

1 L-lactic and 2-ketoglutaric acids, odors from human skin, govern attraction and landing
2 in host-seeking female *Aedes aegypti* mosquitoes

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18 **Keywords** carbon dioxide, Culicidae, wind tunnel, 2-hydroxypropanoic acid, 2-
19 oxopentanedioic acid

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22 **Abstract**

23

24 *Aedes aegypti*, presented with a source of L-lactic and 2-ketoglutaric acid in a wind-

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26 tunnel bioassay, takeoff, fly upwind, and land on the blend at rates comparable those

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28 exhibited by mosquitoes presented with a skin odor stimulus. Addition of carbon dioxide

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30 decreased takeoff latency but was not required to elicit upwind flight nor landings.

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32 Ketoglutaric acid, a recently identified component of human skin odor, combined with

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34 lactic acid elicits the full repertoire of mosquito host-seeking behaviors.

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37 **Introduction**

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39 *Aedes aegypti* (Diptera: Culicidae) is a vector of several consequential arboviruses

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41 including chikungunya, dengue, yellow fever, and Zika. *Aedes aegypti* has invaded much

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43 of the tropics and sub-tropics, making dengue the most prevalent human arbovirus,

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45 infecting 100 million per year and placing nearly half of the world's population at risk

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47 (Bhatt et al. 2013).

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

43 To find a host, *Ae. aegypti* fly upwind when they detect fluctuating levels of CO₂
44 above ambient concentration. Once they are within several meters of a prospective host,
45 they can also sense visual cues and, even closer to the host, thermal cues (Gillies 1980;
46 Cardé and Gibson 2010; van Breugel et al. 2015; Cardé 2015; Sumner and Cardé 2022).
47 The precise distances at which they detect and use host odors other than CO₂ is
48 unresolved (Gillies and Wilkes 1970; Dekker et al. 2005).

49 For *Ae. aegypti*, humans are distinguished from other potential hosts by their skin
50 odor (Steib et al. 2001; Dekker et al. 2002; McBride et al. 2014). Bernier et al. (2002)
51 characterized 279 compounds in the headspace above human skin. A review by Dormont
52 et al. (2021) provides a valuable table of mosquito attractants using Dethier et al.’s (1960)
53 definition of attractant as “a chemical which causes insects to make oriented movements
54 towards its source.” Their list of compounds is organized by species, “co-tested”
55 compounds, and assay type. A challenge is to determine which compounds or
56 combinations of compounds elicit host-finding behaviors. This task is complicated by
57 how different bioassays measure different components of mosquito host-seeking
58 behavior. “Attractiveness” in one assay may measure flying into a port from a still-air
59 chamber, whereas in another, it may measure arrestment after upwind orientation (Cardé
60 2022). Most assays measure attraction by the responder reaching an endpoint of
61 orientation over a set interval.

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

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

80 Lactic acid (2-hydroxypropanoic acid) is thought to be a diagnostic cue used by
81 *Ae. aegypti* (Steib et al. 2001) and *An. gambiae* (Dekker et al. 2002) to distinguish
82 humans from non-human animals. The addition of lactic acid made non-human animal
83 odor more attractive to both species of anthropophilic mosquitoes. Both studies found
84 lactic acid, however, was necessary but insufficient by itself to attract the number of *Ae.*
85 *aegypti* that were attracted to human odor. Smith et al. (1970) tested *Ae. aegypti* in a
86 combined port entry and landing assay. They found that lactic acid reduced landing on a

87 human-worn sock, but increased olfactometer trap catch downwind of the sock,
88 suggesting that the dose of lactic acid reaching the mosquito is important. Healy and
89 Copland (2000) found that *An. gambiae* did not land on a lactic acid source. However,
90 Dekker et al.'s (2002) findings, lactic acid may be an attractant but not a landing cue for
91 *An. gambiae*.

92 Recently, Bello and Cardé (2022) identified 2-ketoglutaric acid (2-
93 oxopentanedioic acid) in human skin odor and demonstrated that a mixture of lactic and
94 ketoglutaric acids in the presence of CO₂ is a landing cue for *Ae. aegypti*. The addition of
95 pyruvic acid, also present in the active fraction of skin odor (Bello and Cardé 2022), to
96 this mixture did not significantly elevate landing rates above those elicited by a blend of
97 lactic and ketoglutaric acids.

98 Carbon dioxide added to ambient air (which has an intrinsic concentration of CO₂
99 of about 0.4%), increases the takeoff rate of *Ae. aegypti* (Daykin et al. 1965), elicits
100 upwind flight (Kennedy 1940), increases heat-seeking (McMeniman et al. 2014), and
101 ultimately leads to increased endpoint capture (Huffaker and Back 1943. In a generally
102 accepted model of mosquito host seeking, formalized by Gillies in 1980, CO₂ is generally
103 considered as the long-range attractant of host-seeking mosquitoes. The model assumes a
104 mosquito detects CO₂, takes off, flies upwind along the plume, and then switches to other
105 host cues. Dekker et al. (2005) found that diluted human skin odor was less attractive to
106 *Ae. aegypti* than undiluted skin odor and that CO₂ sensitizes mosquitoes to other host
107 odors. These models were updated by Cardé and Gibson (2010), van Breugel et al.
108 (2015), and Cardé (2015), but still posit that CO₂ was the long-distance attractant, and
109 that as mosquitoes fly closer to their hosts they would switch to using specific cues such
110 as skin odor.

111 This paradigm may not hold for other mosquitoes. Schreck et al. (1972) found
112 that *Anopheles quadriannulatus* flew to a calf from further distances than to a calf-
113 equivalent quantity of CO₂. It is unknown how far downwind the lactic and ketoglutaric
114 acids are detectable by *Ae. aegypti*. Additionally, it is not known whether there are
115 characteristic behaviors, such as surging upwind or casting crosswind, associated with the
116 detection and subsequent attraction to the landing cue compounds

117 As the blend of lactic and ketoglutaric acids elicited landing in a cage assay (Bello
118 and Cardé 2022), we set out to determine if the blend elicits upwind flight in a wind
119 tunnel in the presence or absence of CO₂. We used wind-tunnel assays with videography
120 allow examination of orientation maneuvers, in addition to landing (Lacey and Cardé
121 2011). Some wind-tunnel assays have used video tracking without landing counts (van
122 Breugel et al. 2015); however, landing is the ultimate measure of successful orientation to
123 a host. If a mosquito does not land, it cannot bite nor transmit pathogens (Reed et al.
124 1900). We measured time from release to takeoff, time from takeoff to the first landing
125 on the odor source, the number of landings, and the duration of landings. Our video
126 tracking system allowed us to examine mosquito flight maneuvers prior to landing.

