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L-lactic and 2-ketoglutaric Acids, Odors from Human Skin, Govern Attraction and Landing in Host-Seeking Female *Aedes aegypti* Mosquitoes

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Abstract *Aedes aegypti*, presented with a source of L-lactic and 2-ketoglutaric acid in a wind-tunnel bioassay, takeoff, fly upwind, and land on the odorant substrate at rates comparable to those exhibited by mosquitoes presented with a skin-odor stimulus. Addition of carbon dioxide decreased takeoff latency but was not required to elicit upwind flight nor landings. Ketoglutaric acid, a recently identified component of human skin odor, combined with lactic acid elicits the full repertoire of mosquito host-seeking behaviors.

Introduction

Aedes aegypti (Diptera: Culicidae) is a vector of several consequential arboviruses including chikungunya, dengue, yellow fever, and Zika. *Aedes aegypti* has invaded much of the tropics and sub-tropics, making dengue the most prevalent human arbovirus, infecting 100 million per year and placing nearly half of the world's population at risk (Bhatt et al. 2013).

Females of the anthropophilic form of *Ae. aegypti* blood feed almost exclusively on humans (Scott et al. 1993). This makes them particularly effective vectors of human pathogens (MacDonald 1952). The cosmopolitan "subspecies" *Ae. aegypti aegypti* diverged from the non-human preferring *Ae. aegypti formosus*, 400 to 550 years ago (Gloria-Soria et al. 2016; Crawford et al. 2017; Powell et al. 2018). Hereafter references to *Ae. aegypti* are to the anthropophilic Orlando strain of *Ae. a. aegypti*.

To find a host, *Ae. aegypti* fly upwind when they detect fluctuating levels of CO_2 above ambient concentration. Once they are within several meters of a prospective host, they can also sense visual cues and, even closer to the host, thermal cues (Gillies 1980; Cardé and Gibson 2010; van Breugel et al. 2015; Cardé 2015; Sumner and Cardé 2022). The precise

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distances at which they detect and use host odors other than CO_2 is unresolved (Gillies and Wilkes 1970; Dekker et al. 2005).

For *Ae. aegypti*, humans are distinguished from other potential hosts by their skin odor (Steib et al. 2001; Dekker et al. 2002; McBride et al. 2014). Bernier et al. (2002) characterized 279 compounds in the headspace above human skin. A review by Dormont et al. (2021) provides a valuable table of mosquito attractants using the definition of attractant proposed by Dethier et al. (1960) as "a chemical which causes insects to make oriented movements towards its source." Their list of compounds is organized by species, "co-tested" compounds, and assay type. A challenge is to determine which compounds or combinations of compounds elicit host-finding behaviors. This task is complicated by how different bioassays measure different components of mosquito host-seeking behavior. "Attractiveness" in one assay may measure flying into a port from a still-air chamber, whereas in another, it may measure arrestment after upwind orientation (Cardé 2023). Most assays measure attraction by the responder reaching an endpoint of orientation over a set interval.

Intermediate steps in orientation also can be monitored. Dekker et al. (2001) in a still-air, port-entry assay and Torr et al. (2008) in a field trapping study used electrocution grids to show that some host odors can lure mosquitoes to the vicinity of the odor source but do not always elicit port or trap entry, respectively. The reasons for such outcomes may relate to the odors being incomplete or containing some antagonistic compounds. Alternatively, the odor plume's structure may be suboptimal for orientation (Geier et al. 1999; Dekker et al. 2001). Another metric to monitor is the rapidity of orientation. Dekker et al. (2001) documented the time to either electrocution or port entry, showing that some odor blends induced more rapid orientation than others, but if the assay's duration was extended to 15 min, this difference faded. Kennedy (1977) discussed the importance of the assay's duration in its outcome and the limitation of endpoint assays in distinguishing undirected movement (kinesis) from directed movement (taxis).

Nonetheless, laboratory endpoint assays are useful for investigations of mosquito responses to host emitted odors. For example, BG-Lure, a human skin-odor mimic, designed for use with the Biogents Sentinel® trap (Biogents, Regensburg, Germany), releases lactic acid, ammonia, and hexanoic acid. This lure was developed using a Y-tube olfactometer with subsequent field-trapping studies (Williams et al. 2006).

Lactic acid (2-hydroxypropanoic acid) is thought to be a diagnostic cue used by *Ae. aegypti* (Steib et al. 2001) and *An. gambiae* (Dekker et al. 2002) to distinguish humans from non-human animals. The addition of lactic acid made non-human animal odor more attractive to both species of anthropophilic mosquitoes. Both studies found lactic acid, however, was necessary but insufficient by itself to attract the number of *Ae. aegypti* that were attracted to human odor. Smith et al. (1970) tested *Ae. aegypti* in a combined port entry and landing assay. They found that lactic acid reduced landing on a human-worn sock, but increased olfactometer trap catch downwind of the sock, suggesting that the dose of lactic acid reaching the mosquito is important. Healy and Copland (2000) found that *An. gambiae* did not land on a lactic acid source. However, Dekker et al. (2002) suggested that lactic acid may be an attractant but not a landing cue for *An. gambiae*.

Recently, Bello and Cardé (2022) identified 2-ketoglutaric acid (2-oxopentanedioic acid) in human skin odor and demonstrated that a mixture of lactic and ketoglutaric acids in the presence of CO_2 is a landing cue for *Ae. aegypti*. The addition of pyruvic acid, also present in the active fraction of skin odor (Bello and Cardé 2022), to this mixture did not significantly elevate landing rates above those elicited by a blend of lactic and ketoglutaric acids.

Carbon dioxide added to ambient air (which has an intrinsic concentration of CO_2 of about 0.04%), increases the takeoff rate of *Ae. aegypti* (Daykin et al. 1965), elicits upwind flight (Kennedy 1940), increases heat-seeking (McMeniman et al. 2014), and ultimately leads to increased endpoint capture (Huffaker and Back 1943. In a generally accepted model of mosquito host seeking, formalized by Gillies (1980), CO_2 is generally considered as the long-range attractant of host-seeking mosquitoes. The model assumes a mosquito detects CO_2, takes off, flies upwind along the plume, and then switches to other host cues. Dekker et al. (2005) found that diluted human skin odor was less attractive to *Ae. aegypti* than undiluted skin odor and that CO_2 sensitizes mosquitoes to other host odors. These models were updated by Cardé and Gibson (2010), van Breugel et al. (2015), and Cardé (2015), but still posited that

CO_2 was the long-distance attractant, and that as mosquitoes fly closer to their hosts they would switch to using specific cues such as skin odor.

This paradigm may not hold for other mosquitoes. Schreck et al. (1972) found that *Anopheles quadriannulatus* flew to a calf from further distances than to a calf-equivalent quantity of CO_2. It is unknown how far downwind the lactic and ketoglutaric acids are detectable by *Ae. aegypti*. Additionally, it is not known whether there are characteristic behaviors, such as surging upwind or casting crosswind, associated with the detection and subsequent attraction to the landing cue compounds.

As the blend of lactic and ketoglutaric acids elicited landing in a cage assay (Bello and Cardé 2022), we set out to determine if the blend elicits upwind flight in a wind tunnel in the presence or absence of CO_2. We used wind-tunnel assays with videography to allow examination of orientation maneuvers, in addition to landing (Lacey and Cardé 2011). Some wind-tunnel assays have used video tracking without landing counts (van Breugel et al. 2015); however, landing is the ultimate measure of successful orientation to a host. If a mosquito does not land, it cannot bite nor transmit pathogens (Reed et al. 1900). We measured time from release to takeoff, time from takeoff to the first landing on the odor source, the number of landings, and the duration of landings. Our video tracking system allowed us to examine mosquito flight maneuvers prior to landing.

