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Behavioral response of the malaria mosquito, *Anopheles gambiae*, to human sweat inoculated with axilla bacteria and to volatiles composing human axillary odor

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Abstract

The responses of *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) to odors from male and female axillary sweat incubated with human axilla bacteria were recorded in a dual-choice olfactometer. *Staphylococcus epidermidis* was selected for its low odor-producing pattern, *Corynebacterium jeikeium* for its strong Nα-acylglutamine aminoacylase activity liberating carboxylic acids including (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid (HMHA) and *Staphylococcus haemolyticus* for its capacity to liberate sulfur-containing compounds including (*R*/*S*)-3-methyl-3-sulfanylhexan-1-ol (MSH). *Anopheles gambiae* behavioral responses were evaluated under (i) its responsiveness to take off and undertake sustained upwind flight and (ii) its discriminating capacity between the two olfactometer arms bearing a test odor in either one or both arms. Experiments were conducted in the presence of carbon dioxide pulses as a behavioral sensitizer. *Anopheles gambiae* clearly discriminated for the olfactometer arm conveying odor generated by incubating any of the three bacteria species with either male or female sweat. Whereas *An. gambiae* did not discriminate between male and female sterile sweat samples in the olfactometer, the mosquito consistently showed a preference for male sweat over female sweat incubated with the same bacterium, independent of the species used as inoculum. Sweat incubated with *C. jeikeium* rendered mosquitoes particularly responsive and this substrate elicited the strongest preference for male over female sweat. Tested on their own, neither HMHA nor MSH elicited a clear discriminating response but did affect mosquito responsiveness. These findings serve as a basis for further research on the odor-mediated anthropophilic host-seeking behavior of *An. gambiae*.

Key words: *Anopheles gambiae*, axilla bacteria, human sweat, malaria mosquito, (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid, (*R*/*S*)-3-methyl-3-sulfanylhexan-1-ol

Introduction

Anopheles gambiae Giles *sensu stricto* (Diptera: Culicidae, hereafter *An. gambiae*) is the main malaria vector in sub-Saharan Africa. There is evidence that its high vectorial capacity, among other important physiological, behavioral, and ecological traits, is due to its marked preference for human hosts (Garrett-Jones 1964). In *Anopheles*

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mosquitoes, which are not active during daylight, host location is strongly olfactory driven (Takken and Knols 1999). Behavioral and electrophysiological investigations have revealed several chemicals found in host effluvia that influence the human host-seeking behavior of *An. gambiae*. Carbon dioxide (CO_2), a major component of expired human breath, is well-known for its role as an activator and sensitizer for anemochemotactic upwind flight behavior, particularly when presented intermittently (Gillies 1980; Mboera et al. 1997; Dekker et al. 2005; McMeniman et al. 2014). Ammonia and volatile organic compounds (VOCs) such as aliphatic carboxylic acids, lactic acid, 1-octen-3-ol, and 4-methylphenol identified in human sweat emanations have also been shown to be important human host cues for *An. gambiae* (Cork and Park 1996; Knols et al. 1997; Braks et al. 2001; Dekker et al. 2002, 2005; Smallegange et al. 2005, 2009; Okumu et al. 2010). There is evidence that selective human host-seeking behavior of *An. gambiae* relies on differences in odors emitted by humans compared with other vertebrate hosts (Costantini et al. 1993; Mboera et al. 1997; Costantini et al. 1998; Dekker and Takken 1998; Dekker et al. 2002) and even on certain human types compared with others (Knols et al. 1995; Dekker et al. 2002; Mukabana et al. 2002). Although it has been established that *An. gambiae* is influenced in its choice between humans over other vertebrate hosts by infochemicals, the identity of the products and their point of action in the complex behavioral sequence leading the insect vector to its final preferred host remains poorly investigated.

Fresh and incubated human sweat has already been tested on several occasions to show that incubated sweat is significantly more attractive than fresh sweat (Braks and Takken 1999; Meijerink et al. 2000; Braks et al. 2001). These studies have underlined the importance of skin bacteria in the odor-mediated behaviors of *An. gambiae*. The involvement of human skin bacteria in the chemical ecology of mosquitoes has been reviewed recently in Verhulst et al. (2010b), Smallegange et al. (2011), and Davis et al. (2013). There is a high degree of variability in the skin microbiota flora between individual humans. Earlier studies indicate how the composition of the microbial community is more stable over time on a single individual than between individuals (Gao et al. 2007; Fierer et al. 2008; Grice et al. 2008; Costello et al. 2009). Past research suggests that there is a correlation between microbial composition (in terms of diversity, quantity, and density) and individual body odor profile (Xu et al. 2007) with a clear correlation between underarm sweat odor intensity and bacterial density (Leyden et al. 1981; Labows 1982; Austin and Ellis 2003; Taylor et al. 2003; James et al. 2004; Rennie et al. 2007). Some individuals can even be differentiated by the odor profile emitted from their hands (Curran et al. 2007). Skin bacteria play a key role in metabolizing skin secretions into the volatile compounds that make up the odorous effluvia emitted by humans and contribute to the specific chemical signature of individuals. With this in mind, recent work in mosquito chemical ecology has brought attention to the role of human skin bacteria in the interaction between humans and *An. gambiae*. In a wind tunnel, traps baited with blood agar plates incubated with skin bacteria from human feet or a reference strain of *Staphylococcus epidermidis* caught significantly more *An. gambiae* than a control trap (Verhulst et al. 2009). The same research group also showed how the skin bacteria spp. *Bacillus subtilis*, *Brevibacterium epidermidis*, and *Corynebacterium minutissimum* produce semiochemicals that affect the behavioral response of *An. gambiae* (Verhulst et al. 2010a).

The axilla or human underarm region is endowed with apocrine and eccrine glands. Apocrine glands are found mainly in the axilla (Wilson 2009). They only become active at puberty and their activity slows down with aging. Earlier studies revealed compounds of steroidal origin (5α-androst-16-en-3-one, 5α-androst-16-en-3α-ol, and 4,16-androstadien-3-one) and volatile carboxylic acids ((*E*/*Z*)-3-methyl-2-hexenoic acid and 7-octenoic acid) as key constituents characteristic human axillary odor (Brooksbank et al. 1974; Zeng et al. 1991, 1992; Van Toller and Dodd 1993; Zeng et al. 1996). Recent analytical work has led to the identification of further human-specific odorous compounds. The proteinaceous liquid secreted by the apocrine glands contains water-soluble products that are transformed by the axilla microflora into, among others, (*R*/*S*)-3-methyl-3-sulfanylhexan-1-ol (MSH) (Hasegawa et al. 2004; Natsch et al. 2004; Troccaz et al. 2004) and (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid (HMHA, the hydrated analogue of (*R*)/(*S*)-3-methyl-2-hexenoic acid) (Natsch et al. 2006). HMHA is released from a glutamine conjugate by the action of a zinc-dependent aminoacylase produced by *Corynebacteria* (Natsch et al. 2003), whereas MSH is derived from a cysteinyl-glycine-*S*-conjugate by the action of *Staphylococci* (Starkenmann et al. 2005).