127

128 **Materials and Methods**

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130 **Mosquito Rearing**

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133 An Orlando strain colony of *Ae. aegypti* was maintained in a L:D 14:10 h photocycle at
134 27 °C and 70 % RH. Approximately 50 larvae were reared in plastic containers (26 x
135 25.6 x 15 cm) with ~1 cm of deionized (D.I.) water and fed Tetramin® pellets (Tetra,
136 Blacksburg, VA, U.S.A). Pupae were held in plastic containers, transferred to screen

137 cages (30 x 30 x 30 cm, BugDorm-1, Megaview Science Co. Ltd. Talchung, Taiwan)
138 before eclosion. Mosquitoes were provided 10 % sucrose solution in D.I. water on a
139 cotton wick. Males and females were held together in the screen cages, and females used
140 in the bioassays were assumed to have mated. Females used in experiments were 3-10
141 days post-eclosion and were not blood fed. Mosquitoes were starved and deprived of
142 water approximately 12 hours prior to experiments. Female mosquitoes were transferred
143 individually to clean cylindrical acrylic release cages (7 × 8 cm diameter) 30 minutes
144 prior to testing; assays were conducted 4-8 h into their photophase.

145

146 **Assay Methods**

147

148 The assay methods were adapted from Sumner and Cardé (2022). The flight and landing
149 of mosquitoes were released in a glass wind tunnel 122 × 30.5 × 30.5 cm and were video
150 recorded (FDR-AX53, Sony, Tokyo, Japan) from above. Air was drawn into the tunnel
151 from an adjacent, uninhabited room (25 °C and 70 % RH). To simulate the presence of an
152 upwind vertebrate host, 100 ml/minute of 4 % CO₂ mixed with tank air (equivalent to
153 1/60 of the exhalation a human, Snow 1970), was carried to the wind tunnel via a 3-m-
154 long Tygon® tube, ensuring temperature equilibration (Pinto et al. 2001). The tube was
155 connected to an L-shaped glass tube (OD 5.5 mm, ID 3.5 mm) that descended 15 cm
156 from the ceiling of the tunnel and extended 20 cm downwind to 60 cm upwind from the
157 release cage. The 4 % CO₂ mix exited at ~0.4 m/s but produced no detectable difference
158 in wind speed (Omega HHF 52 anemometer, Omega Engineering, Inc., Stamford, CT,
159 USA) nor a temperature difference (to within 0.1 °C) 1 cm downwind of the release
160 point. The CO₂ release tube was centered so that the generated plume of CO₂ passed over

161 the beads treated with skin odor and then to the release cage. In trials without the addition
162 of CO₂, tank air was supplied at the same rate through the same equipment.

163 The assay room was maintained at 27 °C and 60 % RH. Illumination for
164 videography was provided by infrared LED lights (AXIS T90A, 850 nm, Axis
165 Communications AB, Lund, Sweden) mounted behind a stainless-steel screen at the
166 downwind end of the tunnel as well as beside the wind tunnel. The infrared light in the
167 camera was turned off to avoid glare. Visible light was provided by incandescent bulbs
168 and measured at ~14 lux inside the tunnel.

169 Treatments were presented on glass beads (black, 10/0 Czech Glass Seed,
170 approximately 2 mm OD toroidal, Precosia Ornela, Zásada, Czech Republic) placed in a
171 clean glass Petri dish (7 cm diameter). Negative control beads are hereafter called clean
172 beads. The blend components, ketoglutaric acid (“KGA” in the figures) (0.5 ml; 10 µl/ml
173 or 100µl/ml in acetone) and lactic acid (“LA” in the figures) (0.5 ml; 10 µl/ml or
174 100µl/ml in acetone) were applied in a dropwise spiral to beads. As Ghaninia et al.
175 (2019) found that acetone was attractive in a flight tube to *Ae. aegypti*, the beads were
176 placed under a fume hood for 10 minutes to ensure that the acetone had evaporated.

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

183 Differing from Sumner and Cardé (2022), the dish of beads was presented on a
184 15-cm high metal stand in the center of the tunnel, 55 cm upwind of the release cage.
185 This ensured that compounds emanating from the beads were detectable by the

186 mosquitoes in their release cage. Assays were run and recorded with video for 6 minutes,
187 commencing with the opening of the release cage. Disposable nitrile gloves were always
188 worn by the experimenter to prevent contamination with skin odors.

189

190 **Treatment Strategy**

191

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

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

205

206 **Data Acquisition**

207

208 Video files were observed, and behavior was scored with BORIS v.5.1.0 (Friard and
209 Gamba 2016). All videos were viewed and scored from the release time until 6 minutes
210 elapsed. The observer recorded release and takeoff as “point” events, whereas landing

211 was scored as a “state” event starting with the landing on the beads and ending with the
212 takeoff from the beads.

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

219

220 **Statistical Analysis**

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222 All data manipulation and statistical tests were conducted using R v.3.5.0 (R Core Team
223 2013) in RStudio v.1.1.463 (RStudio Team 2020).

224 ***Landing Observations***

225

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

230

231 All other tests of manual landing observation data were conducted with
232 generalized linear models (GLMs). A matrix was manually constructed with the
233 independent variable data of each of the treatment combinations. It contained binary
234 values for skin odor and CO₂ as well as a values of 0, 1, or 10 for the dose of the lactic
235 and ketoglutaric acid blend. Instead of considering all treatments as independent, as the
236 Fisher Exact test does, the GLMs used this information about the relationships among the
237 treatments. In particular, the models treat the different doses of the blend as different

237 values of the same independent variable. The GLMs show which treatments were
238 significantly correlated with whichever behavioral outcome was tested. This allowed us
239 to determine which treatments were correlated. If we had relied on testing differences
240 among treatment combinations, we would have potentially masked the importance of
241 some cues. Significant correlations, unlike significant differences, do not translate
242 directly to graphs of whole data sets. Therefore, instead of visually clear asterisks, the
243 outputs of the GLMs are solely listed in the figure captions.

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

252 *Flight Tracks*

253
254 Kruskal-Wallis tests were used to contrast the mean distance of the mosquito from the
255 beads every 1/15 of a second during flight track, the mean velocity of the mosquito
256 during flight, the E_{\max} (track straightness) of the mosquito flight, and the proportion of
257 time the mosquito spent heading ($\pm 20^\circ$) towards the center of the beads. Spearman rank
258 correlations were used to test effects.

259 E_{\max} (a measure of straightness, 1 being a completely straight track) (trajr
260 package, McLean and Skowron-Volponi 2018) was used as a measure of track sinuosity
261 and was calculated (Cheung et al. 2007) using the X and Y coordinates of the subject at

262 each time point. E_{\max} is based on an iterated summing of the expected displacement. As
263 the shortest path between two points is a straight line, a high E_{\max} represents a straight
264 path. This displacement does not necessarily correlate to distance from the beads. A
265 mosquito flying a figure-eight over the beads would have a high E_{\max} and a low mean
266 distance to the beads before landing, whereas a mosquito that flew straight to the beads
267 would have a low E_{\max} and a low mean distance to the beads.

268

269 **Results**

270

271 **Takeoff**

272

273 The presence of CO₂ was the only treatment component that was positively correlated
274 with the proportion of trials with mosquito takeoff (Est. = 0.447, P = 0.022) (Fig. 1).

