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The Genetics of Olfactory and Visually Guided Attractive Behaviors in *Aedes aegypti* Mosquito

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

THE GENETICS OF OLFACTORY AND VISUALLY GUIDED ATTRACTIVE
BEHAVIORS IN *Aedes Aegypti* MOSQUITO

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Joshua Ibukun Raji

2019

To: Dean Michael R. Heithaus
College of Arts, Sciences, and Education

This dissertation, written by Joshua Ibukun Raji, and entitled The Genetics of Olfactory and Visually Guided Attractive Behaviors in *Aedes aegypti* Mosquito, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: September 25, 2019

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Florida International University, 2019

DEDICATION

This dissertation is dedicated to my best friend and wife, Esther. Your soothing words of encouragement, inspiration and emotional support got me through the rigor of the doctoral program. I also dedicate this dissertation to my loving parents, Chief and Mrs. Raji. Your prayers and good wishes paid off in my excellence and accomplishments during the program.

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ABSTRACT OF THE DISSERTATION

THE GENETICS OF OLFACTORY AND VISUALLY GUIDED ATTRACTIVE
BEHAVIORS IN *Aedes Aegypti* MOSQUITO

by

Joshua Ibukun Raji

Florida International University, 2019

Miami, Florida

Professor Matthew DeGennaro, Major Professor

Mosquitoes detect the presence of humans by integrating chemosensory, thermal, and visual cues. Among these, odors are crucial for mosquito host detection. Insects have evolved a diverse repertoire of receptors to detect their plant and animal hosts. Genetic analysis of these receptors in *Drosophila* has set the stage for similar studies in mosquitoes. The diversity of the cues involved in mosquito host-seeking has made designing behavioral control strategies a challenge. The sensory receptors that are most important for mosquito detection of humans can now be determined using genome editing. In this study, we uncovered the behavioral genetics of the IR8a pathway by disrupting the IR8a co-receptor in *Aedes aegypti* using CRISPR/Cas9. The *Ir8a* mutant female mosquitoes are not attracted to lactic acid, a behaviorally active component of human odor, and lack odor-evoked responses to acidic volatiles. The loss of *Ir8a* reduces mosquito attraction to humans and their odor. We show that the CO₂-detection pathway is necessary but not sufficient for IR8a to detect human odor. This study also implicates the IR8a chemosensory pathway in mosquito water seeking behavior. Female *Ae. aegypti* mutant mosquitoes with disrupted IR8a pathway show increased attraction to standing

water, a crucial resource for survival. The strong attraction to water is not mediated by a metabolic need or an activity defect. We hypothesized that *Ir8a* could be part of a neural circuit that represses water sensing. In addition, we also provide evidence that CO₂ sensitization is crucial for yellow fever mosquitoes to track a moving object and describe differences in their visual acuity when compared to *Drosophila*. The CO₂-insensitive mutant female mosquitoes exhibit impaired object tracking behavior and cannot associate CO₂ with visual cues. Similarly, CO₂ elicits increased attention to visual cues in fruit flies. Taken together, this dissertation provides the first comprehensive evidence that the *Ae. aegypti* IR8a pathway mediates attractive behavior to human hosts. It also suggests that the pathway may indirectly regulate attraction to water sources. The CO₂-sensing pathway integrates with the visual system to guide attractive behavior in *Ae. aegypti* mosquitoes. Understanding the molecular targets that drive attractive behavior will provide insights to generate new behavioral control reagents.

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LIST OF ABBREVIATIONS AND ACRONYMS

CAFE	Capillary feeder
CO ₂	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DEET	N, N-Diethyl-meta-toluamide
EAG	Electroantennography
Grs	Gustatory Receptors
IRs	Ionotropic Receptors
LH	Lateral horn
MB	Mushroom body
OBPs	Odorant-binding proteins
<i>ORCO</i>	Olfactory receptor co-receptor
ORN	Olfactory receptor neuron
ORs	Odorant receptor
PNs	Projection neurons
qPCR	Quantitative Polymerase Chain Reaction
SEZ	Suboesophageal zone
ssODN	Single stranded oligo nucleotide
TALENs	Transcription Activator-Like Effector Nucleases
<i>TRPA1</i>	Transient Receptor Potential Cation Channel Subfamily A
WBA	Wing beat amplitude
ZFNs	Zinc Finger Nucleases

CHAPTER 1: Introduction

“If you think you are too small to make a difference, try sleeping with a mosquito.”

