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Effectiveness of Synthetic Versus Natural Human Volatiles as Attractants for Anopheles gambiae (Diptera: Culicidae) Sensu Stricto

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ABSTRACT Females of the African malaria vector, Anopheles gambiae Giles sensu stricto, use human volatiles to find their blood-host. Previous work has shown that ammonia, lactic acid, and aliphatic carboxylic acids significantly affect host orientation and attraction of this species. In the current study, these compounds were tested for their attractiveness relative to human emanations in vivo and in vitro. Emanations from a human hand, incubated sweat, and foot skin residues on a nylon sock were significantly attractive when tested against clean air. In a dual-choice test, foot skin residues were significantly more attractive than emanations from a human hand in vivo. Ammonia alone attracted more mosquitoes than fresh or incubated sweat. However, the odor of a human hand or of foot skin residues were more attractive than ammonia. A known attractive blend of ammonia with lactic acid and carboxylic acids was less effective than natural foot odorants. The results demonstrate that the synthetic blend based on skin odor is attractive for An. gambiae, but that in a choice situation in vitro natural skin odors are still preferred by the mosquito. Differences in volatile organic compound abundances between a worn sock and the synthetic blend may have resulted in stronger attraction to the sock. This suggests that candidate attractants should be evaluated with consideration of the strength of natural odorant sources. The data furthermore suggest that additional unidentified compounds from the human foot are involved in the host-seeking behavior of this mosquito species.

KEY WORDS Anopheles gambiae sensu stricto, olfactometer, host-seeking behavior, human odor, attraction

The African malaria vector, Anopheles gambiae Giles sensu stricto is highly anthropophilic and volatile organic compounds (VOCs) of human origin are the principle cues used by females during their nocturnal quest for blood (Takken 1991, Takken and Knols 1999). This host specificity makes it likely that the females of this mosquito species are able to distinguish and use human-specific odors unlike more catholic feeders such as An. arabiensis Patton, which displays variable degrees of anthropophily across Africa (Le-masson 1997).

Numerous studies have attempted to identify the compounds that constitute human volatile emanations (Cork and Park 1996, Bernier et al. 2000, Meijerink et al. 2000, Braks et al. 2001, Curran et al. 2005, 2007, Penn et al. 2007, Gallagher et al. 2008). Human sweat and human skin residues are highly attractive to An. gambiae (Braks and Takken 1999, Healy and Copland 2000, Qiu et al. 2004a, 2006b, Njiru et al. 2006) and several VOCs that are present in human sweat and/or in human skin emanations such as short-chain carboxylic acids, ammonia, and L-lactic acid, were found to be attractive to An. gambiae either individually or as blends (Knols et al. 1997, Braks et al. 2001, Smallegange et al. 2005, Njiru et al. 2006). These compounds are also attractive to Aedes aegypti L., a mosquito species that is equally anthropophilic and may therefore respond to similar VOCs (Geier and Boeckh 1999, Geier et al. 1999, Bosch et al. 2000, Williams et al. 2006).

The purpose of the current study was to compare the attractiveness of human body emanations in vivo and in vitro with that of synthetic human volatiles to An. gambiae. In particular, we examined the attractiveness of ammonia that plays a key role as kairomone for An. gambiae (Smallegange et al. 2005) and Ae. aegypti (Geier et al. 1999, Bosch et al. 2000, Williams et al. 2006) in relation to the attractiveness of natural human odors. In addition, a blend of ammonia, lactic acid and 12 aliphatic carboxylic acids, which had been shown to have a synergistic effect on this mosquito species, attracting more mosquitoes than ammonia alone (Smallegange et al. 2005), was tested. Emanations of human fingers (Dekker et al. 2001), fresh and incubated human sweat (Meijerink et al. 2000) were used to test the potential of ammonia. In addition, considering the important role of human foot odor in the host location of An. gambiae, and the practical and effective use of human foot odor in vitro (De Jong and Knols 1995a, Qiu et al. 2004b, Njiru et al. 2006, Schmied...
et al. 2008, Spitzen et al. 2008), odors from a sock worn by a human were tested against ammonia and the synthetic blend. The attractive strength of synthetic odors relative to natural odors is discussed with respect to the development of surveillance tools for malaria mosquitoes.

