Anopheles male pheromone increases swarming, female attraction to the swarm, and mating in five main African malaria vectors

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Accumulating behavioural data indicate that aggregation pheromones may mediate formation and sustaining of swarms of mosquitoes. However, chemical cues possibly luring mosquitoes to swarms have not been adequately investigated and the likely molecular incitants of these complex reproductive behaviours remain unknown. Here we show that males of important malaria vector species *Anopheles arabiensis* and *Anopheles gambiae* produce and release aggregation pheromones that attract individuals to the swarm, and enhance mating success. We found that males of both species released significantly higher amounts of 3-hydroxi-2-butanon (acetoin), 6-methyl-5-hepten-2-one (sulcatone), octanal, nonanal, and decanal during swarming in the laboratory. Males fed with stable isotope-labelled glucose, revealed that these five compounds were produced by them. A blend composed of synthetic analogues to these swarming odours proved highly attractive to virgin males and females of both species under laboratory conditions and significantly increased mating in five African malaria vectors: *An. gambiae, Anopheles coluzzii, An. arabiensis, Anopheles merus* and *Anopheles funestus* in semi-field experiments. Our results not only narrow a conspicuous gap in understanding a vital aspect of the chemical ecology of male mosquitoes but also demonstrate fundamental roles of rhythmic and metabolic genes in the physiology and behavioural regulation of these vectors. These identified aggregation pheromones have great potential for exploitation against these highly dangerous insects.

Anopheles males, as among many other mosquito species, form mating swarms[1] that vary in size and temporal stability[2-4]. Wild *Anopheles* males swarm during sunset[1,5-7] and are often found near contrasting shade ground features, presumably guided by that visual cue[1,4,5,8].
Factors and mechanisms of swarming initiation in mosquitoes are still debatable[9]. A few studies have shown that initiation of swarming in mosquitoes is governed by the inherent circadian clock[10,11] and fine-tuned by environmental conditions[12-14]. However, very little is known about chemical cues that may mediate the formation and maintenance of male swarms and attracting females to those swarms in order to copulate[15].2,6,6-trimethylcyclohex-2-ene-1,4-dione was isolated from males and females of *Aedes aegypti* mosquitoes and stimulated swarming behaviour by increasing number of swarming males and extending activity in a dose depending manner[15]. Another study revealed that under laboratory conditions, swarming of *Ae. aegypti* males was triggered with a host odour at the onset of scotophase but chemical cues remain unknown[16]. Behavioural tests showed that volatiles released from alive or dead males of three *Culex* species attracted significantly more conspecific females than the control odour without males[17] pointing out that attraction of females to swarms may be mediated by a sex pheromone. 1-(4-Ethylphenyl) ethanone, produced by both sexes of *Ae. aegypti* mosquitoes, elicited attraction of virgin females

under laboratory conditions but fail to attract males, hence the pheromone did not cause aggregation and attribution of the compound to an aggregation pheromone is debatable[15].

Here, we tested the hypothesis that specific chemicals incite swarming and impact mating behaviours among several species of anophelines responsible for transmitting human malaria in Africa.

Results

In the laboratory, we collected odours released by virgin (4-6 day old) males of *Anopheles arabiensis* (KGB and Dongola strains) and *An. gambiae*, (Keele strain) during swarming (at transition period from photophase to scotophase) as well as non-swarming periods (during mosquito resting at photophase). Odours were collected by solid phase micro-extraction techniques (SPME)[18,19] and control samples were obtained by collecting odours from an empty bottle during photophase. We found that males of both species consistently produced the same five volatile compounds: 3-hydroxi-2-butanon (acetoin), 6-methyl-5-hepten-2-one (sulcatone), octanal, nonanal, and decanal, with significantly higher amounts during swarming compared to non-swarming periods (Fig. 1a-c). To confirm that these compounds were released by male mosquitoes rather than being artefacts occurring in ambient air, adult *An. arabiensis* (Dongola) males were fed on isotope labelled $^{13}C_6$-D-glucose 5% solution in water. Our experiments showed that ratios of labelled versus non labelled ions, collected from the headspace of mosquitoes, differed significantly in acetoin ($\chi^2 = 3.95$, $p < 0.01$), sulcatone ($\chi^2 = 1.79$, $p < 0.01$), octanal ($\chi^2 = 0.44$, $p < 0.05$), nonanal ($\chi^2 = 10.96$, $p < 0.001$), and decanal ($\chi^2 = 4.95$, $p < 0.01$) ratios (Fig. 1d). This demonstrated male mosquitoes as the source of these five compounds. Moreover, we established a positive correlation between amounts of the five compounds and number of swarming males (F value = 106.60, df = 1, $p < 0.001$; Extended data Fig. 1a). We found that the total amount of these five swarming odours was the highest during transition from photophase to scotophase (swarming) compared to that sampled during photophase from an empty bottle (control) ($\chi^2 = 41.74$, $p < 0.001$) and to that sampled during transition from scotophase to photophase ($\chi^2 = 36.67$, $p < 0.001$). Significantly higher amounts of volatiles were also trapped during transition from scotophase to photophase compared to that sampled during control ($\chi^2 = 30.62$, $p < 0.001$) (Extended data Fig. 1b).

The total amount of five swarming odours released by 80 virgin, 4-6 day old *An. gambiae* male mosquitoes was 118 ± 17 ng/h (average ± standard error of mean, n = 3) measured by a dynamic aeration method[20]. Synthetic compounds acetoin, sulcatone, octanal, nonanal, and decanal analogous to naturally produced swarming odours were used at the ratio 1, 4, 13, 50, and 400, respectively, and the dose of 5 μg to produce a synthetic blend similar to the swarming blend

released from 150 swarming males of *An. arabiensis* (KGB strain). To determine bioactivity of the synthetic blend, we tested behavioural responses of males and females in a two-choice olfactometer bioassay. Our data showed that mosquitoes of both *An. arabiensis* and *An. gambiae* exclusively preferred the filter paper impregnated with 5 µg of the blend in 10 µl of methanol placed in one arm versus a control filter paper treated with 10 µl of pure methanol placed in the other arm (methanol was evaporated from the filter papers before placing them in the olfactometer) (Fig. 2a). Significantly more *An. gambiae* (Keele) males were lured to the blend compared to females ($\chi^2 = 4.10$, $p < 0.05$) while males and females of *An. arabiensis* (Dongola) did not show significant preference ($\chi^2 = 3.58$, $p = 0.05$) (Fig 2a). We found equal preference to the blend in males ($\chi^2 = 2.61$, $p = 0.10$) and females ($\chi^2 = 2.20$, $p = 0.13$) comparing *An. arabiensis* versus *An. gambiae* (Fig 2a).

To reveal the effect of the synthetic blend on swarming, we recorded the swarming behaviour of *An. gambiae* males in a wind tunnel under the transition period from photophase to scotophase in the absence and presence of swarming odours released at the rate of 5 ng/min. The results showed that significantly more males swarmed ($\chi^2 = 24.53$, $p < 0.001$) and duration of the activity was three times as long ($\chi^2 = 124.53$, $p < 0.001$) in the presence of the blend compared to the males' activity in the absence of the blend (Fig. 2d).

To determine the effect of the blend on mating rate in African malaria vectors, we compared the percentage of mated females of *An. arabiensis* (KGB), *An. gambiae s.s.* (G3), *An. coluzzii* (COGS), *An. funestus* (FANG), and *An. merus* (MAFUS) under semi-field conditions in the Republic of South Africa. We found that the blend significantly increased mating in these five malaria-vector species compared to solvent alone (Fig 2b). The highest percent of mating during blend exposure was registered for *An. merus* and *An. funestus* (*An. merus:* $\chi^2 = 20.29$, $p < 0.001$; *An. funestus*: $\chi^2 = 64.53$, $p < 0.001$, Fig 2b).

To study the molecular mechanisms that may form the foundation of these behavioural patterns in males, we applied Illumina transcriptome profiling by next-generation sequencing on virgin 5-7 days old *An. gambiae* male head and body separately at scotophase (middle of the period), photophase (middle of the period), and transition from photophase to scotophase (Supplementary Methods). This revealed significant differential expression levels of genes governing the biological processes associated with swarming and pheromone communication such as circadian clock, fatty acid metabolism, olfaction and reproduction (see Supplementary Results).

