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Ammonia as an Attractive Component of Host Odour for the Yellow Fever Mosquito, *Aedes aegypti*

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Abstract

Behavioural responses of *Aedes aegypti* mosquitoes to ammonia were investigated in a modified Y-tube olfactometer. Ammonia was attractive in concentrations from 17 ppb to 17 ppm in air when presented together with lactic acid. Aqueous solutions of ammonia salts in concentrations comparable to those found in human sweat also increased the attractiveness of lactic acid. The role of lactic acid as an essential synergist for ammonia became further apparent by the fact that ammonia alone or in combination with carbon dioxide was not effective, even though the synergistic effect of carbon dioxide and lactic acid was corroborated. An extract from human skin residues, which attracts ~80% of the tested mosquitoes, contains both lactic acid and ammonia. The combination of these compounds, however, attracts no more than 45%, indicating that other components on human skin also play a role in host finding. Preparative liquid chromatography of the skin extract yielded three behaviourally active fractions which work together synergistically. Fraction III contains lactic acid as the effective principle; the compositions of the other two have not been clarified yet. The attractiveness of fraction I was augmented considerably when ammonia was added, whereas the effect of fraction II was not influenced by ammonia. These results suggests that ammonia is part of the effective principle of fraction II and contributes to the attractive effect of host odours.

Introduction

Olfactory cues are widely used by bloodsucking insects to detect and to find their sources for blood meals. Since mosquitoes are one of the most important groups of vectors for human and animal disease, many attempts have been undertaken to explore the attractive blend of host odours. Different mosquito species develop different host preferences, and it is generally assumed that host selection and discrimination is mainly based on olfactory cues (Takken, 1991). Until today, however, only a few attractive components of host odour have been identified and we know little about the role of these volatiles in the complex network of behavioural sequences which lead these insects to their warm blooded hosts. Almost all mosquito species use carbon dioxide, which is given off from hosts with breath, as an alerting and attractive signal. L-(+)-Lactic acid, a major component in breath and on human skin, attracts *Aedes aegypti* in that it acts as an essential synergist when combined with carbon dioxide as well as with volatiles from the skin (Acree, 1968; Smith *et al.*, 1970; Eiras and Jepson, 1991; Geier *et al.*, 1996). Recently, a synthetic mixture of 12 aliphatic fatty acids, identified in the head space of Limburger cheese, has been implicated as attractive for *Anopheles gambiae* (Knols *et al.*, 1997). In the past, certain amines, oestrogens, amino acids and alcohols have also been 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 yellow fever mosquitoes, *Ae. aegypti*, have revealed that other components besides lactic acid contribute to the high attractiveness of human skin residues (Geier *et al.*, 1996). Interestingly, the components were only attractive in combination with lactic acid. These findings indicate that attractive effects of certain compounds can be discovered in a bioassay only in combination with lactic acid. A promising candidate of such an attractant is ammonia, since this compound has been identified in effluvia from vertebrates (Larson *et al.*, 1979; Norwood *et al.*, 1992). Attractive effects of ammonia have already been reported for a range of haematophagous arthropods (Taneja and Guerin, 1997; Hribar *et al.*, 1992). Müller (Müller, 1968), Brown (Brown, 1952) and Rössler (Rössler, 1961) could not find any attractive effect of ammonia in behavioural experiments with *Ae. aegypti*, but they never tested this compound in combination with lactic acid. Taking the synergistic effects of lactic acid and also of carbon dioxide into account, we reinvestigated whether ammonia could be an attractant also for *Ae. aegypti*. In a modified Y-tube olfactometer we tested

