitted by the bacterial species. Six of these compounds were tested and either increased or decreased the attractiveness of the basic blend to *An. gambiae* **(Chapter 7)**.

Analyses of human skin emanations often result in hundreds of compounds (Bernier et al. 2000, Curran et al. 2007, Penn et al. 2007), which makes the identification of the compounds that mediate the host-seeking behaviour of mosquitoes difficult, as each compound potentially may contribute to the overall attraction of the emanations (Smallegange and Takken 2010). The studies described in this thesis demonstrate that the identification of volatiles from human skin bacteria is a novel and effective method for the identification of compounds that affect host-seeking mosquitoes. Analysis of the headspace of skin bacteria *in vitro* resulted in a limited number of compounds **(Chapters 4 & 7)** and thereby reduces the number of potential compounds that disrupt the behaviour of mosquitoes. In addition to this, it is easier to control and standardize the conditions under which headspace samples of skin bacteria are collected than taking headspace samples of human skin.

A study on the attractiveness of volatiles produced by bacterial species present on the human skin that are closely related may not only lead to the identification of new compounds that influence the behaviour of mosquitoes, but also reveal whether the production of these volatiles is species-specific. A change of the constituents of the media on which the bacteria are grown may reveal which metabolic processes are required for the production of the semiochemicals that attract or repel mosquitoes. Such a study may also reveal to what extent this production is affected by the nutrients available to the

bacteria.

Both the concentration and the combination of compounds in an odour blend determine the attractiveness of the blend to mosquitoes (Smallegange et al. 2005, Smallegange and Takken 2010) **(Chapter 6)**. Therefore, the attractiveness of the blends identified in this study can probably be improved by testing more concentrations and combinations of candidate compounds. Attractive blends may be applied in lure and kill strategies, to be used to monitor malaria mosquito populations or in push-pull systems in which an attractive blend may be used to 'pull' mosquitoes into traps and a spatial repellent to 'push' mosquitoes away from human dwellings (Agelopoulos et al. 1999, Cook et al. 2007, Logan and Birkett 2007, Logan et al. 2008, Jawara et al. 2009, Okumu et al. 2010c). In addition to the optimisation of odour blends, trap costs should be lowered substantially to make odour baited traps competitive with current strategies and a valuable tool in integrated vector management (Okumu et al. 2010b).

Future research

Plasmodium infection

Infection of a mosquito with *Plasmodium* parasites increases the efficacy of the mosquito as a vector, because *Plasmodium* favours its own transmission by altering the behaviour of the mosquito. Infected mosquitoes take larger blood meals and the frequency of multiple feeding increases when the mosquito is infected (Koella et al. 1998, Ferguson and Read 2004). Infection of the vertebrate host with *Plasmodium* also influences the behaviour of the mosquito. When mice were infected with either asexual parasites or gametocytes, they were more attractive to *Anopheles stephensi* then uninfected mice (Ferguson and Read 2004). Moreover, children harbouring *Plasmodium* gametocytes attracted about twice as a many mosquitoes as children without infection or with parasites in the asexual stage (Lacroix et al. 2005). This increased attractiveness of infected children could be explained by an increase in body temperature or increased perspiration. However, these factors are less likely to be involved in the observed change in attractiveness, as the infection was asymptomatic in all of the children involved. It is likely that the parasite changes the individual's breath or body odour, which are also indicators of other infections (Lacroix et al. 2005, Prugnolle et al. 2009).

A relation exists between the composition of human skin microbiota and the attractiveness of humans to mosquitoes **(Chapter 8)**. If an infection

with *Plasmodium* affects the composition of the microbiota, this may influence the attractiveness of the infected individual to *Anopheles* mosquitoes (Penn and Potts 1998c). Examples of diseases which affect the skin microbiota are scarce and often focus on skin infections. For example, people infected with HIV have a higher risk of infection with *Staphylococcus aureus* (Weinke et al. 1992) and diabetes patients have a higher risk of a fungal infection with toenail onychomycosis (Gupta et al. 1998).

Alterations in the human odour profile after infection with a vector-borne parasite have been shown before, however, the underlying mechanisms remain largely unknown (O'Shea et al. 2002, Mukabana 2002, Lacroix et al. 2005, Lefèvre and Thomas 2008, Prugnolle et al. 2009). Future studies may identify the possible effect of a *Plasmodium* infection on the human skin microbiota composition and the skin emanations released by the skin microbiota that cause this effect by sampling skin emanations and skin microbiota before and after infection.

Human blood composition

The composition of host blood is important for the development of mosquito oocytes. Protein levels and protein composition both influence mosquito fecundity. When *Aedes* species were fed on human blood or on guinea pig blood, more eggs were produced after feeding on guinea pig blood (Chang and Judson 1979, Briegel 1985). Total protein levels in human blood and guinea pig blood were equal, but variation was found between the amino acid compositions of the two blood sources. Human blood has an insufficient level of the amino acid isoleucine and addition of isoleucine to human blood raised the egg production by *Aedes* mosquitoes to the same level as after feeding on guinea pig blood (Chang and Judson 1979). The fecundity of *An. stephensi* was also affected by the source of blood. Egg production was reduced by half when the mosquitoes were offered human blood as compared to guinea pig blood (Briegel and Rezzonico 1985).

Isoleucine is one of the branched-chain amino acids. In studies on the control of foot odour these amino acids are mentioned as compounds from which fatty acids, which are associated with foot odour, may be derived (James et al. 2004b, James et al. 2004a, Ara et al. 2006). The conversion of branched-chain amino acids into fatty acids appears to be specific for *Staphylococcus* species (James et al. 2004b, James et al. 2004a).

Several volatile fatty acids have been shown to attract *An. gambiae*, in

combination with other compounds (Knols et al. 1997, Smallegange et al. 2005, Smallegange et al. 2009, Smallegange and Takken 2010). The studies described in this thesis have shown that *S. epidermidis*, grown *in vitro*, produces compounds including fatty acids that are attractive to *An. gambiae.* In addition, human individuals which are more attractive to mosquitoes than others, have a higher abundance of *Staphylococcus* spp. on their skin compared to individuals that are less attractive to mosquitoes **(Chapter 8)**. This is a strong indication that volatile fatty acids produced by *Staphylococcus* spp. play a role in mosquito host-seeking behaviour.

If the isoleucine content in human blood influences the odour components produced by the human skin bacteria (*Staphylococcus* spp.*)* then these components are reliable indicators to a female mosquito of the quality of the host (Figure 9.1). Little is known about the relation between branched-chain amino acid levels in human blood and body odour production. However, a disorder in leucine metabolism, called Isovaleric acidemia, causes the accumulation of isovaleric acid (synonym of 3-methylbutanoic acid) in the human body resulting in a strong body odour caused by this fatty acid (Tanaka et al. 1966). An investigation of the relation between branched-chain amino acid levels in human blood, the attractiveness of humans to mosquitoes, and mosquito fitness (Figure 9.1) may reveal whether odour components released from the human skin are indicators of the quality of the host to mosquitoes and

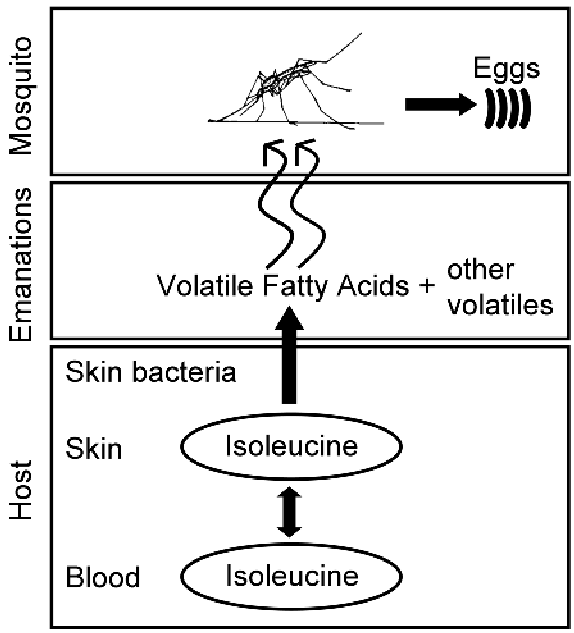


Figure 9.1. Hypothesized influence of human blood composition on the volatiles produced by the skin microbiota and effect on mosquito olfaction and fecundity.

may explain why mosquitoes prefer some hosts more than others.

Personal hygiene

Hand washing with anti-bacterial soap does not change the overall levels of bacterial diversity on the skin of the hands. However, it does alter the composition of the bacterial community (Fierer et al. 2008). The relative abundance of *Staphylococcus* spp., for example, is higher on hands that have been washed recently (Fierer et al. 2008). Washing the feet with anti-bacterial soap alters the biting site preference of *An. gambiae* (de Jong and Knols 1995, Knols and Meijerink 1997) and humans with a lower abundance of skin bacteria are less attractive to *An. gambiae* **(Chapter 8)**.

Reduction of the bacterial abundance on the human skin by washing may reduce someone's attractiveness to mosquitoes. Paradoxically, washing increases the relative abundance of *Staphylococcus* spp. (Fierer et al. 2008) and these bacteria are associated with an increased attractiveness to mosquitoes **(Chapter 8)**.

The increase of personal hygiene during childhood is suggested to attribute to the increase in the prevalence of allergic disorders (Strachan 1989, Yazdanbakhsh et al. 2002).

This negative effect of personal hygiene could possibly also account for the attraction of mosquitoes. I suggest therefore that an increase in personal hygiene may increase an individual's attractiveness to mosquitoes by alterations in the skin microbiota.

CO_2 interference

Carbon dioxide (CO_2) is an important sensory cue in many insects. In *Drosophila*, CO_2 is an odour emitted by stressed flies and fruit flies exhibit olfactory-based avoidance behaviour to CO_2 (Turner and Ray 2009). However, CO_2 is also present in significant quantities in several important food sources that elicit behavioural attraction of *Drosophila*. To block the CO_2 avoidance behaviour, odorants released by ripening fruits block the CO_2 receptors of *Drosophila* (Turner and Ray 2009). In mosquitoes, the CO_2 receptors are located on the maxillary palps and it is assumed that the neurons responsible for human-specific responses are located on the antennae. Recently, inhibitors of CO_2-sensitive neurons in *Culex quinquefasciatus* have been identified and may provide a valuable resource for the identification of volatile compounds

that may reduce mosquito–human contact by blocking the responsiveness to CO_2 (Turner and Ray 2009).

Although inhibitors of CO_2-sensitive neurons in mosquitoes have been identified (Turner and Ray 2009) mosquitoes do not emit CO_2 when stressed as *Drosophila* does. Carbon dioxide is harmful to most organisms in high concentrations. However, CO_2 concentrations in expired air (by vertebrates) are too low to suffocate or kill mosquitoes and constitute one of the main cues for host location (Mboera and Takken 1997, Takken and Knols 1999). Interestingly, five of the 10 compounds identified in the headspace of human skin bacteria (Verhulst et al. 2009) **(Chapter 4)** inhibited the response of palpal neurons of *An. gambiae* to CO_2 (R.A. Suer, pers. comm.).

When *An. gambiae* is in search for its blood meal it follows gradients of CO_2 and host-specific odours (Dekker and Takken 1998b, Takken and Knols 1999, Cardé and Gibson 2010). When the mosquito comes close to the host, the mosquito should divert from the CO_2 plume released from the mouth. I hypothesize that odours released by skin bacteria not only guide *An. gambiae* to the skin, but also inhibit the CO_2 perception at close range. These CO_2 inhibitors could possibly be used as topical repellents (R.A. Suer, pers. comm.). Of course it should be taken into account that many of the odours released by skin bacteria also attract *An. gambiae* and CO_2 inhibitors should, therefore, also be tested for their ability to attract mosquitoes.

Host preference

Anopheles gambiae mainly feeds on humans even when other host species are abundantly present (Garrett-Jones et al. 1980, Costantini et al. 1998, Takken and Knols 1999, Pates et al. 2001). Other members of the *An. gambiae* complex have different host preferences and are more zoophilic or opportunistic, biting a wide range of host species (Figure 9.2). Experiments with field traps baited with human and cow odour have shown that the response of *An. gambiae* to human odours is much stronger than that of its sibling species *An. arabiensis*, which is a more opportunistic species (Costantini et al. 1996a, Costantini et al. 1998). Another sibling species, *An. quadriannulatus* has an anthropophily index lower than 0.25 (Torr et al. 2008) and is more zoophilic (White et al. 1980, Dekker and Takken 1998a, Hunt et al. 1998, Torr et al. 2008). Several studies, however, have also shown that deviations of this index occur depending on the population studied or the conditions under which host preference was tested (Pates et al. 2005, Lefèvre

et al. 2009).

Most mosquito species respond primarily to carbon dioxide. The highly selective response of *An. gambiae* to human odours, mediated by the composition of the human bacterial community (this thesis), may account for the restricted anthropophilic host range of this mosquito species. Studying the link between bacterial communities, the volatiles they produce and sibling species of mosquitoes that differ in host preference will reveal to what extent the composition of bacterial communities of vertebrates determines the mosquito host range and which volatiles are used in the host-seeking process by mosquitoes (Figure 9.2).

Recent studies have shown that skin bacteria may also play a role in the host-seeking behaviour and host-preference of the kissing bug (Ortiz and Molina 2010) and the red poultry mite *Dermanyssus gallinae* De Geer (C.J.M. Koenraadt & M. Dicke, pers. comm.). Studying the effect of skin microbiota on the host preference of mosquitoes within the *An. gambiae* complex will therefore not only reveal the mechanisms underlying the host preference of the most important malaria vectors *An. gambiae s.s.* and its sibling *An. arabiensis*, but may also be used to understand similar mechanisms most likely present in other arthropods of medical and veterinary importance. The identification of the skin bacteria and bacterial volatiles that mediate host

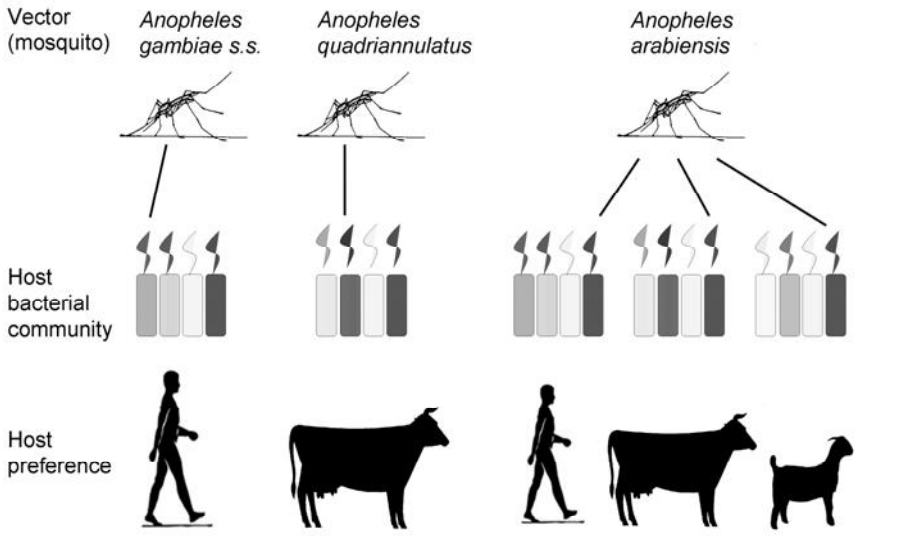


Figure 9.2. Host preference of three mosquito species within the* An. gambiae *s.l. complex and the suggested role of volatiles produced by the host bacterial community.

preference can lead to the development of odour baits that can selectively attract disease vectors with a certain host preference in the field (Kline 2007, Logan and Birkett 2007).

