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Orientation and Landing Responses of Female *Aedes aegypti* Mosquitoes to Natural and Synthetic Sources of Human Skin Odor, and to Heat and Visual Cues

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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by

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Chapter 4

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ABSTRACT OF THE DISSERTATION

Orientation and Landing Responses of Female *Aedes aegypti* Mosquitoes to Natural and Synthetic Sources of Human Skin Odor, and to Heat and Visual Cues

by

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Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2021
Dr. Ring Cardé, Chairperson

Abstract

Mosquitoes that are human-feeding specialists vector several pathogens responsible for hundreds of thousands of deaths each year. Mosquitoes use odor to locate humans and to tell them apart from other potential hosts. By understanding how mosquitoes follow odor plumes, and which odor components they use, we may be able to improve traps used for vector surveillance. First, to examine host finding in female *Aedes aegypti,* a diurnal biting mosquito, we used a small source of skin odor that was visually indistinguishable from its surroundings. Despite being thought of as a visually guided mosquito, we found *Ae. aegypti* landed on a visually indistinguishable patch of odor. A blend of lactic and ketoglutaric acids elicits landings in a cage assay. We found in a wind tunnel assay, that this blend elicits takeoff, upwind flight, and landing of *Ae. aegypti,* even without the addition of CO_2. This property makes the blend of lactic and ketoglutaric acids a

candidate for use in mosquito traps because supplying CO_2 to traps in the field is logistically challenging. Finally, we tested the landing response of wild type *Ae. aegypti* and two genotypes lacking *Ir8a* or *orco* receptors. *Aedes aegypti* lacking the *Ir8a* coreceptor, required for lactic acid detection, landed on the blend of lactic and ketoglutaric acid at half of the rate of wild type mosquitoes. This shows that a receptor other than *Ir8a* is responsible for the detection of ketoglutaric acid. Future studies will be able to determine how *Ae. aegypti* detect ketoglutaric acid.

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CHAPTER 1. HOST SEEKING IN ANTHROPOPHILIC MOSQUITOES

Aedes aegypti, the yellow fever mosquito, vectors several human pathogens including yellow fever, dengue, and chikungunya viruses. To vector a human pathogen, horizontally among humans, a mosquito must land on one human, take a blood meal, incubate the pathogen, and then land on and bite a second human (Reed 1900). As *Ae. aegypti* is anthropophilic, it is likely that it will take consecutive blood meals from humans (Scott et al. 1993). Human pathogen transmission is driven by the square of the probability that a mosquito takes a human blood meal (MacDonald 1952). Therefore, it is essential that we know how *Ae. aegypti* locate, land on, and discriminate humans in contrast to other animals.

Aedes aegypti diverged into *Aedes aegypti formosus* and *Aedes aegypti aegypti* 400 to 550 years ago (Gloria-Soria et al. 2016; Powell et al. 2018; Kotsakiozi et al. 2018). The *aegypti* "subspecies", or form, has lighter scales, different olfactory receptor expression levels, and is found more often in standing water habitats near houses (McBride et al. 2014). It enters houses more readily (Trpis and Hausermann 1978) and is captured in human odor-emitting ports in a ventilated assay more frequently than *Ae. a. formosus* (Gouck 1972). However, McClelland and Weitz (1963), using precipitin and inhibition tests, found "no correlation between the hosts identified and the classes of *Ae. aegypti* distinguished by differences in abdominal scale colour." Until there is a bloodmeal analysis study with modern methods, it is not certain that the *aegypti* form feed on humans more frequently in the field than the *formosus* form. The following references to *Aedes aegypti* are to the *aegypti* "subspecies" unless otherwise noted.

Model of Host Seeking Based on Distances at Which Cues are Detectable

The prevailing model of mosquito host seeking is that CO_2 elicits takeoff and then upwind flight or initiates a turn upwind in an in-flight mosquito (Dekker et al. 2001). The mosquitoes then use other cues closer to the host, as these become detectable. This model will be referred to as the "distance model." The physical dissipation of heat (van Breugel et al. 2015), as well as the eyesight limitations of mosquitoes (Muir et al. 1992), ensure that odors are the only cues presumably detectable by mosquitoes farther than 10 meters from a human host. However, it is far from certain if CO_2 is the only odor that is detectable by mosquitoes at long range (Gillies and Wilkes 1970).

The distance model was formalized by Gillies in 1980. Additional evidence was provided by Dekker et al. (2005) that diluted human skin odor was less attractive to *Ae. aegypti* than full-concentration odor. Following this work, the model was refined by van Breugel et al. (2015) and Cardé (2015).

There is an example in which odors other than CO_2 were attractive at a long range. Gillies and Wilkes (1970) positioned concentric rings of ramp traps at different distances from a calf and from a calf-equivalent source of CO_2. They found that a live calf increased the number of *Anopheles melas* captured in ramp traps positioned far downwind than the release of a calf-equivalent amount of CO_2. This difference persisted in traps up to 45 meters away from the calf or CO_2 canister. It is possible that the distance model of mosquito host seeking accurately describes *Ae. aegypti* behavior, and that with CO_2 is the only long-range cue used by this particular species. Our results in chapters two and three are consistent with this model; however, it should not be assumed that all host-seeking mosquitoes use only CO_2 at long range.

Anopheles melas is known to be captured in ramp traps farther downwind from a calf (Gillies and Wilkes 1969, 1970) and a bird (1974) than from an equivalent quantity of CO_2. This has been observed, to a lesser extent, with *Anopheles ziemanni*, *Mansonia uniformis* and whichever unidentified species of *Aedes* were present near rice fields in Brikama Ba, The Gambia (Gillies and Wilkes 1972). While not stated by Gillies and Wilkes (1972), *Ae. aegypti* is easy to identify, so the omission of this species suggests that it was not captured. *Culex thalassius* were captured farther downwind from a bird than from an equivalent quantity of CO_2 (Gillies and Wilkes 1974). No significant difference was observed between calf and CO_2 maximum attraction distances in *M. africana, C. thalassiu, C. univittatus* (Gillies and Wilkes 1972) and between a bird and CO_2 in *C. decens* (Gillies and Wilkes 1974). While Gillies and Wilkes (1972) captured some *An. gambiae*, they lamented that the numbers (20) were too few for statistical analysis. As they were using calves and CO_2 without human odors, it is unsurprising that this anthropophilic mosquito was not captured in greater numbers. Hosts are known to attract greater numbers of other blood-feeding insects than equivalent sources of CO_2. Mullens and Gerry (1998) found that a calf attracted more than six times as many *Culicoides variipennis sonorensis* into a vicinity than did a calf-equivalent CO_2 source. The maximum distance of attraction elicited by host odors should be studied in other blood-feeding insects as well.

Despite repeatedly finding in 1969, 1970, 1972, and 1974 that CO_2 is not the only possible long-range cue used by mosquitoes, Gillies failed to dispel this notion in his 1980 review. He included the CO_2 trap data from 1970, but not the calf data. It was presented only after amalgamation with data from other studies. Although that review was focused on the role of CO_2 and Gillies mentioned that CO_2 may act in combination with other host odors at “moderate ranges,” it is still misleading without the context of the range studies. Both Healy and Copland (1995) and Dekker (2002b) noted that the findings of Gillies and Wilkes (1970) limit the extent to which the standard distance model of mosquito host seeking may be generalized.

Plume Descriptions

Heat Plumes

Heat plumes differ from chemical plumes in that they dissipate through radiation and conduction, as well as convection. Measuring the distance at which a heat plume is detectable is counterintuitively difficult. If one used a human as a heat source, the mosquitoes may be responding to cues other than heat. One must therefore test an artificial heat source. However, simply matching the temperature only provides an approximation, as the total thermal energy in the plume is also determined by the size of the source. While *Ae. aegypti* exhibit distinct responses on an electroantennogram to temperature changes as small as 0.05 °C, the minimum threshold to which they can respond behaviorally is unknown (Davis and Sokolove 1975). The following are some examples of studies in which heat plumes or mosquito heat seeking were quantified.

The maximum distance at which a heat plume is detectable to a mosquito is quite short, as the temperature of the plume must exceed that of the surrounding air to provide information about a potential host (Kahn 1966; van Breugel et al. 2015; Sumner and Cardé Chapter 2). van Breugel et al. (2015) measured the air temperature at several heights above the wind tunnel floor, which contained their heating pad. At the lowest height (0.5 cm), which is closest to the heating pad and stayed warm the furthest; the air temperature returned to ambient within 40 cm downwind of the 37 °C heating pad. The thermal signature of a human arm dissipated to within 0.2 °C above ambient after 10-15 cm in the wind tunnel as well (van Breugel et al. 2015). The heating plume generated with a heating pad in the wind tunnel used by Sumner and Cardé (Chapter 2) dissipated to within 0.1 °C of the ambient temperature by 30 cm downwind. A small percentage of *Ae. aegypti* in a screen chamber 80 cm above a human temperature heat source landed on the bottom of the chamber (Kahn 1966). Therefore, while it is experimentally difficult to measure the response of mosquitoes to host-equivalent heat sources without providing any other cues, we may assume that mosquitoes only detect host-generated heat over short distances.

Odor Plumes

When air flows over an odor-emitting host, it generates plumes of compounds through turbulent diffusion. Molecular diffusion produces gradients over small distances in still air, increasing in concentration continuously on approach to the source. In contrast, turbulent diffusion produces plumes that may extend tens of meters with packets of compounds interspersed with “clean” air. A mosquito flying toward a human does not detect a CO_2 concentration gradient along its path; instead, it detects filaments of up to 4 % CO_2 intermixed with ambient air containing 1/100th the concentration of CO_2 of that found in host exhalation (Dekker et al. 2005). Mosquitoes can locate their hosts by flying upwind while they detect packets of host odor cues (Cardé and Gibson 2010). The detection of a fluctuating intensity of odor was found to be an integral part of mate seeking in some moths in 1994 (Mafra-Neto and Cardé 1994; Vickers and Baker 1994) and was later found to be an integral part of host seeking in *Ae. aegypti* (Dekker et al. 2011).

CO_2 Plumes

Carbon dioxide is found in the atmosphere at background levels around 400 parts per million (ppm) (Zollner et al. 2004). Carbon dioxide plumes must be above the background concentration when they reach a mosquito to provide information about host presence. Zollner et al. (2004) set up a canister to release CO_2 at a rate equivalent to the exhalation of two oxen. While entirely appropriate for the bovid-seeking tsetse of concern in the study, this is 10 times the CO_2 released by a human (Pinto et al. 2001). By measuring the CO_2 concentration downwind using a near-infrared spectrometer, they were able to find the distance at which a CO_2 plume dissipates to the background level. In a dry riverbed, CO_2 was detectable 64 m downwind, whereas it was detected 32 m downwind in a dense woodland (Zollner et al. 2004). The detector used was sensitive to changes smaller than 10 ppm, while *Ae. aegypti* show electrophysiological responses to CO_2 differences of 50 ppm (Grant et al. 1995) and *An. gambiae* exhibit upwind flight when presented with 100 ppm (Healy and Copland 1995). Therefore, human-emitted CO_2 is likely detectable to mosquitoes at a range substantially less than 60 meters. Replication of the concentric rings of ramp traps used by Gillies and Wilkes (1970) with new tools such as electrocution traps could be used to verify the maximum range of CO_2 attraction on each species of mosquito of interest.

Skin Odor Plumes

Human skin odor is a blend of hundreds of individual compounds. Bernier et al. (1999, 2000) observed 346 gas chromatograph peaks from odor collected on glass beads rubbed on human hands and tentatively identified 279 of these by coupled gas chromatography-mass spectroscopy. Skin odor is a combination of compounds produced by humans endogenously and by the microorganisms that live on skin. This distinction is important because air pumped over sweat incubated for two days elicited more port entry of *An. gambiae* than air pumped over fresh sweat (Braks and Takken 1999).

Odor compounds that elicit mosquito behavior were reviewed by Dormont et al. (2021). They characterized chemicals based on Dethier et al.'s (1962) definition of attractant: "a chemical which causes insects to make oriented movements towards its source." The assay types used in the reviewed studies varied widely and provide different information about maneuvers used in by the mosquitoes attracted by the different compounds. While it is not known which compounds or blends in skin odor anthropophilic mosquitoes rely on for host seeking, several likely candidates are discussed below.

Table 1.1 shows several compounds that have been shown to modify mosquito behavior. It follows the style of Dormont et al.'s table of mosquito "attractants" (2021). Where possible, the "Method" column's abbreviations are identical for ease of comparison. The "Effect" column is our interpretation of the studies listed. For some compounds, we have emphasized different co-tested compounds than listed by Dormont et al. (2021). The abbreviations are: "LA" for lactic acid, "Y-olfactom." for Y-tube olfactometer, "Attract." for attractive, "Syn." for synergist, "2W-olfactom." for dual port entry assay, "SSR" for single sensillum recording, and "BGS" for Biogents Sentinel, which is a commonly used suction trap

Table 1.1 Studies of Compounds Shown to Modify Mosquito Behavior

Compound	Mosquito species	Co-tested compounds	Method	Effect	References
6-Methyl-5-hepten-2-one (Sulcatone)	*Ae. aegypti*	Hand odor	Y-olfactom.	Reduction in flight to both arms of Y-olfactom.	Logan et al. (2008)
6-Methyl-5-hepten-2-one (Sulcatone)	*Ae. aegypti*	Guinea pig skin odor	2W-olfactom. (Port entry)	Nothing	McBride et al. (2014)
6-Methyl-5-hepten-2-one (Sulcatone)	*Ae. aegypti*	Observer's breath	Cage landing assay	"weak attractant" in that 10-30% landed on the compound treated source	Bernier et al. (2002)
2-Oxopentanoic acid	*An. gambiae*	LA, human sweat	Wind tunnel	Attract., Elicited landing	Healy (2002)
Live calf	*An. melas*	n.a.	Ramp traps	(Attract.), Increased capture in downwind ramp traps	Gillies and Wilkes (1970)
CO_2	*An. melas*	n.a.	Ramp traps	(Attract.), Increased capture in downwind ramp traps, over control, but less than live calf	Gillies and Wilkes (1970)

Compound	**Mosquito species**	**Co-tested compounds**	**Method**	**Effect**	**References**
Foot odor	*Cx. quinquefasciatus*	n.a.	Wind tunnel	Landing	Lacey et al. (2011)
Acetone	*Ae. aegypti*	CO_2, Breath	Flight tube, SSR	Attract., Source contact, Detected by the cpA neuron	Ghaninia et al. (2019)
Lactic acid	*Ae. aegypti*	Acetone, dichloromethane, or dimethyl disulfide	3-cages; 2W-olfactom.	Syn. All three acted as binary synergists, eliciting attraction of 4/5 of the mosquitoes	Bernier et al. (2002); Williams et al. (2006)
Lactic acid	*Ae. aegypti*	CO_2	2W-olfactom.	Syn. (CO_2), Increased port entry catch	Acree et al. (1968)
Lactic acid	*Ae. aegypti*	CO_2	Combined port entry and cage landing assay	Attract. Capture in port, but no landing on source	Smith et al. (1970)
Lactic acid, caproic acid, ammonia	*Ae. aegypti*	n.a.	Y-olfactom. + field BGS	Attract. to one side of the Y-tube. No significant increase in BGS trap catch over blank	Williams et al. (2006)

Ketoglutaric acid elicits landing of *Ae. aegypti* when combined with lactic acid and CO_2 (Bello and Cardé in review). It is a component of the Krebs cycle and is found in both fresh and dry sweat (Wishart et al. 2018; Delgado-Povedo et al. 2020). Further research is needed to determine if the ketoglutaric acid in human skin odor is primarily produced by the skin flora or endogenously by humans themselves.

Lactic acid is released by the apocrine sweat glands (Braks and Takken 1999). When sweat is incubated, the total amount of lactic acid decreases (Braks and Takken 1999). This suggests that the lactic acid in the human odor profile is primarily endogenously produced. Further testing should be done to eliminate the possibility that some lactic acid is generated by skin flora from a rapidly exhausted precursor produced endogenously.

Unlike heat and CO_2 plumes from point sources, the structure of skin odor plumes is not well understood. Odor plumes emanating from humans are quite large and likely variable in composition and concentration across body parts. While heat plumes are also generated all over the body, they are more readily quantified, and quickly dissipate. Until the skin odor compounds used by host-seeking mosquitoes are identified, their release rates will also remain unknown. This knowledge gap is intensified as a result of the interplay of exhaled CO_2 and skin odor plumes.

The release rates of lactic and ketoglutaric acid in chapters three and four were not measured. However, their respective volatilities provide a means of estimating the relative abundance of each in the plume. Lactic acid has an experimental vapor pressure of 0.0813 mm Hg at 25 °C (Yaws 1997). The EPA Toxicity Estimation Software Tool

(TEST v.5.1.1) predicted a vapor pressure of 0.0410 mm Hg for lactic acid. Ketoglutaric acid has a predicted vapor pressure of 0.00000517 mm Hg (TEST v.5.1.1). As the difference between the experimental and predicted vapor pressures of lactic acid show, the prediction is only an estimate, but ketoglutaric acid is likely to be substantially less volatile than lactic acid. Although, lactic and ketoglutaric acid are not chemically identical and therefore do not form an ideal solution. The wind tunnel is not a closed system. Despite this, we assume their binary solution behaves roughly in accordance with Raoult's and Dalton's Laws. Combined, these laws state that the vapor from a binary solution will have a higher partial pressure of the more volatile compound. As lactic acid also makes up a greater molar share of the solution on the beads in the assay design of the current study, more lactic acid is probably reaching the mosquitoes than ketoglutaric acid. Because human skin is a complex substrate with many other compounds, empirical sampling of the headspace will be needed to determine the release rate of lactic and ketoglutaric vapor. Nonetheless, in the assay design used in the current study, there is almost certainly more lactic acid reaching the mosquitoes than ketoglutaric acid.

Receptors Used to Detect Host-Cue Plumes

Mosquitoes use several sensory organs for host seeking. The antennae detect heat, humidity, and lactic acid (Davis and Sokolove 1975, 1976; Laursen et al. 2021). The labellum detects many odors, including ketoacids (Kwon et al. 2006), whereas the maxillary palps detect CO_2 (Jones et al. 2007; Kwon et al. 2007).

Insects, including mosquitoes, have three classes of chemoreceptors: olfactory (ORs), gustatory (GRs), and ionotropic (IRs). Olfactory and gustatory receptors share names with mammalian receptors and all cross the cell membrane seven times. However, they do not share amino acid sequence and cross the membrane in the opposite direction of their counterparts (Montell 2009, 2013). Unlike mammalian G-protein-coupled receptors, insect IRs and GRs act as gated ion channels (Benton et al. 2009).

Olfactory Receptors

Olfactory receptors detect skin-odor compounds, such as sulcatone (McBride et al. 2014), as well as floral odors (DeGennaro et al. 2013). Insect olfactory receptors, contrary to their name, are not the only receptors that detect odors. All insect ORs require the expression of a coreceptor, called *orco,* to function (Larsson et al. 2004; DeGennaro et al. 2013).

Gustatory Receptors

Gustatory receptors were first thought to function only as taste receptors (Clyne et al. 2000). The discovery that they are also required for mosquitoes to detect CO_2, made their name a misnomer (Jones et al. 2007; Kwon et al. 2007). *Aedes aegypti* lacking *Gr3* do not exhibit behavioral responses to CO_2 (McMeniman et al. 2014). Unlike the CO_2 detectors of rats (Ferris et al. 2007) and honey bees (Stange 1974), the GRs of mosquitoes directly transduce gaseous CO_2 into a nervous signal (Xu et al. 2020).

Tauxe et al. (2013) found that the cpA neuron that detects CO_2 and that expressed several GRs, detected unknown skin odor compounds in the absence of CO_2. Reception of CO_2 is necessary for normal host-seeking behavior. Therefore, if ketoglutaric acid is only detected by the same GRs involved in CO_2 reception, responses to ketoglutaric acid would be difficult to study separately from the responses to CO_2.

Ionotropic Receptors

Ionotropic receptors are variants of ionotropic glutamate receptors (iGluRs). The rest of iGluRs are found at synapses inside of organisms, but IRs have evolved to sense the outside world. Analogously to ORs, IRs require one of three coreceptors to function, *Ir76b*, *Ir25a*, or *Ir8a* (Benton et al. 2009, Silbering et al. 2011). Ionotropic receptors are also involved in heat and humidity detection (Knecht et al. 2017; Laursen et al. 2021).

Thermoreceptors

Mosquito thermoreception is an area of active research. The sensilla coeloconica of *Ae. aegypti*, found on the tips of the antenna, can detect air temperature changes as small as 0.05 °C (Davis and Sokolove 1975). These sensilla contain the receptor *Ir21a*. This is known as a cooling receptor and mediates heat avoidance in *Drosophila* (Knecht et al. 2016). It has been repurposed in *An. gambiae* to aid in heat seeking. Knockout mosquitoes lacking *Ir21a* have impaired response to heat (Greppi et al. 2020). Transient receptor potential channels are found in many organisms, are used to detect temperature, and evolved before multicellular organisms (Corfas et al. 2015). Mosquitoes use TRPA1 to determine if a potential host is too hot. Knockouts lacking TRPA1 are unable to distinguish host temperature (40 °C) heat sources from those at 50 °C (Corfas et al. 2015).

Humidity detection in *Drosophila* is mediated by a neuron in the sacculus, a chamber near the base of the antennae, that expresses *Ir25a*, *Ir93a*, and *Ir68a* (Knecht et al. 2017). Preliminary findings by Laursen et al. (2021) show that *An. gambiae* lacking *Ir93a* do not respond to moisture and also have an impaired response to heat cues. These new findings show that insect IRs have a broad range of purposes, and are deserving of further study.

Plume Components Used for Host Seeking

Heat and Humidity Detection

It has been known for more than a century that mosquitoes respond to heat sources (Howlett 1910), but how mosquitoes use heat as a cue is not straightforward. There are three types of heat transfer: conduction, convection, and radiation. Conduction is the transfer of thermal energy between objects that are touching. Conduction from a heat source to a mosquito can only occur after landing or other physical contact.

Mosquitoes land on warm surfaces, but how flying mosquitoes use heat as a cue is not settled. Before landing, mosquitoes must detect heat sources by convection or radiation. Any temperature receptor may act as a detector of radiated heat. The remaining question is not whether mosquitoes can detect infrared radiation under some circumstances, but whether they use it for host seeking.

Convection is the transfer of heat by the movement of a heated fluid, such as air. Mosquitoes have sensors which detect air temperature (Davis and Sokolove 1975). A specific type of convection, often called “natural” convention, is sometimes discussed as though it was the only type (Zermoglio et al. 2017). In natural convection, the fluid flow is driven by changes in fluid density produced by the heat source. Human body heat drives natural convection. Mosquitoes can use these plumes generated by natural convection as a host-seeking cue, particularly when it is laden with odors and moisture (Kahn et al. 1966; Eiras and Jepson 1994). Generally, the heat source does not need to be the primary driver of fluid flow. This broader definition is used in common language to describe convection ovens, which use fans to increase the heat transfer rate to food.

Convection in the field also undoubtedly occurs horizontally as well as vertically. The air flow may be due to wind or even indoor fans. The heat plume in chapter two flowed farther horizontally than vertically due to the fan-driven airflow in the wind tunnel. Zermoglio et al. (2017) state that convective currents may only provide a cue to a mosquito above a host. While true in their otherwise still air Y-tube assay, this is not the case in the field or in assays with horizontal airflow. Further references to convective currents employ the general definition of convection.

Thermal radiation is the transfer of thermal energy by electromagnetic waves. *Triatoma* and *Rhodnius* kissing bugs respond behaviorally to vertebrate temperature radiation (Lazzari 2019). *Rhodnius* are capable of measuring air temperature separately from heating by incoming infrared radiation (Zopf et al. 2007). Fire beetles (*Melanophila*) detect the thermal radiation from fires with specialized thermo-mechanoreceptors (Schmitz and Bleckmann 1997). Mosquitoes are not known to possess any such specialized organs, nor is the infrared signature of a fire comparable to that of a human.