275

276 **Latency from Release to Takeoff**

277

278

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

284

285

286 **Time From First Takeoff to First Landing**

287

288 Figure 3 shows the time from takeoff to the first landing on the beads. Skin odor (Est. = -
289 1.88, P < 0.001) and lactic and ketoglutaric acids 50 µg (Est. = -1.061, P = 0.026) reduced

290 the time mosquitoes took to first reach and land on the beads. The presence of CO₂ was
291 not correlated with duration of flight from takeoff to first landing ($P = 0.139$).

292

293 **Proportion of Trials with at Least One Landing**

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295

296 While skin odor with or without supplemental CO₂ elicited the numerically highest
297 proportion of trials with one or more landings on the odor-treated beads, it was not
298 significantly different from the proportion of trials with lactic and ketoglutaric acids 50
299 µg in the presence or absence of CO₂ that elicited one or more landings (Fisher Exact
300 Test, adjusted $P = 0.402$) (Fig. 4).

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

305

306

307 **Repeat Landings**

308

309 Figure 5 shows the number of repeat landings, per trial by treatment, among trials with at
310 least one landing. The presence of skin odor (Est. = 0.3238, $P = 0.0199$) and the high
311 dose blend, 50 µg each, of lactic and ketoglutaric acids (Est. = 0.3244, $P = 0.0311$) were
312 positively correlated with the landings by a single mosquito per trial (Fig. 5).

313

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

315

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

322

323 **Duration of Landing**

324

325 Figure 7 provides the time within each trial that a single mosquito spent on the beads.
326 The skin odor treatment (Est. = 1.626, $P < 0.001$), both doses of the blend of lactic and
327 ketoglutaric acids, 5 μg (Est. = 0.979, $P = 0.028$), and 50 μg (Est. = 1.207, $P = 0.004$)
328 were positively correlated with the duration of landing time of the mosquito (Fig. 7). The
329 presence of CO_2 was not correlated with duration of landing time. Additionally,
330 mosquitoes were observed sticking their proboscises on the beads coated with 50 μg each
331 of lactic and ketoglutaric acids, in the presence of CO_2 , in a manner resembling probing
332 behavior.

333

334 **Analysis of Flight Tracks from Takeoff to First Landing**

335

336 Among the treatments there were no differences in the mean distance of insects from the
337 beads during flight in the wind tunnel ($\chi^2 = 298$, $df = 298$, $P = 0.49$). Selected flight
338 tracks are shown in Fig. 8. Across all treatments, the mean (\pm SE) distance (mm) from the
339 center of the beads during a mosquito flight was 214.15 mm (± 5.47). This mean distance
340 from the beads was negatively correlated with whether an individual landed on the beads
341 ($\rho = -0.36$, $P < 0.001$).

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

347 There were no differences in the proportion of time an insect spent heading (\pm
348 20°) toward the center of the beads during flight in the wind tunnel among treatments (χ^2
349 $= 289.36$, $df = 288$, $P = 0.47$), nor did this proportion of time significantly correlate with
350 whether that individual landed on the beads or not ($\rho = 0.01$, $P = 0.83$). Across all
351 treatments, the mean (\pm SE) proportion of the time a flying mosquito spent heading
352 towards the center of the beads ($\pm 20^\circ$) was 0.16 (\pm 0.0039).

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

358

359 **Discussion**

360

361 The two-component blend of lactic acid and ketoglutaric acid developed by Bello and
362 Cardé (2022) elicited upwind flight and landing. The proportion of *Ae. aegypti* that
363 landed at least once on an upwind odor source of the “high dose” (50 µg of each) of the
364 blend was similar to the proportion that landed on human a source of man odor.
365 Unsurprisingly, the presence of CO₂ also increased the proportion of landings, given the
366 known role of CO₂ in sensitizing mosquitoes to human skin odors (Dekker et al. 2005)
367 and eliciting upwind flight (Kennedy 1940). Individual humans vary in their intrinsic
368 attractiveness to mosquitoes, and some of this variation is likely attributable to the
369 quantitative differences among individuals in their emission of lactic acid and
370 ketoglutaric acid (Thurmon and Ottenstein 1952; Delgado-Povedano et al. 2020).

371 Skin odor and the higher dose of the blend of lactic acid and ketoglutaric acid (50
372 µg), induced rapid orientation and landing on the beads and comparable numbers of
373 repeat landings per individual mosquito. This suggests that this blend elicited the same
374 persistence in mosquito landing behavior as human skin odor. The similarity between the
375 behavioral activity elicited by the high dose and skin odor was further demonstrated in
376 that the blend and skin odor were both correlated with the cumulative duration of landing
377 on the beads. The number of repeat landings, their durations, or latency of first landing
378 were not correlated with the presence of CO₂. This is unsurprising, because in nature a
379 mosquito landing on a skin odor source other than skin on the face would have likely
380 exited the CO₂ plume (Dekker and Takken 1998). Because a long-duration landing
381 reduces the available time for further landings, the numbers and durations of landings
382 were inversely correlated in all trials.

383 The high-dose blend of lactic acid and ketoglutaric acid (50 µg) elicited more
384 landings than lactic acid alone. We found that 50 µg rather than 5 µg of each component

385 of the blend elicited *Ae. aegypti* behaviors akin to skin odor in a wind tunnel. The 5 μ g
386 dose was sufficient to induce numerous landings the cage assay which has little ambient
387 air movement (Bello and Cardé, 2022). The need for a higher dose to evoke upwind
388 source finding in a wind tunnel is consistent with the odor mixture being diluted by
389 turbulent diffusion of the wind-borne plume as it is carried downwind.

390 Carbon dioxide was positively correlated with the probability of takeoff. It was
391 not correlated with the proportion of trials with a landing within the subset of trials with
392 mosquito take off. Our findings support that CO₂ elicits takeoff in *Ae. aegypti* females but
393 does not act as a landing cue. This is consistent with the sequential-distance model of
394 mosquito host seeking (Gillies 1980; Cardé and Gibson 2010; van Breugel et al. 2015;
395 Cardé 2015). In this paradigm a host seeking mosquito takes off and flies upwind in a
396 plume of CO₂ before encountering other cues. The mosquito is then able to detect visual
397 cues, host odors other than CO₂, and finally heat from the host. There are physical limits
398 to the distance at which visual and heat cues should be detectable to mosquitoes (Kahn et
399 al. 1966; Muir et al. 1992).

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

406 Lactic acid has been a controversial candidate as a mosquito attractant (Acree et
407 al. 1968; Smith et al. 1970); Steib et al. (2001) added lactic acid to human and non-
408 human animal odor. The addition of lactic acid resulted in the non-human animals' odor
409 drawing as many *Ae. aegypti* to its arm of the Y-tube as human odor. However, lactic

410 acid alone attracted only 19 % of the mosquitoes tested. Calf and goat odors with added
411 lactic acid attracted 70 % of the *Ae. aegypti* tested.

412 Ketoglutaric acid may be one of the compounds Steib et al. (2001) and Geier et al.
413 (2002) demonstrated existed but did not isolate. Our results corroborate those of Bello
414 and Cardé (2022), that lactic acid is a necessary component but insufficient alone to elicit
415 a rate of mosquito landing equal to that of a blend of human odor compounds.

