

L-lactic acid: a human-signifying host cue for the anthropophilic mosquito *Anopheles gambiae*

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Abstract. Using a dual-choice olfactometer, the role of L-lactic acid was investigated in relation to host-seeking and selection by female *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) mosquitoes in a Y-tube bioassay. L-lactic acid alone was not attractive, but it significantly augmented the attractiveness of CO₂, skin odour and skin-rubbing extracts from humans and other vertebrates. Comparing the left and right index fingers of the same person, one could be made more attractive than the other by adding L-lactic acid to the air stream over that finger. The difference in L-lactic acid concentration between the two air streams offered to the mosquitoes fell within the natural range of variation emanating from a human hand, suggesting that L-lactic acid modulates intraspecific host selection by *An. gambiae*. Analysis of skin rubbings from various vertebrates (carnivores, chickens, primates, rodents, ungulates) indicated that humans have uniquely high levels of L-lactic acid on their skin. Tests with extracts of skin rubbings from cows and humans, with and without added L-lactic acid, suggest that naturally lower levels of L-lactic acid contribute to the lesser attractiveness of non-humans to *An. gambiae* s.s.

Key words. *Anopheles gambiae*, anthropophily, carbon dioxide, cows, host preference, host selection, humans, L-lactic acid, mosquito behaviour, olfactometer, skin odour.

Introduction

For a mosquito to transmit pathogens causing human disease it is necessary to bite different people, i.e. at least two mosquito–human contacts. Therefore, the level of anthropophily, which is the degree to which humans are bitten in preference to other types of host, influences the vectorial capacity of a mosquito species exponentially (Garrett-Jones, 1964). Several studies have shown that mosquitoes base their choice partly on host odours (Costantini *et al.*, 1996, 1998; Mboera *et al.*, 1997; Dekker & Takken, 1998; Dekker *et al.*, 2001b).

The important attractant carbon dioxide, CO₂ (Mboera & Takken, 1997), appears to be of little importance in host discrimination by mosquitoes. Its presence above atmospheric levels does not signify a specific host, although

trap capture of some mosquito species that prefer to bite large bovids increased with increased CO₂ release rates (Reeves, 1953; Costantini *et al.*, 1996; Dekker & Takken, 1998). The anthropophilic mosquitoes *Anopheles gambiae* Giles *sensu stricto* and *Culex quinquefasciatus* Say, however, selected human odour over calf odour, despite similar weights and thus similar CO₂ expiration rates, and selected humans over human CO₂ equivalents. (Costantini *et al.*, 1993, 1998; Mboera *et al.*, 1997; Dekker & Takken, 1998). This suggests that these two species use unknown odours to discriminate between humans and non-humans.

Excretions of sweat glands and sebaceous glands and their microbial breakdown products create the body-odour profile of vertebrates. The composition and distribution of sweat glands and skin microflora vary among vertebrate species and are likely to be responsible for the olfactory signature of a species (Albone, 1984). Eccrine sweat-gland density on human skin is by far highest among all vertebrates (Sokolov, 1982). Human-eccrine sweat contains high levels of L-lactic acid (Sato & Dobson, 1973).

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Among others, Acree *et al.* (1968) and Geier *et al.* (1996) reported that L-lactic acid, although hardly attractive on its own, in combination with other skin odours and CO₂ synergistically increased captures of *Aedes aegypti* (L.).

We examined whether L-lactic acid plays a role in host-seeking behaviour of the anthropophilic species *An. gambiae* s.s., the main Afrotropical vector of malaria. By using the same design of Y-tube olfactometer in which *Ae. aegypti* was tested for its response to L-lactic acid (Geier *et al.*, 1996; Geier & Boeckh, 1999), we could correlate our results with previous tests with *Ae. aegypti*. We also investigated whether L-lactic acid is a human-specific skin component and explored its role in intra- and interspecific host selection by *An. gambiae*.