Discussion

The data we have collected provide direct evidence that the five component blend (natural or synthetic) functions as an aggregation pheromone in *An. arabiensis* and *An. gambiae* mosquitoes and increases mating in another three *Anopheles* species. This is the first study, to our knowledge, demonstrating aggregation pheromones in *Anopheles* mosquitoes. The high similarity of the identified pheromone composition and insignificant differences in the preference for the synthetic pheromone blend by *An. arabiensis* and *An. gambiae* mosquitoes suggest that the swarming odours were not the decisive factor in premating isolation between these two species. The mating enhancement we have observed in another two *An. gambiae* complex species and in more distantly related *An. funestus* indicates structural similarity of swarming odours may extend beyond the *An. gambiae* complex.

Our data on behavioural responses of mosquitoes to swarming odours are in agreement with the published field observations on swarming and mating in sympatric populations of *An. gambiae* complex and *An. funestus* showing various degree of species-specificity of swarms ranging from numerous intra-specific to nearly as many inter-specific as intra-specific swarms[4,14,21-24]. However, very low percentage of hybrids in wild populations and few inter-specific copulae within mixed-swarm indicate the existence of assortative mating, caused by other factors[25,21,23,24] probably functioning simultaneously. Differences between the species of the *An. gambiae* complex, and *An. funestus* in spatial[4,8,26] and temporal[8,24,26] segregation of swarms, short-range acoustic[25,27,28] as well as low-volatility chemical cues[29-32] contribute to minimize interspecific mattings. However a few other studies showed that acoustic behaviour of males in the malarial mosquitoes *An. gambiae s.s.* and *An. coluzzii* did not contribute to reproductive isolation[33,34]. In addition, partitioning of pheromone communication channels due to seasonal differences and habitat preference[35] may lead to reproductive isolation while using chemically similar pheromones. Genomic studies have demonstrated that *An. gambiae s.s.* and *An. coluzzii* separated about 540,000 years ago[36] showing that *An. gambiae* complex species are an evolutionary very young species undergoing rapid speciation by multidirectional development of reproductive isolation mechanisms[35].

In insects, straight-chain C_{10-18} aldehydes are biosynthesized from fatty acid coenzyme A esters by chain shortening steps followed by reduction to alcohols, which are converted to aldehydes during pheromone release[37]. Up to present, no reports describe biosynthesis of octanal and nonanal in insects. Sulcatone is the most widespread norterpene in insects[38] functioning as pheromone in approximately 60 species predominantly in the orders Hymeneoptera and Coleoptera[39]. Sulcatone could be biosynthesized either by insects or by their microbial symbionts de novo through the mevalonate pathway from a few possible intermediates[40,41] or from a prenyl unit and an acetoacetate unit[38]. Acetoin is a well-known product from microbial metabolism and is widespread

in nature[42]. It is known as a pheromone for at least 10 insect species[39], however biosynthetic origin in insects remains unknown.

All five aggregation pheromone components are found in mosquito-host odour profiles[43-46] and elicited behavioural responses in anophelines[45,47-50]. More studies will be needed to confirm if the same compounds mediate different responses depending on the environmental context in which they occur.

Due to the ecological and physiological plasticity of mosquitoes, they show high adaptivity to new environments[51]. It has been hypothesized that extensive use of long-lasting insecticide-treated nets for minimizing human contact with the endophagic night-feeding anophelines induces selection for mosquito feeding earlier in an evening before people go to beds or even biting outdoors[52,53]. Furthermore, mosquito resistance has been accordingly emergent[54,55]. Vector control of desired impacts can't be achieved by any single method and requires integrated control approaches[56]. Most of the adult mosquito control methods focus on females with little attention to targeting males[57,58], perhaps thus overlooking key opportunities to exploit in destabilizing anopheline populations. Field studies have shown that *Anopheles gambiae* s.l. mosquitoes prefer certain sites for swarming[4,8] and application of our identified *Anopheles* aggregation pheromone could contribute to predictability of swarming sites and increase the number of males swarming. This phenomenon may efficiently target mosquito swarms with the aim of disrupting reproduction among populations of important malaria vectors.[58] In addition, the aggregation pheromone may be used in luring mating males and females to baited traps. In contrast using lethal pesticides in control of mosquitoes, this approach may be less vulnerable to acquired resistance. Moreover, implementation of swarming odours into mass production of male mosquitoes for eco-friendly control strategies such as sterile insect techniques[8] may enhance breeding efficiency and increase the success of colonisation of vector species with very low mating success under laboratory conditions. Evaluation of the ability of sterilized males' to produce an aggregation pheromone and respond to it enables determining whether laboratory-bred sterilized males are equally able to locate swarms compared to wild males. Even in the event of apparent minimising the sexual attraction of wild female mosquitoes towards laboratory produced males, the addition of synthetic blend of this pheromone in swarming sites might enhance the trigger of his basic instinct in wild females.

Methods

Ethics: Human blood (type O) was provided in citrate-phosphate-dextrose-adenine anti-coagulant/preservative, and serum (type AB) was obtained from the Blood Transfusion Service at Karolinska Hospital, Solna, Sweden in accordance with the Declaration of Helsinki and approved by the Ethical Review Board in Stockholm (2011/850-32).

Study objects. Mosquitoes used in the identification of swarming odour experiments were from the laboratory colony of *Anopheles arabiensis* (Patton), (KGB) and (Dongola) strains obtained from International Atomic Energy Association (Vienna, Austria). *Anopheles gambiae* (Keele) strain[59] obtained from University of Glasgow, UK. Larvae were reared in incubators under conditions (27 ± 1 °C, 70% humidity, 12 h photophase : 12 h scotophase cycle) and fed on TetraMin® fish flakes (Tetra ltd., Germany).

Pupae were transferred into holding cages for emergence. Adults were fed ad libitum on 10% glucose solution, supplemented with 0.05% (w/v) 4-aminobenzoic acid (PABA, Sigma-Aldrich), through soaked filters on top of the 2 ml tubes and with soaked filter pads inside cages.

Mosquitoes used for testing efficiency of synthetic swarming odour blend were from strains housed at the National Institute for Communicable Diseases (Johannesburg, South Africa)[60]. Mosquitoes were reared at the Botha De Meillon Insectary, Johannesburg, South Africa under standard insectary conditions of 80% humidity, 25˚C, and a 12-hour day/night cycle with 45-minute dusk/dawn transitions[60]. All adult mosquitoes were sustained on a 10% sucrose solution diet.

The male and female mosquitoes were separated as pupae. Sex-separation at the pupal stage was based on observation of the genitalia under a binocular microscope. Anopheline pupae can be reliably sexed by the differences in the genital lobe shape (at the end of the pupal abdominal segments just below the paddles). The male has a spine structure with shorter paddles compare to female pupae. Collected and separated male and female pupae were placed in labelled cages. Each cage was also inspected after the adult emergence (~12 hours) in terms of detecting any false identification to prevent matting before male sexual maturation and genitalia rotation. Male anopheline mosquitoes require a minimum period of 24h postemergence to complete sexual maturation, which includes 180° rotation of the genitalia[61,62].

Sampling and identification of swarming odours. One hour before sampling, virgin 4-6 day old male mosquitoes (20 or 50 individuals of *An. arabiensis* or *An. gambiae*, respectively) were transferred into a glass bottle (1 l volume), which was then placed inside an incubator. In the experiments using *An. arabiensis* males, odours were collected for 1.5 hour during light on stage, not-swarming period (from 3.5 to 2 hours before the transition from photophase to scotophase) and during swarming period (1.5 hour sampling starting at the transition from photophase to scotophase). Control samples were obtained collecting odours from an empty bottle during photophase (from 5.5 to 4 hours before the transition from photophase to scotophase). To decrease the variation caused by SPME fibers, samples from swarming mosquitoes and control were collected at the diferent time periods on the same SPME fiber. To determine the correlation

between the number of swarming mosquitoes and amount of odours trapped, 7, 15, 20, 25, 30, 35, 40, 50, 80 and 90 males of *An. arabiensis* (Dongola) strain were used. In the experiments using *An. gambiae* males, odours were collected for 60 minutes during not-swarming period (from 6 to 5 hours before the transition from photophase to scotophase) and during transition from photophase to scotophase as well as transition from scotophase to photophase. Control samples were obtained collecting odours from an empty bottle during photophase (from 7 to 6 hours before the transition from photophase to scotophase).

