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No evidence for long-range male sex pheromones in two malaria mosquitoes

Serge Bèwadéyir Poda^{1,2,3}, Bruno Buatois⁴, Benoit Lapeyre⁴, Laurent Dormont⁴, Abdoulaye Diabaté¹, Olivier Gnankiné², Roch K. Dabiré¹ and Olivier Roux^{1,3}✉

Cues involved in mate seeking and recognition prevent hybridization and can be involved in speciation processes. In malaria mosquitoes, females of the two sibling species *Anopheles gambiae* s.s. and *An. coluzzii* mate in monospecific male swarms and hybrids are rare. Long-range sex pheromones driving this behaviour have been debated in literature but so far, no study has proven their existence or their absence. Here, we attempted to bring to light their existence. To put all the odds in our favour, we used different chemical ecology methods such as behavioural and electrophysiological assays as well chemical analyses, and we worked with mosquitoes at their optimal physiological mating state that is with swarming males during their natural swarming windows. Despite all our efforts, our results support the absence of long-range sex pheromones involved in swarm detection and recognition by females. We briefly discuss the implications of this finding in ecology, evolution and for control strategies.

Cues involved in mate seeking and mate recognition prevent hybridization and can be involved in speciation processes. In insects, these cues can be visual, acoustical or chemical with a short or a long-range action and are highly species specific^{1,2}.

In mosquitoes, many species mate in flight, within aggregations called ‘mating swarms’^{3–7}. In malaria mosquitoes of the *Anopheles gambiae* complex, mating takes place at sunset in monospecific swarms containing a few males to thousands of them in which virgin conspecific females come to find a mate^{8–14}. In West Africa, *An. coluzzii* and *An. gambiae* s.s. are often found in sympatry but they form distinct swarms spatially segregated and hybrids are rare (roughly 1%)^{13–20}. No evidence for selection against hybrids was found and spermatheca analyses showed that these species mostly mate assortatively^{17,21–25}. This suggests that reproductive isolation between these two sibling species is achieved by strong pre-mating reproductive barriers^{15,23,26}. In addition, as females usually only mate once in their lives²⁷, errors in the choice of mate should be costly and fall under negative selection. As a consequence, one would expect to find specific cues that lead females to conspecific male swarms. However, the way females are attracted to swarms is unknown.

In species of the *An. gambiae* complex, several cues have been identified to play a role in bringing sexes together. Acoustic cues were shown to be involved in close-range recognition, the male and the female adjusting their respective wing-beat frequencies to converge on a shared harmonic frequency^{28,29}. However, it was recently demonstrated that females of some *Anopheles* species are able to detect swarm sounds only in a very close vicinity and are thus, unable to use swarm sound to locate or identify swarms at long range³⁰. Visual cues also play an important role in species segregation with *An. coluzzii* males swarming over contrasted visual ground markers (marker, hereafter) and *An. gambiae* males swarming over bare ground¹³. A recent work showed that, like *An. coluzzii*, *An. gambiae* males also use visual markers but rather to locate their swarm at a distance from the marker³¹. Females also use these markers to form swarms in the absence of males suggesting

that females may use these markers to join the swarm location³¹. Nevertheless, the distance between two heterospecific swarms using the same visual ground marker can be about 2 m in semifield conditions³¹ and it is still unknown from which distance mosquitoes can see such markers, suggesting that females could cross the location of heterospecific swarms by accident. Chemical mating cues have also been under investigation in *Anopheles* species. Heptacosane, a cuticular hydrocarbon, enhances the interaction between males and females³². This compound, however, can only be perceived by contact and can consequently be involved only in mate recognition during courtship and to stimulate acceptance by females. Moreover, the absence of assortative mating in confined heterospecific males and females in laboratory cages or indoor swarms³³, suggests that close-range mating cues, if they exist, cannot ensure total reproductive isolation by themselves. Species isolation is thus likely to occur through long-range and specific swarm recognition cues acting as a first barrier in pre-mating processes that prevent hybridization by limiting contact between sexes of the different species.

To our knowledge, volatile sex pheromones in the *An. gambiae* complex have never been brought to light without ambiguity. Charlwood et al.¹¹ reported an absence of response of *An. gambiae* males in natural swarms to squashed females on filter paper or to living females in a net cage. However, in natural conditions, females are the ones attracted to male aggregation sites^{2,3,10,11,13,14,20,27} and males should be the ones that emit long-range pheromones. Nevertheless, a laboratory study failed to demonstrate any attractiveness of dead males to virgin females in a Y-tube olfactometer³⁴. The difficulties in highlighting the existence of such pheromones might be due to an emission in very low quantities and/or exclusively during swarming. Indeed, in sandfly species, which also mate in large aggregations of males, it was shown that the concentration of male-produced sex pheromones is greater in larger swarms, and hence, the chances for individual males of mating with a conspecific female are also greater^{35,36}. Similarly, Diabaté et al.³⁷ showed that *An. coluzzii* females were more frequently attracted to large swarms,

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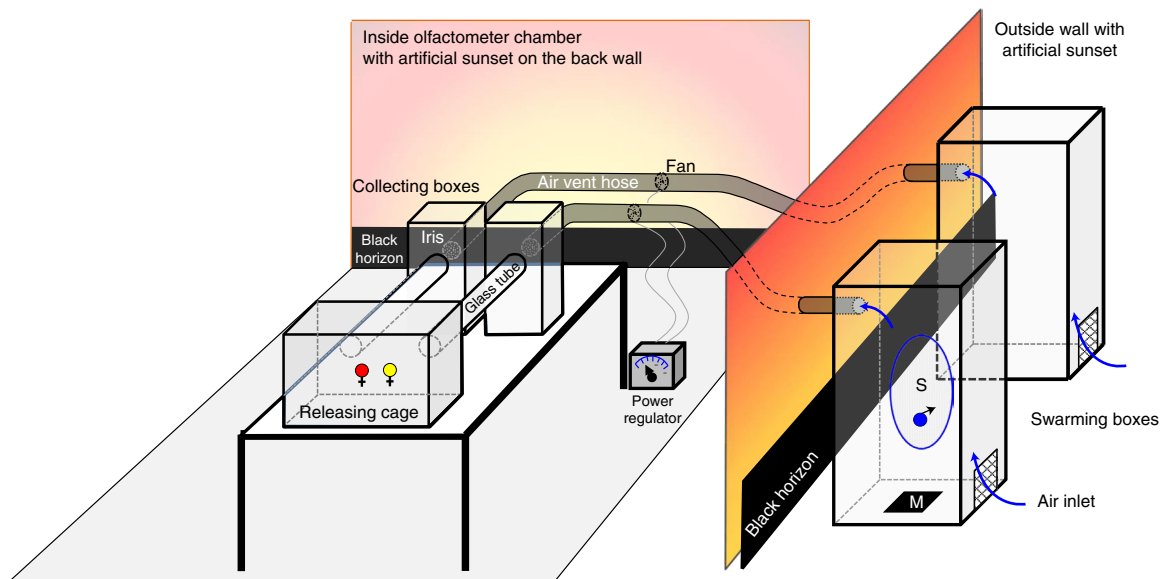


