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Microbial growth enhances the attractiveness of human sweat for the malaria mosquito, *Anopheles gambiae sensu stricto* (Diptera: Culicidae)

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Summary. Behavioural responses of the malaria mosquito *Anopheles gambiae s.s.* to volatiles emitted by pooled and individual sweat samples collected from human volunteers were quantified in a dual-port olfactometer. Both fresh and incubated sweat samples were attractive to *An. gambiae*, but incubated sweat was selected significantly more when the sweat samples were tested against each other. The enhancement of the attractiveness of the sweat following incubation at 37°C appeared to be due to volatiles resulting from bacterial growth during incubation. The pH value of a sweat sample did not affect behavioural responses to sweat. The role of specific bacterial groups and substrates in odour-mediated host-seeking behaviour is discussed.

Key words. Malaria mosquito – *Anopheles gambiae s.s.* – host-seeking behaviour – attraction-human sweat – microbial growth

Introduction

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

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

The specific questions that are being addressed here are as follows. Is there an effect of pH shift *per se* on the attractiveness of sweat to *An. gambiae* and what is the effect of incubation of sweat in the absence of microorganisms? Which bacteria from the skin are present in sweat and what is their development during incubation?

Materials and methods

Insects

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

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Olfactometer

A dual-port olfactometer, consisting of a Perspex flight chamber ($1.60 \times 0.66 \times 0.43$ m) (modified after Braks & Takken 1999) was used to study the attractiveness of sweat samples. Charcoal-filtered, humidified ($65 \pm 5\%$ RH), warm air (27 ± 0.5°C) was led via a Perspex trapping device, which was linked to two ports (diameter 4 cm, 28 cm apart), into the flight chamber with a speed of 20 cm/sec. Light (1 lux) was produced by one light bulb (75 W) and was filtered by a piece of yellow cloth hanging 1 m above the flight chamber.

Collection and treatment of sweat samples

To test the effect of changes in pH value and sterilisation on attractiveness, pooled sweat collected from eight volunteers was used (*Investigation I*). To examine the presence and development of microorganisms and their effects, sweat samples were collected from three volunteers and analysed separately (*Investigation II*).

Investigation I
Droplets of sweat were collected from the foreheads of eight Caucasian human volunteers (five males and three females with ages ranging from 25 to 41 years) with sterile glass Pasteur pipettes and put in glass vials (5 ml). From each volunteer 3 ml of sweat was collected. Sweat production was stimulated by physical exercise in a warm humid room (30°C, 90% relative humidity). Immediately after collection, each sweat sample was divided in two sub-samples of 1.5 ml. One sub-sample was directly stored at -20°C ("fresh" sweat) and the other was incubated under aerobic conditions at 37°C for two days ("incubated" sweat), after which it was also stored at -20°C. The pH value of sweat of each volunteer was determined separately with a micro pH-electrode (Inlab 423, Mettler) and was 5.6 ± 1.0 when fresh and 8.5 ± 0.3 after incubation. Subsequently, 1.0 ml of the sub-samples of each volunteer was pooled resulting in 8 ml of pooled fresh sweat and 8 ml of pooled incubated sweat. Two-third of the pooled fresh sweat was sterilised using a bacterial filter (Millipore, Millex GS, pore size 0.22 μm). Half of the sterilised pooled fresh sweat was directly stored at -20°C; the other sterile half was incubated under aerobic conditions at 37°C for two days, after which it was also stored at -20°C. Half of the pooled incubated sweat was also sterilised using a similar bacterial filter, after which it was stored at -20°C.

The pH value of 0.8 ml of pooled fresh sweat (pH 4.9) was artificially increased by adding small amounts (1–2 μl) of a base (total of 13 μl, 0.1 N NaOH) until the value of the pooled incubated sweat (pH 8.4) was reached. Likewise, 0.8 ml of pooled incubated sweat (pH 8.4) was artificially acidified (16 μl, 0.5% HCL) until the pH value of the pooled fresh sweat (pH 4.9) was reached. During these treatments the pH value of the sweat was constantly monitored with the micro pH-electrode.

Investigation II
Sweat droplets were collected from the foreheads of three Caucasian human volunteers, two males (A and B) and one female (C) with ages of 34, 51 and 28 respectively, as described above. From each volunteer, 3 ml of sweat was collected twice on different days. Immediately after collection, each sweat sample was divided in two sub-samples of 1.5 ml. One sub-sample was directly stored at -20°C and the other was incubated under aerobic conditions at 37°C for two days, after which it was also stored at -20°C.