Oviposition

Although not of human origin, bacterial volatiles also play an important role in the selection of oviposition sites by mosquitoes (Rockett 1987, Huang et al. 2006, Lindh et al. 2008, Ponnusamy et al. 2008, Trexler et al. 2009). Gravid mosquitoes respond to the volatiles produced by microbial metabolic activity in hay or leave infusions (Lindh et al. 2008, Ponnusamy et al. 2008, Ponnusamy et al. 2010). These infusions are often used in ovitraps for monitoring the egg-laying activity of *Aedes* and *Culex* mosquitoes in the field (Elizondo-Quiroga et al. 2006, Lourenço-de-Oliveira et al. 2009, Ritchie et al. 2009).

Since bacterial volatiles play a role in both the host-seeking behaviour (this thesis) and oviposition behaviour (Huang et al. 2006, Lindh et al. 2008) of *An. gambiae*, it would be interesting to compare the bacteria that mediate host-seeking and oviposition. Comparing the volatiles released by these bacteria can lead to the identification of new volatiles that attract or repel mosquitoes. If certain bacterial species produce compounds that attract both host-seeking mosquitoes and mosquitoes searching for an oviposition site, then traps that attract mosquitoes in both physiological stages could be developed.

Inhibitory effects of natural mosquito repellents

Volatile Fatty Acids (VFAs) are associated with human malodour and with the attractiveness of humans to mosquitoes (Knols et al. 1997, James et al. 2004b, Smallegange et al. 2005, Smallegange et al. 2009, Smallegange and Takken 2010) (this thesis). 3-Methylbutanoic acid is one of these fatty acids and the main component responsible for foot malodour (Ara et al. 2006). The compound is produced by skin bacteria in which the enzyme leucine dehydrogenase converts leucine on the skin into 3-methylbutanoic acid. In search for an inhibitor of this enzyme, 60 naturally occurring fragrant agents were tested by Ara et al. (2006). Fragrant agents with the highest inhibitory value of up to 89 % included citral, geraniol, limonene and citronellal. Interestingly, these compounds are known natural mosquito repellents or inhibit the attraction of mosquitoes to humans (Oyedele et al. 2002, Barnard 2004, Omolo et al. 2004, Debboun et al. 2007).

The mode of action underlying the activity of mosquito repellents is still not very clear. It has been suggested that the best known repellent N,N-diethyl-3-methylbenzamide (DEET) masks or jams the olfactory system (Davis 1985, Ditzen et al. 2008), but a more recent study showed that mosquitoes can smell and avoid DEET (Syed and Leal 2008). Moreover, when applied on human skin, DEET alters the profile of emanations by a ''fixative'' effect that may also contribute to repellency (Syed and Leal 2008). Unlike the recently-discovered mode of action of DEET, evidence for the mode of action of natural compounds such as citronellal and limonene is still scarce (Davis 1985, Debboun et al. 2007).

The compounds that inhibit odour production in the study of Ara et al. (2006) may be able to inhibit volatile production or growth of human skin microbiota and thereby reduce a person's attractiveness to mosquitoes. Limonene is one of these compounds that inhibit volatile production by human skin microbiota (Ara et al. 2006) and was associated with individuals which are less attractive to mosquitoes **(Chapter 8)**. If limonene or other natural repellents inhibit growth or volatile production of skin microbiota, it would then be more effective to use these repellents on human skin than on clothing or other materials, as suggested for DEET (Syed and Leal 2008).

Concluding remarks

The results presented in this thesis create new opportunities for both fundamental and applied research. The role of volatiles from skin bacteria in the host-seeking behaviour of other mosquito species and their host preference remains to be investigated and the effect of blood composition, *Plasmodium* infection or personal hygiene on the skin microbiota and volatile production is still unclear.

Volatiles produced by the microbiota on human skin play an important role in the host-seeking behaviour of *An. gambiae* and the abundance and composition of the skin microbiota determine an individual's attractiveness to mosquitoes. The analysis of headspace from skin microbiota grown *in vitro* led to the identification of 16 compounds. The majority of these compounds had a behavioural effect on *An. gambiae*. Optimised blends consisting of these compounds can be used in push-pull strategies, thereby reducing the number of malaria mosquitoes, the human biting frequency, and the intensity of *Plasmodium* transmission. Further research on the role of skin microbiota in the host-seeking behaviour and host preference of biting insects may lead to a better understanding of vector-host interactions and contribute to the fight

REFERENCES

Acree, F., Jr., R. B. Turner, H. K. Gouck, M. Beroza, and N. Smith. 1968. L-Lactic Acid: A mosquito attractant isolated from humans. Science 161: 1346-1347.

Agelopoulos, N., M. A. Birkett, A. J. Hick, A. M. Hooper, J. A. Pickett, E. M. Pow, L. E. Smart, D. W. M. Smiley, L. J. Wadhams, and C. M. Woodcock. 1999. Exploiting semiochemicals in insect control. Pesticide Science 55: 225-235.

Agtmaal van, M. 2006. Masking the attractiveness of human odours to mosquitoes: A dual-port olfactometer study which investigated the putative allomonal effects of several selected human volatiles on Anopheles gambiae s.s host seeking behaviour., pp. 71, Msc-thesis, Laboratory of Entomology. Wageningen University

Allan, S. A., J. F. Day, and J. D. Edman. 1987. Visual ecology of biting flies. Annual Review of Entomology 32: 297-316.

Allan, S. A., U. R. Bernier, and D. L. Kline. 2006. Attraction of mosquitoes to volatiles associated with blood. Journal of Vector Ecology 31: 71-78.

Ansell, J., K. A. Hamilton, G. E. L. Walraven, and S. W. Lindsay. 2002. Short-range attractiveness of pregnant women to *Anopheles gambiae* mosquitoes. Transactions of the Royal Society of Tropical Medicine and Hygiene 96: 113-116.

Anthony, R. M., W. C. Noble, and D. G. Pitcher. 1992. Characterization of aerobic non-lipophilic coryneforms from human feet. Clinical and Experimental Dermatology 17: 102-105.

Ara, K., M. Hama, S. Akiba, K. Koike, K. Okisaka, T. Hagura, T. Kamiya, and F. Tomita. 2006. Foot odor due to microbial metabolism and its control. Canadian Journal of Microbiology 52: 357-364.

Barnard, D. R. 2004. Laboratory evaluation of mosquito repellents against *Aedes albopictus*, *Culex nigripalpus*, and *Ochlerotatus triseriatus* (Diptera : Culicidae). Journal of Medical Entomology 41: 726-730.

Bayoh, M. N., D. Mathias, M. Odiere, F. Mutuku, L. Kamau, J. Gimnig, J. Vulule, W. Hawley, M. Hamel, and E. Walker. 2010. *Anopheles gambiae*: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. Malaria Journal 9: 62.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57: 289-300.

Bernier, U. R., M. M. Booth, and R. A. Yost. 1999. Analysis of human skin emanations by gas chromatography/ mass spectrometry. 1.Thermal desorption of attractants for the yellow fever mosquito (*Aedes aegypti*) from handled glass beads. Analytical Chemistry 71: 1-7.

Bernier, U. R., D. L. Kline, S. A. Allan, and D. R. Barnard. 2007. Laboratory comparison of *Aedes aegypti* attraction to human odors and to synthetic

human odor compounds and blends. Journal of the American Mosquito Control Association 23: 288-293.

Bernier, U. R., D. L. Kline, D. R. Barnard, C. E. Schreck, and R. A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for yellow fever mosquito (*Aedes aegypti*). Analytical Chemistry 72: 747-756.

Bernier, U. R., D. L. Kline, C. E. Schreck, R. A. Yost, and D. R. Barnard. 2002. Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). Journal of the American Mosquito Control Association 18: 186-195.

Bernier, U. R., D. L. Kline, K. H. Posey, M. M. Booth, R. A. Yost, and D. R. Barnard. 2003. Synergistic attraction of *Aedes aegypti* (L.) to binary blends of L-Lactic acid and Acetone, Dichloromethane, or Dimethyl Disulfide. Journal of Medical Entomology 40: 653-656.

Bisno, A. L., and D. L. Stevens. 1996. Streptococcal infections of skin and soft tissues. New England Journal of Medicine 334: 240-246.

Bojar, R. A., and K. T. Holland. 2002. Review: the human cutaneous microflora and factors controlling colonisation. World Journal of Microbiology & Biotechnology 18: 889-903.

Boland, W., P. Ney, L. Jaenicke, and G. Gassmann. 1984. A "closed-loop-stripping" technique as a versatile tool for metabolic studies of volatiles, pp. 371-380. *In* P. Schreier [ed.], Analysis of Volatiles.

Bosch, O. J., M. Geier, and J. Boeckh. 2000. Contribution of fatty acids to olfactory host finding of female *Aedes aegypti*. Chemical Senses 25: 323-330.

Brady, J., C. Costantini, N. Sagnon, Gibson, and M. Coluzzi. 1997. The role of body odours in the relative attractiveness of different men to malarial vectors in Burkina Faso. Annals of Tropical Medicine & Parasitology 91: S121-S122.

Braks, M. A. H. 1999. Human skin emanations in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae*, pp. 118, Laboratory of Entomology. Wageningen University, Wageningen.

Braks, M. A. H., and W. Takken. 1999. Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto*. Journal of Chemical Ecology 25: 663-672.

Braks, M. A. H., R. A. Anderson, and B. G. J. Knols. 1999. Infochemicals in mosquito host selection: Human skin microflora and Plasmodium parasites. Parasitology Today 15: 409-413.

Braks, M. A. H., J. Meijerink, and W. Takken. 2001. The response of the malaria mosquito, *Anopheles gambiae*, to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. Physiological Entomology 26: 142-148.

Braks, M. A. H., E. J. Scholte, W. Takken, and T. Dekker. 2000. Microbial growth enhances the attractiveness of human sweat for the malaria mosquito, *Anopheles gambiae sensu stricto*. Chemoecology 10: 129-134.

Breiman, L. 2001. Random forests. Machine Learning 45: 5-32.

Briegel, H. 1985. Mosquito Reproduction - Incomplete Utilization of the Blood Meal Protein for Oogenesis. Journal of Insect Physiology 31: 15-21.

Briegel, H., and L. Rezzonico. 1985. Concentration of host blood protein during feeding by *Anopheline* mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 22: 612-618.

Brouwer, R. 1960. Variations in human body odour as a cause of individual differences of attraction for malaria mosquitoes. Tropical and Geographical Medicine 12: 186-192.

Bruggemann, H., A. Henne, F. Hoster, H. Liesegang, A. Wiezer, A. Strittmatter, S. Hujer, P. Durre, and G. Gottschalk. 2004. The complete genome sequence of *Propionibacterium acnes*, a commensal of human skin. Science 305: 671-673.

Bruinsma, M., S. van Broekhoven, E. Poelman, M. Posthumus, M. Müller, J. van Loon, and M. Dicke. 2010. Inhibition of lipoxygenase affects induction of both direct and indirect plant defences against herbivorous insects. Oecologia 162: 393-404.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7: 335-336.

Cardé, R. T., and G. Gibson. 2010. Host finding by female mosquitoes: mechanisms of orientation to host odours and other cues, pp. 115-140. *In* W. Takken and B. G. J. Knols [eds.], Olfaction in vector-host interactions. Wageningen Academic Publishers, Wageningen.

Carnevale, P., J. L. Frezil, M. F. Bosseno, F. Le Pont, and J. Lancien. 1978. Etude de l'agressivite d'*Anopheles gambiae* A en fonction de l'age et du sexe des sujets humains. Bulletin of the World Health Organization 56: 147-154.

Chang, Y. Y. H., and C. L. Judson. 1979. Amino-Acid Composition of Human and Guinea-Pig Blood Proteins, and Ovarian Proteins of the Yellow-Fever Mosquito Aedes-Aegypti - and Their Effects on the Mosquito Egg-Production. Comparative Biochemistry and Physiology a-Physiology 62: 753-755.

Coetzee, M., M. Craig, and D. le Sueur. 2000. Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. Parasitology today 16: 74-77.

Cogen, A. L., V. Nizet, and R. L. Gallo. 2008. Skin microbiota: a source of disease or

defence? British Journal of Dermatology 158: 442-455.

Collins, M. D., and D. Jones. 1983. *Corynebacterium minutissimum* sp. nov., nom. rev. International Journal of Systematic Bacteriology 33: 870-871.

Colonna, M., E. Brooks, M. Falco, G. Ferrara, and J. Strominger. 1993. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science 260: 1121-1124.

Cook, S. M., Z. R. Khan, and J. A. Pickett. 2007. The use of push-pull strategies in integrated pest management. Annual Review of Entomology 52: 375-400.

Costantini, C., S. G. Li, A. Della Torre, N. Sagnon, M. Coluzzi, and C. E. Taylor. 1996a. Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a west African Sudan savanna village. Medical and Veterinary Entomology 10: 203-19.

Costantini, C., G. Gibson, N. Sagnon, A. della Torre, J. Brady, and M. Coluzzi. 1996b. Mosquito responses to carbon dioxide in a West African Sudan savanna village. Medical and Veterinary Entomology 10: 220-227.

Costantini, C., N. Sagnon, A. della Torre, M. Diallo, J. Brady, G. Gibson, and M. Coluzzi. 1998. Odor-mediated host preferences of West-African mosquitoes, with particular reference to malaria vectors. American Journal of Tropical Medicine and Hygiene 58: 56-63.

Costello, E. K., C. L. Lauber, M. Hamady, N. Fierer, J. I. Gordon, and R. Knight. 2009. Bacterial community variation in human body habitats across space and time. Science 326: 1694-1697.

Curran, A. M., S. I. Rabin, P. A. Prada, and K. G. Furton. 2005. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. Journal of Chemical Ecology 31: 0098-0331.

Curran, A. M., C. F. Ramirez, A. A. Schoon, and K. G. Furton. 2007. The frequency of occurrence and discriminatory power of compounds found in human scent across a population determined by SPME-GC/MS. Journal of Chromatography B 846: 86-97.

Davis, E. E. 1985. Insect repellents: concepts of their mode of action relative to potential sensory mechanisms in mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 22: 237-243.

de Jong, R., and B. G. J. Knols. 1995. Selection of biting sites on man by two malaria mosquito species. Experientia 51: 80-84.

Debboun, M., S. P. Frances, and D. Strickman. 2007. Insect Repellents. Principles, Methods, and Uses. CRC Press.

Dekker, T., and W. Takken. 1998a. Differential responses of mosquito sibling species *Anopheles arabiensis* and *An. quadriannulatus* to carbon dioxide , a man or a calf. Medical and Veterinary Entomology 12: 136-140.

Dekker, T., and W. Takken. 1998b. The role of carbon dioxide in host seeking

Anopheles gambiae sensu lato (Diptera: Culicidae), pp. 23-28, Proceedings Experimental and Applied Entomology, N.E.V., Amsterdam.

Dekker, T., W. Takken, and R. T. Cardé. 2001a. Structure of host-odour plumes influences catch of *Anopheles gambiae s.s.* and *Aedes aegypti* in a dual-choice olfactomer. Physiological Entomology 26: 124-134.

Dekker, T., W. Takken, and M. A. H. Braks. 2001b. Innate preference for host-odor blends modulates degree of anthropophagy of *Anopheles gambiae* sensu lato (Diptera : Culicidae). Journal of Medical Entomology 38: 868-871.

Dethier, V. G., L. B. Browne, and C. N. Smith. 1960. The designation of chemicals in terms of the responses they elicit from insects. Journal of Economic Entomology 53: 134-136.

Diallo, M., P. Nabeth, K. Ba, A. A. Sall, Y. Ba, M. Mondo, L. Girault, M. O. Abdalahi, and C. Mathiot. 2005. Mosquito vectors of the 1998-1999 outbreak of Rift Valley Fever and other arboviruses (Bagaza, Sanar, Wesselsbron and West Nile) in Mauritania and Senegal. Medical and Veterinary Entomology 19: 119-126.

Dicke, M., and M. W. Sabelis. 1988. Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? Functional Ecology 2: 131-139.

Dickschat, J. S., T. Martens, T. Brinkhoff, M. Simon, and S. Schulz. 2005. Volatiles released by a *Streptomyces* species isolated from the North Sea. Chemistry & Biodiversity 2: 837-865.