Materials and methods

Mosquitoes

The *An. gambiae* colony originated from Suakoko, Liberia (courtesy of Dr W. Takken), and was maintained at 25°C and a LD 12:12 h photoperiod without simulated dusk. Larvae were fed on Tetramin® fish food (Tetra, Blacksburg, VA, U.S.A.) and reared in trays of water (15 × 30 cm area, 7 cm depth). Pupae were collected into bowls placed in cages for adults to emerge: males and females were allowed to consort together for mating. Adults were kept in cubic 30 cm gauze cages at 95–100% r.h., provided with diet of 6% glucose solution and females were routinely offered a bloodmeal twice per week. For olfactometer tests we used non-blood-fed female *An. gambiae* aged 5–14 days.

Experimental set-up and testing procedures

We used a dual-choice olfactometer of Plexiglas® (Geier & Boeckh, 1999). The airflow was 20 cm/s in both upwind arms and 40 cm/s in the downwind arm. Air was purified through an activated charcoal filter, and adjusted to 70 ± 5% r.h. and 28 ± 1°C. Half an hour before each experiment, approximately 18 mosquitoes were lured from the holding cage into a release cage and left in the olfactometer air stream for 20 min to acclimatize. A test was started by slowly opening the release cage. During the 1-min test period mosquitoes could enter either upwind arm. After each test the upwind arms and the release cage were closed and the mosquitoes in each section of the olfactometer were counted. By reversing the air stream the mosquitoes were lured back into the release cage and left for 15 min, after which a new test was started. Each group of mosquitoes was tested for a maximum of six different consecutive treatments. Preliminary tests showed that repeated tests did not change the response of the mosquitoes. The treatments were randomised. Tests were conducted during the last 6 h of scotophase.

Odours

CO₂ and medical air (99.9% purity), both obtained from a pressurized cylinder (Linde, Germany), were mixed to achieve a 0.5% CO₂ plume. The structure of CO₂ plumes strongly influences upwind flight and trap capture of *Ae. aegypti* and *An. gambiae* (Geier *et al.*, 1999; Dekker *et al.*, 2001a). Therefore, we tested both turbulent and homogeneous CO₂ plumes (details in Geier *et al.*, 1999).

We used a homogeneously structured L-lactic acid plume, which at the concentrations used in our study (0.06, 0.6 and 1.7 nmol/L), readily induced upwind flight in *Ae. aegypti* (Geier *et al.*, 1999). The highest concentration roughly equals the amount emanating from a human hand (Smith *et al.*, 1970; Ogawa, 1975).

Skin extracts of humans and cows were obtained by rubbing the skin of an individual with a 1 g cotton swab for 5 min. Each cotton swab was then wrapped in aluminium foil, frozen and transported to the laboratory. By wearing disposable gloves, care was taken not to contaminate the cotton with odours from the person taking the samples. They were packed into a glass column (15 mm in diameter) and extracted with 0.5 mL alcohol for each piece of cotton. Ten µL of the skin extracts was transferred into a pipette. After the alcohol had evaporated, the pipette was inserted into a heating element and clean air was blown through at a rate of 1600 mL/min during the 1 min experimental time (details in Geier *et al.*, 1996).

Skin odour was tested by inserting a test person's fingers (four digits or only the index finger) through a port into the odour chamber. Test persons rinsed their hand with tap water 1 h before each experimental comparison.

Analysis of L-lactic acid content on vertebrate skin

We analysed the L-lactic acid content of skin rubbings of 14 species of vertebrates (Table 1). Skin L-lactic acid content was determined for domestic animals, i.e. possible alternative hosts for *An. gambiae* in a natural setting, and of other taxonomic groups. To avoid contamination, the animals were not touched. A 0.5 g piece of cotton was rubbed gently for 2 min along a 20 × 20 cm skin area on the back of an animal. Animals were rubbed on both the outer hair layer and directly on the skin. Samples were wrapped in aluminium foil, frozen and transported to the laboratory. Each sample was tightly packed in a large volume Pasteur pipette (7.5 mm diameter) and extracted with demineralized water. Repetitive extraction of the same rubbing showed that 90–95% of the L-lactic acid in the rubbing was recovered in the first 1 mL. The extracts were analysed for L-lactic acid content with Sigma Lactate Reagent. L-lactic acid is converted to pyruvate and hydrogen peroxide (H₂O₂) by lactate oxidase. H₂O₂ catalyses the oxidative condensation of chromogen precursors to produce a coloured dye with an absorption maximum at 540 nm. The absorbance was measured with a Diode Array Spectrophotometer (Hewlett Packard 8452A, Palo Alto, CA, U.S.A.) at 540 nm.