Materials and Methods

Mosquito Rearing

An Orlando strain colony of *Ae. aegypti* was maintained in a L:D 14:10 h photocycle at 27 °C and 70% RH. Approximately 50 larvae were reared in plastic containers ($26 \times 25.6 \times 15$ cm) with ~1 cm of deionized (D.I.) water and fed Tetramin® pellets (Tetra, Blacksburg, VA, U.S.A). Pupae were held in plastic containers, transferred to screen cages ($30 \times 30 \times 30$ cm, BugDorm-1, Megaview Science Co. Ltd. Talchung, Taiwan) before eclosion. Mosquitoes were provided 10% sucrose solution in D.I. water on a cotton wick. Males and females were held together in the screen cages, and females used in the bioassays were assumed to have mated. Females used in experiments were 3–10 days post-eclosion and were not blood fed. Mosquitoes were starved and deprived of water approximately 12 h prior to experiments. Female mosquitoes were transferred individually to clean cylindrical acrylic release cages (7×8 cm diameter) 30 min prior to testing; assays were conducted 4–8 h into their photophase.

Assay Methods

The assay methods were adapted from Sumner and Cardé (2022). The flight and landing of mosquitoes released in a glass wind tunnel $122 \times 30.5 \times 30.5$ cm were video recorded (FDR-AX53, Sony, Tokyo, Japan) from above. Air was drawn into the tunnel from an adjacent, uninhabited room (25 °C and 70% RH). Wind speed in the tunnel was 0.2 m/s. To simulate the presence of an upwind vertebrate host, 100 ml/min of 4% CO_2 mixed with tank air (equivalent to 1/60 of the exhalation a human, Snow 1970), was carried to the wind tunnel via a 3-m-long Tygon® tube, ensuring temperature equilibration (Pinto et al. 2001). The tube was connected to an L-shaped glass tube (OD 5.5 mm, ID 3.5 mm) that descended 15 cm from the ceiling of the tunnel and extended 20 cm downwind to 60 cm upwind from the release cage. The 4% CO_2 mix exited at ~0.4 m/s but produced no detectable difference in wind speed (Omega HHF 52 anemometer, Omega Engineering, Inc., Stamford, CT, USA) nor a temperature difference (to within 0.1 °C) 1 cm downwind of the release point. The CO_2 release tube was centered so that the generated plume of CO_2 passed over the beads treated with skin odor and then to the release cage. In trials without the addition of CO_2, tank air was supplied at the same rate through the same equipment.

The assay room was maintained at 27 °C and 60% RH. Illumination for videography was provided by infrared LED lights (AXIS T90A, 850 nm, Axis Communications AB, Lund, Sweden) mounted behind a stainless-steel screen at the downwind end of the tunnel as well as beside the wind tunnel. The infrared light in the camera was turned off to avoid glare. Visible light was provided by incandescent bulbs and measured at ~14 lx inside the tunnel. This lighting facilitated a clear image of mosquitoes. Further details are provided in Sumner and Cardé (2022). *Aedes aegypti* is primarily day active, but it is also crepuscular and active into early evening (Russell

2004 and references therein). This level of lighting is therefore appropriate to conditions it can experience during its normal 24-h rhythm of host seeking.

Treatments were presented on glass beads (black, 10/0 Czech Glass Seed, approximately 2 mm OD toroidal, Precosia Ornela, Zásada, Czech Republic) placed in a clean glass Petri dish (7 cm diameter). Negative control beads are hereafter called clean beads. The blend components, ketoglutaric acid ("KGA" in the figures) (0.5 ml of either: 10 µl/ml or 100 µl/ml in acetone) and lactic acid ("LA" in the figures) (0.5 ml of either: 10 µl/ml or 100 µl/ml in acetone) were applied in a dropwise spiral to beads. As Ghaninia et al. (2019) found that acetone was attractive in a flight tube to *Ae. aegypti*, the beads were placed under a fume hood for 10 min to ensure that the acetone had evaporated.

Human skin odor (BDS) was collected onto glass beads by placing 25 ml of beads into a polyester/cotton blend sock, which was worn for 12 h. Beads were cleaned after use by soaking in a solution of 10% detergent (Micro 90 Cleaning Solution, Cole-Parmer, Vernon Hills, IL, USA) in D.I. water and sonicated for one hour. The beads were then thoroughly rinsed with D.I. water, dried, rinsed twice with acetone (ACS grade, Fisher Scientific, Pittsburg, PA, USA), and heated to 250 °C for 12 h before reuse.

Differing from Sumner and Cardé (2022), the dish of beads was presented on a 15-cm high metal stand in the center of the tunnel, 55 cm upwind of the release cage. This ensured that plume emanating from the beads would engulf the mosquitoes in the release cage. Assays were run and recorded with video for 6 min, commencing with the opening of the release cage. Disposable nitrile gloves were always worn by the experimenter to prevent contamination with skin odors.

Treatment Strategy

We presented the mosquitoes with clean beads, a low dose blend of 5 µg each of ketoglutaric acid (KGA) (2-Keto-glutaric acid 97%, TCI, Tokyo, Japan), and lactic acid (LA) (L-lactic acid 85–90% in water, Honeywell Fluka, Charlotte, NC, USA) (based on Bello and Cardé 2022), a blend of 50 µg each of lactic and ketoglutaric acids, and skin odor-treated beads by being worn in a sock. All four treatments were tested in the presence and absence of a turbulent 4% plume of CO_2 (Table 1).

To confirm the blend was not eliciting mosquito landing solely due to its lactic acid content, we also tested 50 µg of LA alone and the blend of 50 µg each of both compounds in a series of one-choice assays with CO_2. Five mosquitoes were used in each replicate of these assays (Table 1), which enabled a five-fold reduction in the number of assays for these treatments. The potential number of landings was not lowered but criteria such as time to takeoff and time from takeoff to first landing had their sample sizes reduced five-fold.

Data Acquisition

Video files were observed, and behavior was scored with BORIS v.5.1.0 (Friard and Gamba 2016). All

Table 1 Treatment combinations for wind-tunnel assays with female *Ae. aegypti*

Treatment	CO_2	Assays	Assays with takeoff	Mosquitoes per assay	Total mosquitoes
Clean beads	No	46	87%	1	46
Clean beads	Yes	32	88%	1	32
5 µg each of LA+KGA	No	40	75%	1	40
5 µg each of LA+KGA	Yes	39	90%	1	39
50 µg each of LA+KGA	No	32	88%	1	32
50 µg each of LA+KGA	Yes	39	100%	1	39
Skin odor	No	31	74%	1	31
Skin odor	Yes	29	83%	1	29
50 µg each of LA+KGA	Yes	12	All had ≥ 1	5	60
50 µg LA only	Yes	12	All had ≥ 1	5	60

videos were viewed and scored from the release time until 6 min elapsed. The observer recorded release and takeoff as "point" events, whereas landing was scored as a "state" event starting with the landing on the beads and ending with the takeoff from the beads.

Video flight tracking was performed using EthoVision XT v.9.0 (Noldus Information Technology, Wageningen, The Netherlands). Raw numeric data were exported and used in statistical analysis. For data obtained with EthoVision XT, tracking commenced at takeoff and continued until the individual landed on the beads or remained in the upwind section of the wind tunnel, and was therefore indistinguishable, for $\geq$ 30 s.

Statistical Analysis

All data manipulation and statistical tests were conducted using R v.3.5.0 (R Core Team 2013) in RStudio v.1.1.463 (RStudio Team 2020).

Landing Observations

The proportion of trials with at least one landing were compared across all treatments with a Fisher Exact test followed by pairwise Fisher Exact tests with Benjamini–Hochberg correction to reduce the false discovery rate. This method considers all the treatments to be completely independent of each other.