Ditzen, M., M. Pellegrino, and L. B. Vosshall. 2008. Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319: 1838-1842.

Dogan, E. B., and P. A. Rossignol. 1999. An olfactometer for discriminating between attraction, inhibition, and repellency in mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 36: 788-793.

Dwass, M. 1957. Modified randomization tests for nonparametric hypotheses. The Annals of Mathematical Statistics 28: 181-187.

Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. Science 308: 1635-1638.

Egert, M., A. A. de Graaf, H. Smidt, W. M. de Vos, and K. Venema. 2006. Beyond diversity: functional microbiomics of the human colon. Trends in Microbiology 14: 86-91.

Eiras, A. E., and P. C. Jepson. 1991. Host location by *Aedes aegypti* (Diptera Culicidae): a wind tunnel study of chemical cues. Bulletin of Entomological Research 81: 151-160.

Eiras, A. E., and P. C. Jepson. 1994. Responses of female *Aedes aegypti* (Diptera: Culicidae) to host odours and convection currents using an olfactometer bioassay. Bulletin of Entomological Research 84: 207-211.

Elizondo-Quiroga, A., A. Flores-Suarez, D. Elizondo-Quiroga, G. Ponce-Garcia, B. J. Blitvich, J. F. Contreras-Cordero, J. I. Gonzalez-Rojas, R. Mercado-Hernandez, B. J. Beaty, and I. Fernandez-Salas. 2006. Gonotrophic cycle and survivorship of Culex quinquefasciatus (Diptera: Culicidae) using sticky ovitraps in Monterrey, Northeastern Mexico. Journal of the American Mosquito Control Association 22: 10-14.

Eriksson, L., E. Johansson, N. Kettaneh-Wold, J. Trygg, C. Wikström, and S. Wold. 2006. Multi- and megavariate data analysis. Umetrics AB, Umeå, Sweden.

Eschbach, M., K. Schreiber, K. Trunk, J. Buer, D. Jahn, and M. Schobert. 2004. Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. Journal of Bacteriology 186: 4596-4604.

Faith, D. P. 1992. Conservation evaluation and phylogenetic diversity. Biological Conservation 61: 1-10.

Feltman, H., G. Schulert, S. Khan, M. Jain, L. Peterson, and A. R. Hauser. 2001. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. Microbiology 147: 2659-2669.

Ferguson, H. M., and A. F. Read. 2004. Mosquito appetite for blood is stimulated by *Plasmodium chabaudi* infections in themselves and their vertebrate hosts. Malaria journal 3.

Fierer, N., M. Hamady, C. L. Lauber, and R. Knight. 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proceedings of the National Academy of Sciences of the United States of America 105: 17994-17999.

Fierer, N., C. L. Lauber, N. Zhou, D. McDonald, E. K. Costello, and R. Knight. 2010. Forensic identification using skin bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 107: 6477-6481.

Fontenille, D., M. Traore-Lamizana, M. Diallo, J. Thonnon, J. P. Digoutte, and H. G. Zeller. 1998. New vectors of Rift Valley fever in West Africa. Emerging Infectious Diseases 4: 289-293.

Gallagher, M., C. J. Wysocki, J. J. Leyden, A. I. Spielman, X. Sun, and G. Preti. 2008. Analyses of volatile organic compounds from human skin. British Journal of Dermatology 159: 780-791.

Gao, Z., C.-h. Tseng, Z. Pei, and M. J. Blaser. 2007. Molecular analysis of human forearm superficial skin bacterial biota. Proceedings of the National Academy of Sciences of the United States of America 104: 2927-2932.

Garrett-Jones, C., P. F. L. Boreham, and C. P. Pant. 1980. Feeding habits of anophelines (Diptera: Culicidae) in 1971-78, with reference to the human

blood index: a review. Bulletin of Entomological Research 70: 165-185.

Geier, M., and J. Boeckh. 1997. A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours. Entomologia Experimentalis et Applicata. 92: 9-19

Geier, M., O. J. Bosch, and J. Boeckh. 1999. Ammonia as an attractive component of host odour for the Yellow fever mosquito Aedes aegypti. Chemical Senses 24: 647-653.

Gilbert, I. H., H. K. Gouck, and N. Smith. 1966. Attractiveness of men and women to *Aedes aegypti* and relative protection time obtained with DEET. Florida Entomologist 49: 53-66.

Gillies, M. T. 1980. The role of carbon dioxide in host-finding by mosquitoes (Diptera:Culicidae): a review. Bulletin of Entomological Research 70: 525-532.

Gillies, M. T., and T. J. Wilkes. 1969. A Comparison of Range of Attraction of Animal Baits and of Carbon Dioxide for Some West African Mosquitoes. Bulletin of Entomological Research 59: 441-&.

Gillies, M. T., and M. Coetzee. 1987. A supplement to the Anophelinae of Africa South of the Sahara. The south African Institute for Medical Research, Johannesburg.

Githeko, A. K., M. W. Service, C. M. Mbogo, F. K. Atieli, and F. O. Juma. 1994. Origin of blood meals in indoor and outdoor resting malaria vectors in western Kenya. Acta Tropica 58: 307-16.

Githeko, A. K., N. I. Adungo, D. M. Karanja, W. A. Hawley, J. M. Vulule, I. K. Seroney, A. V. O. Ofulla, F. K. Atieli, S. O. Ondijo, I. O. Genga, P. A. Situbi, and Oloo. 1996. Some observations on the biting behavior of *Anopheles gambiae* s.s., *Anopheles arabiensis*, and *Anopheles funestus* and their implications for malaria control. Experimental Parasitology 82: 306-315.

Gotelli, N. J., and R. K. Colwell. 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters 4: 379-391.

Gower, D. B., K. T. Holland, A. I. Mallet, P. J. Rennie, and W. J. Watkins. 1994. Comparison of 16-androstene steroid concentrations in sterile apocrine sweat and axillary secretions: Interconversions of 16-androstenes by the axillary microflora - a mechanism for axillary odour production in man? Journal of Steroid Biochemistry and Molecular Biology 48: 409-418.

Greenwood, B. M., K. Bojang, C. J. Whitty, and G. A. Targett. 2005. Malaria. Lancet 365: 1487-98.

Grice, E. A., H. H. Kong, G. Renaud, A. C. Young, N. C. S. Program, G. G. Bouffard, R. W. Blakesley, T. G. Wolfsberg, M. L. Turner, and J. A. Segre. 2008. A diversity profile of the human skin microbiota. Genome Research 18: 1043-1050.

Grice, E. A., H. H. Kong, S. Conlan, C. B. Deming, J. Davis, A. C. Young, NISC Comparative Sequencing Program, G. G. Bouffard, R. W. Blakesley, P. R. Murray, E. D. Green, M. L. Turner, and J. A. Segre. 2009. Topographical and temporal diversity of the human skin microbiome. Science 324: 1190-1192.

Guerenstein, P. G., and C. R. Lazzari. 2009. Host-seeking: How triatomines acquire and make use of information to find blood. Acta Tropica 110: 148-158.

Gupta, Konnikov, Macdonald, Rich, Rodger, Edmonds, McManus, and Summerbell. 1998. Prevalence and epidemiology of toenail onychomycosis in diabetic subjects: a multicentre survey. British Journal of Dermatology 139: 665-671.

Havlicek, J., and P. Lenochova. 2008. Environmental effects on human body odour, pp. 199-210. *In* J. Hurst, R. J. Beynon, S. C. Roberts and T. Wyatt [eds.], Chemical Signals in Vertebrates. Springer.

Healy, T. P., and M. J. W. Copland. 2000. Human sweat and 2-oxopentanoic acid elicid a landing response from *Anopheles gambiae*. Medical and Veterinary Entomology 14: 195-200.

Helmberg, W., G. Lanzer, R. Zahn, B. Weinmayr, T. Wagner, and E. Albert. 1998. Virtual DNA analysis - a new tool for combination and standardised evaluation of SSO, SSP and sequencing-based typing results. Tissue Antigens 51: 587-592.

Himeidan, Y. E., M. I. Elbashir, and I. Adam. 2004. Attractiveness of pregnant women to the malaria vector, Anopheles arabiensis, in Sudan. Annals of tropical medicine and parasitology 98: 631-633.

Holdeman, L. V., I. J. Good, and W. E. Moore. 1976. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Applied and Environmental Microbiology 31: 359-375.

Howard, J. C. 1977. H-2 and Mating Preferences. Nature 266: 406-408.

Huang, J., J. R. Miller, S.-C. Chen, J. M. Vulule, and E. D. Walker. 2006. *Anopheles gambiae* (Diptera: Culicidae) oviposition in response to agarose media and cultured bacterial volatiles. Journal of Medical Entomology 43: 498-504.

Hunt, R. H., M. Coetzee, and M. Fettene. 1998. The *Anopheles gambiae* complex: a new species from Ethiopia. Transactions of the Royal Society of Tropical Medicine and Hygiene 92: 231-235.

Ibrahim, M. A., P. Kainulainen, A. Aflatuni, K. Tiilikkala, and J. K. Holopainen. 2001. Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests. Agricultural and Food Science in Finland 10: 243-259.

Jackman, J. H. 1982. Body odor - The role of skin bacteria. Seminars in Dermatology 1: 143-148.

Jackman, P. J. H., and W. C. Noble. 1983. Normal axillary skin in various populations. Clinical and Experimental Dermatology 8: 259-268.

Jacoby, R. B., J. C. Brahms, S. A. Ansari, and J. Mattai. 2004. Detection and quantification of apocrine secreted odor-binding protein on intact human axillary skin. International Journal of Cosmetic Science 26: 37-46.

James, A. G., D. Hyliands, and H. Johnston. 2004a. Generation of volatile fatty acids by axillary bacteria. International Journal of Cosmetic Science 26: 149-156.

James, A. G., J. Casey, D. Hyliands, and G. Mycock. 2004b. Fatty acid metabolism by cutaneous bacteria and its role in axillary malodour. World Journal of Microbiology & Biotechnology 20: 787.

Jawara, M., R. C. Smallegange, D. Jeffries, D. C. Nwakanma, T. S. Awolola, B. G. J. Knols, W. Takken, and D. J. Conway. 2009. Optimizing odor-baited trap methods for collecting mosquitoes during the malaria season in The Gambia. PLoS ONE 4: e8167.

Kai, M., M. Haustein, F. Molina, A. Petri, B. Scholz, and B. Piechulla. 2009. Bacterial volatiles and their action potential. Applied Microbiology and Biotechnology 81: 1001-1012.

Kamhawi, S. 2006. Phlebotomine sand flies and Leishmania parasites: friends or foes? Trends in Parasitology 22: 439-445.

Kassir, J., Z. Mohsen, and N. Mehdi. 1989. Toxic effects of limonene against *Culex quinquefasciatus* Say larvae and its interference with oviposition. Anzeiger für Schädlingskunde 62: 19-21.

Kearney, J. N., D. Harnby, G. Gowland, and K. T. Holland. 1984. The follicular distribution and abundance of resident bacteria on human skin. Journal of General Microbiology 130: 797-801.

Kelly, D. W. 2001. Why are some people bitten more than others? Trends in Parasitology 17: 578-581.

Khan, A. A., H. I. Maibach, and W. G. Strauss. 1968. The role of convection currents in mosquito attraction to human skin. Mosquito News 28: 462-464.

Killeen, G., J. Kihonda, E. Lyimo, F. Oketch, M. Kotas, E. Mathenge, J. Schellenberg, C. Lengeler, T. Smith, and C. Drakeley. 2006. Quantifying behavioural interactions between humans and mosquitoes: Evaluating the protective efficacy of insecticidal nets against malaria transmission in rural Tanzania. BMC Infectious Diseases 6: 161.

Kirk, K. M., L. J. Eaves, J. M. Meyer, A. Saul, and N. G. Martin. 2000. Twin study of adolescent genetic susceptibility to mosquito bites using ordinal and comparative rating data. Genetic Epidemiology 19: 178-190.

Kline, D. L. 1998. Olfactory responses and field attraction of mosquitoes to volatiles from Limburger cheese and human foot odor. Journal of Vector Ecology 23: 186-194.

Kline, D. L. 1999. Comparison of two American mosquito traps: the professional and a new counterflow geometry trap. Journal of the American Mosquito Control Association 15: 276-282.

Kline, D. L. 2007. Semiochemicals, traps/targets and mass trapping technology for mosquito management. Journal of the American Mosquito Control Association 23: 241-251.

Kloos, W. E., and M. S. Musselwhite. 1975. Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. Applied and Environmental Microbiology 30: 381-395.

Kluytmans, J., A. van Belkum, and H. Verbrugh. 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clinical Microbiology Reviews 10: 505-520.

Knols, B. G. J. 1996a. On human odour, malaria mosquitoes, and Limburger cheese. The Lancet 348: 1322-1322.

Knols, B. G. J. 1996b. Odour-mediated host-seeking behaviour of the Afro-tropical malaria vector *Anopheles gambiae* Giles, pp. 213, Laboratory for Entomology. Wageningen University.

Knols, B. G. J., and R. De Jong. 1996. Limburger cheese as an attractant for the mosquito *Anopheles gambiae s. s.* Parasitology Today 12: 159-161.

Knols, B. G. J., and J. Meijerink. 1997. Odors influence mosquito behavior. Science and Medicine 4: 56-63.

Knols, B. G. J., R. De Jong, and W. Takken. 1994. Trapping system for testing olfactory responses of the malaria mosquito *Anopheles gambiae* in a wind-tunnel. Medical and Veterinary Entomology 8: 386-388.

Knols, B. G. J., R. De Jong, and W. Takken. 1995. Differential attractiveness of isolated humans to mosquitoes in Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene 89: 604-606.

Knols, B. G. J., J. J. A. van Loon, A. Cork, R. D. Robinson, W. Adam, J. Meijerink, R. de Jong, and W. Takken. 1997. Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. Bulletin of Entomological Research 87: 151-159.

Koella, J. C., F. L. Sorensen, and R. A. Anderson. 1998. The malaria parasite, Plasmodium falciparum, increases the frequency of multiple feeding of its mosquito vector, Anopheles gambiae. Proceedings of the Royal Society of London Series B 265: 763-768.

Kuhn, F., and A. Natsch. 2009. Body odour of monozygotic human twins: a common pattern of odorant carboxylic acids released by a bacterial aminoacylase from axilla secretions contributing to an inherited body odour type. Journal of the Royal Society, Interface / the Royal Society 6: 377-392.

Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, et al. 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature 390: 249-256.

Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K.-i. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, et al. 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. The Lancet 357: 1225-1240.

Lacroix, R., W. R. Mukabana, L. C. Gouagna, and J. C. Koella. 2005. Malaria infection increases attractiveness of humans to mosquitoes. PLoS Biology 3: 1590-1593.

Lefèvre, T., and F. Thomas. 2008. Behind the scene, something else is pulling the strings: Emphasizing parasitic manipulation in vector-borne diseases. Infection, Genetics and Evolution 8: 504-519.

Lefèvre, T., L.-C. Gouagna, K. R. Dabire, E. Elguero, D. Fontenille, C. Costantini, and F. Thomas. 2009. Evolutionary lability of odour-mediated host preference by the malaria vector *Anopheles gambiae*. Tropical Medicine & International Health 14: 228-236.

Lefèvre, T., L.-C. Gouagna, K. R. Dabiré, E. Elguero, D. Fontenille, F. Renaud, C. Costantini, and F. Thomas. 2010. Beer consumption increases human attractiveness to malaria mosquitoes. PLoS ONE 5: e9546.

Lenochova, P., and J. Havlicek. 2008. Human body odour individuality, pp. 189-198. *In* J. Hurst, R. J. Beynon, S. C. Roberts and T. Wyatt [eds.], Chemical Signals in Vertebrates. Springer.

Leyden, J. J., K. J. McGinley, E. Holzle, J. N. Labows, and A. M. Kligman. 1981. The microbiology of the human axilla and its relationship to axillary odor. Journal of Investigative Dermatology 77: 413-416.

Liaw, A., and M. Wiener. 2002. Classification and regression by randomforest. R news 2: 18-22.