Both compounds and their precursors are suspected to be unique to human odor physiology (Natsch et al. 2004, 2006) and, as such, are potential candidates to explain the highly selective and anthropophilic odor-mediated behaviors of *An. gambiae*. Here, we report on the behavioral responses of *An. gambiae* to odors emanating from the incubation of sweat from human axilla with bacteria that are known to generate HMHA and MSH (Troccaz et al. 2004, 2009). We also test the responses of *An. gambiae* to different doses of these two compounds. To investigate the responsiveness of *An. gambiae*, we used a dual choice olfactometer in which the sensitivity of *An. gambiae* to test odors is augmented by the presence of pulsed CO_2 as background stimulus.

Materials and methods

Mosquitoes

The *An. gambiae* colony originated from Lagos, Nigeria (16CSS strain, derived in 1974 from wild-caught adults) and maintained in a walk-in climate chamber under a 14:10 light–dark cycle with 2 h stimulated sunset and sunrise at 28 ± 1 °C and 80 ± 1% relative humidity (RH). A batch of females was offered a blood meal on a Guinea pig once per week. The Guinea pigs used for the mosquito rearing were maintained in an animal house under permit, in compliance with Swiss Federal Regulations. Mosquito eggs were collected in an oviposition bowl filled with deionized water within 2 days of blood meal ingestion and placed in crystallizing dishes. The larvae were reared in trays (25 × 32 × 6 cm) filled with 400 mL water. Each tray contained 250–300 larvae that were fed on Tetramin fish food (Tetra, Blacksburg, VA, USA) according to the following feeding regime: day 1: 36 mg, days 2–5: 72 mg, and days 6–7: 144 mg. Approximately 800 pupae (sex ratio ~1:1) were collected and placed in Plexiglas cages (35 × 35 × 55 cm high) for adult emergence. All pupae transformed to imagoes the following day (age = 0) allowing age determination for behavioral tests in the olfactometer. Adults were provided with 10% sucrose and water on cotton wicks *ad libitum*. Nonblood-fed 5- to 8-day-old female *An. gambiae* were used for olfactometer tests during their last 6 h of the scotophase, that is at an age and a period during which the behavior of the mosquitoes is most likely to be related to appetence for a blood meal (Jones and Gubbins 1978).

Experimental setup and testing procedure

The experimental setup consisted of a dual-choice olfactometer made of Plexiglas as described in Geier et al. (1999b). This type of olfactometer has frequently proven effective to test the responses of mosquitoes to chemostimuli (Geier and Boeckh 1999; Geier et al. 1999b;

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Bosch et al. 2000; Steib et al. 2001; Dekker et al. 2002). In brief, this olfactometer consists of two stainless steel cylinders (11 cm diameter × 15 cm long) lined inside with a 1-mm thick Teflon liner (Angst and Pfister, Zürch, Switzerland) with a 7-cm downwind opening covered by fine polyethylene terephthalate netting (0.8 mm mesh, Sefar, Heiden, Switzerland) connected to the two upwind arms of the olfactometer (7 cm diameter × 17 cm long). These arms lead to an intermediate rectangular chamber (16 × 22 × 7 cm) from which a tube (7 cm diameter × 53 cm long) reaches the mosquito release cage at the downwind end. The airflow source (20 ± 2 cm/s in both upwind arms and 40 ± 2 cm/s in the downwind tube) originated from the institute's pressurized air system. The air was purified through a charcoal filter, humidified (~80% RH) and warmed to ~24 °C, equal in both arms. The olfactometer was surrounded with black curtains to avoid visual cues. Experiments were conducted under 1 kHz low fluorescent light conditions (<1 lux). In order to monitor and evaluate CO_2 pulses (see below) and RH with accuracy, the olfactometer was equipped with a gas analysis system consisting of a pump (Subsampler TR-SS3) connected to an airstream selector (RM8 Multiplexer) controlled and monitored on a computer via a user interface (UI-2). The sampled air (flow: 1.4 L/min) passed through an infrared CO_2 gas analyzer (LI 7000, Li-Cor Inc., Lincoln, NE, USA) and humidity analyzer (RH 300). The airstream selector was programmed to consecutively sample the air passing through each of the 3 compartments of the olfactometer (both upwind arms and the downwind tube) for 20 s each in 1 min loops throughout experiments. A thermistor probe was inserted in the middle of the intermediate chamber to monitor temperature (T°C). All air analysis devices were acquired from Sable Systems International, Las Vegas, NV, USA.

Female *An. gambiae* were activated by briefly exhaling into the rearing cage and lured with the experimenter's hand by placing it over the netting of the rearing cage entrance. From there 18–23 female *An. gambiae* were taken with a mouth aspirator and put into a release cage (7 cm diameter × 11 cm long) that was attached to the downwind tube of the olfactometer (Figure 1) where the mosquitoes were left to acclimatize in the airstream for 20 min. Immediately after stimulus onset, the rotating door of the release cage was opened and the mosquitoes were exposed to test conditions for 1 min. Following this, the rotating doors of both upwind arms and the release cage were closed and the number of mosquitoes in each olfactometer compartment counted. All experimental manipulations were performed with clean textile gloves in order to avoid any contamination of the setup.

