Interindividual variation in the attractiveness of human odours to the malaria mosquito *Anopheles gambiae s.s.*

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**Abstract.** Differences between human individuals in their attractiveness to female mosquitoes have been reported repeatedly, but the underlying mechanisms are not well understood. Skin emanations from 27 human individuals, collected on glass marbles, were tested against ammonia in a dual-choice olfactometer to establish their degrees of attractiveness to anthropophilic *Anopheles gambiae s.s.* Giles (Diptera: Culicidae) mosquitoes. Ammonia was used as a standard odour source because of its proven attractiveness to *An. gambiae s.s.* Skin emanations from most volunteers attracted significantly more mosquitoes than ammonia. There were clear differences in the attractiveness of skin emanations from different volunteers relative to that of ammonia, as well as in the strength of the trap entry response. Consistent differences were observed when emanations from the three most and the three least attractive volunteers were tested pairwise. No gender or age effect was found for relative attractiveness or trap entry response. Emanations from volunteers with higher behavioural attractiveness elicited higher electroantennogram response amplitudes in two pairs, but in a third pair a higher electroantennogram response was found for the less attractive volunteer. These results confirm that odour contributes to the differences in attractiveness of humans to mosquitoes.

**Key words.** *Anopheles gambiae*, electroantennogram, host-seeking behaviour, human odours, malaria mosquito, olfaction.

**Introduction**

Most female mosquitoes require blood meals for reproduction. Physical and chemical cues emanating from the host guide female mosquitoes to these blood sources. Although optical cues and physical cues such as heat and moisture play a role in host location, chemical cues are considered to be the most important (Takken, 1991). Water vapour, carbon dioxide and ammonia are considered to be important cues for guiding mosquitoes to their human hosts (Price *et al.*, 1979; Braks *et al.*, 2001). In addition, organic chemicals such as a number of carboxylic acids, lactic acid, 1-octen-3-ol and acetone have been shown to attract anthropophilic mosquitoes (Takken *et al.*, 1997; Bosch *et al.*, 2000).

It is commonly observed that, when two persons are equally accessible, one person receives more mosquito bites than the other. The mechanisms underlying such discrimination await elucidation. Numerous studies have been published on the differential attractiveness of human individuals to mosquitoes (Muirhead-Thomson, 1951; Smith, 1956; Brouwer, 1960; Mayer & James, 1969; Carnevale *et al.*, 1978; Curtis *et al.*, 1987; Schreck *et al.*, 1990; Lindsay *et al.*, 1993; Knols *et al.*, 1995; Brady *et al.*, 1997; Mukabana *et al.*, 2004). Smart & Brown (1957) observed a higher landing frequency on hands with a darker colour. These authors also reported that when the highly anthropophilic mosquito *Aedes aegypti* (L.) is offered hands by two individuals with different temperatures, it alights more often on the warmer hand. Schreck *et al.* (1990) studied human emanations collected on glass Petri dishes and tested their attractiveness when kept at different temperatures. They demonstrated that although heat is not required to attract mosquitoes, warmed human skin residues attracted higher numbers of *Ae. aegypti*. 

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When hands or forearms with different surface humidities were offered, the drier one was more frequently visited (Smart & Brown, 1957; Gilbert et al., 1966). Positive correlations between human body mass or surface area and mosquito catch were found in several studies (Muirhead-Thomson, 1951; Spencer, 1967; Port et al., 1980). There are also reports of a correlation between pregnancy and attractiveness, with pregnant women being bitten more often than non-pregnant women (Lindsay et al., 2000; Ansell et al., 2002; Himeidan et al., 2004).

In most of these studies the attractiveness of human bodies or human hands from different people were compared. However, in many studies it was not possible to identify olfactory factor(s) as the cause of the differences found because of confounding factors such as body size, hue, heat and moisture. Variations in attractiveness between individuals due to the latter factors can be minimized by collecting human skin residues on glass marbles. Skin volatiles on glass beads or Petri dishes are attractive to Aedes aegypti and Anopheles gambiae Giles sensu stricto (Schreck et al., 1981, 1990; Qiu et al., 2004). This method offers the possibility of comparing airborne human odour from various individuals.