Dalai Lama

Female mosquitoes use a combination of cues to find their vertebrate hosts and blood-feed. Their feeding behavior not only annoys us but also creates a potent pathway for disease transmission. For example, *Aedes aegypti* are vectors of viral diseases such as yellow fever, dengue, chikungunya and Zika [1, 2]. Certain mosquito species like *Ae. aegypti* and the malaria vector, *Anopheles gambiae*, have evolved a preference for humans, which makes them efficient vectors for disease transmission [3, 4]. *Aedes aegypti* mosquitoes have evolved a preference for human hosts (Anthropophily) from an ancestral subspecies that does not prefer humans (Zoophily) [5, 6]. Host discrimination requires olfactory receptor function and has been linked to specific receptors that have increased expression and odor sensitivity in anthropophilic *Ae. aegypti* [6, 7]. From these studies, it is clear that *Ae. aegypti* uses olfaction to find their human hosts. Along with olfaction, other sensory pathways are also likely to participate in the detection of humans by mosquitoes. A comprehensive understanding of the cues that attract mosquitoes to humans, the receptors that detect them, and the neural circuits they activate will provide the necessary insight to develop new strategies to disrupt host-seeking behavior. To achieve this goal, genetic tools are now available.

Our ability to understand the molecular basis of mosquito behavior has been enhanced by the recent development of genome editing tools such as CRISPR-Cas9 RNA-guided nucleases, TALE-effector Nucleases (TALENs) and Zinc Finger Nucleases (ZFNs) in *Anopheles* and *Aedes* [8]. These approaches can be employed to facilitate

targeted mutagenesis at any gene of interest to determine their contribution to host detection and blood feeding. These techniques could also be used to integrate genetic tools to map the neural circuits that enable these behaviors. Targeted mutagenesis of the olfactory receptors has been successfully performed in *Ae. aegypti* and has been linked to a reduction in host attraction [7, 9]. These techniques have already identified multiple genetic pathways that mosquitoes employ to detect their hosts, but many questions remain. The current review seeks to survey the progress made in understanding the molecular mechanism of mosquito host detection.

1.1 Human cues

Odor is a critical cue that signals the presence of a host to mosquitoes [10]. Human odor is a complex blend of chemicals [11]. Skin microbiota plays a large role in generating volatile compounds that attract mosquitoes [12]. In *Ae. aegypti* and *An. gambiae*, odors that elicit both electrophysiological and behavioral responses have been found. Among these compounds are lactic acid, ammonia, ketones, sulfides [13-15], 1-octen-3-ol [16], and carboxylic acids [17]. The odors emanating from a host are sensed via olfactory receptors, which can be found on the mosquito antennae, maxillary palps, and proboscis (Figure 1A, [18]). Exposing female mosquitoes to CO₂ induces flight takeoff and sustained flight [19]. Carbon dioxide is detected by gustatory receptors that are expressed in the capitae peg sensilla of the maxillary palp [20]. Identifying which components of the diverse set of human odor-ligands are the most salient is a key step in understanding how mosquitoes detect humans.

Heat attracts mosquitoes to their hosts at close range [21]. Mosquitoes will land on inanimate objects set at human body temperature in the presence of CO₂ [9, 22]. Electrophysiological studies showed that there is an antagonistic pair of thermosensitive neurons within the coeloconic sensilla of the *Ae. aegypti* antennal tip where one sensillum is tuned to temperature rise and the other is sensitive to cold [23]. The integration of the responses from these two sensilla has been proposed to allow mosquitoes to respond to temperature changes and host thermal cues. The response to thermal cues may depend on the background ambient temperature, which would necessitate that mosquitoes possess a mechanism for sensing thermal contrast. The *TRPA1* receptor allows mosquitoes to avoid warm objects that exceed host body temperature aiding the detection of thermal cues [24]. The sensor(s) that allow mosquitoes to detect attractive heat cues are still unknown. Ionotropic Receptors (IRs) that are temperature responsive have been found in *Drosophila* [25, 26]. Further studies are needed to identify whether these receptors or others are important for mosquitoes to detect the temperature of their hosts.

