

# Laboratory studies on the olfactory behaviour of *Anopheles quadriannulatus*

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## Abstract

The host preference of *Anopheles quadriannulatus* Theobald (Diptera: Culicidae), the zoophilic member of the malaria mosquito complex *Anopheles gambiae* Giles, was investigated in a dual-choice olfactometer. Naïve female mosquitoes were exposed to CO<sub>2</sub>, acetone, 1-octen-3-ol, and skin emanations from cows and humans in various combinations. Their behavioural responses were recorded when they had entered one of either upwind traps from where the odours were being released. The mosquitoes did not respond to CO<sub>2</sub> when released at human or cattle equivalent concentrations. Too few mosquitoes responded to acetone to allow for a statistical analysis. The combination of CO<sub>2</sub> + 1-octen-3-ol was repellent. Cow odour alone was slightly attractive, but there was a synergistic attractive effect of cow odour + CO<sub>2</sub>. Surprisingly, the mosquitoes were attracted to human odour, and in a choice situation human odour was selected above cow odour + CO<sub>2</sub>. Anthropophilic *An. gambiae* Giles s.s. was repelled by cow odour + CO<sub>2</sub> in contrast to *An. quadriannulatus*. In a choice situation, both mosquito species selected human odour above cow odour + CO<sub>2</sub>. The implications of these results are discussed in the light of recent behavioural data from the field.

## Introduction

The *Anopheles gambiae* Giles complex (Diptera: Culicidae) comprises seven sibling species and several 'incipient' species (Coetzee et al., 1982, 1993, 2000; Coetzee, 1986; Gillies & Coetzee, 1987; Dekker & Takken, 1998; Coluzzi et al., 2002). Of the seven species, two are referred to as *An. quadriannulatus* Theobald species A and *An. quadriannulatus* species B. The former is present in southern Africa, while the latter has only been recorded in Ethiopia (Hunt et al., 1998). The identification of sibling species belonging to the *An. gambiae* complex is considered important for practical malaria control (Coetzee et al., 2000), especially in areas where *An. quadriannulatus* occurs in sympatry with a vector sibling species. Very few laboratory or field studies concerning the host-seeking behaviour and host preference of both *An. quadriannulatus* species have been performed. This is

mainly because these mosquitoes are thought to be completely zoophilic and hence of no medical importance (White, 1974; Coetzee et al., 1982, 1993, 2000; Coetzee, 1986; Gillies & Coetzee, 1987; Dekker & Takken, 1998; Prior & Torr, 2002). In the laboratory, this zoophilic behaviour was demonstrated by Dekker et al. (2001a) in an olfactometer by testing the response of *An. quadriannulatus* species A females to human odour, cow odour, or carbon dioxide, where very few mosquitoes were caught with human odour and more were caught with carbon dioxide (delivered at a concentration of 4.5% and rate of 230 ml min<sup>-1</sup>). However, no preference for the cow odour was observed.

The purpose of the present study was to investigate the host preference of *An. quadriannulatus* species A [SKUQUA strain] from South Africa by testing its response to a variety of odours, of both human and animal origin, in a dual-choice olfactometer. Similar laboratory experiments have been performed with *An. gambiae* s.s. to demonstrate the extreme anthropophily exhibited by this sibling species (Pates et al., 2001a). Initial experiments were carried out to assess the response of *An. quadriannulatus* to different

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volumes and concentrations of carbon dioxide; the concentration of carbon dioxide in human and cattle breath is 4.5%, but the volume of carbon dioxide released by a cow is approximately 1000 ml min<sup>-1</sup>, as compared with 230 ml min<sup>-1</sup> from humans. Acetone and 1-octen-3-ol (hereafter termed octenol), also constituents of cattle breath (Hall et al., 1984; Takken et al., 1997), were tested alone or in combination with carbon dioxide.