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

Behavioural assay

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

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

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

Statistics

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

Results

Investigation I

Significantly ($P < 0.001$) more mosquitoes entered trapping devices baited with the differently treated pooled sweat samples than the control (Table 1, Exp. #1). When tested against each other, more mosquitoes responded to incubated than to fresh sweat ($P \ll 0.001$)

Table 1 Results of behavioural experiments testing the effect of pH value and sterilisation on the attractiveness of *An. gambiae* to sweat ($N = 8$)

Experiment	Stimuli		Response[a]		Chi square[b]	N[c]		
	Treatment[d]	Control	Treat	Control	P	T	C	=
1	fresh sweat	water	86	19	***	8	0	0
	incubated sweat	water	108	14	***	8	0	0
	sterilised fresh sweat	water	49	16	***	7	0	1
	incubated sterilised sweat	water	63	16	***	6	1	1
	sterilised incubated sweat	water	105	5	***	8	0	0
2	incubated sweat	fresh sweat	63	12	***	7	0	1
3	incubated sterilised sweat	fresh sweat	12	24	*	1	4	3
	sterilised fresh sweat	fresh sweat	13	16	ns	3	3	2
4	alkalinised fresh sweat	fresh sweat	36	38	ns	3	3	2
	acidified incubated sweat	incubated sweat	36	51	ns	2	4	2

[a] The response is the total number of mosquitoes caught in either the test or control trapping device
[b] Significant differences (*: $P<0.05$, **: $P<0.01$ or ***: $P<0.001$) or no significant differences (ns: $P \geq 0.05$) found between the total number of mosquitoes caught in the test and control trapping device (Chi-squared test)
[c] N = number of replicates. The distribution of the preferences for either the test (T), the control (C) or no preference (=) in the individual replicates are also shown
[d] fresh sweat = pooled fresh sweat; incubated sweat = sweat collected, incubated for two days and subsequently pooled; sterilised fresh sweat = sterilised pooled fresh sweat; incubated sterilised sweat = sweat collected, pooled, sterilised and subsequently incubated; sterilised incubated sweat = sweat collected, incubated, pooled and subsequently sterilised

(Table 1, Exp. # 2). Significantly fewer mosquitoes responded to sweat that had been sterilised and subsequently incubated than to fresh sweat ($P = 0.046$) and no difference was found between the numbers responding to sterilised fresh sweat and fresh sweat ($P = 0.577$) (Table 1, Exp. # 3). When tested against each other, the response to fresh sweat of which the pH value had been artificially modified to 8.4 did not differ from that to untreated fresh sweat ($P = 0.816$). Similarly, the pH-modified incubated sweat was not different in attractiveness from the non-modified incubated sweat ($P = 0.108$) (Table 1, Exp. # 4).

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

Investigation II

On all occasions, viable cell counts (CFU) were formed on the three different selective agar media inoculated with a dilution of defined volumes of fresh sweat or sweat incubated at 37°C for one and two days (Table 2). The results indicated that both aerobe coryneforms and staphylococci grew within the first day of incubation (from day 0 to day 1) but not on the second incubation day (from day 1 to day 2) for the replicate experiments. However this was not the case for propionibacteria, where only one of the samples supported growth and the mean of the data indicated no growth.

In all but one behavioural experiment significantly more mosquitoes were caught in the trapping devices baited with incubated sweat than in the devices with fresh sweat ($P < 0.05$). Although the results from tests with the fresh and incubated sweat of the first collection of volunteer A showed a similar trend, the difference was not significant ($P = 0.057$) (Table 3).

The pH value changed from either slightly alkaline (C) or acidic (A and B) to alkaline (pH > 8) following a 2 d incubation at 37°C (Table 4).

Discussion

Effect of incubation, sterilisation and the pH value on the attractiveness of sweat

Pooled sweat samples were more attractive than water and, therefore, contained components attractive for *An. gambiae*. The responses to incubated sweat, however, seemed to be stronger than those to fresh sweat are. Statistical support for this observation was demonstrated in the second and third experiment where mosquitoes selected the incubated sweat significantly more than fresh sweat. This had also been reported in previous work (Braks *et al.* submitted; Meijerink *et al.* submitted). It was also found that the incubation of sweat in the absence of bacteria did not enhance its attractiveness and even diminished it: sterilised fresh sweat was preferred to sweat that was collected, pooled, sterilised and subsequently incubated. The decrease in attraction might be caused by processes like oxidation or evaporation of volatiles during the incubation procedure. The incubation of sweat, after removal of bacteria, was not accompanied by a shift in pH value, which suggests that the shift in pH value of untreated sweat was the result of bacterial action upon sweat components. Artificial modification of the pH value of fresh and incubated sweat did not affect the attractiveness of sweat. In an earlier report alkalisation of sweat (pH 7–8) to pH 8–9 did not affect the attractiveness of human sweat either while acidification to pH 1.5 caused a slightly repellent response. The latter was probably caused by the release of a high concentration of acidic