The colour intensity was linear with L-lactic acid content in the range 1–500 µg/mL. Below 1.0 µg/mL the test was not able to accurately distinguish L-lactic acid concentrations.

Test series

To test for a possible synergism between L-lactic acid and CO₂ for *An. gambiae*, L-lactic acid was tested alone (1.7 nmol/L) and in combination with 0.5% CO₂. No odour, CO₂ and hand (test person P1) served as controls. Because the structure of CO₂ plume strongly influences upwind flight and trap catch (Geier *et al.*, 1999; Dekker *et al.*, 2001a), we tested a turbulent and a homogeneous CO₂ plume.

To test for the effect of addition of L-lactic acid to human skin emanations, we compared the relative attractiveness of two fingers with L-lactic acid added to one of the air streams. A comparison of the relative attractiveness of the fingers without added L-lactic acid to either air stream served as control. We tested the relative attractiveness of the two fingers of test person 1 with and without addition of 1.7 nmol/L L-lactic acid, and similarly of two different persons (test persons P2 and P3). We also tested different concentrations of added L-lactic acid (test person P4), 0.06, 0.6 and 1.7 nmol/L.

Finally, we tested whether L-lactic acid could augment the attractiveness of extracts of human and cow skin rubbings. In these experiments L-lactic acid (1.7 nmol/L) was added to the air stream containing the extract. The extract without added L-lactic acid and human hand (P1) served as a control.

Data analysis

The olfactometer test results were expressed in terms of two parameters:

- **activation** by a treatment is the percentage of female mosquitoes that left the release chamber;
- **attractiveness** of a treatment is the percentage of female mosquitoes caught in the treatment chamber. Very few mosquitoes were caught in the control arm, unless a stimulus was present.

The data were arcsin transformed and analysed by one-way ANOVA and LSD to sort out differences between means. The relative attractiveness of the two fingers was analysed with a one-sample *t*-test.

Results

CO₂, L-lactic acid and their combination

When no odour was present, 40–50% of the mosquitoes left the release chamber (Fig. 1A) and very few mosquitoes

were caught in one of the upwind arms of the Y-tube (Fig. 1B). L-lactic acid did not increase activation ($P=0.23$ and $P=0.09$ for Fig. 2A,B, respectively) or attraction ($P=0.62$ and $P=0.82$, respectively) of *An. gambiae*. CO₂ activated *An. gambiae* ($P<0.001$ and $P=0.01$, turbulent and homogeneous CO₂ plume, respectively), and significant attraction to CO₂ occurred with a turbulent plume ($P<0.001$) but not with an homogeneous CO₂ plume ($P=0.11$). The attractiveness of CO₂ plus L-lactic acid was slightly higher than the arithmetic sum of the attractiveness of both odours separately, especially with CO₂ presented in a homogeneous plume. The attractiveness of a hand was significantly higher (65% of mosquitoes) than that of the other treatments ($P<0.001$ in all comparisons).

Finger + L-lactic acid compared to finger alone

When two digits were compared directly, they appeared equally attractive ($n=8$, $P=0.96$ for P1; $n=8$, $P=0.34$ for P2 and P3, Fig. 2). Addition of 1.7 nmol/L L-lactic acid to one of the two arms made this arm more attractive than the other ($n=8$, $P<0.001$ for P1; $n=8$, $P=0.003$ for P2 and P3; $n=35$, $P<0.001$ for P4). Differential attractiveness of the fingers (P4) was observed also with the addition of 0.63 nmol/L L-lactic acid ($n=35$, $P=0.01$), but not with 0.17 nmol/L L-lactic acid ($n=35$, $P=0.26$).