## Materials and methods

The same olfactometer and procedure was used as described in Pates et al. (2001a). All experiments were performed at the Laboratory of Entomology in Wageningen University during 1999 and took place towards the end of the scotophase. Thirty 6–10-day-old females, which had not been previously blood-fed, were used in each experiment. Mosquitoes (see below) were randomly picked from their cage 15 h before the experiments began and placed in a releasing cage with access to tap water via damp cotton wool placed on the gauze cover of the cage. The sequence of odour combinations was randomised on the same test day and between days. Test stimuli were also alternated between right and left ports to rule out any positional effects. Experiments with no odours in either port tested the symmetry of the trapping system (anopheline mosquitoes respond with upwind flight to humid airstreams, presumably in response to the variation in moisture gradient within the airstream compared to that of the ambient air). Mosquitoes were left inside the olfactometer for a total of 20 min, after which they were considered to have responded to a test odour (or clean air) if they had entered a trap. Trapped mosquitoes were anaesthetised using 100% carbon dioxide and counted at the end of the experiment. Surgical gloves were worn throughout the experimental procedure to avoid contamination of any of the equipment.

Mosquitoes from a colony of *An. quadriannulatus* species A (SKUQUA strain) kept at Wageningen University (The Netherlands) were used for all experiments described. The colony was established from material collected between December 1995 and May 1996 from Skukuza, South Africa, and laboratory reared for 3.5 years in vivo with blood meals from a human arm. At the time of rearing, this was the only successful method found that resulted in a good number of eggs. However, blood feeding took up to 30 min and females were only induced to feed by breathing into the mosquito cage (as reported by Dekker et al., 2001a). Adults were kept in plastic buckets (width 23 cm) of a minimum height of 25 cm (required for mating) at 27 ± 1 °C, 80% r.h., and a 12 h scotophase with a 30 min dusk/dawn period to allow for mating. Adults were provided with a 6% glucose solution and offered a blood meal five times a

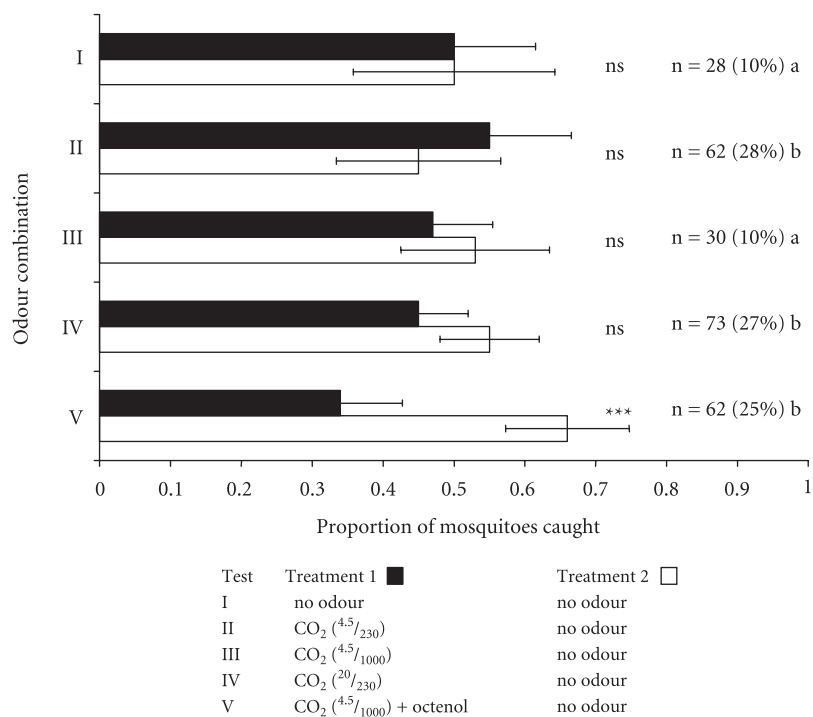
week for 30 min from a human arm during the scotophase. A Petri dish containing soaked black filter paper overlaid with soaked white filter paper was provided for oviposition. Larvae were reared in plastic trays (30 × 10.5 × 8 cm) in demineralised water and fed daily on Tetramin® fish food. Filter paper was placed around the inside of the larval trays to prevent the eggs from drying out. Eggs were then washed down the sides of the tray daily, as hatching can be delayed for up to 8 days. Pupae were removed daily from the trays and allowed to emerge inside the adult cage. Buckets containing adults were cleaned, and the sugar water replaced once a week to minimise the risk of fungi and bacteria accumulating in the cage.