Peterson and Brown (1951) came close to a definitive experiment on mosquito use of host-generated infrared radiation. They constructed an assay with infrared transparent glass over a heat source which showed no attraction of *Ae. aegypti* to that heat source. However, a conclusive experiment would require complete elimination of conduction and convection transfer from the long-wave infrared-transparent glass barrier itself.

The role of physical limitation in insect heat-seeking literature is reviewed by Lazzari (2019). It should be noted that they misstated the direction of approach by writing “approaching from upwind.” The mosquito must be downwind of the heat-plume source to detect it.

Mosquito heat seeking specifically is well-reviewed by Corfas (2016), who focused on Peterson and Brown’s 1951 work with *Ae. aegypti* and an infrared transparent glass window. Corfas (2016) also noted the flaw in Peterson and Brown (1951) that there was no attempt to stop heating of the infrared transparent glass. If their infrared transparent glass warmed up, it would then have provided a heat source that could have driven natural convection and radiated heat. While *Ae. aegypti* did not respond to the glass surface, the experiment could still be improved. As the heat-seeking capabilities of *Ae. aegypti* are important and proving a negative is inherently difficult, it should not be left to chance that one of the flaws is not masking another. I would suggest inverting the rig or placing it in a wind tunnel whereas Corfas suggested active cooling of the infrared transparent glass. Furthermore, Corfas also, reasonably, took issue with Peterson and Brown’s heat source behind the glass being above host temperature.

Zermoglio et al. (2017) presented heat sources in a Y-tube, with long-wave infrared-transparent glass. They corroborated the findings of Peterson and Brown (1951) but did not address the possibility of the infrared-transparent glass warming.

Thus, the evidence suggests that *Ae. aegypti* do not respond to host-generated infrared radiation when presented as a single cue. This is critical to the interpretation of the mosquito behavior in chapter two. If *Ae. aegypti* could detect host-generated infrared radiation, the heat cue would have been detectable throughout almost the entire wind tunnel, rather than the small plume downwind of the heating pad.

After landing, mosquitoes may sense heat through conduction. Heat elicits behavior after landing; Bishop and Gilchrist (1946) found that *Ae. aegypti* fed on heated chicken blood but not ambient temperature chicken blood. Liu and Vosshal (2019) have shown that *Ae. aegypti* behavior after landing was consistent with use of sensors on the head, rather than the tarsi. This suggests that *Ae. aegypti* rely on convection rather than conduction even after landing on a surface.

CO_2 Detection

It has been long known that CO_2 elicits upwind flight of mosquitoes (Kennedy 1940). The sensory cpA neuron expressing *Gr1*, *Gr2*, and *Gr3* that detects CO_2, was discovered by Kwon et al. in 2007. However, knowing which neuron is involved does not explain how gaseous CO_2 is transduced into a sensory signal.

Animals as diverse as rats (Ferris et al. 2007) and honey bees (Stange 1974) are normally capable of detecting CO_2. When treated with carbonic anhydrase inhibitors, they cannot detect CO_2 (Ferris et al. 2007; Stange 1974). Carbonic anhydrase normally converts CO_2 into bicarbonate. This means that the receptors in those diverse animals may detect bicarbonate rather than atmospheric CO_2 directly. Decades went by after the finding that honey bee CO_2 detection could be shut off with a carbonic anhydrase inhibitor, with no further knowledge of how mosquitoes turned CO_2 into a sensory signal.

Xu et al. (2020) inserted mosquito gustatory receptors into *Xenopus* embryos. They first verified the receptors were not general acid detectors. Then they added CO_2 to two vials with the embryos. To one vial, they added acid. The lowered pH shifted the CO_2 to bicarbonate equilibrium to favor CO_2. The receptors in the acidified vial showed a higher amperage than the receptors in the control vial. This shows that *Ae. aegypti* detect CO_2 directly rather than after conversion to bicarbonate.

The cpA neuron on the maxillary palp that expresses *Gr3* and detects CO_2 (Kwon et al. 2007) also expresses *Ir25a* (Younger et al. 2020). Triethyl amine stimulates the cpA neuron, even in mosquitoes lacking *Gr3*. Mutant *Ae. aegypti* lacking *Gr3* responded to lactic acid when provided with triethylamine as readily as wild type individuals provided with CO_2. This suggests that compounds that stimulate the cpA neuron are perceived in a manner similar to how CO_2 is perceived. This is also consistent with the finding that whole human skin odor activates this neuron (Tauxe et al. 2013). This may also partly explain the upwind flight of *Ae. aegypti* and *Culex quinquefasciatus* toward skin odor in the absence of CO_2 (Dekker et al. 2005; Lacey et al. 2011).

Mosquitoes are exquisitely sensitive to fluctuations in concentrations of CO_2 as small as 50 ppm (Grant et al. 1995). Carbon dioxide can "gate" other mosquito behaviors, such as heat seeking in *Ae. aegypti* and odor seeking in *An. colluzzii* (McMeniman et al. 2014; Webster et al. 2015). Carbon dioxide at 150 ppm, in addition to the background (~400 ppm), is sufficient to elicit skin odor-seeking behavior of *Anopheles coluzzii* (Webster et al. 2015)*,* while 100 ppm elicits upwind flight (Healy and Copland 1995). *Aedes aegypti* fly near visual cues after detecting CO_2 (van Breugel et al. 2015).

Anopheles coluzzii, provided with host odors, fly nearer to visual cues than *An. coluzzii* in clean air (Hawkes and Gibson 2016). Their vertical velocity is also greater on approach and when flying away.

Skin Odor Detection

Human skin odor is composed of a myriad of compounds, generated endogenously and by skin flora. Some of compounds found in human skin odor are attractive to mosquitoes, but only in some circumstances. For example, although lactic acid may elicit weak attraction of *Ae. aegypti* on its own, it does not elicit landing (Smith et al. 1970). When presented with CO_2, a small percentage of *Ae. aegypti* land on lactic acid (Bello and Cardé in review). *Anopheles gambiae* do not land on a lactic acid stimulus presented without CO_2 (Healy Copland 2000). Despite being insufficient to elicit the full repertoire of mosquitoes' host seeking, lactic acid is probably a necessary cue for anthropophilic mosquitoes to distinguish humans from other vertebrates.

Adding lactic acid to goat and cow odor drew more than half of *Ae. aegypti* in a Y-tube assay to that arm of the Y-tube (Stieb et al. 2001). This met or exceeded the attraction to human odor with or without supplemental lactic acid. Lactic acid alone attracted less than one fifth of the mosquitoes. While the addition of lactic acid to cow odor did not make it as attractive as a human hand to *An. gambiae* in a Y-tube, it did approximately double its attractiveness from ~15 % to ~30 % (Dekker et al. 2002). Geier et al. (2002) sorted volunteers by the attractiveness of their odor to *Ae. aegypti* in a port entry assay. Addition of lactic acid to odor from less attractive volunteers made *Ae. aegypti* enter that port as frequently as they entered a port which released odor from an “attractive” volunteer without added lactic acid. These findings show that lactic acid is necessary but insufficient to elicit host seeking in both *Ae. aegypti* and *An. gambiae*.

Lactic acid is detected by the antennae of *Ae. aegypti* (Davis and Sokolove 1976). It is not detected by the labellum (Kwon et al. 2006). *Ir8a* is not expressed in the labellum (Saveer et al. 2018). *Ir8a* is a required coreceptor for the detection of lactic acid by *Ae. aegypti* (Raji et al. 2019). The other receptors, possibly IRs, required for lactic acid detection, are not known. This does not rule out other mosquito sensory organs being able to detect lactic acid.

Sulcatone (6-methyl-5-hepten-2-one) is present at higher concentrations in human odor than in odor from other animals (McBride et al. 2014). The expression of *Or4*, an odorant receptor that detects sulcatone, is higher in *Ae. a. aegypti* than in *Ae. a. formosus* (McBride et al. 2014). However, the addition of sulcatone to the odor of a guinea pig did not increase the number of *Ae. aegypti* that enter a port, compared to a port releasing guinea pig odor alone (McBride et al. 2014). Therefore, sulcatone may not be an essential odor component to elicit host seeking by *Ae. aegypti*.

Anopheles gambiae Ir8a and *Ir75k* expressed in *Xenopus* embryos form a complex that detects straight-chain carboxylic acids, including heptanoic, octanoic, and nonanoic acids (Pitts et al. 2017). It is not known how mosquitoes detect ketoglutaric acid, but other ketoacids have been examined. *Anopheles gambiae* were found to land on ketoacids with four to six carbon chains with and without methyl branches, but which receptors the mosquitoes used were not examined (Healy and Copland 2002). To date, all the ketoacids examined in the context of mosquitoes have had one carboxylic group

(Carlson et al. 1973, Healy and Copland 2002, Kwon et al. 2006). Unlike the oxocarboxylic acids presented to mosquitoes in the prior studies, ketoglutaric acid is an oxoacid derivative of a dicarboxylic acid with a carboxyl group on each end.

The labellum of *An. gambiae* detects ketobutyric and ketovaleric acids despite lacking significant *Ir8a* expression (Kwon et al. 2006). In chapter four, we show that *Ae. aegypti* knockouts lacking *Ir8a* still showed a behavioral response to ketoglutaric acid. Despite the differences between ketoglutaric acid and the ketoacids examined in the literature, future studies should examine the role of mosquito labellum in ketoglutaric acid detection (Saveer et al. 2018).

Navigating Along Wind-Borne Plumes

Plumes relevant to host-seeking mosquitoes are structures generated by turbulent diffusion (Cardé and Willis 2008) that carry CO_2, a myriad of skin odor compounds, heat, or humidity downwind (Cardé 2015, 2021). To fly upwind toward the source of a plume, a mosquito must determine the direction of the wind. If it is standing on a surface, it may determine the wind direction with mechanoreceptors. The wind direction at the point of mosquito take off may not point exactly to the host and the wind may change direction after the mosquito takes off. The mosquito may already be flying when it detects a plume carrying a host plume. However, the direction of air flow is difficult to determine when an object, such as a mosquito, is in that air.

Optomotor Anemotaxis

Mosquitoes can fly upwind using optomotor anemotaxis, as first proposed by Kennedy (1940). The optomotor response is triggered by the flow of the visual field around an animal. By turning until the flow of visual cues travel front to back, an insect can ensure that it is flying upwind. If an insect keeps detecting the plume components, it needs only to keep flying upwind to approach the host (Kennedy 1978). The optomotor response does not require all the objects constituting visual flow to be resolved clearly. Zhan et al. (2021) found that standing *Ae. aegypti* will turn in response to a shifting visual field, even when they lack the gene for Op1, which encodes for rhodopsin production, an essential component of the mechanism of vision. Without this gene and the normally abundant rhodopsin it encodes for, the mosquitoes had impaired telotaxis, but not an impaired optomotor response. This means that visual cues farther away than a mosquito can resolve may still be of use for orientation. Further studies with flying insects are needed to determine the minimum required visual capabilities for optomotor anemotaxis.

Aim-Then-Shoot

There is a proposed host-seeking behavior called “aim-then-shoot” that allows insects to determine air flow direction with the benefit of mechanoreception while stationary on a substrate. Upon detecting an airborne cue, the insect takes off into the wind and lands when the plume is no longer detected. If the shifting wind brings the plume back to the insect, it takes off again. By repeating this process an insect can approach to within visual detection distance of the host emitting the plume, even if the plume changes direction. This behavior is probably used by tsetse flies (Griffiths et al. 1995) but is not known to be used by mosquitoes.

Dipping

Gillett (1979) proposed a hypothetical version of “aim-then-shoot” in which the mosquito would “dip” towards the ground without landing. Air near the ground does not flow as quickly as air high above the ground due to friction. Therefore, if the mosquito can sense the horizontal acceleration during the “dip” the mosquito would be able to determine the wind direction. It could then navigate in a way analogous to “aim-then-shoot” without exposing itself to ground-dwelling predators. This proposed mechanism has not been studied.

Odor gradients generated by molecular diffusion only provide the information needed to locate a source within tens of centimeters and are therefore of less use to mosquitoes than turbulent plumes (Murlis et al. 1992). While this has been studied primarily with pheromones emitted by moths, the same physical principles apply to host odors. Minor adjustments for the smaller molecular mass of some host odors compared to pheromone components may be needed. *Aedes aegypti* that lose contact with a plume will cast across the wind until they recontact the plume. *Aedes aegypti* flying upwind begin to cast after losing contact with concentrated CO_2 more quickly than after losing contact with less concentrated CO_2 (Dekker et al. 2011). This is thought to be a mechanism to prevent flying past a host.

Vertical plume following differs from horizontal plume following in that the mosquito may use gravity for orientation. Daykin (1967) found that *Ae. aegypti* moved against the air flow either up or down. This rules out simple geotaxis that is switched on by detection of a cue, such as an odor. Standard geotaxis would have meant that the mosquitoes always went either up or down after detecting an odor. To examine if the mosquitoes were navigating with optomotor feedback, the visual field was moved in the opposite direction of the airflow. This optical flow did elicit flight up or down, irrespective of the airflow direction. However, the response to visual flow may be overriding a response to gravity. This does not rule out orientation to gravity in other circumstances. Until *Ae. aegypti* are tested in an illuminated but visually homogenous vertical flight chamber, the limits of their vertical plume following abilities will remain unknown.

Kinesis

Odor gradients almost certainly do not provide the necessary information for a host-seeking mosquito to orient to a host beyond a few centimeters. Homogeneous plumes of CO_2 do not elicit upwind flight of *Ae. aegypti* (Dekker et al. 2011). However, they could elicit klinokinesis, undirected movements. Undirected movements may explain why homogenous CO_2 elicited as much port entry as turbulent CO_2, but only after twice the time had elapsed (Dekker 2001). An increase in undirected movement upon contacting a homogenous CO_2 plume may be a functional component of the host seeking algorithm of *Ae. aegypti*. A homogenous plume in the field would likely indicate that the mosquito is near the host and increased undirected movements may increase the likelihood that a mosquito detects another host cue such as heat. In the artificial environment of the assay lacking other host cues, an increase in undirected movement may have caused the mosquitoes to enter the port through chance. That *Ae. aegypti* do not exhibit upwind flight in homogenous CO_2 plumes (Dekker et al. 2001) may not be generalizable to all mosquitoes. Some species of moths are known to fly upwind in homogenous pheromone plumes, while others require intermittent contact with pheromone (Justus and Cardé 2002). Therefore, future studies of other mosquito species should examine the possibility that they fly upwind through homogenous odor plumes.

Mosquitoes often land on parts of humans far from the mouth and nose (Knols et al. 1994; Dekker et al. 1998). While some of this may be due to the CO_2 plume covering body parts far from the mouth and nose, mosquitoes can leave a CO_2 plume to follow other cues. van Bruegel et al. (2015) found that mosquitoes would leave a CO_2 plume to fly near a visual cue. Sumner and Cardé (in preparation) found that *Ae. aegypti* would leave a CO_2 plume to land on a skin odor or heat source.

Orientation Other Than Plume Following

Vision in the Context of Telotaxis

At close range, mosquitoes may exhibit telotaxis by flying directly toward their hosts. While the minimum resolvable angle of Aedes eyes is 12.3 °, it has high light sensitivity "optimal for the dim conditions which they prefer" (Muir et al. 1992). This means that a 1.8 m human silhouetted against a high-contrast background would be vertically distinguishable on individual ommatidia at 8.4 m. This human would not be distinguishable in the horizontal plane until the mosquito was much closer. Human clothing and bedding may reduce the visual cue we present in the field. Though a mosquito would not need to perfectly resolve a host visually in order to fly towards it, their limited eyesight ensures that visual telotaxis only occurs at short range.

Hearing

There are no studies on the use of sound for host seeking by human-seeking mosquitoes. Male *Ae. aegypti* do use the sound of female *Ae. aegypti* to find mates (Roth 1948; Cator et al. 2011).

Landing

Behaviors immediately prior to landing in insects have been studied with a focus on visual cues (Goodman 1960; Srinivasan and Zhang 1997; Srinivasan et al. 2000; van

Bruegel and Dickinson 2012). *Aedes aegypti* do not require a visually distinct object in order to land on heat or odor sources (Sumner and Cardé Chapter 2). As mosquitoes must land in order to bite and transmit pathogens, their landing behavior is a crucial component of host feeding and pathogen transmission (Reed et al. 1900).

Traps and Assays

Traps

Trapping remains an important tool for surveillance of both mosquitoes and the pathogens they carry. The Biogents Sentinel® trap, although widely regarded as the "gold standard" for *Ae. aegypti* trapping, has low efficiency (17 % ± 5 %) even when used with its host odor derived lure(Amos et al. 2020 a,b), which affects detectability and potentially results in false negatives (Bau and Cardé 2016; Cardé et al. 2018). A trap is efficient when it catches mosquitoes that enter a predefined area near the trap. If a lure not only attracts mosquitoes to its vicinity but elicits the full landing response, it might increase the trap efficiency.

Assays

The assay used to measure mosquito behavior determines which behaviors are observed. We have picked two diagnostic examples of different assays which provide different types of information about the responses to the same cues. McBride et al. (2014) investigated the role of sulcatone with a port-entry assay. By aerating sleeves worn by humans and guinea pigs, they collected an average of 150 ng of sulcatone from the human worn sleeves and virtually none from the guinea pig worn sleeves. They routed two streams of odor, split from a combination of CO_2 and odor released from a nylon sleeve worn by a guinea pig through two ports. Sulcatone was added to one of the streams but failed to increase mosquito entry into that port. Bernier et al. (2002) found sulcatone in human skin odor. They tested it in a landing assay and found that it was a "weak attractant" in that it elicited landing of 10-30 % of *Ae. aegypti* tested. Logan et al. (2008) tested *Ae. aegypti* in a Y-tube. One arm of the tube was supplied with the odor of a human hand and the other with the odor of a hand plus synthetic compounds previously identified from human odor. With sulcatone, they found a decrease in the "mean proportion of female *Ae. aegypti* mosquitoes showing upwind flight activity." Of those that did fly upwind in trials with sulcatone, there was no difference in mosquito presence between the Y-tube arm with just human odor and the Y-tube arm with human odor and added sulcatone. The findings of McBride et al., Bernier et al., and Logan et al. are all consistent with sulcatone acting primarily as an arrestant rather than an attractant.

The results of some assay types may represent a smaller picture of mosquito host seeking than initially believed. Port entry may not reflect the number of mosquitoes brought into a general vicinity. Torr et al. (2008) used odor-baited entry traps with electrocution grids in front of the entrances. This trap is akin to a field version of a port entry assay, in that the mosquitoes must fly inside to be captured. Across 12 nights of trapping and zapping, they found 35 *An. gambiae* s.l. in the traps, while 1552 were found electrocuted. A nearby CDC Trap, a battery-operated trap that used a fan to physically draw in the mosquitoes, captured 1205. Therefore, the odor-baited entry trap was drawing *An. gambiae* s.l. into the area, but not drawing them all the way in.

Lactic Acid Contention

Smith et al. (1970) reviewed the highly variable findings of lactic acid and mosquito behavior. They stated that the different findings were likely due to the wide array of assays and concentrations used. Their decision to use a port entry and landing combined assay was specifically to avoid reporting an arrestant as an attractant. As digital videography was not available at the time, this was a reasonable compromise. Our wind-tunnel assay with videography allowed us to observe both approach and landing behaviors in chapters two and three.

<u>Current Progress and Challenges</u>

Progress in the Field

CRISPR for Generation of Knockouts

As shown in chapter four, CRISPR has reduced the cost and increased the specificity of genetic manipulation, making new investigations of mosquito behaviors possible.

Leslie Vosshall's laboratory at Rockefeller University recently demonstrated a *Gr3* knockout *Ae. aegypti* with optogenetic control of the cpA neuron that responds to CO_2 (Sorrells et al. 2021). This means that the mosquito cannot detect actual CO_2, but that the experimenter can flash a light which triggers the neuron to fire even in the absence of CO_2. As Younger et al. (2019) found that *Gr3*-lacking *Ae. aegypti* that were provided with triethylamine, which stimulates the glomerulus innervated by the cpA neuron, responded to lactic acid like wild type mosquitoes respond to lactic acid when provided with CO_2, the optogenetic mosquitoes may be perceiving the light as though it were CO_2.

One could revisit the casting and surging behavior of *Ae. aegypti* examined by Dekker et al. (2011). They found that *Ae. aegypti* exiting a more concentrated patch of CO_2 surged shorter distances. This may be to avoid overshooting the host. Instead of testing with plumes of CO_2 at a set concentration, one could vary the artificial concentration perceived by the mosquito. Each mosquito could be given multiple "concentrations" of CO_2 at different points in a wind tunnel. By flooding a wind tunnel with light, the experimenter would not have to wait for the mosquito to make and lose contact with the plume. This would allow a vastly increased sample size and be able to fully explore the role of CO_2 concentration in the surge length of host-seeking *Ae. aegypti*.

Computer Vision

Manual observation of mosquito behavior is labor intensive. It involved a human staring at a screen, scoring a behavior, and then rewinding to ensure the behavior was scored accurately. In chapters two and three, the videos of the entire assay period were watched manually. In chapter four, the videos of the assays were scored at several time points. This saved labor at the cost of being unable to distinguish landing duration from landing frequency. Mosquito behavioral research using computer vision has often ignored landing (van Bruegel et al. 2015). As landing is required for biting and biting is required for pathogen transmission, this leads to an incomplete understanding of mosquito behavior. Improvements to computer vision may soon allow automated landing counts.

Challenges in the Field

Current traps are inefficient (Amos et al. 2020 a,b). While the impacts on monitoring have been discussed, this may also be a hurdle to mosquito research. Lures tested in traps may have elicited flight near the traps, but researchers would have no way of knowing, short of using videography.

Gillies et al. (1978) used electrocution grids mounted next the suction traps to kill mosquitoes and draw them in. By pairing two grids with two suction devices, they could determine if the mosquitoes had been flying upwind or downwind. They did not use these devices to determine the range of attraction. While this was not designed as a surveillance device, the addition of electrocution grids may improve the efficiency of traps.

Findings in *Ae. aegypti* may not be generalizable to all mosquitoes. Future studies with electrocution grids (Gillies et al. 1978; Torr et al. 2008), lidar, or computer vision could replicate the work of Gillies and Wilkes (1970). These techniques do not provide the large visual cue nor disruption to the flow of air presented by the ramp traps used by Gillies and Wilkes (1970).

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CHAPTER 2. VALENCE OF HUMAN-ODOR, VISUAL, AND HEAT CUES INDUCING LANDING IN FEMALE *AEDES AEGYPTI* MOSQUITOES

Abstract

While skin odor is thought to be the cue that mosquitoes use to discriminate us from other animals, the details of how they use skin odor to find humans is unknown. We found that *Ae. aegypti* land on a source of skin odor even without a co-located visual cue. By collecting human odor on glass beads and then using identical glass beads to visually conceal skin odor and heat cues, we were able to study mosquito landing on skin odor, heat, and visual cues separately. Landing is necessary for blood feeding and, therefore, is a diagnostic measure of the epidemiological importance of a host-seeking cue. In two-choice tests in a wind tunnel, a skin odor source had the highest valence for landing, followed by a combination of heat and a visual cue, and finally heat and visual cues presented separately.

Introduction

Female *Ae. aegypti* mosquitoes vector dengue, Zika, chikungunya, and yellow fever viruses by repetitive feeding on humans. Skin odor is thought to be the cue that female *Ae. aegypti* and other anthropophilic mosquitoes use to discriminate humans from other endothermic vertebrates (Gouck 1972; Takken et al. 1997; Dekker et al. 2001, 2002; Besansky et al. 2004; McBride 2016). *Aedes aegypti*, following an encounter with an above-ambient concentration of CO_2, use skin odor to pinpoint a landing site suitable for a blood meal. For example, *Ae. aegypti* in a wind tunnel spent more time near a visual cue than a heat cue without a co-located visual cue (van Breugel et al. 2015).