All other tests of manual landing observation data were conducted with generalized linear models (GLMs). A matrix was manually constructed with the independent variable data of each of the treatment combinations. It contained binary values for skin odor and CO_2 as well as a values of 0, 1, or 10 for the dose of the lactic and ketoglutaric acid blend. Instead of considering all treatments as independent, as the Fisher Exact test does, the GLMs used this information about the relationships among the treatments. In particular, the models treat the different doses of the blend as different values of the same independent variable. The GLMs show which treatments were significantly correlated with whichever behavioral outcome was tested. This allowed us to determine which treatments were correlated. If we had relied on testing differences among treatment combinations, we would have potentially masked the importance of some cues. Significant correlations, unlike significant differences, do not translate directly to graphs of whole data sets. Therefore, instead of visually clear asterisks,

the outputs of the GLMs are solely listed in the figure captions.

First, a GLM was used to compare the number of trials with at least one landing. This test of the same data examined with the Fisher tests allows comparison across methods. The treatment matrix was used again when comparing to the: number of mosquito landings on beads per trial, durations a mosquito remained after landing among the different treatments, latency (time from release to takeoff), and duration of flight from takeoff to first landing. For testing of repeated landings, trials with one landing were converted to arbitrarily small values. The data were square-root transformed to make the residuals acceptably close to normal.

Flight Tracks

Kruskal–Wallis tests were used to contrast the mean distance of the mosquito from the beads every 1/15 of a second during flight track, the mean velocity of the mosquito during flight, the Emax (track straightness) of the mosquito flight, and the proportion of time the mosquito spent heading ($\pm$ 20°) towards the center of the beads. Spearman rank correlations were used to test effects.

Emax (a measure of straightness, 1 being a completely straight track) (trajr package, McLean and Skowron-Volponi 2018) was used as a measure of track sinuosity and was calculated (Cheung et al. 2007) using the X and Y coordinates of the subject at each time point. Emax is based on an iterated summing of the expected displacement. As the shortest path between two points is a straight line, a high Emax represents a straight path. This displacement does not necessarily correlate to distance from the beads. A mosquito flying a figure-eight over the beads would have a high Emax and a low mean distance to the beads before landing, whereas a mosquito that flew straight to the beads would have a low Emax and a low mean distance to the beads.

Results

Takeoff

The presence of CO_2 was the only treatment component that was positively correlated with the proportion of trials with mosquito takeoff (Est.=0.447, P=0.022) (Fig. 1).
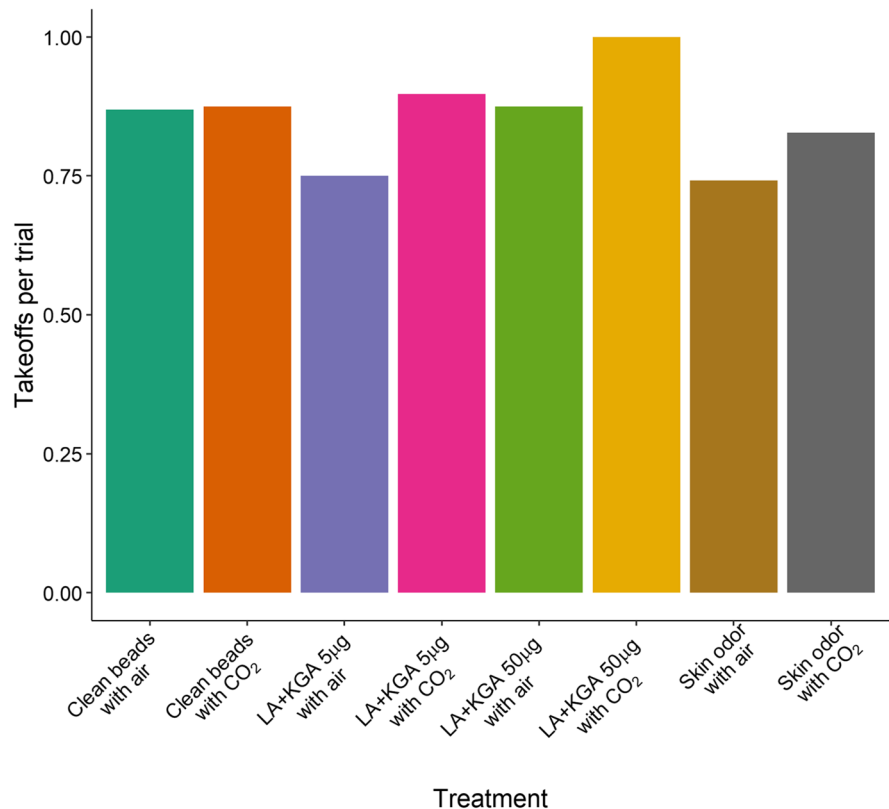
Latency from Release to Takeoff

Among the treatments there were no significant differences in takeoff latencies. Although the 50 µg dose appears to elicit a significant decrease in take-off latency (P = 0.017, Coefficient = -0.3534), the residuals of the GLM (Gamma, link = log) were non-normal (KS P = 0.022), which means that the test cannot be used in this case (Fig. 2). This distribution and link function produced the closest to normal residuals.

Time From First Takeoff to First Landing

Figure 3 shows the time from takeoff to the first landing on the beads. Skin odor (Est. = -1.88, P < 0.001) and lactic and ketoglutaric acids 50 µg (Est. = -1.061, P = 0.026) reduced the time mosquitoes took to first reach and land on the beads. The presence of CO_2 was not correlated with duration of flight from takeoff to first landing (P = 0.139).

Proportion of Trials with at Least One Landing

While skin odor with or without supplemental CO_2 elicited the numerically highest proportion of trials with one or more landings on the odor-treated beads, it was not significantly different from the proportion of trials with lactic and ketoglutaric acids 50 µg in the presence or absence of CO_2 that elicited one or more landings (Fisher Exact Test, adjusted P = 0.402) (Fig. 4).

Only the presence of skin odor (GLM, Est. = 2.88, P < 0.001) and both doses of the blend of lactic and ketoglutaric acids 50 µg (GLM, Est. = 2.434, P < 0.001) and 5 µg (GLM, Est. = 1.192, P = 0.029), were positively correlated with the probability of a mosquito landing at least once during a trial in the wind tunnel.

Repeat Landings

Figure 5 shows the number of repeat landings, per trial by treatment, among trials with at least one landing. The presence of skin odor (Est. = 0.3238,

Fig. 1 The proportions of trials in which *Aedes aegypti* initiated flight. The presence of CO_2 was positively correlated with the proportion of trials with takeoff (GLM, binomial, link = probit, Est. = 0.447, P = 0.022)

