



## A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours

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### Abstract

In a new bioassay a small Y-shaped wind tunnel is used to quantitatively investigate the responses of mosquitoes to host odours. Female yellow fever mosquitoes, *Aedes aegypti* (L.) (Diptera: Culicidae) were tested to (1) a human hand, (2) an extract with human skin residues, (3) L-(+)-lactic acid, and (4) carbon dioxide. The responses to the skin extract followed a sigmoid dose response curve. The most effective dose attracted 80–90% of the mosquitoes within 30 s and was as effective as the human hand. L-(+)-lactic acid was identified in this extract and found attractive for mosquitoes also when presented alone. Carbon dioxide stimulated taking flight and was attractive, an effect which was synergistically enhanced in combination with L-(+)-lactic acid. The presented bioassay is especially suited to test the behavioural effects of synthetic odours as well as of natural odour sources. Due to the fast response of the mosquitoes, the sensitivity, and the simplicity of the testing procedure it is a potent tool in the search for new attractive components.

### Introduction

Olfaction plays an important role in host recognition and orientation of female mosquitoes (Sutcliffe, 1987; Takken, 1991; Davis & Bowen, 1994). Yet, only a few of the odours are known, which are used by mosquitoes for host selection and location. Carbon dioxide, a compound universally emitted with the breath by all kinds of potential hosts, stimulates mosquitoes in that it increases flight activity and duration of flights; it is also an attractant (Gillies, 1980; Eiras & Jepson, 1991). L-(+)-lactic acid, present on human skin as well as in human breath, was repeatedly shown to attract *Ae. aegypti*, but only in combination with carbon dioxide (Acree et al., 1968; Smith et al., 1970; Eiras & Jepson, 1991, 1994). Recently it has been demonstrated that a synthetic mixture of fatty acids identified in Limburger cheese and human sweat attracts *An. gambiae* in a wind tunnel (Knols et al., 1997). Some amines, estrogens, amino acids, and alcohols were also reported to attract mosquitoes, but many of these results remained contradictory. More-

over, mixtures of such compounds never matched the effect of the natural host odour (summaries in Hocking, 1971; Takken, 1991). To identify new attractants and to explore the composition of the attractive natural host odour we have designed a new bioassay, which was supposed to meet the following requirements: (1) Simple and fast testing of many odour samples in a limited time, (2) Easy comparison of extracts from natural odour sources or synthetic attractants with the authentic, natural host odour (Miller & Strickler, 1984), (3) Monitoring of all behavioural sequences in the host finding process as are perception, activation, orientation towards the odour source, and landing (Sutcliffe, 1987), (4) Wide measuring range to differentiate the strength of attractive stimuli, (5) Easy control and avoidance of contamination caused by previous stimuli (Schreck et al., 1967, 1981). In order to prove whether this bioassay fulfils these requirements, the following stimuli were tested: a human hand, an extract from human skin residues, L-(+)-lactic acid, and carbon dioxide. The extract's effectiveness was characterised by dose-response measurements. To as-

sure that no important kairomones were lost in the course of the sampling of active material, we sampled the skin residues from a single person and compared their attractiveness with the one of the same person's hand.

We have chosen the yellow fever mosquito *Aedes aegypti* (L.) for our experiments because this species has been already studied in many laboratories and a substantial amount of knowledge exists about this insect (Takken, 1991, 1996; Davis & Bowen, 1994).

## Materials and methods

### Animals

Female *Ae. aegypti* (10–40 days old), from cultures in the Centre for Plant Research (*Pflanzenschutzzentrum*) at Bayer AG in Monheim (Germany), were used in our experiments. The larvae were fed with Tetramin® fish food. The adult females and males were kept together in a container under the following conditions: 26 °C–28 °C; 60%–70% r.h.; L12:D12. They had constant access to a 10% glucose solution on filter paper.

### Bioassay

**Experimental set-up.** The Y-tube olfactometer consisted of a transparent plexiglass tube (inner diameter: 7 cm, wall thickness: 0.5 cm; Figures 1, 2). Removable chambers were located at all three ends (inner diameter: 7 cm, length: 10 cm, thickness: 0.5 cm). One end of a chamber was covered with gauze while the other had a rotating gauze screen (Figures 1, 2). Both chambers on the branches of the Y-tube fit into PVC® tubes (inner diameter: 10 cm, length: 20 cm) where the odour stimuli are presented (Figures 1p, 2). Air from the institute's pressurised air system was cleaned with a charcoal filter (Figure 1b) before introducing it into the testing apparatus. A bath filled with deionized water was used to humidify the air (Figure 1e). The water was kept at a constant temperature of  $60 \pm 2$  °C by a heating coil with thermostat (Figure 1d). The ratio of moist and dry air was regulated (Figure 1h) so that a certain humidity could be maintained. Before introduction into the stimulus tubes, the moist air was warmed with heating elements (Figure 1i). Temperature in the olfactometer was  $28 \pm 1$  °C, the relative humidity was  $70 \pm 5\%$ . These values were constantly monitored by a thermohygrometer (Conrad Electronics, Wernberg, Germany) and regulated manually. The temperature of the testing room

was 22–26 °C. Pressurised air was permanently introduced into the olfactometer at a rate of 80 l/min. The wind speed in the tunnel, measured using a thermistor, was 0.2–0.3 m/s in the branches and 0.4–0.6 m/s in the stem of the Y-tube. A ventilator (Figure 2m) in front of the stimulus tubes was switched on after the experiment to reverse the air flow in the olfactometer (flow rate in Y-stem: 0.4–0.6 m/s) so that the mosquitoes could be lured back into the release chamber using the hand as an attractant. The olfactometer was placed on a white table, a white cardboard shield of 150 cm × 50 cm was placed on each side to prevent optical stimulation by the experimenter. Two fluorescent light tubes (40 W) served for overhead illumination. No additional visual stimuli were presented.

