

Evolutionary lability of odour-mediated host preference by the malaria vector *Anopheles gambiae*

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Summary

Many species of disease-vector mosquitoes display vertebrate host specificity. Despite considerable progress in recent years in understanding the proximate and ultimate factors related to non-random host selection at the interspecific level, the basis of this selection remains only partially understood. *Anopheles gambiae sensu stricto*, the main malaria vector in Africa, is considered a highly anthropophilic mosquito, and host odours have been shown to play a major role in the host-seeking process of this species. Studies on host preference of *An. gambiae* have been either conducted in controlled conditions using laboratory reared mosquitoes and worn stockings as host-related stimuli, or have been done in the field with methods that do not account for internal (e.g. age of sampled mosquitoes) and/or environmental effects. We explored differential behavioural responses to host odours between two populations of the same sibling species, *An. gambiae* in semi-field conditions in Burkina Faso. The behavioural responses (i.e. degree of activation and strength of anemotaxis) were investigated using a Y-olfactometer designed to accommodate whole hosts as a source of odour stimuli. Two strains of *An. gambiae* (3 to 4-day-old female) from laboratory Kisumu strain, and from field-collected individuals were confronted to combinations of stimuli comprising calf odour, human odour and outdoor air. In dual-choice tests, field mosquitoes chose human odour over calf odour, outdoor air over calf odour and responded equally to human and outdoor air, while laboratory mosquitoes responded equally to human and calf odour, human odour over outdoor air and calf odour over outdoor air. Overall, no effect of CO₂ exhaled by humans and calves neither on the proportion of activated mosquitoes nor on the relative attractiveness to odour stimuli was found. We report for the first time an intraspecific variation in host-odour responses. This study clearly suggests that there may be genetic polymorphism underlying host preference and emphasizes that the highly anthropophilic label given to *An. gambiae s.s.* must be carefully interpreted and refer to populations rather than the whole sibling species.

keywords *Anopheles gambiae*, host preference, anthropophily, olfaction, carbon dioxide, intraspecific variations

Introduction

Knowledge of the degree of contacts between humans and malaria vectors is crucial to predict the intensity of disease transmission (Dye 1992). The frequency of contacts depends on the host choice by the vector, which is influenced by the innate host preference of the insect and environmental factors (e.g. host availability, accessibility) (Lehane 2005). *Anopheles gambiae* Giles *sensu stricto*, one

of the seven sibling species of the *An. gambiae* complex, is the principal vector of malaria in sub-Saharan Africa. The great ability of *An. gambiae s.s.* to transmit the malarial parasite is mainly determined by a strong preference for humans as a source of bloodmeals (Besansky *et al.* 2004).

Many studies have demonstrated that host odours play a major role in *An. gambiae* host choice (e.g. Costantini *et al.* 1996, 1998; Dekker *et al.* 2001a; Duchemin *et al.* 2001; Pates *et al.* 2001). In the laboratory, the preference

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for human odour by *An. gambiae s.s.* has been demonstrated by testing the response of mosquito females to a choice of human odour, cow odour, or clean air in an olfactometer (Dekker *et al.* 2001a; Pates *et al.* 2001).

Natural populations of *An. gambiae s.s.* can show varying degrees of anthropophily (e.g. Diatta *et al.* 1998; Duchemin *et al.* 2001), challenging the notion that *An. gambiae s.s.* should be considered as a strictly anthropophilic species. However, it has been argued that such variations are presumably driven mainly by environmental factors (Zwiebel & Takken 2004). We know little about the intraspecific variability of host preference under controlled conditions, as no study has as yet explored the differential response to host odours between different populations of the same species of the *An. gambiae* complex. Like any other phenotypic traits, host preference can evolve (Futuyma 1998; Gillies 1964). It is therefore likely that two populations of the same species, living in different environmental conditions (e.g. different vertebrate host diversity, laboratory conditions), can be under divergent selective pressures shaping their level of host specificity. Here, we investigated this idea by testing the degree of activation and the anemotactic response of a natural population and a laboratory strain of *An. gambiae s.s.* with an olfactometer designed to accommodate a human and a calf as a source of alternative odour stimuli.