Fig. 2 Latencies in *Aedes aegypti* of takeoff in seconds. While the high dose of the blend (LA + KGA with and without CO_2) was significantly associated with a shorter latency (GLM, Est. = -0.3534, P = 0.017), the residuals of the GLM (gamma, link = log), despite a square root transform of the data, were somewhat divergent from the normal distribution (KS P = 0.022)
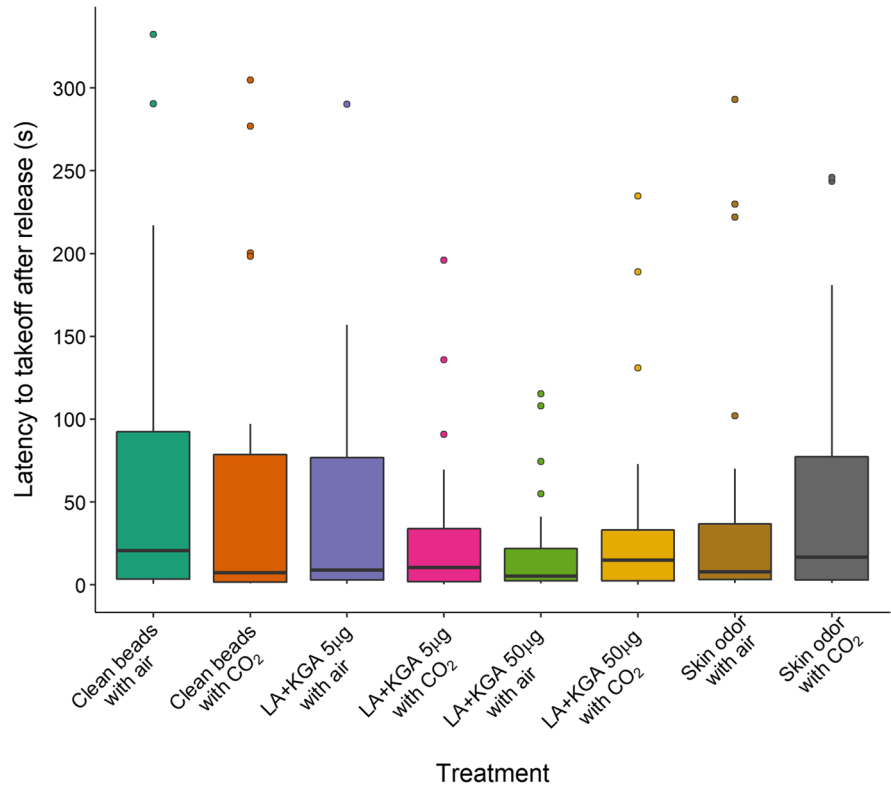
Fig. 3 The durations of *Aedes aegypti* flight from takeoff to the first landing on beads in trials with at least one landing. The presence with and without CO_2 of skin odor (Est. = -1.88, P < 0.001), and the blend of 50 μg each of lactic and ketoglutaric acids (Est. = -1.061, P = 0.026), resulted in shorter flight times from takeoff to first landing. CO_2 was not correlated with flight duration
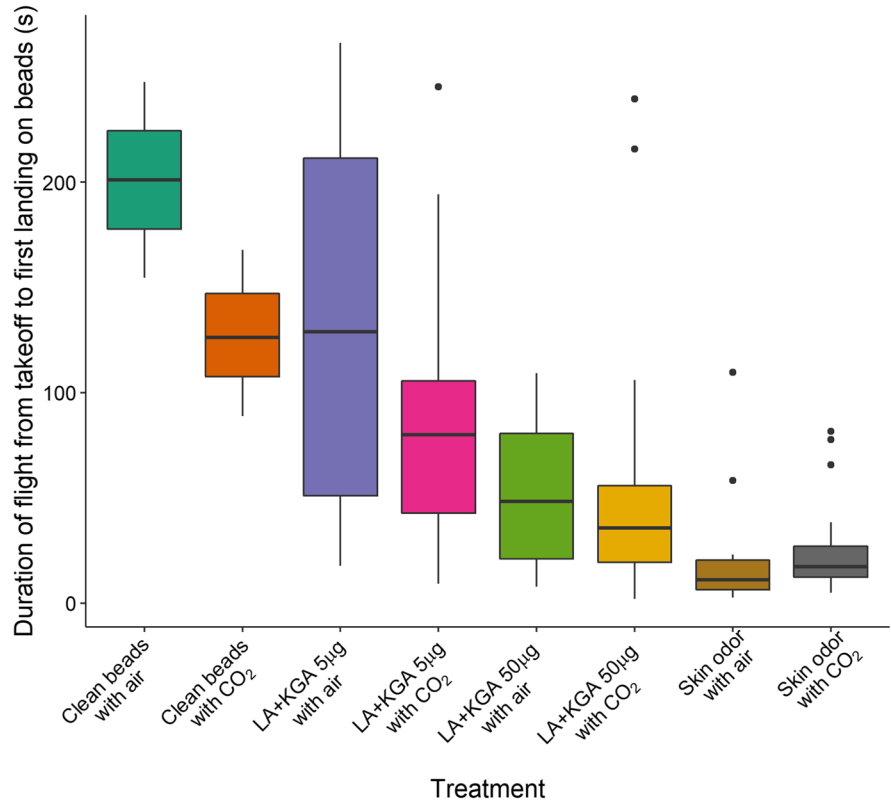
Fig. 4 The proportion of trials in which *Aedes aegypti* landed more than once was tested with a Fisher Exact test followed by pairwise Fisher Exact tests; letters above columns show significant difference (P < 0.05). The GLMs, informed of the relationships among the treatments, found that: the proportion of all trials with ≥ 1 landing is correlated with: CO_2 (Est. = 0.578, P = 0.046), skin odor (Est. = 2.88, P < 0.001), the blend of 5 µg each of lactic and ketoglutaric acids (Est. = 1.192, P = 0.029), and the blend of 50 µg of each (Est. = 2.434, P < 0.001)
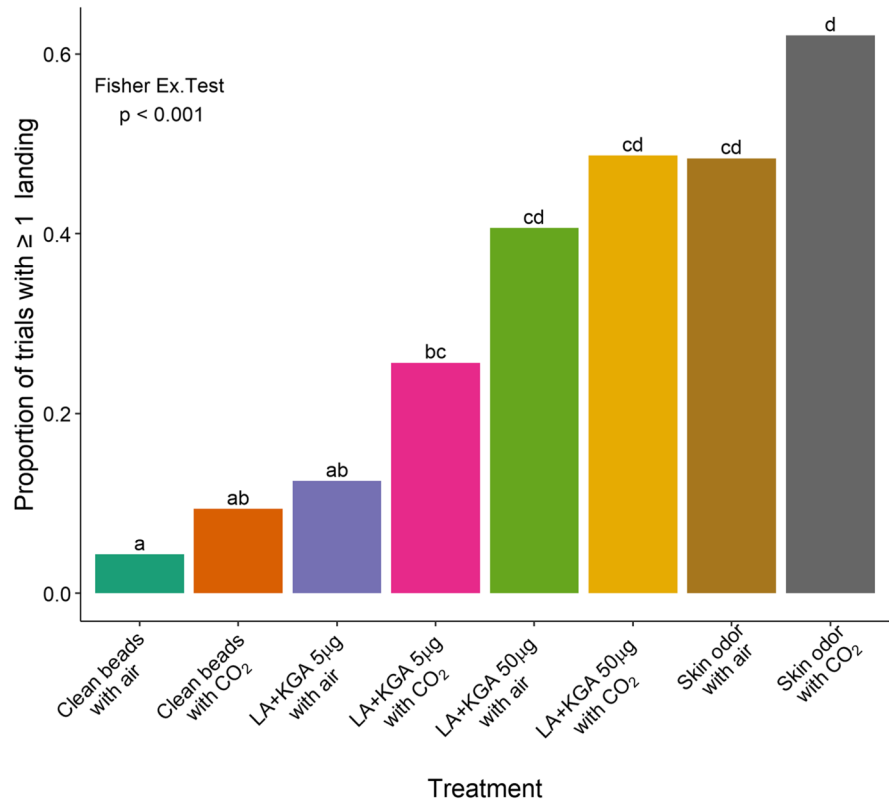
P = 0.0199) and the high dose blend, 50 µg each, of lactic and ketoglutaric acids with CO_2 (Est. = 0.3244, P = 0.0311) were positively correlated with the landings by a single mosquito per trial (Fig. 5).