**Behavioural test.** For a test 20 female mosquitoes were lured out of their container into the release chamber using the human hand as a bait. This procedure ensured that all mosquitoes used in the test were ready to seek for a host. Five minutes after the release chamber had been attached to the olfactometer, the test stimulus was presented in one arm and the control stimulus in the other. At the same moment the release chamber was opened and the mosquitoes were allowed to enter the tube. Thirty seconds later, the rotating screens were closed and the numbers of mosquitoes counted, which were trapped in the release-, test-, and control chamber, respectively. Then the ventilator (Figure 2m) was switched on to lure the mosquitoes back into the release chamber. In this way we determined whether the mosquitoes were still ready to respond to the natural host. Generally a test took no more than 6 min. The time period between two tests for one mosquito group was at least 30 min. Control tests after previous odour stimulation were repeatedly used to check for contamination (see results). In preliminary experiments we had observed strong contamination effects when the inner walls of the olfactometer were touched with the hands; therefore this was carefully avoided in further experiments. As soon as contamination was detected, the olfactometer was thoroughly cleaned with detergents, water, and ethanol. Tests ran from 9:00 a.m. to 6:00 p.m.

Six experiments were conducted independently from each other with different groups of mosquitoes. Within an experiment the groups were randomly tested to each treatment, whereby a given mosquito group was exposed to all treatments. In successive tests the test stimulus was alternately offered in each arm of the olfactometer.

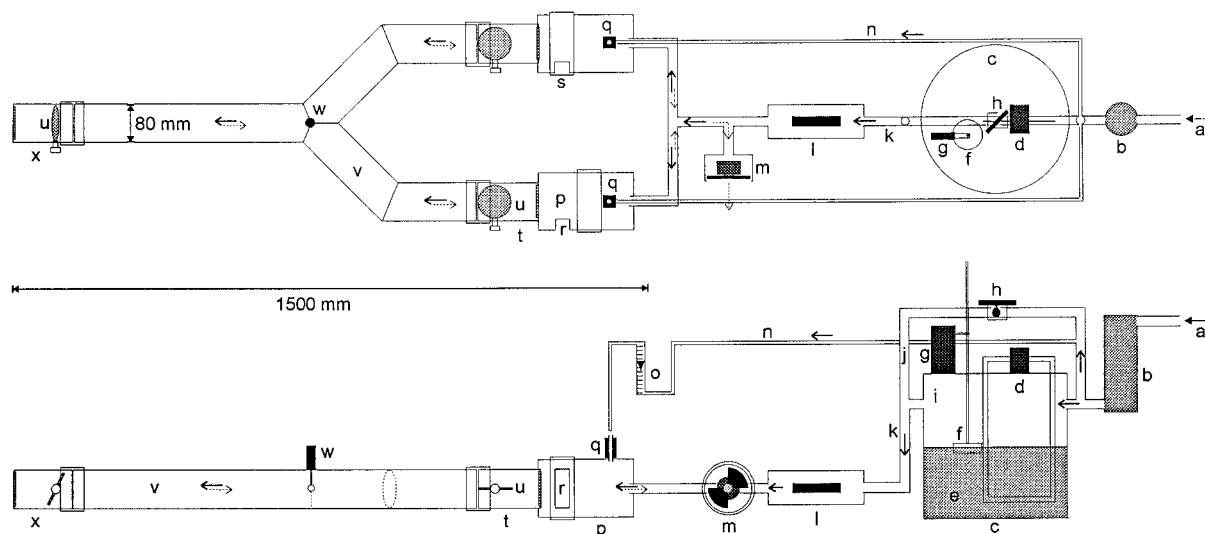


Figure 1. True to scale construction plan of the experimental set-up. Top view (upper picture) and side view (lower picture): pressurised air (a), active charcoal filter (b), stainless steel water container 300 × 300 × 1.5 mm (c), thermostat with heating coil (d), deionized water (e), floater (f) with switch (g) for regulating the water level, valve (h) for adjusting the ratio of moist (i) and dry air (j), plastic tube 23 mm × 25 mm for air supply (k), heating element (l), ventilator (m) for reversing the air stream after completing an experiment, tube for stimulus air stream (n), flow meter (o), stimulus tubes (p), temperature regulated heater with glass cartridge (q) for stimulus application, opening for hand (r), closure for hand opening (s), test and control chamber (t), rotating nylon screen (u), perspex Y-tube (v), temperature and humidity indicator (w), release chamber (x) with rotating screen (u). The arrow indicate standard direction of the air stream during an experiment. In this situation the ventilator (m) is off. The dashed lines symbolise the air stream which has been reversed after the ventilator (m) has been turned on.

