scientific reports

Check for updates

OPEN

# Age-dependent perception of floral emissions and the role of $CO_2$ in regulating nectar-seeking in mosquitoes

Bonaventure A. Omondi[1,3], Betelehem Wondwosen[2,3], Mengistu Dawit[1], Sharon R. Hill[1] & Rickard Ignell[1]✉

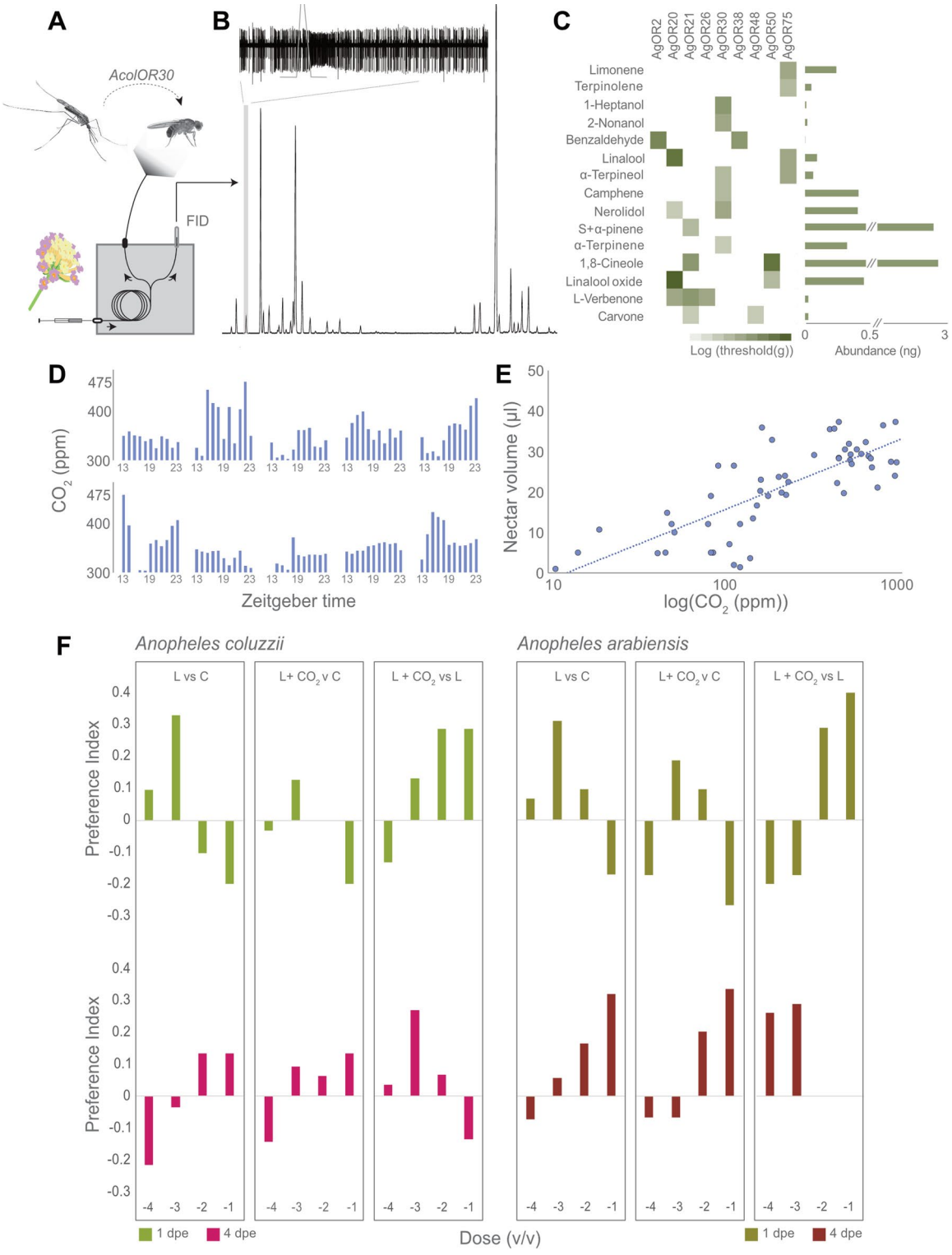
**Mosquitoes require access to nectar for energy and reproduction, a behaviour which varies depending on adult maturation and gonotrophic cycle. To locate and discriminate among nectar resources, mosquitoes may make use of various floral emanations, including volatile organic compounds and carbon dioxide ($CO_2$). In order to identify the bioactive volatile organic compounds (VOCs) in one of the preferred host plants of *Anopheles* mosquitoes, *Lantana camara*, combined electrophysiological and chemical analyses were performed using ectopically expressed *Anopheles coluzzii* odorant receptors (Ors) in the empty neuron system of *Drosophila*. When presented as a synthetic odour blend, and controlled for the detected ratio and emission rate of individual VOCs, the blend elicited an age- and dose-dependent attraction of *An. coluzzii* and of the closely-related *Anopheles arabiensis*. *Lantana camara* demonstrated a differential circadian emission of $CO_2$, which directly correlated with the volume of nectar secreted. Behavioural assays designed to determine the role of ecologically-relevant concentrations of $CO_2$ in regulating nectar seeking, demonstrated a context-dependency, emphasizing that $CO_2$ is used for close-range floral discrimination during foraging. This study demonstrates a mechanism regulating the detection and perception of ecologically-relevant information by mosquitoes during sugar seeking.**