A pre-prepared mixture of 4.5% carbon dioxide in synthetic air (Hoek-Loos, The Netherlands) was used and the carbon dioxide was pumped from a gas cylinder into a 100 L Tedlar® gas sampling bag approximately 20 h before use. Distilled water (10 ml) was added to the bag to obtain a near-saturated humidity level. The carbon dioxide was then pumped (using a membrane pump) through Teflon tubing (5 mm width) via a flowmeter (Sho-rate, Brooks instrumental BV, The Netherlands), into the glass traps with a standard olfactometer air flow of either 230 ml min<sup>-1</sup> (human equivalent) or at 1000 ml min<sup>-1</sup> (cow equivalent). In another experiment, a 20% carbon dioxide mixture was also prepared using 20 l of 100% carbon dioxide and 80 l of clean humidified air, which was pumped into the glass traps with the air flow as described above, at a rate of 230 ml min<sup>-1</sup>.

Both acetone and octenol are found in cattle breath; therefore these chemicals were tested alone, in combination with each other, or in combination with carbon dioxide. Ox breath equivalent concentrations of acetone and octenol (120 µg l<sup>-1</sup> and 5.3 ng l<sup>-1</sup>, respectively) were used (Takken et al., 1997). The exact quantity of acetone needed to fill a 50 l gas bag with vapour was calculated as 7.6 ml, and the exact quantity of octenol needed to fill a 50 l gas bag with vapour at the required concentration was calculated as 3 µl (the concentration of octenol in ox breath is much lower than that of acetone). These quantities were added to a gas sampling bag filled with 50 l of purified, humidified air approximately 20 h before use. The acetone or octenol was then pumped from the gas sampling bag into the glass trap as described above at a rate of 1000 ml min<sup>-1</sup>.

Nylon stockings were used to collect skin emanations from a human foot, based on the known role of human foot odour in the host location of *An. gambiae* s.s. (de Jong & Knols, 1995). The same human (H.V. Pates) was used to collect foot odour for each series of experiments. The stocking was worn for 24 h and placed in a clean glass jar approximately 12 h before use in an experiment. Approximately the same procedure was used to collect cow skin emanations, except that the nylon stocking was tied around the hind leg

**Figure 1** Catches of *Anopheles quadriannulatus* SKUQUA strain with carbon dioxide. The total proportion of mosquitoes caught with either treatment after 10 replicate trials is shown. Asterisks mark significant differences between the total number caught with treatment 1 and treatment 2 ( $\chi^2$  test: ns:  $P>0.05$ , \*\*\* $P<0.001$ ). n = total number of mosquitoes caught with both treatments (also expressed in parentheses as the percentage of mosquitoes caught out of the total number of mosquitoes leaving the release cage in 10 replicate trials). Different letters indicate significant differences in the total number of mosquitoes responding in each experiment. Error bars show 95% confidence limits. The table below the graph indicates the odour combination tested: CO<sub>2</sub> (<sup>4.5</sup>/<sub>230</sub>) = 4.5% carbon dioxide delivered at 230 ml min<sup>-1</sup>, CO<sub>2</sub> (<sup>4.5</sup>/<sub>1000</sub>) = 4.5% carbon dioxide delivered at 1000 ml min<sup>-1</sup>, and CO<sub>2</sub> (<sup>20</sup>/<sub>230</sub>) = 20% carbon dioxide delivered at 230 ml min<sup>-1</sup>.



of a cow just underneath the hock (the most convenient area to tie the stocking), for 12 h before being placed in a clean glass jar for storage before and between experiments. During the experiments, 'cow' and 'human' stockings were laid flat inside the respective glass traps of the olfactometer. The attraction of mosquitoes to individual humans and cows may vary due to differences in volatile emanations but it was not possible to test more than one human and one cow throughout this study. The relative attractiveness of the cow was not known, but the human host (H.V. Pates) had an 'average' level of attractiveness compared with 30 other people in standardised olfactometer experiments with *An. gambiae* s.s. (R.C. Smallegange and Y.-T. Qiu, pers. comm.).