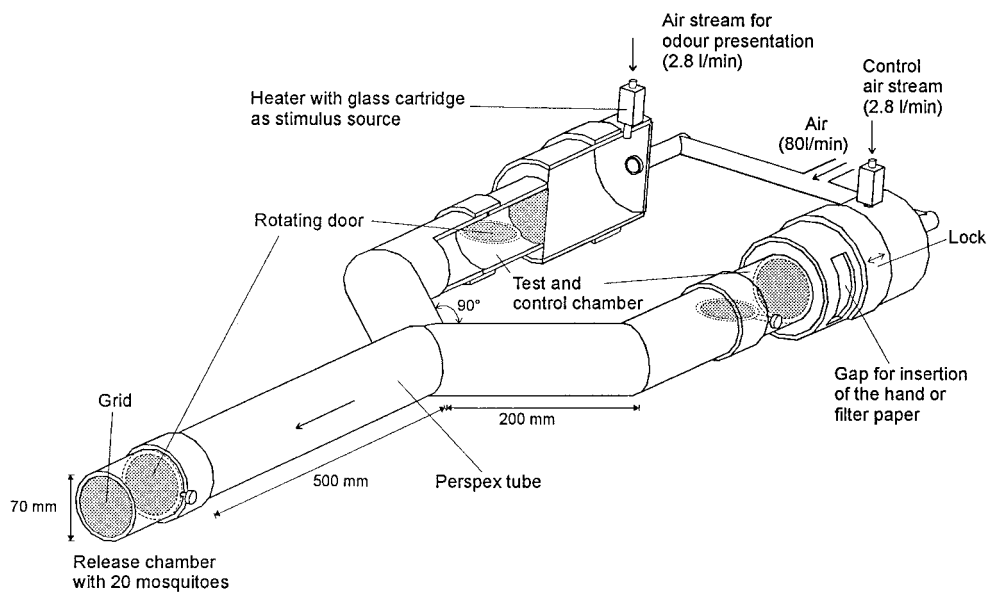


Figure 2. Lay-out of the Y-tube olfactometer.

### *Odours, stimulus delivery, and test program*

**Natural host.** The four fingers of the experimenter's hand were introduced into the olfactometer through a gap in the stimulus tube (Figure 2). This led to an increase of the air temperature in the test arm by  $0.2 \pm 0.2$  °C. Two hours before, the experimenter's hands were rinsed with water, no cosmetics or perfumes were used. The hand of the same person was used in all experiments. In experiment 1 the hand was tested once in a month over altogether 3 months to monitor possible variations of the attractiveness of this standard stimulus over a longer period (see Table 1 for arrangement of stimuli).

**Fluid samples.** Liquid test substances were presented either on filter paper or in glass cartridges: (1) 1 ml of a test solution was pipetted onto a filter paper of 7 cm diameter. After the evaporation of the solvent, the paper was fixed in the middle of the stimulus tube by means of a metal clamp. To avoid contamination, a new clamp was used in each test. A clean filter paper with 1 ml solvent served as control. (2) The test solution was pipetted to the inner side of a glass cartridge of 3.3 mm inner diameter and 5 cm length. After the solvent had evaporated, the cartridge was placed into the heating element on the stimulus tube (Figures 1, 2q). The function of the heater was to increase the evaporation of the test substances. The odour was delivered by blowing air through the cartridge at a rate of 2.8 l/min. This caused an increase of air temperature in the test arm by  $0.2 \pm 0.1$  °C. A cartridge with solvent (airflow: 2.8 l/min; heater: 60 °C) served as control. The flow rate of air streams were controlled by flow meters (Rota, Figure 1o).

**Skin extract.** Odour samples from the person, whose hand had been tested, were obtained by rubbing the hands, forearms, feet, and calves with cotton pads soaked in ethanol (p.A. Fluka, Germany). Each body area was rubbed 20–30 times for 5 min with a separate pad. The pads were then extracted in glass columns of 5 mm inner diameter and 100 mm length with methanol (p.A. Fluka, Germany) at a flow rate of 0.5 ml/min. Preliminary tests revealed that most of the effective material was extracted within the first 5 ml of solvent. Extracts from 50 pads obtained during a period of 2 months were combined, concentrated to 30 ml by evaporation in a rotary evaporator, and then centrifuged at  $-20$  °C with 3000 rpm for 2 h, yielding a clear yellow supernatant. A blank extract made

from 50 clean cotton pads served as control. In experiment 2 (using filter papers for stimulation) and in experiment 3 (using glass cartridges for stimulation) different doses of skin extract were tested (see Table 1). In experiment 4 the skin extract was tested against a hand using glass cartridges for stimulation, because the hand as well as the heated cartridges deliver a comparable thermal stimulus (see Table 1).

**Lactic acid.** The test solution consisted of 8 mg L-(+)-lactic acid (99%, Fluka, Germany) dissolved in 100 ml methanol (p.A., Fluka, Germany). This solution was tested in experiments 5 and 6 (see Table 1).

**Gaseous samples. Carbon dioxide.** The pure gas (99.9%, Linde, Germany; flow rate: 50 ml/min) from a cylinder was mixed with clean air and passed through the heating element while the total airflow through the heater was kept constant at 2800 ml/min. The flow rate of the gas was measured by a flow meter (Rota). The resulting concentration of carbon dioxide in the test chamber was measured with a carbon dioxide analyzer (LI-6251, Li-Cor, Lincoln; Nebraska, U.S.A.) and found at 0.1%. The air in the control chamber contained 0.035% carbon dioxide, the amount generally found in the atmosphere. To prove the synergistic effect between lactic acid and carbon dioxide both stimuli were tested in experiment 6 (see Table 1).

To estimate how the odours are distributed in the olfactometer we visualised the stimulus air stream by using a glass cartridge with 50  $\mu$ l  $\text{TiCl}_4$ . The distribution of smoke was approximately homogeneous in the arms of the Y-tube. Turbulent odour eddies, i.e. odour clouds and filaments, emerged in the stem of the Y-tube where the two air streams clash together.

