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Odor-Mediated Flight Behavior of *Anopheles gambiae* Giles *Sensu Stricto* and *An. stephensi* Liston in Response to CO_2, Acetone, and 1-Octen-3-ol (Diptera: Culicidae)

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The flight behavior of Anopheles gambiae s.s. *Giles and* An. stephensi *Patton exposed to different odor cues was studied in a wind tunnel. Odors consisted of CO_2, CO_2 + acetone (at two concentrations), and CO_2 + 1-octen-3-ol. Mosquitoes were released singly and their behavior was recorded on video. Parameters studied included flight velocity, percentage of time spent flying, percentage of time spent in plume, and number of turns toward the plume. Large differences in behavior toward the odors tested were observed.* An. gambiae *did not respond well to CO_2, whereas* An. stephansi *was positively affected by this compound. In contrast,* An. gambiae *responded significantly to CO_2 + acetone (at a low concentration), but the behavior of* An. stephensi *was completely suppressed by this combination of odor stimuli. CO_2 + a high concentration of acetone or CO_2 + 1-octen-3-ol did not cause significant effects in* An. gambiae *compared to no odor, while these treatments elicited strong behavioral responses in* An. stephensi. *The latter species responded particularly well to CO_2 + 1-octen-3-ol. The results suggest that the observed differences may be inherent to the known differences in host preferences, where* An. gambiae *is highly anthropophilic and* An. stephensi *more zoophilic. This would explain why the latter species responds well to CO_2 and even better to CO_2 + 1-octen-3-ol, a compound readily emitted by bovine ruminants.*

KEY WORDS: mosquito; *Anopheles gambiae*; *An. stephensi*; behavior; olfaction; semiochemicals.

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INTRODUCTION

The host-seeking behavior of bloodfeeding mosquitoes is affected to a large extent by olfactory cues, derived from the host. This behavior, which can be considered as odor modulated upwind anemotaxis (Payne *et al.*, 1986), is generally the same for most mosquito species: females in the appropriate physiological condition detect host odor, carried to them by wind; if the odor is recognized, they fly upwind toward the odor source. Whereas the principle of this behavior is generally accepted (Takken, 1996), studies providing details about the behavior itself are rare. Moreover, there is a little information about the olfactory cues that steer this behavior (Bowen, 1991; Takken, 1991). Many hematophagous insects are attracted to CO_2, a compound universally emitted by all vertebrates on which the insects feed. Since several mosquito species exhibit specialized feeding behavior, having a preference for a particular host species or group of hosts above others, it is unlikely that CO_2 is the only substance to which mosquitoes respond, and other, host-specific, compounds may be involved. Also, natural concentrations of CO_2 often attract only a fraction of the mosquito population that arrives at a natural host, indicating that other cues, presumably of olfactory nature, need to be present to cause a complete olfactory behavior. Recently it was found that *Aedes taeniorhynchus* (Wiedemann) responds to 1-octen-3-ol (henceforth termed octenol), a compound present in the expired air of ruminants (Takken and Kline, 1989). The most exhaustive study on olfactory responses of hematophagous insects to host emanations was done on tsetse flies, where it was found that these insects respond to a group of compounds present in volatile emanations of animals. CO_2, acetone, octenol, and phenols were among the most important chemicals to which tsetse flies respond (Bursell *et al.*, 1988; Willemse and Takken, 1994). The present work was undertaken as part of a larger study on the behavioral responses of mosquitoes to semiochemicals and describes the responses of *Anopheles gambiae s.s.* Giles and *An. stephensi* Liston to carbon dioxide, acetone, and octenol in a wind tunnel. These compounds had been shown previously to cause behavioral responses in other hematophagous insects and were thought to account partially for the recorded differences in host preference between *An. gambiae s.s.* and *An. stephensi.*

MATERIALS AND METHODS

Mosquitoes

The study was done with *An. gambiae s.s.*, originating from Liberia, and *An. stephensi*, which originated from Pakistan. Adult mosquitoes were kept in gauze-covered cages (30 × 30 × 30 cm) at 28°C, 80% RH, and a 12-h sco-

tophase with a sharp transition from light to dark. The mosquitoes had access to a 6% glucose solution. Twice a week they were fed on a human arm. Eggs were deposited on wet filter paper. Larvae were reared in 2-L trays at a density of approximately 2 larvae per cm^2 and fed on Tetramin fish food at a quantity of approximately 2 mg per larva per day. Pupae were collected daily and placed in adult cages for emergence.

