Allomonal effect of breath contributes to differential attractiveness of humans to the African malaria vector *Anopheles gambiae*

Wolfgang R Mukabana*1,2,3, Willem Takken3, Gerry F Killeen4 and Bart GJ Knols5

Address: 1Department of Zoology, University of Nairobi, PO Box 30197, Nairobi, Kenya, 2International Centre of Insect Physiology and Ecology, Mbita Point Research and Training Centre, PO Box 30, Mbita Point, Kenya, 3Laboratory of Entomology, Wageningen University and Research Center, PO Box 8031, 6700 EH Wageningen, The Netherlands, 4Department of Epidemiology and Public Health, Swiss Tropical Institute, Basel, CH-4002, Switzerland and 5Ifakara Health Research & Development Centre, PO Box 53, Ifakara, Tanzania and 6Entomology Unit, Agency's Laboratories Seibersdorf, International Atomic Energy Agency, A-1400 Vienna, Austria

Email: Wolfgang R Mukabana* - rmukabana@uonbi.ac.ke; Willem Takken - willem.takken@wur.nl; Gerry F Killeen - gkilleen@ifakara.mimcom.net; Bart GJ Knols - b.knols@iaea.org

* Corresponding author

Abstract

**Background:** Removal of exhaled air from total body emanations or artificially standardising carbon dioxide (CO₂) outputs has previously been shown to eliminate differential attractiveness of humans to certain blackfly (Simuliidae) and mosquito (Culicidae) species. Whether or not breath contributes to between-person differences in relative attractiveness to the highly anthropophilic malaria vector *Anopheles gambiae* sensu stricto remains unknown and was the focus of the present study.

**Methods:** The contribution to and possible interaction of breath (BR) and body odours (BO) in the attraction of *An. gambiae* s.s. to humans was investigated by conducting dual choice tests using a recently developed olfactometer. Either one or two human subjects were used as bait. The single person experiments compared the attractiveness of a person's BR versus that person's BO or a control (empty tent with no odour). His BO and total emanations (TE = BR+BO) were also compared with a control. The two-person experiments compared the relative attractiveness of their TE, BO or BR, and the TE of each person against the BO of the other.

**Results:** Experiments with one human subject (P₁) as bait found that his BO and TE collected more mosquitoes than the control (P = 0.005 and P < 0.001, respectively), as did his BO and the control versus his BR (P < 0.001 and P = 0.034, respectively). The TE of P₁ attracted more mosquitoes than that of another person designated P₈ (P < 0.021), whereas the BR of P₈ attracted more mosquitoes than the BR of P₁ (P = 0.001). The attractiveness of the BO of P₁ versus the BO of P₈ did not differ (P = 0.346). The BO from either individual was consistently more attractive than the TE from the other (P < 0.001).

**Conclusions:** We demonstrated for the first time that human breath, although known to contain semiochemicals that elicit behavioural and/or electrophysiological responses (CO₂, ammonia, fatty acids) in *An. gambiae* also contains one or more constituents with allomonal (~repellent) properties, which inhibit attraction and may serve as an important contributor to between-person differences in the relative attractiveness of humans to this important malaria vector.
Background
Mosquitoes in search of a blood meal integrate information from host-related visual, physical and chemical cues during the host-seeking process [1-3]. Vision is considered more important among diurnally active mosquitoes [3], whereas physical and olfactory cues are the dominant cues for nocturnal species [1,2]. Many species of blood-feeding insects display non-random host selection at the intraspecific level and, although this has important epidemiological implications, the evolutionary basis for this selection remains poorly understood [4].

Host odour is one of the components influencing host choice. For example, the sandfly Lutzomyia longipalpis responds to hand odour from different humans at significantly different rates [5], and attraction of Simulium blackfly species to total human emanations varies depending on the individual person used as the source of kairomones [6]. Attractiveness of a human arm and hand odour to Anopheles stephensi [7], Aedes aegypti [8-10] and An. quadrimaculatus [10] has been shown to vary substantially between individual human baits. The response of members of the An. gambiae complex to individual humans also varies considerably [11-13], and these intra-specific differences can be observed in traps baited with total body emanations, including those from which the body heat component has been excluded [14,15]. Recently it was shown that either removal of exhaled air from total emanations [5] or artificially standardising CO2 outputs [15] eliminates differential attraction of humans to blackflies and mosquitoes, respectively.

We used a recently developed multi-choice olfactometer [16] to investigate how breath and body odour contribute to and might possibly interact as components of the attractiveness of humans to An. gambiae s.s., one of the most anthropophilic, abundant and efficient vectors of malaria in Africa. Previous work with this system enabled us to rank the attractiveness of nine male Kenyans [16], two of whom were involved in the experiments reported here.