Materials and methods

Mosquitoes

Experiments were conducted using a laboratory strain of *An. gambiae s.s.* (KIS, originating from Kisumu, Kenya, in 1978), and the F₁ progeny of field-collected *An. gambiae* from the Vallé du Kou (VK, southwestern Burkina Faso, see Dabire *et al.* 2006 for details on this location). In this area, the *An. gambiae* complex is composed almost exclusively of *An. gambiae s.s.* (Baldet *et al.* 2003; Diabaté *et al.* 2003). During the experimental period, we however identified (according to the methodology described in Scott *et al.* 1993) 280 field-collected *An. gambiae* to confirm these previous results. We found 277 *An. gambiae s.s.* and only three *An. arabiensis* (T. Lefèvre, L.C. Gouagna, K.R. Dabire, E. Elguero, D. Fontenille, C. Costantini, F. Thomas, unpublished observation). The mosquitoes were reared at 25°C in the insectary. Gravid females were allowed to lay eggs on cups placed inside mesh-covered cages. Eggs were dispensed into plastic trays containing fresh water collected from mineral spring. Larvae were kept in these pans at densities of 100–150 per tray and fed with Tetramin Baby™ (Tetra Werke, Melle, Germany).

Pupae were collected daily and kept in cages containing 6% glucose solution on cotton wool pads. Groups of 3 to 4-day-old adult female mosquitoes without prior access to a blood meal were randomly collected from the rearing cage 6–8 h before the start of the experiments, and placed in a paper cup covered by a gauze.

Experimental procedures

A Y-tube olfactometer of similar design of that of Geier and Boeckh (1999) made with glass was used. The experimental set-up consisted of a source of alternative host odours connected to two collecting boxes (30 × 30 × 30 cm), the Y-shaped flying tunnel, and a release box (30 × 30 × 30 cm) at the downwind end of the olfactometer (Figure 1). Host odours were collected from two polythene tents connected to the two arms of the olfactometer by polythene lay-flat tubing. Fans drew air from the tents to the olfactometer, providing the odour-laden air current against which mosquitoes were induced to fly. Gauze was placed at the junction of the lay-flat tubing with the collecting boxes to restrain responding mosquitoes inside the boxes and avoid that they flew up into the tubing and into the tents. The tents were located outdoors and the olfactometer inside an experimental room. The air speed in the downwind arm of the Y-tube olfactometer was regulated at 20 cm/s using a 435-4 Testo® multi-functional meter (Testo, Forbach, France) equipped with a probe for degree of turbulence [range: 0 to +5 m/s, accuracy: ±(0.03 m/s + 4% of mv)].

One hundred mosquitoes were released into the downwind box and allowed to respond during 30 min. During this timeframe, mosquitoes that were activated by the stimuli left the downwind box and flew upwind into the collecting boxes from which they were retrieved.

Four odour treatment combinations were tested: outdoor air *vs.* outdoor air (O-O), human odour *vs.* outdoor air (H-O); calf odour *vs.* outdoor air (C-O) and, human odour *vs.* calf odour (H-C). Combination O-O was implemented to test whether any bias existed in the symmetry of the response; this test was replicated six times on different days for each mosquito population. The outdoor air treatment consisted of an empty tent with the four side walls rolled up, so that outdoor air was drawn into the olfactometer. In the case of treatment combinations H-O and H-C, the human volunteers acting as odour sources sat on a chair inside the tent, stripped to the waist, and did not use any perfume prior to testing. Participants were volunteer Burkinabe males (aged 20–25 years) from our programme staff. Concerning calf odour, we used zebu of similar mass as that of human volunteers (60–85 kg). Before starting a test, the calf was anchored with