**Keywords** Mosquito, Nectar seeking, Behaviour, Chemical ecology, Volatile organic compounds, Carbon dioxide

Female mosquitoes generally sugar feed shortly after adult emergence to gain energy for flight, increase the probability of survival and storage of reserves for egg production[1,2]. The females feed less regularly on nectar as these develop the capacity to blood feed and enter the gonotrophic cycle[1,3,4]. During the search for sugar and other nutrients, mosquitoes visit predominantly floral nectaries, which are located using a combination of visual and olfactory cues[1,5,6]. High nocturnal emissions of carbon dioxide ($CO_2$) may guide nectar-feeding insects to sugar-rich resources[7–9]. Chemical analysis has provided ample information concerning the complexity of natural floral volatile organic compounds (VOCs)[10,11], and a growing body of literature is demonstrating which VOCs are detected by mosquitoes[1,12], the role $CO_2$ plays in nectar foraging[9], and how detection and perception of floral emanations change as females mature, and how this compares to our current understanding of the cues that drive blood seeking.

The nutritional value of preferred plants appears, in general, to correlate with the ability of these plants to attract mosquitoes, as nectar availability and quality significantly impact mosquito fitness, although evidence opposing this correlation has also been reported[1,2]. *Lantana camara* is visited by several nocturnal mosquito species, including the African malaria vector, *Anopheles gambiae*[1,13]. *Lantana camara*, a flowering plant in the verbena family, is native to the American tropics, which has been introduced and spread across Africa where it is considered an invasive, noxious weed. The nutrients gained by feeding on *L. camara* nectar translate into an increase in survival and fecundity[13–15]. To limit the search for profitable flowers, nectar-seeking insects may use $CO_2$ emissions from nectar-containing flowers as a short- or long-range cue[7,8]. Plants become net $CO_2$ producers during peak nectar foraging of nocturnal insects[7]. Hence, elevated $CO_2$ emission could play

[1]Disease Vector Group, Division of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Alnarp, Sweden. [2]Department of Zoology, Addis Ababa University, Addis Ababa, Ethiopia. [3]Bonaventure A. Omondi and Betelehem Wondwosen Shared first authors. ✉email: rickard.ignell@slu.se




a role as a cue for long-range activation and attraction, as demonstrated for both nectar- and vertebrate host-seeking haematophagous insects[7], although likely diffuse in the diverse background of the wide-diversity of $CO_2$-emitting plants. Alternatively, mosquitoes could use $CO_2$ emission as a true signal for nectar reward, as demonstrated for the moth, *Manduca sexta*[8,16].

Floral scent varies between taxonomically related and diverse species, with more than 1700 VOCs identified to date, of which terpenoids and aliphatics, including fatty acid derivatives, benzenoids and phenylpropanoids, are the major classes[10,11]. Based on available studies, mosquitoes appear to detect only a fraction of these VOCs, predominantly within the major classes of VOCs associated with floral odour[1,12], emphasizing the selective tuning of the female mosquito peripheral olfactory system. This selectivity is determined by the differential sensitivity of the variable odorant receptors (ORs), expressed in the olfactory sensory neurons (OSNs), to, *e.g.*, plant VOCs[17,18]. In response to ageing, and other physiological-state changes, differential expression of mosquito

◀**Fig. 1**. *Lantana camara* floral emanations differentially regulate floral seeking by mosquitoes. (**A**) Schematic of the combined gas chromatography-coupled single sensillum recording (GC-SSR) setup used to identify bioactive volatile organic compounds via the ectopic expression of *Anopheles coluzzii* odorant receptors (ORs) in the empty neuron system of *Drosophila*. FID, flame ionization detector. (**B**) Sample GC-SSR trace, with the SSR trace at the top showing the response of the *AcolOR30*-expressing neuron (large amplitude neuron) to 1-heptanol eluting from the GC. The FID chromatogram showing the elution of *L. camara* odorants. (**C**) Heat map displaying the threshold of response of the Ors, presented numerically, responding to *L. camara* odorants, according to retention time, along with release rates. (**D**) Circadian emission of $CO_2$ from single *L. camara* inflorescences throughout scotophase. (**E**) Nectar volume correlates with $CO_2$ emission from *L. camara* flowers (n = 61). (**F**) Dose-dependent behavioural responses of teneral (1 day post-eclosion (1 dpe)) and mature (4 dpe) female *An. coluzzii* and *Anopheles arabiensis* to the synthetic *L. camara* odour blend with or without $CO_2$ versus a solvent control or the synthetic *L. camara* odour blend alone. A general regression with a beta-binomial distribution demonstrated a significant effect of dose, age, species and context on the behavioural response.

chemosensory genes encoding ORs, and the units forming the $CO_2$ receptor (expressed in the maxillary palps), may alter the sensitivity of the OSNs to select VOCs and $CO_2$, as well as the downstream behavioural response of the female[4]. Such changes may, in turn, alter the downstream coding and perception of information, including odour blend recognition, which is critical for accurate selection and discrimination of a resource.