### *Evaluation*

The percentage of mosquitoes outside the release chamber after 30 s of stimulation was taken as the measure of upwind flight activity. The percentage of mosquitoes trapped in the test- and the control chamber, respectively, was taken as a measure for the attractiveness of the respective stimulus. For each stimulus the mean values ( $\pm$  S.E.) were calculated. Since the data are percentage values they were transformed using angle transformation (Sokal & Rohlf, 1981) for further statistical analysis. To compare the attractiveness of the stimulus side with the control side, a *t*-test for paired samples was used. For comparison of different stimuli, the values for flight activity and

Table 1. Arrangement of stimuli tested in different experiments. Method of stimulation (see Material and methods) is indicated by gc (= glass cartridge) and fp (= filter paper).  $n$  = number of tested mosquito groups; each group with 20 mosquitoes

	Treatment	$n$	Stimulus in test-tube	Stimulus in control-tube
Experiment 1	1	10	hand	empty filter paper (fp)
	2	10	empty filter paper (fp)	empty filter paper (fp)
Experiment 2	1	10	1 ml blank extract (fp)	1 ml solvent (fp)
	2	10	1 ml skin extract:100%vol. (fp)	1 ml solvent (fp)
	3	10	1 ml skin extract: 50%vol. (fp)	1 ml solvent (fp)
	4	10	1 ml skin extract: 25%vol. (fp)	1 ml solvent (fp)
	5	10	1 ml skin extract: 2.5%vol. (fp)	1 ml solvent (fp)
Experiment 3	1	8	0.01 ml blank extract (gc)	0.01 ml solvent (gc)
	2	8	0.0001 ml skin extract (gc)	0.01 ml solvent (gc)
	3	8	0.0005 ml skin extract (gc)	0.01 ml solvent (gc)
	4	8	0.001 ml skin extract (gc)	0.01 ml solvent (gc)
	5	8	0.005 ml skin extract (gc)	0.01 ml solvent (gc)
	6	8	0.007 ml skin extract (gc)	0.01 ml solvent (gc)
	7	8	0.01 ml skin extract (gc)	0.01 ml solvent (gc)
	8	8	0.05 ml skin extract (gc)	0.01 ml solvent (gc)
Experiment 4	1	10	hand	empty glass cartridge (gc)
	2	10	0.05 ml skin extract (gc)	0.05 ml blank extract (gc)
	3	10	0.05 ml skin extract (gc)	hand
Experiment 5	1	10	0.01 ml blank extract (gc)	0.01 ml solvent (gc)
	2	10	0.01 ml lactic acid solution (gc)	0.01 ml solvent (gc)
	3	10	0.01 ml skin extract (gc)	0.01 ml blank extract (gc)
Experiment 6	1	10	0.01 ml lactic acid solution (gc)	0.01 ml solvent (gc)
	2	10	0.01 ml solvent (gc) + CO <sub>2</sub>	0.01 ml solvent (gc)
	3	10	0.01 ml lactic acid solution (gc) + CO <sub>2</sub>	0.01 ml solvent (gc)

the ones for attractiveness of the stimulus side were analysed independently using an One-way ANOVA followed by the Tukey–Kramer HSD *post hoc* test. Dose-response curves were calculated using a Probit analysis (Unkelbach & Wolf, 1985) and a non-linear regression analysis based on the sigmoid logistic function:  $f = (A - B) / [1 + (x/C)^P] + B$ .  $A$  represents the starting value (= threshold),  $B$  the maximal reaction strength (= saturation level of the curve),  $P$  describes the slope of the curve, and  $C$  gives the dose, which elicited 50% of the maximum response (=ED50). The parameters were estimated by using the Levenberg-Marquard Method. The statistics were calculated with the program SPSS 6.0 for Windows, the dose response curves with Origin 4.1 Microcal.

#### *Determination of the solid content, lactate content, and the pH of the skin extract*

In order to determine the solid content of the skin extract, 1 ml of the skin extract was placed in a glass container and nitrogen was blown over it until its weight remained constant for 4 h. This was already obtained after 40 min. The lactate content was determined enzymatically with lactic dehydrogenase from rabbit muscle, (LDH, 550 U/mg, Boehringer, Germany) (Hohorst, 1970). Total lactate, i.e. both, salt and free acid, was measured as lactate at a pH of 9, since free L-(+)-lactic acid (pK value: 3.862 according to Rauen, 1964) is converted to lactate under these conditions. The pH-value of the skin extract was determined by using pH indicator paper (Merck, Germany). From this, the amount of free L-lactic acid in the skin extract was calculated.

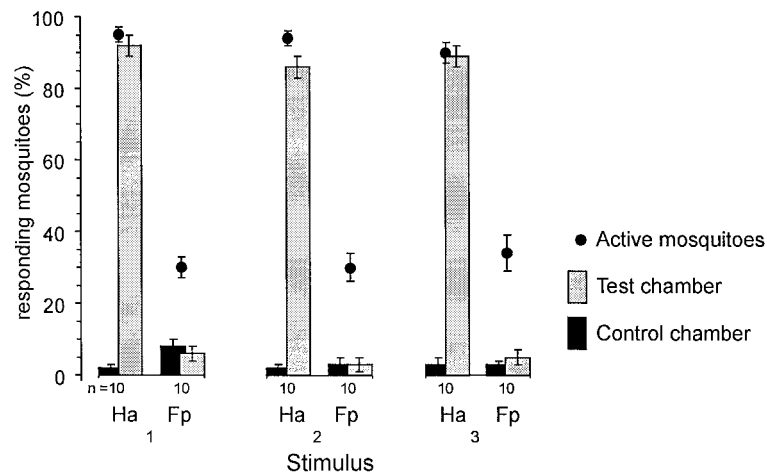


Figure 3. Responses of *Ae. aegypti* to a human hand. Columns show the mean percentage of the mosquitoes attracted to the test and the control chamber, respectively. Dots indicate the mean percentages ( $\pm$  S.E.) of the mosquitoes that had flown out the release chamber. On three different testing days (1, 2, 3) the behavioural responses of the mosquitoes to following stimuli were measured: hand of test person (Ha), clean filter paper (Fp).