Materials and methods
Mosquitoes
Experiments were conducted using the Ifakara strain of laboratory-reared Anopheles gambiae Giles sensu stricto, originally colonized from wild-caught gravid females in Ngage, South-east Tanzania, in 1996. The mosquito larvae were reared under ambient temperature and light conditions in screenhouse insectaries at the Mbila Point Research and Training Centre of the International Centre of Insect Physiology and Ecology (00°25’S, 34°13’E). The larvae were reared using fresh water from Lake Victoria and were fed on Tetramin® fish food three times per day (the total amount of food provided was 0.3 grams tetramin®/100 larvae/day). Pupae were collected from rearing trays and transferred to an insectary where they were kept in mesh-covered 30 cm cubic cages in which rolls of filter-paper soaked in 6% glucose solution were provided. The colony was maintained by routinely offering a human arm to feed upon. Adult females with no prior access to blood were used for experiments when they were four to eight days old and were transferred from the holding cages into release cups six hours before the onset of experiments. Only water-wet cotton wool pads were provided as liquid source on the mesh-topped open ends of the release cups. For further details see [16].

Experimental set-up
Experiments were conducted using two arms of a previously described three-port olfactometer (Figure 1), situated within a large semi-field screenhouse where the ambient atmospheric conditions were not controlled (for details see [16]). Approximately 100 mosquitoes (the exact number was recorded for each experimental release), released into a choice chamber located ~1 metre away from the participants, were used for each experiment. Mosquitoes flying upwind in response to host stimuli were caught in traps, without a chance of entering the tents (for details see [16]).

Human subjects
Two healthy African males, designated person P₁ and person P₈ (numbers refer to the same individuals used in our previous study [16]), were recruited to participate in the experiments. P₁ was aged nineteen years (weight, 80 kg; height, 1.80 m), P₈ was aged 22 years (weight, 79 kg, height 1.85 m). The participants wore only shorts at the time of the experiment and bathed with non-perfumed soap one hour before starting the experiments. No attempt was made to control their daily diet except prohibiting them from ingesting alcohol, a factor that has recently been shown to influence the relative attractiveness of humans for Aedes albopictus [17]. Their malaria infection status was observed daily by microscopic examination of thin and thick smears of finger-prick blood stained with Giemsa. Previous work has demonstrated that P₁ is nearly three times (P < 0.05) more attractive to An. gambiae than P₈ with the mean number of mosquitoes caught during experiments being 20.14 ± 3.17 and 6.78 ± 1.01, respectively [16].

Attraction to total emanations, body odour and breath
P₁ was recruited to assess the response of mosquitoes to his total body emanations (TE) or either his breath (BR) or body odour (BO) alone. In this context TE refers to BR plus all volatile discharges of the skin and BO refers to volatiles discharged solely from the skin. BR and BO were separated using a one-way breathing valve (Harvard-Douglas®). The test person wore the breathing valve as a...
mouthpiece and fitted a sprung nose clip so that he inhaled and exhaled air through the mouth only. Thus, he inhaled air from within the confines of the screen house via a polyvinyl chloride (PVC) pipe and exhaled it via a bendable, corrugated PVC pipe. The BR was discharged to a destination dictated by the needs of each experiment (Figure 1). Separated BR was either recombined with BO to reconstitute the TE (Experiment 1), diverted to the other tent exhaust (Experiment 2), or vented from the apparatus completely through the main air exhaust (Experiment 3).

The first of these three alternative arrangements allowed the response of mosquitoes to TE to be compared with that to a control tent lacking a bait host or body emanations. The second and third of these three arrangements allowed the attractiveness of BO to be compared with BR and with a control tent lacking a bait host or body emanations. The attractiveness of BR, compared with an empty tent, was also assessed. In that case the test person sat outside the tents but exhaled into one of the tents' exhausts (Experiment 4). Experiments were conducted over 30-min test periods, between 19.30–20.00 and 20.30–21.00 hours. After each experiment all mosquitoes were removed from the apparatus and the number trapped counted. Each of the four possible arrangements were repeated 16 (Experiments 1 and 2) or eight (Experiments 3 and 4) times with the human bait switching position so that he occupied each of the two tents for half of the replicates per experiment. Previous experiments found no effect of residual odours on the behavioural responses of An. gambiae [16].

