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Research

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

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Abstract

Background: Removal of exhaled air from total body emanations or artificially standardising carbon dioxide (CO_2) 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 (P_1) 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 P_1 attracted more mosquitoes than that of another person designated P_8 ($P < 0.021$), whereas the BR of P_8 attracted more mosquitoes than the BR of P_1 ($P = 0.001$). The attractiveness of the BO of P_1 versus the BO of P_8 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 (CO_2, 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 intra- and interspecific 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 CO_2 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 Njage, South-east Tanzania, in 1996. The mosquito larvae were reared under ambient temperature and light conditions in screenhouse insectaries at the Mbita 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 P_1 and person P_8 (numbers refer to the same individuals used in our previous study [16]), were recruited to participate in the experiments. P_1 was aged nineteen years (weight, 80 kg; height, 1.80 m), P_8 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 P_1 is nearly three times ($P < 0.05$) more attractive to *An. gambiae* than P_8 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

P_1 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

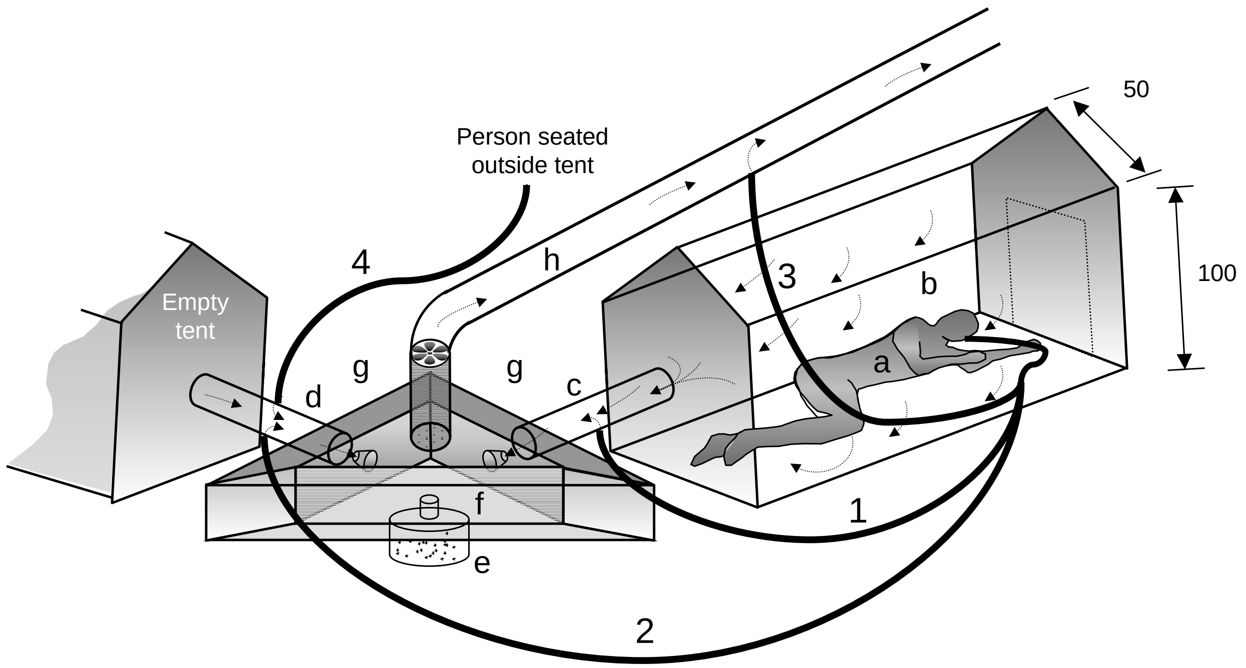


Figure 1
Apparatus used to study the response of *An. gambiae s.s.* mosquitoes to human breath, body odours and a combination thereof. Breath was separated from body odours using a one-way breathing valve and diverted to the exhaust of the same (1) or other tent (2), or vented out through the main air exhaust (3). Alternatively, the test person sat outside the tent and his breath was diverted to one of the tent exhausts (4). a: test person, b: tent, c,d: tent exhaust, e: mosquito release cup, f: choice chamber, g: trapping chamber, h: main air exhaust. Dimensions shown are in cm. Broken arrows depict the direction of movement of air currents. Further descriptive details of the experimental set up see [16].