In the present study, we examined the attractiveness of skin emanations from 27 human individuals to females of the malaria mosquito Anopheles gambiae s.s. (henceforth in this paper termed An. gambiae), which is strongly anthropophilic and the most important malaria vector in Africa (White, 1974). The experiments were performed in a dual-choice olfactometer. The human subjects were ranked according to their index of attractiveness to An. gambiae relative to the response to ammonia, which is a proven kairomone for An. gambiae (Braks et al., 2001). Emanations from three individuals with high and three with low indices were then compared pairwise for the mosquitoes’ behavioural and electroantennogram (EAG) responses.

Materials and methods

Volunteers

We examined the attractiveness of 27 adult humans (16 male and 11 female) aged between 22 and 53 years. Twenty-five were white, one Asian and one Hispanic. All volunteers were non-smokers, free from chronic illnesses and not using any medication on a regular basis. None of the participating women were pregnant. The volunteers were requested to refrain from drinking alcohol on the day before the experiments (Shirai et al., 2002), from peeling garlic or onions, and from eating garlic, onions or spicy food. On the morning of the experiments, volunteers were requested not to use perfumed cosmetics or peel citrus fruits. One hour before the experiments, hands were washed with a standard perfume-free soap (Dermoline liquid soap-free washing emulsion; Tramedico BV, Weesp, the Netherlands), rinsed in tap water and dried on tissue paper. According to their ages, the volunteers were divided into three groups, with nine in each group: those aged 24–27 years were defined as ‘younger’; those aged 28–37 years as ‘middle-aged’, and those aged 40–55 years as ‘older’. The volunteers were coded by gender (M = male, F = female), followed by age group (O = older, M = middle-aged, Y = younger). Each code ended with a unique number from 1 to 27.

Insects

The An. gambiae colony originated from Suakoko, Liberia, and had been reared by blood-feeding on humans since 1988. The mosquitoes were maintained at 27 ± 1 °C, 80 ± 5% RH and at a photo-scotophase of LD 12:12 h.

The experiments were performed during the last 4 h of the scotophase. Groups of 30 mosquitoes were randomly collected from the rearing cage 14–18 h before the start of the experiments and placed in a releasing cage, with access to tap water via a piece of moistened cotton wool placed on the top of the cage. For all experiments, 5–8-day-old female mosquitoes that had not received a blood meal were used.

Olfactometer

A dual-port olfactometer (Pates et al., 2001), consisting of a Perspex flight chamber of 1.60 × 0.66 × 0.43 m, was used to study the behavioural response of the female mosquitoes to different odour stimuli. Charcoal-filtered and humidified, warm, pressurized 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 0.22 ± 0.02 m/s. Dim light was produced by one light bulb (75 watt) and was filtered and scattered through a screen of yellow cloth hanging ~ 1 m above the flight chamber, providing a light intensity of approximately 1 Lux. The experimental room was maintained at a temperature of 28 ± 1.0 °C and a relative humidity of 66 ± 4.0%. The temperature inside the flight chamber was similar to that of the room, and the humidity was maintained at 70 ± 5.0%. The humidity of the air streaming through the ports was maintained above 80% and the temperature was 29 ± 1.0 °C.

Odour stimuli

One day before the experiments, 250 μL of a solution of 2.5% ammonia in water (diluted 10 times with distilled water from a concentrated ammonia solution, 25% in water, analytical grade; Merck, Amsterdam, the Netherlands) was injected into an 80-L Teflon air sample bag (SKC Gulf Coast Inc., Houston, TX, U.S.A.). Subsequently, the bag was filled with 60 L humidified and filtered warm pressurized air at least 17 h prior to the experiments to allow the solution to evaporate. This procedure resulted in an ammonia concentration of 136 p.p.m. in the bag. This concentration was chosen based on previous dose–response experiments with An. gambiae to ammonia (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.) from the air sample bags through Teflon tubes (diameter

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7 mm) into the trapping devices, where it merged with the main air stream of 23.5 L/min. Glass marbles of 16 mm in diameter were cleaned by rinsing in non-perfumed soap (CLY-MAX Heavy Duty Cleaner; Rogier Bosman Chemie B.V., Heijningen, the Netherlands), distilled water and then in ethanol (99.8% purity; Merck). The rinsed marbles were dried in an oven at 200 °C. Skin emanations from the 27 individuals were collected on six glass marbles that the subjects had handled for 10 min.