Mosquitoes are also guided by visual cues to fly towards their hosts [27]. Adult mosquitoes possess compound eyes that are sensitive to varying light intensity [27]. It has been documented that photoreceptors in night-biting mosquitoes, *An. gambiae* adjust to varying light intensity by regulating the rhodopsin levels. The photoreceptors could enhance visual sensitivity to a potential host in low light conditions [28]. Unlike *Anopheles*, visual cues are proposed to be crucial for day-biting mosquitoes including hematophagous *Aedes* and *Culex*, but little is known about visual detection of hosts. Visual cues likely play an intermediate role in host detection by integrating long-range odor plume tracking with shorter-range cues [29]. For instance, CO₂ or human odor can

increase the ability of mosquitoes to focus on visual cues by enhancing visual flight navigation to the host [30, 31]. Understanding the connection between olfactory sensitivity, flight navigation, and visual target selection will help the field identify the behavioral neural circuits that enable mosquito host-seeking.

While female mosquitoes are guided by other cues to fly towards their hosts (Figure 1B), the tastants on the skin likely promote blood feeding once they land. After landing, they soon pierce the skin and draw blood from small blood vessels [31]. The mouthparts of the female mosquito are highly specialized for blood feeding and contain sensory hair cells which help locate blood under the skin [32]. The *An. stephensi* proboscis does not only respond to taste but also detects thermal cues [22]. The transcriptome of the *Ae. aegypti* proboscis has been recently identified [33]. Genetically manipulating the chemoreceptors expressed in the proboscis could provide insight into mosquito biting behavior and possibly provide evidence for a role in host detection during flight. The contact cues on human skin and the receptors that sense them remain for the most part elusive.

Figure 1. Olfaction enables mosquitoes to detect human hosts.

(A) Image of an *Ae. aegypti* mosquito showing the sensory organs involved in host detection and blood feeding. The antenna and maxillary palp detect odors emanating from the host. The proboscis detects taste cues and may also perceive odors. The eyes sense visual features in the environment and allow for flight navigation towards the host. (Photograph courtesy of artist Alex Wild) (B) Mosquitoes detect human hosts using combination of cues. At a distance, they sense CO₂ exhaled from human's breath. As they move closer, they sense odor and heat that emanate from the host, they land on the skin where they taste with the taste receptors on their legs and mouth parts. When they bite, they can either infect the host with pathogens carried by their saliva (white dots) or become infected with the host's blood (red dots).

1.2 Multimodal integration of human cues

Genetic analysis has demonstrated that mosquitoes integrate multiple stimuli to find their hosts. One of the most striking examples is the gating of multiple cues by CO₂ [9]. *Aedes aegypti* mosquito attraction requires at least two cues. For example, neither thermal cues, nor lactic acid are attractive on their own. If CO₂ is combined with either of these cues, mosquito attraction is greatly enhanced. If sensitivity to CO₂ is lost, as in *Gr3* mutants, this synergistic effect of CO₂ does not occur. Host odor may also gate mosquito responses to hosts [7]. Mutant mosquitoes lacking the olfactory receptor co-receptor (*orco*) gene have reduced attraction to human odor, but in the presence of CO₂, there was no difference between the wild-type and *orco* mutant mosquitoes [7]. In addition, both *Gr3* and *orco* mutants respond normally to human arms in olfactometer assays [7, 9]. Taken together, these results demonstrate that mosquitoes have robust and redundant mechanisms to detect human hosts. The disruption of one pathway is unlikely to eliminate mosquito host-seeking. Employing multiple sensory pathways may increase the chance of a mosquito successfully targeting a host.

Mosquitoes may also integrate odor with taste cues in response to hosts. Riabinina et al., [34] recently documented that olfactory receptor neurons (ORN) expressing the *orco* gene project from the labella on the proboscis to the suboesophageal zone (SEZ) of the brain, and suggests that the SEZ may integrate odor and taste cues during blood feeding. This region of the insect brain has been shown to be critical for taste integration in *Drosophila* [35]. In *Ae. aegypti*, *in vivo* calcium imaging revealed the activation of some ORs by some volatile compounds in the blood and removing the function of the ORs in the stylet impairs blood feeding [31]. Clearly, the ability to integrate host sensory

cues represents an essential mechanism employed by mosquitoes to guide host detection and blood-feeding behavior.