The Wind Tunnel and Registration of Behavior

Experiments were conducted in a dark room, the only light source being that of the behavioral assay (see below). Mosquitoes were tested individually in an experimental wind tunnel during a 5-min observation time. The wind tunnel (Fig. 1) consisted of a rectangular flight chamber (200 × 60 × 60 cm) with transparent lexane walls and ceiling and a white trespa floor. Air was drawn from outside by a Fishbach stepless speed-controllable ventilator (Fig. 1, c), filtered (glass-wool filter, Camfil HI-FLO-95; Fig. 1, a), heated (25.5°C; Fig. 1, b), and purified (activated charcoal filter; Fig. 1, d), after which the air entered the wind tunnel through a wire netting screen, at a speed of 20 cm/s. In this way the airstream in the tunnel was nearly laminar. A plume generator

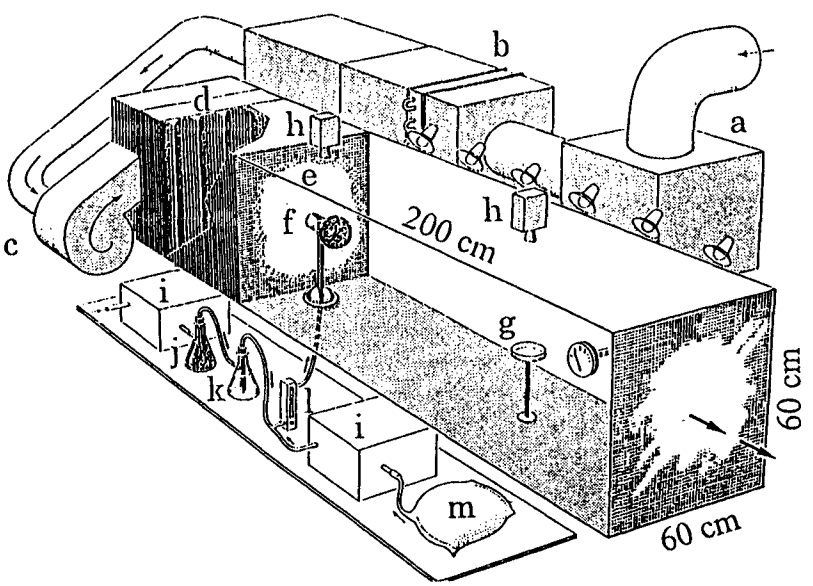

Fig. 1. Diagram of the wind tunnel. a, glass-wool dust filter; b, heater; c, ventilator; d, active charcoal filter; e, mesh screen; f, plume generator; g, release platform; h, video-camera; i, air pump; j, active charcoal filter; k, humidifier; l, flowmeter; m, gas sampling bag.

was placed in front of the wire netting screen (Fig. 1, f). This consisted of a glss funnel (diameter of straight tube, 5 cm; diameter at open top, 15 cm), placed laterally 20 cm above the tunnel floor. Test odors were pumped into the plume generator through a silicon tube which entered the funnel through a glass tube fitted on the straight funnel tube. Test odors were carried along with the conditioned airstream which passed through the plume generator. The tunnel was weakly illuminated by a diffuse light source placed laterally against one of the side walls. Closest to the source the light intensity was 13 lux, and on the opposite end 8 lux. At this light intensity mosquitoes could be observed without affecting their behavior.

Mosquito behavior was registered with two Panasonic CCD videocameras, connected to black-and-white monitors and two Sony super VHS video recorders. The cameras were suspended above the tunnel and each recorded one-half of the tunnel. In addition, one observer, sitting in front of the tunnel, verbally recorded the exact positions of the mosquitoes onto the videotape. At the end of each replicate the mosquito was removed from the wind tunnel by means of a suction tube and a new experiment could start. Afterward the tape was analyzed and the salient behavioral parameters (flight velocity, time spent flying, time spent in plume, number of returns) were extracted.