Materials and Methods

Bioassays. Experiments were conducted in the dual-port olfactometer described by Smallegange et al. (2005). The flight chamber (160 × 66 × 43 cm) had a temperature of 28.1 ± 0.5°C and a relative humidity of 63.5 ± 4.7%. The temperature and relative humidity of the pressurized air flowing out of the ports with a speed of 0.21 ± 0.04 m/s was 28.1 ± 0.6°C and relative humidity exceeding 80%, respectively. The experimental room was maintained at a temperature of 27.8 ± 0.7°C and a relative humidity of 58.8 ± 5.5%.

The mosquitoes used in the bioassays (30 females for each 15 min experiment, aged 5–8 d, which had never received a blood meal before) were obtained from the An. gambiae s.s. colony that originates from Suakoko, Liberia (courtesy Prof. M. Coluzzi), and has been cultured in the laboratory since 1988, at 27 ± 1°C, 80 ± 5% RH, a photo-scotophase of 12L:12D, and was given the opportunity to feed on a human arm twice a week. Larvae were reared in tap water in plastic trays and fed daily with Tetramin baby fish food. Pupae were collected daily and placed in 30 × 30 × 30 cm gauze-covered cages for emergence. Adult mosquitoes had ad libitum access to a 6% glucose solution on filter paper.

Experimental procedures were similar to those described by Smallegange et al. (2005). The mosquitoes were randomly collected from their holding cage 14–18 h before the experiments and placed in a cylindrical release cage (diameter 8 cm, height 10 cm) with water-soaked cotton wool placed on top. In each trial odors were released in the air stream before a group of mosquitoes was set free from a cage that was placed at the downwind end of the flight chamber. 1.6 m from the two ports. Trapping devices were removed at the end of the 15 min experiments. Females remaining in the flight chamber were removed with a vacuum cleaner, whereas mosquitoes that had entered either trapping device were anesthetized with 100% carbon dioxide and counted. Each trial started with new mosquitoes, clean trapping devices, and new stimuli. Experiments were repeated at least four times on different days. The sequence of test odors was randomized on the same day and between days. Test stimuli were alternated between right and left ports in different replicates to rule out any positional effects. Experiments with clean air only in either port were done to test the symmetry of the trapping system. Surgical gloves were worn by the experimenter to avoid contamination of the equipment with human volatiles. Experiments were performed during the last 4 h of the scotophase, when An. gambiae is normally active (Haddow and Ssenkubuge 1973, Maxwell et al. 1998, Kileen et al. 2006).

Odor Stimuli

Complex Human Odor. The sweat was obtained by pooling 3 ml of sweat from the forehead of 14 Caucasian volunteers (nine males and five females, 21–52 yr old) that were cycling on a home trainer in a humidified (70% RH) room at 30°C. Half of the sweat sample was placed in 5 ml glass vials in a freezer (−5°C; ‘fresh sweat’), while the other half, also stored in 5 ml glass vials, was incubated for 42–52 h under aerobic conditions at 37°C and subsequently stored in a freezer (‘incubated sweat’) (Meijerink et al. 2000). An amount of 100 μl of either fresh or incubated sweat was pipetted on a sandblasted glass slide (5 × 2 cm) which was placed in one of the trapping devices (for details see Smallegange et al. 2005). In the opposite trapping device, a glass slide was placed with an equal amount of distilled water or ammonia (2.5%) to test the relative attractiveness of this compound alone compared with human sweat. The two types of sweat were also tested directly against each other.

Human skin emanations were collected from a foot by wearing a nylon sock for 7–14 h the day before it was used in an experiment (by R.C.S., female, 31 yr old) (Njiru et al. 2006, Spitzen et al. 2008). The worn sock was placed in a clean glass jar before use in an experiment. During the experiment, the worn sock was put in one of the trapping devices, whereas a clean sock was placed in the opposite trapping device as a control. Ammonia alone (136 ppm from a gas sample bag; based on the results described in Smallegange et al. 2005) or in combination with lactic acid and 12 carboxylic acids was tested against a worn sock to examine the relative attractiveness of these compounds compared with a complex human foot odor.

The response of An. gambiae toward human skin odor in vivo was tested by inserting four fingers of a human hand (R.C.S.) through an entry slit behind one of the trapping devices into the main air stream (Deker et al. 2001). Contamination of the inner side of the slit was avoided by wearing surgical gloves from which the fingers had been removed. The ‘hand’ odor was tested against clean air or against 136 ppm ammonia from a gas sample bag that was pumped into the other trapping device.