In this study, a reverse-chemical approach was used, providing a high-resolution assay to identify the ORs of the malaria vector, *Anopheles coluzzii,* tuned to the *L. camara* floral scent. A comparative behavioural analysis, in combination with electrophysiological recordings, was then used to assess the potential mechanism regulating differential age-dependent attraction of *An. coluzzii,* and its sibling species *Anopheles arabiensis,* to a synthetic *L. camara* floral odour. Moreover, $CO_2$ emission from *L. camara* flowers was measured, and its association with nectar secretion assessed to explore the role of $CO_2$ in regulating plant foraging.

## Results

### Tuning of *An. coluzzii* ORs to *L. camara* floral scent

Ectopic expression, in the empty neuron system of *Drosophila*, of the near-complete repertoire of the reliably expressed tuning ORs of female *An. coluzzii* (Fig. 1A) provided a high-resolution identification of the ORs involved in the detection of VOCs within *L. camara* floral scent. Extracellular GC-SSR from the OSN ectopically expressing the individual *An. coluzzii* ORs, (Fig. 1B), followed by GC–MS analyses, allowed for the identification of the cognate bioactive odorant ligands. A total of 15 VOCs elicited reproducible responses across 9 of the 49 tested ORs (Fig. 1C; left). The ORs differed in sensitivity to the tested odorants, as determined by dose-dependent SSR analysis of the ectopically expressed ORs, and visualised by a heat plot indicating differences in the threshold of response (Fig. 1C; right). The overall volatile release rate was ca 8 ng $h^{-1}$, with *S* + α-pinene and 1,8-cineole as the most abundant VOCs (Fig. 1C; right). Individual VOCs activated either a single or a small subset of ORs (≤ 3), and individual ORs similarly responded to either a single or a subset (≤ 6) of odorants (Fig. 1C; left).

### Carbon dioxide release and nectar secretion

Using a differential infrared gas analyser, the absolute concentration of $CO_2$ emitted by *L. camara* inflorescences was measured in two separate experiments to assess variation over time and correlation with nectar secretion. The absolute $CO_2$ emission from individual inflorescences varied substantially, with shorter bursts or, more commonly, gradual changes in emission, from ca 300 ppm to up to 475 ppm, occurring throughout scotophase (Fig. 1D), with an average ambient $CO_2$ level in the room of ca 350 ppm. Next, floral $CO_2$ emission was measured from individual or grouped inflorescences, after which all individual flowers were sacrificed and nectar secretion measured, demonstrating that total nectar secretion of individual flowers was positively correlated with the absolute concentration in $CO_2$ emissions ($F = 88.33$, $p < 0.0001$, $r^2 = 0.60$, $y = -18.57 + 7.29(\log_{10}(x))$; Fig. 1E).

### Behavioural response to *L. camara* floral emissions

While *L. camara* floral emissions did not elicit species-dependent behavioural responses, *An. coluzzii* and *An. arabiensis* displayed significant dose-dependent responses ($\chi^2 = 3.73$, $p = 0.05$), which were affected by age ($\chi^2 = 23.27$, $p < 0.0001$) and context ($\chi^2 = 9.26$, $p = 0.0091$), in a two-choice Y-tube olfactometer. When assessed alone, against a solvent control, a synthetic blend composed of the 15 bioactive *L. camara* VOCs, presented at their detected ratios, elicited a similar age- and dose-dependent response in the two species (Fig. 1F; L vs C). Teneral (1 dpe) females were attracted to the lower doses tested, whereas the highest doses tested were aversive (Fig. 1F; L vs C). The opposite was found for 4 dpe females (*post-hoc* $\chi^2 = 9.48$, $p < 0.0021$) (Fig. 1F; L vs C). Pulsed $CO_2$, 100 ppm above ambient $CO_2$ level, representing an ecologically-relevant concentration emitted by a single inflorescence, elicited a mild, but significant aversion in 1 dpe females ($\chi^2 = 9.36$, $p < 0.0025$), whereas 4 dpe mosquitoes were indifferent ($\chi^2 = 1.45$, $p = 0.23$). A similar behavioural response was observed for both species, when the synthetic *L. camara* blend was overladen by pulses of 100 ppm $CO_2$ (*post-hoc* $\chi^2 = 5.63$, $p < 0.0171$) (Fig. 1 F; L + $CO_2$ v C). To assess whether the context in which the mosquitoes perceive the two types of *L. camara* floral emissions affects the behavioural response, females were next provided the choice between the synthetic *L. camara* blend either alone or overladen by pulses of 100 ppm $CO_2$. The presence of $CO_2$ elicited a synergistic response to the synthetic *L. camara* blend, and shifted the dose-dependent attraction of both 1 dpe and 4 dpe mosquitoes to higher and lower doses, respectively (*post-hoc* $\chi^2 = 7.33$, $p < 0.0068$) (Fig. 1F; L + $CO_2$ v L).