Experimental Procedures

Prior to the experiments, 6- to 8-day-old, teneral female mosquitoes were placed individually in 5-cm-diameter petri dishes. To avoid disturbance this was done under low-intensity red light, wearing surgical gloves to avoid contamination of the petri dish with human skin emanations. Petri dishes were stored until use in a closed wooden box. Experiments were done in the last 4 h of the scotophase, which is the period of maximum host-seeking activity of *An. gambiae* and *An. stephensi.* At the beginning of an experiment a petri dish was placed on a release platform, which was standing in the odor plume at a distance 110 cm downwind from the plume generator (Fig. 1, f). A magnet connected to a thin rope was placed on the iron ring on the lid of the petri dish. The experiment started when the mosquito was exposed to the odor plume, by lifting the lid with the rope from outside the tunnel. Care was taken not to disturb the mosquito while lifting the lid. Test odors were released continuously through the plume generator and the release platform was always in the center of the odor plume.

Five treatments were tested. We attempted to simulate human- and animal-equivalent concentrations of acetone, respectively, based on data from Krotoszynski *et al.* (1977) and Vale and Hall (1985). Whereas exhaled breath of humanas and oxen at rest contains similar concentrations of CO_2 ($\pm 5\%$) (Guyton, 1977), the acetone concentration in the breath of a 500-kg ox is about 40×

greater than that of human breath and oxen produce approximately 500× more acetone per unit time than humans. There are no precise data on the concentration of octenol in human breath, and only one concentration of octenol, equivalent to that present in ox breath (Vale and Hall, 1985), was used. Clean moistened air (CMA), obtained by leading room air through activated charcoal and demineralized water, respectively, was pumped into the tunnel with a S-200 Du Pont constant-flow sampler pump at a rate of 230 ml/min. Pure CO_2 from a pressurized gas cylinder was added to the CMA at a ratio of 5:95 to obtain a 5% concentration of CO_2 [CO_2] in clean moistened air and pumped at a rate of 230 ml/min into the plume generator in pulses of 6 s, alternating with 6 s of CMA. Mixtures of 120 ng/L and 120 μg/L acetone and of 5.3 ng/L octenol, respectively, were prepared by filling 100-L Tedlar gas sampling bags with CMA, into which the required amount of acetone or octenol was injected with a Hamilton syringe. Gas sampling bags were left overnight to allow for a thorough mixing of the odors in the bags. Pure CO_2 was mixed with acetone- or octenol-laden air from the gas sampling bags at a ratio of 5:95, similarly as with CMA, and pumped into the plume generator at a rate of 230 ml/min.

One treatment per day was tested with both mosquito species, in order to avoid day effects between the mosquitoes. Between treatments, the tunnel was thoroughly cleaned with soap, demineralized water, hexane, and ethanol, in that sequence.

Data Analysis

For the recording of behavior, the windtunnel was divided into 10 20-cm-wide rectangular sectors starting from the upwind side, defined by black lines drawn on the tunnel floor. An image of the plume shape and spatial position was obtained by pumping artificial smoke, created by mixing ethyl acetate with diethylamine, through the plume generator. It was therefore possible to record the position of a mosquito flying through the tunnel by indicating its position upwind and in relation to the plume. Behavior was divided into flight, sitting, and walking. The 5-min test period of each mosquito was divided into 30 intervals of 10, and the predominant behavior (flight, sitting, walking) for each interval was registered. In addition, flight behavior was also recorded in space, by according spatial flight records for each change in position of the mosquito over the sectors. In this way behavioral intervals and flight records were collected for each mosquito. On the basis of the audio–video registration analysis, the following behavioral parameters were analyzed.

Flight Velocity. The mean flight velocity of all mosquitoes that took off from the release platform was obtained as follows:

$$v\ (\text{cm/s}) = \sum \frac{20 * F_{\text{rec.}})\ \text{cm}}{(F_{\text{int.}} * 10)\ \text{s}} \times 1/n$$

where $F_{rec.}$ is the number of spatial flight records per mosquito per treatment, $F_{int.}$ is the number of flight intervals per mosquito per treatment, and n is the number of mosquitoes per treatment.