Ammonia. When tested against human sweat, which is an aqueous solution, 100 μl of a 2.5% aqueous ammonia solution (Stock: Merck, minimum 25%, 1 l = 0.91 kg) was pipetted on a sandblasted glass slide (5 × 2 cm) which was placed in one of the trapping devices. A glass slide with 100 μl of distilled water was placed in the opposite trapping device when the attractiveness of ammonia alone was tested as a control (Braks et al. 2001).

Gaseous ammonia was used when ammonia (alone or in combination with lactic acid and 12 carboxylic acids) was tested in relation to the attractiveness of human skin emanations from a worn sock or a human hand. This was achieved by injection of 250 μl of an 2.5% aqueous ammonia solution (Stock: Merck, minimum 25%, 1 l = 0.91 kg) in a 80 liters dual stainless steel fitted Tedlar sample bag (SKC Gulf Coast Inc., Hou-
ton, TX) 1 d before the experiments. Subsequently, the bag was filled with 60 liters humidified and filtered warm pressurized air ~17 h before the experiments to allow the ammonia to evaporate and reach equilibrium. This procedure resulted in an ammonia concentration in the sample bag of 136 ppm. Another 80 liters sample bag was filled with 250 μl of distilled water and 60 liters of air and prepared in a similar way to serve as the control stimulus. During the experiments, the air was pumped at 230 ml/min (MG-4 Ametek air pump, Ametek, Paoli, PA) from the sample bags through silicon tubing (diameter 7 mm; Rubber BV, the Netherlands) into one trapping device where it mixed with the main air stream, diluting the ammonia concentration ~100 times (Braks et al. 2001, Smallegange et al. 2005). The two different dispersal methods of ammonia (gaseous and liquid) were also tested against each other to get an impression of possible differences in attraction and therefore concentration. When found to be similar, results of experiments in which ammonia was tested against the three human odor sources could be compared.

Lactic Acid. Based on the results previously obtained by Braks et al. (2001) and Smallegange et al. (2005), the concentration of lactic acid used was 1 μg/ml. A solution of this concentration was made by dissolving l(+)-lactic acid sodium salt (Sigma, 98%) in ethanol (Merk, Ethanol absolute, pro analysis). A filter paper (5 × 2 cm; Whatman 2 or Schleicher & Schuell 985) with 100 μl of the lactic acid solution was placed in an iron clip. The clip with filter paper was put in a trapping device after the ethanol had evaporated. For the control stimulus 100 μl of ethanol was applied in a similar way and placed in the other trapping device (Braks et al. 2001, Smallegange et al. 2005).

Aliphatic Carboxylic Acids. A mixture of 12 aliphatic carboxylic acids (Sigma, minimum 99%) was dissolved in diethyl ether (Merk, minimum 98%) based on the relative amounts of each acid in the acid extracts of Limburger cheese samples, which were shown to be attractive to An. gambiae (de Jong and Knols 1995b, Knols et al. 1997). A stock solution consisting of this mixture of 12 carboxylic acids was prepared and diluted eightfold with diethyl ether (1 × 10^-8 original concentration). A 100 μl aliquot of this synthetic mixture was applied on a sandblasted glass slide (5 × 2 cm). The glass slide was placed in a trapping device after the diethyl ether had been allowed to evaporate. Similarly, a control glass slide with an equivalent amount of diethyl ether was placed in the opposite trapping device (Knols et al. 1997, Smallegange et al. 2005).

Blend of Ammonia, Lactic Acid, and Aliphatic Carboxylic Acids. An odorous blend of gaseous ammonia (136 ppm), lactic acid, and a mixture of 12 carboxylic acids was obtained by combining the methods described above (see also Smallegange et al. 2005). This blend was attractive when tested against clean air during the same experimental period (Smallegange et al. 2005; Table 3).

Statistical Analysis

For each two-choice test a χ²-test was used to analyze whether the total (i.e., sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution.

A Generalized Linear model (GLM; Binomial, linked in logit; Genstat, release 4.2) was used to investigate the effect of the different odor stimuli on the trap entry response (i.e., total number of mosquitoes that entered both the treatment and control trapping device as fraction of the total number of mosquitoes that left the release cage). Two-sided t-probabilities were calculated to test pairwise differences between means. Effects were considered to be significant at P < 0.05.

Results

Symmetry of the Olfactometer. In the absence of odor, 20.1% of the females that left the release cage and entered the flight chamber flew into the trapping devices. Equal numbers of mosquitoes were caught in both trapping devices (64 and 60 mosquitoes of 617 that left the release cage, respectively; χ² test; P = 0.72), which demonstrated that the trapping system was symmetrical.