Odour collections were carried by solid phase micro extraction (SPME) technique[18,19]. Prior to sampling, the polydimethylsiloxane/divinylbenzene-coated SPME fiber was purified for 3 min at 250 °C in a gas chromatograph (GC) (Varian 3400, Varian Scientific Instruments, Palo Alto, CA, USA) injector. Afterwards, the fibre was inserted into the glass bottle and exposed to the headspace. Volatiles were collected for 60 min and analysed immediately by GC coupled to mass spectrometer (MS) (Finnigan SSQ 7000, Finnigan Instrument Corporation, Palo Alto, CA, USA). The volatiles from SPME fibre were desorbed in the injector (splitless mode, 1 min, 225 ^{o}C). Helium was used as the carrier gas with an inlet pressure of 70 kPa. The GC was equipped with a DB-Wax silica capillary column (30 m length, 0.25 mm ID, 0.25 µm film thickness). The GC oven temperature was hold isothermal at 40 ^{o}C for1 min, afterwards increased by 5 ^{o}C min^{-1} up to 150 ^{o}C and then increased by 20 ^{o}C min^{-1} up to 220 ^{o}C and then hold isothermally for 9 min. Electron ionization mass spectra were determined at 70 eV with the ion source at 150 ^{o}C.

Chromatographic profiles of the volatiles were compared and the compounds which occurred in significantly large amounts in the mosquito samples compared to those of blank samples were further analysed. Compounds were identified by comparison of their retention times and mass spectra with those available from NIST mass spectral data base, version 2.0 (National Institute of Standards and Technology, USA) as well as by comparing retention times and mass spectral data of natural products with those of authentic reference standards[63].

To determine total amount of five swarming odours released by mosquitoes, the dynamic aeration method[20] was used. Eighty, virgin, 4-6 day old *An. gambiae* male mosquitoes were transferred into a glass bottle (1.8 L volume), which was placed inside an incubator. The dry air was pushed into the glass bottle with a diaphragm vacuum pump (NMP 830 KNDCB; KNF Neuberger Inc., Freiburg, Germany) through an activated charcoal (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and a humidifier at the flow rate of 0.55 L/min. Air containing swarming odours was withdrawn from the glass bottle through a glass collection tube filled with 50 mg of Tenax TA adsorbent (60/80 mesh; Sigma-Aldrich AB, Sweden) by another diaphragm vacuum pump at the flow rate of 0.5 L/min. By pulling out less air than supplying into the glass bottle, we ensured that no contaminated air from the outside would enter the system. Odours were collected during

swarming period (at the transition from photophase to scotophase) for 1 hour. At the same time, control samples were obtained collecting odours from an empty bottle. Three replicates were obtained. The odours collection tubes were extracted with 350 µL or redistilled diethyl ether (Carlo Erba Reagents SAS, Val-de-Reuil, France) and 50 ng of 10-(*E*)-dodecen-1-yl acetate in 1µL of cyclohexane (Carlo Erba Reagents SAS, Val-de-Reuil, France) was added as internal standard. Extract was concentrated under gentle nitrogen flow up to 1 µL and analysed by GC-MS. For quantitative analysis, calibration solution of synthetic analogous to swarming odours were prepared by stepwise dilution and 1, 10, and 100 ng of each compound were injected in GC-MS system. The same method has been used for odours collection to determine the loading rate and amounts of components to ensure 5ng/min release rate from a wick dispenser.

Determination of the isotope labelled glucose moiety incorporation into swarming odours. After emergence, the adult males were fed with 5% $^{13}C_6$-D-glucose (99% labelled, Cambridge Isotope Laboratories Inc., MA, USA) solution in water[64]. The level of incorporation was determined as the ratio between the abundance of labelled ions *m/z* 44 (*m/z* 41 plus 3 Daltons due to presence of three labelled, by one Dalton heavier ^{13}C atoms) and the non-labelled ions *m/z* 41 (all three ^{12}C atoms non-labelled) in octanal, nonanal, decanal and sulcatone sampled from mosquitoes fed with labelled glucose and from those compounds sampled from mosquitoes fed on non-labelled glucose. For acetoin, the incorporation was determined in the same manner using labelled ions *m/z* 46 and non-labelled ions *m/z* 43.

Two-choice olfactometer bioassay. An Y-tube glass olfactometer[65] (length of the central cylinder and two arms: 25/15/15 cm respectively, inner diameter: 5 cm; an angle of 90° between the two arms) was used. The experiments were carried out under dusk conditions 26 ± 2 °C, 5 lux light intensity. For each experiment, virgin 4-6 day old a glucose-fed males and females of *An. arabiensis* (Dongola) and *An. gambiae* (Keele) species were individually placed in a chamber and release one-by-one. Mosquitoes flew towards the upwind end (purified and humidified air flow 2 l min^{-1}) and entered one of two trapping chambers through which odour was released from a 2 cm^2 filter paper (grade 3, Munktell Filter AB, Falun, Sweden) impregnated with 5 µg of the blend in 10 µl of methanol placed in one arm versus a filter paper treated with 10 µl of pure methanol placed in another arm and considered as a control. Synthetic compounds acetoin, sulcatone, octanal, nonanal, and decanal were used at the ratio 1, 4, 13, 50, and 400, respectively to produce a synthetic blend. Methanol was used as a solvent and was evaporated from the filter papers before placing them into olfactometer. Impregnated filter paper released swarming compounds at the ratio and amount close to 150 swarming mosquitoes and was not used longer than

20 min, as longer use would cause reduced emission. In the first two experiments, the responses of 180 males and 180 females of *An. arabiensis* Dongola strain were tested, and in third and fourth experiment the responses of 180 males and 180 females of *An. gambiae*, Keele strain strain were evaluated. Sixty mosquitoes were tested per day. To control for possible spatial effects, the location of treatment at each olfactometer arm was switched every 20 min (~10 mosquitoes flied one by one in each 20 min). Mosquitoes reaching any of the trapping chambers were considered to have made a choice. The percent of not responding individuals for both sexes and species was 3-4%. Each experiment was repeated six times with in total 180 mosquitoes per treatment. Each individual was tested once.

Estimation of mating stimulation under semi-field bioassay. Assays were carried out in South Africa (Johannesburg-NICD under natural field conditions) at the end of March, a period which normally coincides with the end of the rainy season. Experiments were started exactly one hour before sunset. Semi-field cages made from Anti-Thrip Netting (2.9 m diameter × 2.0 m high with floor) which allowed simulation of prevailing ambient weather conditions were used. A wooden resting box 30 cm × 30 cm × 30 cm lined with black felt with one side having a hinged cover to allow mosquito access was placed in each cage as a resting place. A plastic jar with cotton pad soaked in 10% sucrose solution in each cage provided mosquitoes with an energy source[66]. Custom-made wick type dispensers composed of 1.5 ml glass vial closed with autosampler screw cap bearing PTF septum pierced with a PTF tube (1.5 cm length and 2 mm inner diameter) with inserted cotton wick was used to deliver the odour blend. Swarming odour components namely: acetoin, sulcatone, octanal, nonanal, decanal, and at the loading amounts of 1, 4, 13, 50, and 400 μg mL^{-1} were dissolved in 95% ethanol/water and released from the dispensers at the rate 5 ng min^{-1}. The release rate was assessed trapping emitted odours from the dispenser on a Tenax absorbent. The ratio of the volatiles was close to that identified from swarming *An. arabiensis* KGB strain. In each cage, 100 mosquitoes were released, including 50 unmated females and 50 unmated male (5-7 days old). The morning after each swarming test, we collected and dissected the female mosquitoes for observing the spermateca of the female, inspecting the present of male sperm, and determined percentage of the mated females. For each species, we employed 3 replicates of treatement and solvent as control.

Estimation of the blend effect on swarming behaviour of *An. gambiae* males in the wind tunnel. The number of the males swarming and the duration of swarming behaviour were compared during the light transition period from photophase to scotophase in a wind tunnel with and without swarming blend. A wind tunnel system for insect flight studies under low light intensity was used (Noldus Information Technology, Wageningen, The Netherlands)[67]. In

summary, the system was comprised of: a tracking chamber 160 x 60 x 60 cm (LxWxH); an air purification and delivery system; infrared LED illuminators placed at the downwind end of the wind tunnel, facing upwind; two infrared sensitive Cohu 4722–2000/0000 monochrome CCD video cameras (Cohu, San Diego, CA,USA); and a software package Track3D. Group of 30 virgin males 5-7 days old was released from downwind end of the tunnel at 27 ± 2 ^{o}C, 70 ± 3% relative humidity and a wind speed of 20 ± 1 cm s^{-1}. Three groups of the males were tested in each type of experiment i.e. with and without swarming blend. The experiments lasted for 210 minutes.