This was the minimum flight speed because the flight distance per sector was, by necessity, recorded as a constant of 20 cm, the width of a sector, since the actual path flown could be traced only laterally and not vertically. When it was observed that the insect had passed through x sectors during a 10-s interval, the insect had flown at least $x * 20$ cm during that interval.

Percentage of Time Spent Flying. This was the number of flight intervals multiplied by 10 s as a percentage of the total time spent after takeoff.

Percentage of Time Spent in the Plume. This was the number of flight intervals spent in the plume multiplied by 10 s as a percentage of the total flying time.

Number of Returns. This was the average number of turns directed to redetection of the plume. Often the return would result in a plume entry.

Statistical analyses were done with chi-square tests and Wilcoxon rank-sum tests. Statistical comparisons were made between CMA and CO_2, between CO_2 and CO_2 + acetone, between CO_2 and CO_2 + octenol, and between the two concentrations of acetone.

RESULTS

The proportion of *An. gambiae* responding was higher than that of *An. stephensi* and, with the exception of CO_2 + octenol in *An. stephensi*, was not affected by treatment (Table I). The latter treatment caused a significant increase in the proportion of responding mosquitoes in *An. stephensi*. Significant differences in flight speed between the treatments were found (Table I). Both species also responded differently to the treatments. Whereas the mean flight speed of *An. gambiae* was significantly reduced by the addition of CO_2, that of *An. stephensi* showed a significant increase with CO_2. CO_2 + acetone (mainly at low concentration) caused significantly increased activity in *An. gambiae* compared to the other treatments, while no increase was seen with this treatment for *An. stephensi*. The reverse occurred with CO_2 + octenol, which turned out to be significantly activating for *An. stephensi* but not for *An. gambiae*. In the latter neither CO_2 nor CO_2 + octenol caused an increase in flight velocity.

Taking off from the platform nearly always resulted in flying out of the odor plume, into which the mosquitoes might return farther upwind. The percentage of mosquitoes that stayed in or returned to the odor plume after takeoff is considered a measure of attractiveness of the treatment. Odor had a significant effect on the percentage of mosquitoes that flew through the plume after takeoff, although the effect was markedly different for both species (Table I). In *An. gambiae*, CO_2 caused a 20% increase in plume flights compared to CMA, and this rose by 40% when acetone (low concentration) and CO_2 were combined.

Table I. Number of Mosquitoes that Took Off from the Release Platform, Mean Flight Velocities, and Percentage that Flew Through the Odor Plume

	Treatment				
	CMA	CO_2	CO_2 + $acetone_{low}$	CO_2 + $acetone_{high}$	CO_2 + octenol
An. gambiae					
n released	39	42	39	39	40
n responding (%)	33 (85)	37 (88)	34 (87)	35 (90)	33 (83)
Velocity (cm/s)	6.8 a	5.0 b	10.3 c	8.3 a	6.1 a,b
% that flew in plume	47 a	65 b	88 c	59 a,b	44 a
An. stephensi					
n released	40	45	44	49	40
n responding (%)	25 (60) a	30 (67) a,b	24 (55) a	29 (59) a	31 (78) b
Velocity (cm/s)	7.3 a	9.0 b	9.1 b	8.8 b	10.5 c
% that flew in plume	20 a	63 b	21 a	60 b	52 b

[a]Means in the same row followed by the same letter are not significantly different ($P > 0.05$, Wilcoxon rank-sum test).

This effect was absent with mixtures of CO_2 + acetone (high concentration) or octenol. Only 20% of *An. stephensi* flew in the plume with CMA. This increased significantly, to 63%, with CO_2. The addition of acetone (low concentration) to CO_2 obliterated the effect of CO_2 completely, whereas acetone (high concentration) and ocetenol had no effect in addition to that of CO_2 alone.