Total Landings on Lactic Acid Alone or the Lactic and Ketoglutaric Acid Blend

The number of landings on the 50 µg each blend of lactic and ketoglutaric acids (12.2 mean landings per trial, S.D. = 8.9) were significantly greater than those on lactic acid alone (5.4 mean landings per trial, S.D. = 6.5) (Kruskal-Wallace, P = 0.046) (Fig. 6). The time from takeoff to the first landing was significantly shorter between the two-component blend (mean = 57 s, S.D. = 49.4) and the lactic acid alone (mean = 115 s, S.D. = 99.3) (Kruskal-Wallace, P = 0.039).

Duration of Landing

Figure 7 provides the time within each trial that a single mosquito spent on the beads. The skin odor

treatment (Est. = 1.626, P < 0.001), both doses of the blend of lactic and ketoglutaric acids, 5 µg (Est. = 0.979, P = 0.028), and 50 µg (Est. = 1.207, P = 0.004) were positively correlated with the duration of landing time of the mosquito (Fig. 7). The presence of CO_2 was not correlated with duration of landing time. Additionally, mosquitoes were observed sticking their proboscises on the beads coated with 50 µg each of lactic and ketoglutaric acids, in the presence of CO_2, in a manner resembling probing behavior.

Analysis of Flight Tracks from Takeoff to First Landing

Among the treatments there were no differences in the mean distance of insects from the beads during flight in the wind tunnel ($\chi^2 = 298$, df = 298, P = 0.49). Selected flight tracks are shown in Fig. 8. Across all treatments, the mean (± SE) distance (mm) from the center of the beads during a mosquito flight was 214.15 mm (± 5.47). This mean distance from
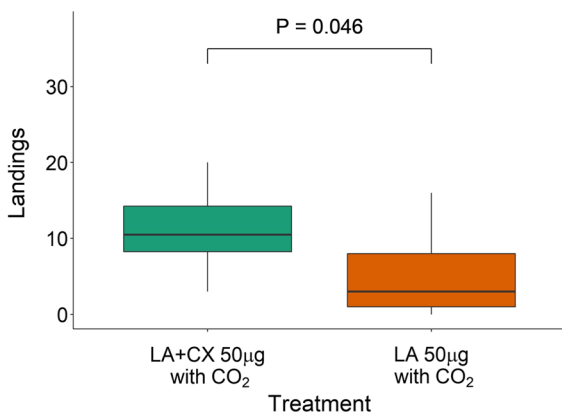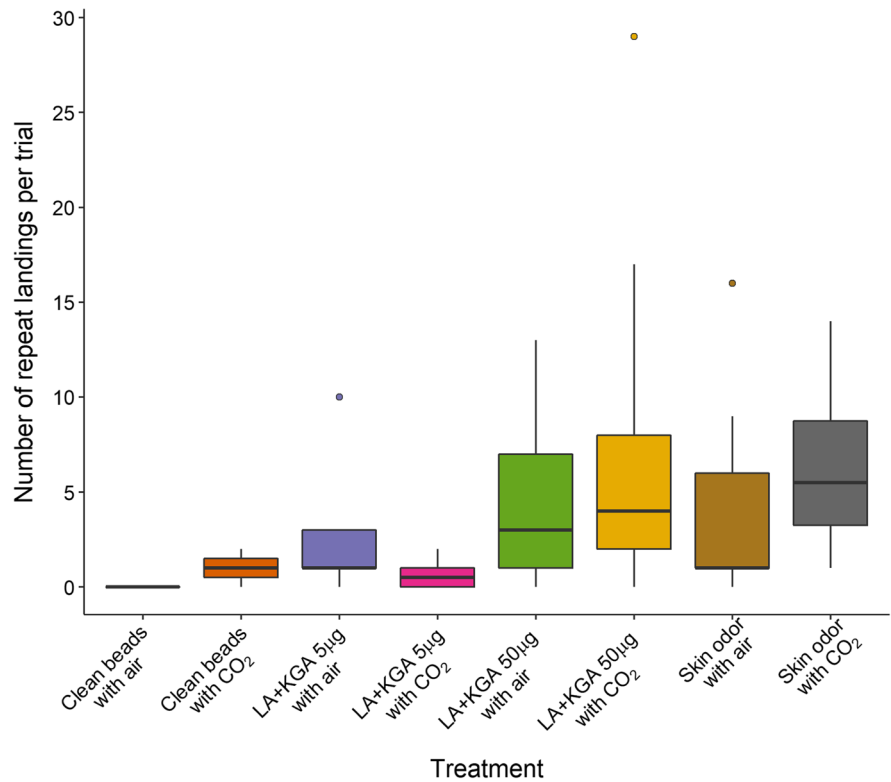
Fig. 6 The total landings of *Aedes aegypti* by trial with the blend versus lactic acid alone. The number of landings on the two-component blend was significantly different than on lactic acid alone (Kruskal-Wallace, P = 0.046)

the beads was negatively correlated with whether an individual landed on the beads ($\rho = -0.36$, $P < 0.001$).

There were no differences in the mean flight velocity (mm/s) of insects during flight in the wind tunnel among treatments ($\chi^2 = 298$, df = 298, $P = 0.49$), nor did velocity of an individual significantly correlate with whether that individual landed on the beads or not ($\rho = -0.12$, $P = 0.05$). Across all treatments, the mean ($\pm$ SE) velocity (mm/s) of mosquito flight in the wind tunnel was 193.15 mm (± 3.67).

There were no differences in the proportion of time an insect spent heading ($\pm 20°$) toward the center of the beads during flight in the wind tunnel among treatments ($\chi^2 = 289.36$, df = 288, $P = 0.47$), nor did this proportion of time significantly correlate with whether that individual landed on the beads or not ($\rho = 0.01$, $P = 0.83$). Across all treatments, the mean ($\pm$ SE) proportion of the time a flying mosquito spent heading towards the center of the beads ($\pm 20°$) was 0.16 (± 0.0039).

The Emax (track straightness; 1 = completely straight track) of insect tracks during flight in the wind tunnel did not differ among treatments ($\chi^2 = 298$, df = 298, $P = 0.49$), nor did the Emax of an individual significantly correlate with whether that individual landed on the beads or not ($\rho = 0.05$, $P = 0.44$). Across all treatments the mean ($\pm$ SE) Emax of mosquito flight in the wind tunnel was 0.35 (± 0.01).