1.3 Molecular sensors of human cues

Insects have evolved complex repertoires of chemosensory receptors to respond to their environment including: odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs) [36]. In *Drosophila*, the ORs are expressed in the dendrites of ORNs. The sensillar lymph surrounding the ORNs is densely packed with odorant-binding proteins (OBPs) that are hypothesized to be involved in odorant uptake and odor delivery to the ORs, but their role remains unclear [37]. The neural processing of olfactory information has been extensively studied in *Drosophila* [38, 39]. The axons of the ORNs project to the antennal lobe where they synapse with projection neurons (PN). In turn, PNs convey olfactory information to the mushroom body (MB) and lateral horn (LH) of the brain which subsequently leads to a behavioral response. How mosquitoes process host cue information has not been well established, but the lessons from *Drosophila* are likely to apply.

In *Drosophila*, ORs form a heteromeric complex with *orco* for their targeting to the cell membrane [40, 41]. The OR pathway plays a critical role in mosquito preference for human hosts and DEET repellency [7, 42]. The ORs from *An. gambiae* have been comprehensively deorphanized using the *Drosophila* empty neuron system [43]. These ORs tested seemed to be narrowly tuned to several odor components that emanate from humans such as 1-octen-3-ol (present in human breath), 2,3-butanedione (by-product of metabolized sweat) and indole (human sweat volatile) [43]. The narrow tuning of these ORs may serve to improve cue salience [44].

The IRs are expressed in the dendrites of the ORNs innervating the coeloconic sensilla [45]. There are at least two IR co-receptors, *Ir8a* and *Ir25a*, and possibly a third, *Ir76b* [45, 46]. These co-receptors form an odor-responsive ion channel complex with odor-selective IRs. *Drosophila* IRs respond primarily to amines and acids while ORs respond to esters and alcohols [47]. Beyond olfaction, *Drosophila* IRs play a role in taste [48, 49], moisture and temperature sensing [25, 26], and possibly in the auditory system [50]. Although GRs are usually involved in taste [35], specialized GRs have been implicated in other sensory modalities in insects including light sensing [51], warmth sensing [52], and CO₂ detection [53]. Excluding the Grs that detect CO₂, it remains unknown whether the orthologues of these receptor genes respond to similar stimuli in mosquitoes [9].

1.4 Behavioral vector control strategies

Given that olfaction is key for mosquitoes to detect their human hosts, identifying new odors that modulate mosquito olfactory receptor function is a promising approach to combatting mosquito-borne disease [54, 55] (Fig. 2). The discovery of novel odors can be accomplished using high-throughput screening assays to identify synthetic and natural compounds that activate receptors associated with repellency or inhibit receptors associated with attraction [56]. This strategy can help develop novel spatial mosquito repellents or create odor baits for traps that reduce mosquito populations. Our current understanding of mosquito host detection makes it likely that effective odor-baited traps would need to include multiple cues such as CO₂ and heat to recreate the multimodal sensory experience that drives mosquitoes to their hosts. Next generation mosquito repellents could block multiple chemosensory pathways to render the human host

“invisible”. As an alternative, new repellents that overstimulate a specific chemosensory pathway could cause mosquitoes to avoid humans. To accomplish this important goal, we will need to know the specific receptors that enable mosquito attraction and repellency.

1.5 Summary

We have reviewed what is known about the multiple cues that attract mosquitoes to humans and their molecular sensors. Host-seeking is not completely abolished even when either the CO₂-sensing or OR pathways were disrupted in *Ae. aegypti* [7, 9]. The redundancy clearly shows that multiple cues must be considered to understand how mosquitoes find their human hosts. Our current knowledge is not sufficient to develop new behavioral control strategies but sets the stage for further studies. To efficiently develop these new strategies, we need to have a comprehensive understanding of how mosquitoes detect their human hosts from the perception of cues at the periphery, to the integration of the information in the central nervous system, and finally, to the motor circuits that drive the behavior. Genome editing tools such as CRISPR-Cas9 and other genetic manipulations such as using the GAL4-UAS system to mark or manipulate neural circuits could play a significant role in addressing how the mosquito’s brain responds to human cues [57]. We have learned so much from the genetic analysis of *Drosophila* behavior. Using similar approaches to understand mosquito behavior may provide the mechanistic insight to break the cycle of mosquito-borne disease transmission.