Plume-leaving resulted sometimes in a behavior directed to bring the mosquito back into the plume. This could be quantified by recording the number of returns after exiting (Table II) and is considered a measure of attractiveness and

Table II. Mean Number of Returns into the Plume

	An. gambiae s.s.[a]		*An. stephensi*[a]	
Treatment	*n*	Mean number of returns	*n*	Mean number of returns
CMA	15	1.0 a	6	0.7 a
CO_2	27	1.0 a	18	2.4 b
CO_2 + $acetone_{low}$	30	3.6 b	9	1.4 a
CO_2 + $acetone_{high}$	19	1.7 a	16	2.5 b,c*
CO_2 + octenol	21	1.3 a	17	3.6 c

[a]Means in the same column followed by the same letter are not significantly different ($P > 0.05$, Wilcoxon rank-sum test).
*$P < 0.1$.

recognition of the odor stimuli under study. While returning behavior seldom occurred without odors, significant increases in returning behavior were seen for some of the odor treatments. With CO_2 alone, the mean number of returns was significantly increased in *An. stephensi* but not in *An. gambiae*. However, this parameter increased signficantly for *An. gambiae* when exposed to CO_2 + acetone (low concentration), while this effect was gone when acetone was used at high concentrations. In contrast, *An. stephensi* showed a significantly lower mean number of returns with CO_2 + acetone (low concentration) compared to CO_2 alone, while with high concentrations of acetone the mean number of returns was significantly increased in comparison with acetone at a low concentration. With CO_2 + octenol, again a difference was seen between *An. stephensi* and *An. gambiae*. The first showed a significant increase in the returning activity when octenol was added compared to CO_2 alone, while the latter's results were not different from those with CO_2.

With some treatments the mosquitoes that returned into the plume after takeoff showed a significantly different behavior when they redetected odor. This can be seen in the differences in the percentage of time spent flying (of the total time after takeoff) between those mosquitoes that did and those that did not return into the plume (Fig. 2). With one exception (*An. gambiae* with CMA), plume-returners flew significantly more than those which had been exposed to the odors only while sitting on the release platform. There was no significant difference in the percentage of time flown for *An. gambiae* that did not return to the plume. However, large differences were observed between mosquitoes which did return to the plume: with CO_2 + acetone (both concentrations) the percentage of time flown was significantly increased. *An. stephensi* showed a different behavior. When mosquitoes had been in touch with CO_2 only on the releasing platform, this already resulted in a significant increase in the percentage of flights. For plume-returners this increased significantly when odors were added, with the exception of CO_2 + acetone (low concentration), which showed a behavior similar to that with no odor. In all odor treatments there was a significant reduction in time flown for those mosquitoes did not fly in the plume (data not shown).

The time spent inside the odor plume as a percentage of the total time flown may be considered as a measure of the recognition of and orientation to the odor: insects that remain inside the plume benefit most from the odor-mediated anemotaxis because they are continuously being stimulated by the kairomones emitted from the source. In *An. gambiae*, only CO_2 + acetone (low concentration) affected the percentage of flight time spent in the plume. The other treatments did not cause an effect compared to that of clean moist air (Fig. 3), where on average 9% of the time flown was spent inside the imaginary plume. In constrast, in *An. stephensi* CO_2 alone already caused a significant increase in this behavior. However, CO_2 + acetone (low concentration) com-