Collection of odorless axilla secretions and regeneration of bacterial odor

The human sterile sweat samples comprised of a mixture of apocrine and eccrine secretions, the latter mainly being water, were collected over a 3-year period with the understanding and written consent of each individual and in accordance with ethical principles involving human subjects, including those of the World Medical Association Declaration of Helsinki. Subjects signed a consent form (approved by Firmenich S.A.) to agree to the experimental protocol before the first sweat sampling occasion. Samples consisted of 3.3 L of sweat collected from 24 men following a total of 140 h in the sauna and of 265 mL of sweat collected from 25 women following a total of 65 h in the sauna. The sweat samples were sterile filtered within 15 min of collection through a double filter consisting of a 1-µm over a 0.2-µm pore filter (Syringe Prefilter Plus, NalgeneTM, Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at −20 °C until use. Male sweat contained 0.22 ± 0.06 mg/mL protein, 3.6 ± 0.9 mg/mL glucose, 70 mg/L of the HMHA precursor *N*α-3-hydroxy-3-methylhexanoyl-(l)-glutamine, and 0.46 mg/L of the MSH precursor *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(l)-cysteinylglycine. Female sweat contained 0.16 ± 0.03 mg/mL protein, 1.0 ± 0.1 mg/mL glucose, 156 mg/L of the HMHA precursor, and 3.5 mg/L of the MSH precursor. Glucose is an important source of carbon for gram-positive bacterial growth. The average pH was lower in female sweat samples (7.5 ± 0.3 vs. 8.0 ± 0.3; see Troccaz et al. 2009).

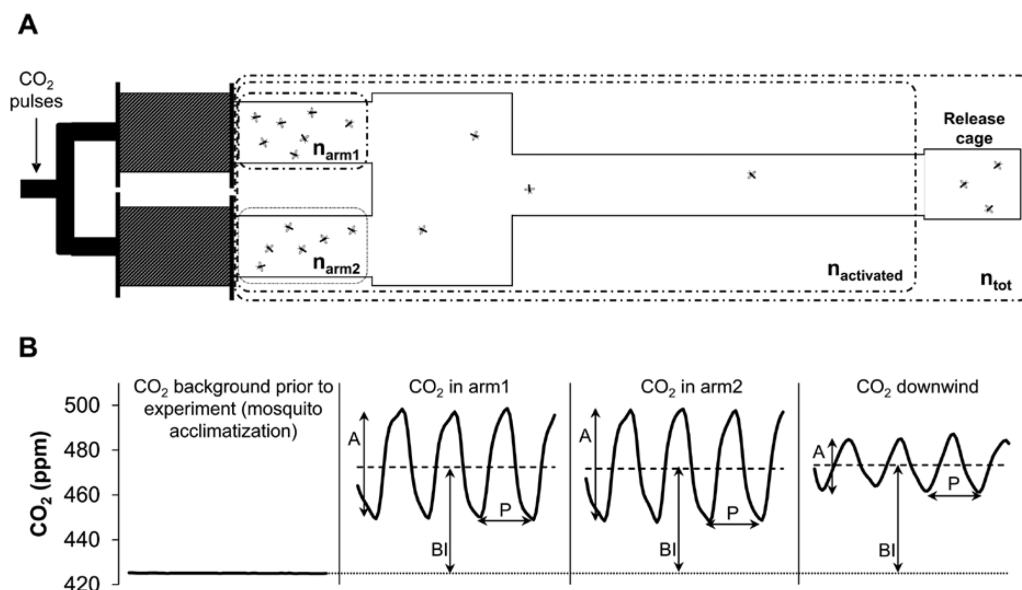

Figure 1. (**A**) Schematic diagram of the dual-choice olfactometer with the mosquito count in compartments (dotted lines) used to calculate the mean percentage of responding mosquitoes (R% = $(n_{arm1} + n_{arm2})/n_{activated} \times 100$) and the mean percentage of mosquitoes flying into each arm (arm1% = $n_{arm1}/n_{total} \times 100$ and arm2% = $n_{arm2}/n_{total} \times 100$). The arrow indicates the point of insertion of the CO_2 pulses into the olfactometer. (**B**) Example of CO_2 pulses as measured prior to and during an experimental trial. CO_2 pulses were characterized using Expedata software in terms of their mean *A*, *P*, and BI in each upwind arm (arm1 and arm2) and downwind near the release cage.

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To regenerate bacteria-specific axillary odor, the sterile sweat samples were inoculated with *Staphylococcus haemolyticus* (Firmenich in-house axilla-isolated CNCM 1-4170), *S. epidermidis* (Firmenich in-house axilla-isolated reference Ax1), and *Corynebacterium jeikeium* (Unilever axilla-isolated reference K411; Microbiology Laboratory, Unilever Research and Development Laboratory, Port Sunlight, Merseyside, UK). *Staphylococcus epidermidis* was chosen for its low odor-producing pattern, *C. jeikeium* for its strong *N*α-acylglutamine aminoacylase activity liberating many carboxylic acids including HMHA, and *S. haemolyticus* for liberating MSH along with other sulfur-containing compounds (Natsch et al. 2006; Troccaz et al. 2009). For this, a 400-µL sweat sample (either male or female) was inoculated with a 50-µL bacterial solution (4×10^8 CFU/mL saline solution) and incubated for 30 min at 37 °C prior to a test in the olfactometer. This procedure gave the sample an odor typical of the bacterium species and gender origin of the sweat sample. It also facilitated the availability of a test substrate when an adult *An. gambiae* population was available for tests in the olfactometer. For controls in experiments with sweat (see below), the bacterial solution was replaced by a 50-µL buffer solution ($NaPO_4$ 0.1 M, pH 6), incubated in the same manner as sweat. Further details on the volunteer pool, the procedure used to obtain sterile sweat samples and the preparation of solutions of each bacterial species are provided in Troccaz et al. (2004, 2009).

Odor delivery

To deliver CO_2 in a pulsed manner in the olfactometer a gas tank with 1% CO_2 (Carbagas, Switzerland) equipped with a manometer was connected to a solenoïd valve (model V301, Sirai, Italy). The valve permitted intermittent delivery of CO_2 at 2 s open, 2 s closed corresponding to 15 cycles/min, which is approximately the breathing rate of a human at rest. The outlet of the valve was equipped with a gas diffuser and inserted into the air supply immediately upwind of the split into the two stainless steel cylinders leading to the arms of the olfactometer (Figure 1). The pressure level on the manometer was adjusted regularly to obtain constant fluctuations in the range of 50 ppm in each arm of the olfactometer. Additional odor delivery was ensured through a cleft in the wall of each stainless steel cylinder located upwind of the olfactometer arms. This allowed insertion of sand-blasted glass slides treated with different doses of test chemicals (below) or odor-laden air from gas wash bottles containing treatments.