Lindh, J. M., A. Kännaste, B. G. J. Knols, I. Faye, and A. K. Borg-Karlson. 2008. Oviposition responses of *Anopheles gambiae s.s.* (Diptera: Culicidae) and identification of volatiles from bacteria-containing solutions. Journal of Medical Entomology 45: 1039-1049.

Lindsay, S., J. Ansell, C. Selman, V. Cox, K. Hamilton, and G. Walraven. 2000. Effect of pregnancy on exposure to malaria mosquitoes. The Lancet 355: 1972.

Lindsay, S. W., J. H. Adiamah, J. E. Miller, R. J. Pleass, and J. R. M. Armstrong. 1993. Variation in attractiveness of human subjects to malaria mosquitoes (Diptera: Culicidae) in The Gambiae. Journal of Medical Entomology 30: 308-

373.

Liu, Z., C. Lozupone, M. Hamady, F. D. Bushman, and R. Knight. 2007. Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic Acids Research 35: e120.

Logan, J. G. 2008. Why do mosquitoes "choose" to bite some people more than others? Outlooks on Pest Management 19: 280-283.

Logan, J. G., and M. A. Birkett. 2007. Semiochemicals for biting fly control: Their identification and exploitation. Pest Management Science 63: 647-657.

Logan, J. G., M. A. Birkett, S. J. Clark, S. Powers, N. J. Seal, L. J. Wadhams, A. J. Mordue, and J. A. Pickett. 2008. Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti m*osquitoes. Journal of Chemical Ecology 34: 308-322.

Lourenço-de-Oliveira, R., J. B. P. Lima, R. Peres, F. da Costa Alves, Á. E. Eiras, and C. T. Codeço. 2009. Comparison of different uses of adult traps and ovitraps for assessing dengue vector infestation in endemic areas. Journal of the American Mosquito Control Association 24: 387-392.

Manichanh, C., L. Rigottier-Gois, E. Bonnaud, K. Gloux, E. Pelletier, L. Frangeul, R. Nalin, C. Jarrin, P. Chardon, P. Marteau, J. Roca, and J. Dore. 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 55: 205-211.

Marshall, J., J. P. Leeming, and K. T. Holland. 1987. The cutaneous microbiology of normal feet. Journal of Applied Bacteriology 62: 139-146.

Marshall, J., K. T. Holland, and E. M. Gribbon. 1988. A comparative-study of the cutaneous microflora of normal feet with low and high-Levels of odor. Journal of Applied Bacteriology 65: 61-68.

Mathenge, E. M., G. O. Omweri, L. W. Irungu, P. N. Ndegwa, E. Walczak, T. A. Smith, G. F. Killeen, and B. G. J. Knols. 2004. Comparative field evaluation of the Mbita trap, the Centers for Disease Control light trap, and the human landing catch for sampling of malaria vectors in Western Kenya. American Journal of Tropical Medicine and Hygiene 70: 33-37.

Maxwell, C. A., J. Wakibara, S. Tho, and C. F. Curtis. 1998. Malaria-infective biting at different hours of the night. Medical and Veterinary Entomology 12: 325-327.

Mayer, M. S., and J. D. James. 1969. Attraction of *Aedes aegypti* (L.): responses to human arms, carbon dioxide, and air currents in a new type of olfactometer. Bulletin of Entomological Research 58: 629-643.

Mboera, L. E. G., and W. Takken. 1997. Carbon dioxide chemotropism in mosquitoes (Diptera: Culicidae) and its potential in vector surveillance and management programmes. Medical and Veterinary Entomology 85: 355-368.

Mboera, L. E. G., W. Takken, and E. Z. Sambu. 2000a. The response of *Culex*

quinquefasciatus (Diptera: Culicidae) to traps baited with carbon dioxide, 1-octen-3-ol, acetone, butyric acid and human foot odour in Tanzania. Bulletin of Entomological Research 90: 155-159.

Mboera, L. E. G., B. G. J. Knols, W. Takken, and A. dellaTorre. 1997. The response of *Anopheles gambiae* sl and *A. funestus* (Diptera: Culicidae) to tents baited with human odour or carbon dioxide in Tanzania. Bulletin of Entomological Research 87: 173-178.

Mboera, L. E. G., B. G. J. Knols, M. A. H. Braks, and W. Takken. 2000b. Comparison of carbon dioxide-baited trapping systems for sampling outdoor mosquito populations in Tanzania. Medical and Veterinary Entomology 14: 257-263.

McClelland, E. E., D. J. Penn, and W. K. Potts. 2003. Major histocompatibility complex heterozygote superiority during coinfection. Infection and Immunity 71: 2079-2086.

McGinley, K. J., G. F. Webster, and J. J. Leyden. 1978. Regional variations of cutaneous propionibacteria. Applied and Environmental Microbiology 35: 62-66.

Meijerink, J., M. A. H. Braks, A. A. Brack, W. Adam, T. Dekker, M. A. Posthumus, T. A. v. Beek, and J. J. A. v. Loon. 2000. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. Journal of Chemical Ecology 26: 1367-1382.

Minakawa, N., C. M. Mutero, J. I. Githure, J. C. Beier, and G. Yan. 1999. Spatial distribution and habitat characterization of anopheline mosquito larvae in Western Kenya. American Journal of Tropical Medicine and Hygiene 61: 1010-6.

Morrison, A. J., Jr., and R. P. Wenzel. 1984. Epidemiology of Infections due to *Pseudomonas aeruginosa*. Reviews of Infectious Diseases 6: S627-S642.

Muirhead-Thomson, R. C. 1951. The distribution of anopheline mosquito bites among different age groups. A new factor in malaria epidemiology. British Medical Journal 1: 1114-1117.

Mukabana, W. R. 2002. Differential attractiveness of humans to the African malaria vector Anopheles gambiae Giles: Effects of host characteristics and parasite infection, pp. 142, Laboratory of Entomology. Wageningen University, Wageningen.

Mukabana, W. R., W. Takken, R. Coe, and B. G. Knols. 2002. Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*. Malaria Journal 1: 17.

Mukabana, W. R., W. Takken, G. F. Killeen, and B. G. J. Knols. 2004. Allomonal effect of breath contributes to differential attractiveness of humans to the African malaria vector *Anopheles gambiae*. Malaria Journal 3: 8.

Mukabana, W. R., W. Takken, G. F. Killeen, and B. G. J. Knols. 2007. Clinical malaria reduces human attractiveness to mosquitoes. Proceedings Experimental and Applied Entomology, N.E.V., 18: 125-129.

N'Guessan, R., V. Corbel, M. Akogbéto, and M. Rowland. 2007. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. Emerging Infectious Diseases 13: 199-206.

Natsch, A., J. Schmid, and F. Flachsmann. 2004. Identification of Odoriferous Sulfanylalkanols in Human Axilla Secretions and Their Formation through Cleavage of Cysteine Precursors by a C&bond;S Lyase Isolated from Axilla bacteria. Chemistry & Biodiversity 1: 1058-1072.

Natsch, A., H. Gfeller, P. Gygax, J. Schmid, and G. Acuna. 2003. A specific bacterial aminoacylase cleaves odorant precursors secreted in the human axilla. Journal of Biological Chemistry 278: 5718-5727.

Njiru, B., W. R. Mukabana, W. Takken, and B. G. J. Knols. 2006. Trapping of the malaria vector *Anopheles gambiae* with odour-baited MM-X traps in semi-field conditions in western Kenya. Malaria Journal 5: 39.

Noble, W. C. 2004. The skin microflora and microbial skin disease. Cambridge University Press, Cambridge.

O'Shea, B., E. Rebollar-Tellez, R. D. Ward, J. G. C. Hamilton, D. El Naiem, and A. Polwart. 2002. Enhanced sandfly attraction to *Leishmania*-infected hosts. Transactions of the Royal Society of Tropical Medicine and Hygiene 96: 117-118.

Okumu, F., L. Biswaro, E. Mbeleyela, G. F. Killeen, R. Mukabana, and S. J. Moore. 2010a. Using nylon strips to dispense mosquito attractants for sampling the malaria vector *Anopheles gambiae s.s*. Journal of Medical Entomology 47: 274-282.

Okumu, F. O., N. J. Govella, S. J. Moore, N. Chitnis, and G. F. Killeen. 2010b. Potential benefits, limitations and target product-profiles of odor-baited mosquito traps for malaria control in Africa. PLoS ONE 5: e11573.

Okumu, F. O., G. F. Killeen, S. Ogoma, L. Biswaro, R. C. Smallegange, E. Mbeyela, E. Titus, C. Munk, H. Ngonyani, W. Takken, H. Mshinda, W. R. Mukabana, and S. J. Moore. 2010c. Development and field evaluation of a synthetic mosquito lure that is more attractive than humans. PLoS ONE 5: e8951.

Olanga, E., M. Okal, P. Mbadi, E. Kokwaro, and W. Mukabana. 2010. Attraction of *Anopheles gambiae* to odour baits augmented with heat and moisture. Malaria Journal 9: 6.

Omolo, M. O., D. Okinyo, I. O. Ndiege, W. Lwande, and A. Hassanali. 2004. Repellency of essential oils of some Kenyan plants against Anopheles gambiae. Phytochemistry 65: 2797-2802.

Ortiz, M. I., and J. Molina. 2010. Preliminary evidence of *Rhodnius prolixus*

(Hemiptera: Triatominae) attraction to human skin odour extracts. Acta Tropica 113: 174-179.

Ouwehand, A., S. Salminen, and E. Isolauri. 2002. Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 82: 279-289.

Oyedele, A. O., A. A. Gbolade, M. B. Sosan, F. B. Adewoyin, O. L. Soyelu, and O. O. Orafidiya. 2002. Formulation of an effective mosquito-repellent topical product from Lemongrass oil. Phytomedicine 9: 259-262.

Park, K. C., and T. C. Baker. 2002. Improvement of signal-to-noise ratio in electroantennogram responses using multiple insect antennae. Journal of Insect Physiology 48: 1139-1145.

Pates, H. 2002. Zoophilic and anthropophilic behaviour in the *Anopheles gambiae* complex, University of London, London.

Pates, H. V., W. Takken, and C. F. Curtis. 2005. Laboratory studies on the olfactory behaviour of *Anopheles quadriannulatus*. Entomologia experimentalis et applicata 114: 153-159.

Pates, H. V., W. Takken, K. Stuke, and C. F. Curtis. 2001. Differential behaviour of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human and cow odours in the laboratory. Bulletin of Entomological Research 91: 289-296.

Pearse-Pratt, R., H. Schellinck, R. Brown, and B. Roser. 1992. Evolutionary and immunological implications of the role of the MHC in olfactory signaling, pp. 167–173. *In* R. L. Doty and D. Muller-Schwarze [eds.], Chemical Signals in Vertebrates. Plenum Press, New York.

Penn, D., and W. K. Potts. 1998a. How do major histocompatibility complex genes influence odor and mating preferences? Advances In Immunology 69: 411-36.

Penn, D., and W. K. Potts. 1998b. Untrained mice discriminate MHC-determined odors. Physiology & Behavior 64: 235-243.

Penn, D., and W. K. Potts. 1998c. Chemical signals and parasite-mediated sexual selection. Trends in Ecology & Evolution 13: 391-396.

Penn, D. J., K. Damjanovich, and W. K. Potts. 2002. MHC heterozygosity confers a selective advantage against multiple-strain infections. Proceedings of the National Academy of Sciences of the United States of America 99: 11260-11264.

Penn, D. J., E. Oberzaucher, K. Grammer, G. Fischer, H. A. Soini, D. Wiesler, M. V. Novotny, S. J. Dixon, Y. Xu, and R. G. Brereton. 2007. Individual and gender fingerprints in human body odour. Journal of the Royal Society, Interface / the Royal Society 4: 331-340.

Ponnusamy, L., D. Wesson, C. Arellano, C. Schal, and C. Apperson. 2010. Species composition of bacterial communities influences attraction of mosquitoes to experimental plant infusions. Microbial Ecology 59: 158-173.

Ponnusamy, L., N. Xu, S. Nojima, D. M. Wesson, C. Schal, and C. S. Apperson.

2008. Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by Aedes aegypti. Proceedings of the National Academy of Sciences of the United States of America 105: 9262-9267.

Port, G. R., P. F. L. Boreham, and J. H. Bryan. 1980. The relationship of host size to feeding by mosquitoes of the *Anopheles gambiae* Giles complex (Diptera: Culicidae). Bulletin of Entomological Research 70: 133-144.

Porter, R. H., J. M. Cernoch, and R. D. Balogh. 1985. Odor signatures and kin recognition. Physiology & Behavior 34: 445-448.

Posey, K. H., D. R. Barbard, and C. E. Schreck. 1998. Triple cage olfactometer for evaluating mosquito (Diptera: Culicidae) attraction responses. Journal of Medical Entomology 35: 330-334.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS ONE 5: e9490.

Prugnolle, F., T. Lefèvre, F. Renaud, A. P. Møller, D. Missé, and F. Thomas. 2009. Infection and body odours: Evolutionary and medical perspectives. Infection, Genetics and Evolution 9: 1006-1009.

Qiu, Y. T. 2005. Sensory and behavioural responses of the malaria mosquito *Anopheles gambiae* to human odours, pp. 207, Laboratory of Entomology. Wageningen University, Wageningen.

Qiu, Y. T., J. Spitzen, R. C. Smallegange, and B. Knols. 2007a. Monitoring systems for adult insect pests and disease vectors, pp. 329-353. *In* W. Takken and B. Knols [eds.], Emerging pests and vector-borne diseases in Europe. Wageningen Academic Publishers, Wageningen.

Qiu, Y. T., R. C. Smallegange, J. J. A. van Loon, and W. Takken. in press. Behavioural responses of *Anopheles gambiae sensu stricto* to components of human breath, sweat and urine. Medical and Veterinary Entomology.

Qiu, Y. T., R. C. Smallegange, J. J. A. van Loon, C. J. F. Ter Braak, and W. Takken. 2006. Interindividual variation in the attractiveness of human odours to the malaria mosquito *Anopheles gambiae s.s.* Medical and Veterinary Entomology 20: 280-287.

Qiu, Y. T., R. C. Smallegange, S. Hoppe, J. J. A. van Loon, E. J. Bakker, and W. Takken. 2004a. Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae Giles sensu stricto* (Diptera: Culicidae) to human skin emanations. Medical and Veterinary Entomology 18: 429-38.

Qiu, Y. T., R. C. Smallegange, H. Smid, J. J. A. van Loon, A. M. S. Galimard, M. A. Posthumus, T. A. v. Beek, and W. Takken. 2004b. GC-EAG analysis of human odours that attract the malaria mosquito *Anopheles gambiae sensu stricto*, pp. 59-64, Proceedings Experimental and Applied Entomology, N.E.V.

Qiu, Y. T., R. C. Smallegange, H. M. Smid, J. J. A. van Loon, W. Takken, A. M.

Galimard, T. A. van Beek, and M. A. Posthumus. 2004c. GC-EAD studies on the malaria mosuito *Anopheles gambiae*. Proceedings of the Netherlands Entomological Society meeting 15: 59-64.

Qiu, Y. T., R. C. Smallegange, C. J. F. Ter Braak, J. Spitzen, J. J. A. van Loon, M. Jawara, P. Milligan, A. M. Galimard, T. A. Van Beek, B. G. J. Knols, and W. Takken. 2007b. Attractiveness of MM-X traps baited with human or synthetic odor to mosquitoes (Diptera: Culicidae) in the Gambia. Journal of Medical Entomology 44: 970-983.

Quince, C., A. Lanzen, T. P. Curtis, R. J. Davenport, N. Hall, I. M. Head, L. F. Read, and W. T. Sloan. 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods 6: 639-641.

Rastall, R. A., G. R. Gibson, H. S. Gill, F. Guarner, T. R. Klaenhammer, B. Pot, G. Reid, I. R. Rowland, and M. E. Sanders. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. FEMS Microbiology Ecology 52: 145-152.