416 The time to takeoff was surprisingly similar across treatments. Even across those
417 with and without CO₂ the differences were not as large as we would have expected. We
418 suspect that the release, while conducted with care, may have mechanically disturbed the
419 mosquitoes enough to influence takeoff. A short latency to takeoff, however, may be an
420 intrinsic character of *Ae. aegypti*. Cilek et al. (2004) described this mosquito as
421 opportunistic or exhibiting “aggressive biting” and found that time to biting after landing
422 averaged 9.8 ± 0.3 s (as opposed to *Culex quinquefasciatus*, which averaged 41.0 ± 1.1
423 s). The test of five mosquitoes at a time with the two-component blend and lactic acid
424 alone was intended to determine if the blend was better at eliciting landing than lactic
425 acid alone. The blend elicited more landings and those mosquitoes landed more quickly
426 than to lactic acid alone.

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

431 Skin odor elicited longer landing durations than either dose of the synthetic lure.
432 This may be in part due to the different suite of cues available after the mosquitoes
433 contacted the beads. Mosquitoes have express gustatory receptors on their tarsi (Sparks et
434 al. 2013). Along with chemoreceptors on the labellum (Saveer et al. 2018), receptors on

435 the tarsi mean that after landing a mosquito may bring chemoreceptors into direct contact
436 with host cues. These may include non-volatile chemicals such as amino acids. Further
437 research, possibly with a cage-landing assay, will be needed to identify possible post-
438 landing cues.

439 Current mosquito traps and lures, including the widely deployed BG Sentinel,
440 have low trapping efficiency (actually captured after being lured to the trap's vicinity)
441 and require a fan (Amos et al. 2020 a,b; Amos and Cardé 2022). By using compounds
442 that elicit landing, it might be feasible to lure the mosquitoes directly into traps. This
443 would boost trap efficiency and perhaps allow the development of traps without fans.
444 We counted the number of repeat landings. If a lure in a trap elicits repeated landing
445 attempt, it would provide multiple opportunities for capture.

446 Many other compounds also are reported to be attractive to *Ae aegypti* mosquitoes
447 (Coutinho-Abreu et al. 2021; Dormont et al. 2021) and should be evaluated to determine
448 if any of these add to the attractiveness of the blend of lactic and ketoglutaric acids.
449 Among these are hexanoic acid, a known attractant of *Ae. aegypti* (Carlson et al. 1973;
450 Williams et al. 2006; Owino et al. 2015), and ammonia (Steib et al. 2001). While known
451 as an attractant of *Anopheles* rather than *Aedes* mosquitoes, 2-oxopentanoic acid, as well
452 as straight-chain carboxylic acids of various lengths (Healy and Copeland 2000; Healy et
453 al. 2002) should be evaluated, although carboxylic acids and short-chain aldehydes can
454 be repellent to *Ae. aegypti* (Logan et al. 2008; Owino et al. 2015). To avoid specious
455 interpretations of which odors mediate natural attraction to human hosts, it will be
456 important to release odors at rates and ratios closely mimicking those that are naturally
457 emitted (Cardé 2022).

458

459 **Conclusion**

460

461 A blend of lactic and ketoglutaric acids discovered by Bello and Cardé (2022) elicits

462 upwind flight and landing of *Ae. aegypti* in a wind tunnel, with and without supplemental

463 CO₂. The effectiveness of this blend without supplemental CO₂ makes this combination a

464 candidate for use in mosquito traps. Ketoglutaric acid may be one of the compounds

465 present in animal odors that when supplemented with lactic acid are highly attractive to

466 anthropophilic mosquitoes.

467

468

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477 **Data Availability** [GitHub link to follow](#).

478 **Code Availability** [GitHub link to follow](#).

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

480 **Ethics Approval** Not applicable.

481 **Consent to Participate** Not applicable.

482 **Consent for Publication** Not applicable.

483 **Open Access**

484 **Authors' Contributions** BDS and RTC designed the experiments, BDS carried out these

485 trials and analyzed our findings. BAA analyzed the flight racks. JB aided in selection of

486 treatments. BDS and RTC wrote the paper.

487

488 **References**

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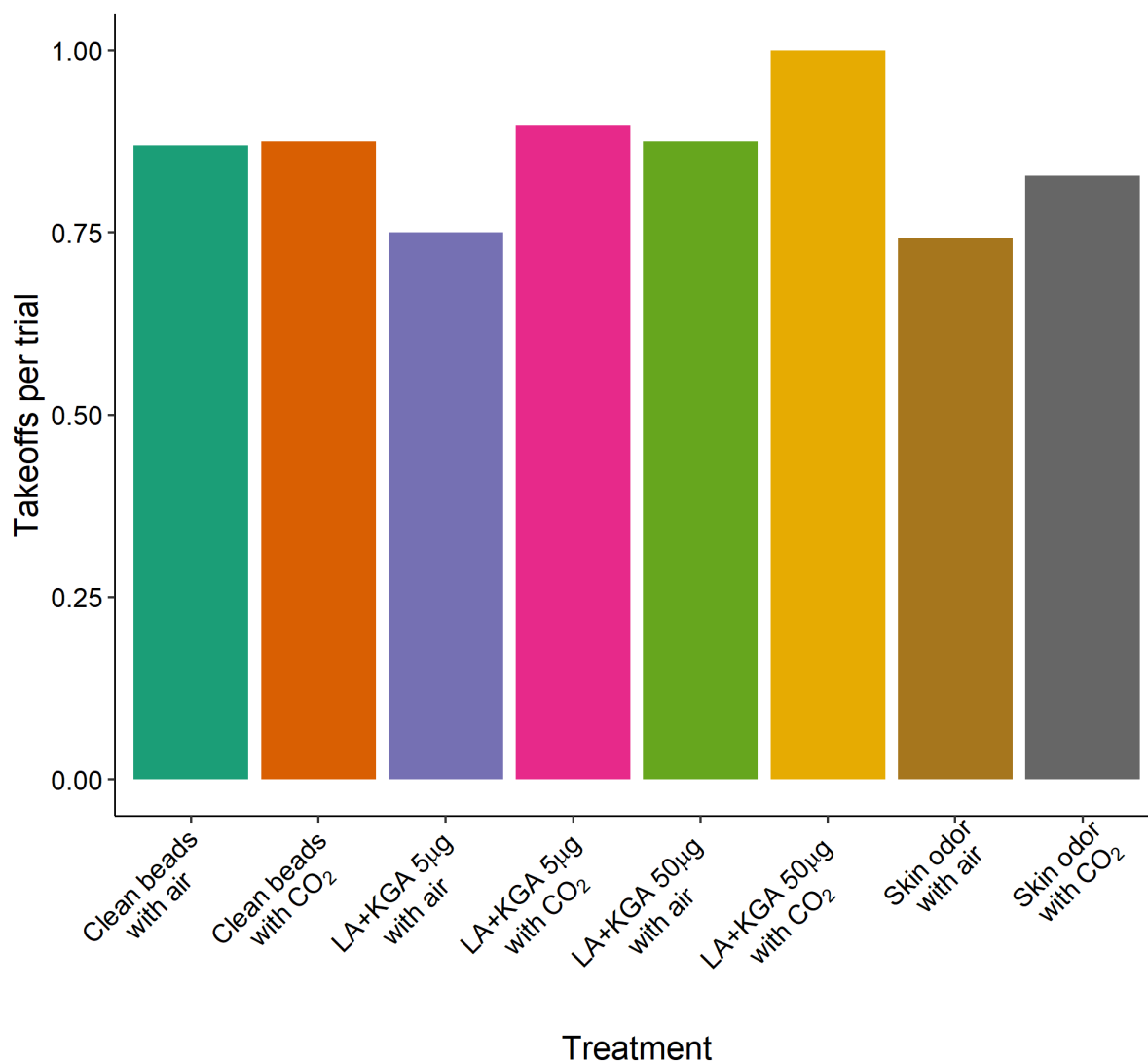
666 **Table 1 Treatment Combinations for Wind Tunnel Assays with Female *Ae. aegypti***