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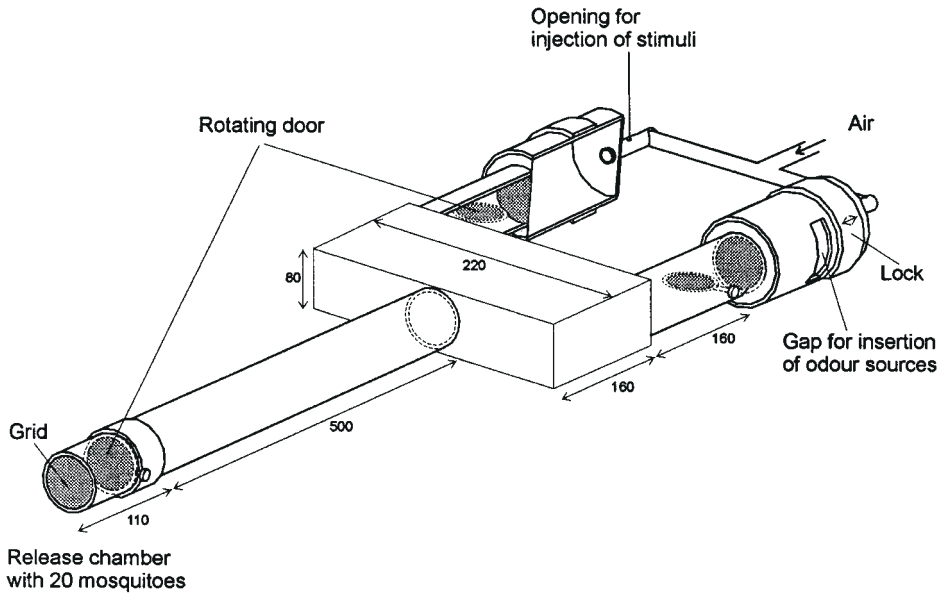

Figure 1 Schematic drawing of the olfactometer. Dimensions are given in mm.

the attractiveness of different sources of ammonia over a wide range of concentrations alone and in different combinations with lactic acid, carbon dioxide and behaviourally active fractions of an extract from human skin residues. Such an extract has been shown to attract *Ae. aegypti* and was separated by means of liquid chromatography in three behaviourally active parts: fractions I and II do not contain lactic acid and have no attractive effect on their own, but they act synergistically together with fraction III, which contains lactic acid as the effective principle (Geier *et al.*, 1996). We tested whether ammonia enhances the effect of one of these fractions.

Materials and methods

Insects

Female *Ae. aegypti*, 5–15 days old, from cultures of the centre for Plant Research at Bayer AG in Monheim, were used in our experiments. The larvae were fed with Tetramin® fish food. Some 300–500 adults were kept in containers (50 × 40 × 25 cm) at 26–28°C and 60–70% relative humidity, with a 12 h:12 h L:D photoperiod. They had access to a 10% glucose solution on filter paper. Since male and females were kept together, we presume that all females had been mated before they were used in the experiments. Shortly before the experiments, we lured the mosquitoes out of their containers by means of the human hand as bait. This ensured that the tested insects were able to respond to the host.

Olfactometer

A modified Y-tube olfactometer (Geier, 1995) was used to measure the attractiveness of odours (Figure 1). The branch of the Y-tube consisted of a rectangular Plexiglas chamber, in which the two arms run into one side and the stem runs into the opposite side (Figure 1). Each of both arms fitted into a PVC® stimulus chamber where the odours were mixed with the air flushing 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 downwind end permit the release or the entrapment of the mosquitoes respectively. A constant airstream (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, 1995). The temperature was 28 ± 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. The olfactometer was placed on a white table with white cardboard shields (height: 20 cm) on both sides to prevent visual stimulation by the experimenter. The room was illuminated by two 40 W light bulbs.