Reeves, W. C. 1951. Field studies of carbon dioxide as a possible host simulant to mosquitoes. Proceedings of the Society for Experimental Biology and Medicine 77: 64-66.

Reisen, W. K., Y. Fang, and V. M. Martinez. 2005. Avian host and mosquito (Diptera : Culicidae) vector competence determine the efficiency of west nile and St. Louis encephalitis virus transmission. Journal of medical entomology 42: 367-375.

Rennie, P. J., D. B. Gower, and K. T. Holland. 1991. *In-vitro* and *in-vivo* studies of human axillary odor and the cutaneous microflora. British Journal of Dermatology 124: 596-602.

Rennie, P. J., D. B. Gower, K. T. Holland, A. I. Mallet, and W. J. Watkins. 1990. The skin microflora and the formation of human axillary odor. International Journal of Cosmetic Science 12: 197-207.

Resh, V. H., C. Lévêque, and B. Statzner. 2004. Long-term, large-scale biomonitoring of the unknown: Assessing the Effects of Insecticides to Control River Blindness (Onchocerciasis) in West Africa. Annual Review of Entomology 49: 115-139.

Ritchie, S. A., L. P. Rapley, C. Williams, P. H. Johnson, M. Larkman, R. M. Silcock, S. A. Long, and R. C. Russell. 2009. A lethal ovitrap-based mass trapping scheme for dengue control in Australia: I. Public acceptability and performance of lethal ovitraps. Medical and Veterinary Entomology 23: 295-302.

Roberts, S. C., L. M. Gosling, T. D. Spector, P. Miller, D. J. Penn, and M. Petrie. 2005. Body odor similarity in noncohabiting twins. Chemical Senses 30: 651-

656.

Rockett, C. L. 1987. Bacteria as ovipositional attractants for Culex pipiens (Diptera: Culicidae). The Great Lakes Entomologist 20: 151-155.

Romero-Garcia, S., C. Hernandez-Bustos, E. Merino, G. Gosset, and A. Martinez. 2009. Homolactic fermentation from glucose and cellobiose using *Bacillus subtilis*. Microbial Cell Factories 8: 23.

Sarkany, I., D. Taplin, and H. Blank. 1962. Incidence and bacteriology of erythrasma. Archives of Dermatology 85: 578-582.

Sastry, S. D., K. T. Buck, J. Janak, M. Dressler, and G. Preti. 1980. Volatiles emitted by humans, pp. 1085-1127. *In* G. R. W. a. O. C. Dermer [ed.], Biochemical Applications of Mass Spectrometry. John Wiley & Sons, New York.

Savelev, S. U., S. Antony-Babu, S. C. Roberts, H. Wang, A. S. Clare, L. M. Gosling, M. Petrie, M. Goodfellow, A. G. O'Donnell, and A. C. Ward. 2008. Individual variation in 3-methylbutanal: a putative link between human leukocyte antigen and skin microflora. Journal of Chemical Ecology 34: 1253-1257.

Schmied, W. H., W. Takken, G. F. Killeen, B. G. J. Knols, and R. C. Smallegange. 2008. Evaluation of two counterflow traps for testing behaviour-mediating compounds for the malaria vector *Anopheles gambiae s.s.* under semi-field conditions in Tanzania. Malaria Journal 7: 230.

Schreck, C. E., and J. James. 1968. Broth cultures of bacteria that attract female mosquitoes. Mosquito News 28: 33-&.

Schreck, C. E., D. L. Kline, and D. A. Carlson. 1990. Mosquito attraction to substances from the skin of different humans. Journal of the American Mosquito Control Association 6: 406-410.

Schreiber, K., N. Boes, M. Eschbach, L. Jaensch, J. Wehland, T. Bjarnsholt, M. Givskov, M. Hentzer, and M. Schobert. 2006. Anaerobic survival of *Pseudomonas aeruginosa* by pyruvate fermentation requires an usp-type stress protein. Journal of Bacteriology 188: 659-668.

Schulz, S., and J. S. Dickschat. 2007. Bacterial volatiles: The smell of small organisms. Natural Product Reports 24: 814-842.

Scott, J. A., W. G. Brogdon, and F. H. Collins. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. American Journal of Tropical Medicine and Hygiene 4: 520-529.

Seyoum, A., G. F. Killeen, E. W. Kabiru, B. G. J. Knols, and A. Hassanali. 2003. Field efficacy of thermally expelled or live potted repellent plants against African malaria vectors in western Kenya. Tropical Medicine & International Health 8: 1005-1011.

Shelley, W. W. B., H. H. J. Hurley, Jr., and A. A. C. Nichols. 1953. Axillary odor; experimental study of the role of bacteria, apocrine sweat, and deodorants. A. M. A. Archives of Dermatology and Syphilology 68: 430-46.

Shirai, O., T. Tsuda, S. Kitagawa, K. Naitoh, T. Seki, K. Kamimura, and M. Morohashi. 2002. Alcohol ingestion stimulates mosquito attraction. Journal of the American Mosquito Control Association 18: 91-6.

Sibson, R. 1978. Studies in the robustness of multidimensional scaling: procrustes statistics. Journal of the Royal Statistical Society. Series B (Methodological) 40: 234-238.

Smallegange, R. C., and W. Takken. 2010. Host-seeking behaviour of mosquitoes - responses to olfactory stimuli in the laboratory, pp. 143-180. *In* W. Takken and B. G. J. Knols [eds.], Olfaction in vector-host interactions. Wageningen Academic Publishers, Wageningen.

Smallegange, R. C., B. G. J. Knols, and W. Takken. 2010. Effectiveness of synthetic versus natural human volatiles as attractants for *Anopheles gambiae* (Diptera: Culicidae) s*ensu stricto*. Journal of Medical Entomology 47: 338-344.

Smallegange, R. C., Y. T. Qiu, J. J. A. van Loon, and W. Takken. 2005. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Chemical Senses 30: 145-152.

Smallegange, R. C., Y. T. Qiu, G. Bukovinszkiné-Kiss, J. J. A. van Loon, and W. Takken. 2009. The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae* sensu stricto. Journal of Chemical Ecology 35: 933-943.

Smallegange, R.C., W.H. Schmied, K.J. van Roey, N.O. Verhulst, J. Spitzen, W.R. Mukabana, and W. Takken. 2010. Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito *Anopheles gambiae s.s*. Malaria Journal. 9:292

Smart, M. R., and A. W. A. Brown. 1957. Studies on the responses of the female *Aedes* mosquito. Part VII. -The effect of skin temperature, hue and moisture on the attractiveness of the human hand. Bulletin of Entomological Research 47: 89-101.

Smid, H. M., J. J. A. van Loon, M. A. Posthumus, and L. E. M. Vet. 2002. GC-EAG-analysis of volatiles from Brussels sprouts plants damaged by two species of Pieris caterpillars: olfactory receptive range of a specialist and a generalist parasitoid wasp species. Chemoecology 12: 169-176.

Smith, T., N. Maire, K. Dietz, G. F. Killeen, P. Vounatsou, L. Molineaux, and M. Tanner. 2006. Relationship between the entomological inoculation rate and the force of infection for *Plasmodium falciparum* malaria. American Journal of Tropical Medicine and Hygiene 75: 11-18.

Sommerville, B. A. 1994. Analysis of human sweat volatiles: An example of pattern recognition in the analysis and interpretation of gas chromatograms. Pesticide science 41: 365-368.

Spencer, M. 1967. Anopheline attack on mother and infant pairs, Fergusson Island. Papua New Guinea Medical Journal 10: 75.

Spielman, A. l., X. N. Zeng, J. J. Leyden, and G. Preti. 1995. Proteinaceous precursors of human axillary odor: isolation of two novel odor-binding proteins. Experientia 51: 40-7.

Spitzen, J., R. C. Smallegange, and W. Takken. 2008. Effect of human odours and positioning of CO_2 release point on trap catches of the malaria mosquito *Anopheles gambiae sensu stricto* in an olfactometer. Physiological Entomology 33: 116-122.

Steib, B. M., M. Geier, and J. Boeckh. 2001. The effect of lactic acid on odour-related host preference of yellow fever mosquitoes. Chemical Senses 26: 523-528.

Stoddart, D. M. 1990. The Scented Ape: The biology and culture of human odour. Cambridge University Press, Cambridge, UK.

Stotzky, G., S. Schenck, and G. C. Papavizas. 1976. Volatile organic compounds and microorganisms. Critical Reviews in Microbiology 4: 333-382.

Strachan, D. P. 1989. Hay fever, hygiene, and household size. BMJ: British Medical Journal 299: 1259-1260.

Svejgaard, A., and L. P. Ryder. 1994. HLA and disease associations: Detecting the strongest association. Tissue Antigens 43: 18-27.

Syed, Z., and W. S. Leal. 2008. Mosquitoes smell and avoid the insect repellent DEET. Proceedings of the National Academy of Sciences 105: 13598-13603.

Takken, W. 1991. The role of olfaction in host-seeking of mosquitoes: a review. Insect Science and its Application 12: 287-295.

Takken, W., and B. G. J. Knols. 1999. Odor-mediated behavior of afrotropical malaria mosquitoes. Annual Review of Entomology 44: 131-157.

Takken, W., and B. G. J. Knols. 2009. Malaria vector control: current and future strategies. Trends in Parasitology 25: 101-104.

Takken, W., T. Dekker, and Y. Wijnholds. 1997a. Odor-mediated flight behavior of *Anopheles gambiae* Giles sensu stricto and *An. stephensi* Liston in response to CO_2, acetone, and 1-octen-3-ol (Diptera: Culicidae). Journal of Insect Behavior 10: 395-407.

Takken, W., B. G. J. Knols, and H. Otten. 1997b. Interactions between physical and olfactory cues in the host-seeking behaviour of mosquitoes: the role of relative humidity. Annals of Tropical Medicine and Parasitology 91: S119-S120.

Tanaka, K., M. A. Budd, M. L. Efron, and K. J. Isselbacher. 1966. Isovaleric acidemia: a new genetic defect of leucine metabolism. Proceedings of the National Academy of Sciences of the United States of America 56: 236-242.

Tauch, A., O. Kaiser, T. Hain, A. Goesmann, B. Weisshaar, A. Albersmeier, T. Bekel, N. Bischoff, I. Brune, T. Chakraborty, J. Kalinowski, F. Meyer, O. Rupp, S. Schneiker, P. Viehoever, and A. Puhler. 2005. Complete Genome

Sequence and Analysis of the Multiresistant Nosocomial Pathogen Corynebacterium jeikeium K411, a Lipid-Requiring Bacterium of the Human Skin Flora. Journal of Bacteriology 187: 4671-4682.

Taylor, D., A. Daulby, S. Grimshaw, G. James, J. Mercer, and S. Vaziri. 2003. Characterization of the microflora of the human axilla. International Journal of Cosmetic Science 25: 137-145.

Téllez, E. A. R. 2005. Human body odor, mosquito bites and the risk of disease transmission. Folia entomologica Mexicana 44: 247-265.

Tomita, B., H. Inoue, K. Chaya, A. Nakamura, N. Hamamura, K. Ueno, K. Watanabe, and Y. Ose. 1987. Identification of dimethyl disulfide-forming bacteria isolated from activated sludge. Applied and Environmental Microbiology 53: 1541-1547.

Torr, S. J., D. R. Hall, R. J. Phelps, and G. A. Vale. 1997. Methods for dispensing odour attractants for tsetse flies (Diptera: Glossinidae). Bulletin of Entomological Research 87: 299-311.

Torr, S. J., A. della Torre, M. Calzetta, C. Costantini, and G. A. Vale. 2008. Towards a fuller understanding of mosquito behaviour: use of electrocuting grids to compare the odour-orientated responses of *Anopheles arabiensis* and *An. quadriannulatus* in the field. Medical and Veterinary Entomology 22: 93-108.

Trexler, J. D., C. S. Apperson, L. Zurek, C. Gemeno, C. Schal, M. Kaufman, E. Walker, D. W. Watson, and L. Wallace. 2009. Role of bacteria in mediating the oviposition responses of *Aedes albopictus* (Diptera: *Culicidae*). Journal of Medical Entomology 40: 841-848.

Turnbaugh, P. J., R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon. 2007. The Human Microbiome Project. Nature 449: 804-810.

Turner, S. L., and A. Ray. 2009. Modification of CO_2 avoidance behaviour in Drosophila by inhibitory odorants. Nature 461: 277-281.

Vale, G. A., and D. R. Hall. 1985. The use of 1-octen-3-ol, acetone and carbon dioxide to improve baits for tsetse flies, Glossina spp. (Diptera: Glossinidae). Bulletin of Entomological Research 75: 219-231.

Verduyn, W., I. I. N. Doxiadis, J. Anholts, J. J. M. Drabbels, A. Naipal, J. D'Amaro, G. G. Persijn, M. J. Giphart, and G. M. T. Schreuder. 1993. Biotinylated DRB sequence-specific oligonucleotides comparison to serologic HLA-DR typing of organ donors in eurotransplant. Human Immunology 37: 59-67.

Verhulst, N. O., W. Takken, and R. C. Smallegange 2008. Structural design affects entry response of mosquitoes in olfactometers. Proceedings of the Netherlands Entomological Society meeting 19: 129-136.

Verhulst, N. O., W. Takken, M. Dicke, G. Schraa, and R. C. Smallegange. 2010. Chemical ecology of interactions between human skin microbiota and mosquitoes. FEMS Microbiology Ecology 74: 1-9

Verhulst, N. O., H. Beijleveld, B. G. J. Knols, W. Takken, G. Schraa, H. J. Bouwmeester, and R. C. Smallegange. 2009. Cultured skin microbiota attracts malaria mosquitoes. Malaria Journal 8: 302.

Vrieze, A., F. Holleman, E. Zoetendal, W. de Vos, J. Hoekstra, and M. Nieuwdorp. 2010. The environment within: how gut microbiota may influence metabolism and body composition. Diabetologia 53: 606-613.

Wallace, P. 1977. Individual discrimination of humans by odor. Physiology & Behavior 19: 577-579.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73: 5261-5267.

Wauters, G., B. Van Bosterhaut, M. Janssens, and J. Verhaegen. 1998. Identification of *Corynebacterium amycolatum* and Other Nonlipophilic Fermentative Corynebacteria of Human Origin. Journal of Clinical Microbiology 36: 1430-1432.

Wedekind, C., and S. Furi. 1997. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? Proceedings of the Royal Society Biological Sciences Series B 264: 1471-1479.

Wedekind, C., and D. Penn. 2000. MHC genes, body odours, and odour preferences. Nephrology Dialysis Transplantation 15: 1269-1271.

Weinke, T., R. Schiller, F. J. Fehrenbach, and H. D. Pohle. 1992. Association betweenStaphylococcus aureus nasopharyngeal colonization and septicemia in patients infected with the human immunodeficiency virus. European Journal of Clinical Microbiology & Infectious Diseases V11: 985-989.

Weisfeld, G. E., T. Czilli, K. A. Phillips, J. A. Gall, and C. M. Lichtman. 2003. Possible olfaction-based mechanisms in human kin recognition and inbreeding avoidance. Journal of Experimental Child Psychology 85: 279-295.

Welsch, M., and J. Thibaut. 1948. A study of corynebacteria from human sources, and especially of their sensitivity to antibiotics. Antonie van Leeuwenhoek 14: 193-213.

White, G. B. 1974. *Anopheles gambiae* complex and disease transmission in Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene 68: 278-299.

White, G. B. 1982. Malaria vector ecology and genetics. British Medical Bulletin 38: 207-212.

White, G. B., F. Tessfaye, P. F. L. Boreham, and G. Lemma. 1980. Malaria vector capacity of *Anopheles arabiensis* and *An. quadriannulatus* in Ethiopia: chromosomal interpretation after 6 years storage of field preparations. Transactions of the Royal Society of Tropical Medicine and Hygiene 74: 683-684.

WHO. 2009. World malaria report, pp. 66. World Health Organisation, Geneva.