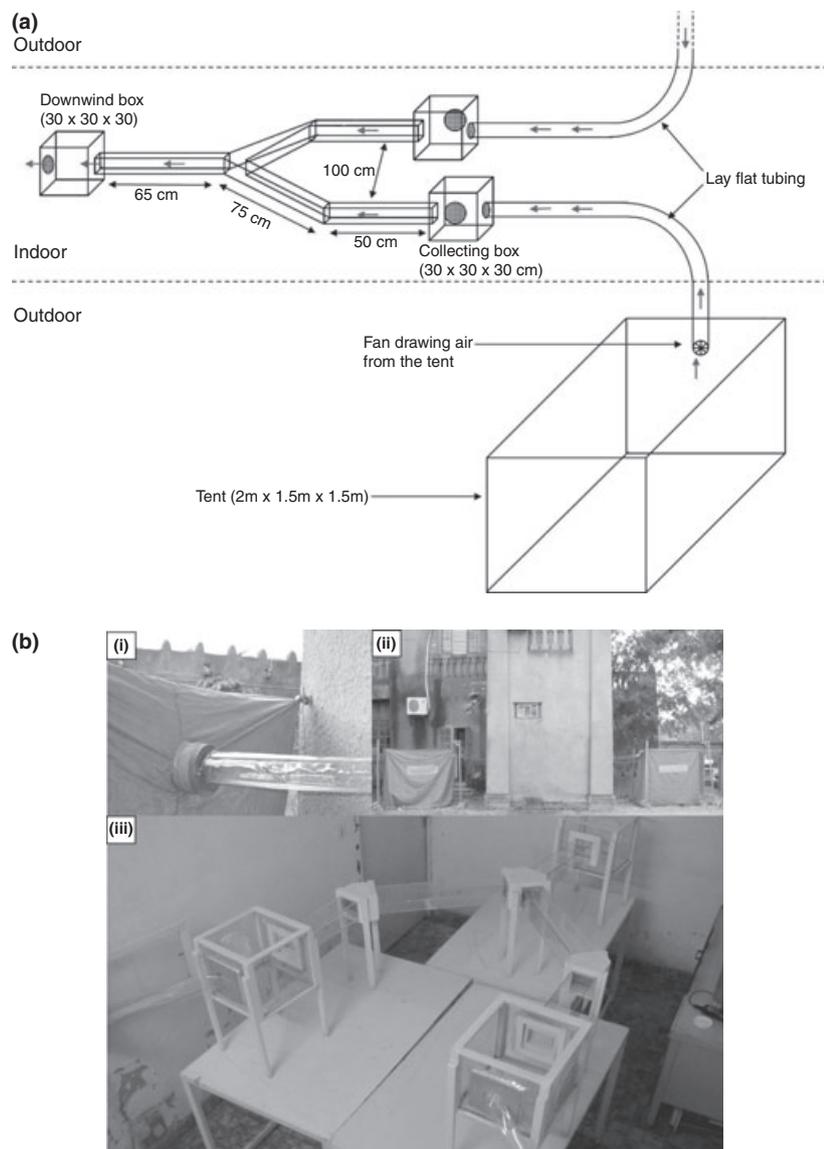


Figure 1 (a). Schematic representation of the Y-olfactometer. The small arrows indicate the odour plume direction. (b). Photos of the device. (i) Fan drawing air from a tent to the olfactometer via lay-flat tubing. (ii) The two polythene tents sets-up outdoors and connected to the two downwind arms of the olfactometer by polythene lay-flat tubing, and the olfactometer room located between the two tents. (iii) The Y-olfactometer with the downwind box (background) and the two collecting boxes (foreground) connected to the tents by lay-flat tubing.

a rope to limit its movements but not prevent it from standing up or lying down.

On each testing day, three combinations were tested: H-O, H-C and C-O, in an independently randomized order. The first treatment combination of the series was tested at 1900 h (sunset, i.e. early scotophase), and the last one at 2100 h. Odour stimuli (human odour, calf odour and outdoor air) were alternated between the right

and left arm to allow for any positional effect. Different combinations of calves and humans were used as odour sources on each testing day to obviate any host effect (i.e. different combinations from a total of 12 participants and six calves). Each batch of mosquitoes was tested once, so that a fresh batch of naive mosquitoes was used per treatment combination. The KIS and VK populations were tested in parallel between May and August, 2007.