Treatment	CO₂	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

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673 **Fig. 1** The proportions of trials in which *Aedes aegypti* initiated flight. Only the presence

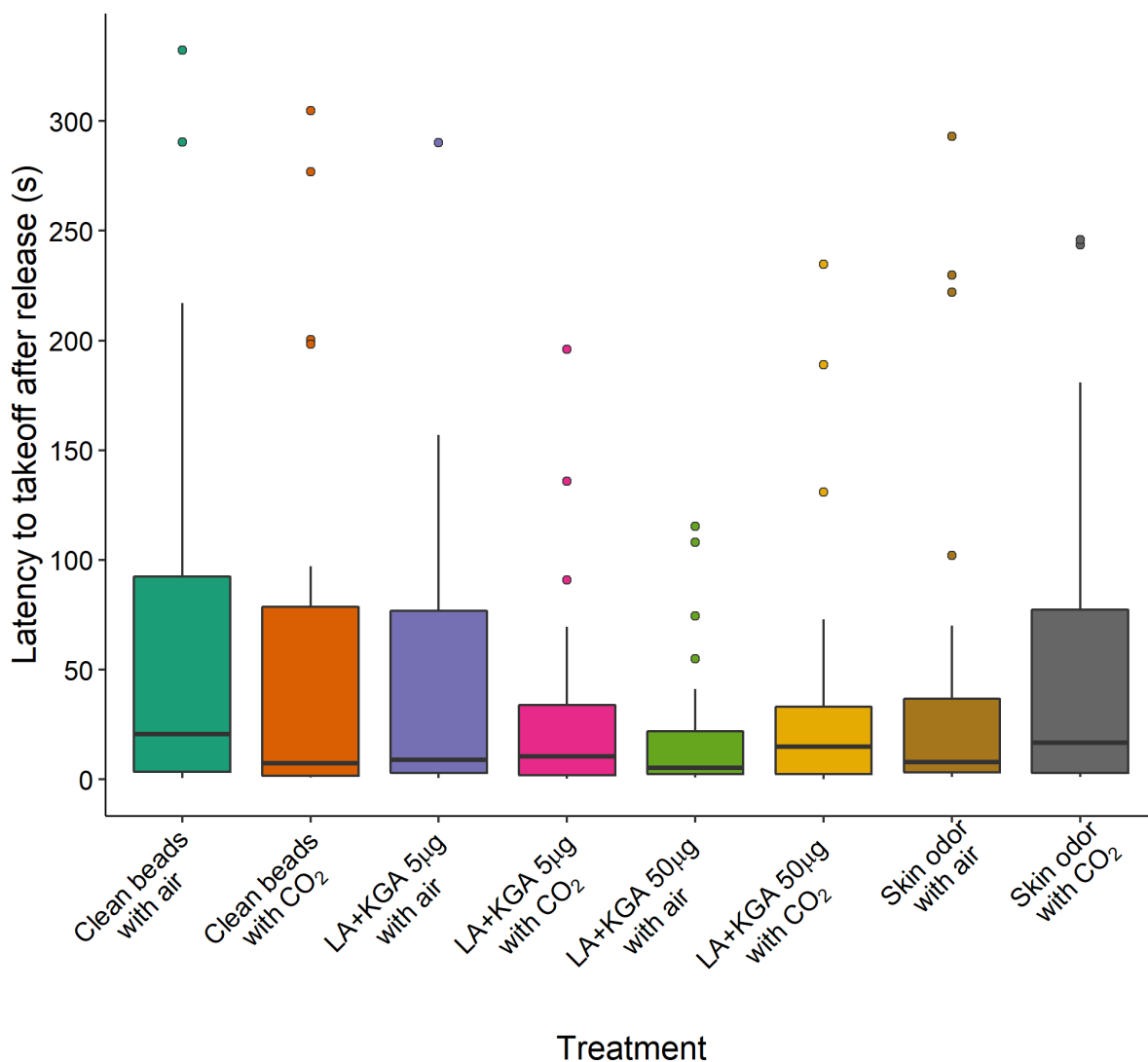
674 of CO₂ was correlated with the proportion of trials with takeoff (GLM, binomial,

675 link = probit, Est. = 0.447, P = 0.022).