Wilson, M. 2008. Bacteriology of humans: an ecological perspective. Blackwell Publishing Ltd, Malden, MA.

Xu, Y., S. Dixon, R. Brereton, H. Soini, M. Novotny, K. Trebesius, I. Bergmaier, E. Oberzaucher, K. Grammer, and D. Penn. 2007. Comparison of human axillary odour profiles obtained by gas chromatography/mass spectrometry and skin microbial profiles obtained by denaturing gradient gel electrophoresis using multivariate pattern recognition. Metabolomics 3: 427-437.

Yamazaki, K., E. A. Boyse, M. H. T. Thaler, B. J. Mathieson, J. Abbott, J. Boyse, Z. A. Zayas, and L. Thomas. 1976. Control of mating preferences in mice by genes in the major histocompatibility complex. Journal of Experimental Medicine 144: 1324-1335.

Yazdanbakhsh, M., P. G. Kremsner, and R. van Ree. 2002. Allergy, parasites, and the hygiene hypothesis. Science 296: 490-494.

Zeng, C., A. I. Spielman, B. R. Vowels, J. J. Leyden, K. Biemann, and G. Preti. 1996. A human axillary odorant is carried by apolipoprotein D. Proceedings of the National Academy of Sciences of the United States of America 93: 6626-6630.

Zoetendal, E. G., E. E. Vaughan, and W. M. de Vos. 2006. A microbial world within us. Molecular Microbiology 59: 1639-1650.

Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. van 't Riet. 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56: 1875-1881.

SUMMARY

Malaria is one of the most serious infectious diseases in the world. One third of the human population lives in a malaria endemic area and each year around 900.000 people die from the disease. *Plasmodium* parasites that cause malaria are transmitted by anopheline mosquitoes. *Anopheles gambiae* Giles *sensu stricto* (henceforth termed *An. gambiae*), is one of the most effective vectors of human malaria because it is highly anthropophilic, endophilic and endophagic and has a wide distribution in tropical Africa.

For *An. gambiae*, human body odours are the most important cues to find its host. Identification of the odours that mediate this behaviour can be used for the development of odour-baited mosquito traps or spatial repellents. Analyses of human skin emanations, however, often result in hundreds of compounds. Since it is assumed that the host-seeking behaviour of mosquitoes is mediated by a selection of these compounds, this high number of components present in human odour makes identification of the selective compounds that play a role in the host-seeking process of *An. gambiae* difficult. Ammonia is one of the few compounds previously identified to be attractive to *An. gambiae,* although alone less attractive than natural human odour. When concentrations of ammonia are too high, it acts as a repellent to *An. gambiae*. Volatile compounds can also have a synergistic effect on mosquito behaviour. The combination of ammonia, lactic acid and a mixture of carboxylic acids is significantly more attractive than any of the three components alone.

Bacteria play an important role in the production of human body odours **(Chapter 2)**. Sweat gets its characteristic smell only after incubation and there is a strong correlation between human body odour and the species composition of bacteria present on the skin. Recent studies on human skin microbiota, using 16S rRNA gene sequencing, found that humans have an individual microbial skin composition that is relatively stable over time **(Chapter 2)**. Several studies have indicated a possible role of skin bacteria in the production of volatiles attractive to mosquitoes. Washing the feet with a bactericidal soap significantly altered the selection of biting sites of *An. gambiae* and human eccrine sweat was found to be more attractive to *An. gambiae* after incubation with skin bacteria for one or two days.

Detailed knowledge on the ecology and genetics of human skin microbiota may unravel the evolutionary mechanisms that underlie the odour-mediated interactions between mosquitoes and their hosts **(Chapter 2)**. The

principle goal of the study presented in this thesis was: *to understand the role of human skin microbiota in mosquito-host interactions and to identify which compounds produced by the micro-organisms are involved in the attraction of* An. gambiae *to humans.*

Several of the behavioural studies described in this thesis were carried out in dual-port olfactometers, which were used to test the behavioural response of mosquitoes to volatiles. In **Chapter 3** it was tested whether the design of the olfactometer could be improved by changing the entrance of the trapping devices used in the olfactometer. The number of mosquitoes caught was significantly higher when funnels instead of baffles were used in both trapping devices. Higher mosquito catches increase the discriminatory power of the system **(Chapter 3)**.

To test whether micro-organisms from the human skin produce substances that affect the behaviour of *An. gambiae* skin bacteria were grown *in vitro* and tested in an olfactometer **(Chapter 4)**. Volatiles released by skin bacteria obtained from human feet and grown on agar plates attracted more *An. gambiae* than volatiles released by sterile agar plates, with a significant effect of incubation time and dilution of the skin bacteria. Agar plates incubated with a reference strain of *S. epidermidis* bacteria caught significantly more *An. gambiae* than sterile agar plates. Headspace volatiles of the bacterial plates were entrained and analysed by gas chromatography – mass spectrometry (GC-MS) and this resulted in the identification of 14 putative attractants. Both, agar plates incubated with human skin bacteria and a synthetic blend of 10 of the identified compounds were attractive to *An. gambiae* in studies using mosquito traps indoors **(Chapter 4)**.

To examine whether human skin bacteria on agar plates or the synthetic blend of 10 compounds could be used in mosquito traps in setups of increasing environmental complexity, experiments were conducted in the laboratory, a large cage in the field (semi-field) and in a Kenyan village **(Chapter 5)**. Carbon dioxide appeared to be an essential component in semi-field experiments to enhance the efficacy of human skin bacteria. Traps baited with skin bacteria caught more *An. gambiae s.s.* than control traps, both in laboratory and semi-field experiments. The field study suggested that skin bacterial volatiles also attract several other disease vectors. Traps baited with a synthetic blend of compounds, identified in the headspace of skin bacteria, caught more mosquitoes than control traps in the laboratory but not in the semi-field experiments.

The synthetic blend identified in the headspace of the skin bacteria was attractive to *An. gambiae* in the laboratory **(Chapters 4 & 5)** but not when tested in semi-field **(Chapter 5)**. Possibly the concentrations of the compounds tested were too low to attract the mosquitoes from a distance in the semi-field experiment or some of the compounds in the blend acted as inhibitors, masking the attractive effect of the other components in the blend. Therefore, the effect of each of the ten compounds on the host-seeking process of *An. gambiae* was tested **(Chapter 6)**. Compounds can have a synergistic effect on mosquito behaviour and therefore the effect of each of the selected compounds to *An. gambiae* was tested in combination with a blend of ammonia, lactic and tetradecanoic acid, which had previously been identified to be attractive to *An. gambiae*. Tests were performed in a three-layer dual-port olfactometer in the laboratory and in a semi-field facility in Kenya, which allowed for high-throughput testing of the compounds in blends and in multiple concentrations in two different systems. 3-Methyl-1-butanol was found to be the best attractant and it is concluded that this compound may be used to increase mosquito trap catches for monitoring or vector control purposes. 2-Phenylethanol decreased mosquito catches in both bioassays and may act as a spatial repellent **(Chapter 6)**. Identified volatiles that influence mosquito behaviour may be used in push-pull systems in which an attractant may be used to 'pull' mosquitoes into traps and a spatial repellent to 'push' mosquitoes away from human dwellings.

Volatiles produced by a natural mixture of human skin bacteria grown on agar attract *An. gambiae* **(Chapter 4)**. To investigate the role of single skin bacterial species in the production of volatiles that mediate the host-seeking behaviour of mosquitoes, the volatiles produced by five bacterial species common on the human skin were tested for their attractiveness to *An. gambiae* **(Chapter 7)**. Volatile blends produced by some bacterial species were more attractive than blends produced by other species. Volatiles produced by *Pseudomonas aeruginosa* did not attract *An. gambiae*. Headspace analysis of bacterial volatiles in combination with behavioural assays led to the identification of six compounds that had a behavioural effect on *An. gambiae* **(Chapter 7)**.

The study described in **Chapter 7** provided further evidence that skin microbiota composition determines the attractiveness of humans to malaria mosquitoes. Next, it was investigated whether a correlation between the microbiota on the skin and human attractiveness to mosquitoes exits. For this

purpose, 48 individuals were tested for their attractiveness to *An. gambiae* **(Chapter 8)**. Because there is a genetic background for human body odour, which is determined, at least partly, by the human leukocyte antigens (HLA) **(Chapter 2)**, also the effect of these genes on the attractiveness of humans to mosquitoes was investigated. From the 48 individuals, blood samples were taken for HLA analysis and skin bacterial samples to determine the skin microbiota composition by both plate counts and 16S rRNA sequencing. The composition of the human skin emanations of each individual was determined by GC-MS and the response of the mosquito olfactory system to the components present in mixtures of human skin emanations determined by gas chromatography – electroantennographic detection (GC-EAD).

The skin emanations from individuals differed significantly in attractiveness to *An. gambiae* **(Chapter 8)**. HLA profile analysis indicated that HLA-Cw*07 may influence this attractiveness. Furthermore, individuals with more bacteria on their skin were more attractive to *An. gambiae*, whereas individuals with a higher diversity of the skin microbiota were less attractive to *An. gambiae*. *Staphylococcus* spp. were associated with individuals that were highly attractive to mosquitoes and *Pseudomonas* spp. *w*ith individuals that were significantly less attractive to mosquitoes. GC-MS and GC-EAD revealed several compounds that were associated with individuals that were attractive or less attractive to mosquitoes **(Chapter 8)**.

The results presented in this thesis create new opportunities for both fundamental and applied research **(Chapter 9)**. The attractiveness of the synthetic blends identified in this study may be improved by testing candidate odourants in more concentrations and other combinations. Mosquito traps should be optimised to reduce costs, preferably by using locally available materials. The specialization of *An. gambiae* on human odours mediated by the composition of the human bacterial community may account for the anthropophilic, restricted, host range of *An. gambiae.* The role of skin bacterial volatiles in the host-seeking process of other mosquito species and their host preference remains to be investigated. The effect of blood composition, *Plasmodium* infection or personal hygiene on the skin microbiota and volatile production is still unclear **(Chapter 9)**.

The main conclusions from this thesis can be summarized as follows:

Volatiles produced by the microbiota on human skin play an important role in the host-seeking behaviour of *An. gambiae* and the abundance and composition of the skin microbiota determine an individual's attractiveness to mosquitoes. The analysis of headspaces from skin microbiota grown *in vitro* led to the identification of 16 compounds, the majority of which had a behavioural effect on *An. gambiae*. Optimised blends consisting of these compounds can be used in push-pull strategies, thereby reducing the number of malaria mosquitoes, the human biting frequency, and the intensity of *Plasmodium* transmission. Further research on the role of skin microbiota in the host-seeking behaviour and host preference of biting insects may lead to a better understanding of vector-host interactions and contribute to the fight against vector-borne diseases.

SAMENVATTING

Malaria is één van de ernstigste infectieziekten ter wereld. Een derde van de wereldbevolking leeft in een gebied waar malaria voorkomt. Ieder jaar komen ongeveer 900.000 mensen om door deze ziekte. De *Plasmodium* parasiet veroorzaakt malaria en wordt overgebracht door anopheline muggen. *Anopheles gambiae* Giles *sensu stricto* (hierna afgekort tot *An. gambiae*), is één van de meest effectieve vectoren van malaria. Deze mug steekt voornamelijk mensen, rust in huizen, gaat in huizen op zoek gaat naar een bloedmaaltijd en is wijd verspreid in tropisch Afrika.

A*nopheles gambiae* vindt haar gastheer voornamelijk door middel van menselijke lichaamsgeuren. Identificatie van de geuren die de mug gebruikt om haar gastheer te vinden, kan helpen bij de ontwikkeling van geurvallen of afstotende stoffen. De analyse van het menselijk geurprofiel leidt echter vaak tot de identificatie van honderden componenten. Aangenomen wordt dat slechts een deel van deze componenten wordt gebruikt door de mug om haar gastheer te vinden. Het hoge aantal componenten dat aanwezig is in het menselijk geurprofiel, maakt het identificeren van de geuren die *An. gambiae* gebruikt voor het vinden van haar gastheer lastig. Ammonia is één van de weinige stoffen die al geïdentificeerd is als aantrekkelijk voor *An. gambiae*. Indien als enige stof gebruikt, is ammonia echter minder aantrekkelijk voor *An. gambiae* dan menselijke geuren. Vluchtige stoffen kunnen ook een synergistisch effect hebben op het gedrag van muggen. De combinatie van ammonia, melkzuur en een mengsel van vetzuren is significant aantrekkelijker dan één van deze stoffen alleen.

Bacteriën spelen een belangrijke rol bij de productie van menselijke lichaamsgeuren **(Hoofdstuk 2)**. Zweet krijgt zijn karakteristieke geur na incubatie met huidbacteriën. Er is een sterke correlatie tussen lichaamsgeur en de bacteriesamenstelling op de menselijke huid. Recente studies naar de menselijke microbiota op de huid, met behulp van 16S rRNA sequentie analyse, hebben aangetoond dat mensen een unieke microbiota samenstelling hebben die relatief stabiel is **(Hoofdstuk 2)**. Verschillende studies hebben aangetoond dat bacteriën een mogelijke rol spelen in de productie van geurstoffen die aantrekkelijk zijn voor muggen. Het wassen van de voeten met antibacteriële zeep verandert de plek waar *An. gambiae* bijt en zweet van eccrine klieren is aantrekkelijker voor *An. gambiae* als het geïncubeerd is met bacteriën gedurende een à twee dagen.

Gedetailleerde kennis van de ecologie en genetica van de microbiota op de menselijke huid kan de evolutionaire mechanismen achterhalen die de

interacties tussen muggen en hun gastheer beïnvloeden **(Hoofdstuk 2)**. Het doel van de studie gepresenteerd in dit proefschrift is: *het begrijpen van de rol van microbiota van de menselijke huid bij de interacties tussen muggen en hun gastheer en de identificatie van geurstoffen geproduceerd door micro-organismen die betrokken zijn bij de aantrekkelijkheid van mensen voor* An. gambiae.

Een gedeelte van de experimenten beschreven in dit proefschrift werden uitgevoerd in een tweekeuze olfactometer. In **Hoofdstuk 3** werd getest of de olfactometer kon worden verbeterd door het ontwerp van de ingang van de vangpotten aan te passen. Het aantal muggen dat gevangen werd was significant hoger als trechters werden gebruikt in de vangpotten in plaats van barrières. Hogere muggenvangsten vergroten het onderscheidend vermogen van de tunnel **(Hoofdstuk 3)**.

Om te testen of huidbacteriën vluchtige stoffen produceren die het gedrag van *An. gambiae* beïnvloeden, werden huidbacteriën *in vitro* gekweekt en getest in een olfactometer **(Hoofdstuk 4)**. De geurstoffen geproduceerd door bacteriën van de menselijke voet en gekweekt op agar platen, trokken meer *An. gambiae* aan dan de geurstoffen van steriele agar platen, met een significant effect van incubatietijd en concentratie van de huidbacteriën. Agar platen geïncubeerd met een referentiestam van *Staphylococcus epidermidis* bacteriën, vingen significant meer *An. gambiae* dan steriele agar platen. Vluchtige stoffen afkomstig van de bacterie platen, werden opgevangen en geanalyseerd met gas chromatografie – massa spectrometrie (GC-MS). Deze geuranalyse resulteerde in de identificatie van veertien stoffen die potentieel aantrekkelijk waren voor *An. gambiae*. Vervolgens werden agar platen geïncubeerd met huidbacteriën en een synthetisch mengsel met tien van de geïdentificeerde stoffen getest in muggenvallen die binnen waren geplaatst. Beide geurmengsels bleken aantrekkelijk voor *An. gambiae* **(Hoofdstuk 4)**.