The role of breath and body odour in between-person differences in relative attractiveness

Behavioural responses of mosquitoes as a result of simultaneous exposure to emanations originating from two human subjects were assessed to determine their relative attractiveness when BR was included or excluded from their TE. BR was removed from the apparatus using a one-
way breathing valve as shown in path 3 (Figure 1). Inclusion of BR did not involve re-direction as shown in path 1; the human subject occupied the tent without using the breathing valve. The following choice tests were carried out: (i) TE of person P1 versus TE of person P8 (Experiment 1), (ii) BR of person P1 versus BR of person P8 (Experiment 2), (iii) BO of person P1 versus BO of person P8 (Experiment 3), (iv) BO of person P1 versus TE of person P8 (Experiment 4) or (v) BO of person P8 versus TE of person P1 (Experiment 5). Experiment 2 was conducted with both participants outside the tents, each of them using a breathing valve in order to direct his BR to a separate tent exhaust. The number of mosquitoes trapped in all comparisons as a result of responses to stimuli originating from person P1 or P8 were counted and noted. Experiments were conducted thrice per night between 19.30–20.00, 20.30–21.00 and 21.30–22.00 hours. Each of the five experiments was repeated 12 or 18 times with the two human subjects switching between the two tents so that half of the tests were conducted with each subject in the two alternative tents. The number of replicates conducted for each experiment is shown in Figure 3.

**Statistical analysis**

Relative attractiveness was calculated as the number of mosquitoes trapped by the emanations of P1 divided by the sum of the number trapped by P1 and P8, thus representing the relative attractiveness of person P1. A relative attractiveness of greater than 0.5 indicates greater attractiveness of person P1, whereas values smaller than 0.5 indicate greater attractiveness of P8. Non-parametric statistical methods were used for analysis because of their robustness and flexibility. The significance of differences in attractiveness between the baits in the two traps in each experiment was assessed by Kendall’s W test for related samples, comparing the catches of person P1 with those of person P8 in the same experiment. The significance of changes in relative attractiveness between experiments was assessed by the Kruskal-Wallis H test for independent samples, comparing the relative attractiveness estimates from repetitions of the same experiments with those of another. In all cases the number of repetitions or releases for each experiment is denoted by N, whereas the total number of mosquitoes which were actually trapped by either human bait is denoted by n. Analysis of the attractiveness of BO of person P1 versus his BR or the control, as well as the attractiveness of his TE or BR versus the control followed the same procedures. Data were analysed using the Statistical Products and Service Solutions (SPSS, version 10.0).

**Results**

Experiments were carried out over a total of forty-eight nights, 24 nights for experiments involving the two human subjects and 24 nights for experiments concerning the single participant. None of the participants presented with malaria parasites over the duration of the study.

**Response to total emanations, body odour and exhaled air**

The attractiveness of the BO of person P1 versus his BR or a control, as well as the attractiveness of his TE or BR versus a control, are shown in Figure 2. His BO (P = 0.005) and TE (P < 0.001) were significantly more attractive than a control (empty tent) as was his BO (P < 0.001) and the control (P = 0.034) over his BR.

**The role of breath and body odour in between-person differences in relative attractiveness**

The between-person differences in relative attractiveness, which was measured with respect to person P1 (i.e. the number of mosquitoes trapped by emanations of person P1 divided by the sum of the number of mosquitoes trapped by emanations of person P1 and person P8) following inclusion or exclusion of BR or BO from their TE, are shown in Figure 3. Person P1 was more attractive than person P8 based on mosquito responses to their TE (P = 0.021) whereas person P8 was more attractive than person P1 based on responses to their BR (P < 0.001). There was no significant difference in the attractiveness of the two persons based on responses to their BO (P = 0.346). The BO of person P1 was more attractive than the TE of person P8 (P = 0.001) and the BO of person P8 was more attractive than the TE of person P1 (P = 0.001). Thus TE without BR (=BO), from either individual, was consistently more attractive than TE from the other. Comparisons of the relative attractiveness of the study subjects between experiments (lower section of Figure 3) were all significant except between experiment 1 and experiment 3 (P = 0.253).

**Discussion**

The behavioural response of *An. gambiae*, as assessed through choice experiments making all possible dual comparisons by total emanations, body odours, breath and a control originating from a single human subject, demonstrated an allomonal effect of breath and an overall kairomonal effect of body odours and total emanations. Whereas the allomonal effect of breath was not known previously, total emanations have been shown to be responsible for over 90% of the attractiveness of humans to *An. gambiae* s.l. [18].

Surprisingly, there was no difference in the number of mosquitoes attracted by the two persons, who were otherwise consistently different in their attractiveness (see [16]), when breath was excluded from their total emanations. Since host seeking is modulated by olfactory cues [1,2], and *An. gambiae* preferentially responds to human rather than other vertebrate-host cues/odours [19,20], regardless of a person’s degree of attractiveness, our
Relative attractiveness (RA) of total emanations (TE) of test person P1 versus a control (Expt. 1), his body odour (BO) versus his breath (BR) (Expt. 2), his body odour versus a control (Expt. 3) and his breath versus a control (Expt. 4).