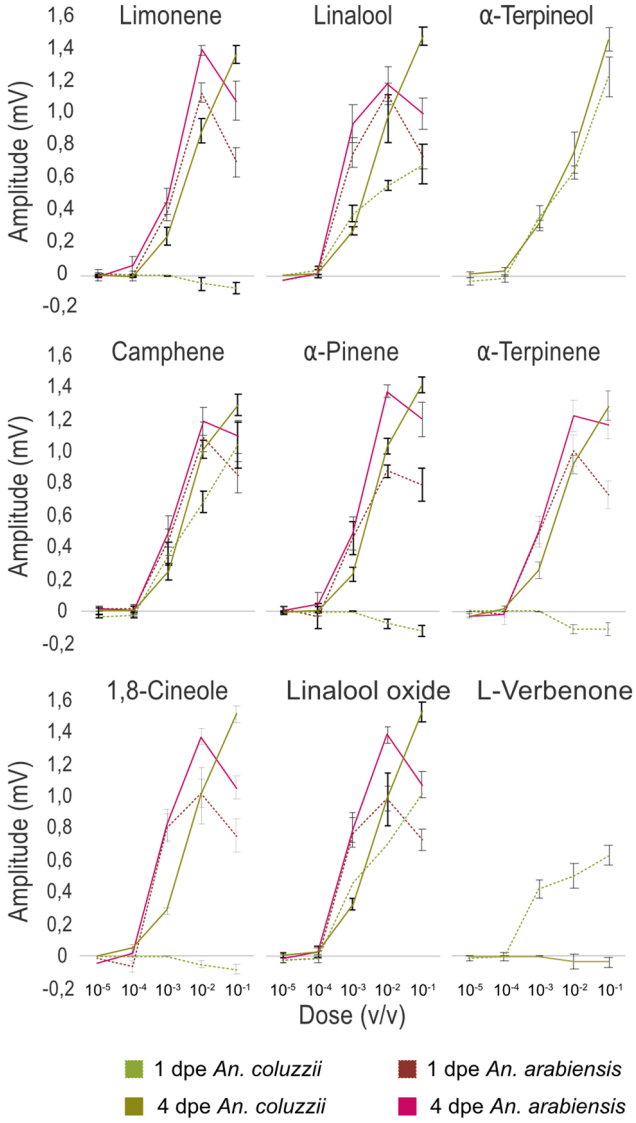



**Fig. 2**. Bioactive volatile organic compounds in *Lantana camara* odour elicit age- and dose-dependent antennal responses in *Anopheles coluzzii* and *Anopheles arabiensis*. Error bars indicate standard error of the mean (n = 10).

## Age-dependent antennal response to *L. camara* VOCs

Electroantennography demonstrated a species-, age- and dose-dependent response to the 9 tested bioactive VOCs originating from *L. camara* floral odour (Fig. 2; Supplementary Table S2). The antenna of 4 dpe females of both species displayed similar sensitivity to the tested VOCs, a response which was generally and significantly enhanced compared to 1 dpe mosquitoes (Fig. 2; Supplementary Table S2). Notably, the antenna of 1 dpe females of *An. coluzzii* did not respond to some of the VOCs tested, including limonene, α-pinene, α-terpinene and 1,8-cineole, whereas these VOCs elicited a significant response in 4 dpe females (Fig. 2). Conversely, verbenone was detected by the antenna of 1 dpe females but not that of 4 dpe females (Fig. 2).

## Discussion

Mosquitoes display an age-dependent behavioural response to resources to obtain nutrients for flight energy, survival and reproduction[1,3,4]. In this regard, sugar-rich plant-derived resources, *i.e.,* nectar and phloem, constitute a critical source of energy for adult mosquitoes of all ages[1,2]. To locate nectar-rich flowering plants and other resources, mosquitoes rely predominantly on blends of VOCs[1,3,12], detected by salient ORs[17,18], in a species-dependent manner, yet resulting in a similar age-dependent behavioural response, as shown in this study for *L. camara* and two *Anopheles* species. Carbon dioxide, which is emitted from individual *L. camara* inflorescences when the nectar rewards are richest, modulates the response of *Anopheles* mosquitoes in a synergistic and context-dependent manner, and thereby may reduce the time needed to spend seeking nectar sources. As a whole, this study provides novel information concerning how mosquitoes detect and perceive

ecologically-relevant information during sugar seeking, as well as indicating potential adaptive mechanisms regulating resource seeking, which are elaborated on below.