**Experimental procedure**

The marbles were put in one of the trapping devices directly after handling. In order to test the attractiveness of the volunteers individually, clean air from an air sample bag was pumped into this trapping device. Gaseous ammonia was pumped into the other trapping device, which contained clean glass marbles. In order to directly compare the attractiveness of the skin emanations from two volunteers, each trapping device contained six marbles handled by one of the two individuals.

In each trial, 30 mosquitoes were released simultaneously from a cage placed at the downwind end of the flight chamber, 1.60 m from the two ports. The trial duration was 15 min. Trapped mosquitoes were counted at the end of the experiments. The odours of each volunteer were tested six times, twice each on three different mornings. Test stimuli were alternated between right and left ports to rule out any positional effects. Experiments without any odour source in either port were performed to test the symmetry of the trapping system. In addition, experiments with ammonia against clean marbles and clean air were performed for control purposes.

Direct comparisons between two individuals were made four times on one specific morning. These experiments were repeated 3–4 times for each pair of volunteers over a 16-month period, from June 2001 to October 2002.

Each trial started with new mosquitoes, clean traps and freshly prepared stimuli. To avoid contaminating the equipment with human volatiles, the operator wore surgical gloves during all experiments. After use, the traps were washed with soapy water (CLY-MAX Heavy Duty Cleaner, Rogier Bosman Chemie B.V., the Netherlands), rinsed with tap water and then cleaned with pure ethanol.

**Electrophysiological experiments**

*Human-handled marbles and their extracts.* On the mornings when odours of paired individuals were tested against one another in the olfactometer, the same individuals also handled small glass marbles (2.9 mm in diameter) for 10 min. Different numbers of handled marbles (8, 16, 24 and 32) were placed into a glass Pasteur pipette and the headspace was blown directly over a mosquito antenna mounted in an EAG measuring circuit (Qiu *et al.*, 2004). The combined surface area of 32 small marbles is equal to that of one large marble (16 mm in diameter) used in the behavioural experiments (see above under ‘Odour stimuli’).

**EAG recordings.** Mosquito head preparations excised from female *An. gambiae* were used for electrophysiological recordings. The excised head was mounted between two glass electrodes filled with 0.1 m KCl. An AgCl-coated silver wire (diameter 0.5 mm) was inserted into a glass capillary (diameter 1.1 mm), placed in a holder, and connected to a DC amplifier (10×; Syntech, Hilversum, the Netherlands). The foramen magnum of a mosquito head was attached to the indifferent electrode, which was grounded. The recording electrode was slid over the tip of the antenna, from which the top segment had been removed. A moistened, charcoal-filtered, continuous air stream (1000 mL/min) was led through a glass tube (diameter 10 mm) ending 0.5 cm from the preparation. Stimulus puffs (4.1 mL in volume) lasting for 0.5 s were applied using a stimulus controller (Syntech) and were injected into the air stream 10 cm from the outlet of the tube delivering the continuous air stream. The amplified potential differences between the electrodes were imported via an IDAC interface box and an A/D converter (Syntech) into an Intel® 486-based personal computer. Recordings were analysed using EAG software Version 2.6 (Syntech).

**Analysis of behavioural data**

For each volunteer, the differences between the number of mosquitoes trapped in the port from which the headspace of the handled marbles was delivered and the number trapped in the port from which ammonia-containing air was delivered were analysed with a generalized linear model (GLM; Binomial, linked in logit; the dispersion was estimated to account for heterogeneity, Genstat, Release 8.11). This method was also used to analyse the results of the tests of symmetry of the olfactometer and of the experiments in which ammonia was tested against clean marbles.

The GLM (Binomial, linked in logit, dispersion estimated) was used to investigate the effect of the individual on two parameters: (1) the relative attractiveness, 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.*, 2004), and (2) 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. Time of testing during the day was included in the model. Two-sided t-probabilities were calculated to test pairwise differences between means. Effects were considered to be significant at $P < 0.05$ (Oude Voshaar, 1994; Sokal & Rohlf, 1998). The results of the experiments with no odour (against no odour) or clean marbles (against ammonia) were included in this GLM analysis. The effects of gender and age on the two parameters mentioned above were also evaluated by a similar GLM model. Because the number of mosquitoes trapped in all experiments was summed in these analyses, the time of testing was not included in the model.