Statistical analysis. General Linear Mixed Model (GLMM) statistical modelling was used to corroborate the validity of results based on the whole data set by including the effect of replications (experimental blocks), including weighting for multiple replications. In all analyses, the effect of the main experimental effects (e.g. treatment [blend or VOCs][36]) was investigated while controlling for variation in experimental replication (random variable). For all results, the significance of all explanatory effects were evaluated by using likelihood ratio test (LRT). Analyses were performed using R statistical software (v.3.2.3 and Rstudio Version 1.1.463 – © 2009-2018). In all analyses, treatment (blend/VOCs vs control) was investigated as the primary effect of interest. Percentage of activity was compared between mosquitoes exposed to blend or not in wind tunnel for around 210 min. In this model (GLMM, Ancova), firstly, we calculate the mean activity of the mosquitoes per each time point using the Noldus software. Then, the model estimated the expected regression line based on the calculated mean activity for each group (with and without blend) separately during 210 min. Experimental replication was treated as a random effect.
Generalised Linear Mixed Models (GLMM, R statistical software v. 3.2.3) assuming a binomial distribution were used to test the effect of blend on the binary response variable of dual choice in the attraction, between different sex (male/female), and species variation (5 main vectors) assays (Logistic regression models, absent or present; lme4 package, glmer, R, v. 3.2.3). Logistic regression is a powerful statistical method for binomial outcome (take the value 0 or 1) with one or more explanatory variables. In this study, we included at least two variables: 1-Treatment, i.e. the blend, (main effect) and 2-Experimental blocks, i.e. the experimental replicates, (random effect). In all analyses, Treatment was investigated as the main effect of interest. All data conformed to the assumptions of the test (normality and error homogeneity). In all mixed models, a maximal model was built that included fixed effects plus the random effects of the experimental replicates.

Data availability

S. Noushin Emami, Raimondas Mozuraitis and Anna Karin M. Borg Karlsson are inventors on a patent application (Sw patent application no. ZSE1077999) submitted by the main applicant, S. Noushin Emami (Zacco- Stockholm University) that covers the attraction effects of aggregation pheremone and the synthetic attractant odour blend.

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Competing interests The authors declare no competing interests.

Additional information

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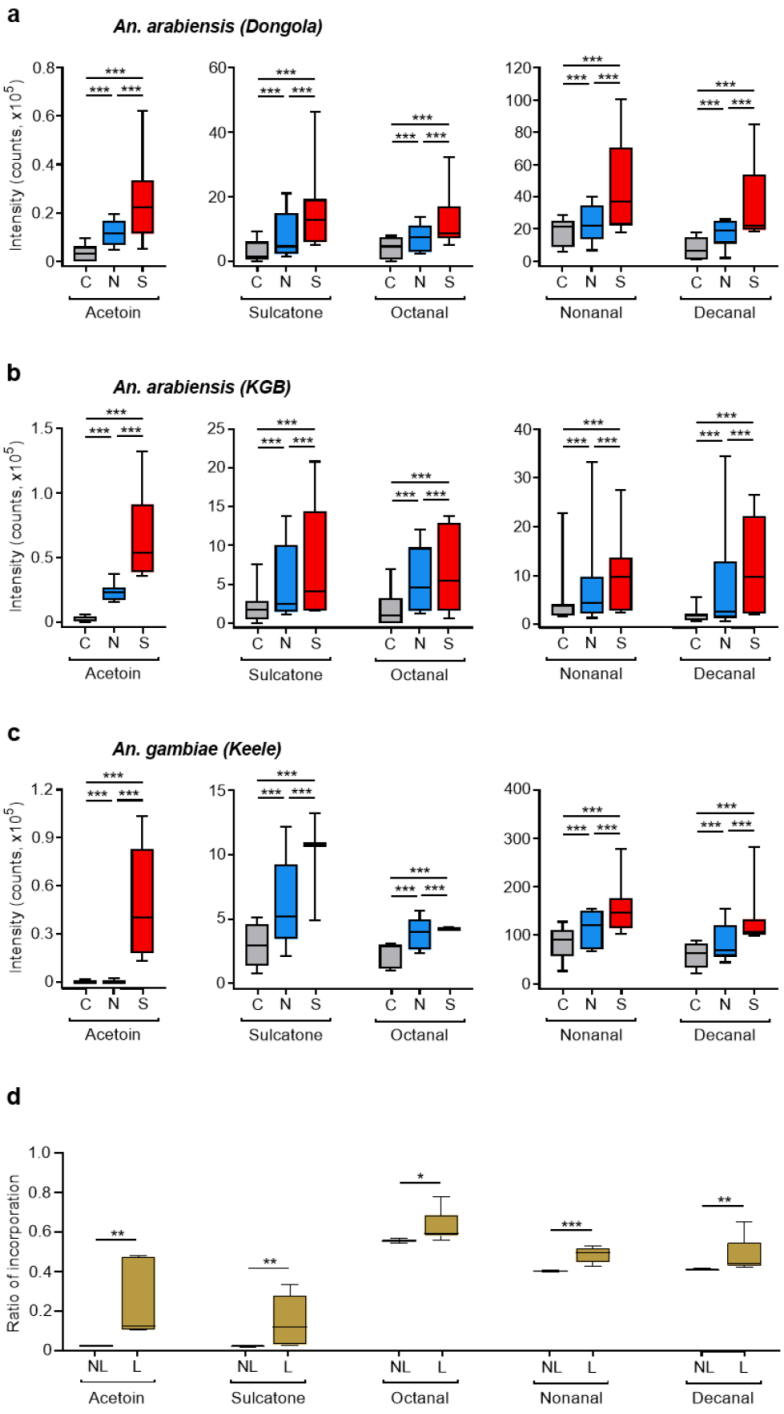


Fig. 1 | Swarming odours of *Anopheles arabiensis* (Dongola) and (KGB) as well as *Anopheles gambiae* (Keele)

a–c, The odours sampled from empty jar are indicated as control (C), and from the males during non-swarming (N) and swarming (S) periods. *An. arabiensis* (Dongola) (n = 11 measurements, 20 males per each measurement), *An. arabiensis* (KGB) (n = 8 measurements, 20 males per each measurement), *An. gambiae* (Keele) (n = 6 measurements, 50 males per each measurement). Intensity values shown on the Y axis are numbers of counts related to the abundance of the ions formed in the mass spectrometer and correspond to the amount of compound analysed. **d**, Ratios of labelled versus non labelled ions in swarming odours collected from the headspace of *An. arabiensis* (Dongola) mosquitoes fed with labelled glucose (L) compare to the ratios measured in the compounds sampled from the headspace of males fed on non-labelled glucose (NL) during swarming. **a–d,** The medians are represented as thick horizontal lines, the boxes represent the upper and lower quartile ranges, the whiskers show the 95% confidence intervals.