Responses to Natural and Synthetic Odors. Incubated but not fresh human sweat was more attractive than clean air (χ² test; P < 0.001 and P = 0.23, respectively; Table 1A). Incubated sweat attracted significantly more females than fresh sweat (χ² test; P = 0.01). The ammonia solution was not significantly attractive when tested against distilled water (χ² test; P = 0.53) although it attracted significantly more mosquitoes than fresh sweat and also more than incubated sweat (χ² test; P < 0.001 and P = 0.005, respectively). The ammonia solution applied on a sandblasted glass slide had a similar attractiveness as 136 ppm gaseous ammonia from a sample bag (χ² test; P = 0.14). No significant differences in the trap entry response between the experiments were found (GLM, P > 0.05; Table 1A).

The results of the tests with gaseous ammonia and volatiles of a human hand or a worn sock are shown in Table 1B. All odor stimuli were more attractive than clean air (χ² test; P < 0.001). The worn sock and the human hand attracted significantly more mosquitoes than gaseous ammonia (χ² test; P < 0.001). When tested directly against each other, significantly more mosquitoes were caught with the emanations from the worn sock than with those from the human hand (χ² test; P < 0.001). The worn sock induced a significantly higher trap entry response of the mosquitoes (78.7%) than ammonia (41.8%) and the human hand (39.8%) (GLM; P < 0.001). This response was not statistically different for the latter two stimuli (GLM; P = 0.39; Table 1B).

The blend of ammonia, lactic acid, and the carboxylic acid mixture was less attractive than a worn sock
tractiveness of humans to other VOCs are involved in the olfaction-based attraction. This suggests that in addition to ammonia, although ammonia alone was attractive when mosquitoes than traps baited with an ammonia solution in Kenya, MM-X traps baited with human skin residues collected on socks caught significantly more mosquitoes than incubated sweat. In addition, in a previous experiment, we found that ammonia constitutes a critical compound in the assemblage of the stimulatory blend: we have no information on the quantity of volatile compounds constructed with these compounds (Bosch et al. 2000). Acetone increased attraction of a blend of ammonia, lactic acid, and hexanoic acid that was attractive on its own, whereas adding ammonia and hexanoic acid improved the attractiveness of a blend consisting of lactic acid, acetone, and dimethyl disulfide (Williams et al. 2006). From previous results in our laboratory (Smallegange et al. 2005) it is apparent that a synthetic blend of volatiles that are naturally present in human odor will lure more mosquitoes than individual components: the combination of ammonia, lactic acid and carboxylic acids was more attractive to female An. gambiae than ammonia or any of the other constituents of the blend alone. Our synthetic blend of ammonia, lactic acid and carboxylic acids, although attractive on its own (Smallegange et al. 2005), however, is incomplete as in the current experiment a blend of natural human skin emanations collected on a nylon sock attracted more mosquitoes than the synthetic blend (Table 1C). This effect may have been caused by differences in quantity of volatiles emitted by each odorant blend: we have no information on the quantity of human odors emanating from the worn sock and this may be considerably higher than that of the synthetic blend, and thus explain the difference in attractiveness. Furthermore, the experiments with the worn socks also suggest that volatiles other than ammonia, lactic acid and carboxylic acids are involved in the host-seeking behavior of An. gambiae. Similar results have been obtained with Ae. aegypti: a synthetic trinary blend consisting of L-lactic acid, acetone and dimethyl disulfide was more attractive than two binary blends to this mosquito species in a
dual-port olfactometer, and the binary blends were more attractive than the individual compounds. Although the trinary blend was attractive, odor of human hands of three volunteers was significantly more attractive. In this case as well, the synthetic blend needs adjustment and most likely addition of other compounds (Bernier et al. 2007).

The question remains which of the many compounds that are known to be present in, for example, human sweat and human skin emanations (e.g., Bernier et al. 2000, Meijerink et al. 2000, Curran et al. 2005, Penn et al. 2007) have to be mixed to obtain a blend that is as attractive to An. gambiae (or Ae. aegypti) as a whole human body. Recent studies on the chemical composition of human skin odors obtained from human individuals with differential attractiveness (Schreck et al. 1990, Bernier 2002, Mukahana et al. 2002, Qiu et al. 2004a, 2006a, Logan et al. 2008) are expected to provide clues to the missing compounds.