Fig. 1 | Schematic of the olfactometer set-up. S, swarm; M, marker. Not at scale. The odour source container was modified to enclose male swarms in boxes made of transparent plexiglass (length, width and height, $60 \times 60 \times 120 \text{ cm}^3$; 'swarming boxes' hereafter). Each swarming box was connected by a 3 m PVC concertinaed air vent hoses (length \times diameter (\varnothing), 6 m when fully extended $\times 10 \text{ cm}$, W3-65014-HQ4) to a collecting glass box (length, width and height, $32 \times 33.5 \times 44 \text{ cm}^3$), which was linked to a mesh-covered releasing cage (length, width and height, $50 \times 40 \times 40 \text{ cm}^3$) by a glass tube (length $\times \varnothing$, $60 \times 10 \text{ cm}^2$). A custom-made electric fan was located at the mid-length of each air vent hose and drew air from the swarming boxes (odour-sources) to the releasing cage, providing an odour-laden air current against which mosquitoes in the releasing cage were induced to fly. The air flow was controlled via two different mechanisms; first, a power regulator (HQ-Power PS1502A, Velleman) connected to fans, and second, iris dampers ($10 \text{ cm } \varnothing$; CIR D100, France air) connecting the air vent hoses to the collecting boxes. The openings of the air vent hoses on both the swarming and collecting boxes sides were covered with nets to prevent mosquitoes from flying into the air vent hoses. The swarming boxes (odour-sources) were located side by side outdoors and the olfactometer inside a room. The air speed in the releasing cage was regulated at $18 \pm 2 \text{ cm s}^{-1}$ using an anemometer (Model 425, Testo) and the room temperature and relative humidity were set at $27 \pm 2^\circ \text{C}$ and $80 \pm 10\%$ relative humidity via an air conditioner and a humidifier (Defensor 505, Condaïr), respectively.

suggesting a potential additive effect of cues released by males. Males gathering in large swarms may increase their 'detectability' and attractiveness in both the male and female's olfactory landscape⁶. In addition, in many insects, pheromone release and receptivity were shown to respond to diel rhythms or even strict time windows of only a few minutes or hours^{38–41} or even to be dependent of the presence of conspecifics^{42,43}.

A recent work by Mozūraitis et al.⁴⁴ and concomitant to a first version of the present work⁴⁵, highlighted the existence of five volatile compounds potentially emitted by *Anopheles* males. According to the authors, these might be involved in aggregation behaviour, attracting both males and females and increasing the insemination rate. Although an interesting biological activity was described by the authors, the claim that these compounds are male swarming aggregation pheromones must be considered with caution and it requires further investigations. Indeed, these five compounds (acetoin, sulcatone, octanal, nonanal and decanal) are very frequently found in nature and, as reported by the authors, have been shown to have a biological activity in *Anopheles* mosquitoes in very different contexts from the reproductive behaviour. They were repeatedly found in human and animal body odour^{46–51}, breath^{52–55} and in host plants⁵⁶. These represent the main sources of food (blood and sugar meals, respectively) for *Anopheles* mosquitoes. As a matter of fact, octanal, nonanal and decanal are part of a blend used in some traps to mimic mammalian host odour for several species of mosquito vectors^{50,57}. Some of these compounds were also found associated to oviposition sites^{58–60} and to ambient air^{61,62} and sulcatone is thought to be responsible for discrimination between humans and animals in human-seeking mosquitoes⁵¹. Thus, it is not surprising that they trigger a strong biological/flight activity in mosquitoes. Nevertheless, the discovery of sex pheromones or a highly attractive blend in malaria

mosquitoes would be a valuable step toward the development of new control and monitoring strategies. Sex or aggregation pheromones could help in designing sexually competitive mosquitoes in sterile insect and gene drive techniques.

Here, we investigated the existence of long-range sex pheromones in *An. gambiae* and *An. coluzzii* mosquitoes that could allow the females to detect, recognize and track conspecific male swarms. On the premise that such pheromones could be produced only by males during swarming windows and to put all the odds in our favour, we used different chemical ecology methods, always with living swarming mosquitoes during their natural swarming windows. First, we investigated the long-range behavioural response of females exposed to headspace of male swarms in an olfactometer. Second, we collected and analysed volatile organic compounds (VOCs) with different methods on both laboratory-induced swarms and natural swarms. Third, we tested for an electroantennographic response of females to male swarm VOCs. We used both recently colonized mosquitoes and large experimental set-ups to ensure males produced a free swarming behaviour. In addition, we replicated the main experiment by Mozūraitis et al.⁴⁴ to search for the five compounds. We specifically added two new controls: one to check whether these compounds were male specific and the other to discard a potential environmental/laboratory pollution.

Results

Behavioural assays. Over 12 replicates of around 200 females for each of the four combinations, a total of 8,918 females were tested (4,423 *An. coluzzii* and 4,495 *An. gambiae*) among which 3,591 flew upwind into the collecting boxes (activation rate $40.2 \pm 1\%$). There was a significant interaction between female species and the four combinations of swarm boxes tested ($\chi^2_3 = 22.19$, $P < 0.001$).

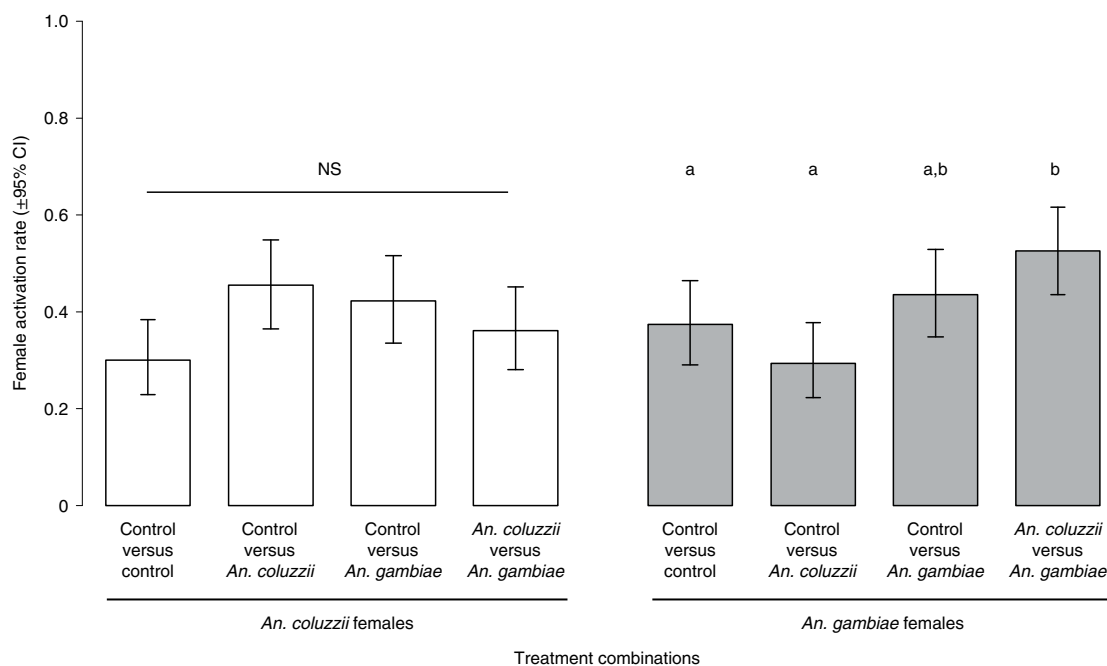


Fig. 2 | Mosquito activation rate, expressed as the proportion of females caught in both collecting boxes out of the total number released females for each of the four tested combinations. For the statistical analyses, binomial generalized linear-mixed models were used considering replicates as random effect (*An. coluzzii* females, $\chi^2_3=5.72$, $P=0.12$; *An. gambiae* females, $\chi^2_3=14.94$, $P=0.001$). For *An. gambiae* females, a subsequent multiple comparison was done using Tukey's all-pair comparison method. Different letters indicate difference at $P < 0.05$ and NS, not a significant difference. Data are presented as percentage values $\pm 95\%$ CI. $n=12$ biologically independent replicates (with 100 females used per species and per replicate).