At the end of a test, the mosquitoes inside each of the two upwind collecting boxes were removed with an aspirator and counted. Two categories of response were assessed: the degree of activation and the strength of anemotaxis, both of which are part of the behavioural sequence leading a mosquito towards its host, and through which host preferences are expressed. On each occasion, the CO₂ concentration in the two boxes was measured using a 435-4 Testo® multi-functional meter equipped with an indoor air quality probe [range: 0 to +10 000 ppm CO₂, accuracy: $\pm(50 \text{ ppm CO}_2 \pm 2\% \text{ of mv})$ (0 to +5000 ppm CO₂)]. The experimental room was at ambient temperature ($28 \pm 2^\circ\text{C}$) and relative humidity ($80 \pm 10\%$). After each trial, the olfactometer was washed with detergent and 70% alcohol to remove odour contaminants left from previous tests. Similarly, latex gloves were worn by the experimenter to avoid contamination of the equipment. The study received formal ethical approval from the national ethical committee of Burkina Faso.

Statistical analysis

Logistic regression by Generalized Linear Models (GLM; binomial errors, logit link; analyzed with the software R version 2.5.1, <http://www.r-project.org/>) were fitted to the data to investigate the effect of the treatments on two behavioural responses:

- (i) Activation, expressed as the proportion of mosquitoes caught in both collecting boxes out of the total number released in the downwind box; this is a measure of how many mosquitoes were induced to take off and fly upwind, leaving the downwind box (Gillies 1980).
- (ii) Relative attractiveness, expressed as the proportion of mosquitoes caught in one collecting box out of the total number retrieved from both collecting boxes; this is a measure of the relative strength of the anemotactic response as modulated by the host odours.

The influence of several other explanatory covariables on the mosquito responses were investigated by including these in the binomial models: influence of mean CO₂ concentration in the device on activation; influence of difference in CO₂ concentration between the collecting boxes on relative attractiveness; position of treatment (i.e. whether the odour was released from the left or right arm of the olfactometer); time of release. We also investigated the between-days variability in mosquito activation by comparing the observed variance with the expected variance under the binomial error distribution.

Results

Baseline response to outdoor air

The symmetry of the response between arms of the olfactometer was tested on 12 days (six for each mosquito strain). In the presence of an airflow without odour cues, the number of VK mosquitoes that left the downwind release box (i.e. activation) was about twice that of KIS mosquitoes (19% *vs.* 9%, respectively; odds ratio (OR) = 2.45, 95% confidence interval (CI) = [1.72, 3.47]; $P < 0.001$) (Figure 2a). When the O-O treatment combination was presented, equal numbers of mosquitoes entered on average each collecting box (OR = 1.03; CI = [0.76, 1.41]; $P = 0.80$), showing that the experimental apparatus did not produce any inherent bias in the response to the tested stimuli (Figure 2b).

Activation in response to host odours

The three host odour treatment combinations (H-O, C-O, and H-C) activated on average twice as many KIS mosquitoes as the O-O control combination (OR = 2.48; CI = [1.83, 3.37]; $P < 0.001$). Activation was not different between the test (H-O) and (H-C) but higher than in (C-O) (OR = 1.25, CI = [1.05, 1.49], $P = 0.012$) (Figure 2a).

Conversely, only the H-O treatment combination, exposing mosquitoes to human odours only, activated significantly more (1.5-fold) VK mosquitoes than the O-O control and the two other treatment combinations C-O and H-C (OR = 2.17; CI = [1.74, 2.72]; $P < 0.001$). Activation did not differ between combinations O-O, C-O and H-C (Figure 2a).

Mosquito activation varied greatly between testing days. For each odour combination and each mosquito population the variance was indeed much higher than expected under the binomial distribution (data not shown).

