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

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Abstract

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 behavior have been debated in literature but to date, 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 favor, we used different chemical ecology methods such as behavioral and electrophysiological assays as well chemical analyses, and we worked with mosquitoes at their optimal physiological mating state *i.e.* 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.

Keywords: *Anopheles*, Male swarm, Mating behavior, Mosquito control.

1. Introduction

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 individual aggregations called "mating swarms" [3–7]. In malaria mosquitoes of the *Anopheles gambiae* complex, mating takes place at sunset in monospecific swarms made of 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 [15–17] but they form distinct swarms spatially segregated [13,14] and hybrids are rare (≈1%) [15,18–20]. No evidence for selection against hybrids was found [21–25] and spermatheca analyses showed that these species mostly mate assortatively [20]. This suggests that reproductive isolation between these two sibling species is achieved by strong pre-mating reproductive barriers [18,23,26]. In addition, since females usually only mate once in their lives [27], errors in the choice of mate should be costly and fall under negative selection. Consequently, 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 have a role in bringing sexes together. Acoustic cues have been 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, mosquitoes are often assumed to only hear over short distances [30] and males are more sensitive than females with males flying toward a source of female flight tones [28,31–33]. Visual cues also play an important role in species segregation with *An. coluzzii* males swarming over contrasted visual markers and *An. gambiae* males swarming over bare ground [13]. However, 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 marker [34]. Moreover, females also used these markers to form swarms in the absence of males suggesting that females may use these markers to join the swarm location [34]. Nevertheless, the distance between two heterospecific swarms using the same marker is about 2 m in semi-field conditions [34] and it is still unknown from which distance mosquitoes can see these markers, suggesting that females could cross the location of heterospecific swarms by accident. Chemical cues have also been under investigation in the *An. gambiae* complex. Cuticular hydrocarbons analyses have shown that their composition allow to discriminate species of the complex on quantitative bases [35–38] and Polerstock et al. [39] found quantitative differences between virgin and mated females (but see [38,40]). These compounds, however, can only be perceived at very short range or by contact and are consequently, at best, involved in mate recognition during courtship or indicators of female receptivity [39,41]. Moreover, the absence of assortative mating in confined heterospecific males and females in laboratory cages or indoor swarms [42], 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 which 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. 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. Consequently, males should be the ones that emit long-range pheromones and should be the attractive sex. Nevertheless, a laboratory study failed to demonstrate any attractiveness of dead males to virgin females in a Y-tube olfactometer [43]. However, 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 has been shown that the larger the swarm, the greater the concentration of male-produced sex pheromones, and hence, the greater the chances for individual males to mate with a conspecific female [44,45]. Similarly, Diabaté et al. [46] showed that *An. coluzzii* females were more frequently attracted to large swarms, suggesting a potential additive effect of cues released by males. Males gathering in large swarms may increase their "detectability" in the female's olfactory landscape [6]. In addition, in many insects, pheromone release and receptivity have been shown to respond to diel rhythms or even strict time windows of only a few minutes or hours [47–50] or even to be dependent of the presence of conspecifics [51,52].

The discovery of sex pheromones in malaria mosquitoes would be a precious step toward the development of new control and monitoring strategies. Furthermore, it could help in designing sexually competitive mosquitoes in sterile insect and gene drive techniques. Here, we attempted to bring to light the existence of long-range sex pheromones in *An. gambiae* and *An. coluzzii* mosquitoes, that may allow the females to detect, recognize and track conspecific male swarms. On the hypothesis that such pheromones could be produced only by males during swarming windows and to put all the odds in our favor, we used different chemical ecology methods always with living swarming mosquitoes during their natural swarming windows.

2. Material and Methods

Mosquitoes

Colonies originated from wild gravid females collected in inhabited human dwellings from Burkina Faso, in 2017 in Bama for *An. coluzzii* and in 2015 in Soumousso for *An. gambiae*. 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* [53,54]. Soumousso 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 [15,55]. Gravid females were placed individually in oviposition cups containing tap water. After oviposition, females were identified to species by routine PCR-RFLP [56]. The larvae were gathered according to their species and reared in tap water, fed with Tetramin® Baby Fish Food (Tetrawerke, Melle, Germany) *ad libitum*. Adult mosquitoes were held in 30 x 30 x 30 cm mesh-covered cages and provided with a 5% glucose solution *ad libitum*. Insectarium conditions were maintained at 27±2 °C, 70±10% RH and 12L:12D. The colonies were repeatedly refreshed 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 osmosed water and the larvae were fed with Tetramin® Baby Fish Food *ad libitum*. Adult mosquitoes were held in 20 x 20 x 20 cm mesh-covered cages and provided with honey diluted at 5% *ad libitum*. Females were fed with rabbit blood on a PS6 Power Unit (Hemotek, Blackburn, UK) for egg production. Mosquitoes were reared in a laboratory climate chamber KBF-S720 (BINDER Gmbh, Tuttlingen, Germany) at 27±2 °C, 70±10% RH and 12L:12D with a sunset time programmed at 3:00pm (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.

