ODOUR-GUIDED HOST FINDING of MOSQUITOES: IDENTIFICATION of NEW ATTRACTANTS on HUMAN SKIN

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Abstract The basic principles of olfaction in mosquito-host interaction is presented using *Aedes aegypti*. Olfactometer bioassays investigated which compounds of the complex blend of human emanations play a role in the attraction, and which factors make some hosts more attractive than others. Beside L-(+)-lactic acid and carbon dioxide, ammonia, and short-chain fatty acids contribute to the attractive. The key compound is lactic acid, which is produced by eccrine sweat glands. Other components also contributed to the high attractiveness of a synthetic mixture. Comparing the hands of different humans we verified that certain individuals are more attractive to mosquitoes than others. The addition of lactic acid to the odour of less attractive people significantly increased their attractiveness and changed the mosquitoes' preference. The importance of lactic acid was confirmed in odour samples from different animals. The samples did not contain significant amounts of lactic acid and elicited only weak behavioural responses. The attractiveness of these animal odours increased when lactic acid was added. These findings emphasise the key role of lactic acid for *A. aegypti*: it is only a weak attractant by itself, but an essential element in the pattern of kairomones. Using this artificial odour blend in CO2 baited mosquito traps could provide an important tool for the surveillance of mosquito population in vector control.

Key Words vector control host finding kairomone olfaction

INTRODUCTION

Blood-sucking mosquitoes are the main vectors for the transmission of human diseases worldwide (Lehane, 1991). The most important vectors are species such as *Anopheles gambiae s.s.* or *A. aegypti*, which show a particular preference for feeding on humans (White, 1974; Scott et al., 1993). While searching for a blood meal, mosquitoes often choose to feed on particular individuals, which may well have implications for disease transmission (Khan et al., 1965; Knols et al., 1995; Lindsay et al., 1993; Burkot, 1988). There is strong evidence that differential host selection is primarily based on the mosquitoes' capability to discriminate host-specific odours (Rahm, 1958; Brouwer, 1960; Schreck et al., 1990; Galun, 1977). A good model organism to study the olfactory host finding is the yellow fever mosquito *A. aegypti*. This species is an important vector of yellow fever and dengue and one of the most intensively investigated mosquito species. Many studies have already shown that both L-(+)-lactic acid, which is present in human sweat at high concentrations, and carbon dioxide, which is emitted with the breath, play a role in the host finding of *A. aegypti* (Acree,1968; Smith et al.,1970; Eiras and Jepson,1991; Eiras and Jepson, 1994; Geier et al., 1996). In the past, certain amines, estrogens, amino acids, fatty acids, or alcohols were also reported to attract mosquitoes, but many of these results were contradictory and synthetic odour blends never matched the effect of the natural host odour (Hocking, 1971; Takken, 1991).

Previous studies in our laboratory with the yellow fever mosquito *A. aegypti* have revealed that besides lactic acid, other not identified components contribute to the high attractiveness of human skin residues (Geier et al.,1996). Interestingly, all these components were only attractive in combination with lactic acid. These findings indicate that in a bioassay, the attractive effects of

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certain compounds can be discovered only in combination with lactic acid. In a modified Y-tube olfactometer we therefore undertook a broad screening for additional attractants, testing aliphatic acids from C1 to C18 and ammonia in combination with lactic acid.

In addition, we investigated if there is a relationship between the attractiveness of human individuals and the quantity of lactic acid given off from their skin. We increased the concentration of lactic acid in skin odour samples from humans and other mammals to investigate whether the quantity of this compound affected their attractiveness.

MATERIALS and METHODS

Insects

Five- to fifteen-day-old female *Aedes aegypti* from cultures from the centre for Plant Research at Bayer AG in Monheim, Germany, were used in our experiments. The larvae were fed with Tetramin® fish food. Three hundred to five hundred adults each were kept in containers (50´40´25 cm) at 26-28 °C, 60-70% relative humidity, and a 12h:12h L:D photoperiod. They had constant access to a 10% glucose solution on filter paper. Since males and females were kept together, we presume that all females had mated before they were used in the experiments. Shortly before the experiments, the mosquitoes were lured out of their containers with the human hand. This ensured that the test insects were able to respond to the host.