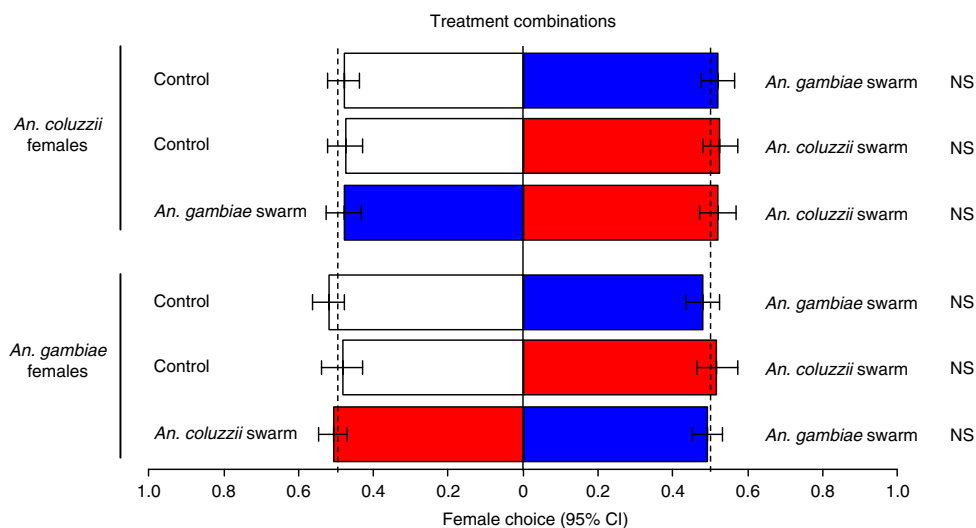


Fig. 3 | Mosquito choice, expressed as the proportion of females caught in one or the other collecting box out of the total number of 'activated' females for each test. For the statistical analyses, binomial generalized linear-mixed models were used considering replicates as random effect (female species, $\chi^2_1=2.45$, $P=0.11$; choice test combinations, $\chi^2_2=1.15$, $P=0.56$). Data are presented as percentage values $\pm 95\%$ CI. $n=12$ biologically independent replicates (with 100 females used per species and per replicate).

Species subset analyses showed that the different test combinations had no effect on the *An. coluzzii* activation rate ($\chi^2_3=5.72$, $P=0.12$; Fig. 2). However, in *An. gambiae*, there was a significant effect of the test combinations on the female activation rate ($\chi^2_3=14.94$, $P=0.001$; Fig. 2) with a higher activation rate when females were exposed simultaneously to both *An. coluzzii* and *An. gambiae* male swarms than when exposed to an *An. coluzzii* swarm alone ($z=4.01$, $P < 0.001$) or to the control (two empty boxes $z=2.57$, $P=0.04$). Nonetheless, no difference was found when females were exposed

to an *An. gambiae* male swarm alone ($z=-1.49$, $P=0.44$). Neither the *An. coluzzii* swarms, nor the *An. gambiae* swarms were attractive for activated females (female species $\chi^2_1=2.45$, $P=0.11$; choice test combinations $\chi^2_2=1.15$, $P=0.56$ and female species \times choice test combination interaction $\chi^2_2=0.34$, $P=0.84$; Fig. 3).

Chemical analyses. No qualitative or quantitative differences were detected between samples of swarming males and their relative controls for both species (see Supplementary Information

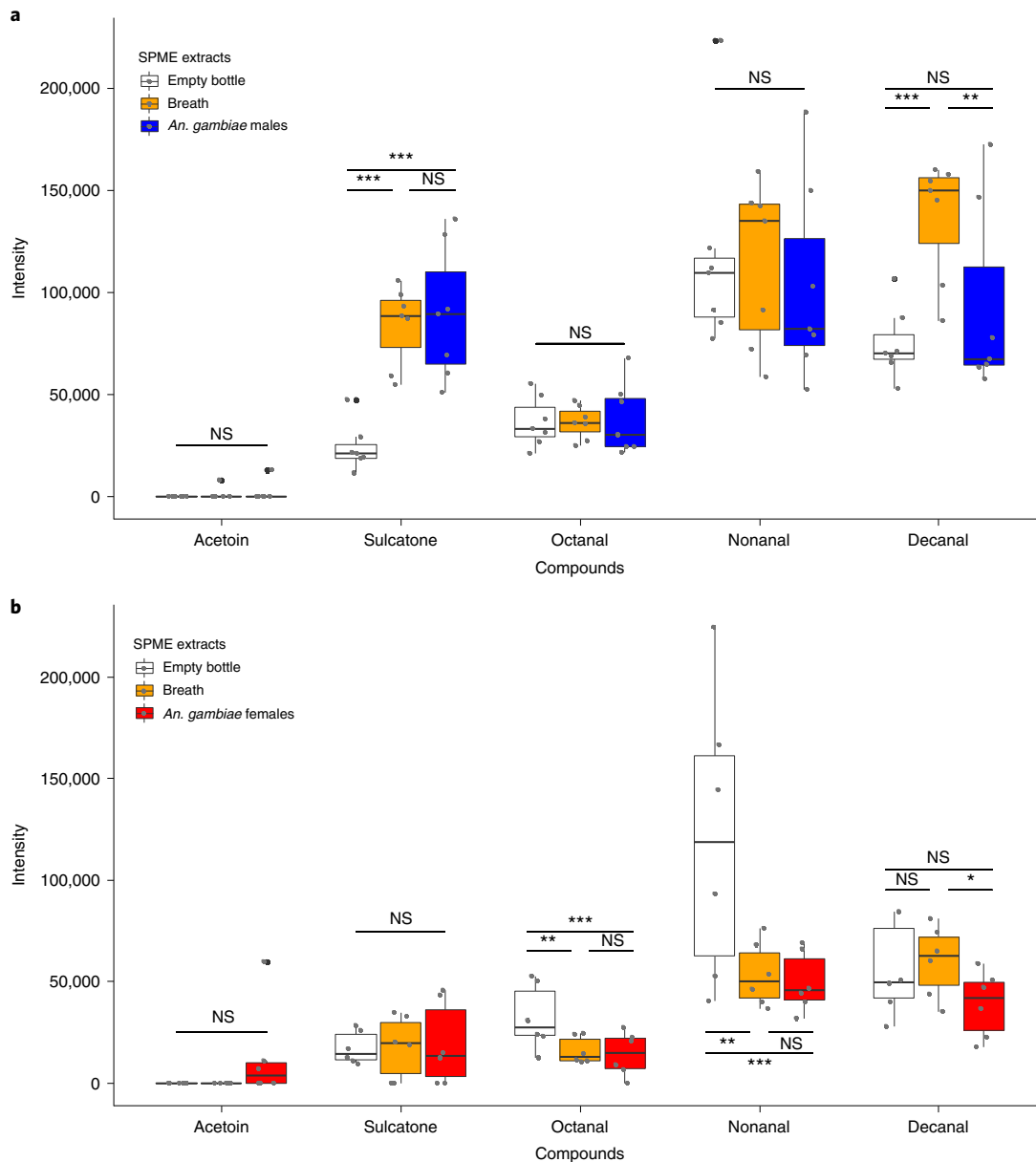


Fig. 4 | Quantities of acetoin, sulcatone, octanal, nonanal and decanal collected with SPME fibres. a,b, Quantities (integration units) contained in the headspace of the empty bottles, the breath of the manipulator and flying males (**a**) and females (**b**) of *An. gambiae* Kisumu and collected with SPME fibres. The intensity values correspond to the counts related to the abundance of the specific ions representative of each molecule formed in the mass spectrometer and correspond to the amount of compound analysed. The box plots indicate the median (wide horizontal bars), the 25th and 75th percentiles (squares) and the minimum and maximum values (whiskers). The black dots represent outliers and grey dots raw data. For the statistical analyses, Gaussian generalized linear-mixed models were used considering replicates as random effect. When necessary, a multiple comparison was done using Tukey's all-pair comparison method. NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 5$ biologically independent samples (with 60–70 mosquitoes used per sample) in **a** and **b**.

and Supplementary Figs. 2 and 3 for details). During the experiment replicating the protocol by Mozūraitis et al.⁴⁴, both males and females started to fly at the expected time (20:00) but due to the small volume it was not possible to determine whether the group was a swarm. Analyses of solid-phase microextraction (SPME) fibres in search for acetoin, sulcatone, octanal, nonanal and decanal showed that acetoin was absent from almost all the male samples (Fig. 4a), but was present on three occurrences in female extracts (over six) (Fig. 4b). Sulcatone, octanal, nonanal and decanal were found in almost all types of extract including controls. However, we did not find significant increased quantities of the five compounds in 'swarming' mosquito extracts compared to the breath controls

(Fig. 4). The control to test the effect of blowing twice in the bottle showed that the headspaces of 'breath' and 'breath ×2' were very similar (Supplementary Fig. 4), demonstrating an absence of accumulation of breath odour between the breath control and the introduction of mosquitoes.