Behavioral assays

Olfactometer setup - Bioassays were conducted in a dual-port olfactometer originally designed to study host preference in the *An. gambiae* complex [57–60]. The odor source end was modified to enclose male swarms in boxes made of transparent plexiglass (LxWxH: 60x60x120cm; "swarming boxes" hereafter). Each swarming box was connected by a PVC air

vent hoses (L x Ø: 600 x 10 cm, W3-65014-HQ4, HQ, USA) to a collecting glass box (L x W x H: 32 x 33.5 x 44 cm) which was linked to a mesh-covered releasing cage (L x W x H: 50 x 40 x 40 cm) by a glass tube (L x Ø: 60 x 10 cm) (Fig. 1). A custom-made electric fan was located at the mid-length of each air vent hose and drew air from the swarming boxes (odor-sources) to the releasing cage, providing an odor laden air current against which mosquitoes in the releasing cage were induced to fly. The air flow was controlled thanks to two mechanisms; first, a power regulator (HQ-Power PS1502A, Velleman, Gavère, Belgium) connected to fans; and second, iris dampers (10 cm Ø; CIR D100, France air, France) 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 (odor-sources) were located side by side outdoors and the olfactometer inside a room (Fig. 1). The air speed in the releasing cage was regulated at 18±2 $cm.s^{-1}$ using an anemometer (Model 425, Testo, Forbach, France) and the room temperature and relative humidity were set at 27±2 °C and 80±10% RH thanks to an air conditioner and a humidifier (Defensor 505, Condair, Croissy-Beaubourg, France), respectively.

Bioassays **-** Bioassays were performed to study the long-range behavioral response of females to whole volatile organic compounds (VOCs) associated with male swarming activity. For each replicate, about 100 2- to 3-day-old virgin females of *An. coluzzii* and *An. gambiae* were used simultaneously. Females were colored the morning of the test with two different colored powders (Luminous Powder Kit, BioQuip, Rancho Dominguez, California, USA) according to species. The matching between the species and the colors was switched between each test. About 500 4- to 5-day-old *An. coluzzii* or *An. gambiae* males were released into a swarming box in the morning about 8 hours before the time of the test 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 odor bias during the test, the empty box (control) was also provided with glucose.

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 a visual marker made of a 20 x 20 cm black cloth were placed either under or next to the boxes for *An. coluzzii* or *An. gambiae*, respectively (see [34]). These markers allowed to trigger swarming behavior and helped 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 [34,61,62]. Inside, females of the two species were released in equal numbers into the releasing cage of the olfactometer allowing them to acclimate for 30 min before sunset. Previously, the fans were switched on in order to purge the air vent hoses and then 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 (2,500K) projected on the wall of the room on the side of the collecting boxes was also provided.

When the males started to swarm (*i.e.* flying in loops at a constant location within the swarming boxes, see [3,34]), the nets covering the glass tube openings and preventing access of females to the collecting boxes were removed thanks to 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 (LEICA S6E) and counted. The remaining mosquitoes inside the releasing cage were also removed. After each test (1 per day), the olfactometer was cleaned with 95% ethanol to remove odor contaminants. All materials were handled with gloves to avoid contamination with skin compounds.

Four combinations of choice tests were performed: i) *An. coluzzii* male swarm *vs.* empty box (hereafter, *An. coluzzii* test); ii) *An. gambiae* male swarm *vs.* empty box (hereafter, *An. gambiae* test), iii) *An. coluzzii vs. An. gambiae* male swarms (hereafter, *An. coluzzii vs. An. coluzzii* test) and iv) empty *vs.* empty box (hereafter, control test). Each combination was repeated 12 times 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. The treatments (mosquitoes or control) between the swarming boxes and the right and left arm of the olfactometer were alternated to avoid cage and side effects, respectively. We assessed the instrumental and side bias through an empty *vs.* empty box choice test (control test). The olfactometer did not present any symmetrical biases (Fig. 2).

Collection of the volatile organic compounds (VOCs), chemical and electrophysiological analysis

Because compounds can be emitted at a given time and only produced in tiny quantities by a single male, we chose to work directly on swarms. Three different sampling methods were used in Burkina Faso; two methods were run in a laboratory setup [62] and one in the field.

Dynamic headspace. The head space volume consisted of a 50 x 50 x 50 cm transparent plexiglass box. Such a volume was a trade-off between a volume large enough to allow males to display a swarming behavior 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, air was purified in a glass charcoal filter and then humidified passing through deionized water. Exit flow passed through an odor trap to adsorb VOCs. Three different odor traps were used for the chemical analyses and electrophysiological analyses. Tenax-TA/Carbotrap sorbent stainless steel 6mm diameter tubes (Gerstell, Mülheim, Germany) and Porapak-Q sorbent VCT glass tubes (ARS, Gainesville, Florida, USA) were used for chemical analyses. Porapak-Q sorbent VCT tubes and home-made sorbent Micro-traps were used for electrophysiological analysis. Micro-traps 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, Munich, Germany)(See [63]). Flow rates and extraction durations were dependent on the odor trap (See Table 1). The entire setup was placed in a room specially designed to stimulate *Anopheles* male swarming behavior thanks to a set of visual stimuli (lights and ground marker, see [62] for details).

About 500 4- to 6-day-old males of *An. gambiae* or *An. coluzzii* were transferred into the 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 human odor before connecting it to the odor trap. Five minutes after males started to swarm (*i.e.* flying in loops at a constant location within the box), the air flow was adjusted at the required rate and the odor trap connected. Forty minutes later, swarming behavior was stopped by turning off the light and the VOC collection continued until the required collection time was reached (see Table 1). The same day and before each collection of VOCs from swarming males, two different control extractions were performed in the same conditions: clean empty box (blank control), and resting (not swarming) male mosquitoes

(resting control) with the same batch of males used for the VOC collection. After extraction, 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 hours to remove the odor contaminants left from the previous extraction. A total of 30 swarm extracts were collected; 17 *An. coluzzii* swarms (5, 8 and 4 with Tenax/Carbotrap tubes, Porapak-Q VCT tubes and Micro-traps, respectively) and 13 *An. gambiae* swarms (6 and 7 with Tenax/Carbotrap tubes, and Porapak-Q VCT tubes, respectively).