As lactic acid is not very volatile, it was applied by bubbling charcoal-filtered air at a flow rate of 40 mL/min through a 250-mL gas wash bottle holding 10 mL of l-(+)-lactic acid solution (90% aqueous solution; Fluka, Switzerland). The resulting output roughly equals the highest rates of lactic acid released from a human hand (Smith et al. 1970; Geier et al. 1999a). HMHA and MSH were provided by Firmenich S.A., Geneva, Switzerland. Dilutions ranging from approximately 10^{-5} to 10^{2} ng/µL of both chemicals were made using tertyl-butyl methylether (TBME, 99%, Merck) as solvent. For each olfactometer test, 10 µL of a given dilution was applied on a sand-blasted glass slide and, after allowing the solvent to evaporate, the slide was inserted into one of the upwind clefts (the same amount of pure solvent applied to a glass slide served as control in the opposite arm). This method has been used successfully elsewhere as an efficient method to vaporize small quantities of scarce volatile compounds (Braks et al. 2001; Qiu et al. 2004). To deliver the odor generated by sweat samples into each arm of the olfactometer, charcoal-filtered compressed air (at 150 mL/min) passed through two 500 mL glass gas wash bottles each containing the desired sweat sample. Both bottles were placed in the center of a heating plate (30 ± 1 °C) to maintain bacterial activity. The sweat sample vial was placed at the center of the base of the bottle. The inlet tube of the gas wash bottle reached the top of the sweat sample in the vial to ensure odor flush out. To economize the odor produced by the sweat samples, the air flow was turned off between experiments. The odor in each arm of the olfactometer was interchanged in order to minimize any bias within a test series and for each mosquito batch tested.

Test series

Preliminarily tests without any chemical stimulus were performed to establish that no mosquitoes responded to the airflow in the olfactometer to which they were acclimatized for 20 min. In all tests with odors, CO_2 pulses were applied as a background stimulus to augment the discriminatory power of the olfactometer bioassay. A test series with lactic acid was performed to establish that the olfactometer could be used to study the role played by host-derived cues on choices made by the mosquito vector. Humans, compared with other vertebrates, release high levels of lactic acid, and this compound has been shown to play an important role in the selective odor-mediated appetence behaviors of *An. gambiae* (Dekker et al. 2002). A concentration series of both HMHA and MSH were tested using TBME as a solvent. To evaluate whether one of the 3 bacteria species could produce volatile metabolites in odorless sweat to which *An. gambiae* responds, female (or male) sterile sweat was tested against female (or male) sweat incubated with a bacterium species. To test for any differential response to female versus male sweat samples, a test series was performed where mosquitoes were presented with odor generated by female sweat against male sweat incubated with the same bacterium. As control for these experiments, sterile female sweat was tested against sterile male sweat. In total, 19 series of experiments testing the responses of *An. gambiae* to treatments were made. Tests where mosquitoes were only exposed to CO_2 pulses in both arms of the olfactometer were made regularly (4–5 cages accompanying groups of experiments with test products) to ascertain that the CO_2 pulses were sensed as being equal in both arms (no mosquito preference for one of the two arms). These repetitions of tests measuring mosquito responses to CO_2 pulses alone generated a large set of experimental data that were pooled and analyzed in detail (see below) to serve as control for experiments where vapors of lactic acid, HMHA, or MSH were added to one arm of the olfactometer.

Data analysis

The behavioral responses of mosquitoes were expressed in terms of two binomial response variables: (i) the mean percentage of responding mosquitoes (R%) defined as the sum of mosquitoes caught in both upwind arms divided by the number of mosquitoes that left the release cage and (ii) the mean percentage of mosquitoes flying into each arm defined as the number of mosquitoes flying into one or the other arm divided by the total number of tested mosquitoes. R% represents the number of mosquitoes that were activated and effectively reached both upwind arms (intensity of anemochemotactic flight, Figure 1). The mean percentage of mosquitoes flying into each arm indicates the preferred test odor (degree of preference for one of the 2 test odors, Figure 1). Using the number of mosquitoes that left the release cage as denominator to calculate R%, pertinent information is provided in Supplementary Table S1 concerning mosquitoes that left the release cage but did not reach the upwind arms of the olfactometer. Descriptive and inferential statistics were performed using R software (version 2.14.0, Copyright © 2011, The R foundation for Statistical Computing). To test whether or not the

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percentage of mosquitoes flying into each arm differed from a 1:1 distribution, proportion tests were made within each treatment. To compare the differences between treatments for each binomial response variable, a generalized linear model (GLM) with a logit link function (binomial distribution) was used followed by a post hoc analysis (Tukey–Kramer). For dose-dependent analyses, the outcome of the experiments performed with CO_2 pulses alone served as zero dose.

CO_2 pulses were sinusoidal in each of the 3 compartments (in each upwind arm and in the downwind arm) of the olfactometer and were evaluated using Expedata software by computing the mean amplitude (A), period (P), and background increase (BI) prior to CO_2 stimulation in each test (see Figure 1 for a detailed description of the parameters). RH% and T°C were averaged over the experimental trial. Because all treatments were accompanied by CO_2 pulses as a sensitizer, it was important to test that these parameters remained constant and insure that they did not influence the response of mosquitoes to the added test odors. To analyze this, the large pool of experiments performed on mosquitoes with CO_2 pulses alone was examined in detail. At first, a t-test comparing the parameters in both upwind arms was performed. Then, GLM analyses were employed to test the effect of airflow conditions and parameters characterizing the CO_2 pulses on the two binomial response variables by assessing the relation between R% with the measured T°C and RH in the olfactometer but also with A, P, and BI downwind (CO_2 pulse mean amplitude near the release cage or at the downwind end [$A_{downwind}$], CO_2 pulse mean period near the release cage or at the downwind end [$P_{downwind}$], and background increase prior to CO_2 stimulation near the release cage or at the downwind end [$BI_{downwind}$]). Similarly, a GLM was used to evaluate the relation between the percentage of mosquitoes flying into either arm with any differences in CO_2 pulses between each arm (represented by the following ratios: CO_2 pulse mean amplitude in one arm/CO_2 pulse mean amplitude in the other arm [A_{arm1}/A_{arm2}], CO_2 pulse mean period in one arm/CO_2 pulse mean period in the other arm [P_{arm1}/P_{arm2}], and background increase prior to CO_2 stimulation in one arm/background increase prior to CO_2 stimulation in the other arm [BI_{arm1}/BI_{arm2}]). A GLM analysis was conducted to test whether any significant variation could be found between the regularly conducted groups of controls without odor treatments (4–5 cages) accompanying groups of experiments (experimental group variable) and to test whether mosquito age had any effect. In all statistical evaluations, significance levels were set to $\alpha = 0.05$ ($^{NS}P > 0.05$ (value provided), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). Values accompanying means in the text and error bars in figures represent standard errors. N always refers to the number of release cages and n the total number of mosquitoes tested.