Fig. 7 The cumulative duration of times female *Aedes aegypti* spent on beads, in trials with ≥ 1 landing. Skin odor (Est. = 1.626, P < 0.001), 5 μg each of lactic and ketoglutaric acids (Est. = 0.979, P = 0.028), and the blend of 50 μg of each (Est. = 1.207, P = 0.004) were correlated with the duration of mosquitoes that remained landed. CO_2 was not correlated with duration landed
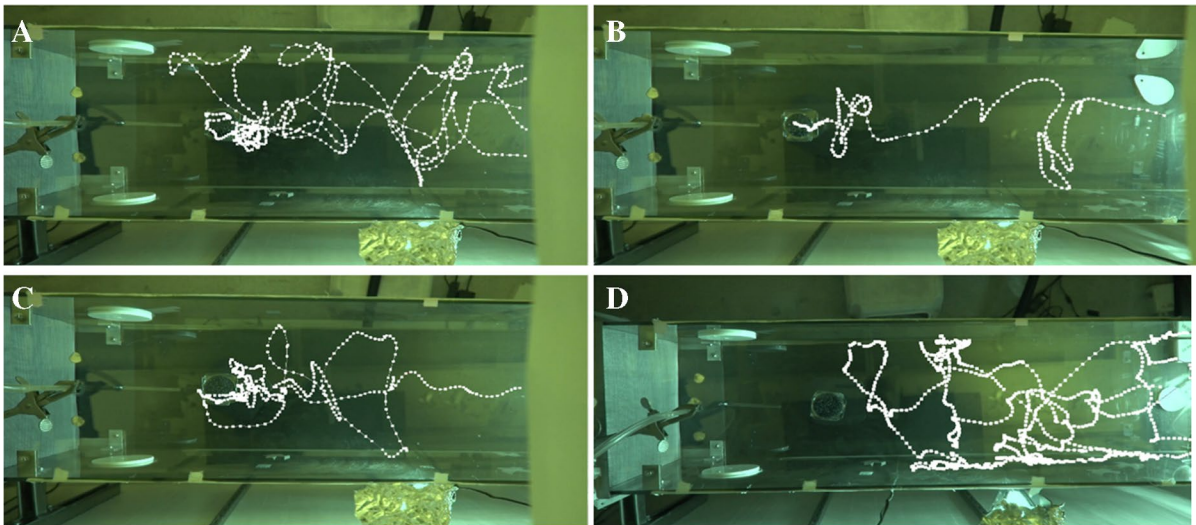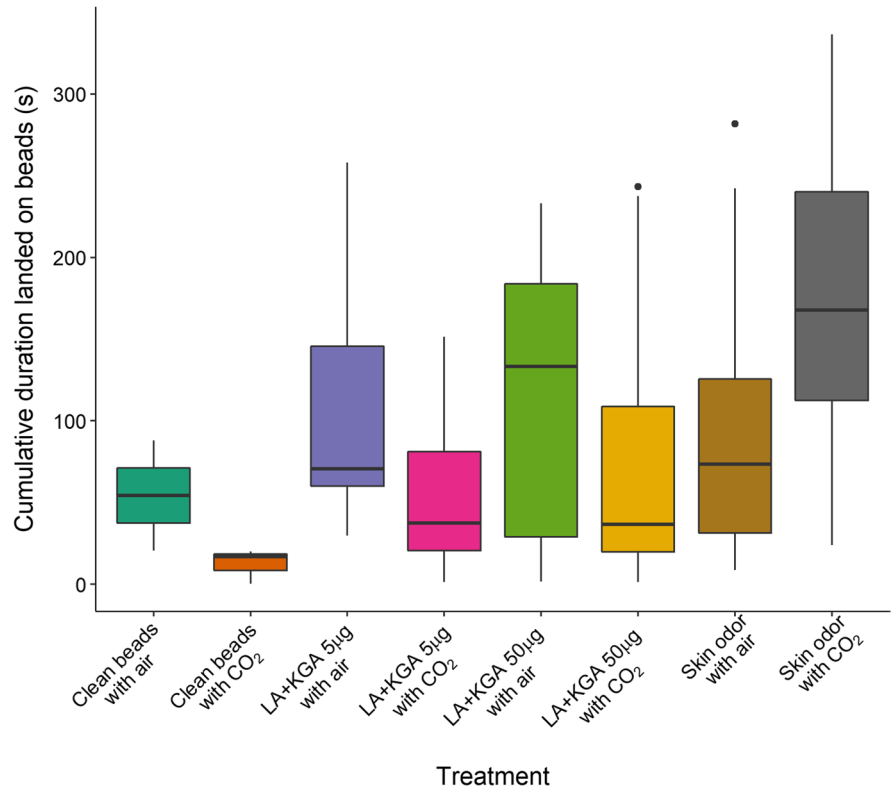
Fig. 8 Top view of selected flight tracks of female *Aedes aegypti*, provided with CO_2, responding to **A** 50 μg of lactic and ketoglutaric acids; **B** 5 μg of lactic and ketoglutaric acids; **C** Skin odor; **D** Clean beads. The mosquito did not land in the track shown in 8D. Airflow was left to right, and the mosquitoes were released on the right

Discussion

The two-component blend of lactic acid and ketoglutaric acid developed by Bello and Cardé (2022) elicited upwind flight and landing. The proportion of *Ae. aegypti* that landed at least once on an upwind odor source of the "high dose" (50 µg of each) of the blend was similar to the proportion that landed on a source of human odor. Unsurprisingly, the presence of CO_2 also increased the proportion of landings, given the known role of CO_2 in sensitizing mosquitoes to human skin odors (Dekker et al. 2005) and eliciting upwind flight (Kennedy 1940). Individual humans vary in their intrinsic attractiveness to mosquitoes, and some of this variation is likely attributable to the quantitative differences among individuals in their emission of lactic acid and ketoglutaric acid (Thurmon and Ottenstein 1952; Delgado-Povedano et al. 2020).

Skin odor and the higher dose of the blend of lactic acid and ketoglutaric acid (50 µg), induced rapid orientation and landing on the beads and comparable numbers of repeat landings per individual mosquito. This suggests that this blend elicited the same persistence in mosquito landing behavior as human skin odor. The similarity between the behavioral activity elicited by the high dose and skin odor was further demonstrated in that the blend and skin odor were both correlated with the cumulative duration of landing on the beads. The number of repeat landings, their durations, or latency of first landing were not correlated with the presence of CO_2. This is unsurprising, because in nature a mosquito landing on a skin odor source other than skin on the face would have likely exited the CO_2 plume (Dekker and Takken 1998). Because a long-duration landing reduces the available time for further landings, the numbers and durations of landings were inversely correlated in all trials.

The high-dose blend of lactic acid and ketoglutaric acid (50 µg) elicited more landings than lactic acid alone. We found that 50 µg rather than 5 µg of each component of the blend elicited *Ae. aegypti* behaviors akin to skin odor in a wind tunnel. The 5 µg dose was sufficient to induce numerous landings in the cage assay which has little ambient air movement (Bello and Cardé, 2022). The need for a higher dose to evoke upwind source finding in a wind tunnel is consistent with the odor mixture being diluted by turbulent diffusion of the wind-borne plume as it is carried downwind.

Carbon dioxide was positively correlated with the probability of takeoff. It was not correlated with the proportion of trials with a landing within the subset of trials with mosquito take off. Our findings support that CO_2 elicits takeoff in *Ae. aegypti* females but does not act as a landing cue. This is consistent with the sequential-distance model of mosquito host seeking (Gillies 1980; Cardé and Gibson 2010; van Breugel et al. 2015; Cardé 2015). In this paradigm a host seeking mosquito takes off and flies upwind in a plume of CO_2 before encountering other cues. The mosquito is then able to detect visual cues, host odors other than CO_2, and finally heat from the host. There are physical limits to the distance at which visual and heat cues should be detectable to mosquitoes (Khan et al. 1968; Muir et al. 1992).

Ketoglutaric acid is a component of the citric acid cycle (Wishart et al. 2018). It is found in fresh and dry sweat (Delgado-Povedano et al. 2020). It is not known how much ketoglutaric acid volatilizes from human skin. Lactic acid is released from human apocrine glands at a rate exceeding that of many non-human animals (Thurmon and Ottenstein 1952). Incubated sweat contains less lactic acid than fresh sweat, suggesting that most is produced endogenously (Braks and Takken 1999).

Lactic acid has been a controversial candidate as a mosquito attractant (Acree et al. 1968; Smith et al. 1970); Steib et al. (2001) added lactic acid to human and non-human animal odor. The addition of lactic acid resulted in the non-human animals' odor drawing as many *Ae. aegypti* to its arm of the Y-tube as human odor. However, lactic acid alone attracted only 19% of the mosquitoes tested. Calf and goat odors with added lactic acid attracted 70% of the *Ae. aegypti* tested.

Ketoglutaric acid may be one of the compounds Steib et al. (2001) and Geier et al. (2002) demonstrated existed but did not isolate. Our results corroborate those of Bello and Cardé (2022), that lactic acid is a necessary component but insufficient alone to elicit a rate of mosquito landing equal to that of a blend of human odor compounds.