The proportion of mosquitoes caught by a stimulus determined the attractiveness of a stimulus in a two-choice test. These proportions were transformed into arcsines of the square roots for analysis. Differences between the response levels to treatments and differences between separate trap catches with a given stimulus were analysed using ANOVA and contrasted by the Tukey test. Differences within each two-choice test were analysed with a  $\chi^2$ -test using the total number of mosquitoes caught after 10 replicates.

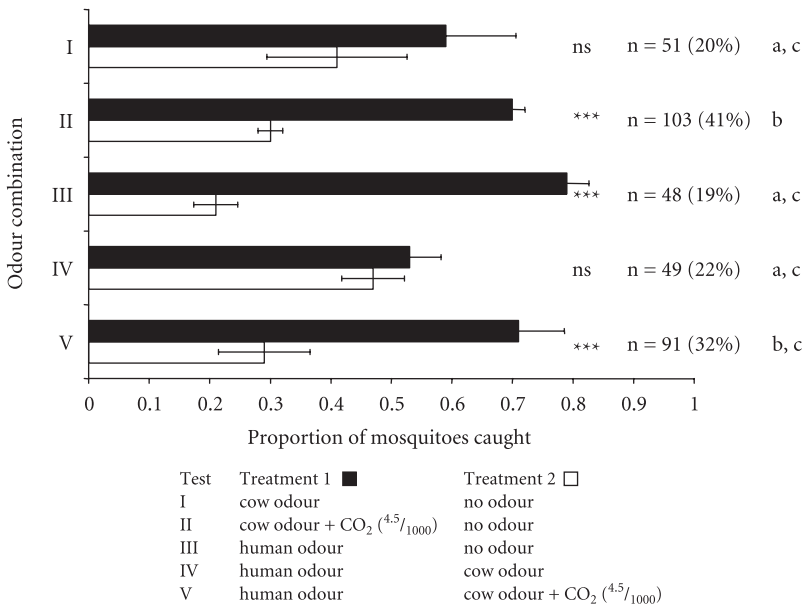
## Results

Most mosquitoes selected for the experiments left their release cage and flew upwind, irrespective of treatment

(85.3% of 3054 mosquitoes tested). There was no effect of day on the total catch size obtained in experiments (ANOVA,  $P>0.05$ ), and control experiments with no odour resulted in similar entry responses to both traps, showing that the olfactometer was symmetrical. Acetone and octenol were tested alone against a control (clean air) but no mosquitoes responded to either stimulus (120 mosquitoes tested). When acetone in combination with carbon dioxide (at animal equivalents) was tested, only 2% of 120 mosquitoes responded, therefore these experiments were excluded from further analysis. Results of the other trials are presented in Figures 1 and 2.

### Response to carbon dioxide and octenol

Significantly fewer mosquitoes responded in the control experiment (no odour vs. no odour) and carbon dioxide delivered at cow equivalents experiment (CO<sub>2</sub> (<sup>4.5</sup>/<sub>1000</sub>) vs. no odour) than in all other experiments (ANOVA,  $P<0.001$ ) (Figure 1, tests I and III). There was no significant difference between the number of mosquitoes entering the port containing carbon dioxide delivered at human equivalents, at cow equivalents, or at the higher concentration of 20%, when 'no odour' was the alternative choice, i.e., carbon dioxide alone did not increase or decrease trap entry. However, significantly more mosquitoes entered the 'no odour' port when carbon dioxide was offered in combination with octenol. These results show that carbon dioxide did not inhibit trap entry unless it was combined with octenol.