Results

Response of *An. gambiae* in the olfactometer in the absence of CO_2 and any treatment

Testing the response of mosquitoes to the airflow in the olfactometer without any chemical stimulus elicited a very low response. Only 5.6 ± 1.5% of female *An. gambiae* flew into one arm and 4.9 ± 1.5% flew into the other (N = 18 cages, n = 350 mosquitoes). Of the mosquitoes that left the release cage, only 20.4 ± 4.9% reached one of the two upwind arms. The low percentage of mosquitoes reaching either arm of the olfactometer includes many experiments where no mosquitoes were observed in one or the two arms at the end of the 1-min test period (11 zero events in the 18 experiments), making it difficult to carry out a statistical evaluation. The data are, however, self-explanatory and indicate that after an acclimation period of 20 min the response to the airflow without any chemical stimulus elicited very low or almost no response from *An. gambiae*.

Response of *An. gambiae* to balanced CO_2 pulses in the olfactometer

When confronted with balanced CO_2 pulses in the two arm of the olfactometer, 82.0 ± 1.3% female *An. gambiae* that left the release cage flew upwind into either of the two arms of the olfactometer. The proportion of mosquitoes flying into each arm was equal and statistically undistinguishable: 34.6 ± 1.2% flew into one arm and 35.1 ± 1.2% into the other arm (Figure 2, lower pair of bars). Regular repetitions of tests measuring mosquito responses to CO_2 pulses alone generated a large set of control experiments (N = 81 and n = 1631) that allowed us to analyze CO_2 pulse characteristics and airflow conditions (T°C and RH) for their repeatability and their effect on the responses of *An. gambiae*. CO_2 pulse characteristics varied within a very narrow range and were observed to be statistically undistinguishable between each arm (Table 1). T°C and RH in the olfactometer remained stable throughout the test series (24.15 ± 0.15 °C and 81.50 ± 0.33%, respectively). GLM analysis revealed no significant correlation between the percentage of responding mosquitoes (R%) with either the CO_2 pulse characteristics measured in the downwind tube or with the airflow conditions in the olfactometer (Table 2). Similarly, the percentage of mosquitoes flying into either arm was not correlated with the small variations between the CO_2 pulse measured in each arm (Table 2) nor with experimental groups (4–5 cages) or with mosquito age (Table 2). Accordingly, data from all the tests with CO_2 pulses alone were pooled and served as control for experiments where lactic acid, HMHA, or MSH were added to one arm.

Response of *An. gambiae* to balanced CO_2 pulses with lactic acid added to one arm

When confronted with equal CO_2 pulses in the two arms but with lactic acid added to one, the percentage of responding *An. gambiae* (R = 81.3 ± 3.3%) remained equal to when CO_2 pulses were presented alone (Figure 2, same lowercase letter). However, the proportion of mosquitoes flying into the arm emitting lactic acid was significantly higher than the proportion flying into the arm without (53.6 ± 4.7 vs. 10.1 ± 2.3%, Figure 2). This finding shows that the addition of lactic acid did not increase activation followed by sustained upwind flight (R%) but was used by *An. gambiae* to redirect its flight toward the arm emitting lactic acid.

Response of *An. gambiae* to female or male sterile sweat tested against female or male sweat incubated with 1 of 3 bacteria species

Testing female sterile sweat (sweat incubated with buffer solution instead of a bacterial solution) against female sweat incubated with 1 of the 3 bacteria species (sweat incubated with a bacterial solution) showed that more mosquitoes flew into the arm with the odor resulting from sweat incubation with a bacterium. This was highly significant for all 3 bacteria species and resulted in the following mean percentages of mosquitoes flying into test and control arms, respectively, of 61.6 ± 4.9% versus 16.0 ± 2.7% with *S. epidermidis*, 63.4 ± 4.8% versus 27.4 ± 4.6% with *C. jeikeium*, and 52.0 ± 3.7% versus 17.3 ± 3.4% with *S. haemolyticus* (Figure 3, bottom panel). These preferences, represented by the distribution in the two arms of the olfactometer, did not differ significantly between

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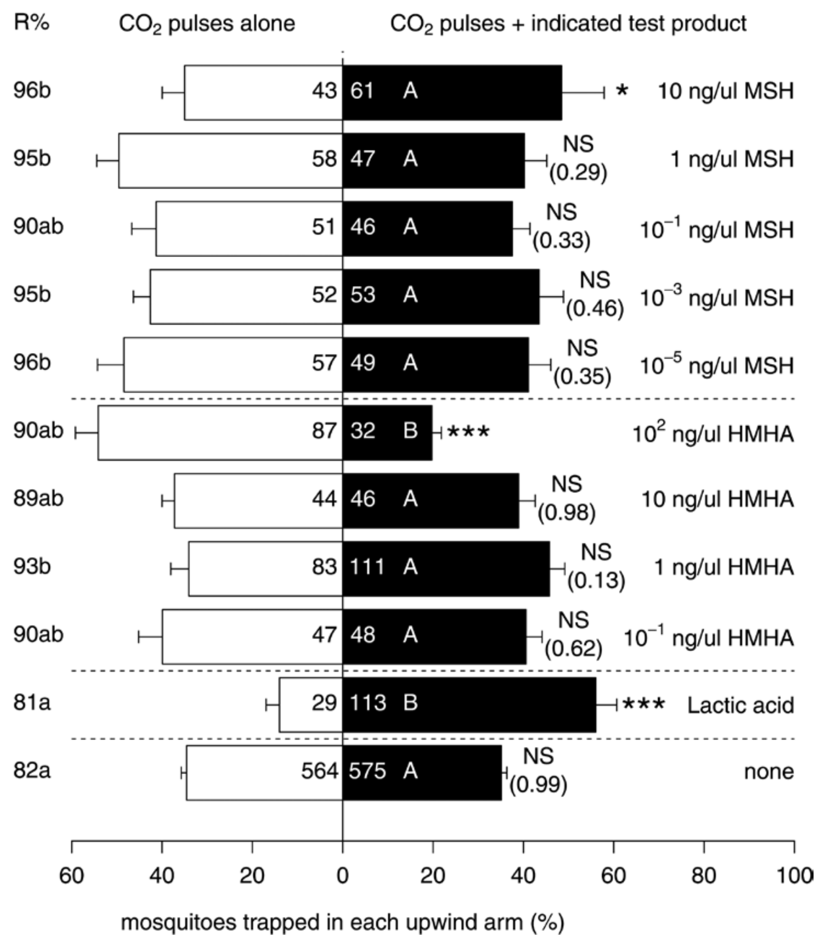