Table 1: Air inlet and outlet rates, and VOC extraction duration according to the type of trap used.

	Air flow rate (L/min)		**VOCs extraction duration (hour)**
	Air inlet	Air outlet	
Tenax/Carbotrap tube	2.5	2.0	2.0
Porapak-Q VCT tube	1.5	1.0	2.5
Micro-trap	0.9	0.4	5.0

VOC collection from natural swarms. VOCs were collected from a natural swarm of *An. coluzzii* in the village of Bama. Static sorptive extractions of volatiles from a non-enclosed swarm were performed with Twisters® (100 µm PDMS; Gerstel, Mülheim, Germany; [64,65]). Twisters were directly introduced into the swarm thanks to a 2 m long glass stick with a metallic tip. Control twisters were placed about 3 m away upwind from the swarm. Extraction lasted for the whole swarm duration (about 20-25 min). We chose a swarm apart from habitations and livestock to limit odor pollutions, containing a large number of males (more than 1,000) and being 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 at different days.

Solvent extraction. About 1,000 4- to 6-day-old males of *An. gambiae* or *An. coluzzii* were introduced into a 30 x 30 x 30 cm 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 x 5 cm black cloth was placed in or out of the cage according to the species as described above to stimulate swarming behavior. 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 24h 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: i) with 1- to 2-day-old virgin males (young male control), ii) with 2- to 3-day-old virgin females (female control) and iii) with solvent mix alone (blank control). A total of 8 swarm extracts were done; 4 of *An. coluzzii* and 4 of *An. gambiae* and 1 replicate of each type of control per species.

Chemical analyzes

Samples were analysed at the "Platform for Chemical Analyses in Ecology" (PACE), technical facilities of the LabEx CeMEB (Centre Méditerranéen pour l'Environnement et la Biodiversité, Montpellier, France). Those obtained on Porapak-Q VCT tubes were eluted with 150 µl of Hexane and injected (1 µl) into a Gas Chromatograph coupled with Mass Spectrometer (GC-MS). Those obtained on Tenax/Carbotrap tubes or Twisters were directly thermo-desorbed into GC-MS. Solvent extracts were injected as is or concentrated under a gentle stream of nitrogen.

Liquid analysis (Porapak elutions and solvent extractions). Liquids were analysed with a GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan). Mass spectra were recorded in electronic impact mode (EI) at 70eV over a m/z mass range from 38 to 350. The temperature of the transfer line and the ion source were programmed to 250 °C 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 x 0.25 mm x 0.25 µm Optima 5-MS (Macherey-Nagel, Düren, Germany) fused silica capillary column with a constant helium flow set close to 1.0 $ml.min^{-1}$. 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, Kyoto, Japan) was used for data processing of these analyses.

Tenax/Carbotrap tubes and Twister analysis. All these three types of sorbent were analysed as described by Souto-Vilaròs et al. [66]. Samples were analysed using a gas chromatograph (GC, Trace™ 1310, Thermo Scientific™ Milan, Italy) coupled with a mass spectrometer (MS, ISQ™ QD Single Quadrupole, Thermo Scientific™ Milan, Italy). The column used was an Optima 5-MS, the same as for liquid analysis. Absorbent traps were handled with a Multi Purpose Sampler (Gerstell, Mülheim, Germany) and desorbed with a double stage desorption system, composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstell, Mülheim, Germany). 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 program was as follow : 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 (EI) at 70eV over a m/z mass range from 38 to 350. The temperature of the transfer line and the ion source were programmed to 250 °C and 200 °C, respectively. Xcalibur™ software (Thermo Scientific™, Milan, Italy) was used for data processing.

Electrophysiological analysis

Electrophysiological activity of female olfactive detection, on the male swarm extracts, was tested with an Electro-Antennografic Detection system, coupled with Gas Chromatography (GC-EAD) at the PACE. Thirty minutes before the mosquito scotophase, both 4- to 6-day-old males and 2- to 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 "mosquito sunset time", mosquitoes were placed in dark. Males were checked for erected antennae, ensuring that change in light and dark rhythm, transport and electrophysiology laboratory conditions did not alter their physiological state 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, Sarasota, USA), pulled and cut using a vertical micropipette-puller (P-30 model, World Precision Instruments, Hertfordshire, United Kingdom) filled with insect Ringer solution (6.0 $g.l^{-1}$ NaCl, 0.4 $g.l^{-1}$ KCl, 0.27 $g.l^{-1}$ $CaCl_2$ and 3.20 $g.l^{-1}$ $NaC_3H_5O_3$) 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 which was connected to an Electro-Antennography Detector setup (EAD, Syntech IDAC-2, Kirchzarten, Germany). One end of the glass electrodes was pulled to a fine point which was then broken to ensure a close fit with the female’s head and antenna.

The above EAD setup was linked to a Gas Chromatograph-Flame Ionization Detector (GC-FID, CP-3800, Varian, Palo Alto, USA) equipped with an Optima 5-MS capillary column, the same as for the GC-MS analyses. Liquid injections after Porapack solvent elution were made in a 1079 PTV injector (Programmed Temperature Vaporizator) at 250°C with a 1:4 split ratio. Microtraps were desorbed in the same injector using a ChromatoProbe sample

introduction device (Varian, Palo Alto, USA) with the same split ratio and temperature programmed as follows: increased from 40 °C to 250 °C at 200 °C.min^{-1}and held for 5 minutes. 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 minutes. 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 x 0.25 mm), one leading to the FID (270 °C) and the other into a heated EAD port (200 °C) (transfer line: Syntech, Kirchzarten, Germany) and leaded to antenna. The GC effluent for antenna was mixed with charcoal-filtered, humidified airflow (300 ml.min^{-1}). StarWorkstation 6.41 software (Varian, Palo Alto, USA) was used for data processing.