Table 2 Natural logarithm of the viable cell count (CFU) per ml sweat collected from three volunteers (A, B and C) on two different occasions (I and II). The CFU was determined on three different media inoculated with sweat which was either fresh (Day 0), incubated for 1 day (Day 1) or incubated for 2 days (Day 2)

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

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

compounds through the rather strong acidification (Braks *et al.* 1997) as also reported for *Aedes aegypti* L. by Thompson and Brown (1955). In contrast Müller (1968) found that attractive sweat from a human arm lost its attractiveness for *Ae. aegypti* after alkalisation and explained this finding by the loss of lactic acid in the headspace. Lactic acid, however, appears to have a limited role in the attraction of *An. gambiae* to sweat (Braks *et al.* submitted). We conclude that the enhancement of the attractiveness of sweat after incubation is due to microbial activity rather than to other chemical or physical processes occurring during incubation.

Microorganisms and their role in the attractiveness of sweat

The absolute number and development of organisms of the three main bacterial groups differed largely between days of incubation, sweat collections and volunteers (Table 2). During the first day of incubation, a distinct growth of the coryneform and staphylococcal species was found in all sweat samples. No general growth was found for these two bacterial groups on the second day of incubation and no significant growth at all was found for the anaerobe diphteroid bacteria. The latter result was expected, as these predominantly lipophilic organisms would not flourish under lipid-poor conditions; thermally induced sweat is stated to consist principally of an aqueous eccrine excretion, which does not contain lipids (Noble & Somerville 1974). In addition, growth of anaerobe microbes was also not expected as the sweat was incubated under aerobic conditions. However, contrary to our expectation, a distinct growth of propionibacteria was seen in some of the sweat samples. This indicates either that the availability of lipids was not limited or that an anaerobic microclimate is present in sweat or that the growing bacteria were not strictly anaerobe. In another study, we reported that the headspace of thermally induced sweat does, indeed, contain components derived from the sebum, next to eccrine components (Meijerink *et al.* submitted). Growth of microorganisms in human sweat samples during incubation was already suggested previously, although not shown by Bergeim & Cornbleet (1943). Using a rather crude categorisation into three large bacterial groups, we showed that bacteria belonging to a wide range of groups are present in fresh sweat and that general bacterial growth occurs during incubation.

Data from the behavioural assay with sweat from three volunteers showed that all incubated sweat samples induced similar responses of the mosquitoes when tested against fresh sweat: incubated sweat was preferred to fresh sweat. These results confirm the outcome of the experiments with pooled sweat (see above). Considering this, together with the observed variation in bacterial populations and proliferation, we propose

Table 3 Results of behavioural experiments testing the preference of *An. gambiae* for fresh or incubated sweat collected from three volunteers (A, B and C) on two different occasions (I and II)

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

[a] The response is the total number of mosquitoes caught in either the trapping device baited with incubated or fresh sweat
[b] See legend under Table 1
[c] N = number of replicates. The distribution of the preferences for either incubated sweat (I), fresh sweat (F) or no preference (=) in the individual replicates are also shown

Table 4 pH-value of fresh (Day 0) and incubated (Day 2) sweat collected from three volunteers (A, B and C) on two different occasions (I and II)

Volunteer	Collection	pH	
		Day 0	Day 2
A	I	5.2	8.8
A	II	4.9	8.7
B	I	5.5	8.9
B	II	5.2	8.8
C	I	7.4	8.6
C	I	7.6	8.3

that the enhancement of the attractiveness of sweat results from a-specific bacterial growth producing attractive volatiles for *An. gambiae*. However, the possibility that the attraction might have been caused by the action of a single bacteria species while the effect of other bacterial development is negligible, cannot be ruled out. The identity of the volatile component(s) responsible for the attraction is not known.

We conclude that, through the action of bacteria, volatile components, which are attractive for *An. gambiae*, are released from the sweat. To our knowledge, only Schreck & James (1968) investigated the role of bacterial volatiles in host-seeking behaviour of mosquitoes and found behavioural preference of *Ae. aegypti* for air led through broth cultures of transient skin bacteria, *Bacillus cereus*, compared to control air. At first sight the attraction to the action of bacterial species not belonging to the resident skin microflora was peculiar since this could never have been responsible for the attractiveness of the human skin. However, considering our data, we suggest that the production of odorous volatiles might be a general process rather than a process specific to a limited range of bacterial species. Many odours emanating from the mammalian skin or its specialised scent glands are thought to be produced by microbial activity (Albone *et al.* 1977). Other examples of insects responding to bacterial-produced volatiles emanating from the mammalian skin have been reported (for a review see Braks *et al.* 1999). Species-specific odours emanating from mammals are probably the result of the substrate and the action of a range of skin bacterial species together. Therefore, a skin-borne odour may be a highly available and reliable kairomone for host-specific mosquitoes that rely on olfactory cues rather than visual cues for the localisation of a blood meal.

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