Figure 2. Responses of *Anopheles gambiae* in a dual-choice olfactometer to CO_2 pulses with and without lactic acid added in its vapor phase to one arm (lower pairs of bars) and to vapors of HMHA (middle pairs of bars) and MSH (upper pairs of bars) released into one olfactometer arm from a sand-blasted glass slide at the indicated dose. The mean percentage of mosquitoes trapped in each upwind olfactometer arm is represented by the pairs of white and black bars (the number within each bar indicates the total number of mosquitoes trapped in the arm). The mean percentage of responding mosquitos (R%) is indicated in the left-hand column for each treatment and significant differences between treatments (GLM analysis) are indicated by different lower case letters. Proportion test statistics for treatments are indicated on the right of each pair of bars (NS not significant, *$P \leq 0.05$, **$P \leq 0.0$, ***$P \leq 0.001$) and error bars represent standard errors. Significant differences between treatments (GLM analysis) in the percentage of mosquitoes counted in the two arms of the olfactometer are indicated by different capital letters.

Table 1. CO_2 pulse characteristics when presented alone to *Anopheles gambiae*

CO_2 pulses characteristics	arm1	arm2	downwind	*P* value of *t*-test between arm1 and arm2
A (Δppm)	48.10 ± 0.36	47.97 ± 0.42	22.54 ± 0.22	0.81
P (s)	4.01 ± 0.01	4.01 ± 0.01	3.99 ± 0.01	0.76
BI (Δppm)	45.78 ± 0.39	45.82 ± 0.37	45.42 ± 0.39	0.95

bacterial treatments (Figure 3, same capital letters). The percentage of responding mosquitoes (R%) was 83.8 ± 4.8% in experiments with *S. epidermidis*, 99.4 ± 0.6% with *C. jeikeium*, and 85.1 ± 3.6% with *S. haemolyticus*. Despite the fact that the R% elicited by *C. jeikeium* was significantly higher than by either *Staphylococcus* species (Figure 3, different lower case letters), the mosquitoes systematically showed a clear preference for the olfactometer arm with bacteria plus female sweat treatments. The outcome of the same experiment but using male sweat incubated with the 3 bacteria species was very similar, resulting in significantly higher mean percentages of mosquitoes flying into test and control arms, respectively, of 51.7 ± 2.9% versus 21.9 ± 2.1% with *S. epidermidis*, 56.8 ± 2.9% versus 30.3 ± 3.4% with *C. jeikeium*, and 55.3 ± 3.6% versus 36.8 ± 4.1% with *S. haemolyticus* (Figure 3, top panel). As with female sweat, *An. gambiae* preferences did not differ significantly between bacterial treatments incubated with male sweat (Figure 3, same capital letters). The percentage of mosquitoes responding (R%) to male sweat incubated with bacteria species differed in that both *C. jeikeium* and *S. haemolyticus* elicited significantly higher R% response values of 95.6 ± 1.9 and 98.0 ± 4.1%, respectively, than the 84.4 ± 2.6% response elicited by *S. epidermidis* (Figure 3, different lower case letters). Despite this, *An. gambiae* females systematically showed a preference for the olfactometer arm with bacteria plus male sweat treatments.

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Response of *An. gambiae* to female sweat tested against male sweat both incubated with the same bacterium species

Presenting mosquitoes in the olfactometer with female sterile sweat (female sweat incubated with buffer solution instead of a bacterial solution) tested against male sterile sweat (male sweat incubated with buffer solution instead of a bacterial solution) induced no preference: 33.6 ± 4.0% of the mosquitoes choose the arm with female sweat and 35.3 ± 3.5% the arm with male sweat (Figure 4, lower pair of bars). When *An. gambiae* was offered to discriminate between male and female sweat both incubated with the same bacterium species, female mosquitoes always showed a significant preference for the arm with male sweat (Figure 4, upper three pairs of bars). This preference was most marked with *C. jeikeium* (64.4 ± 2.7% versus 25.9 ± 3.3% for the arms with male and female sweat, respectively) followed by *S. heamoliticus* (52.3 ± 3.0 % versus 27.6 ± 2.5%) and *S. epidermidis* (49.51 ± 5.9% versus 28.1 ± 5.5%). The same trend was observed in terms of the percentage of mosquitoes that left the release cage and effectively reached both upwind arms with R% = 97.9 ± 0.9 %, 93.1 ± 1.9 %, and 86.4 ± 2.7 %, respectively, for treatments with *C. jeikeium, S. heamoliticus*, and *S. epidermidis*. Only treatment comparisons containing *C. jeikeium* significantly differed from the outcome where male and female sterile sweat samples were compared with each other in terms of both mean percentage of mosquitoes flying into each arm and the percentage of responding mosquitoes (R%, Figure 4, differences in capital and lower case letters).

Table 2. Effect of CO_2 pulse characteristics and airflow conditions on the response of *Anopheles gambiae*

Response variable	Explanatory variable	*P* value of GLMs
Responding mosquitoes (R%)	~$A_{downwind}$	NS (0.79)
	~$P_{downwind}$	NS (0.88)
	~$BI_{downwind}$	NS (0.18)
	~RH	NS (0.26)
	~T°C	NS (0.35)
	~Exp. group[a]	NS (0.78)
	~Age	NS (0.52)
% of Mosquitoes responding in each arm	~A_{arm1}/A_{arm2}	NS (0.09)
	~P_{arm1}/P_{arm2}	NS (0.17)
	~BI_{arm1}/BI_{arm2}	NS (0.23)

[a]Tests with 4–5 cages accompanying a group of test products, usually conducted within an experimental day.