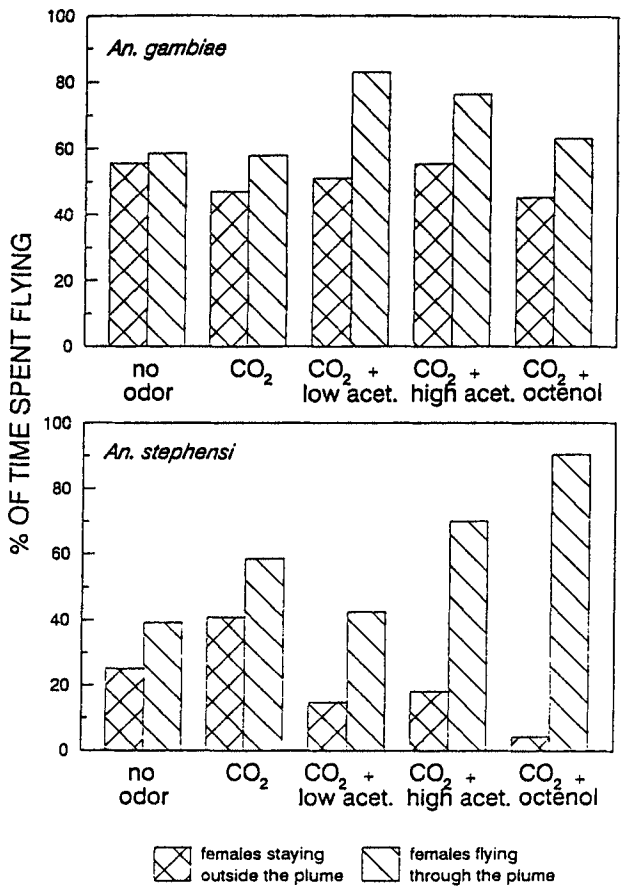

Fig. 2. Percentage of time spent "in flight" of mosquitoes that flew through the plume (hatched bars) and of those that remained outside the plume (cross-hatched bars), respectively. Statistical differences are explained in the text.

pletely suppressed the effect of CO_2 alone. This negative effect was restored with CO_2 + acetone (high concentration) and CO_2 + octenol.

DISCUSSION

The results show that CO_2 has a strong effect on *An. stephensi* but not on *An. gambiae*. The latter species was hardly affected by CO_2 alone, although once exposed to it, it would more often return to the odor plume, which suggests

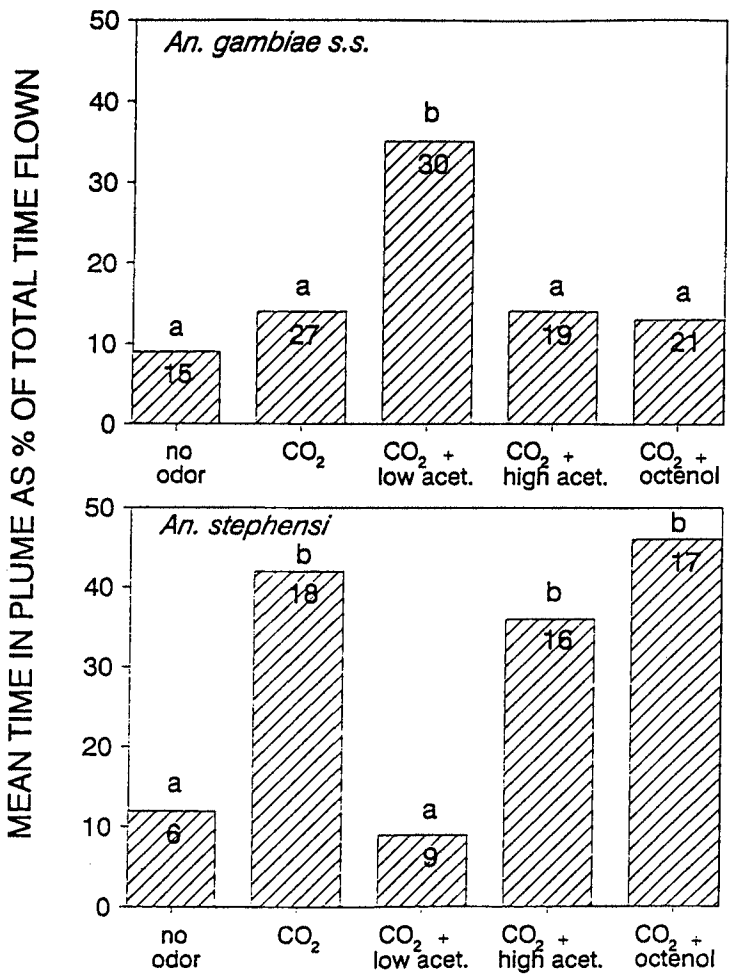

Fig. 3. Mean of the time spent inside the odor plume as a percentage of the total time flown. Figures inside each bar indicate the number of mosquitoes from which the means were derived. Means in each graph with the same lowercase letter above the bars are not significantly different ($P > 0.05$).