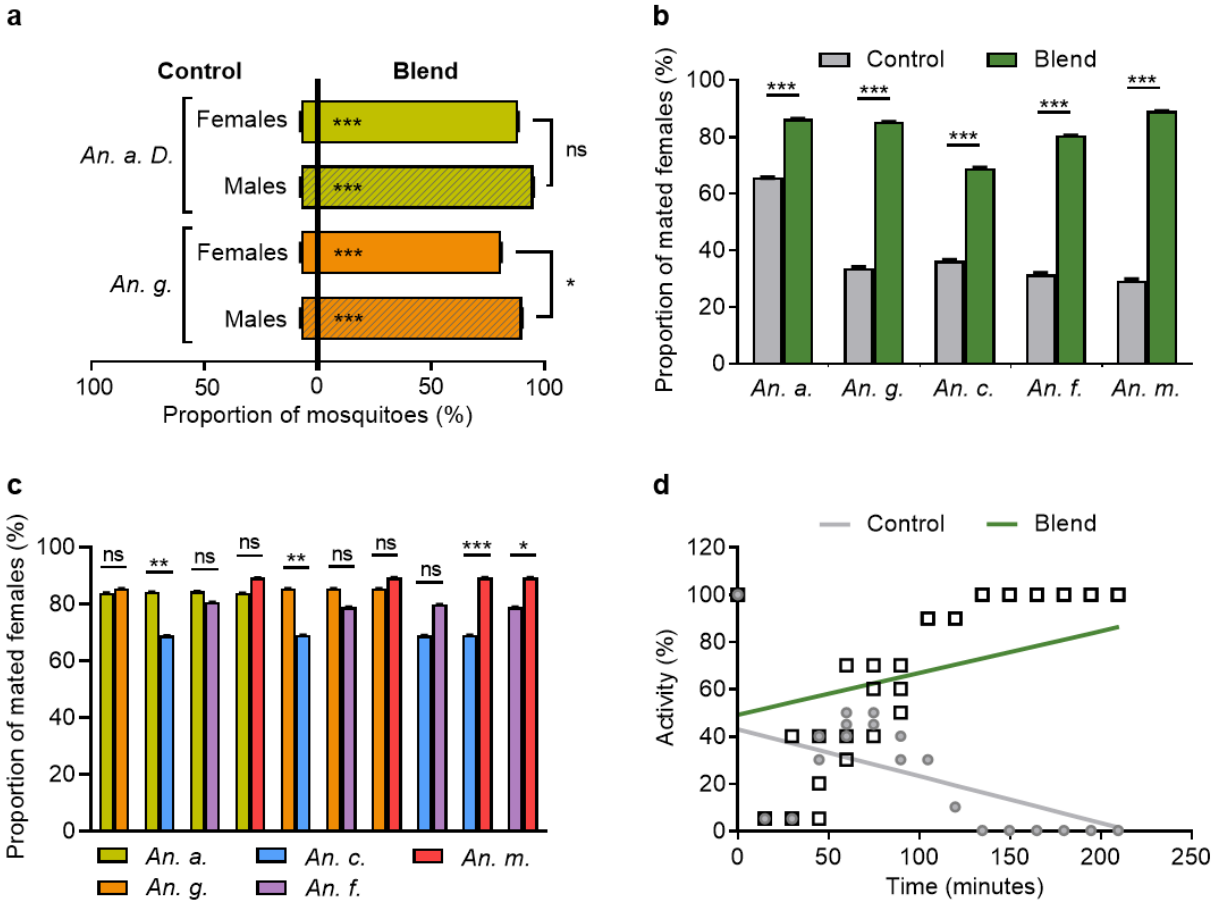
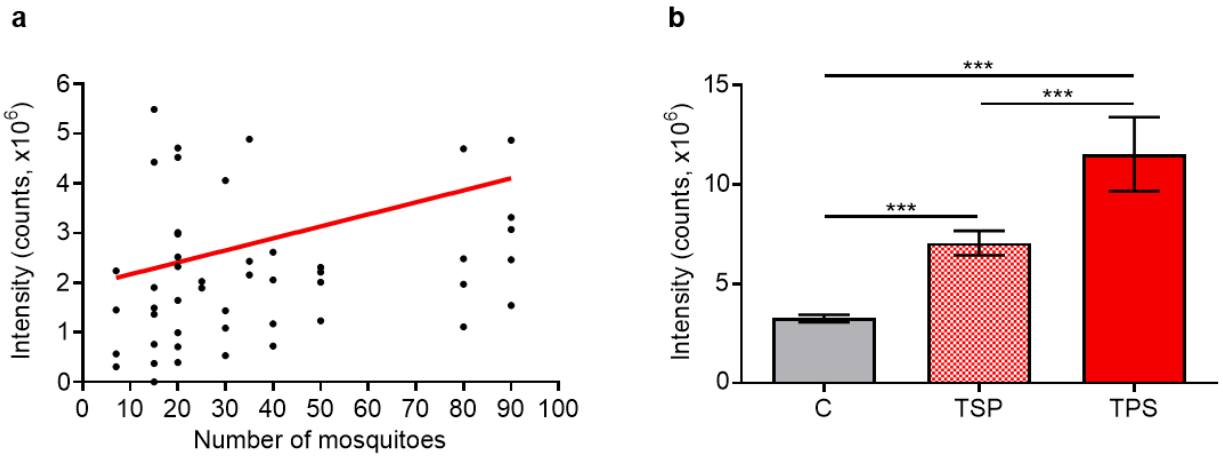


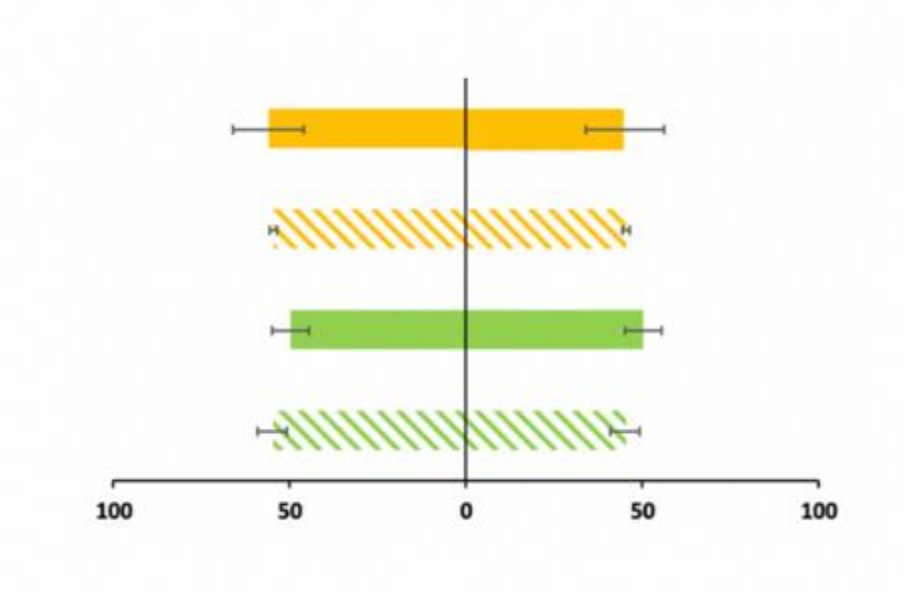
Fig. 2 | Behavioural responses of *Anopheles* mosquitoes to swarming odours. **a**, Responses of males and females of *An. arabiensis* (Dongola) and *An. gambiae* (Keele) in two-choice olfactometer bioassay [*An. gambiae* males/females (χ^2 = 4.10, $p < 0.05$); *An. arabiensis* males/females (χ^2 = 3.58, p = 0.05); males (χ^2 = 2.20, p = 0.13) and females (χ^2 = 2.61, p = 0.10) comparing *An. arabiensis* versus *An. gambiae* species, respectively.] **b**, Effect of the five component blend on mating of *An. arabiensis,* (KGB) *An. gambiae.* (G3), *An. coluzzii* (COGS), *An. funestus* (FANG), and *An. merus* (MAFUS) mosquitoes compare to control (solvent only) under semi-field conditions [*An. arabiensis,* χ^2 = 2.51, $p < 0.001$; *An. gambiae*: χ^2 = 62.56, $p < 0.001$; *An. coluzzii:* χ^2 = 18.02, $p < 0.001$; *An. merus:* χ^2 = 64.53, $p < 0.001$; *An. funestus*: χ^2 = 42.85, $p < 0.001$]. **c**, Par-way comparison of the blend mating stimulation effect among five *Anopheles* species. The values are taken from the logistic regression model estimations and the vertical bars are standard errors. Statistically different comparisons are shown by the asterisks (ns=non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **d**, Effect of the blend on swarming behaviour of *An. gambiae* males (n = 3 measurements, 30 males per each measurement), the release rate of the blend was 5 ng min^{-1}. The lines produced by the GLMM model estimations after considering the random effect of the replication. The green line represents the expected regression line for the mosquitoes exposed to the blend during 210 min, black squares show the percentage of the mean observational activity per each time point calculated via Noldus software. The grey line shows the expected regression line for the control group (without blend). The empty circles represent the proportion of the mean observational activity per each time point in control group calculated via Noldus software.
are shown

Extended Data



Extended Data Fig. 1 | Effect of swarming odours of *Anopheles arabiensis* (Dongola) and (KGB) as well as *Anopheles gambiae* (Keele)

a, The correlation between the number of swarming mosquitoes and amount of odours trapped, males of *An. arabiensis* (Dongola). **b**, Amounts of five swarming odours (VOCs) collected during the control, i.e. photophase (C) and transition periods from scotophase to photophase (TSP) and from photophase to scotophase (TPS). The values are taken from the General Linear Mixed Model estimations (GLMM), including the random effect of experimental replication. Significantly different comparisons are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). In panel b, top of the columns are medians and vertical bars represent standard errors.