Olfactometer

A modified Y-tube olfactometer was used to measure the attractiveness of the test odours. The Y-tube consisted of a rectangular Plexiglas chamber, into which two arms were inserted on one side, and the stem on the opposite side. Each of both arms fitted into a PVC™ stimulus chamber where the odours were mixed with the air flowing into the olfactometer. The release chamber with the mosquitoes was attached to the downwind end of the stem. Rotating screens in the release chamber, as well as in both arms at the upwind end permitted the release and trapping of the mosquitoes. A constant air stream (flow rate: 80 l/min) from the institute's pressurized air system was purified by a filter of activated charcoal, heated, and humidified before passing through the olfactometer. Further details of this experimental arrangement are described elsewhere (Geier et al., 1999; Geier and Boeckh, 1999). The temperature was 28°C ± 1°C, the relative humidity 70% ± 5 %, and the wind speed 0.2 m/s in the arms and 0.4 m/s in the stem, respectively.

Odour Stimuli and Stimulus Delivery

L-(+)-lactic acid was tested at a concentration of 90% and added to the test odours with a flow rate of 15ml/min, which corresponds to a dosage of 3µg/min (Geier et al., 1999).

Three different sources of ammonia stimuli were tested. To measure the dose-response curve, different amounts of ammonia were produced by passing charcoal-filtered air at flow rates between 0.03 ml/min and 300 ml/min through an Erlenmeyer flask filled with 50 ml of an aqueous solution of 0.13 mol/l NH_3 (p.a., Merck, Germany) in distilled water. Charcoal-filtered air at a flow rate of 300 ml/min passing through an Erlenmeyer flask with 50 ml distilled water served as a control. The air was passed over the surface of the solutions.

Flow meters (for flow rates of higher than 3ml/min) or a precision tubing pump (Masterflex, Novodirect GmBH, Kehl/Rhein, Germany; for flow rates of less than 3ml/min) were used to control the air flow.

Fatty acids were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Deisenhofen, Germany), Riedel-de Haën (Seelze, Germany) or Roth (Karlsruhe, Germany). Except for nonanoic acid (97%), tridecanoic acid (98%), and heptadecanoic acid (95%) they were more than 99% pure. Two different dilutions (5 µl and 500 µl each in 50 ml deionised water) of C_1 - C_9 fatty acids were tested at flow rates of 3, 30, and 300 ml/min, respectively. The solid C_{10} - C_{18} fatty acids

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were melted and 2 ml of the pure substances were distributed over the bottom of the Erlenmeyer flask, over which air swept at flow rates of 0.03, 0.3, 3, 30, and 300 ml/min.

Natural odour samples from the hand were obtained by intensively rubbing glass test tubes (16 mm diameter, length 160 mm) between both hands for 30 s. In this way skin residues were transferred to the glass surface (Steib et al., 2001). The glass tube with skin residues was then inserted into a Teflon sheath (inner diameter 17 mm, length 150 mm) and charcoal-filtered air was passed through the thin slit between the glass tube and the Teflon sheath at a flow rate of 2.8 ml/ min. Human donors were 26-32 years of age; individuals A and B were male, C and D female. To compare the lactate content of skin residues, tubes with rubbings from donors A and C were rinsed with ethanol. The total lactate content (salt and free acid) of the dissolved samples was measured enzymatically using Lactate Dehydrogenase (Geier et al., 1996).

Skin extracts from different mammals were collected by rubbing the skin of live animal with 1 g cotton pads. Samples were taken from calf (*Bovis primigenius* f. taurus), goat (*Capra aegagrus* f. hircus), cat (*Felis silvestris* f. catus) and from a human (subject C). After 10 min of rubbing, each pad was placed into a glass column (5 x 80 mm) and extracted with ethanol. Each column filling yielded 0.5 ml of extract. For each behavioural test 5 µl of this extract was applied to the inner surface of a glass cartridge (inner diameter 5 mm). After evaporation of the solvent, the glass cartridge was placed into a heating element on top of the stimulus chamber of the olfactometer, and air was blown through the cartridge at a rate of 2.8 l/min (Geier and Boeckh, 1999).

Bioassay

Bioassays were conducted as described in Geier et al. (1999). Groups of 18 to 22 female mosquitoes were used for the tests. Before stimulation, the mosquitoes were given 20 minutes time to acclimatize. Between the tests, a constant flow of fresh air flushed the olfactometer; the bioassays were conducted between 9:00 a.m. and 6:00 p.m. The odour stimuli were tested in five sets of tests, in which the stimuli were tested repeatedly in random order. A different mosquito population was used for each set of experiments.