Different types of male swarm extracts were tested on several conspecific females' antennae. Antennae of 19 *An. coluzzii* females were tested with solvent extracts, Porapak-Q VCT tube extracts and Micro-trap extracts (8, 9 and 2 females, respectively) and 20 *An. gambiae* females were tested with solvent and Porapak-Q VCT tube extracts (10 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: 5µl of each solution was applied to a 1 cm x 2 cm 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, Hilversum, Netherlands).

For all the chemical and electrophysiological analyses (Liquid, Tenax/Carbotrap tubes, Micro-traps and Twister analysis), a solution of n-alkanes (Alkanes standard solution, 04070, Sigma Aldrich, Darmstadt, Germany) was injected to calculate the linear retention index (LRI) of the compounds of interest [67]. To identify the compounds, we matched the mass spectra with the NIST 2011 MS library, and the LRIs with those reported in the literature.

In all cases, even for the electrophysiological analysis, we studied the chromatograms in a range of LRI between 800 and 1700, 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 organisms [68], were considered as contaminants.

Statistical analysis

All analyses were performed using R (version 3.4.0). Both the activation rate and choice were analyzed using binomial Generalized Linear-Mixed Models (GLMM, "*glmer*" function in "*lme4*" package). Tested combinations (four levels: *An. coluzzii*, *An. gambiae*, *An. coluzzii vs. 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. 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% confident interval.

3. Results

Behavioral assays

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: 40.26±1.01%). There was a significant interaction between female species and the four combinations of swarm boxes

tested (χ_3^2 =22.19, *P*<0.001). Species subset analyses showed that the different test combinations had no effect on the *An. coluzzii* activation rate (χ_3^2=5.72, *P*=0.12; Fig. 3). However, in *An. gambiae*, there was a significant effect of the test combinations on the female activation rate (χ_2^2=14.94, *P*=0.001; Fig. 3) 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: χ_1^2=2.45, *P*=0.11; choice test combinations: χ_2^2=1.15, *P*=0.56 and female species x choice test combination interaction: χ_2^2=0.34, *P*=0.84; Fig. 4).

Chemical analyses

All the peaks observed in the volatile profiles from male swarms were also present in control samples, blank and/or resting mosquitoes, for both species (Figs 5-6). GC-MS traces obtained with the Tenax/Carbotrap tubes showed many peaks identified as pollutants, such as silicones, BTX (mono-aromatic compounds like benzene, toluene and xylene), alkanes. Only few differences, including linear organic acids, could be noted but not reproduced. GC-MS traces from the Porapak-Q samples, Twisters® and the liquid extracts were similar to their respective control.

Electrophysiology analyses

The male swarm VOCs induced a few weak and poorly reproducible depolarizations on conspecific females' antennae. In *An. coluzzii*, 3 females out of 9 weakly responded to a single Porapak-Q extract at the linear retention index (LRI) 1371 (retention time (RT) 12.85min), and

2 females out of 8 responded to solvent extracts at LRI 921, 978 and 1179 (RT 6.40, 7.24 and 10.23min, respectively, Fig. 7). However, these responses did not correspond to noticeable peaks in the GC detector of the GC-EAD apparatus.

Successive GC-MS analyses of the same porapak-Q and liquid extracts did not allow to detect and identify any specific peak in relation to antennae responses. The peak at LRI 1371 that elicited a response in *An. coluzzii* females was identified as an alkane family compound (m/z: 57, 43, 71, 85, 99) also present in the control sample. In liquid extracts, the same type of LRI section was studied, LRI 921 was identified as Hexane-2,5-dione (m/z: 43, 99, 71, 57, 114), a chemical pollutant. The section studied for the LRI 978 and 1179 showed no differences with the control sample.

No reproducible response was recorded from *An. coluzzii* females tested with Micro-traps extracts. In *An. gambiae*, no reproducible antennae response was recorded from the 10 females tested with either Porapak-Q or liquid extracts.

4. Discussion

For the last decades, several authors have suggested the existence of long-range sex pheromones in the attraction of *An. gambiae s.l.* females to male swarms [11,34,46,69] but to date, no reliable experimental evidence has been available. Here, we used behavioral, physiological and detailed chemical approaches to attempt to highlight the existence of such compounds. However, although we used a large set of methodologies and worked according to hypotheses maximizing our chances to highlight the presence of sex pheromones (*i.e.* timing, physiological state, natural behavior), our study failed to provide evidence of the presence of male sex pheromones in both *An. coluzzii* and *An. gambiae*.

Despite a good activation rate in our olfactometer, females 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 heterospecific swarm compared to a single *An. coluzzii* swarm. This was, however, probably without biological significance as no difference was detected between the control-gambiae *vs.* the double control set-up. Moreover, our physiological study supports a lack of detection by females of any volatile chemicals present in swarm extracts as no consistent antennal depolarization was observed across assays. Chemical analyses were also negative and no compounds specific for male swarms could be detected.

Independently, these methods can have weaknesses, such as the sensitivity thresholds of our apparatus which are below the ability of insects to detect low amounts of compounds. However, all together 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 are needed and complementary methodologies such as electro-physiology on palps [70–73], molecular analyses of olfactory protein expression on adequate physiological status, or real time chemical analyses of swarm volatiles with a sensitive apparatus such as a proton-transfer-reaction mass spectrometry could be options.