Figure 2. Designing novel mosquito attractants and repellents.

Novel vector control measures can be designed after identifying the salient cues that mosquitoes use to find their hosts and the receptors that perceive them. (A) Cartoons of single sensillum recordings of olfactory receptor neuron (ORN) responses to novel odor ligands. New volatile compounds that activate (green) or inhibit (red) the activity of the ORNs could be used to control mosquito behavior or reduce mosquito populations. (B) Push-pull strategies for mosquito behavioral control. Mosquitoes can be led to a trap containing odors that attract them (pull). Odors that either mask or constitutively activate olfactory receptor function could cause mosquitoes to avoid humans (mask/push).

1.6 Dissertation objectives and organization

Chapter 1 reviews the progress made in understanding the molecular mechanism underlying host detection in mosquitoes. Current understanding of the salient cues that attract mosquitoes, the receptors used to detect these cues were also reviewed. Chapter 1 also suggests ways forward for novel olfaction-based vector control strategies. This chapter is a reprint of a previously published article in *Current Opinion in Insect Science*. Chapter 2 is a comprehensive genetic and behavioral analysis of genes crucial for mosquito host detection. Our study is the first to implicate a new family of olfactory receptors, the ionotropic receptors as a key driver for mosquito attraction to human odor. Chapter 2 is also a reprint of the published article in *Current Biology*.

In Chapter 3, we explored the role of the Ionotropic co-receptor, IR8a, in mosquito water-seeking behavior. On the basis of our data, *Ir8a* mutants are strongly attracted to water. It is known that water is a crucial resource for mosquitoes to lay their eggs, further examination of the role of the IR8a pathway will provide insight into novel ways of controlling mosquito population. Chapter has been published in *Communicative and Integrative Biology*. Chapter 4 examines the aspect of mosquito vision and how it integrates with odor cues to enhance host seeking process. The study was carried out on *Aedes aegypti* mosquitoes and compared to a non-anthropophilic distantly related fruit fly. Chapter 4 manuscript is currently being written up and intended to be published soon. All the chapters are summarized in chapter 5. Future directions and research gaps in the studies are also discussed.

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CHAPTER 2: *Aedes aegypti* mosquitoes detect acidic volatiles found in human odor using the IR8a pathway

“If only mosquitoes sucked fat instead of blood, then we could sit outside and eat burger”

Anonymous

2.1 Abstract

Mosquitoes use olfaction as a primary means of detecting their hosts. Previously, the functional ablation of a family of *Aedes aegypti* olfactory receptors, the Odorant Receptors (ORs), was not sufficient to reduce host-seeking in the presence of carbon dioxide (CO₂). This suggests the olfactory receptors that remain, such as the Ionotropic Receptors (IRs), could play a significant role in host detection. To test this, we disrupted the *Ir8a* co-receptor in *Ae. aegypti* using CRISPR/Cas9. We found that *Ir8a* mutant female mosquitoes are not attracted to lactic acid, a behaviorally active component of human sweat, and lack odor-evoked responses to acidic volatiles. The loss of *Ir8a* reduces mosquito attraction to humans and their odor. We show that the CO₂-detection pathway is necessary but not sufficient for IR8a to detect human odor. Our study reveals that the IR8a pathway is crucial for an anthropophilic vector mosquito to effectively seek hosts.

2.2 Introduction

Anthropophilic female mosquitoes, such as *Aedes aegypti* and *Anopheles gambiae*, have a strong innate drive to find their human hosts and obtain blood meals, which are required for egg production [1]. As has been shown by the recent Zika outbreak, *Aedes* mosquitoes are efficient vectors for pathogen transmission because of their host-seeking behavior and susceptibility for infection by flaviviruses [2,3]. Female

mosquitoes, like other haematophagous Diptera, integrate an array of sensory information to find their human hosts including carbon dioxide (CO₂), body odor, heat, moisture, and visual cues [4-6]. How these different cues are sensed to enable mosquito host-seeking has only begun to be understood [7]. Among these attractive cues, human body odor is a complex blend of volatile chemicals that distinguishes us from other vertebrate hosts [8-12]. Skin microbiota plays a large role in generating the volatile compounds that attract mosquitoes to human sweat [13-15]. In *Ae. aegypti* and *An. gambiae*, human odors that elicit both electrophysiological and behavioral responses have been identified such as ammonia, amines, carboxylic acids, lactic acid, ketones, sulfides, and 1-octen-3-ol [16-22]. The odors emanating from a host are sensed via olfactory receptors expressed in the mosquito antennae, maxillary palps, and proboscis [23,24].