Due to the special role of skin odor, we presented *Ae. aegypti* with skin odor without co-located visual cues. We also quantified landing, a behavior necessary for mosquito blood feeding. We found that *Ae. aegypti* landed on a source of skin odor presented without a co-located visual cue more frequently than on the visual cue, a heat cue, or even a heated visual cue. The primacy of skin odor contrasts with the view that, during host-seeking, this diurnal mosquito relies primarily on visual cues after following a CO_2 plume (van Breugel et al. 2015). Unlike prior experiments (Goodman 1960; Srinivasan and Zhang 1997; Srinivasan et al. 2000; van Bruegel and Dickinson 2012; Parker et al. 2015), in which visual cues elicited landing or persistent nearby flight, we presented heat and skin odor without co-located visual cues. Mosquitoes nonetheless landed on both visually indistinct source of skin odor and, less frequently, heat stimuli.

To assess the relative valence of host-seeking cues used by female yellow fever mosquitoes, *Ae. aegypti*, we used a free-flight wind tunnel (Fig. 2.1), which allowed us to separate a CO_2 plume from other host cues. Naïve *Ae. aegypti* (Orlando strain) were first exposed to an above-ambient concentration of CO_2 at the tunnel's downwind end and then offered a choice of two competing stimuli on the wind-tunnel floor 50 cm upwind of the release cage. This experiment allowed us to determine which cues host-seeking Ae. aegypti will land on when there is a choice provided.

Materials and Methods

Insects

We used the "Orlando" strain of *Ae. aegypti.* Mosquito colonies were maintained in a L:D 14:10 h cycle, at 25 °C and 70 % RH in the UCR Insectary and Quarantine Facility

(Kuno 2010). The females used for colony maintenance were fed defibrinated bovine blood through an artificial membrane (HemoStat Laboratories, Dixon, CA, USA). Larvae were reared in plastic containers and fed TetraMin Tropical Tablets (Tetra Holding GmbH, Melle, Germany). Approximately 50 larvae were reared in each container. All pupae (male and female) from three containers were allowed to emerge into screen cages (BugDorm 30 × 30 × 30 cm MegaView Science Co., Ltd., Taichung, Taiwan) containing 10 % (v) sucrose solution provided *ad libitum*. All mosquitoes were assumed to have mated and were used in experiments only once. Five female mosquitoes, 3-9 days post eclosion, were transferred to cylindrical acrylic release cages (7 × 8 cm i.d.) three hours before the start of assays. *Aedes aegypti* were assayed 4-8 hours into their photophase.

Wind Tunnel

The flight and landing of mosquitoes were observed in a glass wind tunnel 122 × 30.5 × 30.5 cm (Fig. 2.3A). The exterior of the glass wind-tunnel floor was covered with black construction paper. Yellow tape was applied to the outside of the glass sidewalls in “x” patterns to provide optomotor feedback (shown in Fig. 2.3A, only on one side). Additional visual feedback was available from the wind tunnel’s structural components and the room external to the wind tunnel, the mosquitoes were therefore not in a featureless visual surround. Air was drawn into the wind tunnel from an adjacent uninhabited room (25 °C and 70 % RH). The experimenter did not breathe while loading the release cage into the wind tunnel each trial, so that mosquitoes being transferred into the tunnel were not exposed to a human exhalation of CO_2. Airspeed throughout the wind tunnel was 0.2 m/s. The mosquitoes were video recorded for 6 min using a camera (ICD 48, 6 mm lens; Ikegami, Maywood, NJ, USA) positioned 50 cm above the wind tunnel.

This allowed observation of most of the tunnel including the entire area in which cues were presented. Illumination was provided by four infrared LED lights (AXIS T90A, 850 nm, Axis Communications AB, Lund, Sweden) mounted behind a stainless-steel screen at the downwind end of the wind tunnel. Diffuse room light, provided by incandescent bulbs, measured at ~14 lux in the tunnel. The visible spectrum lights were aimed at the junction of the wall and ceiling opposite the wind tunnel, providing the mosquitoes with light to see. Luminance was measured from a point centered in the wind tunnel and 70 cm from its upwind end, with a Gossen Ultra-Pro (GOSSEN GmbH, Nuremberg, Germany). The luminance at the downwind end was 4 cd/m^2, the upwind end 1 cd/m^2, the room 4 cd/m^2, the wall 8 cd/m^2, and the beads 0.067 cd/m^2.

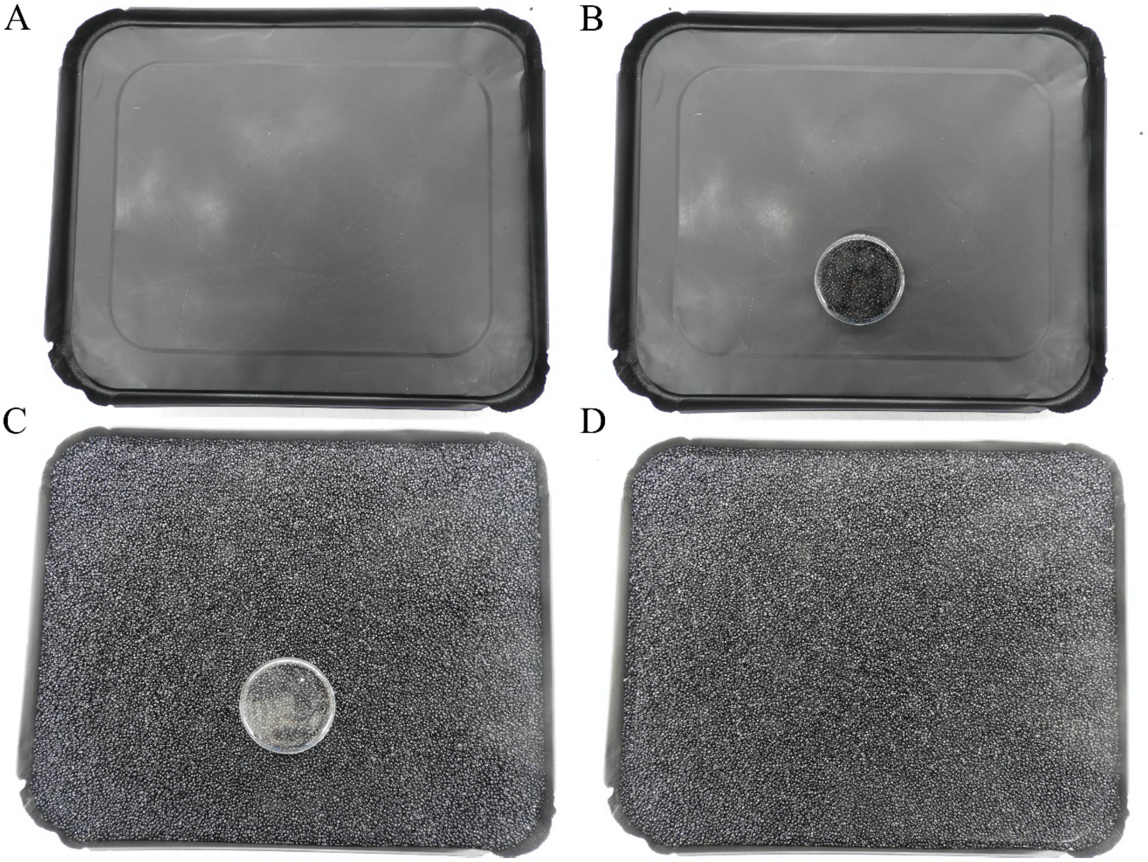

Fig. 2.1 Presentation of Odor Treated Beads

Odor cues were presented on a 5-mm layer of black glass beads (12/0 Czech Glass Seed, approximately 2 mm OD toroidal, Precosia Ornela, Zásada, Czech Republic) in a 32.5 by 26.5 cm black aluminum pan. A) The empty aluminum pan. B) The aluminum pan with a Petri dish containing odor beads. C) The aluminum pan with inverted Petri dish containing odor beads surrounded by clean beads without odor. D) Petri dish removed leaving no visual target of odor beads within the clean beads.

The visually indistinguishable layer of beads allowed heat or skin odor to be presented independent of a co-located visual cue. The two cue presentation areas in each trial were arranged in the middle of the aluminum pan 13.5 cm from the upwind and downwind ends of the aluminum pan and with 5 cm separating each cue from each other and from the lateral edges of the pan (Fig. 2.3A). This consistency of location was crucial as the beads were not visibly distinguishable. The pan was placed with its downwind edge 70 cm from the downwind. Beads were washed between trials.

Carbon Dioxide

To simulate the presence of an upwind vertebrate host, 100 ml/minute of CO_2 at 4 % concentration mixed with tank air was carried to the wind tunnel via a 3 m-long Tygon® tube, ensuring temperature equilibration. The tube was connected to a glass, L-shaped tube (OD 5.5 mm, ID 3.5 mm) that descended 15 cm from the ceiling on the wind tunnel and extended 20 cm downwind to 60 cm upwind from the release cage. The 4 % CO_2 mix exited the inner opening at ~0.4 m/s, but there was no detectable difference in airspeed 1 cm downwind of the CO_2 release point (Omega HHF 52 anemometer, Omega Engineering, Inc., Stamford, CT, USA) and the temperature was identical to the air in the wind tunnel (to within 0.1 °C, same device). The CO_2 release tube was centered so that the generated plume of CO_2 would engulf the release cage. The CO_2 plume was turbulent enough to produce the distinct packets of CO_2 needed to elicit upwind flight (Dekker and Cardé 2011) yet compact enough not to mingle with the skin odor plume below. The CO_2 plume structure was verified with a visible "smoke" plume of titanium dioxide and hydrochloric acid produced by the reaction of $TiCl_4$ with damp air.

We measured the CO_2 concentration with a GasHound CO_2 detector (Model LI-800, LI-Core, Nebraska, USA) at 10 points in line with the CO_2 source as well as 10 points along the floor (Fig. 2.2 and Fig. 2.3B). CO_2 concentrations were recorded after one minute of equilibration. However, as the gas being sampled is drawn through a tube, a pump, and a filter, this instrument produces time-averaged values in contrast to the nearly instantaneous sensing of CO_2 by mosquitoes (Dekker and Cardé 2011). The dashed blue line in Fig. 2.3B shows that the CO_2 did not extend to within 1 cm of the floor 25 cm downwind of the other cue presentation areas. The heights of CO_2 measurements refer to height above the beads. The height was kept the same for measurements downwind of the beads. For measurements downwind of the beads this means the true height was 0.7 cm above the tunnel floor. To land on a floor cue the mosquito must fly both vertically down and upwind of the CO_2 source. A few mosquitoes approached the glass CO_2 release tube. Some cast a few centimeters downwind for several seconds, and others briefly landed on the tip of the CO_2 release tube, but they were not scored.

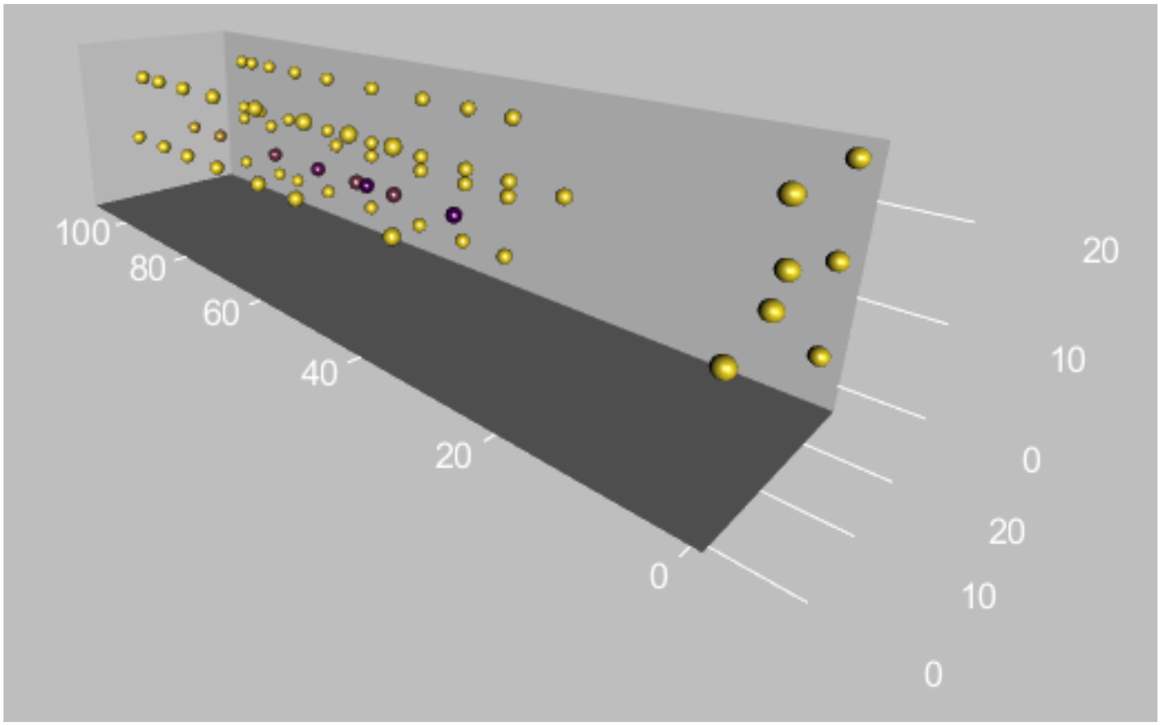

Fig. 2.2 Verification of the Absence of Errant CO_2

Fig. 2.2 is a 3D plot of the CO_2 concentration at several locations in the wind tunnel under assay conditions. The color of each dot varies from yellow (406 ppm) to dark purple (4860 ppm), with intermediate concentrations shown in shades of yellow mixed with purple. The lack of blue or purple dots anywhere other than directly downwind of the CO_2 release point shows the separation of the cues available to the mosquito at any point in its flight. The x-axis shows the distance downwind from the upwind end of the tunnel in centimeters. The y- and z-axes show the displacement from the floor and room-side tunnel wall in centimeters.

The presence of CO_2 flux at the release cage was used to both elicit take off and to sensitize the mosquitoes to other cues. As CO_2 flux occurs in the field host seeking situations, we did not conduct a CO_2 free control. If we had eliminated CO_2, the lower take off rate would have made the sample size small for a reason that is not biologically meaningful. The separation of the CO_2 plume vertically from the skin odor and heat plumes showed that the cues do not need to be encountered simultaneously.

To ensure that elevated concentrations of CO_2 were not present in other parts of the wind tunnel we took additional readings with a portable Amprobe CO2-100 meter (Amprobe, Everett, WA, USA). The readings are shown graphically in Fig. 2.2.

All Cues Presented on the Floor

The orientation of all cues presented on the floor was assigned randomly at the start of each day. To account for possible position effects, their position was switched halfway through the assays being conducted of that type on that day. The beads were put in the same place each time.

Odor Cue

Human skin odor was collected onto black glass beads (12/0 Czech Glass Seed, approximately 2 mm OD toroidal, Precosia Ornela, Zásada, Czech Republic)) by placing 25 ml of beads into a polyester/cotton blend sock, which was then worn by a volunteer (two males and one female) for three hours. One volunteer was used per day. Volunteers refrained from alcohol, spicy foods, vigorous exercise, and scented products for three days before and while wearing the beads. The unavoidable variation in human odor on a day-to-day basis was accounted for by blocking.

The beads treated with skin odor were poured into a 55-mm-diameter plastic Petri dish. Beads were allowed to air dry for one hour before assays. The bead-filled Petri dish was then covered with a black aluminum pan. While pressing the Petri dish into the pan, the whole arrangement was flipped. Clean beads, otherwise identical to those used to collect odor, were then poured into the aluminum pan. Removal of the Petri dish produced a visually indistinguishable patch of beads treated with skin odor surrounded by clean beads.

Between use, beads were sonicated with detergent (Micro-90; Aldrich; St. Louis MO, USA), rinsed with distilled water, rinsed with acetone, and baked at 200 °C for 8 h. One week before assays were conducted, the aluminum pans used to hold the beads (32.5 x 26.5 x 1.6 cm, Catering Tray Lid, Smart & Final, Commerce, CA, USA) were spray painted black (2X Ultracover, Rust-Oleum Corp. Vernon Hills, IL, USA) and baked at 150 °C for 48 hours. Pans containing beads with odor were used for one string of assays and then discarded.

A heated odor cue was not provided, as heat would change the release rates of the skin odor compounds. The odor released by a heated skin odor cue would not have been comparable to unheated odor.

Heat Cue

A 74-mm diameter silicone heating pad (Cole-Parmer, Vernon Hills, IL, USA) was placed in a black aluminum pan and covered with glass beads to a depth of 5 mm. The surface temperature of the beads measured with a thermocouple (BAT-12, Sensorteck Inc., Clifton, NJ) was 34 °C. The power cord was run through an incision in the bottom of the aluminum pan. The heating pad was not visible from above.

Visual Cue Appearance to the Mosquitoes

Aedes aegypti eyes have a minimum resolvable angle of 12.3 °, allowing them to discern an object approximately the size of human-height from ~7 meters away (Muir et al. 1992). Depending on the angle of approach, the black inner circle of our white annulus should be discernible up to 26 cm away. However, as the inner and outer edges of the annulus present ellipses that are narrower from certain angles of approach, the maximum discernible distance varies based on angle of approach. The annulus may be detectable to the mosquito beyond these ranges but would be visualized as shades of gray on several ommatidia. Despite the presumed detectability of the visual cue, we observed few landings on unheated visual cues.

Visual Cue

Few mosquitoes landed on the small, clean, unheated visual cues. A close mimic of human visual cues would have been preferred but would have interfered with the presentation of other cues. The minimal response to the alternative visual cues is shown in Fig. 2.4H and Fig. 2.5H

The visual cues obscured mosquitoes passing over them, which prevented the use of computer vision and thus 3D tracking. Additionally, when a mosquito flew over the annulus and was not visible on the inner beads nor the other side, it was assumed to have landed on the paper and was scored.

Assay-Pairing Strategy

We conducted two sets of assays daily. The first was a two-choice assay in which skin odor was presented alongside another cue (i.e., heat, visual, or heated-visual cue). The second assay, a single-choice assay, presented the same non-odor cue without a competing odor stimulus. This latter assay served as a reference for determining the extent to which mosquitoes are attracted to visually indistinct heat, visual, and heated visual cues. The order of the assays was alternated daily. Mosquitoes used on a given day were from the same emergence cohort.

Cue Presentation

Treatments were presented on a 5-mm layer of black glass beads in a 32.5 by 26.5 cm black aluminum pan. The visually homogeneous layer of beads allowed heat or skin odor to be presented independent of a co-located visual cue (Fig. 2.1).

Fig. 2.3A

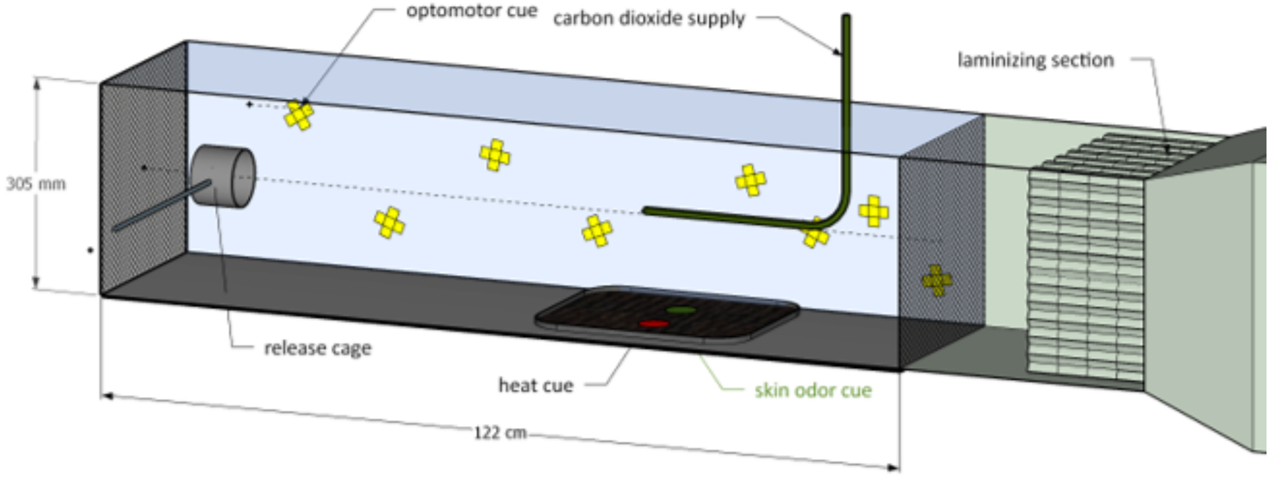

Fig. 2.3B

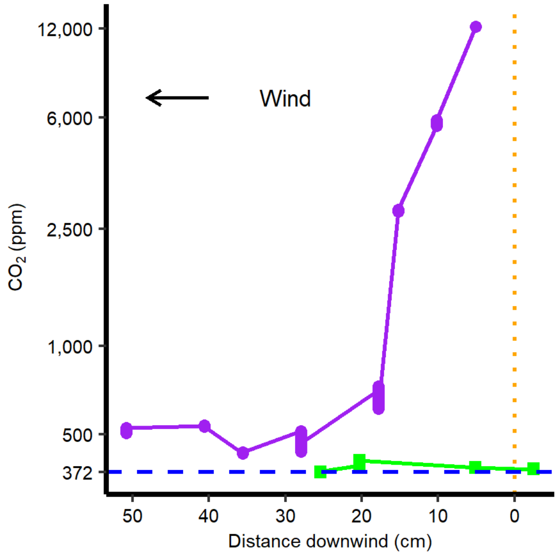

Fig. 2.3C

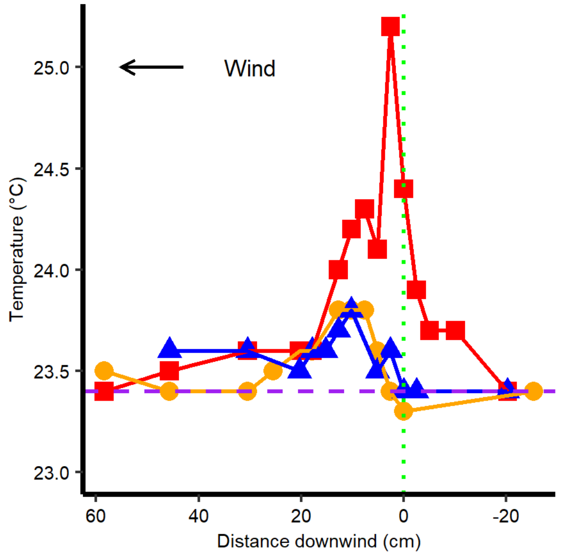

Fig. 2.3 Wind Tunnel and Assay Conditions

(A) Diagram of wind tunnel. Wind flow from right to left. (B&C) The x-axes go right to left, to match the physical arrangement of the wind tunnel. (B) Carbon dioxide concentration in parts per million (ppm) centered in the tunnel at a height of 14 cm (purple circles) and 1 cm (green squares). The dashed horizontal blue line represents concurrent measurements of the ambient CO_2 concentration. Readings of CO_2 concentration at other points in the wind tunnel are shown in Fig. 2.2. (C) The air temperature downwind of the heat pad at three heights. A height of 1 cm is shown in red (squares), 2 cm in orange (circles), and 3 cm in blue (triangles). Concurrent measurements of the ambient air temperature are shown in purple.

In all trials, a 100 ml/min plume of 4 % CO_2 and 96 % tank air was introduced at the same height as the mosquito release cage to simulate the presence of an upwind vertebrate host. This plume was separated from all cues presented on the tunnel floor (Fig. 2.2 and Fig. 2.3B). Human skin odor was transferred onto clean glass beads by placing 25 ml of glass beads into a clean polyester sock, which was then worn for 3 hours by one of 3 volunteers (Bernier et al. 1999; Lacey and Cardé 2011). The beads treated with skin odor were poured into a 55-mm-diameter plastic Petri dish. The bead-filled Petri dish was covered with a black aluminum pan and turned over. Clean beads were then poured into the pan. Removal of the Petri dish produced a visually indistinguishable patch of beads treated with skin odor surrounded by clean beads (Fig. 2.1).