Response of *An. gambiae* to HMHA and MSH applied on sand-blasted glass slides

The dose at which the highest preference for the arm with HMHA was measured was 1 ng/µL. At this dose, 45.8 ± 3.4% of the mosquitoes flew into the arm with HMHA, whereas 34.1 ± 4.0% flew into the control arm, but this difference was not significant (prop. test *P* = 0.13). At the highest dose tested (100 ng/µL), HMHA was clearly repellent (19.7 ± 2.1% in the test arm vs. 54.1 ± 5.1% in the control; Figure 2) and the distribution of mosquitoes between the two arms was also significantly different from the distribution measured for the other HMHA doses tested (Figure 2, different capital letters). Although HMHA did not elicit a clear preference at 1 ng/µL, the percentage of responding mosquitoes (R%) was highest at this dose and clearly distinguishable from the R% value for mosquitoes exposed to CO_2 pulses alone (92.9 ± 2.2% vs. 82.0 ± 1.3%, different lower case letters; Figure 2). R% measured at the other doses of HMHA tested were not statistically different from the control (CO_2 pulses alone). In experiments with MSH, the highest dose (10 ng/µL) elicited a significant preference for the arm with the test product (53.4 ± 5.2 % vs. 31.7 ± 3.6%), but no differences in the mean

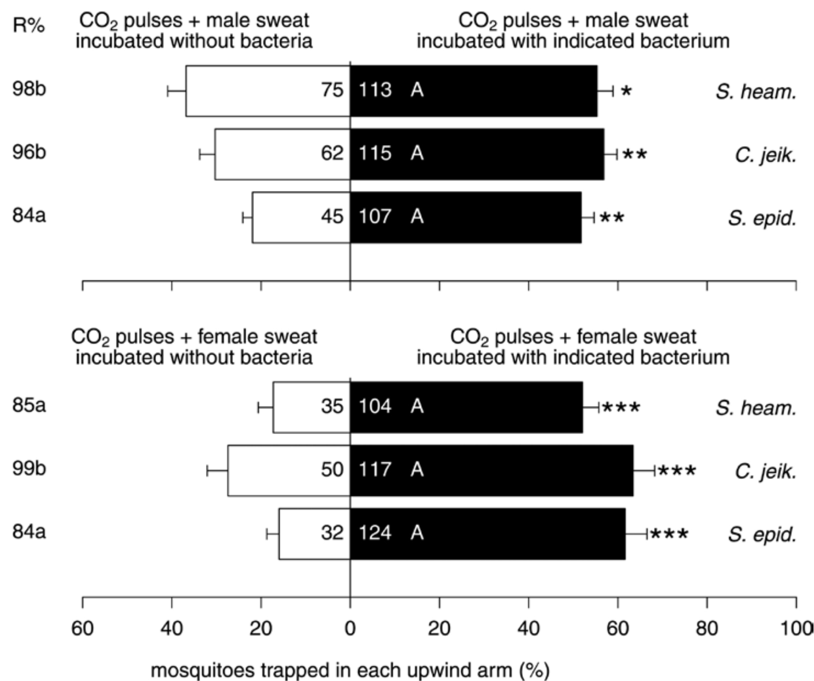

Figure 3. Responses of *Anopheles gambiae* in a dual-choice olfactometer to sterile sweat in one arm and to sweat incubated with 1 of 3 bacteria species in the other arm of the olfactometer. The upper panel presents results for experiments made with male sweat and the lower panel for experiments made with female sweat. All tests were made in the presence of CO_2 pulses as sensitizer. For further explanation, see legend to Figure 2.

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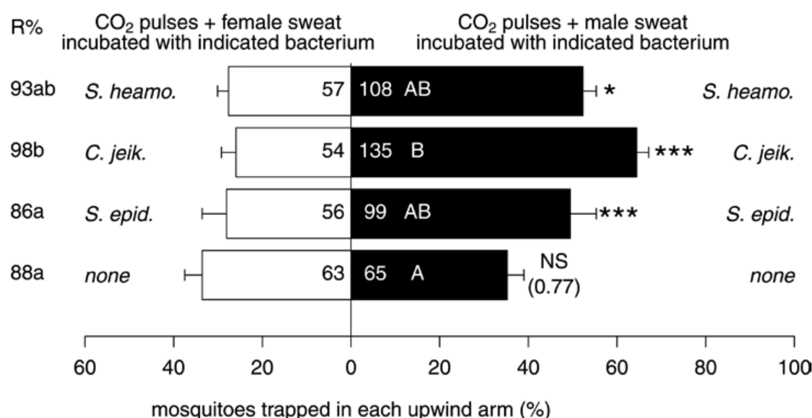

Figure 4. Responses of *Anopheles gambiae* in a dual-choice olfactometer to female sweat incubated with a bacterium species in one arm and to male sweat incubated with the same bacterium species in the other arm of the olfactometer (upper pairs of bars) and to the same sweat samples incubated free of bacteria (lower pairs of bar). All tests were made in the presence of CO_2 pulses as sensitizer. For further explanation, see legend to Figure 2.

percentage of mosquitoes flying into each arm were found between the different doses tested (Figure 2, same capital letters). In contrast to HMHA where an increase in R% was only observed at 1 ng/µL, R% was significantly higher than in the control (CO_2 pulses alone) at all doses of MSH tested with the exception of the 0.1 ng/µL dose (Figure 2, different lower case letters).

Discussion

The olfactory-driven behavioral sequence leading *An. gambiae* to human hosts starts with activation followed by sustained upwind flight (anemochemotaxis). In the dual-choice olfactometer experiments presented here, we evaluated this behavioral step with R%. During sustained upwind flight additional infochemical input may redirect the flight of *An. gambiae* to its preferred host. In our case, we used the preference for an arm emitting the test odor as a proxy to evaluate this behavioral step.

When presented intermittently, CO_2 is well-known for its effect as an activator for mosquitoes and synergist when combined with other odors (Gillies 1980; Mboera et al. 1997; Geier et al. 1999a; Dekker et al. 2002, 2005). In olfactory-driven host-seeking behavior of mosquitoes, rapid CO_2 fluctuations have also been shown to function as a "releaser" of higher sensitivity or responsiveness of mosquitoes to the presence of a potential host (Dekker et al. 2005). We therefore made our experiments using CO_2 pulses as a background sensitizer. In this manner, more than 80% of the 18 to 23 mosquitoes typically present in the release cage could be assessed for their responsiveness to the test odor. It was important to verify that this background stimulation was repeatable and generated a uniform (equal mosquito distribution in both arms) and repeatable response to ensure that any differential response resulted solely from the effect of the test odor.

In a similar olfactometer to the one used here, Dekker et al. (2002) showed that lactic acid does not augment the responsiveness of *An. gambiae* to fluctuating CO_2 but is probably used by the disease vector to discriminate humans from other vertebrate hosts or even between different humans. One must bear in mind that human skin extracts contains particularly high concentrations of lactic acid and that differential attractiveness between humans can be compensated with the addition of lactic acid (Dekker et al. 2002). The findings presented here corroborate these earlier findings in that lactic acid did not augment the responsiveness (R%) of *An. gambiae* but did elicit a flight direction to the arm emitting lactic acid. The experimental paradigm presented here consequently offers a valid approach to test human-related olfactory cues that, on the one hand, potentially influence the responsiveness of the mosquito to a suitable host as simulated with CO_2 pulses and/or, on the other, are used by the mosquito to direct its flight to its favored host odor source. These two aspects of the mosquito's responses are not mutually exclusive.