that this species did perceive CO_2. The mean number of returns into the plume after the mosquito has flown out of it and the percentage of flight time spent in the plume can be considered as a measure of recognition of the odor plume and demonstrate how well the insect can orientate toward an odor source containing attractive emanations (Table II). In parallel studies with *An. gambiae s.s.* in our laboratory, it was found that this anthropophilic species responded to 4.5% CO_2

in an olfactometer but not to 3.6% (Knols *et al.*, 1994; De Jong and Knols, 1995). However, Healy and Copeland (1995) found that, although *An. gambiae s.s.* responds poorly to CO_2, this mosquito is able to distinguish CO_2 at levels 0.1% above background. In a recent field study in Tanzania, it was found that CO_2 is not very attractive for *An. gambiae*, whereas human odor was (Mboera *et al.*, 1997). From these studies it can be concluded that for *An. gambiae s.s.*, CO_2 alone does not play a crucial role in host seeking. However, it may play a role in combination with other odor cues, as a recent study by Costantini *et al.* (1996) suggested. These authors speculate that differences in CO_2 production between human hosts are responsible for observed differences in attraction of *An. gambiae*. Our results show that *An. stephensi*, on the other hand, is strongly affected by CO_2 as evidenced by the data in Fig. 3. This species is less specific in its host preference and readily feeds on nonhuman hosts. Thus it behaves similarly to *An. melas* and culicine mosquitoes that are attracted to CO_2 (Gillies and Wilkes, 1969). *An. gambiae* is highly anthropophilic (White, 1974), whereas *An. stephensi* is a generalist, feeding on a wide range of host species. It is in the latter's interest to find a reliable cue, common to all potential hosts. Clearly, CO_2 satisfies that demand, whereas *An. gambiae* should respond to more host-specific cues, whatever these might be. The recent findings that blends of fatty acids might provide these cues (B. G. J. Knols and R. De Jong, personal communication) and the response to CO_2 + acetone (low concentration) in the present study suggest that indeed odor blends exist to which this mosquito can respond. Both *An. gambiae* and *An. stephensi* respond to acetone, but in a different way. *An. gambiae* is activated by this compound when offered at a human-equivalent physiological concentration, whereas the activating effect of CO_2 in *An. stephensi* is completely masked by this concentration of acetone. Since the latter's effect was not seen with CO_2 + acetone at animal-equivalent concentrations ($acetone_{high}$), we conclude that *An. stephensi* contains receptors that detector differences in concentrations of acetone and that a human-equivalent physiological concentration of acetone blocks the effect of CO_2 completely. It was surprising to note that this effect did not occur when acetone was released in an animal-equivalent physiological concentration. We have not been able to verify in the literature whether such different behaviors to the same olfactory cues have been recorded in other insects. Octenol is a common product emitted by both humans and animals (Hall *et al.*, 1984; Cork, 1996) and has been found to be attractive for a number of mosquito species (Takken and Kline, 1989; Kline, 1994). The results show that *An. stephensi* is strongly attracted to a mixture of CO_2 and octenol. Whether octenol alone is also attractive remains to be seen, since few mosquitoes are attracted to octenol in the absence of CO_2 (Kline *et al.*, 1990; Kemme *et al.*, 1993). *An. gambiae* did not show a behavioral response to octenol, although recently it was reported to show an EAG response to this compound (Cork and Park, 1996). The results suggest that the behavioral

differences between *An. gambiae s.s.* and *An. stephensi* are closely related to their host preferences, which for the former are humans and for the latter unspecified hosts, including bovids. We conclude that *An. stephensi* responds well to animal equivalents of acetone and octenol, in the presence of CO_2. Further studies are required to determine whether this behavior is also expressed with single compounds. It is surprising, however, that human-equivalent physiological concentrations of acetone cause a complete depression of the clear response to CO_2. After all, *An. stephensi* is an infamous malaria vector in Western Asia, suggesting that it frequently bites humans. It is possible that other human emanations may suppress the negative response caused by acetone in this mosquito.

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