To provide a heat cue, a silicone heating pad was buried in the beads and heated to a surface temperature of 34 °C, which matched the skin temperature of the experimenter. To add a visual cue, we cut a white tissue (Kimwipe®) into an annulus with a 75 mm outer diameter and a 55 mm inner diameter and placed it atop the beads. The resulting 55-mm-inner black circle was similar in size to visual cues used in some mosquito traps (Bidlingmayer 1994) and wind-tunnel studies (van Breugel et al. 2015). The Kimwipe® was used to construct the first visual cue to ensure chemical cleanliness. To address concerns that the white Kimwipe® was too reflective, the light gray annuli were cut out of construction paper (Staples Pastel Gray, Staples Inc. Framingham, MA, USA) that was baked for 4 days at 200 °C. To create a facsimile of the visual cue used by van Breugel et al. (2015) an IR-filter (Wratten 2 No. 87, Eastman Kodak Company, Rochester, NY, USA) circle was placed atop a rectangle of gray construction paper that covered half of the bead-filled pan. The inner circles of both annular visual cues were 55 mm in diameter. This matched the diameter of our skin odor cue.

Experimental Procedure

Five female *Ae. aegypti* were transferred to release cages 3 hours before assays. The mosquitoes were allowed one minute to acclimate to the wind tunnel. The release cage was opened at the upwind end and the mosquitoes were allowed to fly freely under video observation for 6 minutes.

Data Collection

Landings were scored when a mosquito stopped movement on one of the 55 mm-diameter cues. Individual mosquitoes are visually indistinguishable and potentially could land multiple times on one or both treatments. All landings on the cues during the 6-minute observation period were counted. Landing was scored manually with BORIS v.5.1.0 (Friard and Gamba 2016). All data manipulation and statistical tests were conducted using R v.3.5.0 (R Core Team 2013; RStudio Team 2015). The times of the following events were recorded: opening of the release cage, and latencies of takeoff, landing upon, and departure from either of the two cue presentation areas. This allowed for the calculation of the duration of each landing. Flying mosquitoes were indistinguishable on an individual basis. Therefore, “take off” considers only the first mosquito to take flight.

Statistical Analysis

The counts on each treatment type were summed by trial and entered into a Wilcoxon signed-rank test. This test treated each trial as a block. As only one odor source volunteer was used each day, this was *de facto* blocking by odor source. This statistical blocking also accounts for daily variations in volunteer odor.

The Wilcoxon signed-rank test has an assumption that mosquito choices are independent events. This means that an animal choosing one option cannot subsequently choose another. Our free-flight wind tunnel allows a mosquito to land on one cue presentation area, take off, and land again on the other cue presentation area. The few observed occurrences of landing on both cues were noted and not used in our analyses.

As we cannot keep track of individual mosquitoes and the camera field of view does not fully cover the assay chamber, a few mosquitoes may have taken off from one cue presentation area, flown out of field of view or into glare, and then landed on the other area. Because only a handful of mosquitoes landed on both cue areas within the camera field of view, we believe that only a small percentage of mosquitoes left the field of view and returned to a different cue presentation area. Therefore, to meet the independence assumptions of the Wilcoxon signed-rank test, 5 % of landings were trimmed from trials in which both cue presentation areas elicited at least one mosquito landing. A 5 % trim was chosen as we believe the percentage of mosquitoes that completed two landings on different cues without being observed and discounted was less than 5 %. This trimming was conducted out of an abundance of caution and the trim level was selected *a priori*. To be invalid, more than one in twenty landings must have been by a mosquito that conducted an improbable maneuver. It is analogous to the "trimmed mean" method described by Tukey and McLaughlin (1963) which is still in common usage (Zhou et al. 2014). Conducting assays of single mosquitoes would have eliminated this problem but would have quintupled the number of assays required from 520 to 2600. We have included Wilcoxon Rank Sum Test outputs generated without the use of this trimming. None of the probability values change across the 0.05 threshold of significance (Table 2.1).

The y-axes of the plots are adjusted by the number of mosquitoes flown. This makes the bar graphs visually comparable within lettered sections. This concession for accuracy does make it harder to view the low numbers of landings on some treatments. A table of the exact data is available (GitHub link in final submission).

Landing durations were contrasted with a Kruskal-Wallis test. As number of landings on a cue is, by definition, the sample size of landing duration, the differences in landing counts on different cues may have limited the statistical power of our test and may explain some of the lack of significant differences between landing durations. This is not unexpected, as the experiment was designed to show a difference in the number of landings on different cues. Landing and remaining landed are separate behavioral categories.

Results

We show that a small source of human skin odor in a larger homogeneous visual background elicited far more mosquito landings than a similarly sized heat ($P < 0.001$), visual ($P < 0.001$), or visually distinct heat cue ($P < 0.001$). The CO_2 plume was vertically separated from the visual, heat and skin-odor cues, so that at the moment of landing, the choice of stimulus was independent of concurrent sensing of fluctuations in CO_2 concentration.

Our study shows that *Ae. aegypti,* following an encounter with an above-ambient concentration of CO_2 as occurs upon approach to a human, use skin odor to pinpoint a landing site suitable for a blood meal. We found that this diurnal mosquito chooses skin odor over heat, and heat over visual cues.

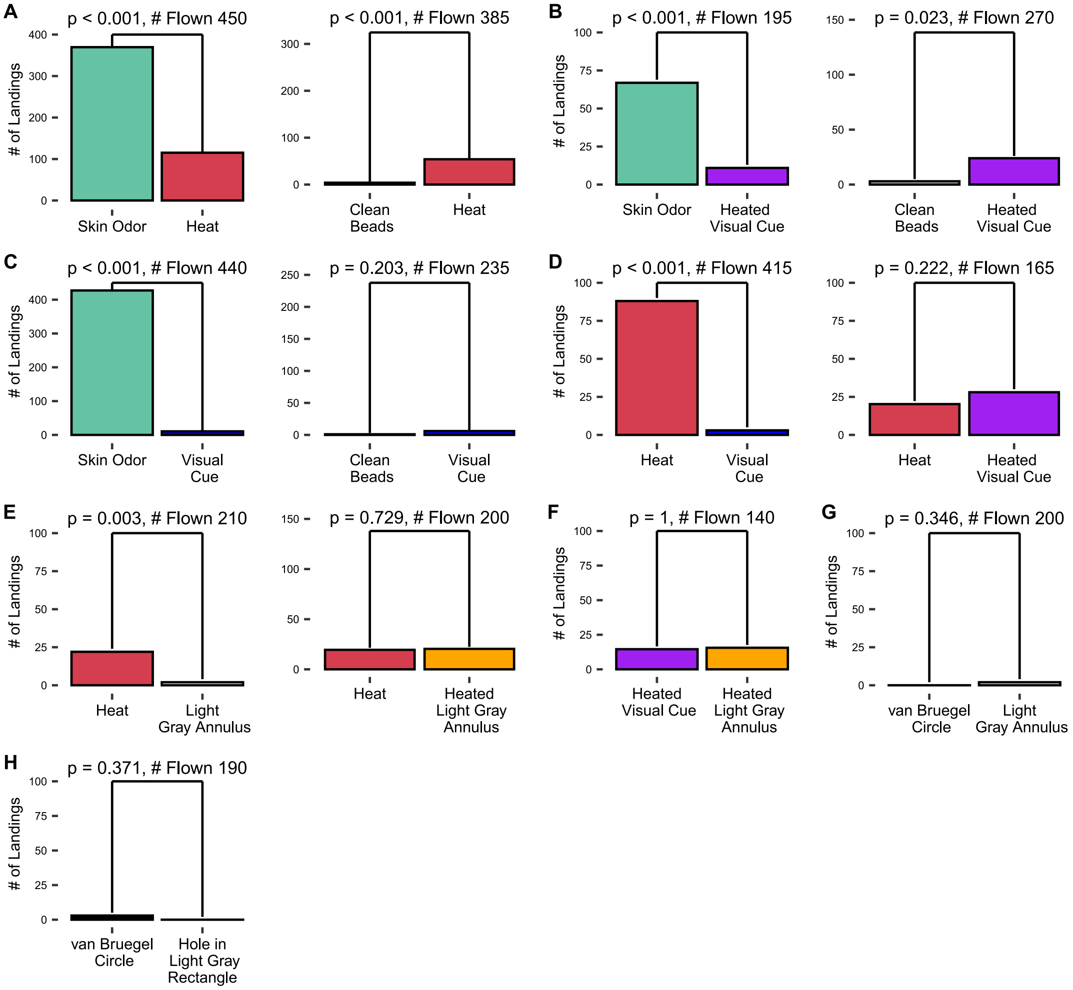

Fig. 2.4 Landing Counts

(A-H) All landings within a lettered box were by mosquitoes from the same rearing cohort and were flown on the same days. Y-axes are adjusted, within each set of days, in proportion to number assayed. This means the bar graphs are comparable within each lettered section. The calculated probabilities (p-values) above the bars show differences within trial type, not between types of trials (Wilcoxon Rank Sum Test).

Mosquitoes landed on skin odor far more frequently than any other stimulus. The heated visual cue and visually indistinct heat cue elicited intermediate numbers of landings. The unheated visual cue elicited the fewest landings (Fig. 2.4A).

Of 450 mosquitoes given a choice of skin odor or a heat cue, there were 369 landings on skin odor and 115 landings on the heat cue ($P < 0.001$). On the same days, 385 mosquitoes were assayed with unheated clean beads versus a heat cue; there were 4 landings on the unheated clean beads and 54 landings on the heated beads ($P < 0.001$) (Fig. 2.4A).

Of 195 mosquitoes assayed with skin odor versus a heated visual cue, there were 66 landings on skin odor and 10 landings on the heated visual cue ($P < 0.001$). On the same days, 270 were assayed with unheated clean beads and a heated visual cue. There were only 3 landings on the unheated clean beads while there were 24 landings on the heated visual cue ($P = 0.023$) (Fig. 2.4B).

We assayed 440 mosquitoes with skin odor versus a visual cue. The skin odor elicited 427 landings while there were 10 landings on the visual cue ($P < 0.001$). On those same days, 235 mosquitoes were assayed with unheated clean beads and a visual cue. There was only one landing on the unheated clean beads and 6 landings on the visual cue (not significantly different ($P = 0.203$) (Fig. 2.4C).

We assayed 415 individuals with a heat cue and a visual cue. The heat cue elicited 88 landings while the visual cue elicited only 3 landings ($P < 0.001$). On those same days, 165 individuals were presented with a heat cue which elicited 20 landings and a heated visual cue which elicited 28 landings (not significantly different) ($P = 0.222$) (Fig. 2.4D).

A light gray annulus alone elicited fewer landings than a visually indistinct heat cue ($P = 0.003$), and a heated light gray annulus did not elicit any more landings than a heated visually indistinct heat cue ($P = 0.729$) (Fig. 2.4E).

To verify that our visual cue was not "repellent," we compared a heated white annulus to a heated light gray annulus. There was no significant difference in landing (Fig. 2.4F). We tested our black glass bead visual cue against an IR-filter as used by van Bruegel et al. (2015). Again, there was no difference in landing (Fig. 2.4H). In this case we provided a neutral gray background that covered one side of the pan. None of unheated visual cues elicited significantly more landings than clean beads.

Treatment	Control	Treatment landings after trim	Control landings after trim	p-value after trim	Treatment landings no trim	Control landings no trim	p-value without trim
Skin Odor	Heat	369.6	115.2	0.00000176	376	118	0.00000156
Clean Beads	Heat	4	54	0.00012234	4	54	0.00012234
Skin Odor	Heated Visual Cue	66.9	10.95	0.00030519	67	11	0.00029541
Clean Beads	Heated Visual Cue	3	24	0.02320554	3	24	0.02320554
Skin Odor	Visual Cue	427.25	10.55	0	430	11	0
Clean Beads	Visual Cue	1	6	0.20309179	1	6	0.20309179
Heat	Visual Cue	88	3	0.00000054	88	3	0.00000054
Heat	Heated Visual Cue	20.25	28.05	0.22150949	21	29	0.25334915
Heat	Light Gray Annulus	22	2	0.00284787	22	2	0.00284787
Heat	Heated Light Gray Annulus	19.45	20.4	0.72859961	20	21	0.88378219
Heated Visual Cue	Heated Light Gray Annulus	14.55	15.6	1	15	16	0.96344182
van Bruegel Circle	Light Gray Annulus	0	2	0.34577859	0	2	0.34577859
van Bruegel Circle	Hole in Light Gray Rectangle	3	0	0.37109337	3	0	0.37109337

Table 2.1 Landing Count Full Statistical Results

The landing counts and probability values on the left side are the same as those shown in Fig. 2.4 although they are not abbreviated here. The landing counts and probability values on the right side were produced with the same Wilcoxon Rank Sum Test, but without the removal of 5% of the landings from trials with landing on both cues. The trimming procedure did not change the significance ($P < 0.05$) of any assay result

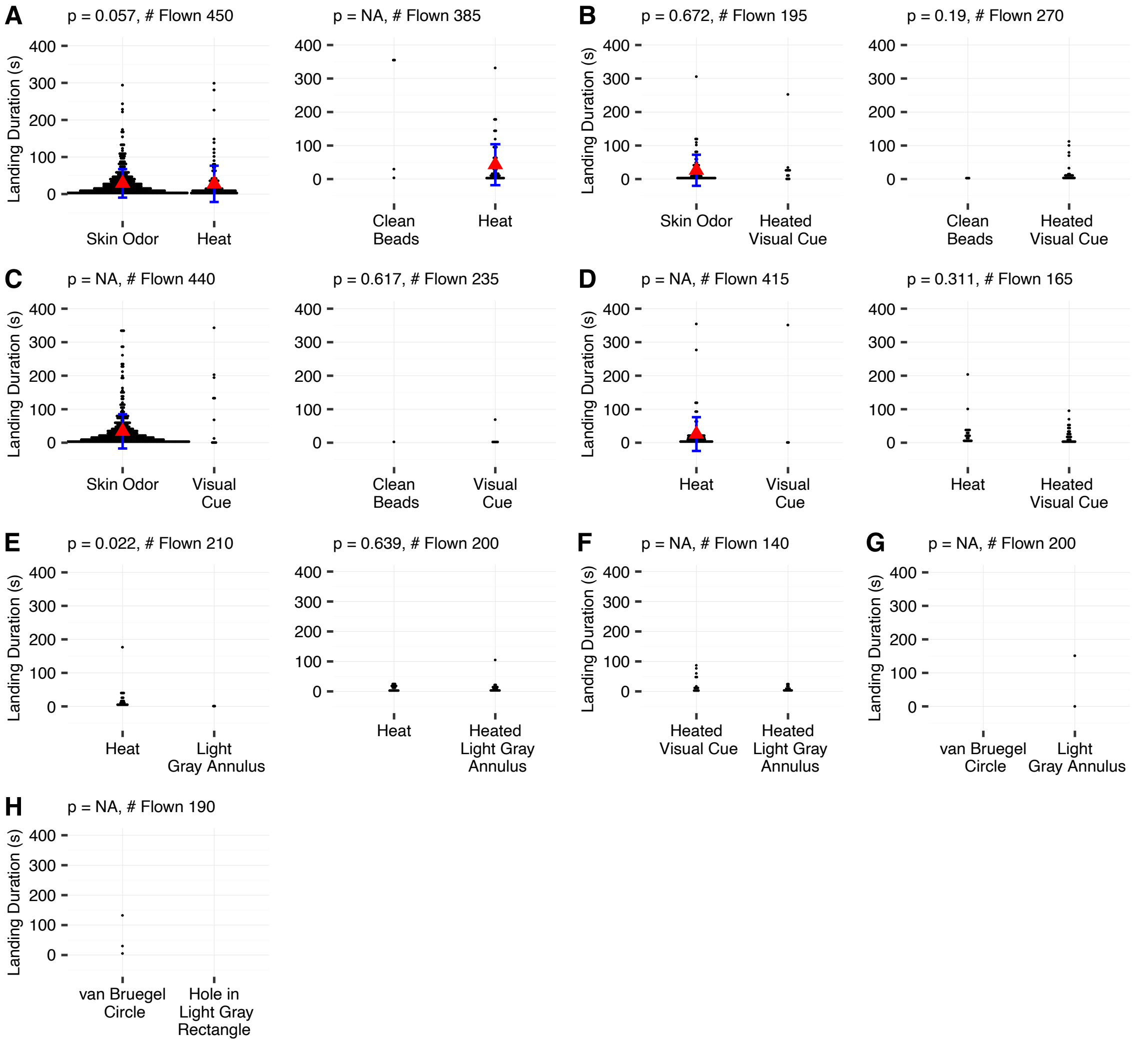

Fig. 2.5 Landing duration

(A-H) All landings within a letter were by mosquitoes flown on the same days. The p-values above the dot plots show differences within trial type, not between the two types of trials in each lettered section (Kruskal-Wallis test, no correction). The x-axis in each graph is the treatment presented. The lateral displacement of the dots is to ensure that the dot does not overlap with its neighbors. The more landings had a given duration, the more lateral displacement was graphically required. The red triangles indicate the median. A p-value of 'NA' indicates an insufficient sample size.

Landing Durations

Most pairs of cues, even those in which one elicited vastly more landings than the other, did not have significantly different landing durations (Figs. 3A, 3B, and 3C). Some of these were not statistically comparable due to insufficient sample size for at least one cue (Figs. 3C, 3G, and 3H). There were no statistical differences between heat versus heated visual cues (Figs. 3D and 3G). This was mirrored by the lack of significant difference between durations of landings on heat versus light gray annulus (Fig. 2.5E).

Discussion

Host-seeking in mosquitoes is traditionally understood to be a series of sequential orientation maneuvers. As a mosquito approaches a host, it encounters cues, produced by the host, that release a series of discrete maneuvers (Cardé and Gibson 2010; van Breugel et al. 2015; Cardé 2015). The maneuvers include flight upwind along plumes laden with host cues and orientation toward visual cues. In this sequential-distance model, the mosquito is presumed to encounter progressively more host-specific cues, on the assumption that additional cues that are host-specific become detectable as the mosquito closes the distance to the host. The sequential-distance model does not explain choice when more than one cue is available. *Aedes aegypti* land on a heat source when CO_2 is elevated above background, but rarely on the CO_2 source itself (Lacey et al. 2014; McMenamin et al. 2014). The malaria mosquito *Anopheles coluzzii* landed on a nylon mesh patch imbued with skin odor rather than a CO_2 source provided several centimeters away. However, the mosquitoes only landed on the mesh when a plume of elevated CO_2 concentration was present (Webster et al. 2015). In both species, (Lacey et al. 2014;

McMenamin et al. 2014; Webster et al. 2015) the mosquitoes choose to land on the skin odor or heat over the CO_2 source.

Other versions of the sequential-distance model suggest that the pairing of cues in addition to the distance between a mosquito and its host also plays a part in determining the mosquito's response. For instance, one cue may lower the response threshold for another such as skin odor or visual cues (Dekker et al. 2005; van Breugel et al. 2015; Cardé 2015). We propose that in addition to host seeking based on pairs of cues that are detectable a given point, *Ae. aegypti* has an innate hierarchy of cue preference.

Mosquitoes track odor plumes upwind toward their source by optomotor aenemotaxis (Kennedy 1940; Dekker and Cardé 2011). How far mosquitoes are able to track odor plumes is not precisely known. Using rings of traps around a source of ~1 L/min CO_2, which is equivalent to ~4 sedentary adult humans (Snow 1970), Schreck et al. (1972) captured mosquitoes from many genera, including *Aedes.* Of these, 92% were captured within 18 m of the CO_2 source, suggesting that a human CO_2 plume would be attractive to mosquitoes only within ~18 m. This finding has a source of bias, as distant traps cover a smaller angle from the CO_2 source.

After following a plume of CO_2, the sequential-distance model assumes that host-seeking *Aedes* mosquitoes orient toward visual cues (Cardé and Gibson 2010; van Breugel et al. 2015). The eyes of *Ae. aegypti* have a minimum resolvable angle of 12.3 °, allowing them to discern a human-sized object from ~7 meters away (Muir et al. 1992). The black inner circle of our white annulus would be discernible up to 26 cm away, depending on the angle of approach. However, we observed very few landings on unheated visual cues (Fig. 2.4). Although the visual cue provided was obviously different from that provided by a human it was similar to those presented by other researchers (van Breugel et al. 2015).

The scarcity of landings on our unheated visual cues contrasts with the finding of van Bruegel et al. (2015) that *Ae. aegypti* spent more time near a visual than a heat cue. However, they did not report whether the mosquitoes that flew within “an 8 × 8 × 4 cm volume above and downwind” of the black disk, made out of near-infrared transparent plastic, actually landed on it. Others have observed that *An. coluzzii* often fly near visual cues without landing on them (Hawkes and Gibson 2016).

Liu and Vosshall (2019) found that magnetically tethered *Ae. aegypti* orient towards black vertical stripes, which would support that visual cues are important prior to landing. They also quantified mosquito occupancy, i.e., landings and remaining landed, after landing on an unheated visual cue. Although a vertically oriented black dot elicited more mosquito occupancy than the surrounding white paper, it was less than half of mosquito occupancy of heat without a visual cue.

To address this ambiguity, we replicated the black disk used by van Bruegel et al. (2015) (Supplemental Methods), and presented it, 5 mosquitoes at a time, to 390 mosquitoes. Only two landed on it, although many flew near the disk. We also tested a black circle of beads exposed through a hole cut into gray paper. This provided a small black cue in a larger area of neutral color yet still did not elicit landings (Figs. 2H and 3H). This means that all of or visual cues provided on the floor, which were approximately similar to those tested in the literature, failed to elicit substantial mosquito landing.

Our results affirm that even in the presence of elevated CO_2, visual cues elicit few landings. As landing is a prerequisite for blood feeding and therefore pathogen transmission, it is an important diagnostic measure of mosquito host-seeking behavior.

In studies of insect landing in which stereotyped maneuvers precede contact with the substrate, insects were provided with a distinct visual cue. Leg extension, body saccades (turns) and pitch change (van Breugel and Dickinson 2012) are triggered by objects covering an expanding portion of the insect visual field. Honey bees can maintain a fixed angle relative to a visual cue in order to execute a smooth descent path toward and landing upon the cue (Srinivasan and Zhang 1997; Srinivasan et al. 2000). In contrast to those studies with prominent visual cues, we presented heat and skin odor without co-located visual cues. The visual cues available to the mosquito for optomotor feedback were lateral and above the mosquito (Fig. 2.3A). Our mosquitoes still executed landings, using a mechanism different from those previously studied.

In addition to following odor plumes, *Ae. aegypti* at close range can follow convective heat plumes. *Aedes aegypti* landed frequently on a nylon mesh cone placed 5 cm above a 43.3 °C black billiard ball but failed to land when the convection was interrupted by a long-wave, infrared-transparent KRS-5 filter (Peterson and Brown 1951). The tips of the antennae of *Ae. aegypti* are equipped with coeloconic neurons that exhibit phasic shifts in response to air temperature changes as small as 0.05 °C (Davis and Sokolove 1975). The human-temperature heating pad in our assay produced a plume 0.2

°C above ambient ~30 cm downwind. Therefore, the heat plume could have been detectable to the mosquitoes throughout a large swath of the wind tunnel and yet they still preferred to land on a source of skin odor.