Evaluation of the Behavioural Responses

The percentage of mosquitoes trapped at the upwind end of the test- and the control chamber, respectively, was taken as a measure for the attractiveness of the test- and control odour. For each stimulus the means (± SE) of the attractiveness values were calculated. Since the data are percentage values, they were transformed using angle transformation (Sokal and Rohlf, 1981) for further statistical analysis. The transformed means were analysed independently by a one-way ANOVA using the LSD method as post hoc test for the comparison of the treatments. Values for the two-choice tests were compared using a t-test for paired samples. All calculations and statistics were performed with the statistic program SPSS 8.0 for Windows.

RESULTS

Lactic acid at the dose of 3µg/min, which is in the range of the evaporation rates from human hands {179 /id Smith, Smith et al., 1970}, attracted approximately 20 % of the mosquitoes. The attractiveness of this stimulus was significantly increased by the addition of ammonia over a wide concentration range (Figure 1). The effective concentration range of ammonia was between 0.7 nmol/l and 700 nmol/l air (17ppb - 17ppm). The lowest ammonia concentration of 0.07 nmol/l (1.7 ppb) did not affect the response to the lactic acid stimulus, indicating a threshold between 0.07 and 0.7 nmol/l air (1.7 - 17 ppb). Addition of 7 nmol ammonia per liter air (170 ppb) doubled the percentage of mosquitoes attracted to lactic acid alone. Higher concentrations did not further

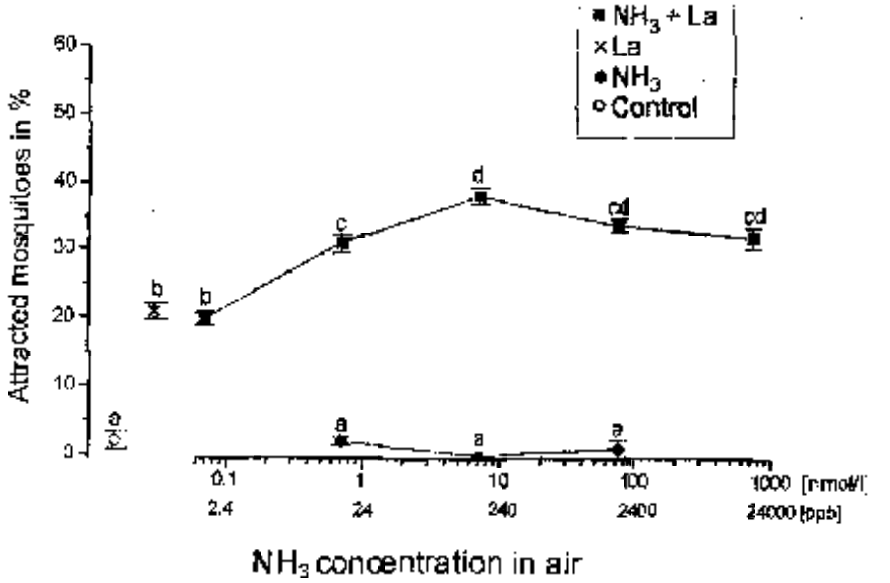

Figure 1. Mean (± S.E.) percentages of mosquitoes trapped in the test chamber of the olfactometer in relation to different concentrations of ammonia in the olfactometer air. Distilled water served as the control stimulus. Each dot shows the mean of 28 tests with 18-22 mosquitoes each. Means were compared using the one-way ANOVA and the LSD post hoc test; the letter code on top of the dots indicate significant differences: means with no common letter code are significantly different (P<0.05).

raise the attractiveness. Between 0.7 nmol/l and 70 nmol/l air (17-1700 ppb), ammonia alone was not attractive compared to the controls.

The attractiveness of lactic acid was also significantly increased by the addition of saturated fatty acids from C_1 to C_3, C_5 to C_8, and C_{13} to C_{18} at certain concentrations (Figure 2, 3). No increase was observed with butyric acid (C_4), nor with the fatty acids from C_9 to C_{12}. A slight, however not significant, decrease of attractiveness was observed at the addition of certain concentrations of C_9 and C_{11} to lactic acid, compared to lactic acid alone. In general, the attractive effect of the fatty acids from C_{13} to C_{18} increased with increasing concentrations, whereas the lower chain fatty acids were most effective at lower (C_1 to C_3) or medium (C_5 to C_8) concentrations.