Relative attractiveness of host odours

In the presence of odours from a single host, KIS mosquitoes responded more strongly to the odour-laden air current compared to outdoor air (for human odours: OR = 3.97; CI = [3.04, 5.19]; $P < 0.001$; for calf odours: OR = 3.87; CI = [2.84, 5.23]; $P < 0.001$; Figure 2b). When the odours from both hosts were presented simultaneously, KIS mosquitoes distributed equally among the collecting boxes regardless of host type (OR = 0.99; CI = [0.74, 1.10]; $P = 0.34$, Figure 2b).

The response of VK mosquitoes was substantially different. In the presence of only human odour, there was

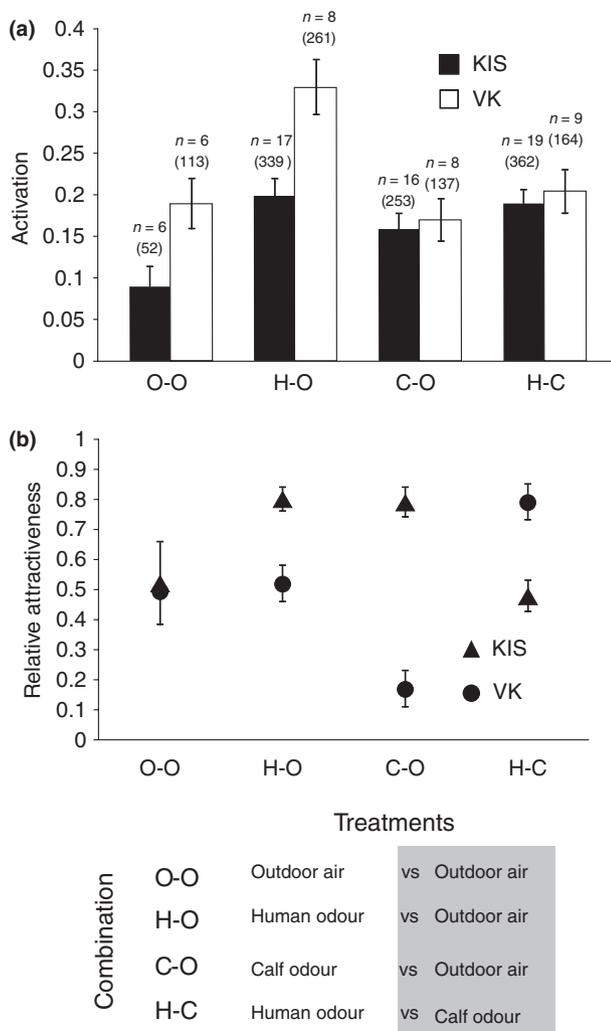
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Figure 2 (a) Activation, expressed as the proportion of mosquitoes caught in both upwind collecting boxes out of the total number released in the downwind box for each treatment combinations and for both mosquito populations. n = number of replicates. In parentheses are indicated the total numbers of mosquitoes entering both traps. Error bars show 95% confidence limits. (b) Relative attractiveness, expressed as the proportion of mosquitoes caught in one collecting box (that pertaining to the unshaded treatment on the left in the above list of treatment combinations) out of the total number retrieved from both collecting boxes. Error bars show 95% confidence limits. Below the graph are indicated the treatment combinations tested.

no significant difference in the number of VK mosquitoes retrieved from the two collecting boxes (OR = 1.10; CI = [0.87, 1.41]; P = 0.42, Figure 2b), indicating that human odours did not produce a stronger anemotactic response than outdoor air. Conversely, when calf odours were presented, VK mosquitoes responded more strongly

to outdoor air (OR = 4.95; CI = [3.16, 7.75]; P < 0.001; Figure 2b), suggesting that calf odours modified the anemotactic response of this population in a negative way. When both human and calf odours were presented simultaneously, VK mosquitoes responded more strongly to human rather than calf odours (OR = 3.82; CI = [2.63, 5.54]; P < 0.001; Figure 2b).

Effect of other experimental covariables

The mean concentration of CO₂ during the outdoor air treatment combination was 353 ppm. The mean concentration of CO₂ exhaled by calves (806 ppm) was significantly higher than that exhaled by human hosts (710 ppm) (Mann–Whitney test; P = 0.03).