A posteriori, we also quantified acetoin, sulcatone, octanal, nonanal and decanal in our previous gas chromatography–mass spectrometry (GC–MS) analyses in search for similar variations as described in Mozūraitis et al.⁴⁴. We did not find acetoin in our chromatograms. Indeed, as we used a low polarity Optima 5-MS column, acetoin (if present) was eluted before the linear retention index (LRI) 800. In the samples obtained from natural swarms of

An. coluzzii with the twister method, sulcatone and octanal were absent. The quantities of both nonanal and decanal were not significantly higher in the swarm than in the control (Fig. 5a). In laboratory swarm samples, sulcatone, octanal, nonanal and decanal were found inconsistently. Nevertheless, swarms did not show higher quantities of these compounds compared to the empty box (Fig. 5b) excepted in samples obtained with the Porapack tubes (Fig. 5c). However, this difference was not statistically significant. Whatever, the method used, we can note a high quantitative variability between the samples. This was true for both the control and the mosquito samples.

Electrophysiology analyses. The male swarm VOCs induced a few weak and poorly reproducible depolarizations on the antennae of conspecific females. These responses did not correspond to noticeable peaks in the GC detector of the GC–electroantennographic detection system (GC–EAD) apparatus (Fig. 6) nor to any detectable specific peak in the GC–MS analyses at the corresponding LRI (see Supplementary Information and Supplementary Figs. 2b and 3b for details).

Discussion

For the past decades, several authors have suggested the existence of long-range sex pheromones in the attraction of *An. gambiae* s.l. females to male swarms^{11,31,37,63} but so far, no reliable experimental evidence is available. Here, we used behavioural, physiological and detailed chemical approaches to attempt to highlight the existence of such compounds. However, despite a large set of methodologies and working hypotheses to maximize the chances of highlighting the presence of pheromones (that is, timing, physiological state, natural behaviour, recently colonized mosquitoes), our study failed to provide evidence of the presence of long-range male sex pheromones in both *An. coluzzii* and *An. gambiae*. We also replicated an experimental set-up that recently showed an increase in quantity of five compounds in ‘swarming’ males compared to controls. However, unlike in Mozūraitis et al.⁴⁴, we did not find any significant difference with our controls.

During our behavioural tests in the olfactometer, females were not attracted to air currents passed over male swarms regardless of the species. During the experiment, the males released in the large plexiglass boxes and exposed to natural light started to fly randomly at sunset. Then, about 150–200 males out of the 500 released (<50%) gathered in the upper half of the box flying in loops without touching the box walls. They also reacted to the movements of the visual marker, meaning they were indeed swarms. The other males flew randomly in the box, bouncing against the walls. Females showed a good activation rate but they did not show a particular choice for any arm, containing a swarm or not. Nevertheless, *An. gambiae* females showed an intriguing higher activation rate when exposed simultaneously to both a conspecific and a hetero-specific swarm compared to a single *An. coluzzii* swarm. This was, however, probably without biological merit as no difference was detected in the test providing *An. gambiae* females with a choice between the control and *An. gambiae* swarm, versus a test offering two controls.

Our behavioural result was consistent with our physiological study. Indeed, despite the fact that we checked for both behavioural responsiveness of mosquitoes and for receptivity of mounted antennae, no consistent antennal depolarization was observed in females across assays when exposed to swarm extracts. This suggests a lack of response by females to any volatile chemicals present in our swarm extracts.

Chemical analyses were also negative and no compounds specific for male swarms could be detected whatever the method used. Moreover, unlike in Mozūraitis et al.⁴⁴, acetoin, sulcatone, octanal, nonanal and decanal were found inconsistently across swarming mosquito samples. When they were found, their quantities were not larger in mosquito extracts compared to controls, making it difficult to support the assumption according to which they could actually be emitted by males. These divergent results could be explained by the fact that, unlike in the experiments of Mozūraitis et al.⁴⁴, we used a control that considered the potential introduction of pollutions at the same time as mosquitoes. This showed that breath was responsible for most of the variability. The most convincing result showing that sulcatone, octanal, nonanal and decanal are probably not pheromones emitted by males is the one obtained with the twister method. Indeed, twisters are coated with the same adsorbent phases as SPME fibres used both in this study and in the one by Mozūraitis et al.⁴⁴. According to the manufacturer, twisters are up to 1,000 times more sensitive than SPME fibres partly due to a larger sorbent volume. Moreover, we exposed the twisters directly in a natural swarm formed by more than 1,000 males (probably up to 6,000 males according to the estimation of trained technical staffs). Despite this sensitivity, the number of mosquitoes and the natural biological context, sulcatone and octanal were not found and both nonanal and decanal had similar quantities in the swarm and outside the swarm (control twister placed 3 m upwind from the swarm). In addition, the high quantitative variability found in the laboratory and the fact that these compounds are frequently found in controls suggest that they could be laboratory and/or human pollutions that are difficult to control for.

Our results contrast with those of Mozūraitis et al.⁴⁴ probably for several reasons. First, in their laboratory experiments, they reported a simple flight activity instead of a swarming activity in which males should fly in regular loops with erected antennae^{3,31}. This can be explained by the absence of adequate visual stimuli such as ground markers that are mandatory to trigger swarming behaviour^{9,10,31,64–66}. Second, this flight activity was extraordinary long for a swarming flight, exceeding 200 min in the presence of the five compounds. Indeed, swarming behaviour is known to last for only 20–30 min in nature^{9,10,14,20,67} and only up to 60 min in artificial conditions^{9,31}. In the latter, the number of males in swarms decreased over time while the others flew randomly, bouncing on the flight arena walls³¹. Indeed, swarming is an activity with a high energy demand, consuming 50% of sugar and glycogen reserves in 25 min (refs. 68,69). Mosquitoes probably switched to a more random flight to try to escape and search for a sugar meal to refuel their reserves. This behaviour can be stimulated by acetoin, sulcatone, octanal, nonanal and decanal, as they are frequently emitted from nectar sources and fermented sugar^{56,70,71}. Finally, they also reported that these five

Fig. 5 | Quantities of sulcatone, octanal, nonanal and decanal collected with Twistlers, Tenax/Carbotrap or Porapack tubes. **a–c**, Quantities (integration units) contained in a natural swarm sampled with twistlers (**a**); in the headspace of the empty box, swarming *An. coluzzii* males and *An. gambiae* males sampled with Tenax/Carbotrap tubes (**b**) or collected with Porapack tubes (**c**). The intensity values correspond to the counts related to the abundance of the specific ions representative of each molecule formed in the mass spectrometer and correspond to the amount of compound analysed. The box plots indicate the median (wide horizontal bars), the 25th and 75th percentiles (squares), and the minimum and maximum values (whiskers). The black dots represent outliers and grey dots the raw data. For the statistical analyses, Gaussian generalized linear-mixed models were used considering replicates as random effect. When necessary, a multiple comparison was done using Tukey’s all-pair comparison method. NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NA, the analytical procedure for these analyses did not allow detection of acetoin. $n = 5$ biologically independent samples (with more than 1,000 mosquitoes used per sample in **a**), $n = 5$ or 6 (with 500 mosquitoes used per sample in **b**) and $n = 7$ or 8 (with 500 mosquitoes used per sample in **c**).