**Figure 2** Response of *Anopheles quadriannulatus* SKUQUA strain to skin emanations. ( $\chi^2$  test: ns: P>0.05, \*\*\*P<0.001). See Figure 1 for full explanation.

**Response to skin emanations**

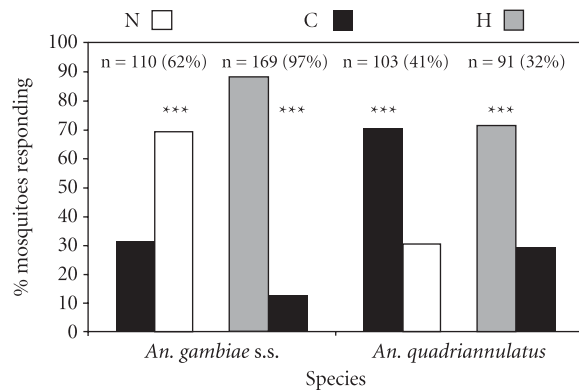
Significantly more mosquitoes responded to cow odour + carbon dioxide than when the cow odour was tested alone (ANOVA, P<0.001). There was no significant difference in the number of mosquitoes entering the cow odour port when ‘no odour’ was the alternative choice. However, in the human odour vs. no odour experiment, significantly more mosquitoes entered the human odour port (P<0.001; Figure 2, test III). There was no significant difference between the number of mosquitoes entering the human odour port when tested against cow odour. Significantly more mosquitoes entered the cow odour + carbon dioxide port when tested against no odour (P<0.001). However, significantly (P<0.001) more mosquitoes entered the port containing human odour when tested against cow odour + carbon dioxide.

**Comparison of *Anopheles gambiae* s.s. and *Anopheles quadriannulatus* behaviour**

*Anopheles gambiae* s.s. and *An. quadriannulatus* responded differently to combinations of no odour vs. cow odour + CO<sub>2</sub> (4.5/1000) or human odour vs. cow odour + CO<sub>2</sub> (4.5/1000) (Figure 3). *Anopheles gambiae* s.s. was strongly repelled by cow odour + CO<sub>2</sub> (4.5/1000), with more mosquitoes entering the no odour port than the port of the test odour (P<0.001). By contrast, with this combination *An. quadriannulatus* preferred the port with cow odour + CO<sub>2</sub> (4.5/1000) (P<0.001). Both species significantly preferred human odour above cow odour + CO<sub>2</sub> (4.5/1000). The entry response of *An. gambiae* was significantly (P<0.001) greater than that of *An. quadriannulatus*.

**Discussion**

The results show that carbon dioxide alone did not have an effect on trap entry. Fewer mosquitoes were caught compared to the control when carbon dioxide was combined with octenol, but significantly more mosquitoes were caught when carbon dioxide was combined with cow odour, except when this odour combination was tested against human odour. The results from these experiments



**Figure 3** A comparison of the behaviour of *Anopheles gambiae* s.s. and *Anopheles quadriannulatus* in the olfactometer (data from this paper and from Pates et al., 2001a,b). N = no odour; C = cow odour + CO<sub>2</sub> (4.5/1000); H = human odour. Asterisks mark significant differences between the total number trapped with each treatment ( $\chi^2$  test: \*\*\*P<0.001). n = total number of mosquitoes caught with both treatments (also expressed in parentheses as the percentage mosquitoes of the total number of mosquitoes released). CO<sub>2</sub> (4.5/1000) = 4.5% carbon dioxide delivered at 1000 ml min<sup>-1</sup>.