**Analysis of EAG data**

Electroantennogram response was transformed into a percentage relative to the standard stimulus, 0.1% 1-octen-3-ol. Differences between treatments were analysed by GLM univariate procedure (spss for Windows, Release 10.0.5). The effect of individual volunteers was set as a fixed factor in the
linear regression model. The other two factors considered in each model were the number of marbles and different mosquito antennae (replicates). Interactions between the fixed factors were excluded from the model when the interaction effect was not significant. The effect of a fixed factor was tested by the $F$ statistic and was considered to be significant when $P < 0.05$. The differences between means were compared pairwise by Tukey’s honestly significant difference test. Effects were considered significant when $P < 0.05$.

Results

Symmetry of olfactometer

Without odours there was no significant difference in the number of mosquitoes collected in both trapping devices (GLM, $P = 0.757, n = 18$), showing that the olfactometer was symmetrical.

Comparison of skin emanations with ammonia

Ammonia at the chosen dose attracted more mosquitoes than clean marbles (GLM, $P < 0.002$). Skin emanations from 22 of 27 individuals were more attractive than ammonia (GLM, $P < 0.05$). Skin emanations from MO9, FO4, MM15, MY8 and MY14 attracted similar numbers of mosquitoes as ammonia (GLM, $P > 0.05$) (Fig. 1).

Differences between individuals in terms of relative attractiveness

The relative attractiveness of skin emanations from each volunteer when tested against ammonia was significantly higher than that of clean marbles tested against ammonia (GLM, $P < 0.001$). There were clear differences in attractiveness between the volunteers when tested individually (GLM, $P < 0.001$, d.f. $= 26$) (Fig. 1). The relative attractiveness was not affected by gender adjusted for age (GLM, $P = 0.89$) or by age adjusted for gender (GLM, $P = 0.44$).

Differences between individuals in terms of trap entry response

When clean marbles were tested against ammonia, the trap entry response was 41% (Fig. 1), a proportion that was
significantly higher than the 23% observed in the test on symmetry when no odour was offered (GLM analysis, \( P = 0.003 \)). When skin emanations from the volunteers were tested individually against ammonia, the mean trap entry response of all experiments was 51 ± 21\% (Fig. 1; \( n = 162 \)). The latter proportion was significantly higher than found in experiments testing ammonia against clean marbles for five of the 27 volunteers (GLM, \( P < 0.05 \)), namely MO7, MY14, MY19, FM21 and MY25. There were also differences in the trap entry response between the skin emanations from the volunteers (GLM, \( P < 0.001 \)). The trap entry response was affected neither by gender adjusted for age (GLM, \( P = 0.54 \)), nor by age adjusted for gender (GLM, \( P = 0.11 \)).

**Pairwise comparisons between the most and least attractive individuals**

The skin emanations from the three least attractive and three most attractive persons of the same gender were tested pairwise in the olfactometer (FO4 against FO3; MY8 against MO7; MY14 against MM5). In all three pairs, there were significant differences in relative attractiveness and trap entry responses between the two individuals when ammonia was the alternative odour source (Fig. 1). For all three pairs, the skin emanations from the persons who had been found to be the most attractive in the previous experiment (skin emanations from 27 humans tested against ammonia), attracted more mosquitoes than did the skin emanations from the persons who had been found to be least attractive in the former experiments (GLM, \( P < 0.05 \)) (Fig. 1).

We subsequently conducted three series of pairwise tests among these six volunteers over a 16-month period (Fig. 3). Because volunteer MM5 was not available for our later experiments, we replaced him with MM22, who showed high attractiveness in the first set of behavioural assays (Fig. 1). Two pairs of volunteers (FO4, FO3 and MY8, MO7) showed consistent patterns of relative attractiveness, whereas the third pair (MY14 and MM22) did not have results consistent with the results shown in Fig. 1.

**EAG responses**

With regard to the emanations of the two female volunteers differing widely in attractiveness, strongly attractive FO3 and weakly attractive FO4, the EAG responses of female *An. gambiae* to the former showed higher amplitudes than EAG responses to the latter (Fig. 4). Likewise, for emanations from one pair of male volunteers, MO7 and MY8, EAG responses to the more attractive emanations of MO7 were higher than EAG responses to the less attractive emanations of MY8. However, the relative attractiveness of the other two male volunteers, MM22 and MY14, was not consistent in the repeated tests (Figs 3 and 4). Skin volatiles from MY14 were more attractive than those from MM22 in the bioassay that ran simultaneously with the EAG tests, whereas EAG responses to skin emanations from MY14 were lower than EAG responses to emanations from MM22.