Odour stimuli and stimulus delivery

Three different sources of ammonia stimuli were tested. To measure the dose–response curve (experiment 1) different amounts of ammonia were produced according to the procedure of Ough and Stone (Ough and Stone, 1961). Charcoal-filtered air at flow rates of 0.03–300 ml/min was passed through an Erlenmeyer flask filled with 50 ml of an aqueous solution of 0.13 mmol/l NH_3 (p.A., Merck, Darmstadt, Germany) in distilled water. Charcoal-filtered air at a flow rate of 300 ml/min passed through an Erlenmeyer flask filled with 50 ml distilled water served as a control. The air was passed over the surface of the solutions. To determine

the output of NH_3, the air of four different flow rates, 0.3, 3, 30 and 300 ml/min, was trapped in gas wash flasks with a solution of 0.01 N HCl for 23 h, 1 h, 30 min and 12 min respectively. At the highest flow rate the trapping flasks were filled with 50 ml of the HCl solution; at the lower flow rates they were filled with 10 ml. We connected up to four trapping flasks in series to monitor any breakthrough from a previous one. The amount of ammonia was determined by titration with 0.01 N NaOH solution (Poethke, 1987). Figure 2 shows the relationship between the flow rate through the stimulus source and the total amount of NH_3 trapped per minute. In experiments 2, 3 and 5 the ammonia was delivered in the same way with a flow rate of 3 ml/min, which resulted in an output of 5 µg/min and a concentration of 7 nmol/l air (170 ppb) in the test chamber of the olfactometer.

In experiment 4 we tested an aqueous solution of 10 mmol/l ammonium chloride (p.A., Merck, Darmstadt, Germany) in distilled water. The pH value of this solution, determined by means of a pH indicator paper (Merck), was found to be 6.5. Small open glass vials (height: 30 mm; inner diam.: 16 mm) were filled with 2 ml of this solution and placed into the stimulus chamber. A 10 mmol/l NaCl solution served as control. In this experiment a L-(+)-lactic acid/ammonia buffer which simulates the composition of human sweet was also tested. For this, 400 mg of L-(+)-lactic acid (p.A., Merck) and 270 mg of 25% ammonia were dissolved in 50 ml of distilled water. This resulted in a lactate/ammonium salt solution of 89 mmol/l lactic acid and 80 mmol/l ammonia with a pH value of 5 and a surplus of free lactic acid; 0.5 ml of this solution was applied to filter paper discs (7 cm diam.) and the wet discs were put into the stimulus chamber. A lactic acid/sodium lactate buffer with 450 mg of L-(+)-sodium lactate (p.A. Merck) and 40 mg of L-(+)-lactic acid dissolved in 50 ml of distilled water served as a control. The pH value of this solution (80 mmol/l sodium lactate and 9 mmol/l lactic acid) was also 5, indicating the same amount of free lactic acid as in the lactate/ammonium salt solution.

Lactic acid stimuli were generated using a set-up similar to one based on the design of Ough and Stone (Ough and Stone, 1961), described above. Charcoal-filtered compressed air at a flow rate of 15 ml/min was passed through a 250 ml Erlenmeyer flask filled with 10 ml of L-(+)-lactic acid solution (90% in aq. sol.; Merck, Darmstadt, Germany). According to the calibration of Geier *et al.* (Geier *et al.*, 1999), at this flow rate an output of 3 µg/min lactic acid was generated and led into the stimulus chamber. This dose is in the range of the lactic acid given off from human hands (0.4–2.22 µg/min) after data from Smith *et al.* (Smith *et al.*, 1970).

The carbon dioxide used in experiment 3 was taken from a gas cylinder having the trade-standard purity of 99.9% (Linde, Nürnberg, Germany). The gas was injected into the stimulus chamber at a flow rate of 1600 ml/min and homogeneously mixed with the olfactometer air, yielding a concentration of 4% in the test arm of the olfactometer.