Extended Data Fig.2 | Behavioural responses of mosquitoes to blend vs blend and control vs control. Responses of male and female mosquitoes [*An. arabiensis* (Dongola) and *An. gambiae* (Keele)] in two-choice olfactometer bioassay was evaluated. The yellow bars show *An.arabiensis*, and green bars represent *An.gambiae* panel. It is shown comparison of Control vs Control: [*An. arabiensisi* (Dongola) male: $\chi^2_1 = 0.26$, $P = 0.60$; *An. arabiensis* (Dongola) female: $\chi^2_1 = 0.34$, $P = 0.56$; *An. gambiae* (Keele) male: $\chi^2_1 = 0.33$, $P = 0.45$; *An. gambiae* (Keele) female: $\chi^2_1 = 0.40$, $P = 0.33$].

a

An. arabiensis (Dongola)															
	Control vs Not Swarming					Control vs Swarming					Not Swarming vs Swarming				
	Estimate		SD		p value	Estimate		SD		p value	Estimate		SD		p value
	C	N	C	N		C	S	C	S		N	S	N	S	
Acetoin	3886	11549	1266	1266	p<0,001	3886	24483	3724	3724	p<0,001	11549	24483	3801	3801	p<0,001
Sulcatone	346656	759936	159641	159641	p<0,001	346656	1532956	277942	277942	p<0,001	759936	1532956	303255	303255	p<0,001
Octanal	414799	703342	116016	116016	p<0,001	414799	1195981	185171	185171	p<0,001	703342	1195981	194435	194435	p<0,001
Nonanal	1752591	2364563	309784	309784	p<0,001	1752591	4381540	609920	609920	p<0,001	2364563	4381540	632097	632097	p<0,001
Decanal	815065	1694378	222888	222888	p<0,001	815065	3744481	510942	510942	p<0,001	1694378	3744481	522723	522723	p<0,001

b

An. arabiensis (KGB)															
	Control vs Not Swarming					Control vs Swarming					Not Swarming vs Swarming				
	Estimate		SD		p value	Estimate		SD		p value	Estimate		SD		p value
	C	N	C	N		C	S	C	S		N	S	N	S	
Acetoin	2880	23257	1850	1850	p<0,001	2880	64664	8643	8643	p<0,001	23257	64664	8805	8805	p<0,001
Sulcatone	226352	497790	137632	137632	p<0,001	226352	763110	193839	193839	p<0,001	497790	763110	222445	222445	p<0,001
Octanal	179654	412961	129885	129885	p<0,001	179654	585175	152947	152947	p<0,001	412961	585175	181694	181694	p<0,001
Nonanal	495134	826002	319413	319413	p<0,001	495134	1021211	275743	275743	p<0,001	826002	1021211	334947	334947	p<0,001
Decanal	205483	800963	294915	294915	p<0,001	205483	1139678	249946	249946	p<0,001	800963	1139678	382862	382862	p<0,001

c

An. gambiae (Keele)															
	Control vs Not Swarming					Control vs Swarming					Not Swarming vs Swarming				
	Estimate		SD		p value	Estimate		SD		p value	Estimate		SD		p value
	C	N	C	N		C	S	C	S		N	S	N	S	
Acetoin	546,7	829,7	702,6	702,6	p<0,001	600,1	48441,8	16979,8	13154,7	p<0,001	829,7	48441,8	16988,6	13159,3	p<0,001
Sulcatone	299129	610424	129661	129661	p<0,001	299129	1009568	125076	155615	p<0,001	610424	943657	160236	180243	p<0,001
Octanal	226932	383008	51459	51459	p<0,001	226932	423982	37088	47881	p<0,001	383008	423982	46593	60151	p<0,001
Nonanal	8516676	11320696	1724782	1724782	p<0,001	8516676	16329120	2199822	2006726	p<0,001	11320696	15540409	2302241	1981715	p<0,001
Decanal	5939757	8470897	1573064	1573064	p<0,001	5939757	13381965	2427656	2109990	p<0,001	8470897	13179546	2578946	2320681	p<0,001

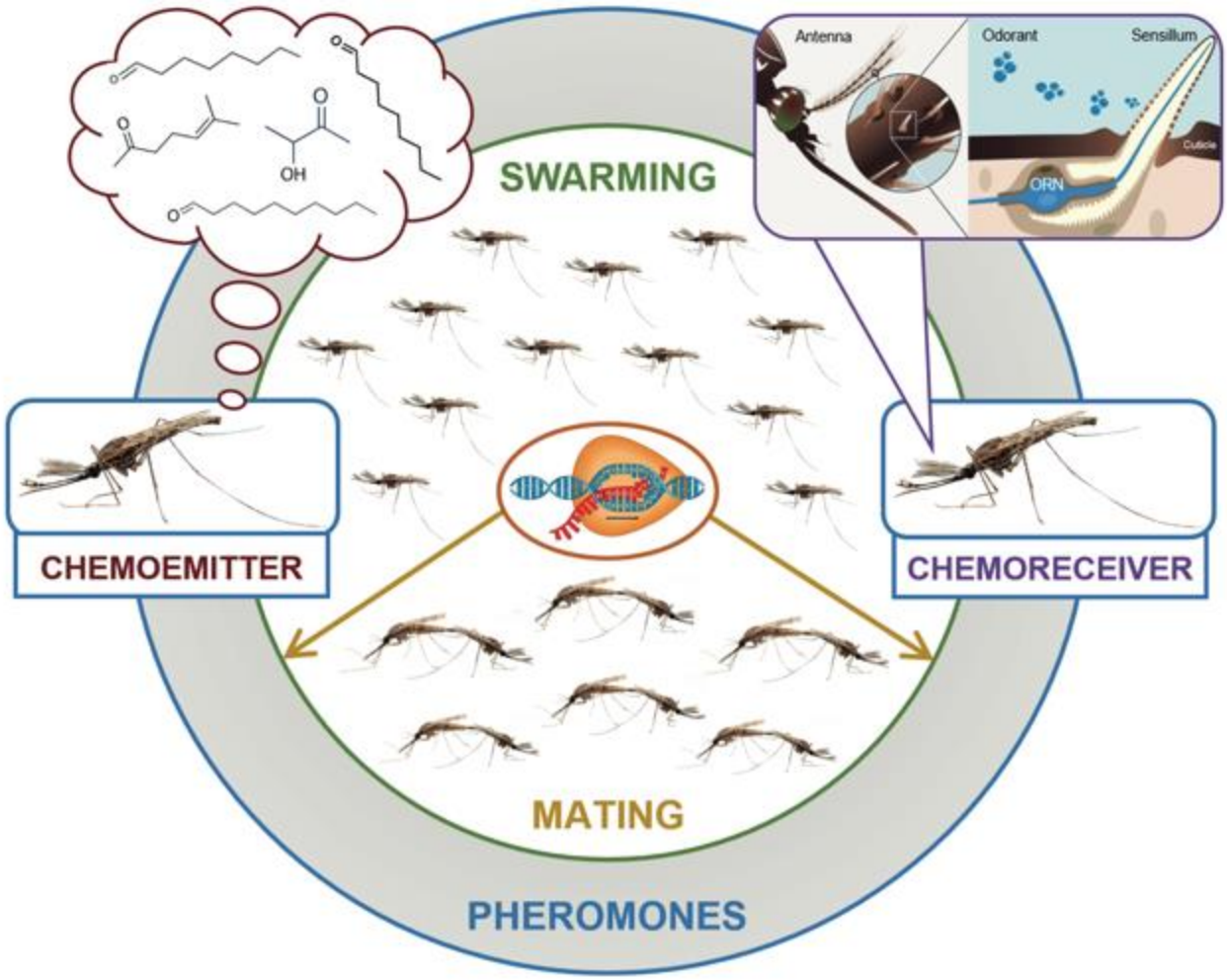
d

Labelled glucose experiment					
	Estimate		SD		p value
	NL	L	NL	L	
Acetoin	0.03120	0.23006	0.04995	0.04995	p<0,01
Sulcatone	0.02529	0.14857	0.03227	0.03227	p<0,01
Octanal	0.55533	0.63614	0.02330	0.02158	p<0,05
Nonanal	0.4030	0.4864	0.0100	0.0100	p<0,001
Decanal	0.41086	0.48100	0.02279	0.02279	p<0,01

Extended Data Table 1 | All comparisons, and model estimations, and Standard Errors (SE) for swarming odours of three different *Anopheles* species a-c, d shows the model estimation model estimations, and Standard deviation (SD) for labelled glucose experiment. The values are taken from the GLMM models using R statistical software (v.3.2.3). The models which used in these analyses were "lmer" and "glth" due to treating the random effect of experimental replicates (N=6). These models analysed using lmer followed by glth (Tukey) pairwise comparisons. β estimated (Estimates), standard error (SEs), and p values extracted from the model. In each replicate, 20-50 single male mosquitoes have been used.