The time to takeoff was surprisingly similar across treatments. Even across those with and without CO_2 the differences were not as large as we would have expected. We suspect that the release, while conducted with care, may have mechanically disturbed the mosquitoes enough to influence takeoff. A short latency to takeoff, however, may be an intrinsic character of *Ae. aegypti*.

Cilek et al. (2004) described this mosquito as opportunistic or exhibiting "aggressive biting" and found that time to biting after landing averaged 9.8 ± 0.3 s (as opposed to *Culex quinquefasciatus*, which averaged 41.0 ± 1.1 s). The test of five mosquitoes at a time with the two-component blend and lactic acid alone was intended to determine if the blend was better at eliciting landing than lactic acid alone. The blend elicited more landings and those mosquitoes landed more quickly than to lactic acid alone.

The lack of statistical differences in flight tracks among treatments was unexpected, as there are large differences in the landing propensities among treatments. It may be that such tracks are inherently "messy," and they do not differ in flight characteristics in our assay or in our method of analysis.

Skin odor elicited longer landing durations than either dose of the synthetic lure. This may be in part due to the different suite of cues available after the mosquitoes contacted the beads. Mosquitoes express gustatory receptors on their tarsi (Sparks et al. 2013). Along with chemoreceptors on the labellum (Saveer et al. 2018), receptors on the tarsi mean that after landing a mosquito may bring chemoreceptors into direct contact with host cues. These may include non-volatile chemicals such as amino acids. Further research, possibly with a cage-landing assay, will be needed to identify possible post-landing cues.

Current mosquito traps and lures, including the widely deployed BG Sentinel, have low trapping efficiency (actually captured after being lured to the trap's vicinity) and require a fan (Amos et al. 2020a, b; Amos and Cardé 2022). By using compounds that elicit landing, it might be feasible to lure the mosquitoes directly into traps. This would boost trap efficiency and perhaps allow the development of traps without fans.

We counted the number of repeat landings. If a lure in a trap elicits repeated landing attempt, it would provide multiple opportunities for capture.

Many other compounds also are reported to be attractive to *Ae aegypti* mosquitoes (Coutinho-Abreu et al. 2022; Dormont et al. 2021) and should be evaluated to determine if any of these add to the attractiveness of the blend of lactic and ketoglutaric acids. Among these are hexanoic acid, a known attractant of *Ae. aegypti* (Carlson et al. 1973; Williams et al. 2006; Owino et al. 2015), and ammonia (Steib et al. 2001). While known as an attractant of *Anopheles* rather than *Aedes* mosquitoes, 2-oxo-pentanoic acid, as well as straight-chain carboxylic acids

of various lengths (Healy and Copland 2000; Healy et al. 2002) should be evaluated, although carboxylic acids and short-chain aldehydes can be repellent to *Ae. aegypti* (Logan et al. 2008; Owino et al. 2015). To avoid specious interpretations of which odors mediate natural attraction to human hosts, it will be important to release odors at rates and ratios closely mimicking those that are naturally emitted (Cardé 2022).

Conclusion

A blend of lactic and ketoglutaric acids discovered by Bello and Cardé (2022) elicits upwind flight and landing of *Ae. aegypti* in a wind tunnel, with and without supplemental CO_2. The effectiveness of this blend without supplemental CO_2 makes this combination a candidate for use in mosquito traps. Ketoglutaric acid may be one of the compounds present in animal odors that when supplemented with lactic acid are highly attractive to anthropophilic mosquitoes.

Authors' Contributions BDS and RTC designed the experiments, BDS carried out these trials and analyzed our findings. BAA analyzed the flight racks. JB aided in selection of treatments. BDS and RTC wrote the paper.

Data Availability GitHub link to follow.
https://github.com/bdsumner/KGA-LA-Windtunnel

Code Availability GitHub link to follow.
https://github.com/bdsumner/KGA-LA-Windtunnel

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare that they have no conflicts of interest.

References

Acree F Jr, Turner RB, Gouck HK, Beroza M, Smith N (1968) L-Lactic acid-a mosquito attractant isolated from humans. Science 161:1346–1347

Amos BA, Cardé RT (2022) Verifying the efficiency of the Biogents Sentinel trap in the field and investigating microclimatic influences on responding *Aedes aegypti* behavior. J Vect Ecol 47:166–170

Amos BA, Ritchie SA, Cardé RT (2020a) Attraction versus capture II: efficiency of the BG-Sentinel trap under semifield conditions and characterizing response behaviors of male *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 57:1–11

Amos BA, Staunton KM, Ritchie SA, Cardé RT (2020b) Attraction versus capture: efficiency of BG-Sentinel trap under semi-field conditions and characterizing response behaviors for female *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 57:884–892

Bello JE, Cardé RT (2022) Compounds from human odor that induce landing and attraction in female yellow fever mosquitoes (*Aedes aegypti*). Sci Rep 12:15638. https://doi.org/10.1038/s41598-022-19254-w

Bernier U, Kline D, Schreck C, Yost R, Barnard D (2002) Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). J Am Mosq Control Assoc 18:186–195

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O et al (2013) The global distribution and burden of dengue. Nature 496:504–507

Braks MA, Takken W (1999) Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae* sensu stricto. J Chem Ecol 25:663–672

Cardé RT (2015) Multi-cue integration: how female mosquitoes locate a human host. Curr Biol 25:R793–795

Cardé RT, Gibson G (2010) Host finding by female mosquitoes: mechanisms of orientation to host odours and other cues. In: Takken W, Knols BGJ (eds) Olfaction in Vector-Host Interactions. Wageningen Academic Publishers, Wageningen, pp 115–140

Cardé RT (2023) Wind tunnels and airflow-assays: methods for establishing the cues and orientation mechanisms that modulate female mosquito attraction to human hosts. Cold Spring Harb Protoc (in press)

Carlson DA, Smith N, Gouck HK, Godwin DR (1973) Yellowfever mosquitoes: compounds related to lactic acid that attract females. J Econ Entomol 66:329–331

Cheung A, Zhang S, Stricker C, Srinivasan MV (2007) Animal navigation: the difficulty of moving in a straight line. Biol Cybern 97:47–61

Cilek JE, Petersen JL, Hallmon CE (2004) Comparative efficacy of IR3535 and deet as repellents against adult *Aedes aegypti* and *Culex quinquefasciatus*. J Am Mosq Control Assoc 20:299–304

Coutinho-Abreu IV, Riffell JA, Akbari OS (2022) Human attractive cues and mosquito host-seeking behavior. Trends Parasitol 38:246–264

Crawford JE, Alves JM, Palmer WJ, Day JP, Sylla M, Ramasamy R, Surendran SN, Black WC, Pain A, Jiggins FA (2017) Population genomics reveals that an anthropophilic population of *Aedes aegypti* mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector. BMC Biol 15:16. https://doi.org/10.1186/s12915-017-0351-0

Daykin PN, Kellog FE, Wright RH (1965) Host finding and repulsion of *Aedes aegypti*. Can Entomol 97:239–263

Dekker T, Takken W (1998) Differential responses of mosquito sibling species *Anopheles arabiensis* and *An. quadriannulatus* to carbon dioxide, a man or a calf. Med Vet Entomol 12:136–140

Dekker T, Takken W, Cardé RT (2001) Structure of host-odour plumes influences catch of *Anopheles gambiae* s.s. and *Aedes aegypti* in a dual-choice olfactometer. Physiol Entomol 26:124–134

Dekker T, Steib B, Cardé RT, Geier M (2002) L-lactic acid: a human-signifying host cue for the anthropophilic mosquito *Anopheles gambiae*. Med Vet Entomol 16:91–98