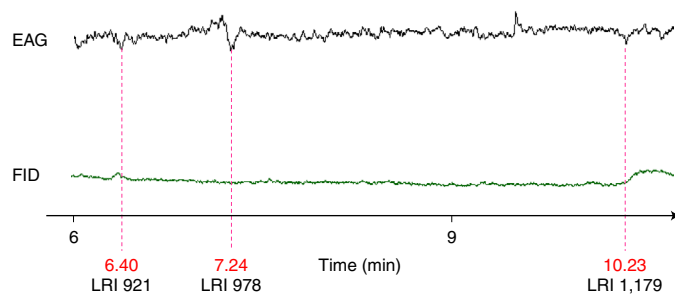


Fig. 6 | Mean electrophysiological responses of female *An. coluzzii* antennae to male swarm solvent extracts, recorded by GC-EAD. Dashed lines indicate retention times (and related LRI) for which the most significant EAG responses were recorded. Only two females in eight tested produced this pattern. EAG, electroantennogram; FID, flame ionization detector.

compounds increased insemination rates in five different species in semifield cages. However, instead of stopping the experiment after the swarming time, they collected the females in the morning. As they left both a dark box in the arena as resting site and a sugar source, it is likely that males and females also mated in or around these two resources during the night. Indeed, it was shown that mating can occur indoors, outside swarming times and locations³³. Moreover, because some of the five compounds can be related to nectar odours, they could have been attractive after the swarming time, gathering mosquitoes in a hotspot and thus inducing more inseminations than in the control.

Independent from this, our methods can also have weaknesses. Even if the number of swarming males was biologically relevant compared to natural swarms, a higher number of males might have been required in experimental set-ups or the sensitivity thresholds of our chemical and electrophysiological apparatus might be below the ability of insects to detect low amounts of compounds. In addition, although the air flow used in our VOC collection in laboratory was not unconventional, it might have been too high inducing a leak of compounds of interest. Likewise, the air quality could have masked some compounds emitted in minute quantities even though our behavioural experiment and VOC collection in natural swarms led to negative results too.

Altogether and keeping in mind that an absence of evidence is not an evidence of absence, our results support the absence of long-range sex pheromones emitted by male swarms. However, further investigations and complementary methodologies are needed. Electrophysiological analyses on palps^{72–75}, molecular analyses of olfactory protein expression on adequate physiological status or real-time chemical analyses of swarm volatiles with a proton-transfer-reaction mass spectrometry could be options. The eventuality that organic compounds with low volatility might be involved in mating process other than long-range attraction cannot be discarded.

The question of how *Anopheles* females seek male swarms is still open. The lack of post-mating barrier^{21–25} and the low rate of hybrids in this geographical area^{15–18} necessarily involve a reasonably distant process that prevents females from entering heterospecific swarms. As acoustic cues have shown some limitations at long range³⁰, and because long-range chemical cues are still disputable, visual cues such as ground markers^{13,14,31} could be good candidates, but their effective range and level of specificity is poorly known.

Methods

Mosquitoes. Mosquitoes used in behavioural experiments and for both VOC collection and electrophysiological analyses were from colonies raised from wild gravid females collected in inhabited human dwellings in Burkina Faso

(West Africa). *Anopheles coluzzii* were collected in 2017 in Bama and *An. gambiae* in 2015 in Soumouso. Bama is a village located in a rice-growing area located 30 km North of Bobo-Dioulasso (11° 24' 14" N; 04° 24' 42" W) where previous studies showed that the *Anopheles* population is almost exclusively composed of *An. coluzzii*^{6,77}. Soumouso is a typical Guinean savannah village located 30 km North-East of Bobo-Dioulasso (11° 00' 46" N, 4° 02' 45" W) where *An. coluzzii* and *An. gambiae* coexist, with a predominance of the latter^{18,78}. Gravid females were placed individually in oviposition cups containing tap water. After oviposition, females were identified to species by routine PCR-RFLP⁷⁹. The larvae were gathered according to their species and reared in tap water, fed with Tetramin Baby Fish Food (Tetrawerke) ad libitum. Adult mosquitoes were held in 30 × 30 × 30 cm³ mesh-covered cages and provided with a 5% glucose solution ad libitum. Insectarium conditions were maintained at 27 ± 2 °C, 70 ± 10% relative humidity and 12 h light/12 h dark. The colonies were refreshed twice a year with F1 from mosquito females caught in the wild.

Mosquitoes used in the electrophysiological study were transferred as eggs from Burkina Faso to France. On their arrival, eggs were allowed to hatch in osmoted water and the larvae were fed with Tetramin Baby Fish Food ad libitum. Adult mosquitoes were held in 20 × 20 × 20 cm³ mesh-covered cages and provided with honey diluted at 5% ad libitum. Females were fed with rabbit blood on a PS6 Power Unit (Hemotek) for egg production. Mosquitoes were reared in a laboratory climate chamber KBF-S720 (BINDER GmbH) at 27 ± 2 °C, 70 ± 10% relative humidity and 12 h light/12 h dark with a sunset time programmed at 15:00 (synchronization of swarming/mating time and electrophysiological test time to ensure an optimal receptivity of females). Mosquitoes used for the experiments were sexed early after emergence and sexes were kept in separate rearing cages to prevent mating.

Mosquitoes used to replicate the experiment by Mozūraitis et al.⁴⁴ were from a 15-year-old colony of *An. gambiae* (Kisumu strain) reared in the IRD laboratory of Montpellier. They were maintained at 27 ± 2 °C, 80 ± 10% relative humidity, with a photoperiod cycle of 12 h light/12 h dark and reared as described above.

Long-range behavioural response of virgin females to swarm VOCs. Test preparation. Bioassays were conducted in a dual-port olfactometer originally designed to study host preference in the *An. gambiae* complex^{80–83} (Fig. 1). In the morning, about 8 h before the time of the test, 100 2–3-day-old virgin females of *An. coluzzii* and *An. gambiae* were coloured with two different coloured powders (Luminous Powder Kit, BioQuip) according to the species. In addition, about 500 4–5-day-old *An. coluzzii* or *An. gambiae* males were released into a swarming box to allow them to acclimate. Both males and females were provided with a 10% glucose solution and were kept under insectary conditions until the time of the test. To avoid biases during the test due to both humidity and odour as a result of manipulation or bacterial proliferation, the empty swarming box (control box) was also provided with glucose.

Test execution. Bioassays were carried out at sunset, when males naturally swarm. About 30 min before sunset time, the swarming boxes were moved outside, the cups containing the glucose pads were removed and visual markers made of a 20 × 20 cm² black cloth were placed either under or next to the boxes for *An. coluzzii* or *An. gambiae*, respectively (ref. ³¹). These markers allowed to trigger swarming behaviour and to stabilize swarms in the middle of the box. For the same purpose, we provided an artificial twilight horizon made of a 40 W incandescent bulb (2,500 K) located on the floor between a white wall and a 50 cm high black horizon^{31,64,65}. Inside, about 10 min before introducing females, the fans were switched on to purge the air vent hoses and the air flow was set at 18 ± 2 cm s⁻¹. The glass tube openings on the releasing cage side were covered with nets to prevent female mosquitoes from flying into the collecting boxes before the test began. The complete olfactometer, except the end of the releasing cage, was covered with a white cloth to eliminate visual bias during the test and to provide a diffused light. Sunset light was allowed to enter the olfactometer room until dark and an additional artificial twilight made of a 40 W incandescent bulb (2,500 K) projected on the wall of the room on the side of the collecting boxes was also provided. Thirty minutes before sunset, about 100 females of each species ($n = 200$, one replicate) were released simultaneously into the releasing cage of the olfactometer allowing them to acclimate.

When the males started to swarm (that is, flying in loops at a constant location within the swarming boxes^{3,31}), the nets covering the glass tube openings and preventing access of females to the collecting boxes were removed via threads that were attached to the nets. The females were allowed to respond for 20 min (swarm duration), then the glass tube openings were covered again with nets and females that reached the upwind collecting boxes were removed with a mouth aspirator, killed by freezing and identified according to their coloration (species) under a binocular microscope (LEICA S6E) and counted. The remaining mosquitoes inside the releasing cage were also removed. After each test (one per day), the swarming boxes were disconnected from the olfactometer and the air flows were increased up to 50 cm s⁻¹ for 1 h to purge the air vent hoses and the olfactometer. Then the olfactometer was cleaned with 95% ethanol to remove odour contaminants. All materials were handled with gloves to avoid contamination with skin compounds.