Insects respond to volatile chemicals in the environment with a complex repertoire of olfactory receptors that are evolutionarily distinct from vertebrate olfactory receptors [25-27]. Two families of odor-gated ion channels have been identified in insects that respond to a diverse set of molecules, the odorant receptors (ORs) and the ionotropic receptors (IRs) [28-31]. In addition, there are also gustatory receptors (GRs) that are highly sensitive to CO₂ [32-34] and whose activity can be altered by other odors [35,36]. Odor-tuned ORs rely upon the obligate olfactory co-receptor Orco to form an odor-gated ion channel complex [37-41]. In *Ae. aegypti*, loss of *orco* results in a loss of electrophysiological responses to some but not all odorants [42]. In the presence of CO₂, attraction to human odor was not significantly different from wild-type controls in female *Ae. aegypti orco* mutants. However, in the absence of CO₂, *orco* mutants lose strong attraction to human odor. *Ae. aegypti* mutants lacking *Gr3*, a subunit of the heteromeric

CO₂ receptor complex, show no electrophysiological or behavioral responses to CO₂ [43]. Behavioral analysis of *Gr3* mutants showed that CO₂ can gate multiple cues that are sensed by mosquitoes including heat and human odor.

Drosophila IRs are expressed in olfactory sensory neurons (OSNs) that are distinct from OR and GR lineages [44]. There are at least two IR co-receptors including IR8a and IR25a, and a putative third, IR76b [30,45]. These co-receptors form an odor-responsive ion channel with other odor-tuned IRs. For example, some *Drosophila* odor-tuned IRs require IR8a as a co-receptor, while some other odor-tuned IRs form a functional complex with IR25a and/or IR76b, but not all the three co-receptors. Similarly, some OSNs have been shown to express both IR8a and IR25a protein [45]. There are 30 putative odor-tuned IRs expressed in the *Ae. aegypti* antennae that could potentially form an odor-responsive ion channel with any of the IR co-receptors [24]. The combinatorial pattern of expression observed in IRs may allow flexibility to respond to more diverse olfactory cues with fewer odor-tuned receptors than the Orco pathway [45], which encompasses at least 117 odor-tuned OR [46].

Insect IRs have been reported to detect amines, aldehydes, ketones, and acids [22,30,44,47,48]. As many of these compounds are not represented in the OR chemical space, which includes alcohols and esters, the IR odor-ligands are largely complementary and not overlapping with OR odor-ligands [44,49,50]. In *Drosophila*, *Ir25a* and *Ir76b* are necessary for odor-evoked electrophysiological responses to amines [51], whereas receptor neurons expressing *Ir8a* are tuned to volatile acids [45]. IR8a forms a functional complex with AgIR75k to elicit odor-evoked inward currents in response to carboxylic acids including heptanoic acid, octanoic acid and nonanoic acid [22]. Lactic acid is

enriched in human skin emanations compared to other vertebrates and may be one of the cues that signals to the mosquito that the target host is a human [52,53]. How lactic acid is sensed in mosquitoes is unknown, but it is likely to be IR-dependent.

Whereas *Drosophila Ir25a* is involved in many functions, including olfaction, taste, hygrosensation, thermosensation, and attraction to CO₂ [30,45,51,54-62], *Ir8a* appears to function exclusively in detecting odors and is not necessary for attractive responses to CO₂ [30,45,47,48,62]. In *Ae. aegypti*, *Ir8a* expression is localized to the antenna and cannot be detected in other chemosensory tissues, whereas *Ir25a* is broadly expressed in multiple chemosensory tissues and three-fold more abundant in sugar fed female mosquito antennae than *Ir8a* [24]. Similarly, *Ir8a* transcript abundance was detected only in the antenna of *An. gambiae* adults and larvae [23,63,64]. These results make *Ir8a* a likely candidate receptor for odor detection during mosquito host-seeking behavior. *Ir25a* and *Ir76b* have broader expression patterns that are consistent with these receptors being involved in other sensory modalities in addition to olfaction [24,45,51,54].