The distance at which skin odor plumes are detectable to a mosquito in the field is unclear. Dekker and Cardé (2011) found that skin odor, supplied by an odor stream from an enclosed arm, ~1 meter upwind, readily elicited upwind flight of *Ae. aegypti*. However, when the skin odor plume was diluted five-fold, it elicited proportionally fewer flights. When a plume of CO_2 was added, the mosquitoes were "instantly sensitized" to the diluted skin odor and surged upwind. Such sensitization clearly would have occurred in our trials, and also would have contributed to landing on a skin odor patch in *An. coluzzii* (Webster et al. 2015).

Skin odor is thought to be the cue that anthropophilic mosquitoes use to tell humans apart from other endothermic vertebrate hosts (Gouck 1972; Takken et al. 1997; Pates et al. 2001; Dekker et al. 2001, 2002; Besansky et al. 2004; McBride 2016). Degennaro et al. (2013) found that *orco* mutant *Ae. aegypti* lose their strong preference for human odor over that of guinea pig in a cage assay. By lacking *orco*, the olfactory coreceptor, these mosquitoes lost the function of all of their olfactory receptors. Anopheline mosquitoes (Ribbands 1946) and *Ae. aegypti* (Trpis and Hausermann 1978) also appear to use human scent for house-entering, which can occur well before biting. Once inside a human dwelling, host seeking could be triggered by a fluctuating concentration of CO_2 (Dekker and Cardé 2011). Our findings show that human skin odor not only provides a human-specific cue, but also provides a direct host-seeking and landing cue.

Landing duration is a potential measure of cue salience. Mosquitoes have chemoreceptors on their tarsi (Sparks et al. 2013) and labella (Saveer et al. 2018). Once a mosquito's tarsi or labella contact beads treated with skin odor, the mosquito could detect non-volatile chemicals such as amino acids.

Conclusion

Here we establish that following exposure to a plume of CO_2, heat stimulus, and a heated visual cue, all evoke at least some orientation and landing. However, the use of a choice bioassay allowed these cues to be ordered in a valance hierarchy. Naïve *Ae. aegypti* had a high preference for landing on on a skin odor source; heated cues were the second most effective cue for eliciting landing, whereas the unheated visual cues elicited virtually no landings. This demonstrates that *Ae. aegypti*, a day-biting mosquito, are able to locate and land on skin odor and heat without a co-located visual cue. The order, primacy, and interaction of cues used by *Ae. aegypti* and other mosquitoes during host finding in the field remain to be firmly established. However, our findings suggest that *Ae. aegypti* track cues based on an innate hierarchy. This hierarchy appears to rank cue types in descending order of human specificity. The primacy of skin odor contrasts with the view that this diurnal mosquito relies primarily on vision following detection of a fluctuating concentration of CO_2.

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CHAPTER 3. L-LACTIC AND 2-KETOGLUTARIC ACIDS, ODORS FROM HUMAN SKIN, GOVERN ATTRACTION AND LANDING IN HOST-SEEKING FEMALE *AEDES AEGYPTI* MOSQUITOES

Introduction

Aedes aegypti Blood-Feeding Preferences

Aedes aegypti (Diptera: Culicidae) are a vector of several consequential arboviruses including Zika, chikungunya, yellow fever, and dengue (Bhatt et al. 2013). *Aedes aegypti* have 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).

Female *Ae. aegypti* blood feed almost exclusively on humans (Scott et al. 1993). This makes them, and other human specialist mosquitoes, particularly effective vectors of human pathogens (MacDonald 1952). The cosmopolitan "subspecies" *Ae. aegypti aegypti* split 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, unless otherwise noted, references to *Ae. aegypti* in the present work are to the anthropophilic Orlando strain of *Ae. a. aegypti* (see Materials and Methods).

To find a host, *Ae. aegypti* fly upwind when they detect fluctuating levels of CO_2 above ambient. Once they are within several meters of a prospective host, they can also sense visual cues and, once closer to the host, thermal cues (Gillies 1980; Cardé and Gibson 2010; van Breugel et al. 2015; Cardé 2015). The precise distances at which they detect and use particular 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. (2000b) characterized 279 compounds in the headspace above human skin. A review by Dormont et al. (2021) provides a useful table of mosquito attractants using Dethier et al.'s (1962) definition of attractant "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. The 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 host-seeking behaviors of mosquitoes. "Attractiveness" in one assay may be a measure of flying into a port from a still-air chamber, whereas in another, it may be a measure of arrestment after upwind orientation.

Assay and Trap Design for Characterizing Host-Seeking Behavior

Sulcatone (6-methyl-5-hepten-2-one) is a human skin odor component that has been found to be attractive in one assay, inactive in another, and perhaps even "repellent" in a third. Bernier et al. (2003) presented sulcatone in a continuously observed landing assay, ranking it as a "weak attractant" because it elicited landing of 10-30 % of the *Ae. aegypti* tested. McBride et al. (2014) found that the addition of sulcatone to guinea pig odor and CO_2 did not alter the number of *Ae. aegypti* captured in a port. Logan et al. (2008) found that the addition of sulcatone to human hand odor on one side of a Y-tube reduced the total number of *Ae. aegypti* that flew upwind to either side. However, there was no difference in mosquito presence between the Y-tube side with a clean hand and the side with a hand and sulcatone. Taken together, these findings are all consistent with sulcatone acting as an arrestant. An arrestant is a compound that makes an animal stay in an area it has already arrived at, rather than elicit “oriented movements towards its source” (Deither et al. 1962).

The different results among these assays which tested sulcatone’s “attractiveness” demonstrate the limitations of end-point assays used by McBride et al. (2014) and Logan et al. (2008). End-point assays record the number of mosquitoes that end up in a designated area containing a compound of interest. End-point assays including those using baited traps or flight chamber entry ports (port-entry assay) do not establish the underlying mechanism of orientation.

To improve upon the basic port-entry assay design, Dekker et al. (2001) added electrocution grids near the ports. If *Ae. aegypti* or *Anopheles gambiae* mosquitoes were found below a grid but not in the nearby port, it was interpreted as the cues tested having brought the mosquitoes into the vicinity of the entry port but failed to elicit port entry. A homogenous plume offered to *An. gambiae* did not elicit port entry but resulted in many mosquitoes beneath the electrocution grid (Dekker et al. 2001). A turbulent plume of CO_2 and a plume of skin odor elicited both port entry and hovering, resulting in electrocution. Torr et al. (2008) used similar methods in the field, finding that 35 *An. gambiae* s.l. entered the traps while 1,552 were collected below the electrocution grid. Their odor-baited entry trap may have provided a homogenous plume of host odors.

Dekker et al. (2001) also examined the times at which *Ae. aegypti* entered the ports. Skin odor combined with CO_2 quickly lured *Ae. aegypti* into the trap by the three-minute mark. Carbon dioxide alone lured half the *Ae. aegypti* more slowly, requiring six minutes after release to capture half the *Ae. aegypti*. The implication is that long duration end-point assays may be unable to distinguish undirected movement (kinesis) from directed movement (taxis) was discussed explicitly by Kennedy (1977). Both findings suggest that homogeneous plumes elicit flight in a general vicinity but fail to elicit flight quickly into the ports used by Dekker et al. (2001) or much at all into the trap portion of the odor-baited entry trap used by Torr et al. (2008).

Nevertheless, laboratory end-point assays may be useful for initial investigations of mosquito responses to host emitted odors, particularly if followed with other assays. For example, BG-Lure, a human skin odor mimic, designed for use with the Biogents Sentinel® trap (Biogents, Regensburg, Germany), which releases lactic acid, ammonia, and caproic acid (Williams et al. 2006). This lure was developed using Y-tube olfactometers with subsequent field-trapping studies (Williams et al. 2006).

Given the inconsistent "attractiveness" results of cues and ignorance to mosquito approach behaviors with the end-point assays illustrated above, we chose a wind-tunnel assay with videography and landing quantification. Wind-tunnel assays with videography allow examination of orientation maneuvers, in addition to landing (endpoint). Some wind-tunnel assays have used video tracking without landing counts (van Bruegel et al. 2015); however, landing counts are important because if a mosquito does not land, it cannot bite nor transmit pathogens (Reed 1900).

Lactic Acid as a Host-Seeking Cue

Lactic acid (2-hydroxypropanoic acid) is thought to be a cue used by *Ae. aegypti* (Steib et al. 2001) and *An. gambiae* (Dekker et al. 2002) to distinguish humans from non-human animals. Both studies found lactic acid was necessary but insufficient to attract the number of *Ae. aegypti* that were attracted to whole human odor. The addition of lactic acid made non-human animal odor more attractive to both species of anthropophilic mosquitoes. Smith et al. (1970) tested *Ae. aegypti* in a unique 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, considering Smith et al.'s (1970) findings with *Ae. aegypti* and those of Dekker et al. (2002), lactic acid may be an attractant but not a landing cue for *An. gambiae*. Lactic acid should be presented to *An. coluzzii* in a wind tunnel with landing quantification to resolve its role in eliciting *An. coluzzii* host seeking.

Recently, Bello and Cardé (in review) identified 2-ketoglutaric acid (2-oxopentanedioic acid) in human skin odor and demonstrated a mixture of lactic and ketoglutaric acids in the presence of CO_2 is a landing cue for *Ae. aegypti*. While also found in the attractive fraction of skin odor, pyruvic acid did not significantly elevate landing rates above those elicited by the blends of lactic and ketoglutaric acids.

As the blend of lactic and ketoglutaric acids elicited landing in a cage assay, we set out to determine if the blend elicits upwind flight in a wind tunnel in the presence or absence of CO_2. We measured time from release to takeoff, time from takeoff to the first landing, the number of landings, and the duration of landings. Our video tracking system also allowed us to examine mosquito flight maneuvers prior to landing, unlike the cage assay used by Bello and Cardé (in review).

Carbon dioxide increases the takeoff rate of *Ae. aegypti*, elicits upwind flight (Kennedy 1940), increases heat-seeking behavior (McMeniman et al. 2014), and ultimately leads to increased end-point capture (Huffaker and Back 1943) compared to normal air. Carbon dioxide is generally considered as the only 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. This model was formalized by Gillies in 1980. Following the findings by Dekker et al. (2005) 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, the model was updated by van Bruegel et al. (2015) and Cardé (2005, 2015). The updated models 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. There have not been any studies on the maximum distance specific skin odor compounds may attract mosquitoes.

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. By using a wind tunnel with videography and landing quantification we sought to examine the behaviors elicited by the blend of lactic and ketoglutaric acids. We also wanted to find out how these compounds acted on *Ae. aegypti* with and without supplemental CO_2.

Materials and Methods

Mosquito Rearing

A colony of *Ae. aegypti*, Orlando strain, was maintained in a L:D 14:10 h photocycle at 27 °C and 70 % RH. Approximately 50 larvae were reared in each container (26 x 25.6 x 15 cm, TakeAlongs Large Rectangular Food Storage Containers, 1 Gallon, Rubbermaid, Atlanta, GA, USA) filled with ~1 cm of deionized (D.I.) water and fed Tetramin® pellets (Tetra, Blacksburg, VA, U.S.A) *ad libitum*. Pupae were collected into plastic containers, transferred to screen cages (30 x 30 x 30 cm, BugDorm-1, Megaview Science Co. Ltd. Talchung, Taiwan) before eclosion, and provided 10 % sucrose solution in D.I. water on a cotton wick. Both males and females were kept together in the screen cages, and the females used in the bioassays were assumed to have mated prior to the experiments. Females used in experiments were 3-10 days post-eclosion and were not blood fed. Mosquitoes were starved and deprived of water approximately 12 hours prior to experiments. Female mosquitoes were transferred individually to clean cylindrical acrylic release cages (7 × 8 cm diameter) 30 minutes prior to testing.

Assay Methods

The flight and landing of mosquitoes were released in a glass wind tunnel 122 × 30.5 × 30.5 cm and were video recorded (FDR-AX53, Sony, Tokyo, Japan) (Sumner and Cardé in preparation). Air was drawn into the tunnel from an adjacent, uninhabited room (25 °C and 70 % RH). To simulate the presence of an upwind vertebrate host, 100 ml/minute 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 a 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 built into the camera was turned off to avoid producing glare. Visible light was provided by incandescent bulbs and measured at ~14 lux inside the tunnel.

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; 10 µl/ml or 100µl/ml in acetone) and lactic acid ("LA" in the figures) (0.5 ml; 10 µl/ml or 100µl/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 minutes to ensure that the acetone had evaporated.

Human skin odor was collected onto glass beads by placing 25 ml of beads into a polyester/cotton blend sock, which was worn by BDS for 12 hours. Beads were cleaned 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, Pennsylvania), and heated to 250 °C for 12 hours before reuse.

The dish of beads was presented on a metal stand (15-cm high, Fig. 3.1) in the center of the tunnel, 55 cm upwind of the release cage. Assays were run and recorded with the video camera for 6 minutes, commencing with the opening of the release cage. Disposable nitrile gloves were always worn by the experimenter to prevent contamination with skin odors.

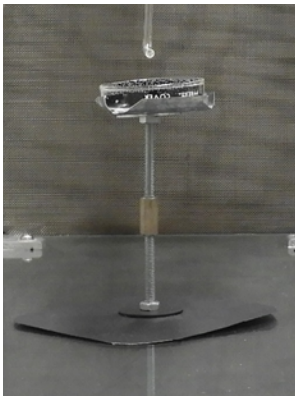

Fig. 3.1 The Stand Holding the Beads

The stand holding the Petri dish of beads as viewed from the downwind end of the wind tunnel. The CO_2 releasing tube is also visible behind the Petri dish.

Treatment Strategy

We presented the mosquitoes with clean beads (acetone solvent only), 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, North

Carolina) (based on Bello and Cardé in review), a high dose blend of 50 μg each of lactic and ketoglutaricacids, 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 3.1).

Table 3.1 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

To confirm the blend was not eliciting mosquito landing solely due to its lactic acid content, we 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. Each of these assays contained five mosquitoes (Table 1), which enabled a five-fold reduction in the number of assays for these treatments. The potential number of landings was not adversely affected, 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. All videos were viewed and scored from the release time until 6 minutes 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, Leesburg, VA). 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 ≥ 30 seconds. All data manipulation and statistical tests were conducted using RStudio v.1.1.463 (2009–2018 RStudio, Inc.) and R v.3.5.0 (R Core Team 2017).

Statistical Analysis

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 allows 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 the: number of mosquito landings on beads per trial, durations a mosquito remained after landing amongst 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 was root ten 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 E_{max} (track straightness) of the mosquito flight, and the proportion of time the mosquito spent heading (± 20 °) towards the center of the beads. Spearman rank correlations were used to test effects.

E_{max} (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. E_{max} is bases on an iterated summing of the expected displacement. As the shortest path between two points is a straight line, a high E_{max} 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 E_{max} and a low mean distance to the beads before landing, whereas a mosquito that flew straight to the beads would have a low E_{max} 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. 3.2).

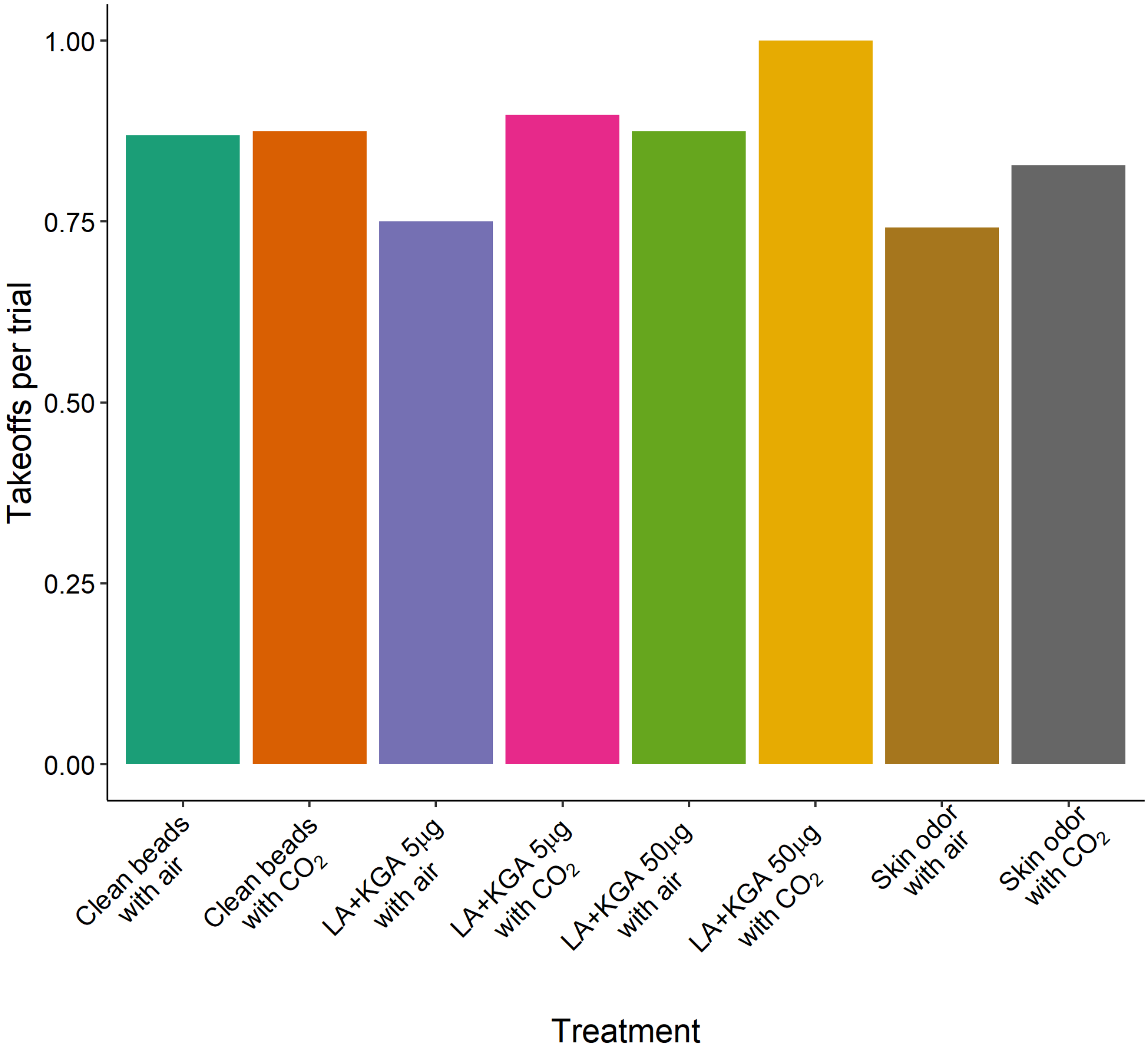

Fig. 3.2 Takeoffs of Female *Ae. aegypti* by Treatment Combination per Trial

The proportions of trials in which the mosquito took off. Only the presence of CO_2 was correlated with the proportion of trials with takeoff (GLM, binomial, link = probit, Est. = 0.447, P = 0.022).

Latency from Release to Takeoff

Among the treatments there was not a significant difference in the latency from release to mosquito takeoff. Although the 50 μg dose appears to elicit a significant decrease in the latency from release to take off ($P = 0.017$, 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. 3.3).

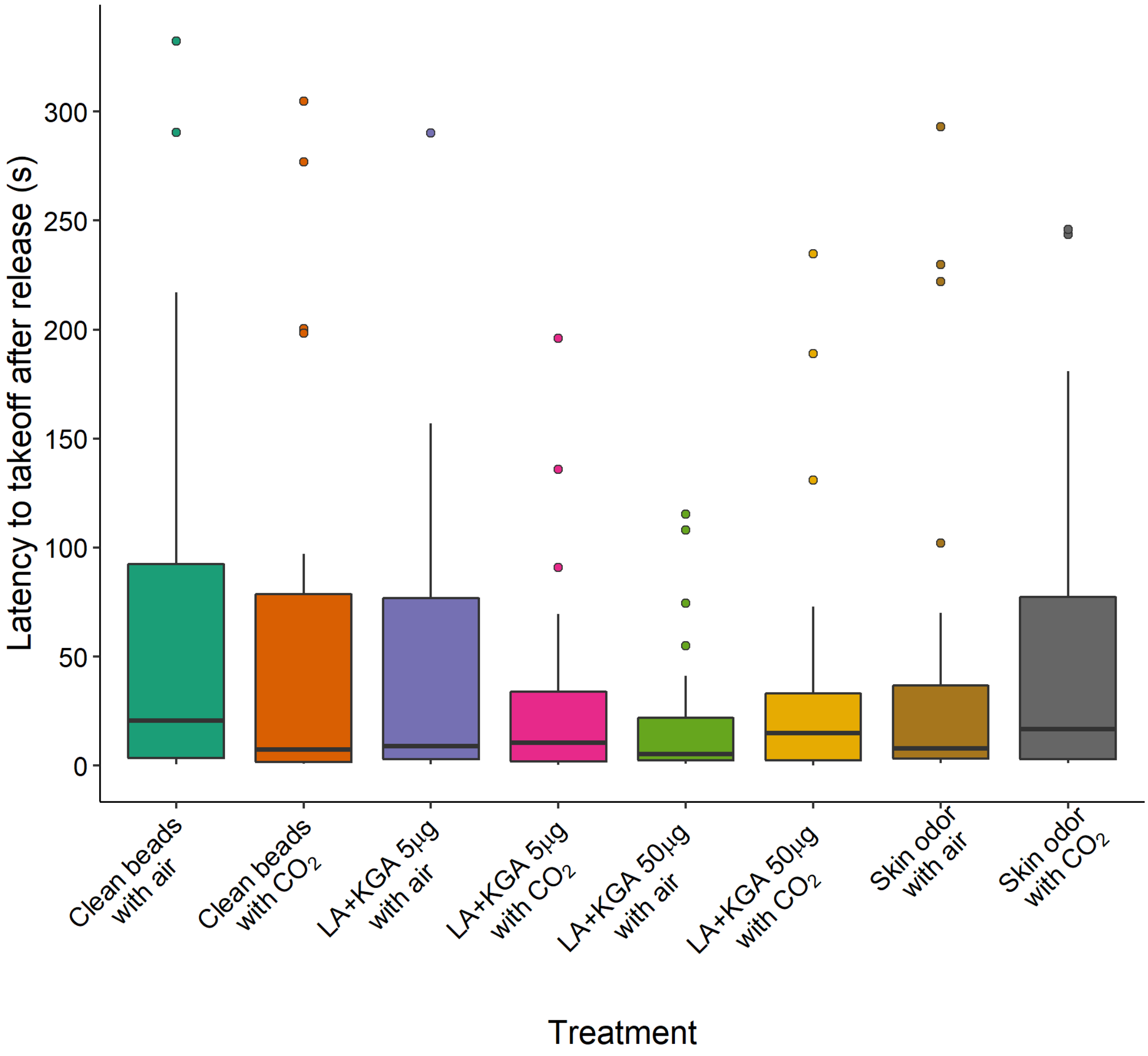

Fig. 3.3 Boxplot of the Latency from Female *Ae. aegypti* Release to Takeoff

The latencies from release to takeoff in seconds across treatment combinations. While the high dose of the blend was statistically significantly associated with a lower latency (GLM, Est. = -0.3534, P = 0.017), we do not believe this is biologically meaningful. 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).

Time From First Takeoff to First Landing

Fig. 3.4 shows the time elapsed 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, $P = 0.026$) were each negatively correlated with the duration of flight from takeoff to first landing. This means that these two treatments 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 ($P = 0.139$).