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 P_1 versus TE of person P_8 (Experiment 1), (ii) BR of person P_1 versus BR of person P_8 (Experiment 2), (iii) BO of person P_1 versus BO of person P_8 (Experiment 3), (iv) BO of person P_1 versus TE of person P_8 (Experiment 4) or (v) BO of person P_8 versus TE of person P_1 (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 P_1 or P_8 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 P_1 divided by the sum of the number trapped by P_1 and P_8, thus representing the relative attractiveness of person P_1. A relative attractiveness of greater than 0.5 indicates greater attractiveness of person P_1, whereas values smaller than 0.5 indicate greater attractiveness of P_8. 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 P_1 with those of person P_8 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 P_1 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 P_1 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.

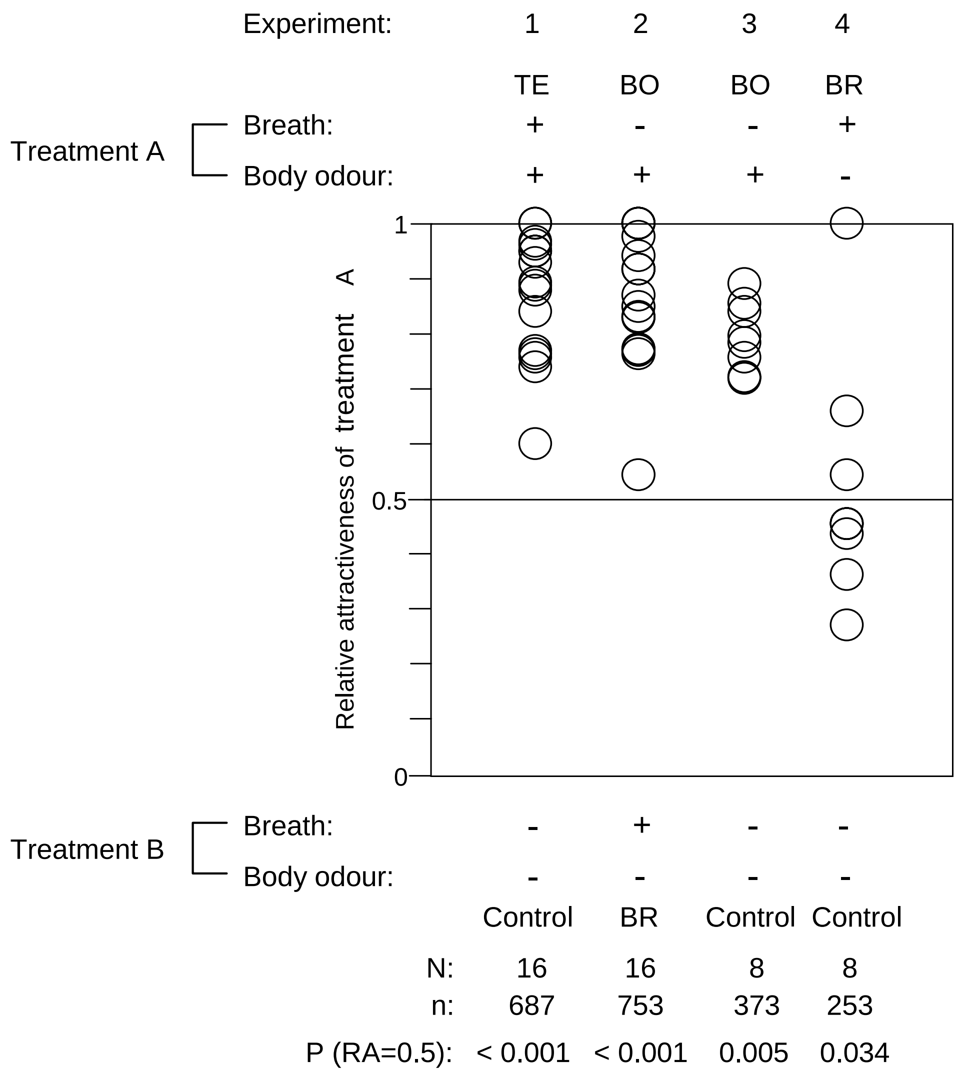
The role of breath and body odour in between-person differences in relative attractiveness

The between-person differences in relative attractiveness, which was measured with respect to person P_1 (i.e. the number of mosquitoes trapped by emanations of person P_1 divided by the sum of the number of mosquitoes trapped by emanations of person P_1 and person P_8) following inclusion or exclusion of BR or BO from their TE, are shown in Figure 3. Person P_1 was more attractive than person P_8 based on mosquito responses to their TE ($P = 0.021$) whereas person P_8 was more attractive than person P_1 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 P_1 was more attractive than the TE of person P_8 ($P = 0.001$) and the BO of person P_8 was more attractive than the TE of person P_1 ($P = 0.001$). 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