**Figure 2**
Relative attractiveness (RA) of total emanations (TE) of test person P1 versus a control (Expt. 1), his body odour (BO) versus his breath (BR) (Expt. 2), his body odour versus a control (Expt. 3) and his breath versus a control (Expt. 4). N: number of replicates, n: total number of mosquitoes collected by both treatments in each experiment. P: statistical significance level of (i) differences between the catches of treatment A and treatment B (RA = 0.5) or (ii) the change in the relative attractiveness of treatment A between different experiments (RA1 = RA2).
Figure 3
Relative attractiveness (RA) of person P₁ in choice experiments evaluating mosquito responses to total emanations (TE) of P₁ versus total emanations of person P₈ (Expt. 1), breath (BR) of P₁ versus breath of P₈ (Expt. 2), body odour (BO) of P₁ versus body odour of P₈ (Expt. 3), body odour of P₁ versus total emanations of P₈ (Expt. 4) and total emanations of P₁ versus body odour of P₈ (Expt. 5). N: number of replicates, n: total number of mosquitoes collected from both treatments in each experiment. P: statistical significance level of (i) differences between the catches of P₁ and P₈ (RA = 0.5) or (ii) the change in the relative attractiveness of P₁ between different experiments (RA₁ = RA₂).
present results suggest that breath is a key factor responsible for variability in human attractiveness to An. gambiae. Our data also show a clear interaction between components of breath and body odour as the attractiveness of person P1, who was significantly more attractive than person P2 based on responses to their total emanations, was reversed when mosquitoes were allowed to make choices between their breaths.

These findings corroborate findings for other blood-feeding insects: removal of breath from total emanations has been shown to eliminate individual differences in attractiveness of humans to Simulium species [6] and artificially standardising outputs of carbon dioxide, a major component of breath, has been shown to equalise human attractiveness to An. gambiae s.l. and An. funestus [15].

Human breath has been reported to be attractive to Anopheles mosquitoes [21,22] and Aedes aegypti [23]. Krotoszynski et al. [24] identified 102 organic compounds of endogenous and exogenous origin in human breath, obtained from a group of 28 carefully selected healthy individuals. Since then several hundred additional volatile organic compounds have been identified [25]. Carbon dioxide is by far the most abundant compound, and 97% of the remaining chemicals have a mean concentration between 0.06 and 9.5 ng L⁻¹. Acetone, isoprene and acetoinitrile, with concentrations of 120, 33 and 24 ng L⁻¹, respectively, account for 51% of the mean organic contents. Compounds of bacterial origin, such as dimethylsulphide or methanethiol have also been found [26].

Several of the above compounds have been shown to influence the host-seeking process of An. gambiae. CO₂, for example [27], contributes to the overall attractiveness of humans in the range of 9–40% [18,28] and human equivalents (300–500 ml min⁻¹) yield significant responses both in the laboratory [29,30] as well as in the field [31]. Acetone, present in concentrations ranging from 293–870 ppb [32], has also been shown to affect An. gambiae behaviour [33], as does ammonia [34,35], for which concentrations of 422–2389 ppb have been recorded from breath samples [32]. The precise nature of such behavioural responses remains largely unknown, and breath has only been shown to be directly responsible for influencing the selection of a landing/biting site by An. atroparvus [36] and An. albimanus [37].

Considering the importance of breath in affecting host selection by An. gambiae, three interesting findings emerge. First, given the minute concentrations at which most of these compounds occur, one or more very potent repellent(s) may be isolated, microgrammes of which may suffice to inhibit the host-seeking response. Second, given the intense exchange of metabolic gases at the alveolar interface between the bloodstream and lung cavity, malarial disease may alter the composition of exhaled breath and as such affect the host-selection process [38]. Third, our results show that mosquito host selection is not just a matter of ‘attractiveness’, but also determined by a person’s repellency, the sum of which may affect the threshold level for a mosquito to initiate close-range and biting behaviour once near a host.

**Conclusions**

We have identified an allomonal effect of human breath for An. gambiae s.s. and shown this to contribute to between-person differences in relative attractiveness. Unfortunately, whereas factors that might be responsible for the allomonal effects remain unknown, it is interesting to contrast our observations with those who have noted that CO₂, a major component of breath, is a potent activator for An. gambiae [27]. It is also interesting to note that the orientation behaviour of such a specialised human-feeding mosquito as An. gambiae is inhibited by breath and that this species prefers to bite the ankles and feet of its chosen host [36]. We hypothesise that the allomonal properties of human breath combined with the attraction to such extremities as the feet and ankles may represent a mechanism that facilitates successful, undetected feeding by An. gambiae upon their favoured human hosts.

**Authors’ contributions**

WRM designed the olfactometer, conducted all the experimental work, and drafted the original manuscript. GFK assisted with statistical data analysis and interpretation. BGJK conceived the study, obtained funding for it, supervised the experimental work and edited the final version of the paper in collaboration with WRM, GFK and WT.

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