## Results

**Responses to a human hand.** On three different days the hand consistently yielded very high values of flight activity and attractiveness (90%–95% and 86%–92%, respectively). Figure 3 shows the responses to the hand and to an empty filter paper. The differences between the test and the control chambers were always highly significant ( $P < 0.001$ ;  $t = 12.76, 17.63, \text{ and } 19.01$ ;  $df = 9$ ;  $t$ -test for paired samples). The behavioural responses to the hand, as well as to the controls, did not differ significantly between the different days (one-way ANOVA,  $P > 0.05$ , for flight activity as well as attractiveness values of test and control chamber, respectively). Most of the mosquitoes left the release chamber just a few seconds after stimulation and flew upwind. They then moved towards the test side of the Y-tube and landed on the gauze screen which was located directly in front of the hand. There, some ran back and forth and repeatedly probed through the screen or flew to another place on the screen. No mosquito was observed to fly back to the release chamber. When a clean filter paper was used instead of a hand, only a small percentage (3% and 8%) of mosquitoes was found in the test and the control chamber, respectively and there was no significant preference for a particular chamber ( $P_1 = 0.252, t_1 = -1.22$ ;  $P_2 = 0.642, t_2 = 0.48$ ; and  $P_3 = 0.397, t_3 = 0.89$ ;  $df = 9$  each,  $t$ -test for paired samples). In these control experiments the number of activated mosquitoes was significantly lower than with stimulation by the

hand ( $P < 0.0001, F[5:54] = 70.12$ , one-way ANOVA; Tukey–Kramer HSD:  $P < 0.05$ ). The mosquitoes never attempted to probe through the gauze of either the test or the control chamber.

**Responses to the skin extract.** In order to determine the effectiveness of the skin extract, the dose response relationship was measured with heated glass cartridges as well as with unheated filter papers as stimulus sources. Figure 4 shows the results for both stimulation methods. The commonly used probit model in pharmacological dose-response relationships (Unkelbach & Wolf, 1985), did not yield an adequate curve fit ( $\chi^2$  model adaptation test:  $T = 19.85, df = 4, P = 0.001$ ), whereas a sigmoidal logistic function of a non-linear regression analysis yielded an acceptable fit (Figure 4). The curve for the filter papers is determined by the parameters  $A = 11.0, B = 100.0, c = 294.4$ , and  $p = 1.5$ , whereas the curve for the glass cartridges is determined by the parameters  $A = 4.0, B = 88.6, c = 0.8$ , and  $p = 1.4$ . With both methods of stimulus delivery the maximum percentage of attracted mosquitoes was around 90%, a value which was also attained in previous experiments with the human hand. To directly compare the effect of both stimuli, 50  $\mu\text{l}$  skin extract from one person and the hand of the same person were tested separately as well as simultaneously (Figure 5). In the first case the attractiveness and the flight activity for stimulation with the hand and the skin extract, respectively, were not significantly different (flight activity:  $P = 0.207, t = 1.31, df = 18$ ;

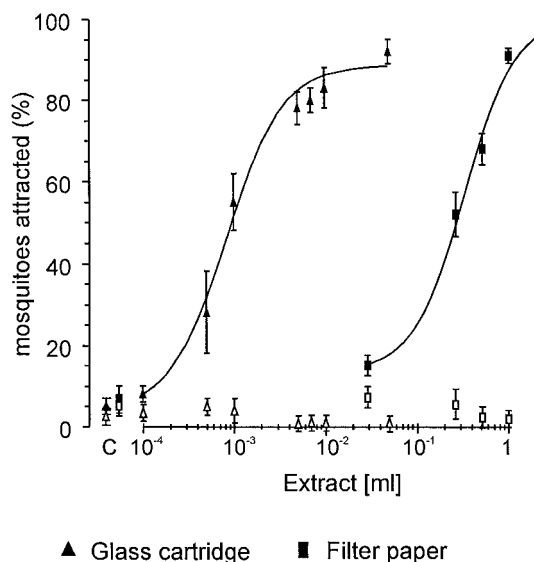


Figure 4. Dose-response curves of responses of *Ae. aegypti* to skin extract. Rectangular symbols represent the mean percentages ( $\pm$  S.E.,  $n = 8$ ) of tests with heated glass cartridges as stimulus source, triangular symbols the mean percentages ( $\pm$  S.E.,  $n = 10$ ) of tests with unheated filter papers as stimulus source. Filled symbols stand for mean percentages of the test side, empty symbols for the mean percentages of the control side. X-axis represents the amount of skin extract in  $\mu$ l in the glass cartridge or on the filter paper, respectively. C = control treatments with blank extract (10  $\mu$ l on glass cartridge and 1 ml on filter paper, respectively). The curve fittings yielded from regression analysis using the sigmoidal logistic function:  $f = a \cdot (x/c)^b / [(x/c)^b + 1]$ .

attractiveness test chamber:  $P = 0.447$ ,  $t = 0.78$ ,  $df = 18$ ;  $t$ -test for unpaired samples). In the direct comparison the mosquitoes were evenly distributed in both chambers at the upwind side ( $P = 0.991$ ,  $t = 0.01$ ,  $df = 9$ ;  $t$ -test for paired samples), which again indicates that both stimuli were equally attractive.