Addition of L-lactic acid to skin-rubbing extracts

Cow skin rubbing extract was less attractive than human skin rubbing extract ($n=28$, $P<0.001$, Fig. 3). This difference disappeared when 1.7 nmol/L L-lactic acid was added to the air stream with the cow skin rubbing extract ($P=0.886$). In contrast, addition of 1.7 nmol/L L-lactic acid did not enhance attractiveness of human skin rubbing extract ($P=0.157$). The hand was more attractive than any of these treatments ($P<0.001$).

L-lactic acid on human and non-human skin

Table 1 shows that human skin rubbing extracts had higher levels of L-lactic acid than any other vertebrate sample, with a mean of 151 µg/mL, averaging 10-fold more than the next highest, chimpanzee. Some cow skin rubbing extracts had elevated levels of L-lactic acid, but the highest was still 2.5-fold lower than the lowest level found on humans. We detected a five-fold range of difference in L-lactic acid content of rubbing extracts from 10 humans.

Discussion

We found that L-lactic acid is an important host stimulus for the anthropophilic mosquito *Anopheles gambiae*, increasing

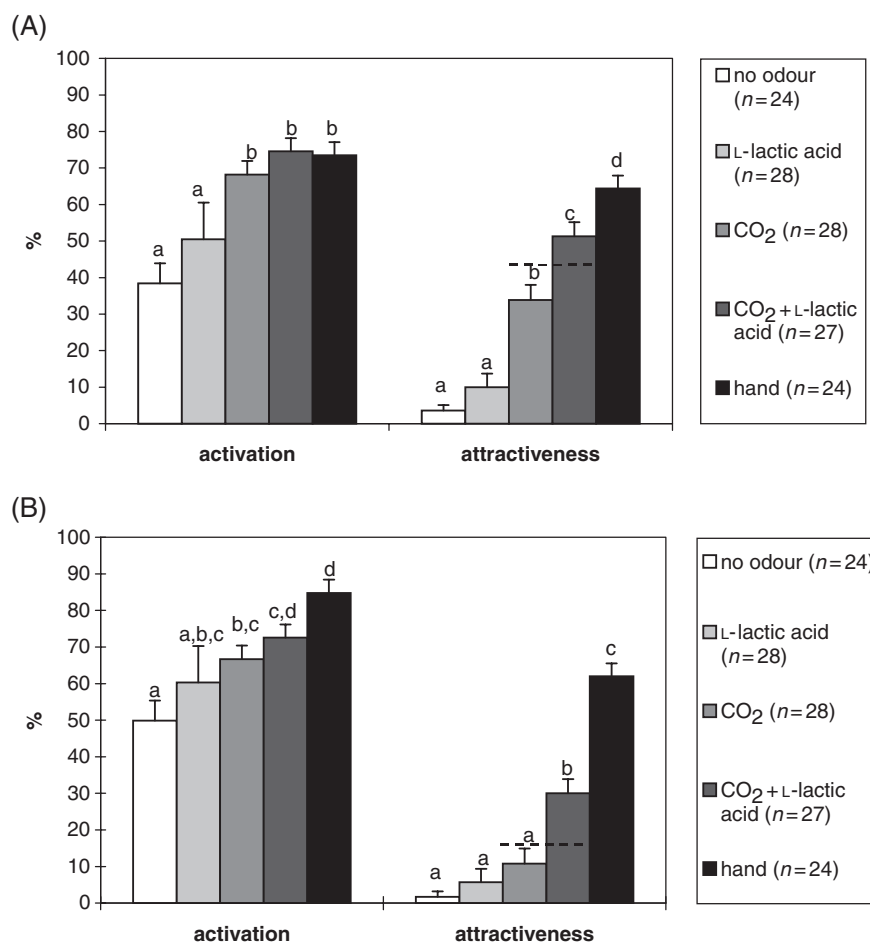


Fig. 1. *Anopheles gambiae* activation by and attractiveness of five treatments of the air flowing through one arm of the olfactometer. (A) CO₂ presented as a turbulent plume. (B) CO₂ presented as a homogeneous plume. Dotted line shows the level for arithmetic sum of the attractiveness of L-lactic acid and of CO₂. Bars that share no letters above are significantly different at $P < 0.05$.