**Discussion**

In our experiments, skin emanations from individual volunteers differed significantly in attractiveness to female *An. gambiae* mosquitoes. Because the skin emanations were collected on glass marbles that were tested in the olfactometer, the effect of skin odour alone on differential attractiveness was examined, while physical factors, such as body heat and humidity, were excluded. Using a similar method (collecting human skin emanations on a glass surface), Schreck *et al.* (1990) demonstrated that skin emanations from different individuals vary in attractiveness to another highly anthropophilic mosquito, *Ae. aegypti*. Nevertheless, our results show that the ranking order of the volunteers differs when trap entry response or relative
Variation in human attractiveness to mosquitoes

Our previous research demonstrated that ammonia is attractive to female *An. gambiae* mosquitoes in an olfactometer (Braks et al., 2001; Smalllegange et al., 2005). In the present study, we found a relative attractiveness (Fig. 1) similar to that previously found for the same concentration of ammonia (82.4 ± 17.5%) (Smalllegange et al., 2005). Here, we demonstrate that the skin emanations from most (22 of 27) volunteers were significantly more attractive to *An. gambiae* than ammonia alone. If we had chosen another ammonia concentration for comparison, the proportion of volunteers whose skin emanations were more attractive than ammonia may have been different. Unfortunately, the natural emanation rate of ammonia from human skin is still unknown. The amount of ammonia on the glass beads handled by volunteer FY18 was much lower than the total amount of ammonia injected in the trapping device with the clean beads (personal communication A. M. S. Galimard). This suggests that in addition to ammonia, other chemicals are involved in the attraction of this mosquito species to humans. Meanwhile, the fact that ammonia was as attractive as the odours from several of the volunteers demonstrates again that ammonia is an important kairomone for *An. gambiae*.

The results of the pairwise comparisons confirmed the consistency of the ranking of human individuals in the tests of the attractiveness of their odours relative to that of ammonia (Figs 1 and 2). The order of attractiveness was consistent over time for two of the three pairs of individuals, whereas such consistency was not found for the third pair (MM22 and MY14) (Fig. 3). The more attractive skin emanations from MO7 and FO3 elicited higher EAG responses than the less attractive skin emanations from FO4 and MY8, whereas the less attractive skin emanations from MM22 elicited higher EAG responses than the more attractive skin emanations from MY14 (Fig. 4). It is known that both attractants and repellents elicit depolarization measured as EAG responses (Blackwell et al., 1997). Therefore, a possible explanation for these results is that a human skin emanation is more attractive to mosquitoes either because it contains higher amounts of attractants or because it contains lower amounts of repellents (Skinner et al., 1965; Maibach et al., 1970; Bernier et al., 2002). This explanation needs to be verified in future experiments.

In this study, we demonstrated that chemical cues alone result in differential attractiveness of human subjects to mosquitoes. The differences in odour production by human individuals might be quantitative or qualitative in nature, or a combination of both (Sastry et al., 1980). A comparison of the odour profiles of human individuals with different levels of attractiveness to mosquitoes will contribute towards elucidating the chemical basis of mosquito host-seeking behaviour, by identifying key host-seeking kairomones. These kairomones could be used to improve the efficiency of the existing odour-baited mosquito traps used for the surveillance and control of the malaria mosquito *An. gambiae*. The odour profiles of the three pairs of volunteers with different levels of attractiveness to mosquitoes have been compared by chemical analysis and subsequent statistical analysis. These results will be reported elsewhere (A. M. S. Galimard, unpublished data).

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Fig. 4. Electroantennogram and corresponding behavioural responses to odours from two human individuals with different levels of attractiveness to mosquitoes. The three figures on the left show EAG amplitudes elicited by various numbers of marbles handled by human volunteers relative to a standard stimulus, 0.1% 1-octen-3-ol. The error bars are SE of means (n = 10). The figures on the right present mean relative attractiveness (i.e. the number of mosquitoes caught in a trapping device baited with human skin emanations as a percentage of the total number of mosquitoes caught in both trapping devices) of odours from three pairs of volunteers when tested against one another. Bars indicate mean values and SE based on four experiments. Bars indicated by an asterisk represent significantly higher values of mean relative attractiveness. * = P < 0.05, ** = P < 0.01 (GLM analysis of the number of mosquitoes attracted by odours from two volunteers). The trap entry response (%) and the number of mosquitoes that left the releasing cage (N) are shown.

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