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 [15,18–20] necessarily involve a reasonably distant process which prevents females entering heterospecific swarms. The use of visual cues such as ground markers [13,14,34] or acoustic cues emitted by the gathering of males [28,29] are good candidates, but their effective range and level of specificity is poorly known.

Our results show that the absence of long-range sex pheromones is of prime importance. From an evolutionary and ecological point of view, it demonstrates that the ecological barriers that allow these two species to mate assortatively are probably not of chemical origin. Regarding control strategies, thanks to our findings, research programs working on the sterile

insect technique (SIT) or genetically-modified mosquitoes (GMM) will avoid to focus on the eventuality that the induced modifications may alter long-range sex pheromones making their mosquitoes less competitive than wild individuals.

Authors' 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 and SBP and BB 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., B.L. drafted the manuscript and L.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

We have no competing interests.

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Figure legends

Figure 1: Schematic of the olfactometer set up. S: swarm; M: marker. Not at scale.

Figure 2: Test for olfactometer symmetry. Female choice, expressed as the proportion of female mosquitoes caught in one or the other collecting box out of the total number retrieved from both collecting boxes for the test with empty boxes (control *vs.* control combination).

Figure 3: 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. Different letters indicate difference at $P<0.05$.

Figure 4: 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.

Figure 5: a. Chromatograms obtained with eluted Porapak extracts from headspaces of the control, an *An. coluzzii* swarm and an *An. gambiae* swarm (linear retention index (LRI) 800 to 1700). **b.** Enlarged view of the retention time (RT) interval containing the LRI at which *An. coluzzii* antennal responses were detected in GC-EAD. No difference was detected between the control and the swarm extracts. All the peaks correspond to emissions born from the material.

Figure 6: a. Chromatograms obtained with the desorption of Twisters obtained with the static sorptive extraction methods under natural conditions in the field (linear retention index (LRI) 800 to 1700). The twister was either located within a non-enclosed swarm of *An. coluzzii* or 3 m away upwind (control). **b.** Enlarged view of the retention time (RT) interval containing the LRI at which *An. coluzzii* antennal responses were detected in GC-EAD. No difference was

detected between the control and the swarm extracts. All the peaks correspond to environmental emissions.

Figure 7: Mean electrophysiological responses of female *An. coluzzii* antennae to male swarm solvent extracts, recorded by coupled gaz chromatography – electroantennography detection (GC-EAD). Dashed lines indicate retention times (and related LRI) for which the most significant EAG responses were recorded. Only 2 females in 8 tested produced this pattern.

Figure 1.

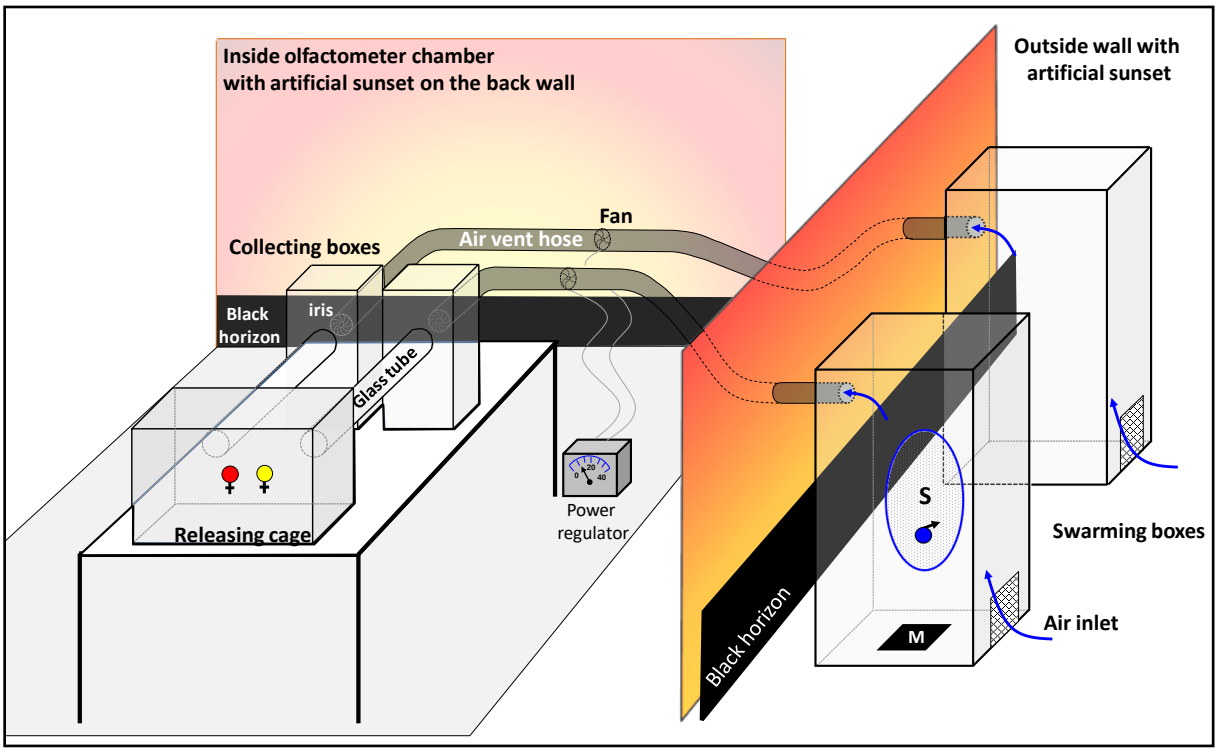


Figure 2.