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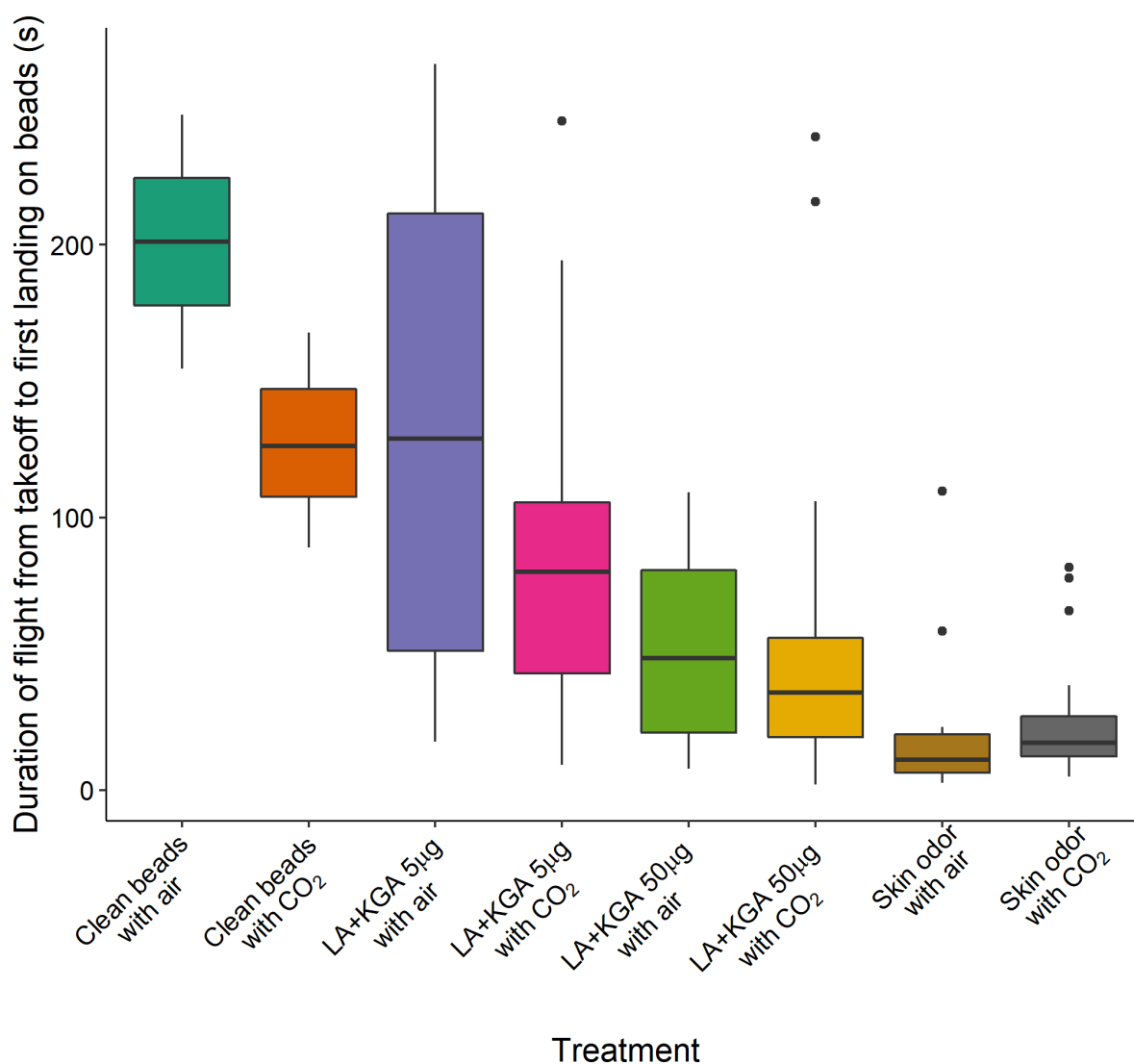
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680 **Fig. 2** Latencies in *Aedes aegypti* of takeoff in seconds. While the high dose of the blend
681 was significantly associated with a shorter latency (GLM, Est. = -0.3534, P =
682 0.017), the residuals of the GLM (gamma, link = log), despite a square root
683 transform of the data, were somewhat divergent from the normal distribution (KS
684 P = 0.022).

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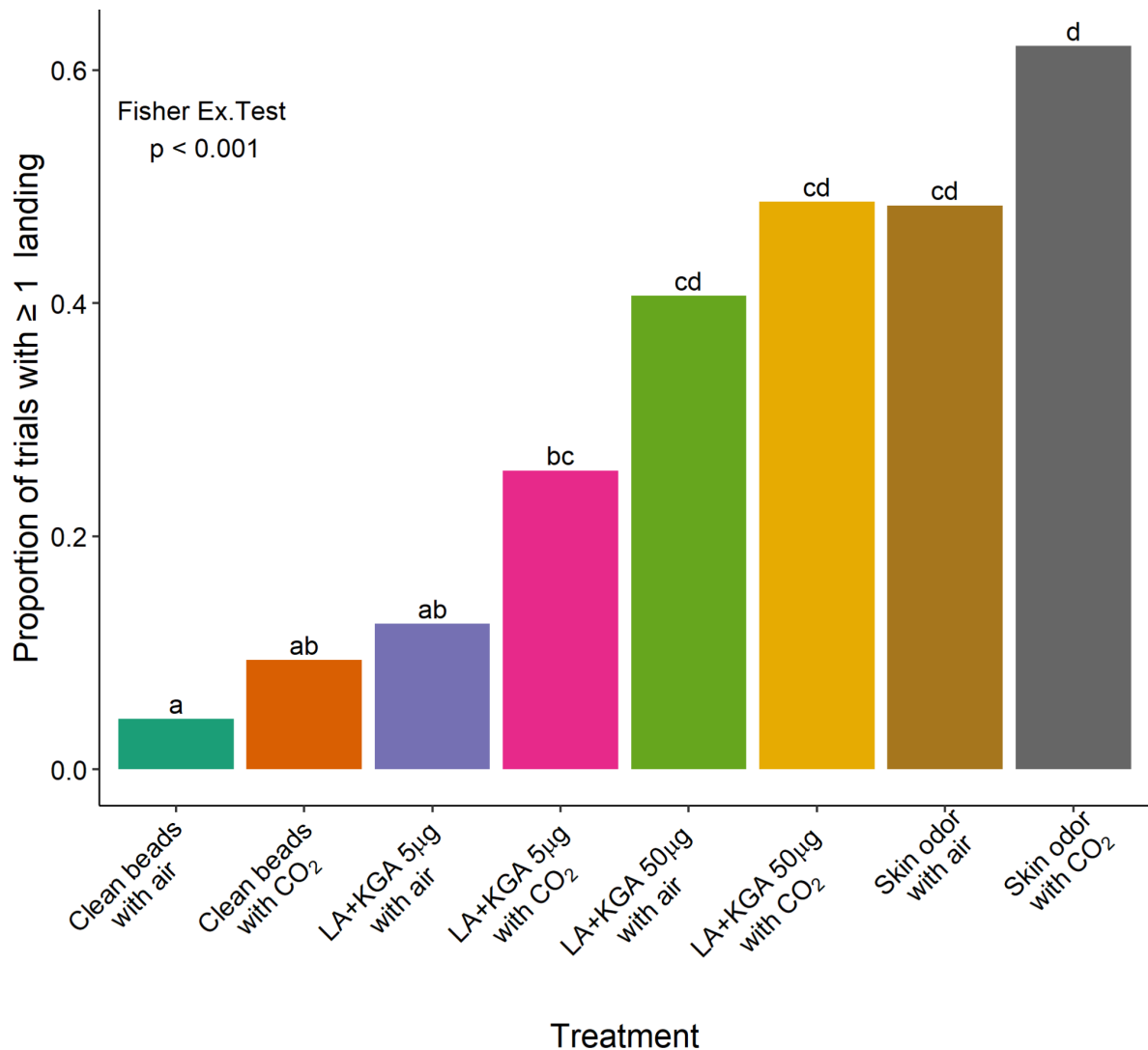
689 **Fig. 3** The durations of *Aedes aegypti* flight from takeoff to the first landing on beads in
690 trials with at least one landing. The presence of skin odor (Est. = -1.88, $P < 0.001$)
691 and the blend of 50 µg each of lactic and ketoglutaric acids (Est. = -1.061, $P =$
692 0.026) resulted in shorter flight times from takeoff to first landing. CO₂ was not
693 correlated with flight duration.