showed a consistently low overall response (23.1%) of *An. quadriannulatus* to the odour stimuli offered in the olfactometer. Therefore each experiment was replicated 10 times. In the absence of odour stimuli, only 10% of mosquitoes leaving the release cages responded by entering either the right or left port. Under the same circumstances, more than three times as many *An. gambiae* s.s. responded by entering either port (Pates et al., 2001a). This may reflect inherent differences in the mosquito's behaviour. In general, *An. gambiae* s.s. is endophilic whereas *An. quadriannulatus* species A is markedly exophilic, although indoor house resting *An. quadriannulatus* specimens have been recorded in southern Africa (Hunt & Mahon, 1986). *Anopheles quadriannulatus* species B in Ethiopia tends to be more endophilic, which is probably a reflection of high altitude and low temperatures (White, 1974). Mukwaya (1976, 1977) observed that the response of a non-anthropophilic strain of *Ae. simpsoni* (Theobald) from Bwayise, Uganda seemed to be inhibited by the enclosed chamber of an olfactometer and the mosquitoes could not fly inside a Y-tube. The reduced overall response observed with *An. quadriannulatus* was also reflected in the proportion of mosquitoes that left the release cage (85% vs. 99% of *An. gambiae* s.s.; see Pates et al., 2001a).

The effect of carbon dioxide on *An. quadriannulatus* was consistent in that there was no preference for the carbon dioxide port or the no odour port, regardless of the concentration or release volume of carbon dioxide (Figure 1). Carbon dioxide released from traps in the field very often increases the catch; this may well be because of the plume structure, which is encountered in discrete 'packets' rather than as a continuous stream (Murlis et al., 2000). Recently, Dekker et al. (2001b) demonstrated that the response of *An. gambiae* s.s. to carbon dioxide in an olfactometer was strongly affected by the plume structure. A homogeneous plume of carbon dioxide caused a reduced entry response, whereas a turbulent plume had no effect or a positive effect on the entry response, depending on the presence of human skin emanations. A similar effect was also recorded for *Ae. aegypti* L. (Geier et al., 1999; Dekker et al., 2001b). In our experiments we used the same olfactometer as Dekker et al. (2001b), and recent simulation studies of the odour plume with smoke revealed that the carbon dioxide plume as presented in our investigations was highly filamentous, and should therefore not have caused inhibited trap entry (W. Takken, unpubl.). A field study in South Africa found that significantly more *An. quadriannulatus* were collected in traps baited with carbon dioxide than with a human host (Dekker & Takken, 1998). Costantini et al. (1996) compared dose-response to carbon dioxide with a standard human bait catch. The highest dose of carbon dioxide

did not attract more *An. gambiae* s.l. than one human bait catch, whereas the three highest doses of carbon dioxide tested caught significantly more *Mansonia uniformis* (Theobald) (a more generalist feeder) than did one human bait. The response of *Ae. aegypti* to carbon dioxide has been tested many times and generally shows no effect of this gas when tested alone, but a synergistic effect when tested with skin emanations or with L-lactic acid (Acree et al., 1968; Gillies, 1980). From these studies it follows that carbon dioxide can be considered an activator for *An. gambiae* s.s. (Dekker et al., 2001b) and *An. quadriannulatus* species A (this study) when present in discrete filaments of air, and as the olfactory stimulus alone increased trap catch in the olfactometer. Apparently it is not the actual concentration but the difference from background carbon dioxide concentration that is being perceived by the insect (Grant et al., 1995) and which activates it to engage in upwind anemotaxis.

The effects of carbon dioxide as a mixture with animal emanations was entirely different from carbon dioxide alone; carbon dioxide alone did not cause an effect, but carbon dioxide + cow odour was attractive to *An. quadriannulatus*. These results provide further support to the hypothesis that carbon dioxide is a more important cue for zoophilic species when it is present in combination with other odours, such as skin emanations. However, there was no significant difference between the number of mosquitoes choosing the cow odour port when human odour or no odour (in the absence of carbon dioxide) were the alternative choices. This appears anomalous for a zoophilic species, because one would have expected significantly fewer mosquitoes to enter either the no odour port or the human odour port in these circumstances. Furthermore, when human odour was tested alone or against cow odour + carbon dioxide (Figure 2, tests III and V), significantly more mosquitoes were attracted to the human odour source. These results were unexpected, as *An. gambiae* s.s. showed similar behaviour under similar conditions (see Pates et al., 2001a).