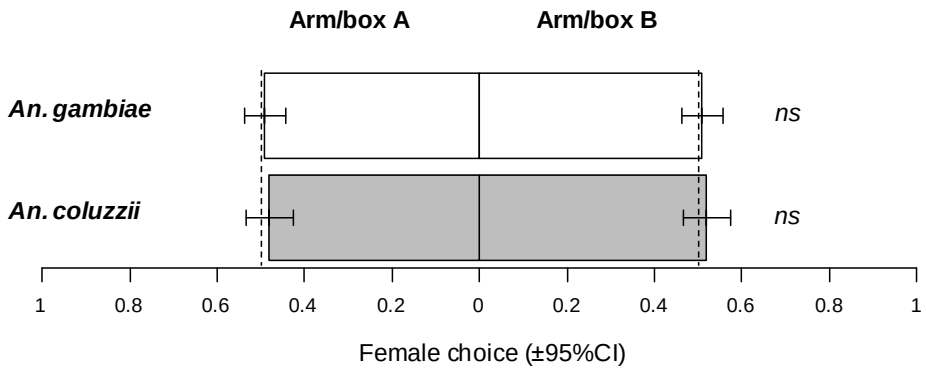


Figure 3.

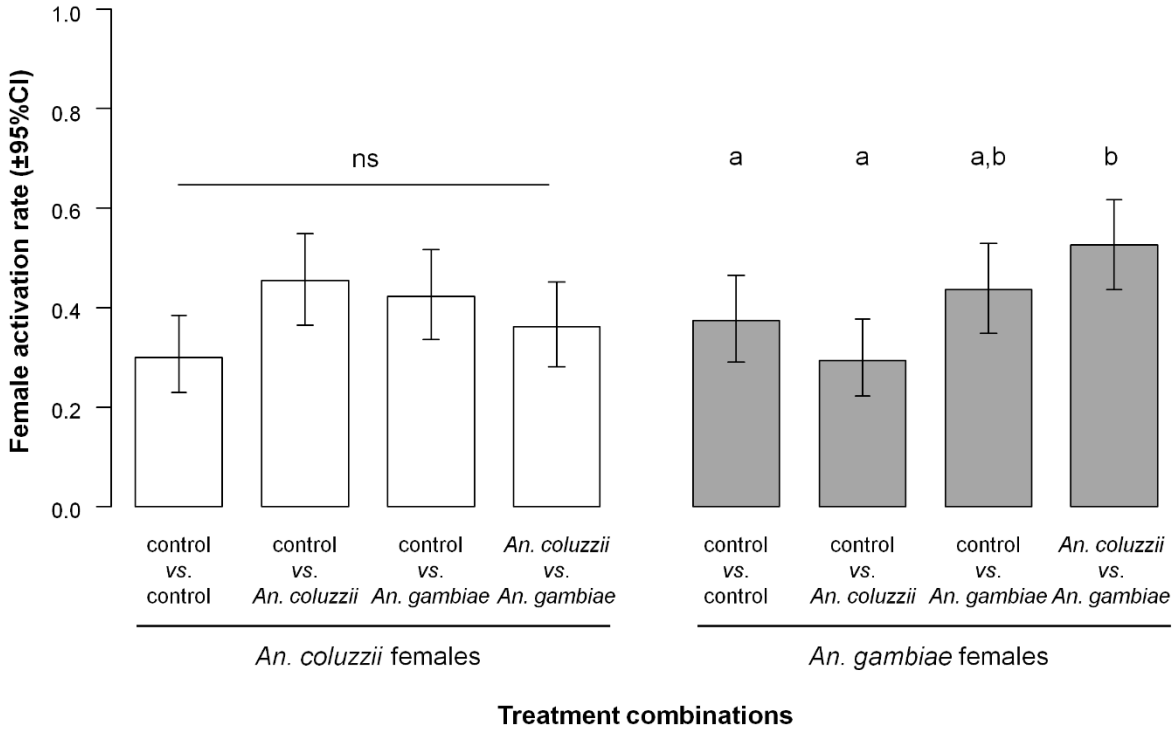


Figure 4.

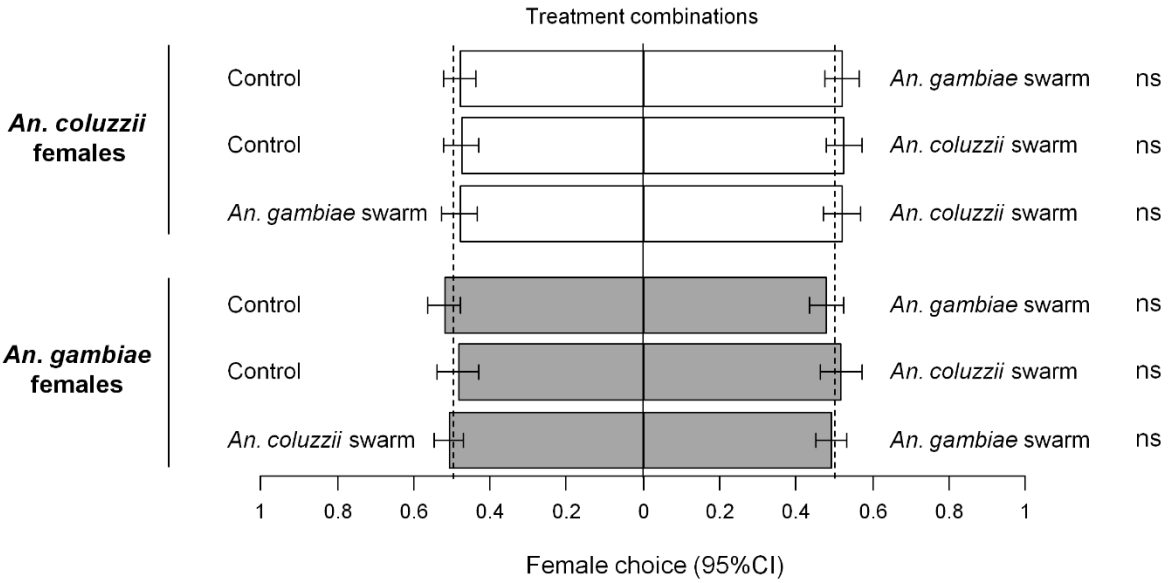


Figure 5.