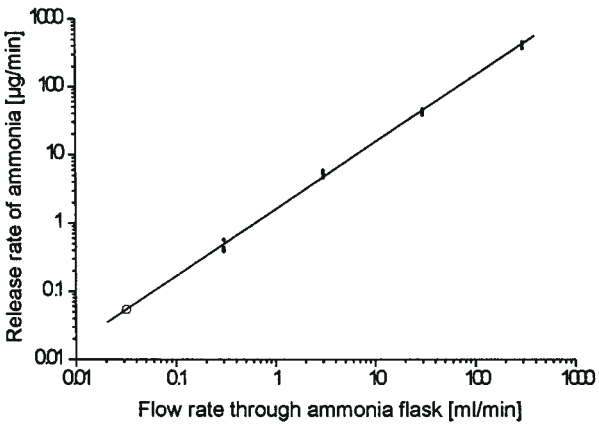

Figure 2 Calibration curve of ammonia output from an Erlenmeyer flask filled with 50 ml of an aqueous solution of 0.13 mmol/l NH_3 in distilled water. Each dot shows the amount of ammonia trapped in solutions of HCl at flow rates between 0.3 and 300 ml/min. Since we used these flow rates in the bioassays, the amount of ammonia released at a flow rate of 0.03 ml/min was extrapolated.

A skin extract was obtained according to a method described in detail by Geier *et al.* (Geier *et al.*, 1996). Hands, forearms, feet and calves were rubbed for 5 min with pads, which were then extracted with methanol (p.A. Fluka, Germany). The extracts from 50 pads sampled within a period of 2 months were combined, concentrated to 30 ml by evaporation in a rotary evaporator, and then centrifuged at –20°C (950 *g*; 2 h) to yield a clear yellow supernatant extract with a concentration of free L-(+)-lactic acid of 6 mmol/l (Geier *et al.*, 1996). The NH_3 concentration was 7.4 mmol/l, measured using a gas-sensitive NH_3 electrode after the method of standard addition in the research laboratory of the Bayer AG (Camman, 1979).

The skin extract was fractionated on a preparative silicagel column with acetonitrile and ethanol, yielding three separate fractions—fractions I, II and III (Geier *et al.*, 1996). A blank extract made from 50 cotton pads served as a control. For stimulus delivery a volume of 0.01 ml of skin extract or fractions, respectively, were applied to the inner side of a glass cartridge (inner diam.: 3.3 mm; length: 5 cm). After the solvent had evaporated, the glass cartridge was placed into a heating element on top of the stimulus chamber and air was blown through it at a rate of 2.8 l/min to deliver the odours from the surface of the glass cartridge as described elsewhere (Geier and Boeckh, 1999). The flow rate of the airstream was regulated and controlled by flow meters (Rota GmbH, Germany).

Odour distribution

Since we know that the spatial distribution of odours influences the attractiveness of odour sources (Geier *et al.*, 1999), we visualized the distribution of the odorants by

means of $TiCL_4$ smoke. In the arms of the olfactometer smoke was equally distributed similar to the homogeneous plume type outlined by Geier *et al.* (Geier *et al.*, 1999). More turbulent odour eddies, i.e. odour clouds and filaments, emerged in the rectangular Plexiglas chamber and the stem of the Y-tube respectively, where the two airstreams of the arms come together.

Bioassay

Bioassays were conducted as described in detail elsewhere (Geier *et al.*, 1999). Groups of 18–22 female mosquitoes were used for the tests. Before stimulation, the mosquitoes were given 20 min to acclimatize. Between the tests a constant flow of fresh air flushed the olfactometer; the bioassays ran from 9:00 a.m. to 6:00 p.m. The odour stimuli were tested in five blocks of tests, in which the stimuli were tested repeatedly in random order. For each block of experiments a different mosquito population was used.

Evaluation of activation and attraction

In each test we distinguished two behavioural categories of responses: (i) the percentage of mosquitoes found outside the release chamber after 30 s was taken as a measure for activation, which included taking flight and short upwind progress. (ii) The percentages of mosquitoes trapped at the upwind end of the test- and control chambers, respectively, were taken as measures for attractiveness of the test- and control odours. For each stimulus the means (± SE) of activation and attractiveness 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 a *post hoc* test for comparison of the treatments. All calculations and statistics were performed with the statistics program SPSS 8.0 for Windows.