For all treatment combinations in VK mosquitoes and for combination (C-O) in KIS mosquitoes, we found no effect of mean CO₂ concentration on mosquito activation. We however found that the more the hosts released CO₂, the less KIS mosquitoes activated during combinations (H-O) and (H-C) (respectively OR = 0.999; CI = [0.997, 1]; P < 0.05 and OR = 0.998; CI = [0.997, 0.999]; P < 0.001) (Figure 3a).

For both strains, we found no effect on the relative attractiveness of a treatment mediated by differences in CO₂ concentration when testing the H-O and C-O treatment combinations (OR = 0.9995; CI = [0.9987, 1.003]; P = 0.24; and OR = 0.9994; CI = [0.9983, 1.0004]; P = 0.25, respectively). Interestingly, a slight effect of CO₂ concentration was found only for KIS mosquitoes submitted to the H-C treatment combination (OR = 1.001; CI = [1.0003, 1.002]; P = 0.013), indicating that this population responded more strongly to the air current with a higher CO₂ concentration (Figure 3b). We found no positional or temporal effects on either mosquito activation or the relative attractiveness of the treatments.

Discussion

A Y-tube olfactometer was used to measure the odour-mediated behavioural responses associated to host preference of a natural population and a laboratory strain of *An. gambiae* s.s. This set-up allowed the study of responses from total host emanations instead of fractions thereof, such as skin extracts adsorbed on worn stockings (Dekker *et al.* 2001a; Pates *et al.* 2001). The two populations of *An. gambiae* displayed significant differences in their response to air currents without host odours or with odours from a human or a calf. The overall expression pattern of host preference was consistent with a more anthropophilic behaviour of the natural population

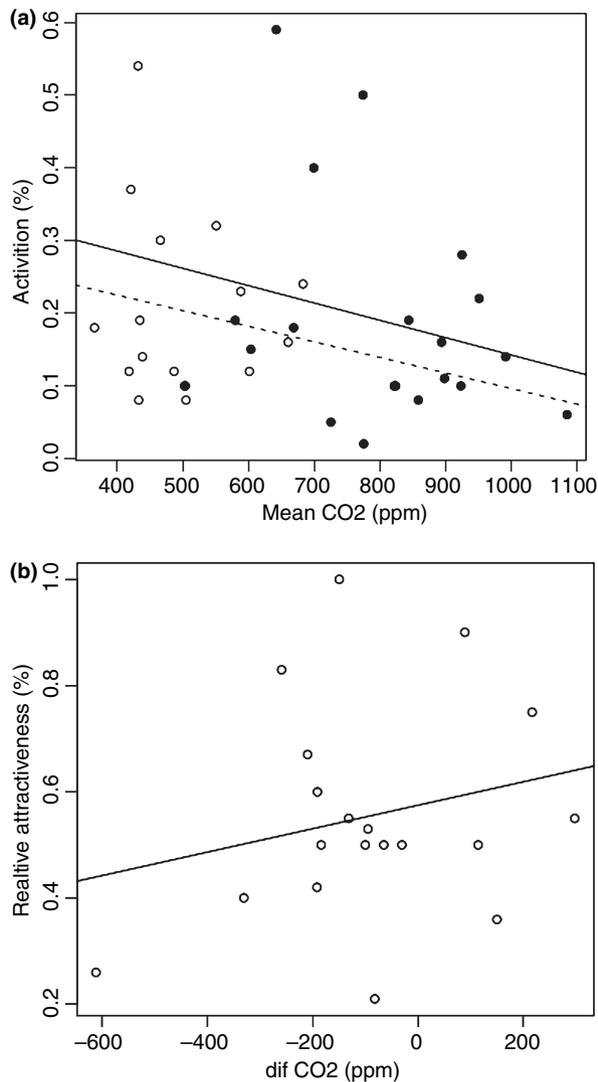


Figure 3 (a) Influence of mean CO₂ concentration in the device on the activation of KIS mosquitoes during H-O treatment combination (open circles and dash line) and during H-C treatment combination (black-filled circles and solid line). (b) Influence of the difference in CO₂ concentration between the two collecting boxes during the H-C treatment combination (CO₂ in box baited with human odour – CO₂ in box baited with calf odour) on the relative attractiveness.

compared to the laboratory strain. Specifically, the former preferred human odour over calf odour, outdoor air over calf odour and did not exhibit preference between outdoor air and human odour, while the latter responded equally to calf odour and human odour, chose calf odour over outdoor air and human odour over outdoor air.