comparisons	Statistical model estimation
An. arabiensis (An.a)/An.gambiae (An.g):	$\chi^2 = 0.11$, $p = 0.73$
An. arabiensis (An.a)/ An. coluzzii (An.c):	$\chi^2 = 0.60$, $p = 0.43$
An. arabiensis (An.a)/ An. coluzzii (An.c):	$\chi^2 = 7.02$, $p < 0.01$
An. arabiensis (An.a)/ An. funestus (An.f):	$\chi^2 = 0.60$, $p = 0.43$
An. arabiensis (An.a)/ An. merus (An.m):	$\chi^2 = 1.51$, $p = 0.21$
An. gambiae (An.g)/ An. coluzzii (An.c):	$\chi^2 = 8.42$, $p < 0.01$
An. gambiae (An.g)/ An. funestus (An.f):	$\chi^2 = 1.65$, $p = 0.19$
An. gambiae (An.g)/ An. merus (An.m):	$\chi^2 = 0.82$, $p = 0.36$
An. coluzzii (An.c)/ An. funestus (An.f):	$\chi^2 = 2.72$, $p = 0.09$
An. coluzzii (An. c)/ An. merus (An. m):	$\chi^2 = 12.56$, $p < 0.001$
An. funestus (An.f)/ An. merus (An.f):	$\chi^2 = 4.20$, $p < 0.05$

Extended data Table. 2 | Behavioural responses of *Anopheles* mosquitoes to swarming odours
Par-way comparison of the blend mating stimulation effect in five *Anopheles* species. The values are taken from the logistic regression model estimations and the vertical bars are standard errors. Statistically different comparisons are shown by the asterisks (ns=non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).



Extended Data Fig. 3 | Behavioural response of *Anopheles* mosquitoes upon reception of the pheromones. (1) Stimulated *Anopheles* males secrete an aggregation pheromones (Chemoemiter) which are a mixture of five volatile compounds including acetoin, sulcatone, octanal, nonanal and decanal. (2) The pheromones mediate formation and sustenance of swarm comprised of tens of thousands of flying males. (3) Males respond to the pheromones through antennal sensory organs (Chemoreceiver) with peak of swarming activity during the photoperiod transition(through the diel and circadian gene regulation). (4) After the male swarm a critical swarm size is initiated achieved, the pheromones enhance female attraction to the swarm and increase mating activity (the section between two arrows). Females respond to the mal pheromones (our data), and acoustic signal as an essential signal for coupling (previous literature) with characteristic agitated flight, which serves as attraction stimuli to males in the swarm, inducing males to copulate with females while flying.

Supplementary Information

Anopheles male pheromone increases swarming, female attraction to the swarm, and mating in five main African malaria vectors

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1. Supplementary, gene expression profiling of *An. gambiae* males

Results and Discussion

Apart from the chemical cues described in the main Results section, we also aimed to investigate the global transcriptome of male Anopheles mosquito in order to identify additional gene transcript during this complex behavioural phase of the male mosquito. In depth studies of these transcripts may provide insights into the eco-evolutionary molecular mechanism of male pheromone and metabolite roles in mating success and reproduction. Specifically, we used Illumina RNA sequencing to profile gene expression of virgin 5-7 days old *An. gambiae* male head and body separately at scotophase (middle of the period), photophase (middle of the period), and transition from photophase to scotophase (Supplementary Methods, below).

The canonical clock genes, *Period* (*agPER*, AGAP001856), *timeless* (*agTIM*, GAP008288), *Par-domain protein 1 (agPDP1*, AGAP006376), *Clock (agCLK*, AGAP005711) *and cycle (agCYC*, AGAP005655) were found to be rhythmically expressed under transition and scotophase in both

heads and bodies, consistent with findings in female *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*[68]. Moreover, these genes were rhythmic with high amplitude change and with predicted phase relationships (i.e., *agTIM*, and *agPER* sharing a similar peak phase and in antiphase with *agCYC*, and *agCLK*).

There are multiple published studies focusing on trascriptome of the female mosquitoes, and the main role of mating lipid transporter genes (such as *Lipophorin*; Lp)[69]. Particularly, it has been reported that a steroid hormone, 20-hydroxyecdysone (20E) injected by male mosquitoes during mating up-regulated Lp expression in female vectors[70]. Regulation of these genes is involved in delivering nutrients to the oogenesis process, and also has a dramatic impact on midgut transcription in the female prior to a blood meal[71]. By performing a global transcriptome study of the male mosquitoes, we sought to understand if the lipid transporter genes would mimic similar expression patterns at the time point when mating takes place and if this pattern is mirrored in the transcriptional changes observed in the mated females. During mating, male metabolites are transferred to the female and result in a transcriptional change of nearly 500 genes in female midgut alone[71]. In addition, mating enhances female susceptibility to the human malaria parasites. We found genes involved in glycolysis, the citric acid cycle, oxidative phosphorylation, and fatty acid oxidation, among other processes, to be differentially expressed in the transition period compared to the photophase. These genes include *Lipophorin* (*Lp*; AGAP00182), and fatty acid transport proteins (*Fatp*; AGAP001763). Fatty acid transport (*Fatp*) isoforms alone or in concert with specific long chain acyl CoA synthetase (Acyl) isoforms function to drive energy-dependent processes. It is reported that fatty Acyl reductases are specifically involved in biosynthesis of sex pheromones including aldehydes in moths[72]. Moreover, their roles in lipid metabolism may also lead to elevated levels of circulating free fatty acids[73], which could be used in *An. gambiae* pheromone and/or hormone biosynthesis. Furthermore, *CRP* genes were highly up-regulated in mosquito heads during the transition period. These genes are highly conserved among mosquitoes and play a role in the metabolism of xenobiotic compounds. We identified three known odorant-binding proteins (OBPs), and an odorant receptor, *OR52 (AGAP000230)*. Interestingly, the known OBPs oscillate at the end of the transition period from photophase to scotophase and at early scotophase in female mosquitoes. That has been viewed as a mechanism for preparing the system for an increase in arrival of odour molecules during the nocturnal host- and nectar-seeking behaviours[68]. The *OR52* (*AGAP000230*) was also rhythmic under transition conditions, peaking similarly to the OBPs near the end of the photophase. This result highlights the possibility of a synchronised rhythm in male olfactory activity and therefore gating of olfactory sensitivity occurring additionally downstream of the OBPs and olfactory receptors during swarming and mating activities.

Recent improvements in gene editing tools aimed at controlling malaria vectors, and our current proof of principle of male rhythmic transcriptional combined with chemo-behavioural variation, highlighted the need for further studies on male mosquito functional genomics for at least two main reasons. First, genetic control methods intend to interfere with mosquito mating success, using target genes that have a role in male rhythmic activity, pheromone/hormone biosynthesis and/or flight activity. Second, currently the majority of the genetic control techniques under development focus on female transcriptomics data.

Methods

Mosquito dissection and RNA extraction. Experiments were performed using 4-6 days old An. gambiae males. RNA samples were collected at three photophase-scotophase cycle time points: i) mid-photophase in climate chamber; ii) at transition from photophase to scotophase); iii) 2 hours after beginning of scotophase. Three replicates each comprised of 10 mosquitoes were collected and analysed for olfactory, circadian and neuroimmunological gene expression. Ten mosquitoes collected from each time point were pooled, with heads (including antennae) separated from bodies (including thorax and abdomen, with wings and legs). Dissected tissues were stored in 100 µl TRIzol (Life Technologies, Darmstadt, Germany) at -80 °C until RNA extraction. Mosquito samples were homogenised using NucleoSpin Bead Tubes Type B (heads) and Type D (bodies), respectively (Macherey-Nagel, Duren, Germany). Total RNA was extracted using NucleoSpin RNA Plus XS kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions.

Library preparation and sequencing. RNA integrity was estimated with a Bioanalyser 2100 system using an RNA 6000 Nano kit (Agilent Technologies, Santa Clara, US). RNA concentration was measured using a Qubit RNA Assay with a Qubit 2.0 Fluorometer (Life Technologies). Fifty ng to 1 µg-1 of the mosquito total RNA were used for strand-specific cDNA library preparation using TruSeq Stranded mRNA Library Prep kit (Illumina) and prepared as described in the manufacturer's low sample poly-A isolation protocol (Illumina). The libraries quality was assessed using a DNA 1000 kit on a Bioanalyser 2100 system (Agilent Technologies). DNA concentration was measured using a Qubit dsDNA Broad Range Assay with a Qubit 2.0 Fluorometer (Life Technologies). Libraries were normalized to 4nM, and then pooled; 75-bp paired-end sequencing was carried out on an Illumina NextSeq 500.

Bioinformatics analysis. The sequencing Fastq files were evaluated for overall quality using FastQC. Adapter sequences and low quality reads were removed using Trimmomatic (version 0.36; parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 SLIDINGWINDOW:4:15 MINLEN:70).