Results

Lactic acid at the dose of 3 μg/min, which is in the range of evaporation rates from human hands (Smith *et al.*, 1970), attracted ~20% of the mosquitoes (Figure 3). The attractiveness of this stimulus was significantly increased by addition of ammonia over a wide range of concentrations (Figure 3). The effective concentration range of ammonia was between 0.7 and 700 nmol/l air (17 ppb–17 ppm). 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/l air (170 ppb) ammonia doubled the percentage of mosquitoes attracted to lactic acid alone. Higher concentrations did not further raise the attractiveness. From 0.7 to 70 nmol/l air (17–1700 ppb) ammonia alone was not attractive compared with controls.

In a direct choice situation, when ammonia and lactic acid were tested simultaneously as separate stimuli, all mosquitoes preferred significantly the lactic acid source (experiment 2, Table 1). A combination of lactic acid and ammonia, however, was significantly more attractive than lactic acid alone. When the stimulus in both chambers was lactic acid, both chambers were equally attractive.

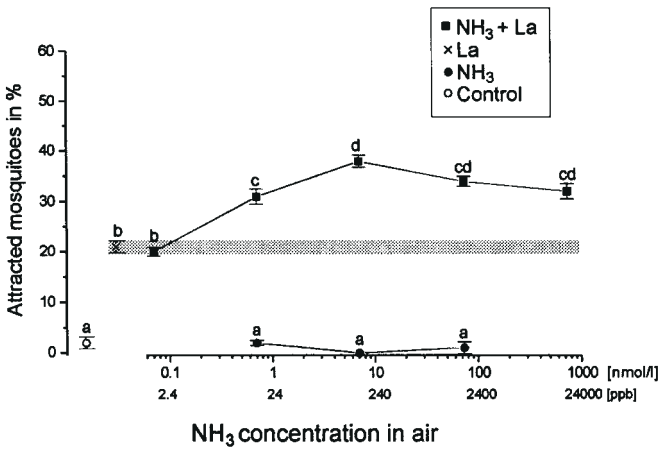

Figure 3 Experiment 1: relationship of mean (± SE) percentages of mosquitoes trapped in the test chamber of the olfactometer and different concentrations of ammonia in the olfactometer air. The ammonia concentrations are calculated by the amounts released from the stimulus source and the flow rate of air flushing the test chamber. Distilled water served as the control stimulus. Each dot shows the mean of 28 tests, each using with 18–22 mosquitoes. Means were compared with each other using a one-way ANOVA and the LSD *post hoc* test; the letter code above the dots indicates significant differences: means with no letter in common are significantly different ($P < 0.05$).

In contrast to the synergism found between ammonia and lactic acid, no such effect was observed when ammonia was added to carbon dioxide (experiment 3, Table 1). A concentration of 4% carbon dioxide had a strong activating as well as a slight attractive effect upon the mosquitoes. By adding ammonia at a concentration of 7 nmol/l air (170 ppb) neither the percentage of activated mosquitoes nor the percentage of attracted mosquitoes was significantly higher than with carbon dioxide alone. In the same experiment, however, a combination of carbon dioxide and lactic acid attracted nearly 80% of the mosquitoes, indicating a strong synergistic effect between these stimuli.

In experiment 4 we tested two other ammonia sources, which mimic the composition of human sweat. The results summarized in Table 1 show that an aqueous solution of ammonium chloride increased the attractiveness of the lactic acid standard stimulus. In addition, the aqueous buffer system of lactic acid and ammonia attracted a significant higher percentage of mosquitoes than a buffer of lactate/lactic acid at the same pH.

Both fractions I and II, obtained by means of preparative liquid chromatography of a highly attractive skin extract, have been shown to increase the attractiveness of lactic acid, but they had no effect on their own (Geier *et al.*, 1996).