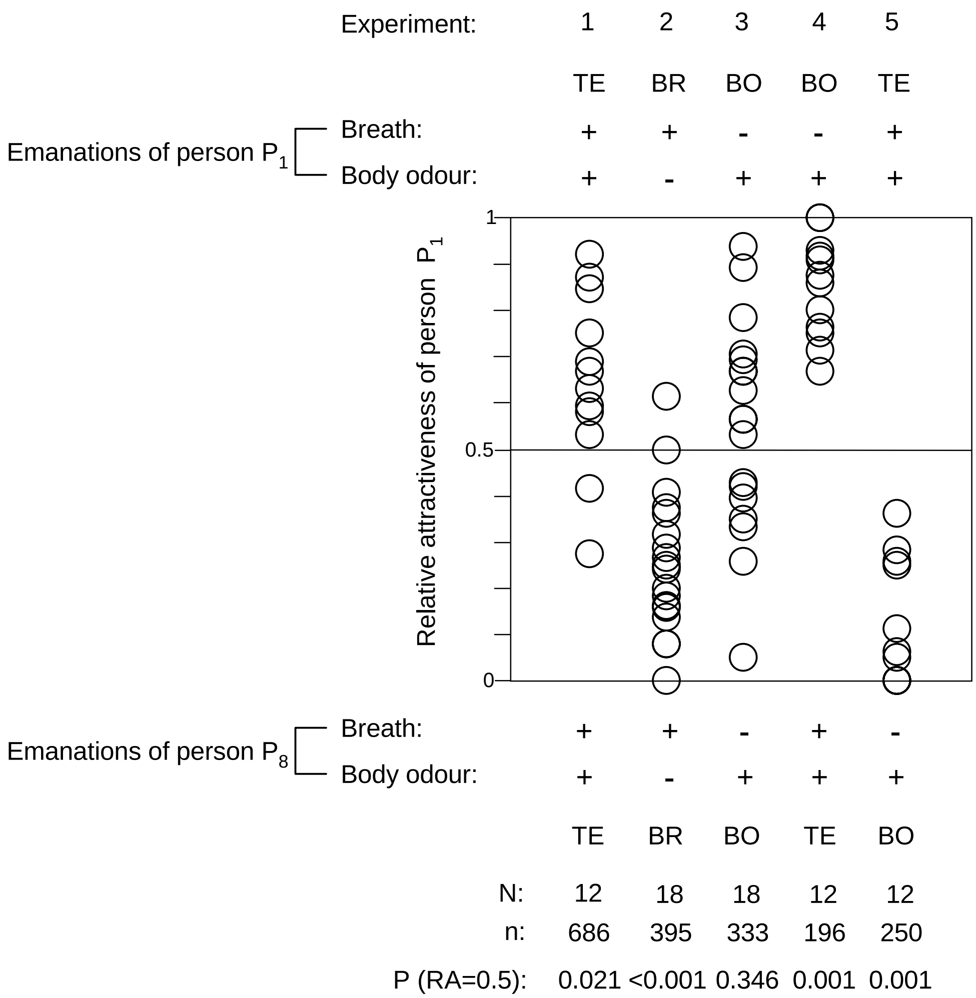


P ($RA_1 = RA_2$):

RA_1 \ RA_2	2	3	4
1	0.734	0.071	0.003
2		0.050	0.003
3			0.012

Figure 2

Relative attractiveness (RA) of total emanations (TE) of test person P_1 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 ($RA_1 = RA_2$).



P ($RA_1 = RA_2$) :

RA_1 \ RA_2	2	3	4	5
1	<0.001	0.253	0.006	<0.001
2		<0.001	<0.001	<0.017
3			<0.001	<0.001
4				<0.001

Figure 3
Relative attractiveness (RA) of person P_1 in choice experiments evaluating mosquito responses to total emanations (TE) of P_1 versus total emanations of person P_8 (Expt. 1), breath (BR) of P_1 versus breath of P_8 (Expt. 2), body odour (BO) of P_1 versus body odour of P_8 (Expt. 3), body odour of P_1 versus total emanations of P_8 (Expt. 4) and total emanations of P_1 versus body odour of P_8 (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 P_1 and P_8 (RA = 0.5) or (ii) the change in the relative attractiveness of P_1 between different experiments ($RA_1 = RA_2$).

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 P_1, who was significantly more attractive than person P_8 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]. Krotozynski 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 acetonitrile, 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*. CO_2, 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 CO_2, 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 hypothesize 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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