Mosquitoes generally rely on distinct blends of VOCs for locating and discriminating among potential resources, including flowering plants[3,5,12]. The vast majority of bioactive VOCs identified in the headspace of *L. camara* were isoprenoids, including terpenoids and a sesquiterpenoid, to which at least seven ORs are tuned, with members of other chemical classes including two alcohols and benzaldehyde, also being detected by select *An. coluzzii* ORs [this study]. As such, this study confirms and extends our understanding of the function of these tuning ORs[17–19], and emphasises the important role of the detection of isoprenoids for host plant location by mosquitoes. Isoprenoids constitute a major part of floral scent in general[10,11], but are also associated with vertebrate hosts and breeding sites[21,22], thereby demonstrating that the peripheral olfactory system of mosquitoes is tuned to parsimonious VOCs[3], and that select tuning ORs appear to be under a strong selection pressure to detect select members of this highly diverse chemical class. The species- and age-dependent physiological responses to terpenoids may correlate with differences and changes, respectively, in the transcript abundance of the genes encoding for the salient ORs, which translate into an altered behavioural sensitivity to the synthetic *L. camara* odour blend, as previously demonstrated for the response to human host emanations in various mosquito species, including *An. coluzzii*[4,23,24]. In summary, the behavioural response of the two species appears to be conserved, however, the demonstrated differences in the physiological tuning to individual floral VOCs suggests a conservation of information processing at the higher olfactory centres that does not necessarily correlate with a conservation of the peripheral olfactory pathways.

Both *Anopheles* species displayed an age-dependent response to $CO_2$ alone, ranging from aversive to neutral, translating into a context-dependent additive or synergistic response when $CO_2$ was presented in the background of the synthetic *L. camara* floral odour blend. The differential behavioural response to $CO_2$ alone emphasises a change in the innate valence of this well-characterised cue, which may be regulated by central nervous circuits, as the peripheral $CO_2$-sensitive neurons in the maxillary palps of both age groups has a similar threshold of response[20]. The observed context-dependent response to $CO_2$ and the synthetic *L. camara* floral odour blend does not lend support to the hypothesis that mosquitoes use $CO_2$ released from single flowers as a cue for nectar seeking[9], rather as a true signal for nectar reward, which was initially demonstrated in the moth, *M. sexta*[8,16]. However, as opposed to *M. sexta*[8], *Anopheles* mosquitoes do not respond to floral emanations in a scale-dependent manner. The demonstrated context-dependent response to *L. camara* floral emanations thus emphasises taxon-dependent differences in blend recognition and perception of floral emanations, and may be an adaptation of *Anopheles* mosquitoes to reduce foraging time and metabolic cost, as well as predation risk. The increased sensitivity to $CO_2$ in older mosquitoes, as a result of the maturation of neuronal responsiveness, could further reduce the cost and risk.

Nectar seeking and feeding by mosquitoes, which constitutes an essential source of nutrition for adults, has received considerably less attention than the mechanisms regulating human host seeking. While the sensory cues regulating nectar seeking in mosquitoes have gained some attention in recent times, the underlying neuronal and molecular mechanisms of how these cues are detected and perceived, as well as the relative contribution of each cue, are poorly understood. This study provides a novel insight into these mechanisms, which could be exploited in the future development of attractive toxic sugar bait technology, as well as garnering further insight into the effect of plant-derived sugars on vectorial capacity.

## Methods

### Drosophila

The 49 available UAS-AcolOR transgenes were crossed into the ΔHalo genetic background of *Drosophila melanogaster* containing the Or22a-Gal4 construct (courtesy of Prof. John Carlson, Yale University)[18]. All AcolORs were functionally validated through single sensillum recording analysis[19].

### Mosquito rearing

*Anopheles coluzzii* (Suakoko strain) were reared and maintained at 27 ± 1 °C and 65 ± 5% relative humidity under a 12 h light: 12 h dark regimen. Eggs were hatched in larval trays (30 cm × 15 cm × 5 cm), half-filled with distilled water, and emerging larvae (approximately 200 per tray) fed every other day with Tetramin Baby fish food (1 mg larvae$^{-1}$ day$^{-1}$; Tetra GmbH, Melle, Germany). Pupae were transferred to Bugdorm cages (30 cm × 30 cm × 30 cm; MegaView Science, Taichung, Taiwan), and after emergence, adult mosquitoes were provided ad libitum access to a 10% sucrose solution. For colony maintenance, females were fed on sheep blood (Håtunalab AB, Bro, Sweden), using a membrane feeding system (Hemotek, Blackburn, UK). Non-blood fed mosquitoes, 1-day post-emergence (dpe) or 4 dpe, were used for the experiments.

### Collection of floral *Lantana camara* headspace odour

The headspace odour of *L. camara* flowers was collected under field conditions in Kisumu, Kenya. During mid-day, individual flowers were covered with fine mesh to prevent nectar foraging by other insects. Shortly before sunset, a single inflorescence was enclosed in a 1 l polyamide roasting bag (Toppits Cofresco, Frischhalteprodukte GmbH & Co., Minden, Germany). For the closed loop volatile headspace collection, an activated charcoal-filtered airstream (100 ml min$^{-1}$) was circulated through the polyamide bag and an adsorbent column, using a diaphragm vacuum pump (KNF Neuberger, Freiburg, Germany), for 3 h. A total of ten replicates were collected, 1 h-to-3 h after sunset. As a control, headspace collection from an empty polyamide bag was performed in parallel with the other collections. The adsorbent column was made of Teflon tubing (70 mm × 5 mm i.d.), holding 40 mg Porapak Q (50/80 mesh; Waters Associates, Milford, USA) between glass wool plugs. The columns were rinsed with 1 ml each of methanol (> 99.8%, Sigma-Aldrich, Stockholm, Sweden), acetone (> 99.5%, Sigma-Aldrich) and pentane (99.0%, Sigma-Aldrich) prior to use. Following headspace collections, the columns were placed in

a 10 ml vial covered with aluminium foil, and then kept at -20 °C before being transported to Sweden. Adsorbed volatiles were eluted with 400 µl pentane, pooled, concentrated under a gentle stream of nitrogen to contain 0.25 min equivalents $\mu l^{-1}$ and then stored at -20 °C until further analyses. Before concentration, heptyl acetate (1 µg, 99.8%; Sigma-Aldrich) was added to the pooled extract as an internal quantification standard.