Table 1 Behavioural responses of *Ae. aegypti* to ammonia stimuli

Experiment	Test chamber			Control chamber			Active	
	Stimulus	% T[1]	SE	Stimulus	% C[2]	SE	% A[3]	SE
2	lactic acid + NH_3	25	4.8*	NH_3	8	2.1	66.9[a]	6.4
	lactic acid	14.6	2.7*	NH_3	0.4	0.4	63.3[a]	4.3
	lactic acid	10.4	1.6	lactic acid	10.9	2.4	57.6[a]	4.8
3	CO_2	10[a]	1.7*	no	0	0	91[a]	2.8
	lactic acid + CO_2	79[b]	2.7*	no	0	0	97[a]	1.3
	NH_3 + CO_2	7[a]	2.1*	no	0	0	86[a]	3.4
4	solvent	3[a]	1.6	solvent	1	2	35[a]	4.1
	NaLa	19[b]	2.7*	solvent	2	3	69[bc]	2.8
	AmLa	39[c]	3.7*	solvent	3	4	80[c]	5.2
	lactic acid + NaCl	22[b]	2.1*	solvent	2	1.5	64[b]	2.1
	lactic acid + AmCl	44[c]	2.9*	solvent	1	1.1	83.5[c]	2.3
5	lactic acid	20[a]	2*	solvent	0	0	65[a]	2.2
	lactic acid + FrI	50[b]	3.6*	solvent	0	0	84[b]	1.8
	lactic acid + NH_3 + FrI	67[c]	3.3*	solvent	0	0	87[b]	2.3
	lactic acid + FrII	67[c]	3*	solvent	0	0	85[b]	1.9
	lactic acid + NH_3 + FrII	65.8[c]	3.2*	solvent	0	0	81[b]	2.2
	lactic acid + FrI + FrII	82[d]	1.9*	solvent	0	0	90.2[b]	1.3
	skin extract	85[d]	2*	solvent	0	0	94.2[b]	1.4

Means from 20 tests per treatment; each test with 18–22 mosquitoes. Abbreviations: NaLa = L-(+)-lactic acid/sodium lactate buffer, AmLa =L-(+)-lactic acid/ammonia buffer, AmCl = aqueous solution of ammoniumchloride, NaCl = aqueous solution of sodium chloride, FrI and FrII are two fractions of liquid chromatography separation of the skin extract.

[1]Mean percentage of mosquitoes trapped in the test chamber.

[2]Mean percentage of mosquitoes trapped in the control chamber.

[3]Mean percentage of mosquitoes which left the release chamber.

*Significant difference ($P < 0.01$) of mean percentage in test- and control chamber: *t*-test for paired samples. Within all experiments means in the test- or active columns followed by the same letter are not significantly different (P 0.05, one-way ANOVA: LSD *post hoc* test).

When ammonia was added to a combination of either one with lactic acid, a significant increase of attractiveness was observed only with fraction I, but not with fraction II (experiment 5, Table 1). The combination of fraction I, lactic acid and ammonia, however, was less attractive than the combination of fractions I and II and lactic acid.

Discussion

The data presented here clearly demonstrate an attractive effect of ammonia on *Ae. aegypti* in concentration ranges which exist around or downwind from human hosts.

The concentration of this compound in human breath has been found by several investigators to be between 120 and 3170 ppb (Larson *et al.*, 1979; Norwood *et al.*, 1992). The lowest concentration which caused a significant behavioural response in the olfactometer was found to be 0.7 nmol/l air (17 ppb), which is clearly below the concentration of ammonia in breath. 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) ammonia and 3.9–67.7 mmol/l (235–4000 mg/l) urea, which is quickly decomposed to ammonia by the bacterial microflora on the skin surface (Fiedler, 1968; Ciba-Geigy, 1977). 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 this pH value most of the ammonia is bound as salts and composes a buffer system together with lactate/lactic acid. Since we do not know the evaporation rate of gaseous ammonia above the human skin surface, we tested an aqueous solution of ammonium chloride (pH 6.5) and an aqueous lactic acid/ammonia buffer (pH 5) in con- centration ranges similar to that found in human sweat. Both stimuli significantly enhanced the responses to lactic acid, indicating a considerable evaporation of ammonia from these sources. These results and also the dose–response characteristics (Figure 3) indicate that *Ae. aegypti* is sensi- tive to ammonia at the levels which are given off by humans with their breath as well as from their skin.