Dekker T, Geier M, Cardé RT (2005) Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. J Exp Biol 208:2963–2972

Delgado-Povedano MM, Castillo-Peinado LS, Calderon-Santiago M, Luque de Castro MD, Priego-Capote F (2020) Dry sweat as sample for metabolomics analysis. Talanta 208:e120428

Dethier V, Browne BL, Smith CN (1960) The designation of chemicals in terms of the responses they elicit from insects. J Econom Entomol 53:134–136

Dormont L, Mulatier M, Carrasco D et al (2021) Mosquito attractants. J Chem Ecol 47:351–393

Friard O, Gamba M (2016) BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol Evol 7:1324–1330

Geier M, Bosch OJ, Boeckh J (1999) Influence of odour plume structure on upwind flight of mosquitoes towards hosts. J Exp Biol 202:1639–1648

Geier M, Bosh O, Steib B, Rose A, Boeckh J (2002) Odour-guides host finding mosquitoes: identification of new attractants on human skin. Proc Int Conf Urban Pests. Regensburg, Germany, pp 37–46

Ghaninia M, Majeed S, Dekker T, Hill SR, Ignell R (2019) Hold your breath - Differential behavioral and sensory acuity of mosquitoes to acetone and carbon dioxide. PLoS One 14:e0226815

Gillies M (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. Bull Entomol Res 70:525–532

Gillies MT, Wilkes TJ (1970) A comparison of the range of attraction of animal baits and of carbon dioxide for some West African mosquitoes. Bull Entomol Res 59:441–456

Gloria-Soria A, Ayala D, Bheecarry A, Calderon-Arguedas O, Chadee DD et al (2016) Global genetic diversity of *Aedes aegypti*. Mol Ecol 25:5377–5395

Healy T, Copland M (2000) Human sweat and 2-oxopentanoic acid elicit a landing response from *Anopheles gambiae*. Med Vet Entomol 14:195–200

Healy T, Copland M, Cork A, Przyborowska A, Halket J (2002) Landing responses of *Anopheles gambiae* elicited by oxo-carboxylic acids. Med Vet Entomol 16:126–132

Huffaker CB, Back RC (1943) A study of methods of sampling mosquito populations. J Econ Entomol 36:561–569

Kennedy JS (1940) The visual responses of flying mosquitoes. Proc Zool Soc Lond Ser A 109:221–242

Kennedy JS (1977) Behaviorally discriminating assays of attractants and repellents. In: Shorey HH, McKelvey JJ (eds) Chemical control of insect behavior. Wiley, New York, pp 215–229

Khan AA, Maibach HI, Strauss WG (1968) The role of convection currents in mosquito attraction to human skin. Mosq News 28:462–464

Lacey ES, Cardé RT (2011) Activation, orientation and landing of female *Culex quinquefasciatus* in response to carbon dioxide and odour from human feet: 3D flight analysis in a wind tunnel. Med Vet Entomol 25:94–103

Logan JG, Birkett MA, Clark SJ et al (2008) Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes. J Chem Ecol 34:308

Macdonald G (1952) The objectives of residual insecticide campaigns. Trans R Soc Trop Med Hyg 46:227–235

McBride CS, Baier F, Omondi AB, Spitzer SA, Lutomiah J, Sang R, Ignell R, Vosshall LB (2014) Evolution of mosquito preference for humans linked to an odorant receptor. Nature 515:222–227

McLean DJ, Skowron-Volponi MA (2018) trajr: an R package for characterization of animal trajectories. Ethology 124:440–448

McMeniman CJ, Corfas RA, Matthews BJ, Ritchie SA, Vosshall LB (2014) Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell 156:1060–1071

Muir LE, Thorne MJ, Kay BH (1992) *Aedes aegypti* (Diptera: Culicidae) vision: spectral sensitivity and other perceptual parameters of the female eye. J Med Entomol 29:278–281

Owino EA, Sang R, Sole CL, Pirk C, Nbogo C, Toroto B (2015) An improved odor bait for monitoring populations of *Aedes aegypti*-vectors of dengue and chikungunya viruses in Kenya. Parasit Vectors 8:253

Pinto MC, Campbell-Lendrum DH, Lozovei AL, Teodoro U, Davies CR (2001) Phlebotomine sandfly responses to carbon dioxide and human odour in the field. Med Vet Entomol 15:132–139

Powell JR, Gloria-Soria A, Kotsakiozi P (2018) Recent history of *Aedes aegypti*: Vector genomics and epidemiology records. Bioscience 68:854–860

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/ Accessed June 3, 2018.

Reed W, Carroll J, Agramonte A, Lazear JW (1900) The etiology of yellow fever—a preliminary note. Public Health Pap Rep 26:37–53

RStudio Team (2020) RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/ Accessed June 3, 2018.

Russell R (2004) The relative attractiveness of carbon dioxide and octenol in CDC- and EVS-type light traps for sampling the mosquitoes *Aedes aegypti* (L.), *Aedes polynesiensis* Marks, and *Culex quinquefasciatus* Say in Moorea, French Polynesia. J Vect Ecology 25:158–163

Saveer AM, Pitts RJ, Ferguson ST, Zwiebel LJ (2018) Characterization of chemosensory responses on the labellum of the malaria vector mosquito, *Anopheles coluzzii*. Sci Rep 8:5656. https://doi.org/10.1038/s41598-018-23987-y

Schreck CE, Gouck HK, Posey KH (1972) The range of effectiveness and trapping efficiency of a plexiglas mosquito trap baited with carbon dioxide. Mosq News 32:496–501

Scott TW, Chow E, Strickman D, Kittayapong P, Wirtz RA, Lorenz LH, Edman JD (1993) Blood-feeding patterns of *Aedes aegypti* (Diptera: Culicidae) collected in a rural Thai village. J Med Entomol 30:922–927

Smith CN, Smith N, Gouck HK, Weidhaas DE, Gilbert IH, Mayer MS, Smittle BJ, Hofbauer A (1970) L-lactic acid as a factor in the attraction of *Aedes aegypti* (Diptera: Culicidae) to human hosts. Ann Entomol Soc Am 63:760–770

Snow WF (1970) The effect of a reduction in expired carbon dioxide on the attractiveness of human subjects to mosquitoes. Bull Entomol Res 60:43–48

Sparks JT, Vinyard BT, Dickens JC (2013) Gustatory receptor expression in the labella and tarsi of *Aedes aegypti*. Insect Biochem Mol Biol 43:1161–1171

Steib BM, Geier M, Boeckh J (2001) The effect of lactic acid on odour-related host preference of yellow fever mosquitoes. Chem Senses 26:523–528

Sumner BD, Cardé RT (2022) Valence of human-odor, visual, and heat cues inducing landing in female *Aedes aegypti* mosquitoes. J Insect Behav 35:31–43

Thurmon FM, Ottenstein B (1952) Studies on the chemistry of human perspiration with special reference to its lactic acid content. J Invest Dermatol 18:333–339

Torr S, Della Torre A, Calzetta M, Costantini C, Vale G (2008) Towards a fuller understanding of mosquito behaviour: use of electrocuting grids to compare the odour-orientated responses of *Anopheles arabiensis* and *An. quadriannulatus* in the field. Med Vet Entomol 22:93–108

van Breugel F, Riffell J, Fairhall A, Dickinson MH (2015) Mosquitoes use vision to associate odor plumes with thermal targets. Curr Biol 25:2123–2129

Williams CR, Bergbauer R, Geier M, Kline DL, Bernier UR, Russell RC, Ritchie SA (2006) Laboratory and field assessment of some kairomone blends for host-seeking *Aedes aegypti*. J Am Mosq Control Assoc 22:641–647

Wishart DS et al (2018) HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 46:608–617