## Carbon dioxide concentration and nectar secretion

Potted *L. camara* shrubs (Flyinge Plantshop, Flyinge, Sweden) were used to measure $CO_2$ emission and nectar secretion from individual inflorescences. Intact inflorescences were individually enclosed in a 1 l polyamide roasting bag, carefully fitted and sealed around the stem, ca 30 min before the onset of the analysis. Ethylene vinyl acetate tubing, lined with polyethylene, serving as air input and output, connected the bag with a differential infrared gas analyser (LI-7000, LI-COR Biosciences, Lincoln, USA). Between measurements, ambient air, scrubbed of $CO_2$ using ascarite II (LI-COR) and de-humified using magnesium perchlorate, was pumped continuously ($0.9\ l\ min^{-1}$) through the bag. For absolute measurement of $CO_2$ concentration, a solenoid valve diverted the airflow to a closed loop that included the chamber and the LI-7000. The transient increase in $CO_2$ in the loop was recorded for 1 min. Measurements from individual inflorescences (n = 10) were taken to assess circadian change, from 19h00 to 5h00, in $CO_2$ concentration, together with measurements of the ambient level of $CO_2$ in the room. Moreover, $CO_2$ concentration was measured from inflorescences, different from those in the previous experiment but from the same plants, which were then sacrificed for nectar collection. Nectar was collected and measured from individual flowers of the inflorescences, using micro capillaries. After removal from the inflorescence, a capillary was gently moved into contact with the bottom of the flower. Care was taken to avoid damage to the floral tissue. Log-linear relationship between nectar volume and $CO_2$ concentration (ppm) was determined using general regression (JMP®, Version 17. SAS Institute Inc., Cary, NC, 1989–2023).

## Combined GC-SSR and GC–MS analyses

Tentative ligands for the 49 AcolORs, expressed in the *Drosophila* empty neuron system, were determined using combined gas chromatography (GC) and single sensillum recording (SSR) analysis, as previously described (Omondi et al., 2019). In short, an Agilent 6890 GC (Agilent Technology, Santa Clara, USA), fitted with a fused silica capillary column (30 m × 0.25 mm i.d.) coated with a non-polar HP-5 stationary phase (d.f. = 0.25 µm), was used to separate the VOCs within the pooled headspace extract of *L. camara* flowers. The GC was fitted with a make-up hydrogen-fed four-way cross at the end of the column, delivering half of the effluent to the flame ionization detector, and the other half to the air stream passing over the antenna of the fly via a heated transfer line. Responses to eluting compounds were verified through at least three independent injections.

The bioactive VOCs determined through GC-SSR analysis were identified using combined GC (Agilent Technology 6890N GC) and mass spectrometry (Agilent Technology 5975 MS). The GC was fitted with the same type of non-polar column, and ran under the same conditions as for the GC-SSR analysis. The bioactive VOCs were identified according to Kovats' retention indices and mass spectra, by comparison with reference mass spectra in a commercially available library (NIST05, Agilent). Bioactive VOCs were confirmed by parallel injections of synthetic reference compounds and authentic samples on the GC-SSR and GC–MS (Supplementary Table S1). For each bioactive VOC, the detected release rate, normalised to the internal standard, was calculated. To determine the detection threshold for the bioactive VOCs, serial dilutions of synthetic compounds were tested using GC-SSR, with each respective AcolORs expressed in the *Drosophila* empty neuron system. The detection limit was identified as the lowest $\log_{10}$ dilution at which a response significantly higher than the solvent control was recorded. The detection threshold is expressed as the negative log of the dilution of the lowest detection level.