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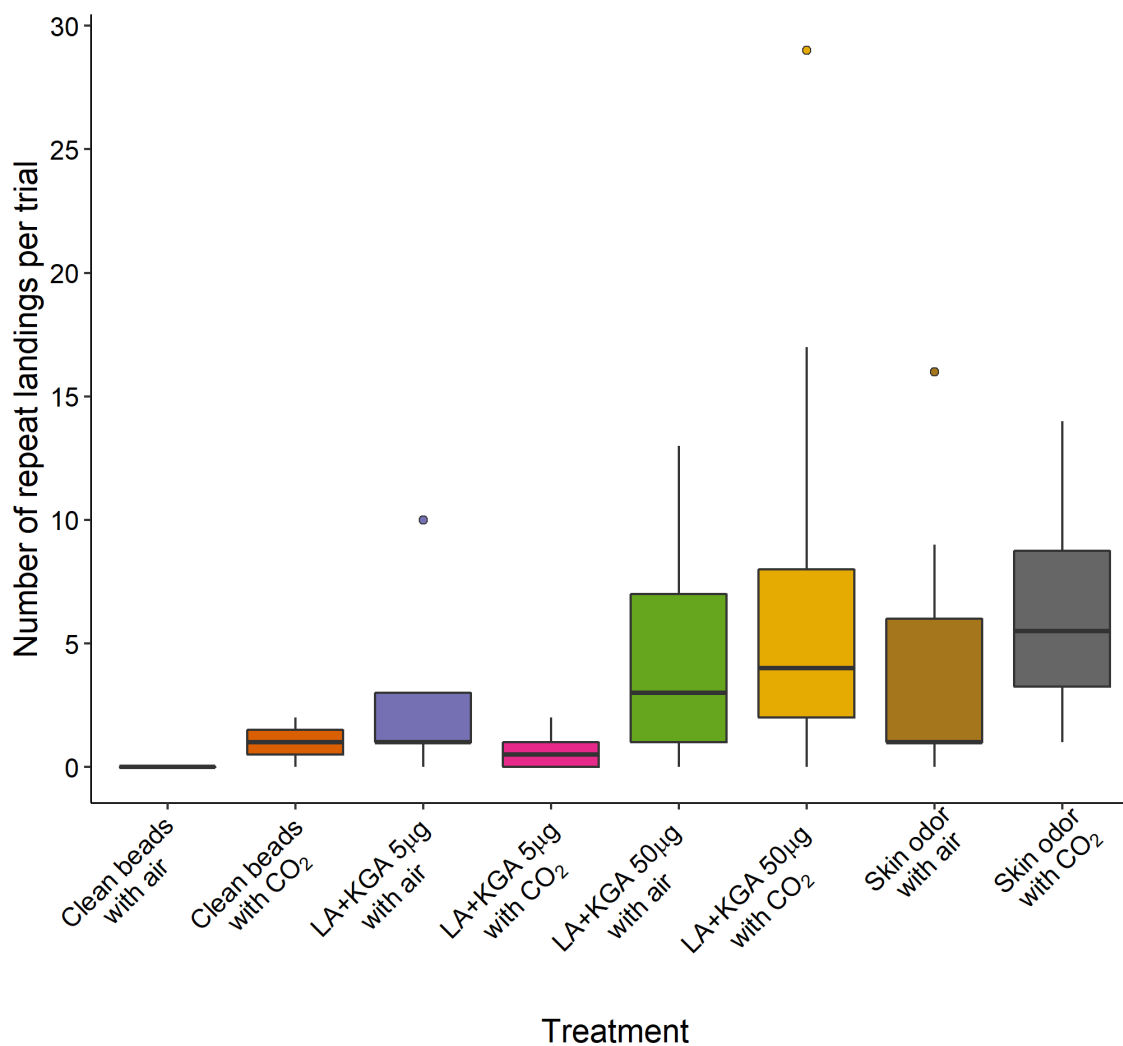
701 **Fig. 4** The proportion of trials in which *Aedes aegypti* landed more than once was tested
702 with a Fisher Exact test followed by pairwise Fisher Exact tests; letters above
703 columns show significant difference ($P < 0.05$). The GLMs, informed of the
704 relationships among the treatments, found that: the proportion of all trials with ≥ 1
705 landing is correlated with: CO₂ (Est. = 0.578, $P = 0.046$), skin odor (Est. = 2.88, P
706 < 0.001), the blend of 5 µg each of lactic and ketoglutaric acids (Est. = 1.192, $P =$
707 0.029), and the blend of 50 µg of each (Est. = 2.434, $P < 0.001$).

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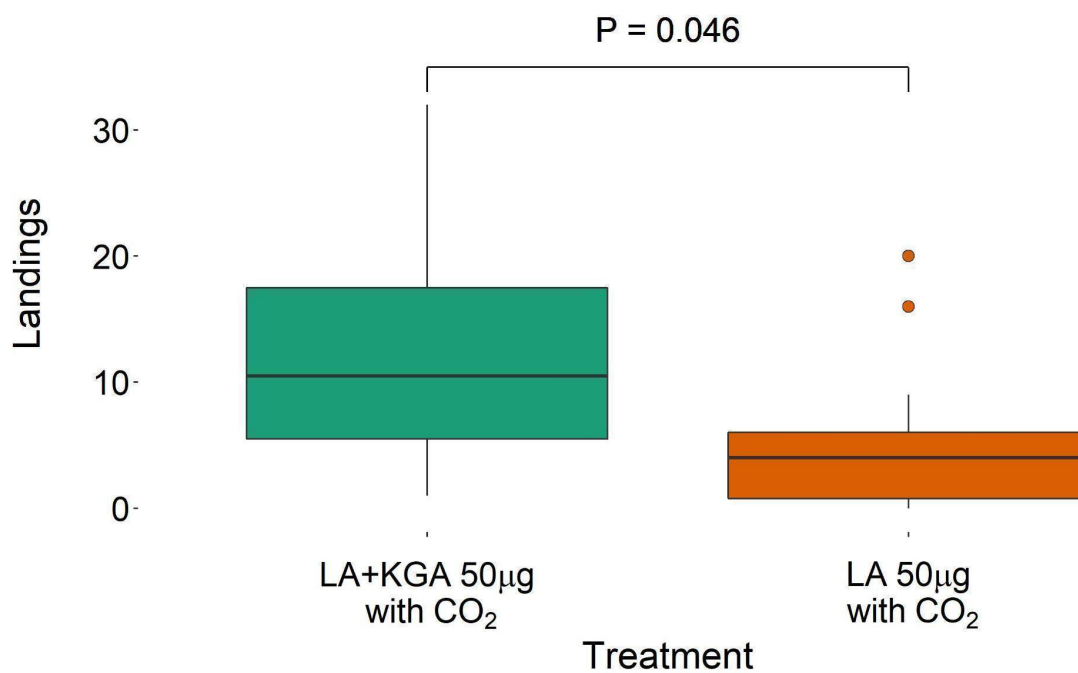
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713 **Fig. 5** The number of repeat landings by female *Aedes aegypti* per trial among trials with
714 at least one landing. Only skin odor (Est. = 0.3238, P = 0.0199) and 50 µg each of
715 lactic and ketoglutaric acids (Est. = 0.3244, P = 0.0311) were correlated with the
716 number of landings per trial.