**Responses to L-(+)-lactic acid and carbon dioxide.** Since L-(+)-lactic acid has been shown an attractive component of acetone washes from human skin (Acree, 1968), we determined the amount of L-(+)-lactic acid in our skin extract. Using an enzymatic lactate test, a concentration of 11 mg/ml lactate was determined (total solids content: 67 mg/ml). The pH was approx. 5. Given that L-(+)-lactic acid has a pK value of 3.862 we calculated that 7% of the total lactate was free L-(+)-lactic acid (i.e. a concentration of 0.8 mg/ml free lactate acid). To compare the effect of lactic acid with the complete skin extract the following stimuli were tested: (1) blank extract, (2) skin extract, and (3) an equivalent amount of L-(+)-lactic acid. The results are shown in Figure 6. L-

(+)-lactic acid activated more mosquitoes ( $P < 0.001$ ,  $F[2:27] = 31.96$ ; One-way ANOVA; Tukey-Kramer HSD:  $P < 0.05$ ) than the blank extract and was significantly attractive ( $P < 0.001$ ,  $t = 8, 17$ ,  $df = 9$ ,  $t$ -test for paired samples) in contrast to the blank extract ( $P = 0.077$ ,  $t = 2.0$ ,  $df = 9$ ;  $t$ -test for paired samples). However, the skin extract was considerably more attractive than an equivalent L-(+)-lactic acid stimulus ( $P < 0.001$ ,  $F[2:27] = 62.79$ , One-way ANOVA; Tukey-Kramer HSD:  $P < 0.05$ ).

In an additional experiment we investigated the effects of carbon dioxide alone and in combination with L-(+)-lactic acid. The following stimuli were tested: (1) solvent, (2) carbon dioxide, (3) L-(+)-lactic acid, and (4) a combination of carbon dioxide and L-(+)-lactic acid. Carbon dioxide activated significantly more mosquitoes than L-(+)-lactic acid ( $P < 0.001$ ,  $F[2:27] = 23.76$ , one-way ANOVA; Tukey-Kramer HSD:  $P < 0.05$ ) and was also more attractive ( $P < 0.001$ ,  $F[2:27] = 37.17$ , one-way ANOVA; Tukey-Kramer HSD:  $P < 0.05$ ) (Figure 7). Furthermore, the attraction to the combined stimuli (86%) was higher than the mere added responses to the single stimuli (61%), indicating a synergistic effect. The combined stimuli also increased the flight activity, but not in a synergistic manner (Figure 7).

Even after multiple tests with the same mosquitoes during a day, the behavioural responses to L-lactic acid were not changed remarkably. Pooling the data of many tests, the mean percentage of attracted mosquitoes which were used for the first time, was 28% S.E. = 2.1, and the mean percentage of activated mosquitoes was 61% S.E. = 2.4 ( $n = 48$  mosquito groups). After these mosquitoes had been tested 6–10 times with various stimuli (control, hand, carbon dioxide, or lactic acid), 24% S.E. = 2.3 were attracted to lactic acid and 64% S.E. = 2.8 were activated ( $n = 48$  groups) by this compound.

In order to find out possible contamination control runs without odour were performed directly after stimulation with the hand or lactic acid, respectively. Table 2 shows that previous presentation of such stimuli in an arm of the Y-tube did not result in a higher attraction to this arm or in an increase of flight activity in successive control tests.

## Discussion

In the described Y-tube odour stimuli like carbon dioxide, lactic acid, and human skin residues attracted

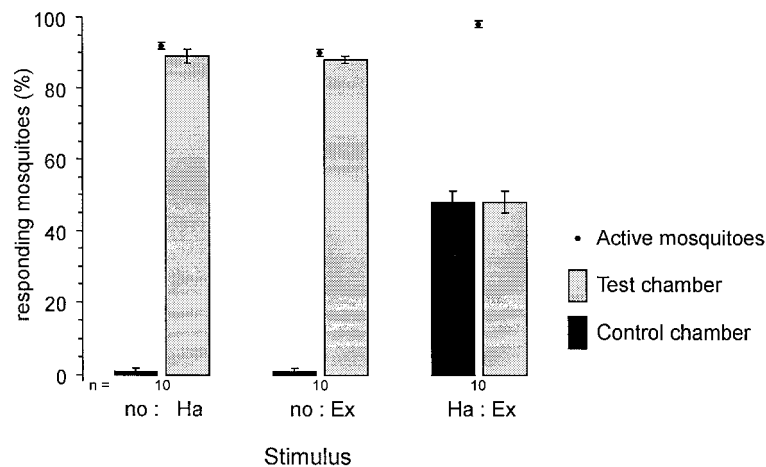


Figure 5. Comparison of responses of *Ae. aegypti* to hand and skin extract. Columns show the mean percentage ( $\pm$  S.E.) of the mosquitoes attracted to the test and the control chamber, respectively. Circles indicate the mean percentages ( $\pm$  S.E.) of the mosquitoes that had flown out of the release chamber. Stimuli tested: hand in the test chamber (Ha), no stimulus in the control chamber (no); 50  $\mu$ l skin extract in the test chamber (Ex), no stimulus in the control chamber (no); 50  $\mu$ l extract in the test chamber (Ex), hand in the control chamber (Ha).