Odour samples taken from the hands of four human subjects (A, B, C, D) were tested against each other, two at a time (Figure 4). This revealed differences among individuals regarding their attractiveness to yellow fever mosquitoes. The order of attractiveness was A>B>D>C. Rubbings from A and C differed in their attractiveness by 35% absolute. The amount of lactate in the skin rubbing samples was determined on three test days where significant differences in the attractiveness were observed. The rubbings of donor A had a 2.9-4.2 times higher lactate content than of donor C (A mean SD 58.4 15.9 µg; C mean 16.2 4.1 µg lactate per rubbing). However, when lactic acid (3.1 µg/min) was added to the less attractive skin rubbing of person C, its attractiveness increased far beyond that of person A.

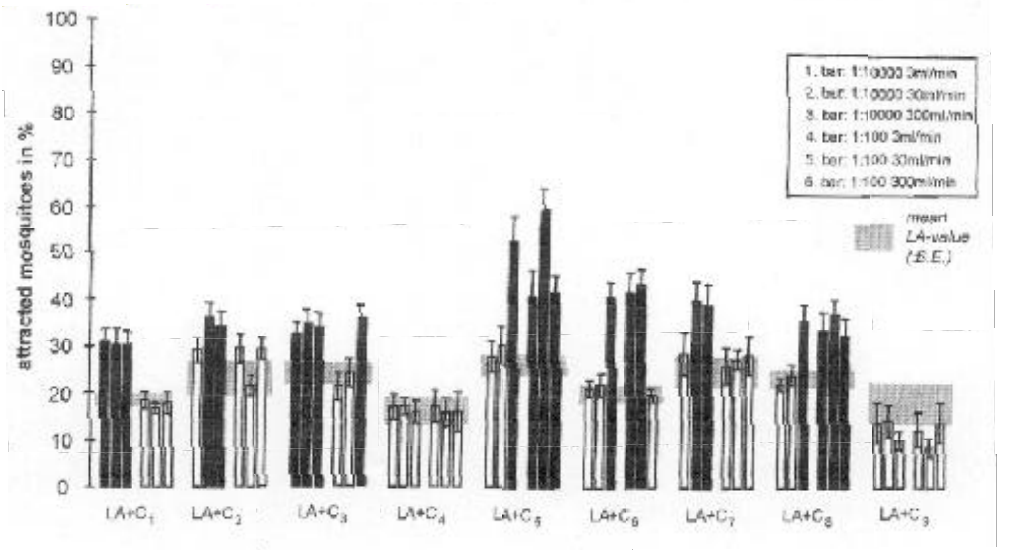

Figure 2. Effect of C_1–C_9 fatty acids added alone at different concentrations on the attractivenss of lactic acid for A. aegypti*. The columns represent the mean percentages of mosquitoes trapped in the test chamber. Since no mosquitoes were trapped in the control chamber, the data are not shown in the diagram. Horizontal bars show the mean attractiveness ± S.E. (n = 16 repetitions) of lactic acid for each experiment. Black bars indicate significant difference to the mean value of lactic acid at P = 0.05. Means were compared using the Duncan test post hoc to a one-way ANOVA.*