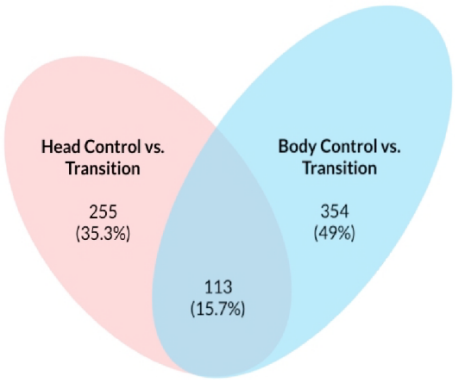
Reads retained after quality control were mapped to the mosquito reference genome using HISAT2 (version 2.1.0, using default parameters). Reference genome sequence and annotation were downloaded from Vectorbase.org (AgamP4, version12). Gene read counts were generated using HTSeq-count (version 0.9.1; parameters: -s no -t exon -i gene_id -r pos -m intersection-nonempty) and custom bash scripts (available on demand). Differential gene expression analysis was performed in R using DESeq2 package with default parameters. Visualisations were performed using various graphical packages in R.

2. Supplementary Figures

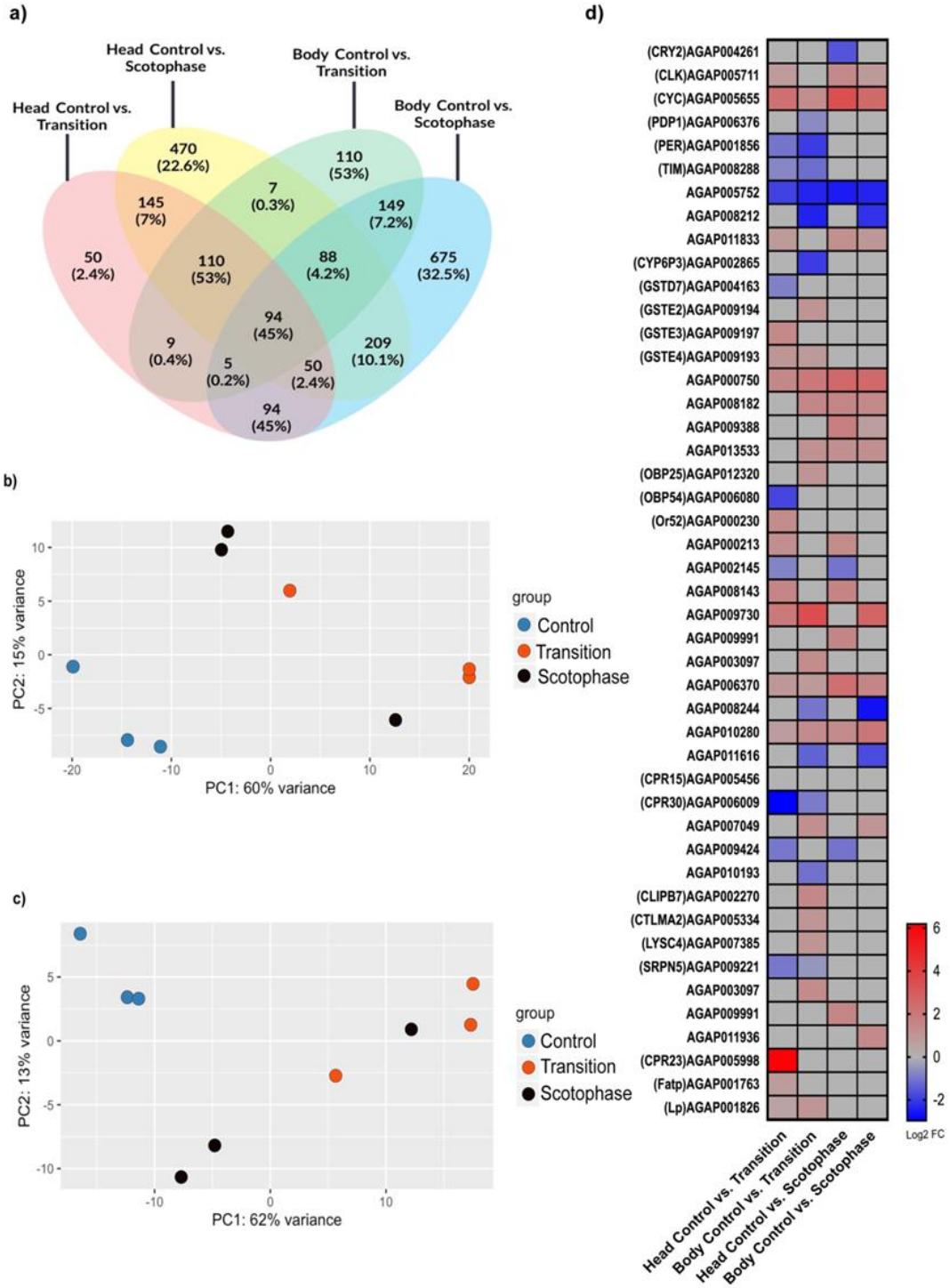
d)

Gene name	Head Control vs. Transition	Body Control vs. Transition
(CLK)AGAP005711	0,78	0,00
(CYC)AGAP005655	2,27	1,25
(PDP1)AGAP006376	0,00	-0,67
(PER)AGAP001856	-1,04	-1,98
(TIM)AGAP008288	-0,74	-1,14
(CYP6P3)AGAP002865	0,00	-1,97
(GSTD7)AGAP004163	-0,82	0,00
(GSTE2)AGAP009194	0,00	0,98
(GSTE3)AGAP009197	1,41	0,00
(GSTE4)AGAP009193	1,03	0,81
(OBP25)AGAP012320	0,00	0,97
(OBP54)AGAP006080	-1,82	0,00
(Or52)AGAP000230	1,32	0,00
(CPR30)AGAP006009	-2,97	-0,93
(CLIPB7)AGAP002270	0,00	1,48
(CTLMA2)AGAP00533	0,00	1,03
(LYSC4)AGAP007385	0,00	1,00
(SRPN5)AGAP009221	-1,01	-0,49
(CPR23)AGAP005998	6,18	0,00
(Fatp)AGAP001763	0,75	0,00
(Lp)AGAP001826	0,52	0,98

c)



Supplementary Fig. 1 | Differential expression of genes in *Anopheles gambiae* (Keele) males during swarming (transition from photophase to scotophase) compare to resting (photophase). The head and body of *An. gambiae* transcriptome was compared in two light-dark cycle stages: transition from photophase to scotophase; and the middle of photophase (control). Differential expression levels of genes including those involved in, biological processes such as metabolism, detoxification, olfaction, vision, cuticle regulation, and immunity, are listed (displayed as log2 fold changes over control, $p < 0.05$). The accession numbers and acronyms of annotated genes are shown. **a-b,** The principal component analysis (PCA) of expression level in Head and Body. The percentage of the variation explained by the principal components is shown after axis title. **c,** Venn diagram shows the total number of transcripts in head and body of *An. gambiae* identified as rhythmic in transition period. **d,** The whole body *An. gambiae* male transcriptome (5-7 days after emergence), at scotophase (middle of the period), photophase (middle of the period), and transition from photophase to scotophase. Differential expression levels of genes including those involved in, glycolysis, the citric acid cycle, oxidative phosphorylation, and fatty acid transportation, and oxidation, metabolic fluctuations, as well as rhythms and olfactory activity are listed (displayed as log2 fold changes over control [photophase], $p < 0.05$). The accession numbers and acronyms of annotated genes are shown



Supplementary Fig. 2 | Differential expression of genes in *Anopheles gambiae* Keele strain males in three photophase-scotophase cycle stages. The head and body of *An. gambiae* transcriptome was compared in three photophase-scotophase cycle stages: transition from photophase to scotophase, scotophase (2 hours after transition), and middle of photophase (control). Differential expression levels of genes including those involved in, biological processes such as metabolism, detoxification, olfaction, vision, cuticle regulation, and immunity, are listed (displayed as log2 fold changes over control, $p < 0.05$). The accession numbers and acronyms of annotated genes are shown. **a,** Venn diagram shows the total number of transcripts in head and body of *An. gambiae* identified as rhythmic in transition and dark stages compare to control. **b-c,** The PCA of expression level in Head and Body. The percentage of the variation in two conditions explained by the principal components. **d,** Hierarchical clustering of genes found rhythmic using the pattern matching algorithm in head and body. Red indicates higher expression, and blue indicates lower expression.

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