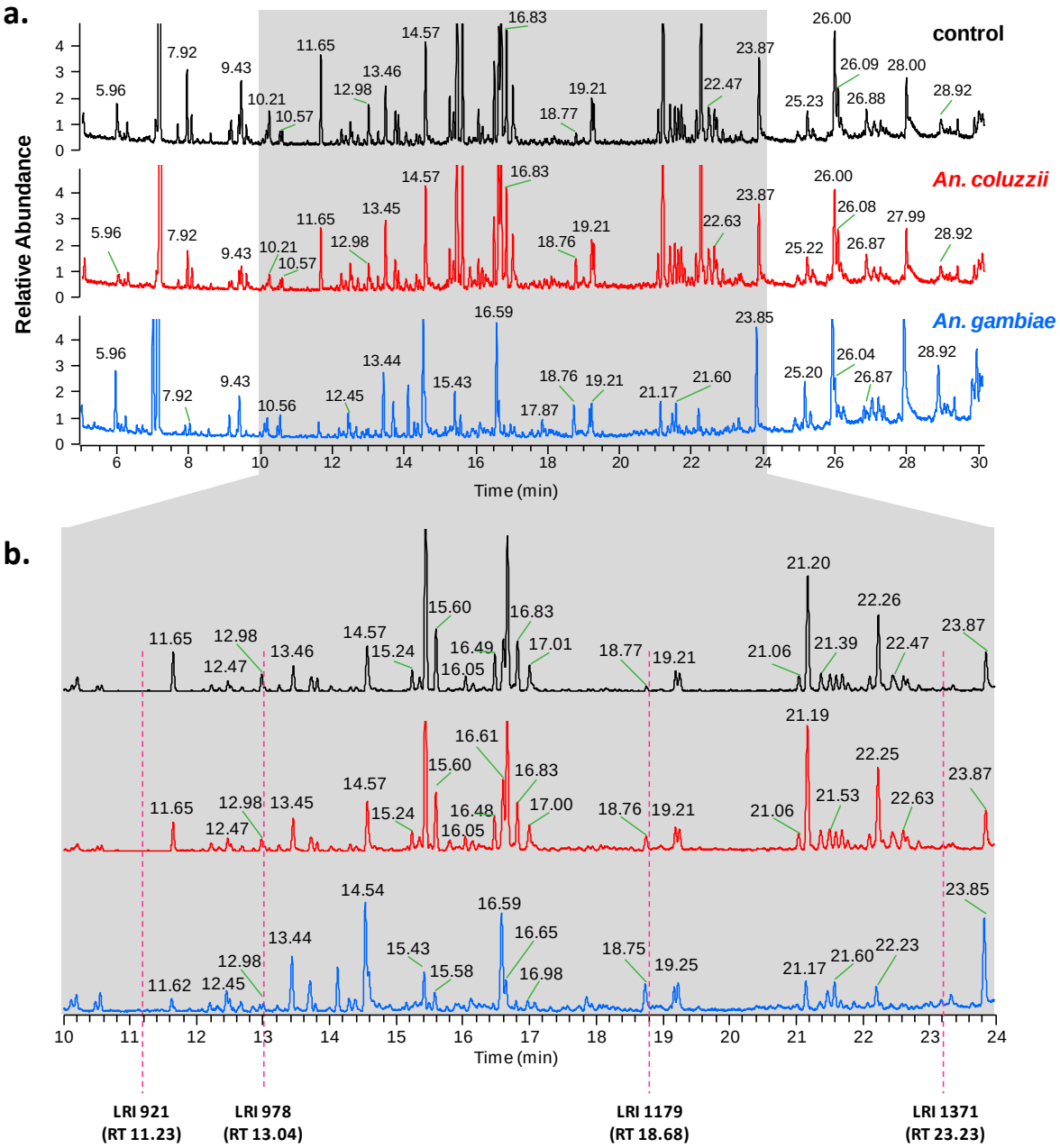
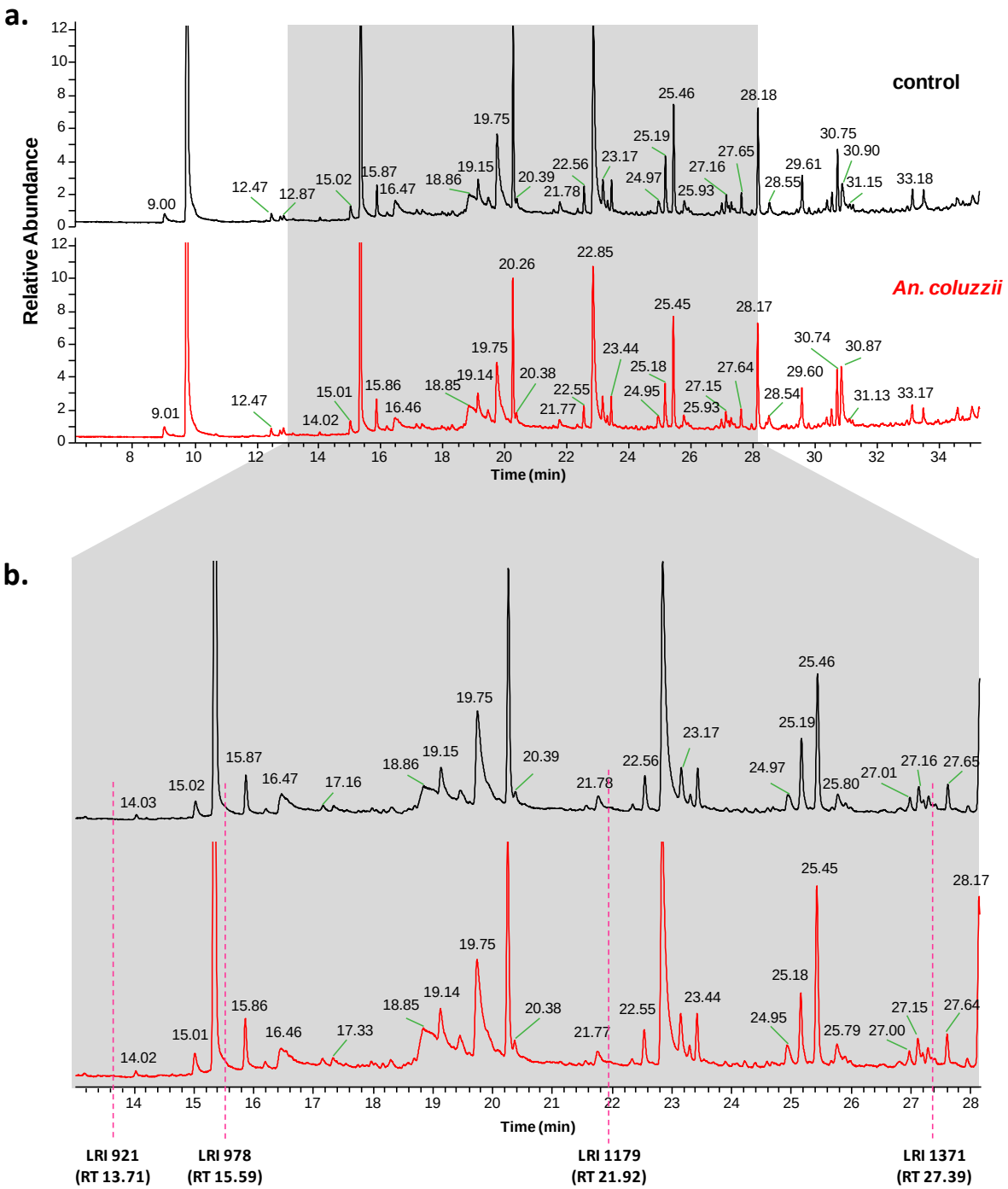