Test combinations. Four combinations of choice tests were performed: (1) *An. coluzzii* male swarm versus empty box (hereafter, *An. coluzzii* test), (2) *An. gambiae* male swarm versus empty box (hereafter, *An. gambiae* test), (3) *An. coluzzii* versus *An. gambiae* male swarms (hereafter, *An. coluzzii* versus *An. coluzzii* test) and (4) empty versus empty box (hereafter, control test). We ran 12 replicates per combination and females were used only once. Males were used twice in two consecutive days. In that case, a 10% glucose solution was introduced into the two swarming boxes after the test and boxes were kept under insectarium conditions until the next day. The swarming boxes were cleaned with ethanol between each batch of males. To avoid biases, the matching of species and colours was switched between each test, and we alternated the treatments (mosquitoes or control) between the swarming boxes and the right and left arm of the olfactometer. We assessed the instrumental and arm bias through an empty versus empty box choice test (control test). The olfactometer did not present any symmetrical biases (Supplementary Fig. 1).

Collection of swarm VOCs. Because compounds can be emitted at a given time and only produced in tiny quantities at individual scale, we chose to work directly on swarms. Three different sampling methods were used; two methods were run in a laboratory set-up⁶⁵ and one in the field.

Dynamic headspace collection in the laboratory. VOC collection set-up. The collection of swarm VOCs was performed in a room specially designed to stimulate *Anopheles* swarming behaviour via a set of visual stimuli (see ref. ⁶⁵ for details). The headspace volume consisted of a 50 × 50 × 50 cm³ transparent plexiglass box (extraction box). Such a volume was a trade-off between a volume large enough to allow males to display a swarming behaviour without too much constraints and a volume small enough to be sucked up in a reasonable time. Entrance and exit flow rates were maintained by two pumps and regulated by flowmeters. The entrance flow was higher than the exit flow ensuring that the system was continuously purged to compensate for the inevitable leaks, and that no contaminated outside air would enter the system. At the entrance side, ambient air was purified in a glass charcoal filter and then humidified passing through deionized water. Exit flow passed through an odour trap to adsorb VOCs. Three different odour traps were used for the chemical analyses and electrophysiological analyses. Tenax-TA/Carbotrap sorbent stainless steel 6 mm diameter tubes (80 and 40 mg, respectively, Gerstel) and Porapak-Q sorbent VCT glass tubes (30 mg of Porapak, ARS) were used for chemical analyses. Porapak-Q sorbent VCT tubes and home-made sorbent Microtraps were used for electrophysiological analysis. Microtraps were constituted by ChromatoProbe quartz microvials of Varian Inc. (length 15 mm; inner diameter 2 mm), previously cut closed-end and filled with 3 mg of a 1:1 mix of Tenax-TA and Carbotrap (60–80 and 20–40 mesh, respectively; Sigma Aldrich, ref. ⁴⁸). Flow rates and VOC collection durations were dependent on the odour trap (Supplementary Table 1). VOC collection was performed under constant temperature and humidity (27 ± 1 °C and 70 ± 5% relative humidity, respectively).

VOC collection. About 500 4–6-day-old males of *An. gambiae* or *An. coluzzii* were transferred into the extraction box about 30 min before swarming time. Clean air was pushed into the box at a rate of 10 l min⁻¹ to purge the box from both human and mosquito container odours before connecting it to the odour trap. Five minutes after males started to swarm (that is, flying in loops at a constant location within the box), the air flow was adjusted at the required rate and the odour trap connected. Forty minutes later, swarming behaviour was stopped by turning off the light and the VOC collection continued until the required collection time was reached (Supplementary Table 1). After VOC collection, samples were stored at 4 °C until analysis. After males were removed, the box was cleaned with 95% ethanol and then flushed with clean air for at least 5 h to remove the odour contaminants left from the previous extraction. A total of 30 swarm extracts were collected; 17 *An. coluzzii* swarms (five, eight and four with Tenax/Carbotrap tubes, Porapak-Q VCT tubes and Microtraps, respectively) and 13 *An. gambiae* swarms (six and seven with Tenax/Carbotrap tubes, and Porapak-Q VCT tubes, respectively). Control consisted of a clean empty box. Before they were sent and used in Burkina Faso, adsorbents were cleaned as follows. Tenax-TA/Carbotrap tubes were heated at 250 °C for 30 min under a 30 ml min⁻¹ flow then sealed. Porapak-Q VCT tubes were eluted with 3 ml of hexane then packed individually in a nalophan bag. Chromatoprobe tubes were heated for 2 h at 270 °C by 100 chromatoprobe tubes under a 100 ml min⁻¹ nitrogen flow, then packed in a glass vial with a Teflon cap.

VOC collection from natural swarms. VOCs were collected from a natural swarm of *An. coluzzii* in the village of Bama. Static sorptive collections of volatiles from a non-enclosed swarm were performed with Twisters (100 µm PDMS stir bar, Gerstel^{44,65}). The swarm flew about 2.5 to 3.5 m above the ground. Thus, we used a 1.5 m long glass stick (Glaswarenfabrik Karl Hecht GmbH & Co.) with a metallic push pin head glued at the tip and covered with nalophan to introduce the magnetic twisters directly into the swarm. Control twisters were placed about 3 m away upwind from the swarm. VOC collection lasted for the whole swarm duration (about 20–25 min). We chose a swarm far from habitations and livestock to limit odour pollutions, containing a large number of males (more than 1,000)

and attractive for females (observation of a large number of couples). After VOC collections, twisters were individually placed into glass vials and stored at 4 °C until analysis. Five replicates were performed on the same swarm on different days.

Solvent extraction. About 1,000 4–6-day-old males of *An. gambiae* or *An. coluzzii* were introduced into a 30 × 30 × 30 cm³ cage and kept under insectarium conditions. The cage was made of unpainted metallic frames covered by white nets and the bottom was covered with a white paper. Thirty minutes before sunset time, the cage was placed outside and mosquitoes were observed. A 5 × 5 cm² black cloth was placed in or out of the cage according to the species as described above to stimulate swarming behaviour. About 5 min after mosquitoes started to swarm, the entire cage was quickly placed into a freezer at –20 °C for about 10 min. Then, mosquitoes were transferred into a 20-ml glass tube and covered with a 1:1 mix of hexane and dichloromethane. The tube was sealed and mosquitoes were kept in the solution for 24 h at 27 ± 2 °C. Then, the solution was filtered on glass wool to remove mosquito scales and stored at 4 °C until analysis. Three different controls were made: (1) with 1–2-day-old virgin males (young male control), (2) with 2–3-day-old virgin females (female control) and (3) with solvent mix alone (blank control). A total of eight swarm extracts were done; four of *An. coluzzii* and four of *An. gambiae* and one replicate of each type of control per species.