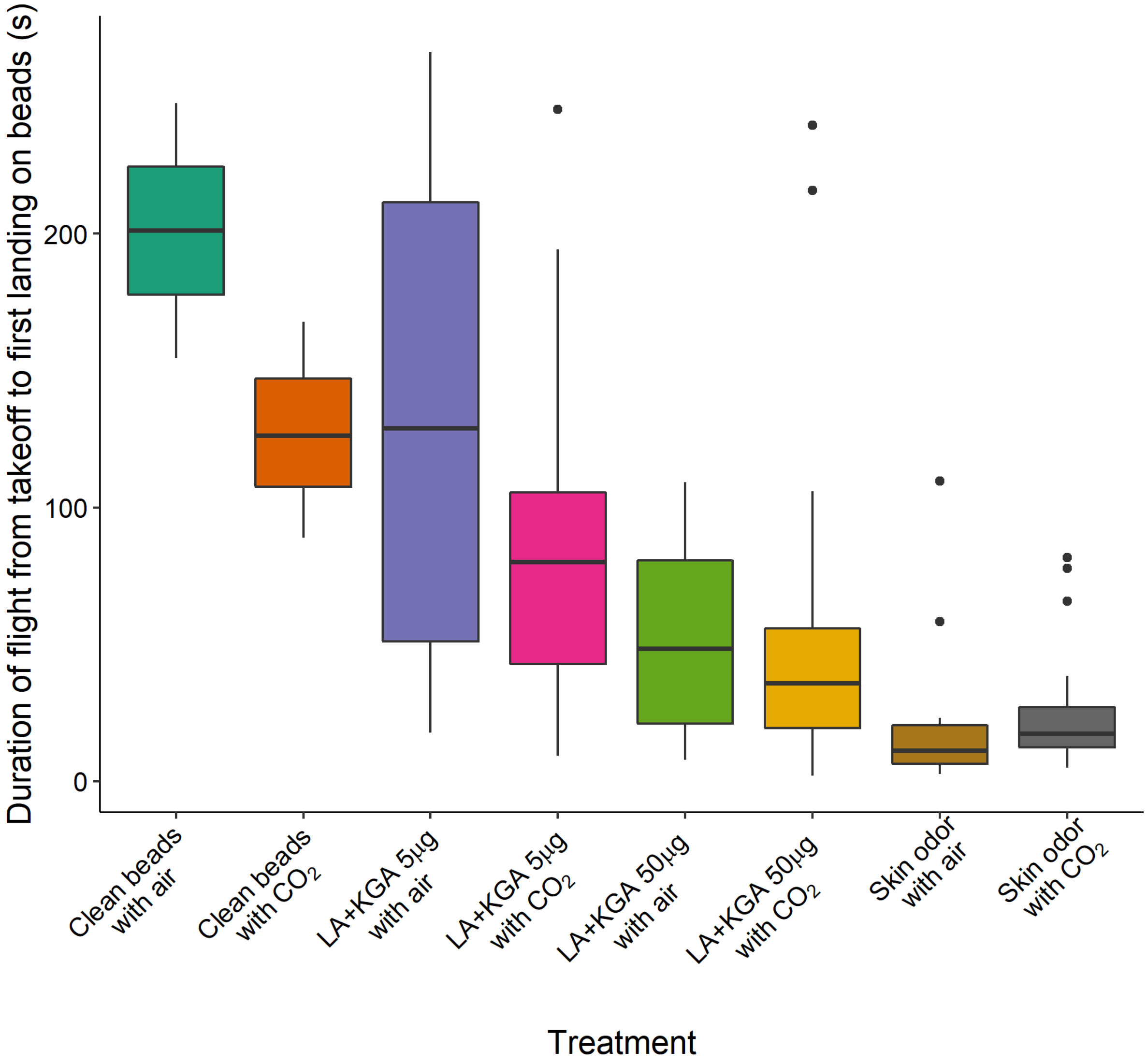

Fig. 3.4 Boxplot of the Duration of Female *Ae. aegypti* Flight from Takeoff to First Landing, i.e., a Measure of Rapidity of Response to the Lure

The durations of female *Ae. aegypti* flight from takeoff to the first landing on beads in trials with at least one landing in a wind tunnel. The presence of skin odor (Est. = -1.88, $P < 0.001$) and the blend of 50 µg each of lactic and ketoglutaric acids (Est. = -1.061, $P = 0.026$) were negatively correlated with the duration of flight from takeoff to first landing. CO_2 was not correlated with flight duration.

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 statistically 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. 3.5).

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.

When examining only those trials in which mosquito takeoff occurred (Figure 3.1), the presence of CO_2 was not significantly correlated with trials in which the mosquito landed at least once (Est. = 0.256, $P = 0.161$). Within this subset of trials, the proportion of those with at least one landing is correlated with skin odor (Est. = 1.974, $P < 0.001$), and the blends of lactic and ketoglutaric acids 50 µg (Est. = 1.364, $P < 0.001$) and 5 µg (Est. = 0.688, $P = 0.016$).

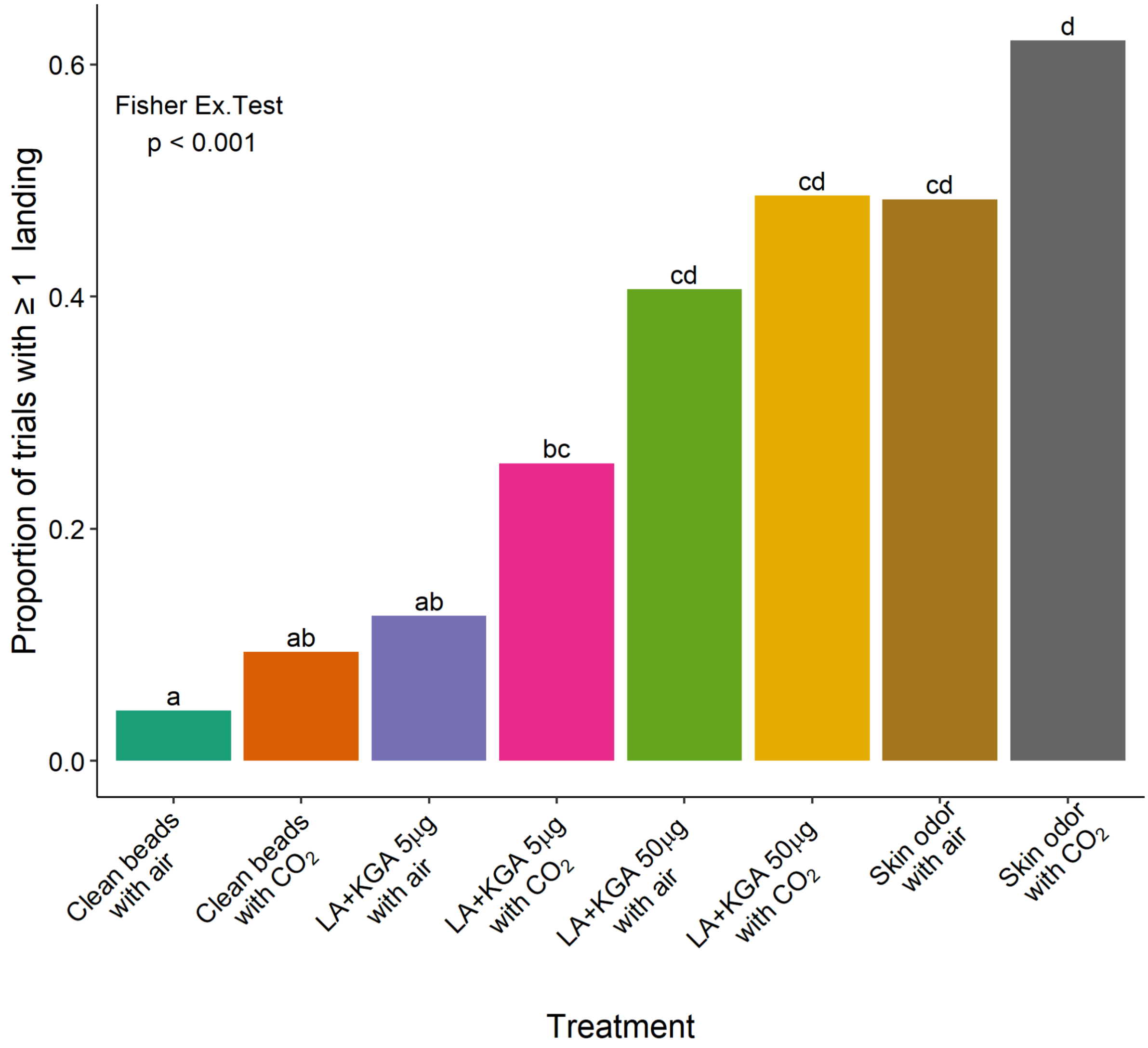

Fig. 3.5 Proportion of Wind Tunnel Trials in which the Female *Ae. aegypti* Landed on Beads Once or More

The proportion of wind tunnel trials in which the female *Ae. aegypti* landing on beads 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, $P < 0.001$).

Repeat Landings

Fig. 3.6 shows the number of repeat landings, on beads per trial by treatment, among trials with at least one landing. The presence of skin odor (Est. = 0.3238, P = 0.0199) and the high dose blend, 50 µg each, of lactic and ketoglutaric acids (Est. = 0.3244, P = 0.0311) were positively correlated with the landings by a single mosquito per trial (Fig. 3.6).

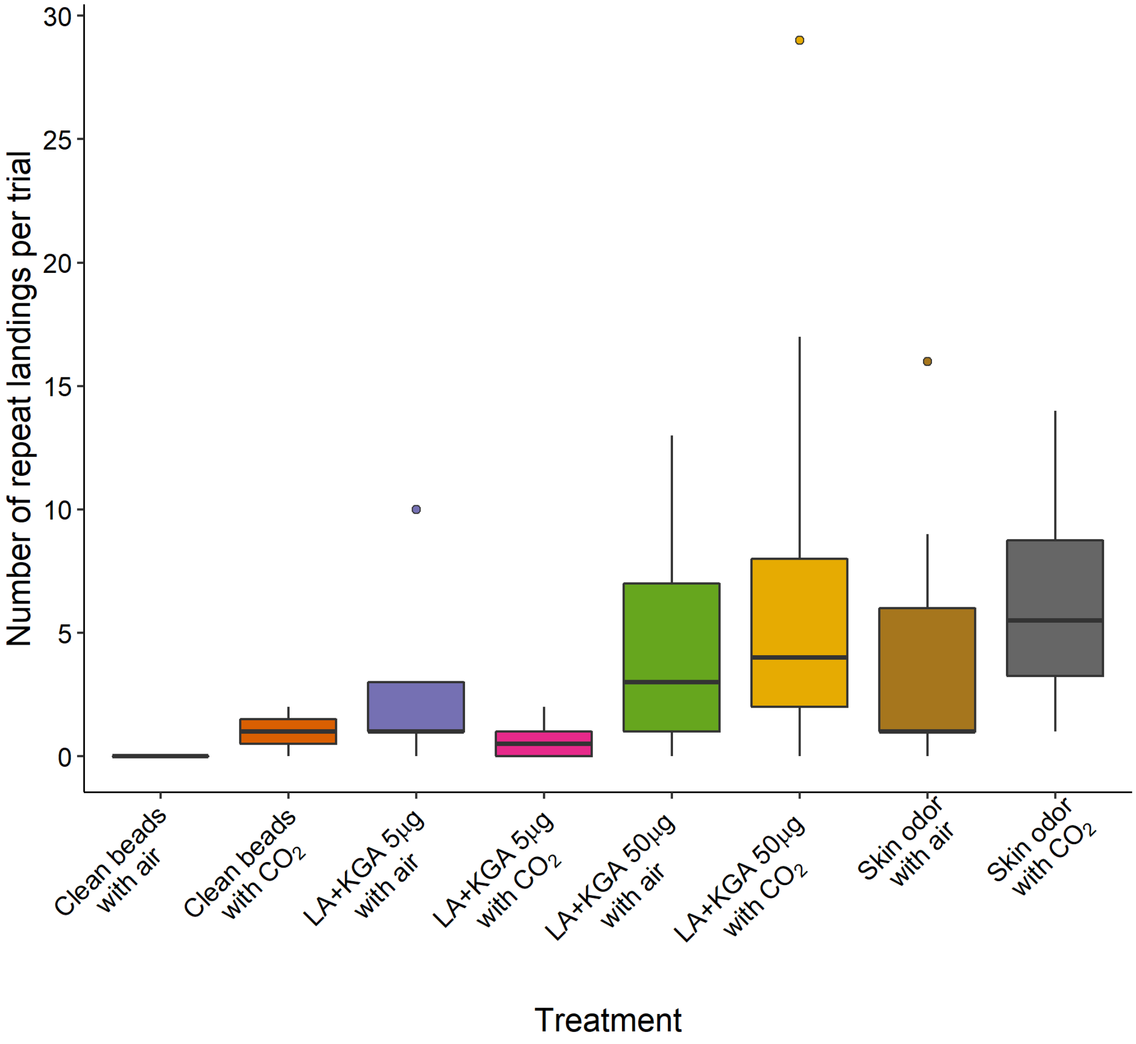

Fig. 3.6 Boxplot of the Number of Repeat Landings on Beads by Female *Ae. aegypti* per Trial among Trials with at Least One Landing

In trials with at least one landing by the mosquito, only skin odor (Est. = 0.3238, P = 0.0199) and 50 µg each of lactic and ketoglutaric acids (Est. = 0.3244, P = 0.0311) were correlated with the number of landings per trial.

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. 3.7). Despite the reduced sample size due to five mosquitoes being assayed at a time, the time from takeoff to the first landing was significantly shorter between the two-component blend (mean = 57 seconds, S.D. = 49.4) and the lactic acid alone (mean = 115 seconds, S.D. = 99.3) (Kruskal-Wallace, P = 0.039).

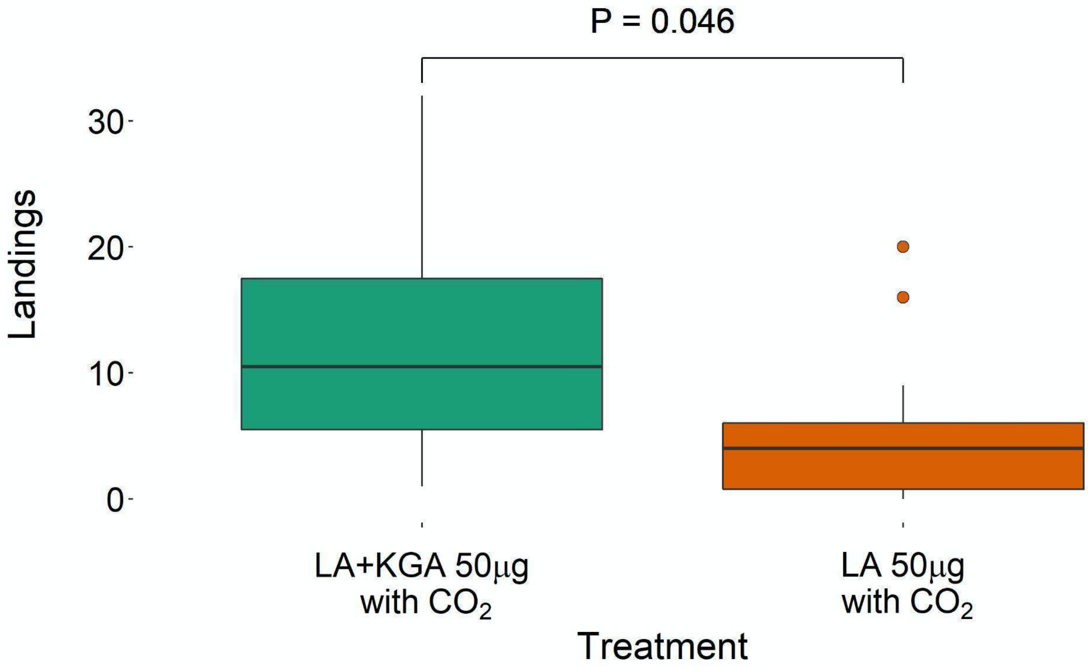

Fig. 3.7 Boxplot of the Total Landings of Female *Ae. aegypti* by Trial on the Blend Versus Lactic Acid Alone

The number of landings on the two-component blend was significantly different than on lactic acid alone (Kruskal-Wallace, P = 0.046).

Duration

Fig. 3.8 provides the time within each trial that the 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. 3.8). 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.

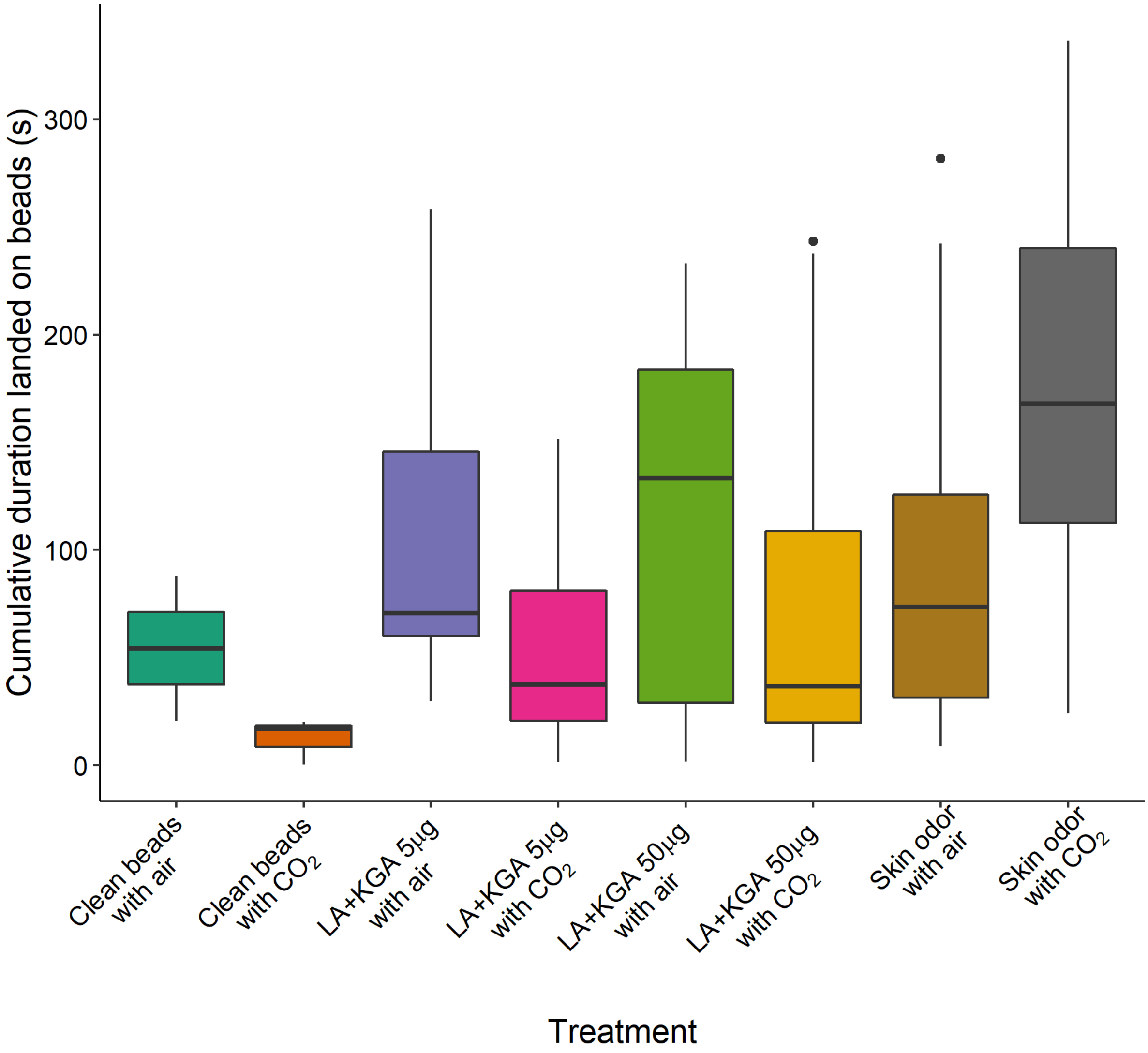

Fig. 3.8 Cumulative Duration Female *Ae. Aegypti* Stayed Landed on the Beads, Summed by Trial

The cumulative duration of times female *Ae. aegypti* spent landed on beads, in trials with ≥ 1 landing in a wind tunnel. 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 mosquitoes remained landed. CO_2 was not correlated with duration landed.

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 (χ^2 = 298, df = 298, P = 0.49). Selected flight tracks are shown in Fig. 3.9. 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 the beads was negatively correlated with whether an individual landed on the beads (ρ = -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 (χ^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 (ρ = -0.12, P = 0.05). Across all treatments, the mean (± 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 (± 20°) toward the center of the beads during flight in the wind tunnel among treatments (χ^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 (ρ = 0.01, P = 0.83). Across all treatments, the mean (± SE) proportion of the time a flying mosquito spent heading towards the center of the beads (± 20 °) was 0.16 (± 0.0039).

The E_{max} (track straightness; 1 = completely straight track) of insect tracks during flight in the wind tunnel did not differ among treatments (χ^2 = 298, df = 298, P = 0.49), nor did the E_{max} 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 (± SE) E_{max} of mosquito flight in the wind tunnel was 0.35 (± 0.01).

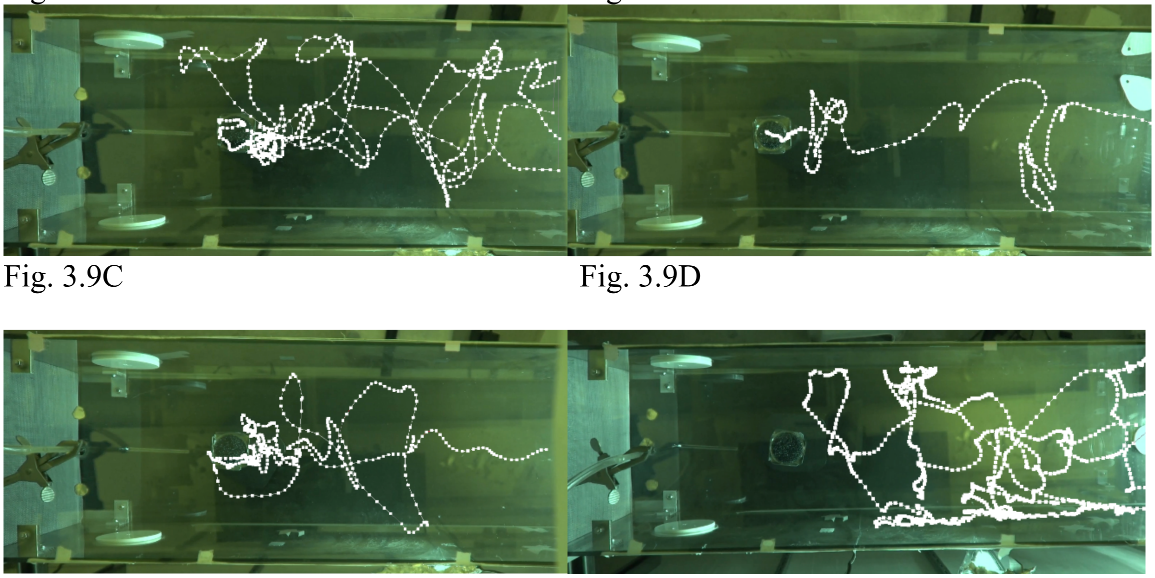

Fig. 3.9A

Fig. 3.9B

Fig. 3.9C

Fig. 3.9D

Fig. 3.9 Selected Flight Tracks by Female *Ae. aegypti*

Top view of selected flight tracks of female *Ae. aegypti* in a wind tunnel, 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 this track shown in 3.8D. The view is from the top, the airflow was left to right, and the mosquitoes were released on the right.

Discussion

For female *Ae. aegypti*, the two-component blend of lactic acid and ketoglutaric acid developed by Bello and Cardé (in review) elicited upwind flight and landing in a wind tunnel. The higher dose of the blend used in this study (50 µg of each) gave comparable results to skin odor in terms of the proportion of *Ae. aegypti* females that landed at least once on an upwind odor source in a wind tunnel. Unsurprisingly, the presence of CO_2 also positively affected the proportion of landings, given the known role of CO_2 in sensitizing mosquitoes to human skin odors (Dekker et al. 2005).

Skin odor and the high dose of the blend of lactic acid and ketoglutaric acid (50 µg each) also elicited comparable numbers of repeat landings per individual mosquito.