## Behavioural analysis

To assess the behavioural response of 1 dpe and 4 dpe mosquitoes, a Y-tube olfactometer (100 mm i.d. × 1200 mm total length), illuminated from above with red light at 2–5 lx, was used. A charcoal-filtered and humidified laminar air stream (26 ± 1 °C, RH 75 ± 5%) flowed through the olfactometer at $30\ cm\ s^{-1}$. The odour blend tested mimicked the composition and ratio of all VOCs identified in the *L. camara* floral headspace (Supplementary Table S1), diluted in pentane and released by diffusion from wick dispensers, using 1.5 ml glass vials (GeneTec AB, Västra Frölunda, Sweden). The wicks were constructed of 5 cm × 1.5 mm Teflon™ tubing lined with an unbleached cotton thread, and inserted through the lid of the vial to allow for the release of all VOCs in constant proportions throughout the experiment[25]. As a control, pentane was released in a similar manner. The wick dispensers were inserted into glass wash bottles (500 ml; Lenz Laborglas, Wertheim, Germany), and delivered to the upwind end of the olfactometer through Teflon™ tubing, by passing charcoal-filtered and humidified air, at $0.5\ l\ min^{-1}$, through the wash bottles. In experiments designed to assess how an ecologically-relevant concentration of $CO_2$ regulates attraction and discrimination of mosquitoes towards floral odour, the odour blend was embedded within a background of pulsed $CO_2$ (500 ppm; 100 ppm above ambient $CO_2$ level, at 0.5 Hz), as previously described[20]. The $CO_2$ level was monitored at the downwind end of the bioassay using a $CO_2$ analyser (LI-820, LI-COR Biosciences). As a negative control, pentane was used and introduced as described above into either of the arms in the Y-yube olfactometer, demonstrating a non-bias in the behavioural response.

Female mosquitoes were kept individually in 6 cm × 10 cm i.d. release cages for 6 h before the experiments. During this acclimatisation period, the mosquitoes were provided ad libitum access to water. The release cages were placed at the downwind end of the olfactometer, and the mosquitoes were allowed 5 min to acclimatise, after which the door of the release chamber was opened. Thereafter, the mosquito was provided 5 min to make a choice. Mosquitoes that did not move were considered as non-responding and were not included in subsequent analysis. A minimum of 30 single mosquitoes per age group was tested in each experiment. All mosquitoes were tested 0 h – 4 h into scotophase, *i.e.*, at peak flight activity. For visualisation of the behavioural response,

a preference index was calculated as (T – C)/(T + C), in which T is the number of mosquitoes associated with the test odour and C the number of mosquitoes associated with the control. General regression with a beta-binomial distribution was used to analyse the behavioural responses of 1 dpe and 4 dpe mosquitoes, in which age, species and context were categorical factors, dose a continuous factor and the number of mosquitoes the response variable (JMP®, Version 17).

### Electroantenographic analysis

To assess the overall antennal response of sugar-deprived (12 h prior to experiments) 1 dpe and 4 dpe mosquitoes, stand-alone electroantennography (EAG) was used. Select individual bioactive VOCs were delivered randomly to the antenna in a dose-dependent manner, starting with the lowest dose to avoid habituation. To this end, serial dilutions of each VOC were prepared v/v in re-distilled hexane (99.9% purity, Sigma-Aldrich), and 10 µl aliquots loaded onto pieces of filter paper (20 mm × 10 mm) inserted into Pasteur pipettes on the day of recording. Each pipette was used only once. Each EAG recording included control stimulations by the solvent (hexane) at the beginning and at the end of every individual odorant series. For electrophysiological recordings from the mosquito antenna, the head of either a 1 dpe or 4 dpe mosquito was excised, and a pulled glass microcapillary, serving as the reference electrode, filled with Beadle–Ephrussi ringer solution, was inserted into the foramen. The distal segment of the antenna was then cut and inserted into a recording electrode filled with the same ringer solution. The recording electrode was connected to a pre-amplifier (10×), which in turn was connected to a high impedance DC amplifier interface box (Ockenfels Syntech, Buchenbach, Germany). For the EAG recordings, the individual antennal responses were normalised by subtracting the average control response from the neighbouring hexane stimulations. A General Linear Model, followed by Tukey's *post-hoc* test, was employed to compare the antennal responses, between the two age groups of *An. coluzzii* and *An. arabiensis*, to each of the bioactive VOCs tested. The dependent variable was the weighted response of the antenna, with age as the independent fixed factor and replicates as the random effect. Data analysis was performed using IBM SPSS Statistics for Windows (v. 22, IBM Corp., Armonk, USA).

## Data availability

All data generated or analysed during this study are included in this published article.

Received: 28 November 2024; Accepted: 26 February 2026
Published online: 08 March 2026