VOC collection with SPME fibre. We replicated the experiment from the study by Mozūraitis et al.⁴⁴ in which they collected mosquito headspace with a SPME fibre in a 1-l bottle. However, because most methods used to introduce mosquitoes into a bottle may potentially result in entering pollutants as well, we added a supplementary control. One way to easily introduce the mosquitoes is to blow them from a mouth aspirator into the bottle. This classic method may bring breath volatiles within the recipient, which can contain the five compounds described in Mozūraitis et al.^{44,52–55}. Consequently, we added a 'breath control'. In addition, we also replicated the experiment with females to check for sex specificity. In the morning, the two glass bottles were cleaned with acetone and dried under a clean air flow, then closed with nalophan. Three hours before swarming time (17:00), we collected the headspace of empty bottles with polydimethylsiloxane/divinylbenzene-coated SPME fibres for 1 h (step 1, empty bottle). Then, we simulated the introduction of mosquitoes by blowing three times for 3 s with a mouth aspirator into the bottles. These were then closed with nalophan (18:00) and the headspace of the breath of the experimenter was collected for 1 h with SPME fibres (step 2, breath control). We then introduced 60–70, 5–7-day-old unmated males in each bottle with the mouth aspirator, closed the bottles with nalophan (19:00) and let the mosquitoes acclimate for 1 h. During the acclimation period, we simulated a light transition from photophase to scotophase to trigger crepuscular flight. Then, when males started to fly (20:00), SPME fibres were introduced for 1 h (step 3, 'swarm' extract). Six different fibres were used and cleaned at 250 °C during 5 min in the injector of a gas chromatograph (HP 6890 Series PLUS, Agilent) just before use. Each fibre was always used for the same type of extract and in the same bottle. Before the experiment, we made sure that the fibres had similar sensitivity by exposing them to a mix of the five compounds. Only minor quantitative differences were detected, and this was considered during subsequent statistics. Second, we performed the same three steps but with 60–70, 3–4-day-old virgin females instead of males. Finally, as during the introduction of mosquitoes, we blew a second time into the bottles and repeated the three steps of headspace collection but this time, instead of introducing mosquitoes in the third step, we blew again into the bottles (breath ×2) to check for potential accumulation of breath compounds. We kept track of the day of the experiment, the bottles and fibre identities for statistical analyses.

Chemical analyses. Samples obtained on Porapak-Q VCT tubes were eluted with 150 µl of Hexane and injected (1 µl) into a GC–MS. Those obtained on Tenax/Carbotrap tubes, twisters or SPME fibres were directly thermo-desorbed into GC–MS. Both solvent and eluted Porapak extracts were injected as was and after concentration under a gentle stream of nitrogen. All the analyses were run at the Platform for Chemical Analyses in Ecology (PACE), the technical facilities of the LabEx CeMEB (Centre Méditerranéen pour l'Environnement et la Biodiversité, Montpellier, France).

Liquid analysis (Porapak elutions and solvent extractions). Liquids were analysed with a GC–MS QP2010 Plus (Shimadzu). Mass spectra were recorded in electronic impact mode at 70 eV over a *m/z* mass range from 38 to 350. The temperature of the transfer line and the ion source were programmed to 250 and 200 °C, respectively. The injections were made with an injector temperature of 250 °C, and a 1:4 split mode ratio. Analyses were performed using a 30 m × 0.25 mm × 0.25 µm Optima 5-MS (Macherey-Nagel) fused silica capillary column with a constant helium flow set close to 1 ml min⁻¹. The oven temperature was programmed as follows: 40 °C (held 5 min) to 250 °C at 6 °C min⁻¹, and finally to 300 °C at 14 °C min⁻¹ and held 2 min. GC–MS Solution software (Shimadzu) was used for data processing of these analyses, with the NIST 2011 as the spectrum database.

Tenax/Carbotrap tubes and twister analysis. These sorbents were analysed as described by Souto-Vilarós et al.⁸⁶. Samples were analysed using a gas

chromatograph (Trace 1310, Thermo Scientific) coupled with a mass spectrometer (ISQ QD Single Quadrupole, Thermo Scientific). The column used was an Optima 5-MS, the same as for liquid analysis. Absorbent traps were handled with a Multi Purpose Sampler (Gerstell) and desorbed with a double stage desorption system, composed of a Thermal Desorption Unit and a Cold Injection System (Gerstell). The sorbents were desorbed in splitless mode at 250 °C on a trap cooled at –80 °C by liquid nitrogen. Then, the trap was heated to 250 °C with a 1:4 split ratio to inject the compounds in the column. The carrier gas used was helium at 1 ml min⁻¹. Oven temperature programme was as follows: held at 40 °C for 3 min, then increased to 220 °C at 5 °C min⁻¹ and finally to 250 °C at 10 °C min⁻¹, and held for 2 min. Mass spectra were recorded in electronic impact mode at 70 eV over a *m/z* mass range from 38 to 350. The temperature of the transfer line and the ion source were programmed to 250 and 200 °C, respectively. Xcalibur software (Thermo Scientific) was used for data processing and the NIST 2011 as the spectrum database.

SPME fibre analysis. We analysed SPME fibres as described in Mozūraitis et al.⁴⁴ with a GC–MS QP2010-SE (Shimadzu). Volatiles were desorbed into the injector at 225 °C for 1 min in split mode (1:4 ratio). Analyses were performed using a 30 m × 0.25 mm × 0.25 µm J&W DB-Wax silica capillary column (Agilent) with a constant helium flow at 1 ml min⁻¹. The oven temperature was programmed as follows: 40 °C (held 1 min) to 150 °C at 5 °C min⁻¹, and finally to 220 °C at 20 °C min⁻¹ and held 9 min. Mass spectra were recorded in electronic impact mode at 70 eV over a *m/z* mass range from 38 to 350. The temperature of the transfer line and the ion source were 250 and 200 °C, respectively. GC–MS Solution software (Shimadzu) was used for data processing of these analyses.

Electrophysiological analysis. Electrophysiological activity of female olfactory detection, on the male swarm extracts, was tested with a GC–EAD at the PACE. Thirty minutes before the mosquito scotophase, both 4–6-day-old males and 2–3-day-old virgin females were placed separately in cardboard cups (Ø = 75 mm, *h* = 100 mm), provided with a 5% honey solution and transferred from the insectarium to the electrophysiology laboratory at PACE. At mating time, mosquitoes were placed in the dark. Males were checked for erected antennae as a proxy to ensure that change in the light/dark cycle, transport and electrophysiology laboratory conditions did not alter mosquito motivation for mating. Two minutes after male antennae were erected, a female was removed from the cardboard for the antennal preparation.

The female's head was gently excised with a pair of surgical scissors and mounted between two glass capillary tubes of 76 mm in length and 1.12 mm in diameter (World Precision Instrument), pulled and cut using a vertical micropipette-puller (P-30 model, World Precision Instruments) filled with insect Ringer solution (6.0 g l⁻¹ NaCl, 0.4 g l⁻¹ KCl, 0.27 g l⁻¹ CaCl₂ and 3.20 g l⁻¹ Na₂H₂O₄) and connected to silver wires. The indifferent electrode was inserted into the back (the foramen) of the isolated head and the intact tip of one antenna was inserted into the recording electrode that was connected to an EAD set-up (Syntech IDAC-2). One end of the glass electrodes was pulled to a fine point that was then broken to ensure a close fit with the female's head and antenna.

The above EAD set-up was linked to a GC–flame ionization detector (CP-3800, Varian) equipped with an Optima 5-MS capillary column, the same as for the GC–MS analyses. Liquid injections after Porapak solvent elution were made in a 1079 PTV injector (Programmed Temperature Vaporizer) at 250 °C with a 1:4 split ratio. Microtraps were desorbed in the same injector using a ChromatoProbe sample introduction device (Varian) with the same split ratio and temperature programmed as follows: increased from 40 to 250 °C at 200 °C min⁻¹ and held for 5 min. Oven temperature was held at 50 °C for 0.40 min, then increased to 180 °C at 10 °C min⁻¹ and finally to 220 °C at 12 °C min⁻¹, for a run duration less than 17 min. The carrier gas used was helium at 1 ml min⁻¹. The effluent was split equally into two deactivated fused silica capillary columns (100 cm × 0.25 mm), one leading to the flame ionization detector (270 °C) and the other into a heated EAD port (200 °C) (transfer line, Syntech) and led to antenna. The GC effluent for antenna was mixed with charcoal-filtered, humidified air flow (300 ml min⁻¹). StarWorkstation v.6.41 software (Varian) was used for data processing.