This suggests that this blend elicits 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 demonstrated in the finding that the blend and skin odor were each correlated with the cumulative duration of landing on the beads. Neither the number of repeat landings nor landing durations were correlated with the presence of CO_2. This is somewhat 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 et al. 1998). The number and durations of landings are at odds with each other. A long duration landing reduces the available time for further landings.

Skin odor and the high dose of the blend of lactic acid and ketoglutaric acid (50 µg), were also negatively correlated with the duration of mosquito flight from takeoff to the first landing on the beads. These treatments both induced more rapid orientation and landing on the beads in the wind tunnel. The presence of CO_2 did not change the time from takeoff to the first landing.

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 more akin to skin odor in a wind tunnel. The 5 µg dose was sufficient in the cage assay used by Bello and Cardé (in review), but the need for a higher dose is consistent with our hypothesis that more of the lactic and ketoglutaric acid blend would be required to elicit landing due to turbulent diffusion in our wind tunnel than in the cage assay. Future studies may examine how much lactic and ketoglutaric acid volatilize from human skin.

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 is not acting directly as a landing cue. This is consistent with the sequential-distance model of mosquito host seeking (Gillies 1980; van Breugel et al. 2015; Cardé 2015). In this model 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 (Muir et al. 1992; Kahn et al. 1966).

Ketoglutaric acid is a component of the citric acid cycle (Wishart et al. 2018). It is found in fresh and dry sweat (Delgado-Provedo et al. 2020). It is not known if the majority of ketoglutaric acid in human skin odor is produced by humans directly or possibly by skin flora. While the mechanism of detection of ketoglutaric acid is unknown, other ketoacids have been investigated. The whole labellum of *An. gambiae* (G3 strain) can detect ketovaleric and ketobutyric acids (Kwon et al. 2006). While not specified, we understand these to be 2-ketovaleric and 2-ketobutyric acids. A neuron of the labellum expressing *Ir76b* and *Ir25a* but lacking *Ir8a* responds to 2-ketovaleric acid (Saveer et al. 2018). That neuron does not show a response to 2-ketobutyric acid.

Dekker et al. (2005) and Lacey et al. (2011) found that *Ae. aegypti* and *Culex quinquefasciatus* surge upwind when provided with human skin odor in air with ambient CO_2. Tauxe et al. (2013) found a sensillum on the capitate peg of the maxillary palps that responded to both CO_2 and some human odors. Younger et al. (2020), found that the neurons expressing *Gr3*, which is needed for CO_2 detection, also expresses *Ir25a*. Sumner and Cardé (in preparation) found that *Ir8a* is likely not involved in detection of ketoglutaric acid. This means that some host odors, such as ketoglutaric acid, may be detected by mosquitoes in a way like that of CO_2. This may explain the small difference in landings between the blend lure when presented in the presence and absence of CO_2. Further studies with *Ir25a* and *Ir76b* knockout mosquitoes will be necessary to resolve this possibility.

Lactic acid has been a contentious candidate as a mosquito attractant (Acree et al. 1968; Smith et al. 1970). Lactic acid is released from human apocrine glands at a variable 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).

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 many as *Ae. aegypti* to its arm of the Y-tube as human odor did. 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. This means that while our blend of ketoglutaric acid and lactic acid is particularly attractive, lactic acid may still be the human signifying cue. Dekker et al. (2002) found that human skin rubbings contained higher quantities of lactic acid than several species of non-human animals. The human rubbings were found to attract more *An. gambiae* to one arm of a Y-tube olfactometer than did the rubbings from non-human animals.

Geier et al. (2002) found that lactic acid was necessary but not sufficient alone to attract mosquitoes as frequently as the most attractive human volunteers in a y-tube. Addition of lactic acid to the odor from an unattractive volunteer made them as attractive as the volunteers that were previously classified as most attractive. There were compounds produced by all the volunteers, not identified by Geier et al. (2002), that were needed to elicit the maximum mosquito attraction. Our findings suggest that ketoglutaric acid may be one of the compounds Geier et al. demonstrated existed but did not isolate. The results corroborate those of Bello and Cardé (in review), that lactic acid is necessary but insufficient to elicit a rate of mosquito landing equal to that of a blend of human odor compounds.

Smith et al. (1970) found that lactic acid elicited entry into a port, but that the same mosquitoes would not proceed to land on a treated sock. While this finding probably represents lactic acid alone being merely insufficient to elicit landings, the possibility of repellency at high doses should not be excluded. Dose-dependent termination of attraction is known in other systems. Male oriental fruit moths cease upwind flight when presented with high concentrations of attractive pheromone (Baker and Roelofs 1981). We suggest that future studies using these compounds in field traps test a wide range of doses.

The type of assay used is important (Kennedy 1977). While a cage assay was an entirely appropriate tool to guide the chemical fractionation steps of Bello and Cardé’s (in review) investigation, it may not reveal the full suite of behaviors that the blend elicits. The cage assay only tells how many mosquitoes were observed landed at several time points. It does not specify how the mosquitoes arrived, nor how long they stayed. As stated in the methods, our assay allowed us to analyze time to takeoff, time from takeoff to the first landing, as well as the number and duration of landings. As the wind-tunnel assay tests elicitation of landing behavior as well as upwind flight from a distance, it measures a more complete repertoire of host-seeking behavior than does the cage assay.

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 elicit takeoff. The test of five mosquitoes at a time with the two-component blend and lactic acid alone was strictly intended to test if the blend was better at eliciting landing than lactic acid alone. The short latency to takeoff may be partly an intrinsic character of *Ae. aegypti*. They have been described as opportunistic or exhibiting "aggressive biting" (Cilek et al. 2004). Cilek et al. (2004) found that time to first bite for *Ae. aegypti* averaged 9.8 ± 0.3 s (as opposed to *Cx. quinquefasciatus*, which averaged 41.0 ± 1.1 s).

The lack of statistical differences in the flight tracks among treatments is surprising, as there is a large difference in the landing behavior among treatments. While this reinforces the need to quantify landing, future studies may consider analyzing subsets of trials with individual behaviors. The simplest test would be to reexamine only tracks from trials with landings. The main problem is that the sample sizes among treatments that elicit different numbers of landings will be inherently unequal. Such a study may only become feasible with effective and reliable multiple mosquito tracking.

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 the previously mentioned chemoreceptors on the labellum (Saveer et al. 2018), the 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, probably with a cage landing assay, will be needed to identify post-landing cues.

Current mosquito traps and lures, even the "gold standard" BG Sentinel, have low trapping efficiency and require an electric fan (Amos et al. 2020 a,b). By finding compounds that elicit landing instead of attraction to a general vicinity, we can lure the mosquitoes directly into trap entrances. This would boost trap efficiency immediately and allow the development of traps without fans.

We counted the number of repeat landings. If a lure in a trap elicits repeated landing attempts by mosquitoes, it would provide the accompanying trap with repeated opportunities to capture that mosquito. The blend also elicited significantly more landings than lactic acid alone.

Conclusion

We found that the blend of lactic and ketoglutaric acid discovered by Bello and Cardé (in review) elicits upwind flight and landing of *Ae. aegypti* in a wind tunnel. This was the case with and without supplemental CO_2. The lack of a need for supplemental CO_2 makes this blend a strong candidate for use in mosquito traps. Ketoglutaric acid may be one of the compounds present in non-human animal odors that when supplemented with lactic acid are highly attractive to mosquitoes.

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CHAPTER 4. FEMALE *AEDES AEGYPTI* MOSQUITOES DO NOT REQUIRE THE *IR8A* PATHWAY TO RESPOND TO 2-KETOGLUTARIC ACID, AN ODORANT INDUCING LANDING

Introduction

Female *Aedes aegypti*, the yellow fever mosquito, transmit several pathogens: yellow fever, dengue, and chikungunya viruses. Because *Ae. aegypti* specializes in feeding on humans, it is particularly effective at transmitting human pathogens (Scott et al. 1993; MacDonald 1952).

Lactic acid may be a cue that anthropophilic mosquitoes use to discriminate humans from other hosts (Steib et al. 2001; Dekker et al. 2002; Geier et al. 2002). L-lactic acid (2-hydroxypropanoic acid) henceforth referred to as lactic acid. Several studies have shown that the addition of lactic acid to human and animal odors dramatically increased the number of anthropophilic mosquitoes attracted (Steib et al. 2001; Dekker et al. 2002; Geier et al. 2002). However, lactic acid alone does not elicit landing of *An. gambiae,* another anthropophilic mosquito (Healy and Copland 2000). When presented with CO_2, lactic acid elicits some but not many *Ae. aegypti* to land on the lactic acid source (Bello and Cardé, in review).

Humans release substantial, although variable, quantities of lactic acid directly from the apocrine sweat glands (Thurmon and Ottenstein 1952). Incubated sweat contains less lactic acid than fresh sweat, which suggests that endogenous production as the primary source of lactic acid on human skin (Braks and Takken 1999). Acree et al. (1968) isolated lactic acid from human skin and found that it increased the number of *Ae. aegypti* captured by a funnel trap inside a ventilated flight chamber compared to clean air. The exact role of lactic acid in mosquito host seeking has been contentious. Smith et al.

(1970) attributed the lack of mosquito attraction to lactic acid in all studies prior to 1968 to be due to the assay types and cue presentations used. Presentation of lactic acid alone in a wind-tunnel assay with CO_2 is not sufficient to elicit a landing rate of *Ae. aegypti* comparable to skin odor (Sumner et al. Chapter 3, in preparation).

Bello and Cardé (in review) isolated lactic acid and 2-ketoglutaric acid (2-oxopentanedioic acid; hereinafter ketoglutaric acid) from a fraction of human skin odor that elicited landing of *Ae. aegypti* in a cage assay. More mosquitoes were observed to land on a blend of synthetic ketoglutaric and lactic acids than on lactic acid alone. The same blend presented to *Ae. aegypti* in a wind tunnel elicited landings at a higher rate than lactic acid alone and similar to the landing rate elicited by whole human skin odor (Sumner et al. Chapter 3, in preparation). The blend of ketoglutaric and lactic acids elicited landing as quickly as skin odor, and even elicited landing without the provision of CO_2. Ketoglutaric acid is an intermediate in the Krebs cycle (Wishart et al. 2018), and is present in both fresh and dry sweat (Delgado-Provedo et al. 2020). Both endogenous production and bacterial fermentation are possible sources of ketoglutaric acid.

Mosquito Chemoreception

Mosquitoes have three types of chemoreceptors: ionotropic (IR), gustatory (GR), and olfactory (OR). Insect olfactory receptors are gated ion-channels. Insect olfactory receptors are always found in a complex with *orco*, an obligate coreceptor (Larsson et al. 2004). Insect GRs have a wide range of functions. They were first thought to detect chemicals on contact (taste), as they are expressed in the labellum (Clyne et al. 2000) but have since been shown to detect (smell) CO_2 (Jones et al. 2007; Kwon et al. 2007) and temperature changes (Ni et al. 2013). Neither insect olfactory nor gustatory receptors share homology or evolutionary history with mammalian odorant and gustatory receptors. All four types cross the cell membrane seven times. However, both receptor types in insects have their C-terminus on the outside of the cell, the opposite of the mammalian arrangement (Montell 2009, 2013). Insect ORs are not G-protein coupled chemoreceptors like the ORs of mammals, but instead act as cation channels (Sato et al. 2008; Wicher et al. 2008; Butterwick et al. 2018).

Ionotropic receptors are a variant of ionotropic glutamate receptors (iGluRs) (Benton et al. 2009; Silbering et al. 2011). The rest of iGluRs are found at synapses inside of organisms. The IRs have evolved to detect compounds from outside the body. Insect IRs serve as ion channels just like other iGluRs (Benton et al. 2009; Mayer et al. 2011). Their ion channel function and shared evolutionary history enable the IRs to be functional when inserted into *Xenopus* embryos (Kwon et al. 2007). All IRs require one of three coreceptors, that are themselves IRs, *Ir8a, Ir25a,* or *Ir76b,* to function (Benton et al. 2009; Silbering et al. 2011). Insect IRs are evolutionarily plastic; within the last quarter million years, *Drosophila seychellia* has developed a modified version of *Ir75b* which detects hexanoic acid (Prieto-Godino et al. 2017).

The detection of lactic acid by mosquitoes is well-studied. *Aedes aegypti* detect lactic acid with their antennae (Davis and Sokolove 1976). Whereas an electrolabellogram of *An. gambiae* showed no response to lactic acid (Kwon et al. 2006). Raji et al. (2019) found that *Ir8a* is a necessary coreceptor for lactic acid detection by *Ae. aegypti* by testing knockout mosquitoes in a uniport assay and with electroantennograms.

The detection of and behavior responses to ketoacids by mosquitoes have been examined in less detail. Pitts et al. (2017) expressed *An. gambiae* ionotropic receptors in *Xenopus* embryos. They found that *Ir8a* and *Ir75k* formed a complex that detected straight-chain carboxylic acids such as: heptanoic, octanoic, and nonanoic acids. The complex also showed statistically insignificant responses to hexanoic and decanoic acids. None of the IR complexes tested in the *Xenopus* embryos detected 2-ketovaleric acid, which is detectable by the labellum of *An. gambiae* (Kwon et al. 2006).

Healy et al. (2002) found that *An. gambiae* land on four to six-carbon chain-length, 2-ketoacids, but they did not examine the receptors involved. The labellum of *An. gambiae* responds to ketovaleric and ketobutyric acids (Kwon et al. 2006). A neuron on the labellum of *An. gambiae* is able to detect 2-ketovaleric acid but not 2-ketobutyric acid. The neuron expresses *Ir76b* and *Ir25a*, but **not** *Ir8a* (Saveer et al. 2018). Antennal electrophysiological response to butyric, heptanoic, octanoic acid, and nonanoic acids also disappeared in the *Ir8a* mutants. Because of the necessity of *Ir8a* in detection of lactic, butyric, heptanoic, octanoic, and nonanoic acids, our initial hypothesis was that *Ir8a* would be a necessary coreceptor for detection of ketoglutaric acid.

In this study we investigated the receptors that *Ae. aegypti* use to detect both lactic and ketoglutaric acids. We did this using *Ir8a* and *orco* knock-out mosquitoes in a cage landing assay to verify that *Ir8a* is needed to detect lactic acid, but found it is not necessary to detect ketoglutaric acid.

Methods

Insects

Aedes aegypti eggs of three genotypes provided by the DeGennaro laboratory were reared and prepared with methods adapted from those in Sumner et al. (Chapter 2, in preparation). Mosquito colonies were reared in a room with a L:D 14:10 h cycle, kept at 25 °C and 70 % RH in the UCR Insectary and Quarantine Facility. Larvae were reared in 1 cm of deionized (D.I.) water in plastic containers (26 x 25.6 x 15 cm, TakeAlongs Large Rectangular Food Storage Containers, 1 Gallon, Rubbermaid, Atlanta, GA, USA) with ~50 larvae per container. They were fed TetraMin Tropical Tablets (Tetra Holding GmbH, Melle, Germany). The mosquitoes were allowed to emerge into a Bug Dorm (30 × 30 × 30 cm MegaView Science Co., Ltd., Taichung, Taiwan) with 10 % (v) sucrose solution available. Thirty female mosquitoes, 3-9 days after eclosion and presumed to be mated, were aspirated into small cylindrical acrylic release cages (7 × 8 cm i.d.) one hour before the start of assays. *Aedes aegypti* were assayed 4-8 hours into their photophase.

Assay Procedure

In all assays, mosquitoes were presented with a one-choice assay consisting of a 55-mm diameter glass Petri dish containing black glass beads treated with a synthetic odor blend and provided with CO_2 (4% CO_2 mixed with tank air, 0.1 l/min). The CO_2 mixture was released 2 cm from the middle of a screen side of the cage. The synthetic blend consisted of 50 µg of lactic acid and 50 µg of ketoglutaric acid. Acetone solutions of each component (10 µg/ml) were placed dropwise onto the beads. As acetone elicits *Ae. aegypti* flight, the beads were then dried for 15 minutes in a fume hood (Ghaninia et al. 2019).

Mosquitoes were released from their holding cages into the assay cage and given 5 minutes to acclimate. The assay chamber was a Bug Dorm with the top panel replaced with a glass panel to allow viewing. The assay chamber was derived from that used by Webster et al. (2015) though the glass top was made of laser-cut borosilicate glass with beveled edges for safety. The CO_2 mixture was supplied through a 3-meter-long tube, which ensured it was the same temperature as the assay chamber. Following mosquito acclimation, the Petri dish with treated beads was inserted into the assay chamber. The CO_2 supply was turned on and marked in the video recording by the experimenter waving their hand in front of the camera. The mosquitoes were allowed to behave freely for 6 minutes under observation using an FDR-AX53 Handycam (Sony Inc., Tokyo, Japan) in “Nightshot” (Near-IR sensitive) mode, mounted above, and with infrared lighting from the side (AXIS T90A, 850 nm, Axis Communications AB, Lund, Sweden). To avoid the infrared light source providing a heat plume for the mosquitoes, the light was mounted in between the assay cage and the room exhaust vent.

Genotypes and Treatment Strategy

We compared three genotypes of mosquitoes, the Orlando strain (wild type) and two mutants (*orco* and *Ir8a* knockouts) derived from this strain. The *Ir8a* mutant mosquitoes were generated from the Orlando strain using CRISPR/Cas9 RNA-guided gene-editing (Raji et al. 2019). The *orco* mutants were derived from the Orlando strain using a pair of zinc-finger nucleases (DeGennaro et al. 2013). If odorant receptors are not involved in the detection of lactic acid or ketoacids, the *orco* mutant mosquitoes should not have been impaired in their response to the lactic and ketoglutaric acid blend. We thought it prudent to eliminate the possibility that odorant receptors were detecting the ketoglutaric acid. A response like that of the wild type mosquitoes allowed the *orco* mutants to serve as a positive control, ensuring that the genetic modification process did not destroy the behavioral response of the mosquitoes. As the *orco* mutants were observed on the lure as frequently as the wild type mosquitoes, the *orco* mutants were not included in the second round of assays.

We tested *Ir8a* mutants with the expectation that they would be unable to detect the blend of lactic and ketoglutaric acids and would not land on the beads treated with the blend. However, based on our initial finding that the *Ir8a* mutants had a low but non-zero response to the lure, we rejected our hypothesis that *Ir8a* mutants would not detect the lactic and ketoglutaric acid blend, and instead hypothesized that the *Ir8a* mutants could be detecting ketoglutaric acid alone. Therefore, we devised a second round of assays to test wild type*, orco* and *Ir8a* mutants with the two-component lure and also with ketoglutaric acid alone.

Video Scoring

Videos were viewed and scored with BORIS v.7.10.7. The number of mosquitoes that were observed on the beads was recorded at 30 second intervals over a 6 min period that began when the CO_2 was first turned on. Mosquitoes were scored as landed if they remained on the beads for at least one second. Our assay scoring procedure does not distinguish between long landing duration and repeated short landings. For example, one mosquito that landed once at the 20 second mark and took off at 100 second mark would receive a score of 2. A mosquito that landed at the 27 second mark, took off on the 32 second mark, landed again on the 88 second mark, before finally taking off on the 93 second mark would also receive a score of 2. The necessitates the rather ungainly use of the phrase “observed landed” rather than “landing,” as our assay scoring never quantified landing directly

Data Processing

Each observation name in BORIS was standardized to contain the strain, observation date, trial number, and the treatment information. Underscores and zeros were used to ensure that the information could be found at consistent character lengths. This prevents the need for text reading functions and the possibility of ambiguity in treatment interpretation by computer code.

BORIS generates a table of events. Each event is assigned a separate row. The row contains all the information BORIS has about that event: the behavior, the time, etc. Our R code took the event data and cleaned it up by discarding columns that would not be used later. The code then extracted the date of the observation from the text of the observation name. The BORIS generated column called “Observation.date” is the date the video was scored, not the date the mosquitoes flew.

As each behavioral event was missing its respective start time of the trial, we needed to add it to the row using R. For example, a behavioral event, in BORIS’s default output, reads that five wild type mosquitoes landed on the beads at 96 seconds on January 1, 2020. However, the assay could have started at 6, 36, or 66 seconds. Each trial has a start time recorded as a unique “Behavior,” even though it is marked on the video with the experimenter’s hand wave. The R code fills in the “Start” time for each trial using the standardized information we included in the observation name, the “Observation.id.” Once the start time is entered for every event, simple subtraction between columns produces the true time for every event. This part of the code is built with loops that do not function if there is missing or duplicate data. This helps to reduce human error through manual entry.

Statistical Methods

Behavioral events were exported from BORIS and processed with an R script (R v.4.0.4). The mosquitoes within the same trial are not independent of each other. Therefore, we used a linear mixed model. The output is analogous to that of a repeated measures ANOVA but allows for within-group analysis. Next, we ran a series of pairwise estimated marginal means comparisons. We used the “fdr” post-hoc correction. It is less aggressive than the Tukey-HSD correction. Our correction was done with all 630 comparisons, as is required by good statistical practice. This pairwise analysis incurs a cost in statistical resolving power due to the large number of pairwise comparisons for which the probability values must be adjusted. However, comparing one strain at the one-minute timepoint to another strain at the three-minute timepoint is not biologically informative. Therefore, the “cldList” package was run only on the subset of landing counts within each time point. The “cldList” output was used to generate the brackets shown in Fig. 4.1B.

Results

Mosquito Landing on Blend

There was a significant difference in the number of mosquitoes landed among the genotypes (KW, P = 0.0007) (Fig. 4.1A). The Wilcox pairwise comparisons show that the *orco* mutant and wild type mosquitoes, with 801 and 750 landings each, were not significantly different from each other. The *Ir8a* mutants, with 357 landings, were significantly lower than the other genotypes.

Fig. 4.1B displays the number of landed mosquitoes at each time point. Although the time effect was not significant (P = 0.2698), the genotype effect (P < 0.0001) and the genotype:time interaction effect (P = 0.0004) were significant. The brackets show significant differences **within** each observation time point. At later time points, the *orco* mutants are observed on the beads more than the wild type mosquitoes.

First Round of Assays

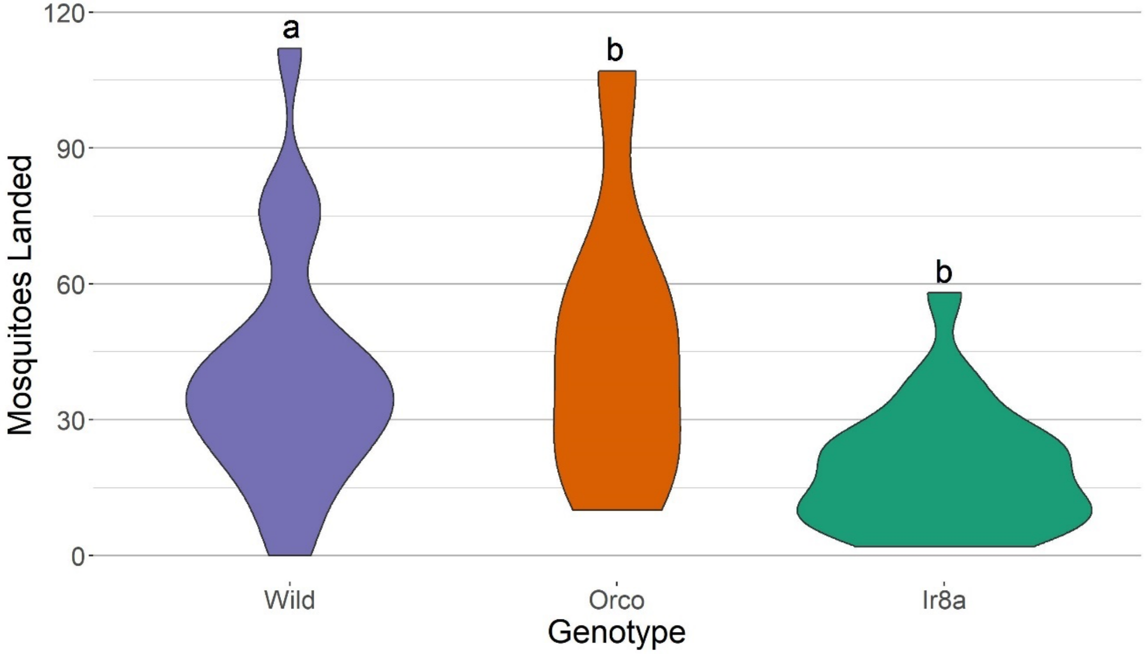

Fig. 4.1 The Total Numbers of Mosquitoes Observed Landed by Treatment per Trial

The total numbers of Wild type Orlando, *Ir8a* mutant, and *orco* mutant mosquitoes observed on the treated beads, by trial. Each trial had 12 observation points, every 30 seconds, until 6 minutes elapsed. The mosquitoes observed landed, summed across time points within each trial, were compared across the three genotypes with a Kruskal-Wallace test (P = 0.0007). The genotypes were contrasted pairwise with Wilcox tests, followed by a "holm" correction (*Ir8a*: *orco*, P = 0.0024; *Ir8a*: wild type, P = 0.0054). The width of a violin shape on the plot at a given y-axis value indicates the number of trials with that total number of landed mosquitoes summed across all time points. The bandwidth, which determines the smoothing along the y-axis, is set to 1.