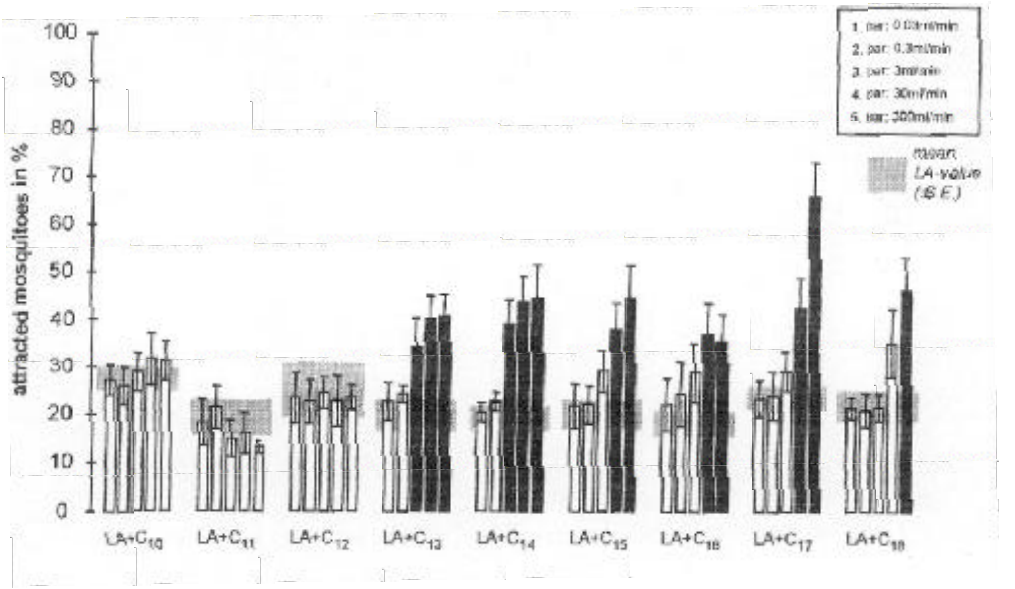

Figure 3. Effect of C_{10}–C_{18} fatty acids added alone at different concentrations on the attractiveness of lactic acid for A. aegypti*. For further explanations, see legend to Fig. 2.*

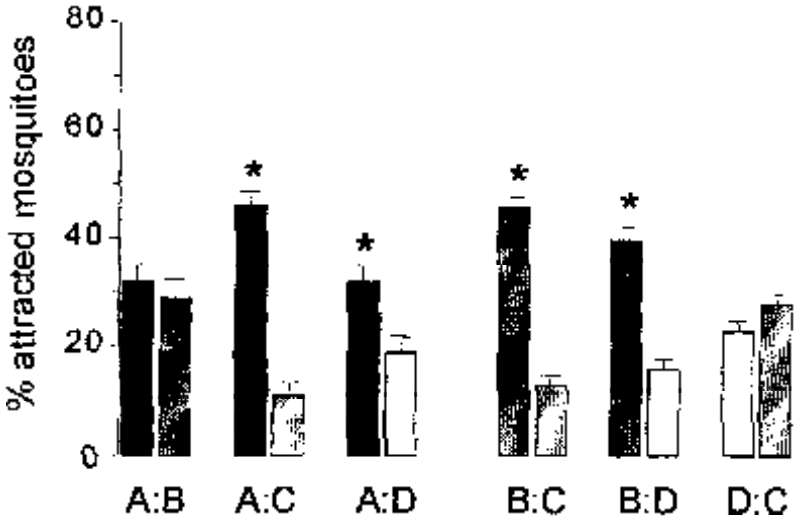

Figure 4. Comparison of odour samples obtained from four different humans (A, B, C, D) with respect to their attractiveness for yellow fever mosquitoes. Values are means ± S.E. of 56 repeated bioassays with 20 mosquitoes, respectively. Asterisks show significant differences (t-Test for paired samples, P < 0.05).

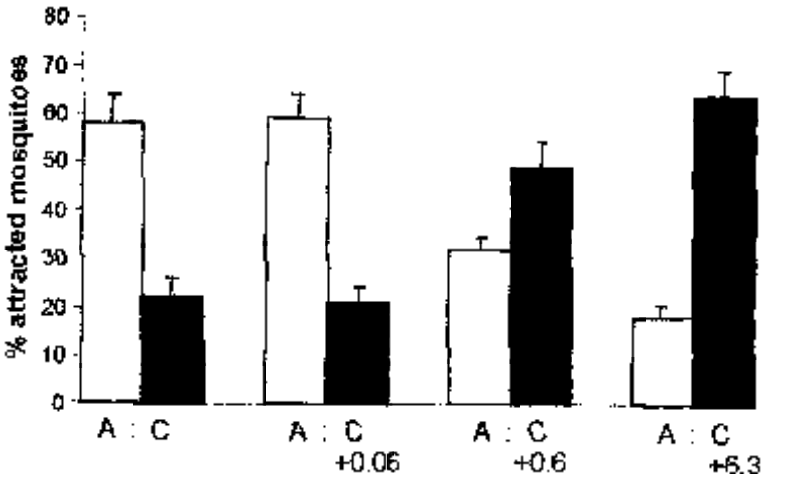

Figure 5. Effect of lactic acid added to the less attractive odor sample of donor C when compared with donor A. Values are means ± S.E. of 16 repeated bioassays with 20 mosquitoes respectively. Asterisks show significant differences (t-Test for paired samples, P < 0.05).