Different types of male swarm extract were tested on the antennae of several conspecific females. Antennae of 19 *An. coluzzii* females were tested with solvent extracts, Porapak-Q VCT tube extracts and Micro-trap extracts (eight, nine and two females, respectively) and 20 *An. gambiae* females were tested with solvent and Porapak-Q VCT tube extracts (ten females each). Before GC–EAD analysis of the VOC extracts, we checked the living antenna for good activity using a negative (solvent) and a positive (mix of many VOCs) stimulus solutions. We proceeded as follows: 5 µl of each solution was applied to a 1 × 2 cm² filter paper contained in a Pasteur pipette. The pipette was then placed in an air pump system and the volatiles were directly sent to the antennal preparation with a pulse duration of 0.5 s and a flow rate of 890 ml min⁻¹ regulated by a CS-55 Stimulus Controller (Syntech).

Identification of specific and active VOCs. For all the chemical analysis, we compared the chromatograms of swarming mosquito extracts with those of their corresponding controls to search for any qualitative or quantitative differences. When such a difference was pinpointed, we matched the mass spectra of the

compounds of interest with the NIST 2011 MS library via built-in algorithms. A solution of *n*-alkanes (alkanes standard solution, 04070, Sigma Aldrich) was also injected to calculate the LRI ±10 error points (LRI) of these compounds of interest⁸⁷ and their LRIs were compared with those reported in the literature and databases (PUBCHEM, ADAMS, FLAVORNET, PHEROBASE and mVOC2.0).

In the electrophysiological analysis, we studied the chromatograms in a range of LRI between 800 and 1,700, including compounds present at very low and trace amounts. When a depolarization was observed in the electrophysiological analysis, a section of 20 points of LRI was studied around the compound's LRI on the GC–MS trace. Comparison of the samples with their respective controls collected on the same day, allowed to subtract potential contaminants from the samples. Non-natural compounds, such as industrial chemicals and/or compounds not naturally produced by living organisms⁸⁸, were considered as contaminants.

We also particularly searched for the presence of acetoin, sulcatone, octanal, nonanal and decanal reported by Mozūraitis et al.⁴⁴. With that aim, we defined a LRI window of 20 points on both sides of the LRI of these compounds on the bases of injected standards (for SPME fibres; acetoin, sulcatone, octanal, nonanal and decanal, ≥98, 99, 99, ≥98 and ≥98% GC, respectively, Sigma Aldrich) or literature (for other extraction methods). LRIs for the five compounds were: 711, 981, 998, 1,100 and 1,201 on the Optima 5-MS column and 1,272 to 1,295, 1,319 to 1,365, 1,307, 1,402 and 1,447 on the DB-Wax column. Then, we searched for their specific ions at those LRI as follows: 88, 108, 84, 98 and 112 for acetoin, sulcatone, octanal, nonanal and decanal, respectively. Then, when a peak corresponding to both LRI and specific ion was found, we compared its spectra with the NIST 2011 MS library before quantification with integration units.

Statistical analysis. Both the activation rate and choice were analysed using binomial generalized linear-mixed models ('glmer' function in 'lme4' package). Tested combinations (four levels: *An. coluzzii*, *An. gambiae*, *An. coluzzii* versus *An. gambiae* and control tests), female species (two levels: *An. coluzzii* and *An. gambiae*) and their interaction were considered as fixed effects, and replicates as random effects. All analyses were performed using R (v.3.4.0).

We analysed separately the quantities using integration units of acetoin, sulcatone, octanal, nonanal and decanal. When pairing between the controls and the mosquito extracts was possible (with SPME and twister) and to account for repeated measurements, we used Gaussian generalized linear-mixed models. In those cases, the extract (three levels for SPME fibres: empty bottles, breath and flying mosquitoes (either male or females or breath ×2) and two levels for twisters: control and swarm) was considered as fixed effect. The replicates (that is the day for twisters and the SPME) and fibre nested within the bottle for SPME fibres were considered random effects. Considering the SPME fibres as a random effect made it possible to account for their minor differences of sensitivity in the model. When pairing between the controls and the mosquito extracts was not possible (Tenax-TA/Carbotrap and Porapak-Q VCT tubes), Gaussian generalized linear-mixed models were used. The extract (empty box, *An. coluzzii* swarm or *An. gambiae* swarm) was considered as fixed effect. In some occasions, some compounds were not found in the extracts or only in two samples. Consequently, it was not possible to perform statistical analyses and we considered the extracts as not significantly different. No statistical analysis was carried out with liquid extracts because of the small number of replicates. For model selection, we used the stepwise removal of terms, followed by likelihood ratio tests. Term removals that significantly reduced explanatory power (*P* < 0.05) were retained in the minimal adequate model. All percentages are provided with their 95% confidence interval.

Ethical approval. The use of rabbit blood was approved by the Ministry of Higher Education, Research and Innovation under the registration no. APAFIS#13817-2018022712203932 v.4.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw datasets are available online at <https://doi.org/10.5281/zenodo.4719568>.

Code availability

Script and codes are available online at <https://doi.org/10.5281/zenodo.4719568>.

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Author contributions

O.R. conceived the study. O.R., S.B.P., B.B., B.L. and L.D. designed the chemical and electrophysiological experiments. O.R. and S.B.P. performed chemical extractions. S.B.P. and B.B. performed the chemical analysis. S.B.P. and B.L. performed the electrophysiological experiments. O.R. and S.B.P. designed olfactometric experiments and S.B.P. performed data collection. S.B.P. and O.R. performed statistical analyses. S.B.P., O.R., B.B. and B.L. drafted the manuscript and L.D., O.G., A.D. and R.K.D. critically revised the manuscript. All authors revised the manuscript, gave final approval for publication and are accountable for the work performed therein.

Competing interests

The authors declare no competing interests.

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Data collection GC-MS Solution software (Shimadzu, Kyoto, Japan) and Xcalibur™ software (Thermo Scientific™, Milan, Italy) were used for data processing of the GC-MS analyses. The NIST 2011 was used as spectrum database. StarWorkstation 6.41 software (Varian, Palo Alto, USA) was used for data processing of the GC-EAD analyses.

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Sample size	For the bioassays, 200 females and about 500 males were used for each replicate and 12 replicates per combination (4 combinations) were performed. For the collection of Volatile Organic Compounds (VOCs) from swarm in the laboratory, about 500 males (dynamic headspace collection) or 1,000 males (solvent extraction) were used for each replicate and a total of 30 swarm extracts (17 for <i>An. coluzzii</i> and 13 for <i>An. gambiae</i>) were analyzed. For the extraction of VOC with SPME fibers, 60-70 males or female were used for each replicate and 6-7 replicates were performed for each treatment (swarm extract, empty control, breath control and female control). For the collection of VOC's from a natural swarm in the field, a swarm of more than 1,000 males was used and 5 replicates were performed on different days (5 swarms and their respective controls). No sample size calculation was performed. Numbers of samples were determined based on maximum feasibility and previous experience.
Data exclusions	No data were excluded from the analyses.
Replication	see "sample size" section above
Randomization	Mosquitoes used in the experiments were collected from large colonies and randomly allocated to the different treatments. The natural swarm used for VOC collection in the field was selected on the basis of its size (>1 000 males) and because numerous mating events were observed (attractive for females).
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Laboratory animals	Recent (less than one-year-old) colonies of <i>Anopheles coluzzii</i> and <i>Anopheles gambiae</i> from Burkina Faso (West Africa) were used for bioassays and VOC collection. A 15-year-old colony of <i>Anopheles gambiae</i> (Kisumu strain) was used for VOC collection with SPME. 2- to 3-day-old virgin females and 4- to 6-day-old males were used.
Wild animals	VOCs from a wild <i>Anopheles coluzzii</i> swarm were collected in Burkina Faso. A Twister (stir bar coated with an adsorbant phase) was placed into the swarm for 20-25 min.
Field-collected samples	No field-collected animal was involved in our study
Ethics oversight	No ethical approval was required for our study. Colonized and field mosquitoes are not yet under our institutional ethics committee

oversight. The use of rabbit blood to feed mosquitoes was approved by the Ministry of Higher Education, Research and Innovation under the registration No. APAFIS#13817-2018022712203932 v4.

Note that full information on the approval of the study protocol must also be provided in the manuscript.