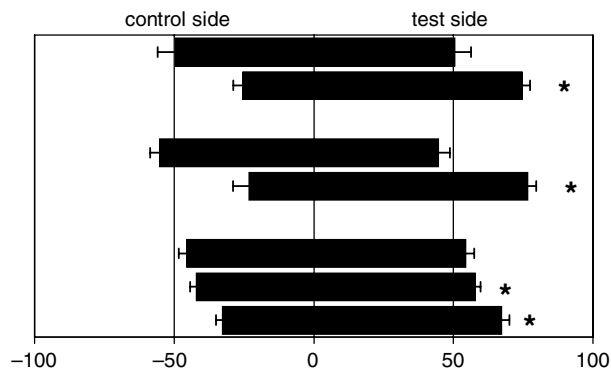


Fig. 2. Proportions of *Anopheles gambiae* caught in the two upwind arms (test and control) of the olfactometer Y-tube. Upper pair of bars: relative attractiveness of the fingers of one test person (P1), before and after addition of L-lactic acid to one of the air streams. Mid pair of bars: as above, comparing fingers of two different test persons (P2 and P3). Lower three bars: index fingers of test P4 with and without added L-lactic acid at different concentrations. Significant difference at $P < 0.05$ indicated by *.

their response to other human skin components, although L-lactic acid was not attractive on its own. Moreover, our results indicate that humans have an unusually high level of L-lactic acid on their skin. Cow skin extract was relatively less attractive to *An. gambiae*, but addition of L-lactic acid to the air stream made cow skin as attractive as human skin extract. Together these findings indicate that *An. gambiae* uses L-lactic acid in host-odour recognition and host selection.

Anopheles gambiae females showed strong preference for hum between L-lactic acid and other skin odours and suggesting that differential levels of L-lactic on the skin cause differential attractiveness of humans to mosquitoes. Whereas responses of *Aedes aegypti* show strong synergism between CO₂ and L-lactic (Geier & Boeckh, 1999), the catch of *An. gambiae* with L-lactic acid plus CO₂ was only slightly more than the sum of catches with L-lactic acid and CO₂ presented separately.

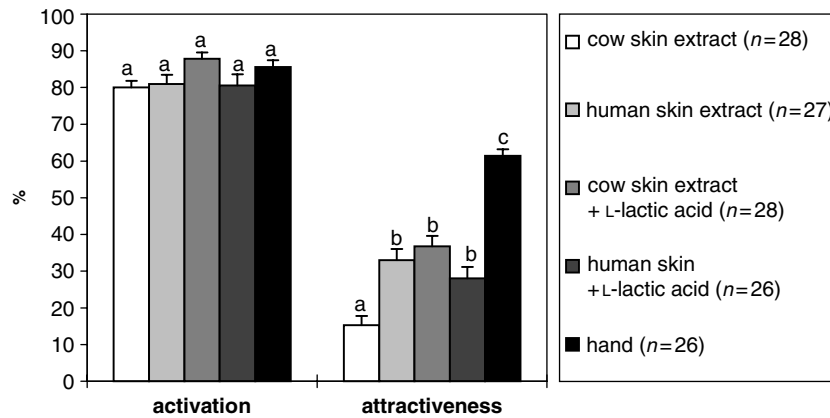


Fig. 3. Attractiveness to *Anopheles gambiae* of extracts of human skin rubbings and cow skin rubbings, with and without the addition of 1.7 nmol/L L-lactic acid. Bars that share no letters above are significantly different at $P < 0.05$.

L-lactic acid on human and non-human skin

L-lactic acid on the human skin is an end-product of glycolysis during anaerobic metabolism of the myoepithelial cells of the eccrine sweat glands (Sato & Dobson, 1971, 1973). Eccrine glands are, in contrast with apocrine glands, not associated with hair follicles and only in humans, where they occur all over the body, do they play an important role in thermoregulation (Robertshaw, 1991). Non-human primates have a much lower density of eccrine sweat glands than humans (Sokolov, 1982), which may explain the low level of skin L-lactic acid on rhesus monkey and chimpanzee.