a.
control
An. coluzzii
An. gambiae
Relative Abundance
Time (min)
b.
Time (min)
LRI 921
(RT 11.23)
LRI 978
(RT 13.04)
LRI 1179
(RT 18.68)
LRI 1371
(RT 23.23)

Figure 6.


a.
control
An. coluzzii
Relative Abundance
Time (min)
b.
Time (min)
LRI 921
(RT 13.71)
LRI 978
(RT 15.59)
LRI 1179
(RT 21.92)
LRI 1371
(RT 27.39)

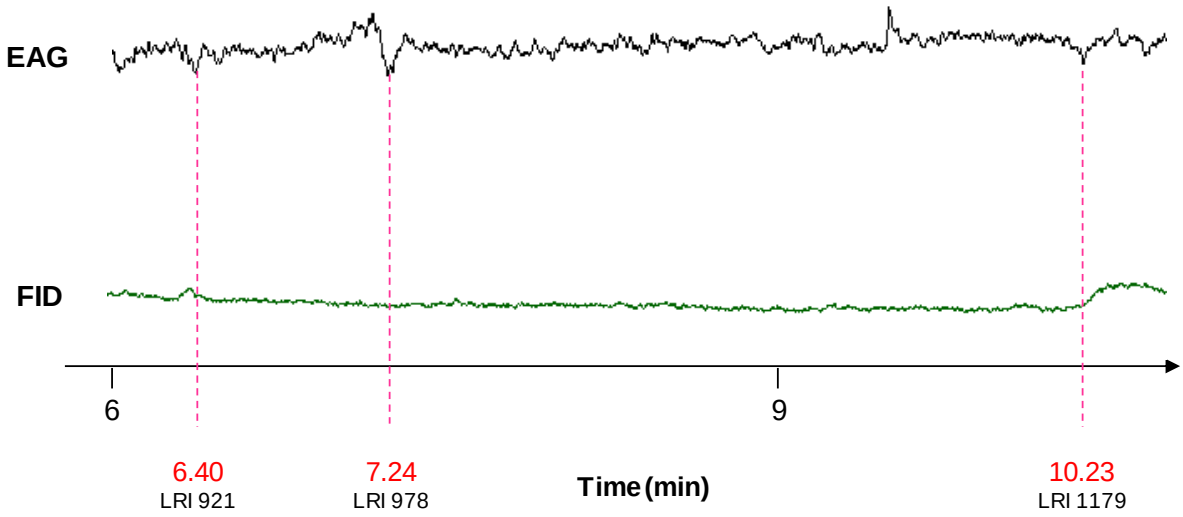



Figure 7.