From our data, we assume a sensory threshold to am-

monia in a concentration range between 2 and 17 ppb, which is similar to the one found in the haematophagous bug *Triatoma infestans* (Taneja and Guerin, 1997). Nymphs of these bugs were attracted to concentrations of 3 and 17 ppb on a servosphere, whereas no significant response was found at 0.3 ppb. Other examples of attraction or aggregation to ammonia sources have been documented for a variety of both haematophageous and non-haematophagous arthropods, such as the horse-fly *Hybomitra lasiophtalma* (Hribar *et al.*, 1992), the human body louse *Pediculus humanus* (Mumcuoglu *et al.*, 1986), the cockroach *Blatella germanica* (Sakuma and Fukami, 1991) and the Mediterranean fruit fly (Mazor *et al.*, 1987). Female fruit flies use ammonia as an attractive odour cue in a similar context as female *Aedes* mosquitoes: they are attracted towards ammonia-releasing proteinaceous sources in order to retrieve protein for egg maturation. In contrast to yellow fever mosquitoes, which respond to ammonia only in combination with lactic acid, fruit flies and *Triatoma* bugs are attracted by ammonia alone (Taneja and Guerin, 1997). This might reflect the different behavioural contexts in which ammonia is used by mosquitoes with their narrow host range on the one hand and by more opportunistic insects on the other. Only in combination with a specific human skin component such as lactic acid might ammonia contribute to the host recognition of the anthropophilic mosquito *Ae. aegypti*. The opportunistic bug *T. infestans*, however, might make more versatile use of the same stimulus, e.g. for finding their refuges, which are marked with ammonia-releasing faeces, and also for host finding (Taneja and Guerin, 1997). The finding that ammonia is attractive to yellow fever mosquitoes only in combination with lactic acid explains the results of Brown (Brown, 1952), Rössler (Rössler, 1961) and Müller (Müller, 1968), who could not find behavioural responses to ammonia stimuli because they did not test this compound together with lactic acid.

Previous studies on *Ae. aegypti* in our laboratory showed that enzymatic decomposition of lactic acid abolished the attractive effect of human skin residues (Geier *et al.*, 1996). An attractive effect was regained by combining synthetic lactic acid with the lactic acid-deprived residues. This implies that all components which contribute to the attractiveness of skin odour are only effective when lactic acid is present concurrently. The observed behavioural responses to ammonia correspond with these findings. Lactic acid seems to play the key role in odour-mediated host finding of yellow fever mosquitoes. It acts synergistically together with carbon dioxide, as well as with ammonia and other unidentified compounds on human skin. The fact that no synergistic effects were found between ammonia and carbon dioxide shows that neither ammonia nor carbon dioxide can substitute lactic acid as a synergist in attracting *Ae. aegypti*. Therefore we suggest an olfactory host recognition pattern in which different compounds of the attractive odour might act together at distinct levels of synergism. The highly attractive skin extract contained, beside many other compounds, considerable amounts of free lactic acid (6mmol/l) and ammonia (7.4 mmol/l). While the complete skin extract attracted 80–90% of the mosquitoes, a mixture of lactic acid and ammonia attracted at most 45%. Since this attractiveness did not increase even with higher doses of ammonia, it is obvious that additional components of the extract play a role. This is further confirmed by the results from the combinations of two behaviourally active fractions of the skin extract with ammonia. Both fractions and lactic acid combined are as effective as an equivalent amount of skin extract. Ammonia increased the attractiveness of fraction I plus lactic acid whereas no increase was observed with fraction II plus lactic acid. The combination of fraction I, lactic acid and ammonia, however, was less attractive than the combination fractions I and II and lactic acid. This suggests that ammonia is an effective principal in fraction II, but it is obviously not the only one.

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