Apocrine glands also often contain myoepithelial cells, and those seem to play a role in sweat excretion and thermoregulation in some large mammals (Allen & Blight, 1969; Jenkinson *et al.*, 1979; Robertshaw, 1991). Our data show, however, that there is far less L-lactic acid on the skin of cattle than on humans. Possibly aerobic glucose metabolism predominates over anaerobic metabolism in apocrine

sweating. Both types of glucose metabolism exist in primate eccrine glands (Sato & Dobson, 1971).

Contribution of L-lactic acid to differential attractiveness of humans

Odour-based differential attractiveness of humans to mosquitoes has been reported frequently (Schreck *et al.*, 1990; Lindsay *et al.*, 1993), and was associated with age (Carnevale *et al.*, 1978) and sex (Gilbert *et al.*, 1966). Differential attractiveness of humans also seems to vary among mosquito species (Curtis *et al.*, 1987). Knols *et al.* (1995) found equal attractiveness of three human subjects to opportunistic *Mansonia* spp., but one of them was more attractive to the anthropophilic *Cx. quinquefasciatus*, *Anopheles funestus* and *An. gambiae*. Acree *et al.* (1968) and Smith *et al.* (1970) found that test persons with high

Table 1. Amount of L-lactic acid in skin-rubbing extracts from humans compared with 12 other mammals and chickens. ND, no L-lactic acid detected. <1.0: test could not accurately separate concentrations lower than 1.0 µg/mL L-lactic acid.

Species	Common name	n	L-lactic acid (µg/mL)	
			mean	SD
<i>Homo sapiens</i>	Human	12	151	63
<i>Pan troglodytes</i>	Chimpanzee	4	13.5	3.5
<i>Macaca mulatta</i>	Rhesus Monkey	2	9.7	0.3
<i>Sus scrofa</i>	Pig	4	6.8	4.5
<i>Bos taurus</i>	Cow	13	9.4	9.6
<i>Capra hircus</i>	Goat	2	2.5	3.5
<i>Ovis aries</i>	Sheep	4	9.7	8.4
<i>Equus caballus</i>	Horse	3	3.3	4.5
<i>Llama glama</i>	Llama	2	5.6	3.2
<i>Canis familiaris</i>	Dog	2	5.7	3.6
<i>Felis catus</i>	Cat	3	ND	
<i>Rattus norvegicus</i>	Rat	2	ND	
<i>Oryctolagus cuniculus</i>	Rabbit	2	ND	
<i>Gallus gallus</i>	Chicken	4	< 1.0	

amounts of L-lactic acid on the skin and in the headspace generally were more attractive to *Ae. aegypti*. Other skin odours, however, may also have caused this correlation. Because we tested recently washed fingers of one test person, it is unlikely that the two fingers differed substantially in odour profile, which is supported by the 1 : 1 distribution before adding L-lactic acid. Therefore, the shift in preference can be ascribed to the addition of L-lactic acid to the air stream.

Humans differ in their level of L-lactic acid in both skin rubbings and skin headspace by at least a factor of 5 (Table 1; Acree *et al.*, 1968; Smith *et al.*, 1970). Factors contributing to variation in L-lactic acid levels between humans include eccrine sweat gland density and their activity (Sokolov, 1982) and differential skin pH (Albone, 1984). In our study the amount of added L-lactic acid needed to induce a shift in preference between fingers was approximately 2–6 times the amount from an average finger. This is within the order of magnitude of naturally encountered differences in L-lactic acid levels on the skin of humans (see above). Nearly identical results were obtained with *Ae. aegypti* (Steib *et al.*, 2001), which indicates that L-lactic acid may contribute to intraspecific selection by several anthropophilic mosquitoes species.