Table 2. Behavioural responses of *Ae. aegypti* as an indication for possible contamination effects after previous treatments with odour stimuli. Mean percentages ( $\pm$  S.E.) of mosquitoes found in the test chamber<sup>a</sup>, control chamber<sup>b</sup>, and outside the start chamber<sup>c</sup> in tests with the mentioned odour treatments, and in subsequent control tests without odour stimulation. The data of  $n$  tests (20 mosquitoes per test) from different experiments were pooled

Previous stimulus ( $n$ )	Previous tests			Subsequent control tests		
	Test <sup>a</sup>	Control <sup>b</sup>	Activity <sup>c</sup>	Previous test <sup>a</sup>	Previous control <sup>b</sup>	Activity <sup>c</sup>
Hand (10)	81 (3.7)*	2 (1.1)	93 (1.9)a	7 (1.5) NS	3 (1.3)	39 (5.1)a
Skin extract (10)	73 (3.2)*	3 (1.3)	89 (3.2)a	6 (1.5) NS	2 (1.1)	41 (4.2)a
Lactic acid (9)	23 (4.2)*	5 (1.4)	53 (5.6)b	3 (1.2) NS	6 (2.6)	35 (5.5)a
No odour (8)	6 (1.8)NS	11 (2.9)	34 (5.0)c	3 (1.3) NS	5 (2.7)	35 (5.7)a

\*Significantly attractive compared to the control chamber ( $t$ -test,  $P < 0.05$ ; NS = non significant). Means of the activity values in one column followed by same letter do not differ significantly (ANOVA,  $P < 0.05$ , Tukey-Kramer HSD).

female yellow fever mosquitoes *Ae. aegypti* within 30 s towards the stimulus outlet or source. The percentage of responding mosquitoes depended upon the quality as well as on the dose of the stimuli. Carbon dioxide stimulated the upwind flight activity and was significantly attractive, but considerably fewer mosquitoes (ca. 40%) were attracted by this compound than by a human hand (ca. 90%). Addition of lactic acid increased the attractiveness of carbon dioxide in a synergistic manner. The reactions to both compounds correspond closely to those described by other authors (Eiras & Jepson, 1991; Gillies, 1980; Takken, 1991; Davis & Bowen, 1994; Bowen, 1991; Sutcliffe, 1987; Acree, 1968; Smith et al., 1970; Carlson et al., 1973). Acree et al. (1968), Smith et al. (1970), and Eiras

& Jepson (1991, 1994) found lactic acid attractive only in combination with carbon dioxide concentrations above atmospheric level. In our experiments, however, lactic acid was significantly attractive without an increase of the concentration of carbon dioxide in the olfactometer air. We do not entirely exclude the possibility that the mosquitoes from our culture might have responded more sensitive to lactic acid than the ones used in other investigations. Mukwaya (1977) found indeed differences in the host finding behaviour of different cultures of the same mosquito species. However, preliminary tests with offsprings from a culture of *Ae. aegypti* from the Swiss Tropical Institute revealed also a significant attractiveness of lactic acid alone. Our experiments were performed at

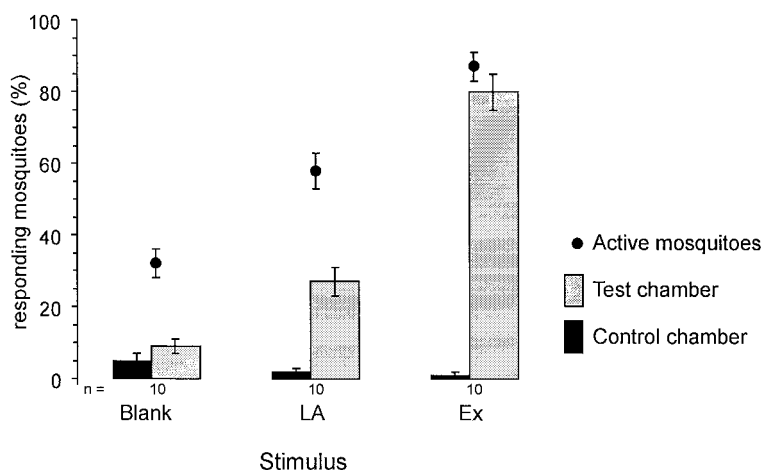


Figure 6. Comparison of responses of *Ae. aegypti* to skin extract and L-(+)-lactic acid. Columns show the mean percentage ( $\pm$  S.E.) of the mosquitoes attracted to the test and the control chamber, respectively. Circles indicate the mean percentages ( $\pm$  S.E.) of the mosquitoes that had flown out of the release chamber. Stimuli tested: glass cartridge with 10  $\mu$ l blank extract (Blank); glass cartridge with 8  $\mu$ g L-(+)-lactic acid (LA); glass cartridge with 10  $\mu$ l skin extract containing 8  $\mu$ g L-(+)-lactic acid (Ex).

similar temperature, humidity, and wind speed as were present in other investigations, and slight variations of these parameters do not seem to effect the responses of the mosquitoes (Rössler, 1961). Our choice to use only mosquitoes, which had been lured by the human hand, cannot be the reason for the attractive effect of lactic acid in our study. Other authors (Eiras & Jepson, 1991; Smith et al., 1970), who did not observe a significant response to lactic acid, selected the mosquitoes in the same way. The fact that we observed an attractive effect of lactic acid alone might be due to certain geometrical properties of the Y-tube, to the special mode of stimulus presentation, or to a certain the distribution of the odours in the air stream. The fast responses of the mosquitoes and the strong attraction to a human hand or to the skin extract support this interpretation.