## References

1. Foster, W. A. Chapter 7: Behavioural ecology of plant-mosquito relations. In *Sensory Ecology of Disease Vectors* (eds. Ignell, R, Lazzari, C. R., Lorenzo, & Hill, S. R) 171–234. (Wageningen Academic Publishers, 2022).
2. Foster, W. A. Mosquito sugar feeding and reproductive energetics. *Annu. Rev. Entomol.* **40**, 443–474 (1995).
3. Ignell, R. & Hill, S. R. Malaria mosquito chemical ecology. *Curr. Opin. Insect Sci.* **40**, 6–10 (2020).
4. Hill, S. R. & Ignell, R. Modulation of odour-guided behaviour in mosquitoes. *Cell Tissue Res.* **383**, 195–206 (2021).
5. Barredo, E. & DeGennaro, M. Not just from blood: Mosquito nutrient acquisition from nectar sources. *Trends Parasitol.* **36**, 473–484 (2020).
6. Peach, D. A. & Gries, G. Mosquito phytophagy: Sources exploited, ecological function, and evolutionary transition to haematophagy. *Entomol. Exp. Appl.* **168**, 120–136 (2020).
7. Guerenstein, P. G. & Hildebrand, J. G. Roles and effects of environmental carbon dioxide in insect life. *Annu. Rev. Entomol.* **53**, 161–178 (2008).
8. Goyret, J., Markwell, P. M. & Raguso, R. A. Context- and scale-dependent effects of floral $CO_2$ on nectar foraging by *Manduca sexta*. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 4565–4570 (2008).
9. Peach, D. A., Gries, R., Zhai, H., Young, N. & Gries, G. Multimodal floral cues guide mosquitoes to tansy inflorescences. *Sci. Rep.* **9**, 3908 (2019).
10. Knudsen, J. T. & Gershenzon, J. The chemical diversity of floral scent. In *Biology of Plant Volatiles* (eds Pichersky, E. & Dudareva, N.) 57–78 (CRC Press, 2020).
11. Knudsen, J. T., Tollsten, L. & Bergström, L. G. Floral scents: A checklist of volatile compounds isolated by head-space techniques. *Phytochemistry* **33**, 253–280 (1993).
12. Shannon, D. M., Richardson, N., Lahondère, C. & Peach, D. Mosquito floral visitation and pollination. *Curr. Opin. Insect Sci.* **65**, 101230 (2024).
13. Impoinvil, D. E. et al. Feeding and survival of the malaria vector *Anopheles gambiae* on plants growing in Kenya. *Med. Vet. Entomol.* **18**, 108–115 (2004).
14. Manda, H. et al. Discriminative feeding behaviour of *Anopheles gambiae* s.s. on endemic plants in western Kenya. *Med. Vet. Entomol.* **21**, 103–111 (2007).
15. Manda, H. et al. Effect of discriminative plant-sugar feeding on the survival and fecundity of *Anopheles gambiae*. *Malar. J.* **6**, 113 (2007).
16. Thom, C., Guerenstein, P. G., Mechaber, W. L. & Hildebrand, J. G. Floral $CO_2$ reveals flower profitability to moths. *J. Chem. Ecol.* **30**, 1285–1288 (2004).
17. Wang, G., Carey, A. F., Carlson, J. R. & Zwiebel, L. J. Molecular basis of odor coding in the malaria vector mosquito **Anopheles gambiae**. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 4418–4423 (2010).
18. Carey, A. F., Wang, G., Su, C. Y., Zwiebel, L. J. & Carlson, J. R. Odorant reception in the malaria mosquito **Anopheles gambiae**. *Nature* **464**, 66–71 (2010).
19. Omondi, A. B., Ghaninia, M., Dawit, M., Svensson, T. & Ignell, R. Age-dependent regulation of host seeking in **Anopheles coluzzii**. *Sci. Rep.* **9**, 9699 (2019).
20. Omondi, B. A., Majeed, S. & Ignell, R. Functional development of carbon dioxide detection in the maxillary palp of **Anopheles gambiae**. *J. Exp. Biol.* **218**, 2482–2488 (2015).
21. Hinze, A., Hill, S. R., & Ignell, R. Chapter 9: Odour-mediated host selection and discrimination in mosquitoes. In *Sensory Ecology of Disease Vectors* (eds. Ignell, R, Lazzari, C. R., Lorenzo, & Hill, S. R) 253–276. (Wageningen Academic Publishers, 2022).
22. Khan, Z., Ignell, R., & Hill, S. R. Chapter 14: Odour-mediated oviposition-site selection by mosquitoes. In *Sensory Ecology of Disease Vectors* (eds. Ignell, R, Lazzari, C. R., Lorenzo, & Hill, S. R) 373–417. (Wageningen Academic Publishers, 2022).

23. Hill, S. R., Taparia, T. & Ignell, R. Regulation of the antennal transcriptome of the dengue vector, **Aedes aegypti**, during the first gonotrophic cycle. *BMC Genomics* **22**, 71 (2021).
24. Hajkazemian, M. et al. Mosquito host-seeking diel rhythm and chemosensory gene expression is affected by age and *Plasmodium* stages. *Sci. Rep.* **12**, 18814 (2022).
25. Birgersson, G., Dalusky, M. J., Espelie, K. E. & Berisford, C. W. Pheromone production, attraction, and interspecific inhibition among four species of *Ips* bark beetles in the Southeastern USA. *Psyche* **2012**, 532652 (2012).

## Acknowledgements
This study was funded by grant from the Swedish University of Agricultural Sciences and the Linnaeus initiative 'Insect Chemical Ecology, Ethology and Evolution, IC-E3'.

## Author contributions
RI conceived and designed the study, interpreted the data, as well as wrote the article. BAO acquired and analysed the data for the GC-SSR analysis of ectopically expressed Ors. BW acquired and analysed the EAG data. MD acquired data on CO2 emission and behaviour. SRH performed statistical analyses. All authors read and approved the final version of the article.

## Funding
Open access funding provided by Swedish University of Agricultural Sciences.

## Declarations

## Competing interests
The authors declare no competing interests.

## Additional information
**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-026-42694-7.

**Correspondence** and requests for materials should be addressed to R.I.