Significance of L-lactic acid in host selection by mosquitoes

The amount of L-lactic acid obtained from human skin was found to be much greater than from the skin of non-human vertebrates of various types (Table 1). Cow skin rubbings had low attractiveness to *An. gambiae*, but could be made as attractive as human skin rubbings when L-lactic acid was added (Fig. 3). Attractiveness of animal extracts to *Ae. aegypti* was even more strongly dependent on the addition of L-lactic acid (Steib *et al.*, 2001). In contrast, the addition of L-lactic acid to human skin extract did not enhance its attractiveness (Fig. 3), presumably because of the naturally high level of L-lactic acid in the extract. This suggests that, although cow odour contains compounds attractive to *An. gambiae* and *Ae. aegypti*, the low level of L-lactic acid compared to humans contributes to its relatively low attractiveness. Bloodmeal analysis from field-collected *An. gambiae* reveals that indeed a low percentage of *An. gambiae* feeds on other animals, especially cattle. This percentage increases with the ratio of cattle to humans (Garrett-Jones *et al.*, 1980).

Conversely, L-lactic acid may repel zoophilic haematophagous insects. Decreased capture rates of the bovine-biting tsetse *Glossina pallidipes* and *G. morsitans* (Clausen *et al.*, 1998) were observed when human odour was presented simultaneously with ox odour. L-lactic acid presented together with cow odour or cows sprayed with L-lactic acid gave similar results (Vale, 1979).

Human breath (Smith *et al.*, 1970) and presumably animal breath also contain L-lactic acid. One could argue therefore that L-lactic acid cannot be used as a human-specific cue. Although the precise instantaneous

concentrations of L-lactic acid from both sources are not known, the rate of L-lactic acid volatilization is several orders of magnitude higher from a human body than from human breath (Smith *et al.*, 1970). Our results also indicate that mosquitoes are able to discriminate between intermingled odour filaments from different sources. In the section of the olfactometer where the air streams come together a turbulent plume is created. The choice of the mosquitoes for the finger with higher levels of L-lactic acid indicates their ability to discriminate between closely intermingled skin-odour filaments with high and low L-lactic acid concentrations. Recent studies demonstrated such ability in other insects. Several moth species are able to discriminate between pulses of pheromone separated by less than 0.05 s (Mafra-Neto & Cardé, 1994; Vickers & Baker, 1994). *Helicoverpa zea* males were able to distinguish alternate pheromone and antagonist puffs separated in time by only 0.001–0.003 s (Fadamiro *et al.*, 1999). We suggest that under natural conditions mosquitoes might discriminate between breath- and skin-derived odour pulses, despite overlap in odour profile from both sources.

Conclusions and prospects

Odours that attract mosquitoes could be useful in the development of new trapping methods for either epidemiological research or for control of anthropophilic vectors by mass trapping (Takken & Knols, 1999). Together with CO₂, acetone and ammonia (Takken *et al.*, 1997; Braks *et al.*, 2001), L-lactic acid is the fourth compound shown to play a role in host-finding by *An. gambiae*. More importantly, the uniquely high levels of L-lactic acid detected on human skin could influence intra- and interspecific selection by anthropophilic mosquitoes and other haematophagous arthropods.

Although aedine and anopheline mosquitoes diverged long before *Homo sapiens* appeared (Lutz, 1985), evidently anthropophilic species representing these lineages are similarly responsive to L-lactic acid. Questions arise as to the distribution of receptors for L-lactic acid among Culicidae, what their response characteristics are and how such receptors affect host preference. Inter-specific differences in quantity, compartmentalization, sensitivity and hormonal control of sensitivity have been recorded for these receptors (both L-lactic acid-excited and L-lactic acid-inhibited cells) in several culicine mosquitoes: *Aedes aegypti*, *Ae. atropalpus*, *Ae. bahamensis*, *Ae. epactius* and *Culex pipiens* (Bowen, 1995, 1996). Comparative sensory physiology of mosquito species showing contrasted degrees of anthropophily may reveal a relationship between host preference and L-lactic acid receptor-cell characteristics.

Acknowledgements

We are indebted to Dr W. Takken of Wageningen University, the Netherlands for supplying *An. gambiae*.

Ms M. Müller helped with the experiments. We appreciated the cooperation of many people in taking the skin rubbing samples, particularly Ms C. Szijs, Dr L. Parr from Yerkes Regional Primate Research Center, Atlanta, Georgia, and Lizze Customer Processing Inc., Ontario, California. This research was supported in part by the University of California System-Wide Mosquito Research Program.

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Accepted 12 July 2001