The dose-response characteristics of lactic acid was determined by adding three different doses (ranging from 0.06-6.3 µg/min) to odour samples from subject C and testing them against subject A (Figure 5). The unaltered samples as well as those modified with the lowest dosage of 0.06 µg/min lactic acid were significantly less attractive than subject A. When lactic acid was added in a 10-fold higher amount (0.6 µg/min), the attractiveness of the modified stimulus was doubled and the altered rubbings were significantly preferred to those of subject A. The addition of a higher amount of lactic acid (6.3 µg/min) increased the attractiveness even further: more than three times as many mosquitoes chose the modified odour source compared to the unaltered odour sample of subject A. In general, lactic acid enhanced the attractiveness of subject C in a dose-dependent manner.

Extracts from skin rubbings of different mammals were tested alone and in combination with lactic acid. All skin extracts were presented to the mosquitoes in successive trials and against pure air. Without addition of lactic acid, clear differences were found between the attractiveness of human and animal odours (Figure 6). Human skin extracts were attractive, with about half of the mosquitoes responding to them. Animal skin odour extracts were either only marginally attractive or not attractive at all. The amounts of lactic acid in a human extract was 803 µg/ml, whereas no lactic acid was found in extracts from the other mammals (sensitivity of the lactic acid determination: 15µg/ml extract). When lactic acid was added to the animal extracts, their attractiveness increased enormously and reached the same levels as that obtained with human extracts.

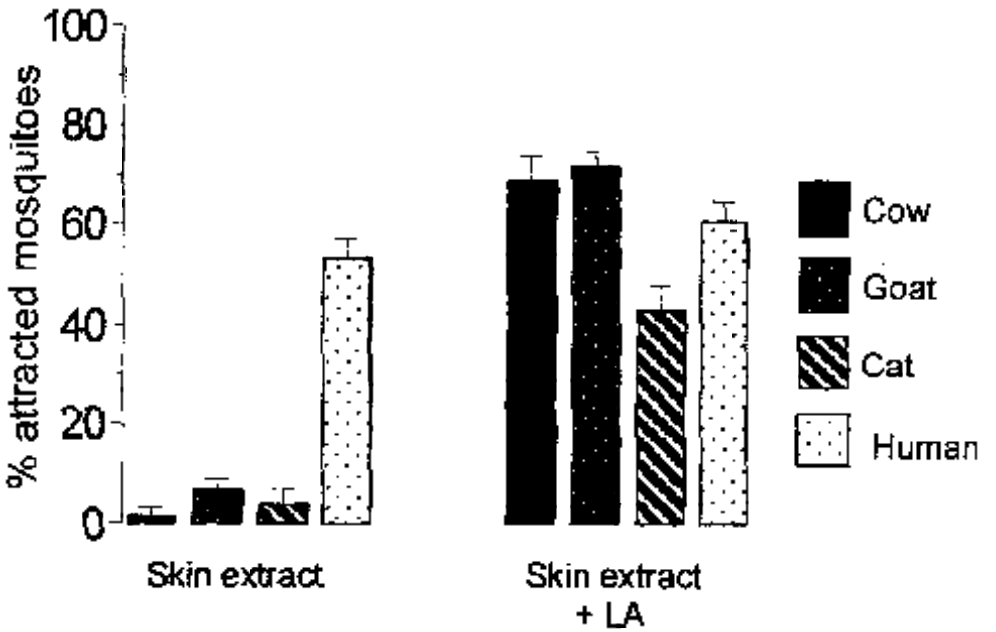

Figure 6. Attractiveness of skin rubbing extracts from four different mammals (calf, goat, cat, and the least attractive human donor C) with and without lactic acid added at a dose of 3.1 µg/min. All stimuli were tested against blank air. Values are means ± S.E. of 12 repeated bioassays with 20 mosquitoes, respectively. Means with no letter code in common are significantly different (ANOVA, P< 0.05).

DISCUSSION

The Key Role of Lactic Acid

Lactic acid acts as a synergist with carbon dioxide in breath (Acree,1968) and with components from human skin (Geier et al., 1996) in attracting mosquitoes. Previous studies in our laboratory have demonstrated that an enzymatic decomposition of lactic acid eliminates the attractive effect of human skin residues for *A. aegypti* (Geier et al., 1996). The attractiveness of these residues could be completely regained by adding synthetic lactic acid. This implies that all components which contribute to the attractiveness of skin odour are only effective when lactic acid is present. The behavioural responses described here correspond with these findings.