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721 **Fig. 6** The total landings of *Aedes aegypti* by trial with the blend versus lactic acid alone.

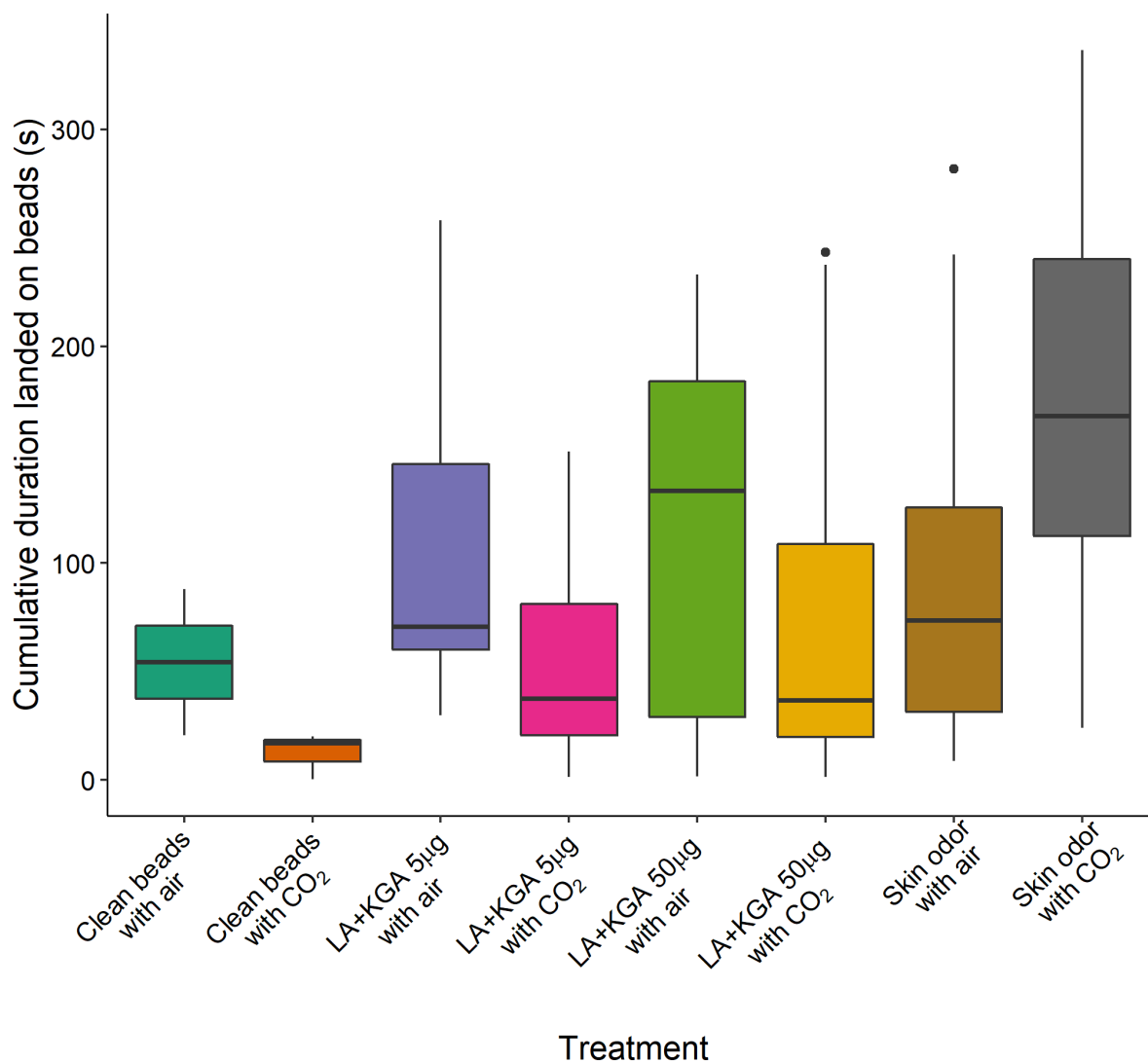
722 The number of landings on the two-component blend was significantly different

723 than on lactic acid alone (Kruskal-Wallis, P = 0.046).

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728 **Fig. 7** The cumulative duration of times female *Aedes aegypti* spent on beads, in trials

729 with ≥ 1 landing. Skin odor (Est. = 1.626, $P < 0.001$), 5 μg each of lactic and

730 ketoglutaric acids (Est. = 0.979, $P = 0.028$), and the blend of 50 μg of each (Est. =

731 1.207, $P = 0.004$) were correlated with the duration mosquitoes remained landed.

732 CO₂ was not correlated with duration landed.

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735 **Fig. 8A**

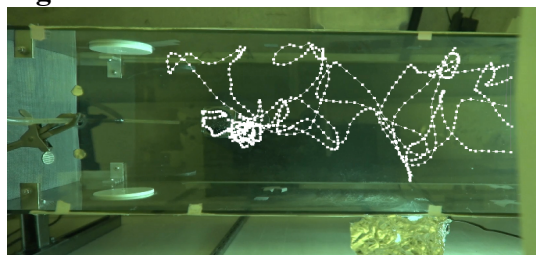
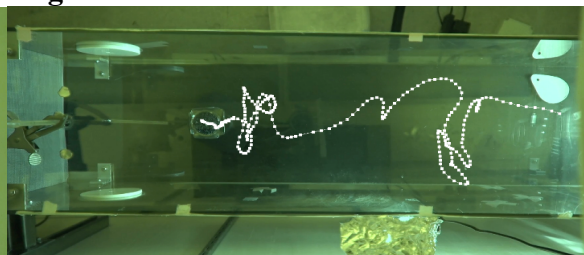


Fig. 8B



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737 **Fig. 8C**

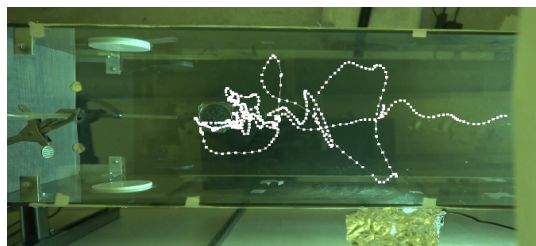
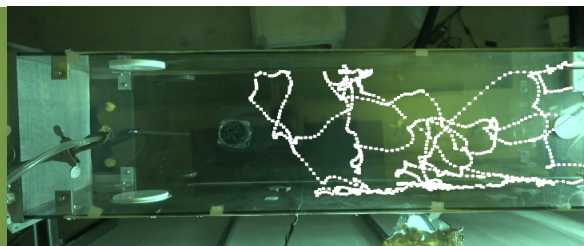


Fig. 8D



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739 **Fig. 8** Top view of selected flight tracks of female *Aedes aegypti*, provided with CO₂,
740 responding to A) 50 µg of lactic and ketoglutaric acids; B) 5 µg of lactic and
741 ketoglutaric acids; C) Skin odor; D) Clean beads. The mosquito did not land in
742 the track shown in 8D. Airflow was left to right, and the mosquitoes were released
743 on the right.