The design of the Y-tube olfactometer permits the use of the human hand as a source of natural host odour. Considering the fact that the moisture and warmth given off by a human hand may enhance the attractiveness of the skin odours (Davis & Bowen, 1994), the hand represents the most natural set of attractive stimuli. According to Miller & Strickler (1984) such a natural stimulus source is important for a critical evaluation of the effect of attractants. Since the mean attractiveness of the hand of one test person averaged over one day remained constant over 2 months, this stimulus source seems to be a simple reference to assess the effect of sampled odoriferous material from human skin. In the past host odours from dif-

ferent sources like human perspiration (Parker, 1948; Rössler, 1961; Skinner, et al., 1965, 1968; Müller, 1968; Eiras & Jepson, 1991), urine (Rössler, 1961), blood (Rudolfs, 1922; Schaerffenberg & Kupka, 1959; Brown & Carmichael, 1961), skin washings (Acree et al., 1968; Smith et al., 1970; Schreck et al., 1981, 1990), and headspace samples from humans (Bar-Zeev et al., 1977) or mice (McCall et al., 1996) were demonstrated to be attractive for mosquitoes, but in all these studies the odoriferous material was never compared with the reference of the natural host from which the material was sampled. Our results show that the odour from skin residues of an attractive person attracts yellow fever mosquitoes almost as well as the hand of this person. This indicates that the extract contains at least the most important kairomones. Threshold, saturation level, slope, and the ED 50 value of the sigmoidal dose-response curve characterise the extract's effectiveness; by these parameters, extracts from other sources (e.g., either from different human test subjects or different host species) can be compared in future.

To present such an extract in the bioassay, we tested two different methods: (1) the odourants were blown into the olfactometer from a heated glass cartridge (60 °C) coated with extract, and (2) a non heated filter paper loaded with extract was placed directly in the air stream of the olfactometer. The comparison of the according dose-response curves show that for similar behavioural responses a more than 300 fold higher dose of extract is required on filter papers than

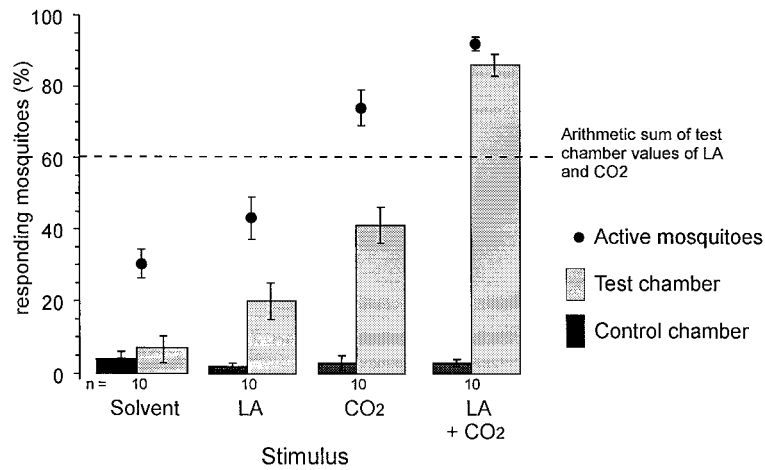


Figure 7. Synergistic effect of L-(+)-lactic acid and carbon dioxide. Columns show the mean percentage ( $\pm$  S.E.) of the mosquitoes attracted to the test and the control chamber. Circles indicate the mean percentages ( $\pm$  S.E.) of the mosquitoes that had flown out of the release chamber. Stimuli tested: glass cartridge with 10  $\mu$ l ethanol (Solvent); glass cartridge with 8  $\mu$ g L-(+)-lactic acid (LA); 0.1% CO<sub>2</sub> in the test chamber (CO<sub>2</sub>); simultaneous introduction of these stimuli (LA + CO<sub>2</sub>).

in heated glass cartridges (ED 50<sub>glass cartridge</sub>: 0.8  $\mu$ l, ED 50<sub>filter paper</sub>: 250  $\mu$ l). Mosquitoes are known to respond sensitively to temperature fluctuations (Davis & Bowen, 1994). Nevertheless, the heat stimulus which is given off together with the odour from the glass cartridges is not a requirement for strong behavioural responses to host odours. The results with non-heated filter papers as odour source indicate that the same responses can be evoked by the odour stimulus alone. Moreover, the heat stimulus of an empty glass cartridge was never attractive. By heating the extract, the output of attractive compounds is increased by either an increase of evaporation of odour components or by acceleration of chemical reactions and decomposition. This is probably the main effect of heating the stimulus source, although it is also possible that the heat stimulus synergizes the effect of an attractive odour. The principal advantage of heating is the reduced consumption of test material. In this way, the bioassay becomes a more sensitive tool for chemical analysis to detect and identify attractive components.

Schreck et al. (1981, 1990) reported strong contamination effects caused by touching the test equipment with hands. We also observed these effects shortly after construction of the olfactometer. Once the apparatus was thoroughly cleaned with detergents, hot water, and ethanol we did not observe any contamination effects provided neither test substances nor human skin came directly into contact with the inside of the stimulus tubes. This indicates that contamination is probably caused by vestiges of solid or fluid

material adhered to surfaces rather than by adsorption processes in the gaseous phase. We gauged our procedure by repeatedly performing control experiments and found no hints for contamination effects created during the test program.

The bioassay used in these experiments has a relatively high testing capacity (six behavioural tests can be performed in one hour using one olfactometer). It allows sensitive measurements of attraction to odours, and provides for a comparison with the natural stimulus. For this reason, it appears to be well suited for routine tests of various odour samples and as a tool for the identification of attractive components in these samples. It is clear that besides L-(+)-lactic acid, other compounds contribute to the attractiveness of the human skin residues. Further identification of these compounds will be a matter of chemical analysis in close interaction and co-operation with biological testing.

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