Human axillary sweat originates from sebaceous, eccrine, and apocrine glands. The composition of this sweat is also unique among living organisms. The water-soluble forms of HMHA, a *N*-glutamyl derivative, and the soluble form of MSH, a cysteinyl-glycine-*S*-conjugate, have not been found to date in any other species or primate (Natsch et al. 2006). These precursors are excreted in various ratios and concentrations by humans. The microflora of individuals varies considerably (Troccaz et al. 2015) with the consequence that an individual that is a high producer of precursors can release more HMHA and MSH depending on the type of microflora present. Incubation of sterile sweat with isolated bacteria colonies was preferred in this study for the selective odor release profiles obtained: *S. epidermidis* for its low odor-producing pattern, *C. jeikeium* for its strong Nα-acylglutamine aminoacylase activity that liberates many carboxylic acids in addition to HMHA, and *S. haemolyticus* for it capacity to liberate MSH from sweat along with other sulfur-containing compounds (Natsch et al. 2006). Sweat incubated with one of the three bacteria species was always preferred over sterile sweat of the same gender although the degree of preference by *An. gambiae* for sweat inoculated with either of the bacteria species did not differ significantly. Nevertheless, the percentage of responding mosquitoes (R%) was consistently higher in experiments performed with *C. jeikeium* as inoculum, irrespective of whether male or female sweat was used as substrate. The R% was also higher with *S. haemolyticus* as inoculum but only when male sweat was used as a substrate. When *An. gambiae* was offered a choice between male and female sweat samples both inoculated with the same bacterium species mosquitoes systematically showed a preference for male sweat. In these experiments, the arm of the olfactometer conveying odor from male sweat incubated with *C. jeikeium* induced the strongest preference and the highest percentage of R%. This suggest that *C. jeikeium* with its strong aminoacylase activity that liberates carboxylic acids including HMHA from human sweat is of particular importance in sensory ecology of *An. gambiae*. On the other hand, *S. epidermidis* with its low odor-producing capacity in sweat systematically induced the

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lowest percentage of responding mosquitoes. Sweat samples incubated with bacteria induced differential R% values but always permitted discrimination between the olfactometer arms.

Overall, our findings demonstrate that axillary odor metabolized by the 3 tested bacteria species is sensed by *An. gambiae* and plays an important role in the odor-mediated behaviors of *An. gambiae* toward human hosts. It also shows that both the bacterium species, that is the agents of transformation, and sweat constituents, that is the substrate, can contribute to different behavioral criteria in terms of percentage responding mosquitoes R% and the preference by *An. gambiae* for one or the other arm of the olfactometer. A diverse community of bacterial species dominated by *Staphylococci* and *Corynebacteria* populates axillary sweat (Shehadeh and Kligman 1963; Leyden et al. 1981; Troccaz et al. 2015) and this diversity may be required to maximize behavioral response of *An. gambiae*. Nevertheless, here we show that incubating axillary sweat with a single bacterium species is sufficient to elicit a strong behavioral response in *An. gambiae*. The high responsiveness of *An. gambiae* to *C. jeikeium* sweat incubations is of particular interest because earlier investigators have correlated human axillary odor intensity with higher *Corynebacterium* density (Leyden et al. 1981; Jackman and Noble 1983). Moreover, in Verhulst et al. (2010a), the behavioral response of *An. gambiae* to different bacteria species grown *in vitro* revealed a *Corynebacterium* (*C. minutissimum*) that produces VOCs that are pertinent to the host odor-mediated behaviors of this mosquito. It seems that *Corynebacteria* play a particularly important role in odor-mediated and anthropophilic host-seeking behaviors of *An. gambiae*.

Preferences by *An. gambiae* for odors emanating from incubation of male sweat inoculated with different bacteria species over female sweat incubated with same bacteria was not due to the intrinsic content of male sweat because mosquitoes offered a choice between male and female sterile sweat showed no preference. Thus, the action of bacteria was required to elicit a preference for male sweat over female sweat. This could be the result of two effects or a combination of both. Firstly, sweat originating from the male pool, in terms of its quantitative composition (Troccaz et al. 2009), provides a better substrate for bacteria resulting in male sweat producing odors preferred by higher numbers of *An. gambiae* than incubated female sweat. The sweat samples showed notable differences in pH and glucose content, the latter being an important source of carbon for gram-positive bacteria (Troccaz et al. 2009). Because male sweat contained more glucose than female sweat, it cannot be excluded that bacterial growth was higher on the former, in which case the observed preference would have a quantitative origin. Secondly, the presence of male-specific product(s) could be responsible for the more attractive nature of male sweat incubated with bacteria. To reach a conclusion as to the origin of the stronger response of *An. gambiae* to male sweat, a detailed chemical analysis would need to be made, providing an opportunity for further research on the topic.

Among other carboxylic acid and sulfur-containing compounds, *C. jeikeium* and *S. haemolyticus* produce two human-associated compounds, HMHA and MSH (Troccaz et al. 2009; Natsch et al. 2006), which have not previously been tested on *An. gambiae*. Accordingly, the responses of *An. gambiae* to different doses of these two compounds were tested here. The proportion test indicated an effect for both MSH and HMHA at the highest doses tested, namely a preference for the olfactometer arm bearing MSH and an avoidance response in the test with HMHA. However, the doses tested at 100 ng for MSH and 1 µg for HMHA are arguably too high in terms of physiological relevance. Meanwhile, and in contrast to lactic acid, it must be noted that the percentage of responding mosquitoes (R%) was higher than in control experiments (CO_2 pulses alone) at almost all doses of MSH tested, including the lower ones and for one dose of HMHA. In view of this, one might cautiously suggest that these compounds may serve to activate and enhance upwind flight in *An. gambiae*. However, neither MSH nor HMHA presented alone is responsible for the discriminative responses expressed by the mosquitoes for the arm of the olfactometer conveying human sweat samples incubated with bacteria.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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Conflict of interest statement

Both M.T. and C.S. are employees of Firmenich S.A., Geneva, Switzerland, and the other authors declare they have no competing interests.

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