Human hand odor was tested using the method described by Dekker et al. (2001). However, subsequent temperature measurements showed that the temperature in the port increases ≈0.7°C when four fingers are held in the air stream behind this port for 15 min. Therefore, the preference for the human hand over ammonia (Table 1B) may have been an effect of temperature instead of the result of olfactory cues only. Temperature gradient may result in thermotaxis (Laarman 1958) and thus may have influenced our results. A pilot study showed that inserting four fully gloved fingers in the slit behind a trapping device also increased the temperature at this port with 0.7°C without adding attractive odors (R.C.S., unpublished data). However, a worn sock was also preferred over ammonia and the experiments in which the hand odors were tested directly against the worn sock showed that the combination of human emanations with a higher temperature does not necessarily be more attractive than human odors at room temperature (Table 1C). In fact, the results of this experiment suggest that foot odors are more attractive than hand odors, possibly caused by a greater abundance of microbiota on the foot than on the hand (Noble 1982), causing a higher odor intensity of feet (Taylor et al. 2003). In addition, differences in microbiota species present on human foot and hand exist (Grice et al. 2009), likely resulting in differences in odor production (Xu et al. 2007).

Using a worn sock as material to absorb human skin emanations excludes the confounding effect of heat. Unfortunately, worn socks also contain many nonhuman contaminants (Qiu et al. 2004b), which constitute a technical obstacle when using GC-MS analysis for studying composition of odor blends (Labows et al. 1979, Bernier et al. 2000). Qiu et al. (2004a) showed that glass beads onto which skin emanations from human hands had been transferred by rubbing the beads elicit a level of attraction to An. gambiae females similar to a human hand, despite the absence of physical (i.e., elevated temperature and high moisture content) cues. This method appeared to be a very useful one for testing human odors behaviorally, electro-physiologically, and chemically to identify the chemicals responsible for the attractiveness of humans to An. gambiae females (Schreck et al. 1981, Bernier et al. 1999, Smallegange et al. 2003, Qiu et al. 2004a, 2006a).

Current research focuses on enhancing the attractiveness of the synthetic blend of ammonia, lactic acid and aliphatic carboxylic acids. Other compounds of human origin to which electrophysiological responses have been found (Qiu et al. 2006b) will be tested in combination with ammonia, lactic acid, and aliphatic carboxylic acids for their ability to improve the attractiveness of the blend. Meanwhile, several studies are conducted to identify more candidate chemicals to be tested in the olfactometer. The efficacy of promising blends will be validated in semifield systems similar to Njiru et al. (2006) and Schmied et al. (2008). Finally, field experiments have to be done to reveal the effectiveness of different blends under divergent environmental conditions.

The work described here shows that An. gambiae is attracted to emanations from various body parts (ecr ine sweat from the forehead, hand and foot emanations). Interestingly, human skin residues collected on a nylon sock, appeared more attractive than emanations from a human hand in vivo (of the same person). This emphasizes the major role of foot odors in the host-seeking process of this mosquito species. In addition, ammonia attracted more mosquitoes than fresh or incubated sweat, which underlines the importance of this kairomone. Although the effect of the tested candidate odor blend cannot be compared with that of natural human volatiles, this knowledge can nevertheless be used to direct our search for additional kairomones. Further studies in progress are likely to produce a series of novel compounds that are expected to be part of the An. gambiae kairomone complex. Bernier et al. (2007) showed that a relatively simple synthetic blend approaches the attractiveness of a human being to Ae. aegypti. Recent studies in Wageningen and Tanzania suggest that such blends, although of different composition, can also be developed for An. gambiae (Smallegange et al. 2009, Okumu et al. 2010).

Odor baits can be used in surveillance studies for epidemiological purposes, and are expected to attract a fixed proportion of the wild mosquito population. The application of bait technology would be a significant advantage above currently available sampling tools for mosquitoes such as human landing catch, CDC-light traps (combined with bed nets) or collection of resting mosquitoes (reviewed by Qiu et al. 2007). All depend on the skill or smell of the operator (=host), which varies considerably between individuals (e.g., Service 1993, Knols et al. 1995, Qiu et al. 2006a, Logan et al. 2008) and hence cause variation in trap collections. Odor-baited traps provide a constant degree of attractiveness, and their efficacy is independent of the operator. Odor-baited traps can also be used for population reduction (Vale 1993). To make this work, a fraction of the population needs to be removed, preferably at a constant rate. In both situations, surveillance and population reduction, odor
baits can be made operational, even when not fully competitive with natural hosts.

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