Experimenten werden uitgevoerd in het laboratorium, in een grote kooi in het veld (semi-veld opstelling) en in een Keniaans dorp om te testen of menselijke huidbacteriën en de synthetische blend van tien stoffen gebruikt konden worden in muggen vallen in set-ups met verschillende omgevingscomplexiteit, **(Hoofdstuk 5)**. Kooldioxide bleek een essentiële component in de semi-veld experimenten om de effectiviteit van de menselijke huid bacteriën te verhogen. Vallen met huidbacteriën vingen significant meer *An. gambiae* dan de controle vallen, zowel in de laboratorium als de semi-veld experimenten. De resultaten van de veldstudie suggereerden dat de

geurstoffen van huidbacteriën ook andere vectoren van ziekten aantrekken. Vallen met het synthetische mengsel, geïdentificeerd in het geurprofiel van de bacteriën, vingen meer muggen dan de controle vallen in de laboratorium, maar niet in de semi-veld experimenten.

Het synthetische mengsel, geïdentificeerd door middel van geuranalyses van huidbacteriën, was aantrekkelijk voor *An. gambiae* in laboratorium experimenten **(Hoofdstuk 4 en 5)**, maar niet in semi-veld experimenten **(Hoofdstuk 5)**. Wellicht waren in de semi-veld omstandigheden de concentraties van de geteste geurcomponenten te laag om muggen van een afstand aan te trekken, of fungeerden sommige stoffen als remmers die het aantrekkelijke effect van de andere stoffen in het mengsel maskeerden. Daarom werd het effect van elk van de tien stoffen op *An. gambiae* getest **(Hoofdstuk 6)**. Geurstoffen kunnen een synergistisch effect in het gedrag van muggen veroorzaken en daarom werd het effect van elk van de tien componenten op *An. gambiae* getest in combinatie met een mengsel dat bij een vorige proef aantrekkelijk was gebleken, bestaande uit ammonia, melkzuur en tetradecaanzuur. Testen werden uitgevoerd in een tweekeuze olfactometer in het laboratorium en in een semi-veld kooi in Kenia. De combinatie van deze twee systemen maakte “high-throughput” testen van deze componenten mogelijk in verschillende concentraties. 3-Methyl-1-butanol was één van de meest aantrekkelijke stoffen en kan wellicht gebruikt worden om muggenvangsten in vallen te verbeteren. Deze vallen kunnen gebruikt worden voor het monitoren of controleren van muggen populaties. 2-Phenylethanol verlaagde de muggenvangsten in beide biotoetsen en kan wellicht gebruikt worden als afstotende stof **(Hoofdstuk 6)**. De geïdentificeerde stoffen die het muggengedrag beïnvloeden kunnen gebruikt worden in “push-pull” systemen, waarin een afstotende stof gebruikt wordt om de muggen van menselijke nederzettingen weg te houden (“push”) en een aantrekkelijke stof gebruikt kan worden om muggen in de val te lokken (“pull”).

Geurstoffen geproduceerd door een natuurlijk mengsel van menselijke huidbacteriën zijn aantrekkelijk voor *An. gambiae* **(Hoofdstuk 4)**. Om de rol van individuele bacteriesoorten in de productie van geurstoffen die *An. gambiae* beïnvloeden te onderzoeken, werden vijf bacteriesoorten die algemeen voorkomen op de huid getest op hun aantrekkelijkheid voor *An. gambiae* **(Hoofdstuk 7)**. De geurmengsels geproduceerd door enkele bacteriesoorten waren aantrekkelijker dan die van andere soorten. Geurmengsels geproduceerd door *Pseudomonas aeruginosa* waren niet

aantrekkelijk voor *An. gambiae*. Een analyse van de vluchtige stoffen geproduceerd door de verschillende bacteriesoorten in combinatie met gedragstudies, resulteerde in de identificatie van zes chemische stoffen die elk een gedragseffect op *An. gambiae* veroorzaakten **(Hoofdstuk 7)**.

De studie beschreven in **Hoofdstuk 7** bevatte verdere aanwijzingen dat de compositie van de microbiota op de menselijke huid de aantrekkelijkheid van de mens voor malariamuggen beïnvloedt. Vervolgens werd onderzocht of er een correlatie bestaat tussen de microbiota op de huid en de aantrekkelijkheid van mensen voor muggen. Om dit te onderzoeken werd de aantrekkelijkheid van 48 vrijwilligers voor *An. gambiae* bepaald. Omdat menselijke lichaamsgeuren gedeeltelijk genetisch bepaald worden en deze eigenschap geassocieerd is met humane leukocytenantigenen (HLA) **(Hoofdstuk 2),** werd het effect van deze genen op de aantrekkelijkheid van mensen voor muggen onderzocht. De compositie van het geurprofiel van de menselijke huid werd bepaald door middel van GC-MS. De respons van het olfactorisch systeem op de componenten aanwezig in de geurprofielen werd bepaald door middel van gas chromatografie – electroantennogram detectie (GC-EAD).

De geurprofielen afkomstig van de huid van de individuen verschilden significant in aantrekkelijkheid voor *An. gambiae* **(Hoofdstuk 8)**. HLA analyse resulteerde in één gen (HLA-Cw*07) dat deze aantrekkelijkheid zou kunnen beïnvloeden. Individuen met meer bacteriën op hun huid waren aantrekkelijker voor *An. gambiae;* echter mensen met een hogere bacterie diversiteit op hun huid waren minder aantrekkelijk voor *An. gambiae*. *Staphylococcus* spp. werd geassocieerd met individuen die sterk aantrekkelijk waren voor muggen en *Pseudomonas* spp. met mensen die significant minder aantrekkelijk waren voor muggen. Door middel van GC-MS en GC-EAD analyses konden verschillende geurstoffen worden geassocieerd met mensen die sterk aantrekkelijk of minder aantrekkelijk waren voor muggen **(Hoofdstuk 8)**.

De resultaten gepresenteerd in dit proefschrift creëren nieuwe mogelijkheden voor zowel fundamenteel als toegepast onderzoek **(Hoofdstuk 9)**. De aantrekkelijkheid van de synthetische mengsels, geïdentificeerd in deze studie, kunnen verbeterd worden door kandidaatgeurstoffen in meer concentraties en in meer combinaties te testen. Muggenvallen moeten geoptimaliseerd worden om de kosten te reduceren, bij voorkeur door het gebruik van materialen die lokaal te verkrijgen zijn. Het antropofiele karakter van *An. gambiae* kan wellicht verklaard worden door de voorkeur voor

menselijke geuren, bepaald door de compositie van de microbiota op de huid. De rol van geurstoffen geproduceerd door huidbacteriën in het gastheer zoekgedrag van andere muggensoorten en hun gastheervoorkeur is een onderwerp voor verder onderzoek. Het effect van de samenstelling van het bloed, een *Plasmodium* infectie of persoonlijke hygiëne op de microbiota van de huid en geurproductie is nog onduidelijk **(Hoofdstuk 9)**.

De conclusies van dit proefschrift kunnen als volgt worden samengevat:

Geurstoffen geproduceerd door microbiota op de menselijke huid, spelen een belangrijke rol in het gastheerzoekgedrag van *An. gambiae*. De aantrekkelijkheid van een individu voor muggen wordt bepaald door de hoeveelheid en compositie van deze microbiota. Analyse van de geurstoffen geproduceerd door *in vitro* gekweekte huid bacteriën resulteerde in zestien chemische componenten, waarvan het merendeel een effect had op *An. gambiae.* Geoptimaliseerde mengsels van deze componenten kunnen gebruikt worden in “push-pull” strategieën om het aantal malaria muggen te reduceren evenals het aantal muggenbeten en daarmee de intensiteit van de *Plasmodium* transmissie. Verder onderzoek naar de rol van de microbiota op de huid in het gastheerzoekgedrag en gastheervoorkeur van bloedzuigende insecten, kan leiden tot een beter begrip van deze vector-gastheer interacties en bijdragen aan de strijd tegen vectorziekten.

DANKWOORD

Na vier jaar ligt er een proefschrift dat niet tot stand had kunnen komen zonder de hulp en steun van vele mensen. De eerste die ik dan wil bedanken is mijn vrouw Anneke. Ze heeft me op alle fronten gesteund en geholpen tijdens mij PhD. Ik kon niet alleen mijn ideeën ventileren en frustraties bij haar kwijt, maar ze heeft me ook regelmatig geholpen bij het verzorgen van de muggen of het voorbereiden van experimenten in het weekend. Ze heeft de layout van dit proefschrift voor haar rekening genomen en mij zo veel werk bespaard in een drukke tijd.

"Anneke, de keren dat ik weg was voor veldwerk of een congres waren niet gemakkelijk al probeerde je dit zo min mogelijk te laten blijken. Jouw onvoorwaardelijke steun was van grote waarde bij het tot stand komen van dit proefschrift en ik ben blij dat ik daar bij toekomstige projecten op kan blijven vertrouwen."

Mijn begeleiders tijdens mijn PhD, Renate Smallegange, Willem Takken en Marcel Dicke, hebben zich stuk voor stuk ingespannen om dit proefschrift mogelijk te maken. Renate was degene die mij intensief heeft begeleid en waar ik ontzettend veel van heb geleerd.

"Renate, vaak kwam je even langs en zaten we al snel meer dan een uur te praten. Je oog voor details is verbluffend en door jou leerde ik logisch nadenken over zowel de opzet als het opschrijven van mijn experimenten. Je hebt me de afgelopen tijd ook geholpen met projecten die niet met mijn proefschrift te maken hadden en ik hoop dat ik ook in de toekomst nog regelmatig gebruik kan maken van je expertise."

"Willem, je gedegen kennis van de literatuur en ervaring in de wetenschap zijn vaak erg nuttig geweest. Vaak wist je de kern van mijn onderzoek naar voren te halen en hiermee de juiste nadruk te leggen in de discussies van mijn publicaties."

"Marcel, ik was vaak verbaasd hoe snel ik mijn stukken van je terugkreeg, voorzien van gedegen commentaar. Met je ervaring heb je bijgedragen aan meerdere hoofdstukken in dit proefschrift en was je nauw betrokken bij de afronding van het geheel."

Zonder muggenkweek geen muggen. Frans van Aggelen, André Giddink, Leo Koopman, Dennis Veldhuizen en Léon Westerd hebben er al die jaren voor gezorgd dat er muggen waren. Muggen hebben bloed nodig en vóór de overstap op de membraanvoeding hebben Frans van Aggelen, Fedor

Gassner, André Giddink, Leo Koopman, Sander Koenraadt, Jeroen Spitzen en Willem Takken de muggen gevoed op hun eigen arm. Ik weet uit eigen ervaring dat het niet altijd een pretje is om je arm in een kooi met tweehonderd vrouwtjesmuggen te steken en wil deze mensen dan ook heel erg bedanken voor hun hulp.

During my PhD I have worked together with people from several research groups, all experts in their field. I would like to thank Rob Knight and Noah Fierer and their research teams of Colorado University for the bacterial analysis and Stefan Schulz and his team from the University of Braunschweig for the analysis of the bacterial volatiles. Without their expertise I could not have completed large parts of the work presented in this thesis.

For chapter five of this thesis I spent one month at the Thomas Odiambo station of ICIPE in Mbita Point, Kenya. Richard Mukabana, Phoebe Mdadi, Tony Njeru , Mike Okal , Evelyn Odinga and many others, not only made this trip to a scientific success, but they also gave me a very warm welcome and made me feel at home. In the future I hope to visit this beautiful place again.

De twee MSc studenten die ik begeleid heb, Rob Andriessen en Björn van Loon, hebben ook mij veel geleerd. Ik wil ze bedanken voor hun enthousiasme, inzet en bijdrage aan dit proefschrift.

Achtenveertig vrijwilligers hebben meegewerkt aan het onderzoek in hoofdstuk acht van dit proefschrift. Ik wil ze ontzettend bedanken voor het dragen van pantysokken, afstaan van bloed en het rollen met knikkers over de voeten, zonder dat daar iets tegenover stond. Met Hans Beijleveld, Roland Mumm, Yu Tong Qiu, Renate Smallegange en vele anderen hebben we aan dit project gewerkt en ik wil ze hartelijk bedanken voor de tijd die ze in de buurt van stinkende voeten hebben moeten doorbrengen.

Bart Knols was een grote bron van inspiratie en wetenschappelijke kennis.

“Bart, je enthousiasme aan het begin van mijn PhD, toen ik voorstelde onderzoek te doen naar huidbacteriën en de relatie tot muggen, heeft me toen definitief doen besluiten mijn PhD aan dit onderwerp te wijden. Ik ben nog altijd blij met die beslissing en ik hoop in de toekomst nog eens samen te kunnen werken.”

Sociale ontspanning is ook belangrijk tijdens een PhD en ik wil dan ook iedereen bedanken die hieraan heeft bijgedragen zowel binnen als buiten de Leerstoelgroep Entomologie in de vorm van koffiepauzes, kroegbezoeken, lab-uitjes en feestjes. Mijn squash maatjes Sander Koenraadt, Fedor Gassner en Arno Hoetmer wil ik bedanken voor de vele maandagen waarop ik eventuele frustraties eruit kon slaan op de squashbaan.

Natuurlijk is er ook altijd veel support geweest van familie, schoonfamilie en vrienden. Opa Wolter Hijlkema wist vaak beter dan vele anderen waar ik mee bezig was en ik kreeg regelmatig krantenberichten van hem opgestuurd.

"Paps en mams, bedankt voor alle gesprekken, overnachtingen in hotel Hoofddorp en het corrigeren van de Nederlandse teksten."

Niels

CURRICULUM VITAE

Niels Verhulst was born on May 29, 1979 in Zwanenburg, The Netherlands. He finished secondary school and started the study Plant Breeding and Crop Protection at the Wageningen University in 1997. He focused his MSc projects on Entomology at the Laboratory of Entomology, Wageningen University. The subject of the second MSc project was on the age structure of wild *Encarsia formosa* and was conducted in Costa Rica during a stay of three months. He obtained his MSc degree in Plant Breeding and Crop Protection, specialization Ecological Crop Protection in 2003. As a researcher at the Laboratory of Entomology, Wageningen University, he continued the work of his third MSc project on the blood meals of Dutch malaria mosquitoes. After nine months as a field researcher for the, Instituut voor Rationele Suikerproductie (IRS) he travelled through Central and South America for half a year. Back in The Netherlands in 2004, he worked at the Plant Protection Service, The Netherlands, as an analyst phytosanitary law and at Tropenzorg B.V. as a researcher preparing the registration of the products of Tropenzorg B.V. under the new European Union Biocide Directive. In 2006 he worked as a junior researcher at the Laboratory of Entomology, Wageningen University on a project about the distribution and population dynamics of arthropod vectors of infectious diseases in The Netherlands, with a focus on Bluetongue. In 2006 he started his work on skin bacterial volatiles that mediate the behavior of *Anopheles gambiae s.s.*, of which the results are presented in this thesis. After defending his PhD dissertation, Niels will continue to work on skin bacterial volatiles and their effect on mosquito behaviour as a post-doc at the Laboratory of Entomology, Wageningen University.

PUBLICATIONS

Verhulst, N.O., P.A. Mbadi, G. Bukovinszkiné Kiss, W.R. Mukabana, J.J.A. van Loon, W. Takken and R.C. Smallegange. Improvement of a synthetic lure for *Anopheles gambiae sensu stricto* using compounds produced by human skin microbiota. Submitted

Verhulst, N.O., R. Andriessen, U. Groenhagen, G. Bukovinszkiné Kiss, S. Schulz, W. Takken, J.J.A. van Loon, G. Schraa and R.C. Smallegange. Differential attractiveness to malaria mosquitoes of volatile blends produced by human skin bacteria. Submitted

Verhulst, N.O., W.R. Mukabana, W. Takken and R.C. Smallegange. Skin microbiota and their volatiles as odour baits for the malaria mosquito *Anopheles gambiae*. Submitted

Smallegange, R.C., **N.O. Verhulst** and W. Takken. Sweaty skin: an invitation to bite? Trends in Parasitology. Submitted

Gassner, F., W. Takken, A. Van Vliet, S. Burgers, F.H.H. Jacobs, P. Verbaarschot, M. Hovius, S. Mulder, **N.O. Verhulst** and van L. Overbeek. Geographic variation in population dynamics of *Ixodes ricinus* and associated *Borrelia* infections in the Netherlands. Vector-Borne and Zoonotic Diseases. In press

Smallegange, R.C., W.H. Schmied, K.J. van Roey, **N.O. Verhulst**, J. Spitzen, W.R. Mukabana, and W. Takken. 2010. Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito *Anopheles gambiae s.s.* Malaria Journal. 9:292

Verhulst, N.O., W. Takken, M. Dicke, G. Schraa and R.C. Smallegange. 2010. Chemical ecology of interactions between human skin microbiota and mosquitoes. FEMS Microbiology Ecology, 74:1-9

Verhulst, N.O., H. Beijleveld, B.G.J. Knols, W. Takken, G. Schraa, H.J. Bouwmeester and R.C. Smallegange. 2009. Cultured skin microbiota attract malaria mosquitoes. Malaria Journal, 8:302.