As naive mosquitoes were used, controlling at the same time for physiological and environmental factors modu-

lating host selection (Takken 2005), the differential host preferences between the populations are likely to reflect genetic differences. Such intraspecific variation is presumably the result of different selective pressures and/or genetic drift acting on the two populations, indicating that *An. gambiae*, a mosquito generally considered as strictly ‘anthropophilic’, can evolve toward less anthropophilic behaviour. More than forty years ago, Gillies (1964) showed, from a wild anthropophilic population of *An. gambiae* that after several generations of laboratory selection for feeding on calves, it was possible to induce changes in feeding preferences (i.e. from human- to calf preference). The KIS colony used in this study originates from a wild population in Kisumu, western Kenya, that is highly anthropophilic (Petrarca *et al.* 1991; Githeko *et al.* 1994, 1996 but see also Service *et al.* 1978 and Gillies & Coetzee 1987 indicating that this mosquito can be zoophagic when sampled in granaries). This suggests that rearing these mosquitoes in the laboratory, where they are generally fed on animals may have generated changes in innate host preferences. Such changes, and the intraspecific variation demonstrated in this study, confirm that there is an underlying genetic polymorphism for behavioural responses associated to host preferences in *An. gambiae*.

Our study emphasises the long-established lore that results of behavioural studies using laboratory-reared mosquitoes are difficult to extrapolate to natural populations. This is so not only because environmental conditions to which laboratory and wild mosquitoes are exposed are different, but also because, in the process of colonization, laboratory populations can evolve fundamental changes in their innate behaviours.

Mosquitoes from the natural population exhibited higher levels of baseline responsiveness to an air current free of host odours than those from the laboratory strain. In nature, flying insects use optomotor anemotaxis as an optimal strategy to detect host odours (Bell *et al.* 1995). As laboratory-reared mosquitoes do not need to actively find a source of bloodmeal, one can hypothesise that their ability to initiate optomotor anemotaxis has been reduced during colonisation and/or maintenance.

The presence of human odour markedly increased the activation of the VK population, but it did not bias their anemotactic response towards the odour-laden current. This is an example of odour-mediated anemotaxis, i.e. a positive response to an air current in the presence of host odours. In this case, host odours do not provide any directional cue to orient the flying insect toward their source, but they do switch on the anemotactic response. This is a well-known phenomenon in moths, and biting flies, among others (Bell *et al.* 1995; Gibson & Torr 1999). In the absence of visual and other host-related short-range

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cues, odour-mediated anemotaxis is probably a sufficient strategy to bring *An. gambiae* close to its host from a distance in open habitats like the rice fields the VK mosquitoes originated from. At closer distance from the host, additional cues (physical, visual) probably modulate the orientation toward the host. Eventually, endophagic mosquitoes like *An. gambiae* enter the closed space of a house to bite the host, hence in the absence of wind they must switch to other 'host-seeking' strategies. Our bioassay can only measure the activation and strength of the anemotactic response, thus it does not provide information on behaviours that operate on the final closer-range phase of approach to the host.

When calf odour was presented, the activation of VK mosquitoes fell to the baseline level, suggesting that VK *An. gambiae* did not respond to calf volatiles. However, the mosquito distribution in the two arms of the olfactometer was markedly biased toward the outdoor air end, demonstrating that the anemotactic response of VK mosquitoes was indeed negatively affected by calf odours. When calf odour was presented, the degree of bias in the mosquito distribution between the olfactometer arms was similar whether outdoor air or human odour was the alternative treatment. This result confirms the interpretation that VK mosquitoes did not use human emanations to orient their flight, whereas they did steer away from calf volatiles.