Ammonia

In synergism with lactic acid, ammonia was clearly shown to have an attractive effect in concentration ranges which exist around or downwind from human hosts. The lowest concentration of ammonia causing a significant behavioural response in the olfactometer was found at 0.7 nmol/l air (17 ppb). This is clearly below the concentration found in human breath, which was determined by several investigators to lie between 120 and 3170 ppb (Norwood et al., 1992; Larson et al., 1979). Another major source of ammonia is the human skin. Sweat produced by the eccrine sweat glands contains 0.7-25 mmol/l (12-425 mg/l) of ammonia and 3.9-67.7 mmol/l (235-4000 mg/l) of urea, which is quickly decomposed to ammonia by the bacterial microflora on the skin surface (Fiedler, 1968; Ciba-Geigy, 1977). However, the high lactic acid concentration (27-37 mmol/l, = 2.5-3.4 g/l) of human sweat sets the pH value of human skin between 5 and 6.8 (Fiedler, 1968). At these pH values most of the ammonia is bound as salt and composes a buffer system together with lactate/lactic acid. It is therefore difficult to estimate the rate of ammonia evaporation from human skin.

From our data, we assume the mosquitoes' sensory threshold for ammonia to lie in a concentration range between 2 and 17 ppb. This is similar to the one found in the haematophagous bug *Triatoma infestans*, whose nymphs were attracted to concentrations of 3 ppb and 17 ppb on a servosphere, whereas no significant response was found at 0.3 ppb (Taneja and Guerin, 1997). The finding that ammonia is attractive to yellow fever mosquitoes only in combination with lactic acid explains the results of Müller (Müller, 1968), Brown (Brown, 1952), and Rössler (Rössler, 1961), who could not find behavioural responses to ammonia stimuli. They had not tested this compound together with lactic acid.

Fatty Acids

Our experiments demonstrate that the attractive effects of fatty acids in the host finding of *Ae. aegypti* depend on two factors: their chain length and their specific combination in the blend. Their attractive effect was also only apparent when lactic acid was present. The mosquitoes were able to discriminate between fatty acids of distinct chain lengths. The attractiveness of mixtures of fatty acids varied greatly with the specific composition of the blend. We were able to demonstrate that the attractive host odour for mosquitoes consists not only of a single substance, but rather of a blend of odorants which are not or only slightly attractive by themselves. Our data suppose that several compounds contribute to the attractive blend emitted from human hosts: short chain fatty acids, medium-length fatty acid, ammonia, and lactic acid as the indispensable synergist. Whether fatty acids with longer carbon chains (C_{13} - C_{18}) also play a role in the host finding is not yet clear. Each of these fatty acids significantly increased the attraction to lactic acid, but only in very high doses. Our most effective artificial blend so far was however not as attractive as a human skin extract (Bosch et al., 2000). This might indicate that some additional attractants have not yet been identified. It is, however, also conceivable that the concentrations and proportions of the synthetic compounds tested so far were not optimal.

Inter-Individual Variations in Attractiveness

Inter-individual variations in the attractiveness of human hosts for mosquitoes have been reported earlier (Brouwer, 1959; Lindsay et al., 1993; Knols et al., 1995). Our two-choice tests with odour samples from different human test subjects indicate that olfactory cues may indeed cause significant differences in the level of attractiveness among individuals. So far, it is not yet understood which components of human body odour are responsible for these differences. Our results suggest that for *A. aegypti*, the amount of lactic acid in the host odour contributes to host preferences in a dose-dependent manner.

Lactic acid acts synergistically together with carbon dioxide, as well as with ammonia and fatty acids from human skin, emphasising its key role as an essential element in the pattern of kairomones emitted from the human host. This is also in accordance with the test of animal-derived odour samples. These were not, or only slightly attractive to yellow fever mosquitoes. An enzymatic lactic acid determination revealed that the high amount of this compound found in human skin residues is not present in skin residues from the tested animals. This could explain the low degree of attraction to these samples: when lactic acid was added, the attractiveness increased enormously to between 7.2-fold and 68-fold. This demonstrates that other compounds which are required for the host finding of the anthropophilic mosquito *A. aegypti* are not necessarily human-specific.

These findings open the door to the composition of artificial odour blends that mimic the particular human scent for anthropophilic mosquitoes. Such blends could increase the efficacy of the existing mosquito traps enormously and would also be effective without the addition of carbon dioxide, which is the most important commercially used attractant so far. At the moment, we evaluate the optimum blend composition, as well as the best ratio of the compounds, to create a odour mixture that is as attractive as the natural human scent.

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