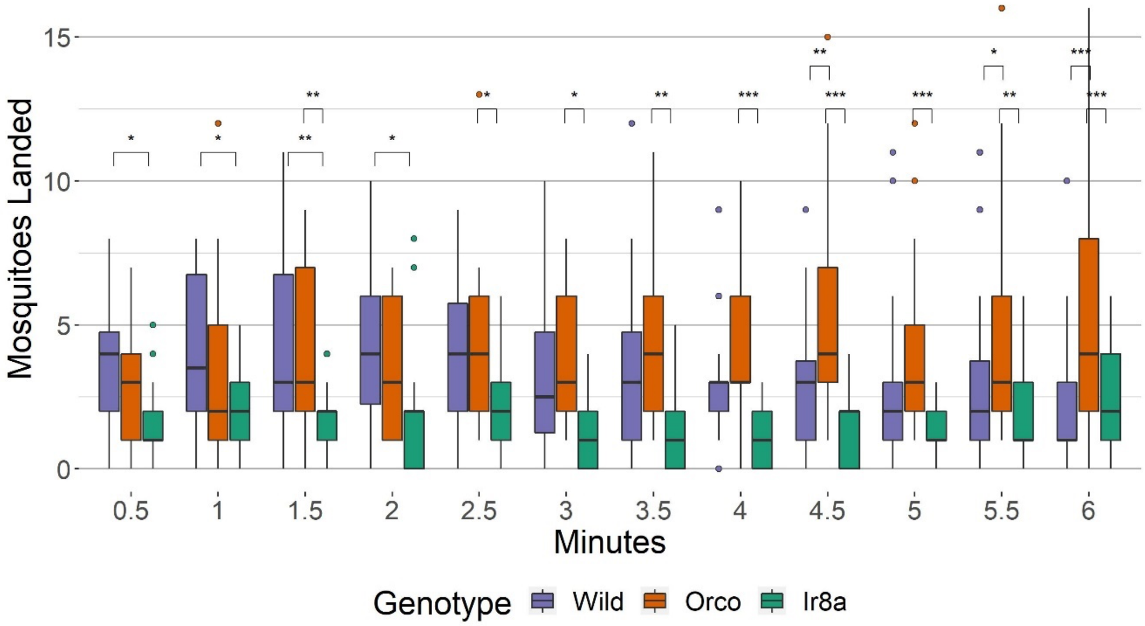

Fig. 4.2 Boxplot of Mosquitoes Landed at each Time Point by Genotype

Linear mixed model effect probability values: time P = 0.2698, genotype P < 0.0001, genotype:time P = 0.0004. Brackets show significant differences across genotypes **within** each time point, generated with pairwise estimated marginal means. The "fdr" correction was implemented across all strains and times. Lower brackets are between *Ir8a* mutants to wild type, middle brackets are between *Ir8a* mutants to *orco* mutants, and upper brackets are between *orco* mutants to wild type. (*P < 0.05, **P < 0.01, ***P < 0.001).

Mosquito Landing on Ketoglutaric Acid Alone Compared to the Blend

There were significant differences among the genotype and treatment combinations (Kruskal-Wallace test P = 0.0408). However, the pairwise Wilcox tests followed by the "holm" correction did not find a significant difference among any of the pairs. While the probability values among the wild type with ketoglutaric and lactic acids and the other three combination were smaller than among the other pairs, none were less than 0.05.

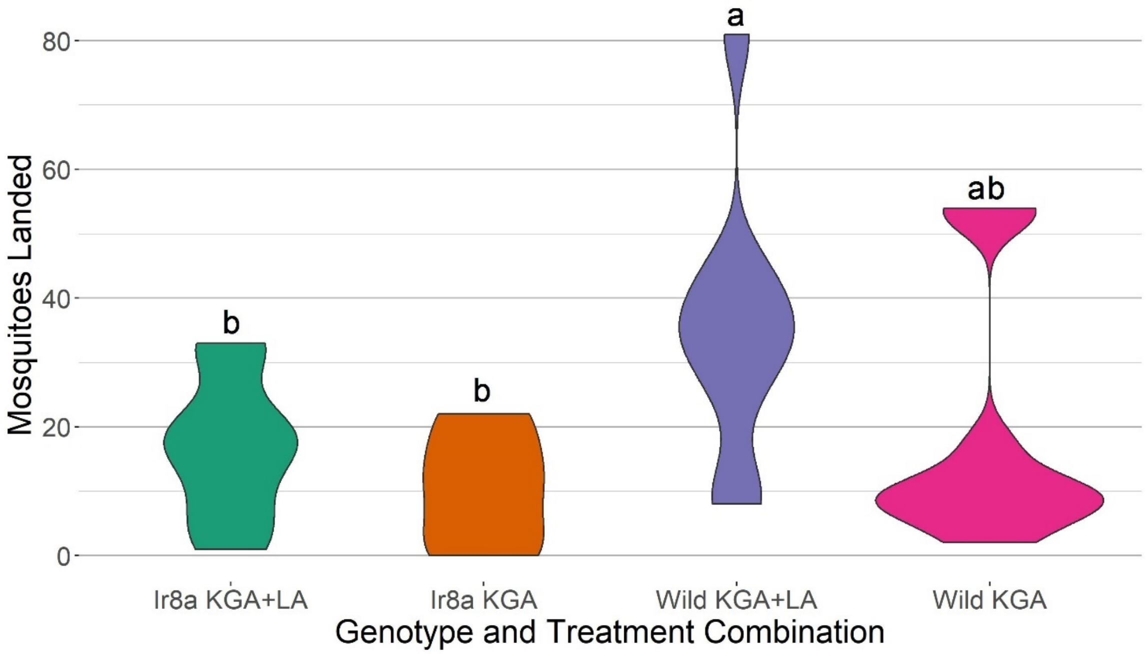

Fig. 4.3 *Ir8a* mutants and Wild Type Mosquitoes Presented with the Lactic and Ketoglutaric Acid Blend or Ketoglutaric Acid Alone

Trial totals were compared across the four genotype and treatment combinations with a Kruskal-Wallace test (P = 0.0408). Whereas, pairwise with Wilcox tests followed by a "holm" correction were not significant (*Ir8a* lactic and ketoglutaric acids: wild type lactic and ketoglutaric acids, P = 0.1510; *Ir8a* ketoglutaric acid: wild type lactic and ketoglutaric acids, P = 0.0520, wild type lactic and ketoglutaric acids: wild type ketoglutaric acids, P = 0.4063).

Discussion

Mosquito Detection of Ketoacids

The *Ir8a* mutants landed on the lactic and ketoglutaric acid blend at a lower rate relative to the wild type and *orco* mutants. However, in the first round of assays, there was no difference in the landing rate of the *Ir8a* mutants to ketoglutaric acid versus the blend of lactic and ketoglutaric acids, which is consistent with the hypothesis that *Ir8a* mutants are able to detect ketoglutaric acid but not lactic acid.

Kwon et al. (2006) showed that a sensory neuron on the labellum of *An. gambiae* detects both ketobutyric and ketovaleric acids. They termed this neuron "the small amplitude S1 ORN" because an odorant receptor was expressed within it. While not specified, we believe these detected compounds to be 2-ketobutyric and 2-ketovaleric acids. This study predates knowledge of gustatory and ionotropic receptors being necessary to detect some volatile compounds (Jones et al. 2006; Kwon et al. 2007). In contrast, Saveer et al. (2018) found that 2-ketovaleric acid is detected by a sensillum on the labellum of *An. gambiae*, but that this same receptor did not detect 2-ketobutyric acid. They found that the expression of *Ir8a* in the labellum was negligible, whereas *Ir76b* and *Ir25a* were both expressed in the labellum. Ketoglutaric acid differs from all other ketoacids tested for mosquito attraction (Carlson et al. 1973, Healy and Copland 2002, Kwon et al. 2006), in that it has two carboxyl groups. Despite this difference, it is likely that ketoacids are detected by an ionotropic receptor that requires one of the two other known ionotropic coreceptors *Ir76b* or *Ir25a*.

Younger et al. (2020) found that *Ir25a* is expressed in neurons that connect to the same glomerulus as the cpA neuron which is responsible for CO_2 detection. They also found expression of *Gr3* and *Ir25a* in some of the same neurons. This neuron is located on the maxillary palp (Kwon et al. 2007). Presenting triethylamine to *Gr3* mutants elicited a response to lactic acid like that observed in wild type mosquitoes presented with lactic acid and elevated CO_2. This suggests that the mosquitoes did not discriminate based on which receptor was stimulating the glomerulus. Tauxe et al. (2013) found that the cpA neuron responded to both CO_2 and human odor extracts. The whole human odor extracts presumably contained ketoglutaric acid (Bello and Cardé in review). As the neuron expressed *Ir25a* but not the other two IR coreceptors, it would be logical to test

Ir25a knockout mosquitoes in future studies (Younger et al. 2020). Other ketoacids are known to be detected by the labellum; nonetheless, until the appropriate tests are conducted, the maxillary palps cannot be ruled out as involved in the detection of ketoglutaric acid (Kwon et al. 2006; Saveer et al. 2018).

While the number of mosquitoes landing on ketoglutaric acid alone versus lactic and ketoglutaric acids in cage assays in this study was not significantly different, numerical differences were noted and a larger number of replicates may show differences. However, future efforts would be better spent using other methods such as electrophysiological testing of *Ir25a* and *Ir76b* knockout mosquitoes. While the *Ir8a* mutants responded to ketoglutaric acid in our cage assay, electrophysiology could determine if *Ir8a* is required for a full neuronal response to ketoglutaric acid.

Continued presence of *orco* Mutants on the Beads after the Wild Type Mosquitoes Left

Unexpectedly, the *orco* mutants were observed landed numerically less frequently than the wild type mosquitoes early in the assay and statistically significantly more frequently at later time points. The simplest explanation would be that the *orco* mutants are deficient in a way other than their odorant coreceptor expression. For example, if the mutants had limited locomotion, a delayed response would be expected. However, we believe that sufficient steps were taken to ensure the mosquitoes were not otherwise deficient. The *orco* and *Ir8a* mutants are heteroallelic mutants, in that they have two different mutations on the same allele. The production of heteroallelic mutants reduces the chance of off-target mutations being expressed, as any off-target mutations in the two parent mutant genotypes are unlikely to be for the same off-target allele (DeGennaro et al. 2013).

Heterozygous *orco* mutants, which still express *orco* but have had half of their genome modified by the same procedure as the heteroallelic mutants, showed a normal response to honey odor (DeGennaro et al. 2013). The *orco* and *Ir8a* mutants were also backcrossed with wild type Orlando mosquitoes extensively (DeGennaro et al. 2013; Raji et al. 2019). The mutant genotypes were also checked for normal locomotor activity; in that they interrupted an infrared beam aimed through a glass chamber as frequently as did wild type mosquitoes.

Another possibility is that odorant receptors are used by landed mosquitoes to determine whether that potential host is suitable, rather than for initial host finding. Some insect ORs have low affinity, in that they only detect a given odor when present at high concentrations (Kreher et al. 2008). An OR with low affinity for either lactic or ketoglutaric acids would be well suited for determining the concentrations of one of those compounds after landing. If this is the case, the *orco* mutants would not have been able to evaluate the lure. Future work with computer vision may be able to observe and score the full videos of the assays. This would allow separation of landing versus remaining landed behavior.

Possible Crowding

To test for crowding, we tested fifteen wild-type mosquitoes in a cage assay with the two-component blend. Twenty-eight (with repeated individuals) were observed landed across all time points in the trial. This is greater than the 20.1 that would be expected with a linear decline from the mean number in trials with 30 mosquitoes. This suggests moderate crowding. While the simple size was one, and we did not analyze this statistically, we would recommend future researchers consider using fewer than thirty mosquitoes per trial.

Conclusion

Our findings support that *Ir8a* mutants can detect ketoglutaric acid. They also demonstrate that ketoglutaric acid is necessary but insufficient to elicit the full rate of being landed on the beads demonstrated by the wild type mosquitoes presented with the lactic and ketoglutaric acid blend. The findings suggest a receptor, possibly one requiring *Ir25a* or *Ir76b*, is responsible for the detection of ketoglutaric acid. Determining which receptor detects this compound of newfound importance will enable improved research on and monitoring of mosquitoes. Future work with *Ir25a* and *Ir76b* knockout mutants in behavioral and electrophysiological assays will hopefully identify the receptor for ketoglutaric acid.

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CHAPTER 5: CONCLUSION

Overview

Aedes aegypti carry several human pathogens (Reed et al. 1900; Bhat et al. 2013) and specialize in feeding on humans (Scott et al. 1993), which makes them effective at transmitting those pathogens (MacDonald 1952). Despite the public health problems stemming from mosquito-borne disease and behavioral research dating back to 1910 (Howlett 1910), we still do not fully understand how mosquitoes find hosts.

In chapter two, we sought to define the parameters of *Ae. aegypti* orientation to an odor source. Not knowing which specific chemicals mosquitoes use, we presented them with crude human skin odor. We found that *Ae. aegypti* will land on a source of human skin odor, even when presented on a visually homogenous background. This finding challenges the preeminence of direct visual (telotactic) host seeking in the day biting mosquito *Ae. aegypti* (van Breugel et al. 2015).

Mosquitoes in fly upwind to following odor plumes from potential hosts. Many insects fly upwind to follow pheromone plumes generated by potential mates. The basic principles of moth pheromone-plume following are understood; however, pheromone communication within a species is an example of a co-evolved signal. The host of a mosquito is not selected evolutionarily to produce cues to attract the mosquito, unlike many female moths which must produce a pheromone signal to mate. While numerous insect pheromones have been identified, the definitive blend of human odors that mosquitoes use to find us is not known.

Human odor is a set of volatile compounds with no evolutionary pressure in favor of mosquito attraction. Unlike moth pheromones in which a few compounds may be released, human skin odor is comprised of many compounds. So far, this has hampered efforts to distinguish which of these hundreds of compounds mosquitoes use to find human hosts. Bello and Cardé (in review) verified the importance of ketoglutaric acid in combination with lactic acid as an *Ae. aegypti* “attractant” and elicitor of landing. They used classical chemical ecology, with a cage assay similar to that used in chapter four, to guide fractionation of human skin odor extracts. After they determined that a blend of ketoglutaric and lactic acids elicited landing in their cage assay, we decided to determine if these compounds would elicit takeoff, upwind flight toward the source, and landing on the odor source in a wind tunnel. This fuller set of behaviors is more relevant to natural mosquito host seeking. We found that the blend of these compounds does elicit this full suite of behaviors in a wind tunnel.

The use of homing endonucleases enables the production of mosquitoes that are missing one gene required for the function of a receptor but that are otherwise functional mosquitoes. These knockout mosquitoes can be tested in bioassays just like their wild counterparts. For example, Raji et al. (2019) knocked out *Ir8a* from *Ae. aegypti*. This receptor, *Ir8a,* is a coreceptor required by many ionotropic receptors. They found that the elimination of *Ir8a* stopped detection of and behavioral response to lactic acid. Because the mosquitoes are otherwise normal, we were able to test the same genotype mosquitoes in a cage assay.

We decided to test these knockout mosquitoes in our cage assay with the blend of lactic and ketoglutaric acids. As *Ir8a* is required for the detection of lactic acid (and other acidic volatiles), we hypothesized that it would be required for the detection of ketoglutaric acid as well. To our surprise, the *Ir8a* knockouts showed a landing response consistent with ketoglutaric acid detection but not detection of lactic acid. Thus, *Ir8a* is apparently not involved in ketoglutaric acid detection. Although the results in chapter four have not fully elucidated how mosquitoes detect ketoglutaric acid, they have demarcated what needs to be done. The following sections review potential follow-up studies delineated by chapter.

Chapter Two

In chapter two we found, *Ae. aegypti* can find and land on a patch of odor in a visually homogenous background. Other blood-feeding insects should be tested for this capability. The first insect of interest would be the night biting *Anopheles coluzzii* because, like *Ae. aegypti*, they are an anthropophilic mosquito. However, they encounter their hosts while the hosts are asleep and under dimmer light (Hawkes and Gibson 2016).

Synthetic Lures

Testing of synthetic lures, such as those discussed in chapters three and four, on a visually homogenous background similar to that used to present crude human skin odor in chapter two would be interesting. The hypothesis is that these lures which elicit landings on a visually distinct set of beads would still elicit landings when presented on a visually homogenous background. Future studies would likely require large numbers of assays, as did the study in chapter two.

Leg Extension

In preliminary trials in which the experimenter manually viewed from the side, the mosquitoes appeared to extend their legs prior to landing on the visually homogenous presentation of skin odor. How they know to extend their legs prior to landing is not well understood. Landing in other insects has been studied as a telotactic visual behavior (Goodman 1960; Srinivasan and Zhang 1997; Srinivasan et al. 2000; van Bruegel and Dickinson 2012).

Chapter Three

In chapter three, while presenting a blend of lactic and ketoglutaric acids, skin odor, or clean beads in a wind tunnel to *Ae. aegypti*, we found little difference in the time from release to takeoff across all treatments. Future studies should use a mosquito-release device that is actuated in a way to eliminate the possibility that mosquitoes are being mechanically disturbed at the beginning of the assay.

The synthetic lure, containing lactic and ketoglutaric acids, in chapter three, both with and without CO_2, elicited mosquito takeoff, upwind flight, and landing. The lure should be evaluated in field traps. If we can eliminate the need for CO_2 in field traps, the logistical requirements of traps will be dramatically reduced.

As discussed in chapters one and three, the range at which host odors attract mosquitoes is not known. The lactic and ketoglutaric acids used in chapters three and four are likely the components in human skin odor responsible for a substantial component of the attractiveness of human skin odor to *Ae. aegypti*. It should now be possible to determine the ranges at which these chemicals elicit mosquito host-seeking behaviors. As mentioned at the end of chapter three, new tools could be used to examine the maximum range at which cues elicit mosquito behaviors. Fieldwork using computer vision, electric nests, and lidar, arranged in concentric rings in the manner used by Gillies with ramp traps in 1970, may yield the definitive answers for the maximum range of mosquito attraction. These techniques could be used with live hosts, CO_2 alone, and the lactic and ketoglutaric acid blend. These field methods might be cost-prohibitive; however, laboratory wind tunnel tests with a wider spread of lure doses than were tested in chapter three may shed light on this question. Real-time computer vision coupled with a chemical release device may allow future researchers to present isolated packets of natural and synthetic odors to mosquitoes that are already airborne. This could reasonably approximate the conditions far downwind from a host.

The lack of differences in flight tracks among all treatments in this experiment, even those that elicited tremendously different numbers of landings, was unexpected. The large number of comparisons may have cost statistical resolving power. Future studies of flight tracks may examine subsets of trials that meet a criterion such as having at least one landing.

Testing of Lactic and Ketoglutaric Acid in Combination with other “Attractants”

Many compounds in some bioassay settings elicit attraction of mosquitoes (reviewed by Dormont et al. 2021). Testing these compounds in combination with lactic and ketoglutaric acid would be worthwhile. Such an empirical approach may yield improvements to trap lures for mosquitoes of medical interest. Ketoglutaric acid may be necessary but insufficient by itself to elicit attraction across many mosquito species.

Octenol (oct-1-en-3-ol), thought to be a ruminant specific cue, would be a good first candidate (Takken and Kline 1989).

Probing

We observed mosquitoes sticking their proboscises onto the beads in a manner that appeared like probing. The glass beads were impenetrable which prevented further examination of this behavior that is required for pathogen transmission with our assay. Future work should examine the effects of the lactic and ketoglutaric acid blend on this behavior. To determine if the blend elicits probing with and without CO_2 or heat, a landing assay could be constructed with a conductive screen, an insulating penetrable membrane, and a conductive plate. Once the mosquitoes land on the conductive screen, they could probe through the membrane and complete a circuit by touching the plate. The use of synthetic blood with an electrical penetration graph would be useful in determining the details of mosquito feeding behavior following probing. While knowing the details of mosquito host feeding has no obvious practical applications, the behaviors of these important disease vectors should be examined.

Chapter Four

In chapter four, we showed that *Ir8a* is not needed to detect ketoglutaric acid. The next step is to find which mosquito sensory organ uses which receptors to detect ketoglutaric acid. The maxillary palps and the labellum are two likely candidates. As mentioned in the conclusion of chapter four, the maxillary palps express *Ir25a* and may be involved in the detect of ketoglutaric acid. Future electrophysiological studies with wild type and *Ir25a* knockouts should determine the role of *Ir25a* and the maxillary palps in ketoglutaric acid detection. If the maxillary palps, specifically the cpA neuron, are involved in ketoglutaric acid detection, behavioral assays that present ketoglutaric acid as though it is CO_2 would be the next step. Such an assay is described below. Should the maxillary palps and *Ir25a* be found not to be involved in detecting ketoglutaric acid, electrophysiological studies of wild type and *Ir76b* mutants should be focused on the labellum. In the unlikely event that the electrophysiological studies of the maxillary palps, labellum, and antennae do not answer these questions, behavioral studies of *Ir25a* and *Ir76b* knockouts would be able to determine if any known IRs are involved in the detection of ketoglutaric acid.

Course of Action if the Maxillary Palps Detect Ketoglutaric Acid

The first test would be analogous to that used by Webster et al. (2015) in which CO_2 was piped in through the side of a BugDorm in which a human-worn fabric was placed on the bottom. By separating the cues by 45 degrees and 19 cm Webster et al. (2015) were able to separate out the attraction to a CO_2 source directly from CO_2 "gating" landing on the skin odor. They found that *An. coluzzii* landed on the source of skin odor on the bottom of the cage, only when CO_2 was supplied from the side, and that the mosquitoes did not fly to the CO_2 source itself. The same separation was used in chapter four between CO_2 and the blend of lactic and ketoglutaric acids.

A follow-up study could be to place lactic acid-treated beads on the bottom of a metal BugDorm-sized cage and to pipe ketoglutaric acid-laden air in through the side. Sorrells et al. (2021) found that mutant *Ae. aegypti* with optically stimulated cpA neurons landed on lactic acid as frequently as wild type mosquitoes exposed to CO_2. As a result, we would expect our wild type mosquitoes to land on the lactic acid. Analogously, one could also test a heat source on the bottom of a chamber with ketoglutaric acid piped in from the side (McMeniman et al. 2014). Either of these combinations would require complete cleaning between assays, as ketoglutaric acid cannot simply be exhausted from the cage in a few minutes.

Overall Findings

We have found that *Ae. aegypti* will land on a visually indistinct source of human skin odor rather than a visually indistinct heat cue and either of those rather than a visual cue. We found that the blend of lactic and ketoglutaric acid will elicit upwind flight and landing of *Ae. aegypti* in a wind tunnel. Finally, we found that the *Ir8a* pathway is probably not required for the detection of ketoglutaric acid by *Ae. aegypti*.

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