Takken, W., **N.O. Verhulst**, E.J. Scholte, F. Jacobs, Y. Jongema, and R. van Lammeren. 2008. The phenology and population dynamics of *Culicoides* spp. in different ecosystems in The Netherlands. Preventive Veterinary Medicine 87: 41-54.

Verhulst, N.O., W. Takken and R.C. Smallegange. 2008. Structural design affects entry response of mosquitoes in olfactometers. Proceedings of the Section Experimental and Applied Entomology of the Netherlands Entomological Society (NEV) 19: 129-135.

Verhulst, N.O., C.F. Curtis and N. Hill. 2007. Personal protection against European disease vectors, pp. 355-368. *In* W. Takken and B. Knols [eds.], Emerging Pests and Vector-borne Diseases in Europe. Wageningen Academic Publishers, Wageningen.

Takken, W., E.M.A. van Rooij, **N.O. Verhulst**, F. Jacobs, S. Huijben, J. Beeuwkes, N. Groot, V.C.A. Vos, J. Spitzen, R.C.G. Heutink and P.A. van Rijn. 2007. Bluetongue: an emerging vector-borne disease outbreak in North-western Europe, pp. 113-121. *In* W. Takken and B. Knols [eds.], Emerging Pests and Vector-borne Diseases in Europe. Wageningen Academic Publishers, Wageningen.

Takken, W., **N.O. Verhulst**, E.J. Scholte, F.H.H. Jacobs, Y. Jongema, R.J.A. van Lammeren, A.R. Bergsma, T.C. Klok, H.J.W. van Roermund, A.A. de Koeijer and F.H.M. Borgsteede. 2007. Distribution and dynamics of arthropod vectors of zoonotic disease in the Netherlands in relation to risk of disease transmission, pp. 59. Wageningen University.

EDUCATION CERTIFICATE

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
Chemical ecology of human skin microbiota and mosquito interactions

Writing of project proposal (4.5 ECTS)
Disruption of malaria transmission by chemical manipulation of anopheline olfactory responses

Post-graduate courses (6.5 ECTS)
Advanced statistics; PE&RC (2007)
Insect chemical ecology; Alnarp, Sweden (2007)
Statistical analysis of ~omics data; WUR (2008)
Multivariate data analysis basic course; Umetrics, Sweden (2009)

Laboratory training and working visits (1.7 ECTS)
PhD-excursion; Rothamsted, Imperial College London School of Hygiene and Tropical Medicine, University of Southampton, UK (2007)
Correlation between skin microbiota composition and attractiveness to mosquitoes; University of Colorado, Department of Chemistry and Biochemistry, USA (2010)

Deficiency, refresh, brush-up courses (1.5 ECTS)
Basic statistics; PE&RC (2007)

Competence strengthening / skills courses (4.5 ECTS)
PhD Competence assessment; WGS (2009)
Writing of a research proposal for ALW funding, including writing a rebuttal : skin microbiota as determinant of host specificity in mosquitoes (approved, three years post-doc funding) (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)
PE&RC Introduction weekend (2007)
PE&RC Day (2007, 2009)
PE&RC Current themes in ecology (2008)

Discussion groups / local seminars / other scientific meetings (7.6 ECTS)
Grand Challenge for Global Health meetings; Tanzania and Wageningen (2007 and 2008)
Annual Dutch Entomology Meeting (2007 and 2009)
PhD Discussion group Entomology (2007-2010)
Local monthly seminars at the department of Entomology (2007-2010)

International symposia, workshops and conferences (7.1 ECTS)
25th International Meeting ISCE; Neuchatel, Switzerland (2009)
5th International SOVE Congress; Antalya, Turkey (2009)
Keystone Symposium Molecular Targets for Control of Vector-Borne Diseases; Bridging Lab and Field Research, Copper Mountain, USA (2010)

Lecturing / supervision of practical 's / tutorials (6.2 ECTS)
Ecology II; 2 days (2006)
Molecular and Evolutionary Entomology; 12 days (2007)
Ecological aspects of bio-interactions; 0.5 day (2007-2008)
Ecology I ; 5 days (2007-2009)

Supervision of 2 MSc students; 4 months (6 ECTS)
The attractiveness of different species of skin microorganisms to the malaria vector *Anopheles gambiae s.s.*
The role of skin bacteria in mosquito behaviour: is host specificity dependent on skin microflora?

SUPPLEMENTAL TABLE

Supplemental Table S7. Compounds identified in the headspace of broths of skin bacteria

Compounds	RI	Medium	*Bac* Eg	*Bac* St	*Bre* Eg	*Bre* St	*Cor* Eg	*Cor* St	*Sta* Eg	*Sta* St	*Pse* Eg	*Pse* St
3-Methyl-1-butanol	n.d. [1]			++			++			+++		
Acetoin	n.d. [1]		++	++						+++		
Dimethyldisulfide	n.d. [1]			++		++				+++		+++
Toluene	n.d. [1]		+++ +		+++	+++		++				
1-Butanol	n.d.[1]	++++			++		++		+++		++	
Butyl acetate	826		+++	+++ +	++	+++ +		+++ +		+++ +		+++
m/z: 88, 43, 55	854			+++								
3-Methylbutanoic acid	863					++		++				
m-Xylene	872				+							
p-Xylene	876				+							
Cyclohexanone	901	++	+++		++	++	++	++	++	++	++	+++
3-(Acetyloxy)-2-butanone	903			++								
2,5-Dimethylpyrazine	915	+++	+++	++	++	+++	++	+++ +	++	+++	++	+++ +
2-Hydroxy-5-methyl-3-hexanone	947			++								
3-Hydroxy-5-methyl-2-hexanone	952		++	++		++		++				
Butyl isobutyrate	958			++		++		++				+
Benzaldehyde	963	++++	+++ +	++	+++ +	++	+++ +	+++ +	+++ +	+++ +	+++ +	+++ +
Dimethyltrisulfide	968		+++ +	+++ +	+++	+++ +	++	+++ +	+++	+++ +	+++	+++ +
1,2-Dithiolane	978				++							
Butyl butyrate	997	++					+		+	++	++	++
Trimethylpyrazine	999	+++	++	++	++	++	++	+++	+	++	++	+++ +
Cyclosiloxane[2]	1003				++							
2-Acetylthiazole	1016	++	++	++		++	+	++		++		++
2-Ethyl-1-hexanol[2]	1029		+++				+			++	++	
2,3-Dimethyl-2-cyclopenten-1-on	1037				++							
2-Hydroxybenzalde-hyde	1040	+										
Butyl 2-methylbutanoate	1042			++		++		+++		++		
Ethyl 2-isopropyl-2-butenoate	1042		++							+		
Pyrazine M: 136	1050	++	++	++	+	++	+	++	+	++	++	++

=> See next page

Compounds	RI	Medium	*Bac* Eg	*Bac* St	*Bre* Eg	*Bre* St	*Cor* Eg	*Cor* St	*Sta* Eg	*Sta* St	*Pse* Eg	*Pse* St
S-Methyl methanethiosulphonate	1062				++		+			+		
Acetophenone	1063	++	++	++	++	++	++	++	++	++	++	++
5-Nonanone	1073								++	+		
2-Ethyl-3,6-dimethylpyrazine	1075	++	++	++	++	++	++	+++	++	++	++	+++
2-Methyl-5-(1-methyl-ethyl) pyrazine	1082	++	+	++		++		++		++	+	++
2-Methoxyphenol	1087			++								+++
1-Undecene	1088											+
m/z: 123,138	1090		+	++		+		++	+	++		
2-Nonanone	1090							++				
Methyl benzoate	1091											+++
Nonanal	1104	++							+	+	+	
m/z: 43, 123, 101, 138	1111	+	+	++	+	++	+	++	+	++		++
Ethyl-3-acetoxy-butyrate	1113	+										
2,3-Diethyl-5-methylpyrazine	1151	++	+	+		+				+		+
2,6-Di-(1-Methylethyl) pyrazine	1153	++	+	+		++		++		++		+
8-Methyl-2-nonanone	1155									++		
2-Ethyl-3,5,6-trimethylpyrazine	1155					+	+	++				+
Siloxane[2]	1156	++	+	+	+		++		+	++	++	
7-Methyl-2-nonanone	1160					+		++		+		
Naphthalene	1176	++	+									
Tetrathiolane	1187					++						
2,5-Dimethyl-3-(1-methylethyl) pyrazine	1193	++					+		+		+	
2,5-Dimethyl-(2-methylpropyl)-pyrazine	1196	+		++		+	+		+	++		+
Decanal	1201	+						++		++	+	
Verbenone	1203	+										
Dimethyltetrasulfide	1208		++	+++ +		+++ +		+++ +		+++		+++ +
2-Butyl-3,6-dimethylpyrazine	1253	++	++	++	+	++	+	++	+	++	+	++
1-Chlorodecane[2]	1263				++							

=> See next page

Compounds	RI	Medium	*Bac* Eg	*Bac* St	*Bre* Eg	*Bre* St	*Cor* Eg	*Cor* St	*Sta* Eg	*Sta* St	*Pse* Eg	*Pse* St
α-Ethylidene benzene acetaldehyde	1272	++										
m/z: 145, 76, 120, 46, 192	1277	++										
Isobornyl acetate	1285	++	++	+			+					
m/z: 145, 118, 192, 46	1289	++										
2-Undecanone	1292			+		+		++		+		++
Indole	1294					+				+		
m/z: 57, 143, 87, 69, 159	1301				++		+		+	+	+	
2-(2-Methylbutyl)-3,6-dimethylpyrazine	1302	++	+			+	+	+	+	+	+	
2,5-Dimethyl-3-(3-methylbutyl)-pyrazine	1313	++	++	++	++	++	+	++	+	++	++	++
m/z: 69, 43, 85, 111, 154	1321							+			+	
Siloxane[2]	1329	++	+	+	++	+	+	+	++	+	++	+
m/z: 138, 95, 83, 193, 57	1340		+	+	++		+		++		+	
10-Methyl-2-undecanone	1359					++		+		+		
9-Methyl-2-undecanone	1363					++		++		+		
m/z: 91, 71, 99, 117, 55	1379			+	++							
Pyrazine	1388			+		+				+		
Geranyl methyl thioether	1396											+++
Tetradecane	1397				+	+	+	+	+	+	+	
m/z: 71, 117, 43	1428			++								
Diphenylmethane[2]	1430											+
Isobutyl 2-phenylacetate	1432			++		+		++				
Pentathiane	1437			++		++		++				
Dimethylpentasulfide	1455			++		++		++		++		++
Cyclohexadien-1,4-dione derivative[2]	1470		+	+	+				+	+	+	+
Hexasulfur	1487	++			+	+	+					
5-Methyl-2-phenyl-2-hexenal	1488		+		+	+	+	+	+	++	+	
2-Tridecanone	1496					+				+		

=> See next page

Compounds	RI	Medium	*Bac* Eg	*Bac* St	*Bre* Eg	*Bre* St	*Cor* Eg	*Cor* St	*Sta* Eg	*Sta* St	*Pse* Eg	*Pse* St
Pentadecane	1498	+		+								
Cyclosiloxane[2]	1499	+	+		+	+	+	+	+	+	+	
2,4-bis(1,1-dimethylethyl)-phenol[2]	1509		++	++	++	++	+	++	++	++	++	++
Methyl dodecanoate	1530								+	++		
Calamene	1535								+			
12-Methyl-2-tridecanone	1557					++		+		+		
Nerolidol	1562								+			
m/z: 69, 93, 41, 107, 71	1563									+		
11-Methyl-2-tridecanone	1567					++		++		+		
m/z: 107, 163, 56, 79, 41	1586									+		
Hexadecane	1596	++	++			+	+	++		++	++	+
m/z: 71, 43, 139, 224, 97	1609				+							
Lenthionine	1623							+				
α-Methyl-α-phenyl-benzene-methanol[2]	1639								+			
13-Methyl-2-tetradecanone	1662					+						
Cyclosiloxane[2]	1669	+	+		+	+		++	+		+	+
Acorenone	1692									+		+
2-Pentadecanone	1695			++		++		++		+		
Hexathiepane	1707			++		++		++		+		+
Siloxane[2]	1745	+										+
14-Methyl-2-pentadecanone	1760		+			++		++		+		
13-Methyl-2-pentadecanone	1771		+			++						
Methyl 13-methyltetradecanoate	1786		+									
Octadecane	1796							++				
2-Hexadecanone	1800					++						
Diterpene	1802							++	+	+	+	
Diterpene	1857	+					+		++	+	+	
15-Methyl-2-hexadecanone	1865					++						
14-Methyl-2-hexadecanone	1866					++						

=> See next page

Compounds	RI	Medium	*Bac* Eg	*Bac* St	*Bre* Eg	*Bre* St	*Cor* Eg	*Cor* St	*Sta* Eg	*Sta* St	*Pse* Eg	*Pse* St
Bis(2-methylpropyl) phthalate[2]	1866	++	++	+	++	++	++	++	+	++	++	
Branched Hepta-decen-2-one	1874							++				
Diterpene	1884									+		
Nonadecane	1896							++				
2-Heptadecanone	1902					++						
Siloxane[2]	1903	+				+						
Diterpene	1905								++	+	+	
Di-2-pyridinylethanedione[2]	1906							++				
m/z: 205, 217, 220, 175	1917	+										
m/z: 160, 145, 270, 95, 105	1943	+			+				++	++	+	
m/z: 243, 91, 258, 147, 133	1950									+		
Dibutylphthalate[2]	1961	++	++		++	++	+	++	++	++	+	++
16-Methyl-2-heptadecanone	1968					++						
15-Methyl-2-heptadecanone	1976					++						
Diterpene	1988	+	+		+		+		++		+	
Eicosane	1995							++				
2-Octadecanone	2004					+				+		
				+++		+++		+++				
Octasulfur	2025			+		+		+		++	+	+++
Cyclosiloxane[2]	2063					+						
2-Ethylhexyl do-decanoate[2]	2099	++	+									
2-Nonadecanone	2105					+						
m/z: 121, 179, 137, 292	2120	++										
m/z: 197, 282, 254, 239, 141	2121								+	++		
Diterpene	2168								++	++		
m/z: 149, 91, 121, 79	2253	++	++									
m/z: 149, 91, 121, 79, 243	2257									++		

Bacillus subtilis *(Bac),* Brev. epidermidis *(Bre),* C. minutissimum *(Cor),* S. epidermidis *(Sta) and* P. aeruginosa *(Pse) headspace volatiles were grown in standard medium and analyzed in their exponential growth (Eg) and stationary (St) phase. Broths were attractive (grey columns) or unattractive (blank columns) to mosquitoes in olfactometer bioassays. Abundance: + 0 – 0.5%; ++ 0.5 – 10%; +++ 10 – 30%; ++++ 30 – 100%, relative to the total ion count. RI = Retention Index. [1]not determined, [2]artefact*

This project was funded by a grant from the Foundation for the National Institutes of Health (NIH) through the Grand Challenges in Global Health Initiative (GCGH#121).

Lay-out: Anneke Verhulst-Venbrucx
Cover design: Hans Smid
Cover picture + page 10: Hans Smid, www.bugsinthepicture.com

Printed by: Wöhrmann Print Service, Zutphen, The Netherlands