In spite of the overruling effect of human odour compared to cow odour + carbon dioxide, it is clear from these results that the behaviour of *An. gambiae* s.s. and *An. quadriannulatus* under the experimental conditions used was very different (Figure 3), but these differences are not as stark as was previously thought. This renders the proposal for attempting to select genes involved in zoophily by cross-mating and backcrossing (Curtis, 1994; Curtis et al., 1999) unlikely to be successful, unless an authentic zoophilic mosquito is used to produce the initial F<sub>1</sub> hybrid generation.

We considered whether selection within the *An. quadriannulatus* colony for a preference for human odour due to feeding females on a human arm may have occurred. This



phenomenon was observed by Laarman (1958), who fed *An. atroparvus* van Thiel on rabbits in the laboratory (this mosquito feeds on pigs in nature). When laboratory reared and wild *An. atroparvus* were tested in an olfactometer, it was found that the odour from a rabbit was significantly less attractive to the wild mosquitoes than to the laboratory reared specimens. It was concluded that the laboratory strain, which had been fed on rabbits for at least 20 generations, had developed an adaptation to that host. However, a field experiment in which *An. quadriannulatus* species A and *An. gambiae* s.s. were allowed to choose unhindered between a calf and a human host, clearly showed that *An. quadriannulatus* was significantly less anthropophilic than *An. gambiae*. This experiment ruled out the selective effect of exposure to a human arm, as a different strain of *An. quadriannulatus* species A (SANQUA) that had never fed on a human arm exhibited a similar behaviour as the SKUQUA strain (Pates et al., 2001b). From this we conclude that the observed response of *An. quadriannulatus* to human odours has a genetic basis.

The repellent effect of the binary mixture of carbon dioxide and octenol was surprising, as octenol has been identified in cow breath and has been effective in trapping a fairly wide range of species of mosquito when combined with carbon dioxide (Takken & Kline, 1989; Kline, 1994; Van den Hurk et al., 1997). Octenol is present in leguminous plants and is probably released during the process of rumination (Hall et al., 1984). It is possible that the observed repellent response of *An. quadriannulatus* to octenol was the result of using an inappropriate concentration of octenol, and further studies testing a range of octenol concentrations, in combination with carbon dioxide, would be needed to investigate this.

Studies on the host preference of both species of *An. quadriannulatus* are limited because of its observed zoophilic behaviour in the field, which has led to the opinion that this species is not a vector of malaria. Furthermore, the fact that Ethiopian *An. quadriannulatus* species B has been regarded as the same species as its southern African namesake led to the assumption that both species share similar feeding habits and vectorial capacity (Coetzee et al., 2000). However, White (1974) recognised that *An. quadriannulatus* from Ethiopia was more endophilic than in South Africa and he observed that 'some females willingly bite humans, indoors or outdoors, especially when located confusingly close to cattle'. It is considered that the most reasonable interpretation of the results presented in this paper is that *An. quadriannulatus* species A is not exclusively zoophilic, but is actually an opportunistic species although such opportunistic behaviour has not yet been observed in the field. Humans constitute a minor fraction of the potential blood hosts of *An. quadriannulatus*

in its natural habitat in southern Africa (Gillies & de Meillon, 1968; Prior & Torr, 2002), and we may therefore assume that selective pressure favouring anthropophilic characteristics are entirely absent in field populations of this mosquito species. Other members of the *An. gambiae* complex are also opportunistic (Coluzzi, 1984), feeding rapidly on humans when the opportunity arises, particularly when animal hosts are scarce. These opportunistic species can in fact switch from being largely zoophilic to expressing a high degree of anthropophily between seasons (Charlwood et al., 2000). It is thus clear that results from laboratory experiments as reported here should be complemented by field experiments, in order to understand the plasticity of presumed behavioural traits.

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