The overall expression pattern of these behavioural mechanisms was that VK mosquitoes 'preferred' human odour to calf odour, i.e. expressed an 'anthropophilic' behaviour. The interpretation underlying this conclusion, however, is paradoxically rather reversed: the 'preference' for human odour resulted from a lack of orientation to human odour and a negative response to calf odour. In a sense, VK mosquitoes were 'repelled' by calf odour. This is in agreement with results from field choice-tests performed with odour-baited traps and other trapping devices suggesting that odours from non-preferred hosts orient *An. gambiae* toward the preferred one (Costantini *et al.* 1998; Dekker *et al.* 2001a). Torr *et al.* (2008) have argued that the use of blanket terms like 'anthrophily' or 'preference' can hinder our understanding of mosquito behaviour, unless they are attached to specific behavioural endpoints. Our results reiterate their point.

The response of KIS mosquitoes to the same host odour treatments was fundamentally different. Both activation and the strength of the anemotactic response were positively correlated with the presence of host odours, in a similar way regardless of host type. In the presence of odours from both hosts, KIS mosquitoes could not orientate towards any of the two hosts.

A possible interpretation of these results is that, in the course of strain colonization and maintenance, KIS mosquitoes have lost the ability to respond to host-specific emanations. A conservative interpretation is that these mosquitoes were activated and oriented towards a chemical signature that was common to humans and calves. Carbon dioxide is one of the volatile compounds released at comparable levels from both hosts, but the role of other chemicals present in a comparable range of concentrations in both humans and calves cannot be completely ruled out. It would be informative to investigate the response of KIS mosquitoes to other kind of hosts such as rabbits (i.e. the laboratory animals that were used to rear the KIS colony), or other mammals to measure the extent to which the response of this strain can be generalised across hosts.

We found a high variability in mosquito activation between the different testing days. Previous studies (e.g. Brady *et al.* 1997) showed that such variability could be attributed to differences in CO₂ emanating from vertebrate hosts. It is indeed generally accepted that CO₂ can be involved in mosquito activation (Gibson & Torr 1999; Guerenstein & Hildebrand 2008). However in our study, an higher mean CO₂ concentration in the device did not result in higher trap catch and the activation variability observed is probably the consequence of different climatic conditions (e.g. relative humidity, barometric pressure, Steinberg *et al.* 1992; Dethier 1974) between testing days and/or cues other than CO₂ emanating from the different combinations of host individuals (Mukabana *et al.* 2002, 2004; Qiu *et al.* 2006).

We found for KIS mosquitoes, that the more the hosts exhaled CO₂, the less activated were the mosquitoes during the combinations H-O and H-C. Such an inhibitory or neutral effects of CO₂ corroborates previous findings (Takken *et al.* 1997; Dekker *et al.* 2001a,b; Mukabana *et al.* 2004) and it is, for instance, possible that beyond a certain threshold, a higher CO₂ concentration did not increase activation. Interestingly we found, only for KIS mosquito and during the combination H-C, an effect of the difference in CO₂ concentration between the collecting boxes on the relative attractiveness: this population responded more strongly to the air current with a higher CO₂ concentration. These results are difficult to interpret and clearly more research is needed to fully understand the role of CO₂ in the activation and relative attractiveness of both populations. The fine-scale structure of the CO₂ plume strongly influences the behavioural responses of mosquitoes (Guerenstein & Hildebrand 2008) with a homogeneous plume reducing activation (Dekker *et al.* 2001b). In the future, it would be interesting to study this structure (continuous *vs.*

discrete stream) in our device as it could help to better understand the role of CO₂.

This study reported differential responses to host odours between two populations of the same sibling species of the *An. gambiae* complex. Our results confirm that there is polymorphism underlying *An. gambiae* host preference. In the search of host preference determinism, molecular genetic analysis of differences between several populations of *An. gambiae* s.s. showing different response to host odours is likely to bring fundamental knowledges as well as to contribute to the design of vector control measures.

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