Since host-seeking is not completely ablated in *orco* or *Gr3* mutants [42,43], we reasoned that the IR olfactory receptors retained in these mutants are crucial for host-seeking. Here, we used the CRISPR/Cas9 system to disrupt *Ae. aegypti Ir8a*. We test the relative contribution of *Ir8a* in human odor detection and its genetic interaction with other olfactory receptor pathways that have been previously implicated in *Ae. aegypti* host-seeking. We find that *Ir8a* mutants are not attracted to lactic acid, a behaviorally active component of human odor, or able to detect acidic components of human odor. In membrane blood-feeding assays, *Ir8a* mutants have reduced responses to human odor,

but not heat and CO₂, when compared to wild-type controls. *Ir8a* mutants are also less responsive to humans and human odor than wild-type controls in uniport olfactometer assays. The genetic interactions of *Ir8a* and *orco* as well as *Ir8a* and *Gr3* suggest a crucial role for CO₂ to sensitize mosquitoes to human odor and highlights the importance of human acidic volatile detection during mosquito host-seeking.

2.3 Materials and methods

2.3.1 Experimental model and subject details

All research was conducted in compliance with the NIH guidelines and the Florida International University Environmental Health and Safety guideline. Laboratory practices, facilities and equipment were reviewed and approved by the Florida International University Institutional Biosafety Committee (IBC-18-004) and Institutional Review Board (IRB-16-0388 & IRB-16-0386-CR02). Informed consent was obtained from human subject volunteers before their participation in this study.

Aedes aegypti mosquitoes were reared and maintained at 25-28°C, 75% relative humidity under a 14:10 light-dark cycle (lights on at 8 am). All mosquitoes used in these experiments were generated from the Orlando laboratory strain. Mosquito eggs were hatched in deionized, deoxygenated water containing dissolved tablets of Tetramin tropical fish food (catalog #16152, Tetra, Melle, Germany), which served as food for the emerged larvae. Adult mosquitoes were given *ad libitum* access to 10% sucrose solution. About 1 to 2-week-old adult females were fed on defibrinated sheep blood to generate eggs. Before behavioral assays, 5 to 7 day old sugar-fed mosquitoes were sorted and sexed under hypothermic (4°C) conditions and fasted for up to 24 hours on water. All mosquitoes were tested only once in the behavioral assays and then sacrificed.

A total of 18 human subject volunteers participated in the host-seeking experiments (Table S2). However, three subjects were later excluded because they failed either, to complete all the trials or their body odor was not attractive to wild-type mosquitoes. Subject was tested twice to assess their attractiveness to mosquitoes. The volunteers were diverse in age (19 to 41 years), sex (Male = 7, Female = 8), and race (White = 5, Hispanic = 7, Asian = 1, Black = 2).

2.3.2 CRISPR/Cas9 nuclease reagents

CRISPR short guided RNAs (sgRNA) were designed according to standard protocols [43]. The sgRNAs sequences for the mutagenesis of exon 2 and exon 3 of *Ir8a* gene were chosen by the presence of protospacer-adjacent motifs (PAMs) with the sequence NGG. Generated sgRNA sequences were checked for potential off-target binding sites using Zifit (<http://zifit.partners.org/ZiFiT/>). Double-stranded DNA templates for specific sgRNAs were produced by performing a template-free PCR with two overlapping oligonucleotides. One containing the specific target sequences (*Ir8a^{dsRED}* exon2: GGGCGGACAAAATGGCGTAT and *Ir8a^{attP}* exon 3: GGACATCTGTCGACGATAAC), and the universal CRISPR reverse primer (Table S1). The forward primers used were IR8aExon2CRISPRF and IR8aExon3CRISPRF. The reverse primer used was sgRNArev. Both sgRNAs were produced using MEGAscript T7 Transcription Kit (Catalog #AM1334, Life Technologies, Carlsbad, CA, USA). Following incubation, sgRNA transcripts were purified using MEGAclean Transcription Clean-Up Kit (Catalog #AM1908, Life Technologies) and verified on the Agilent bioanalyzer (Agilent, Santa Clara, CA, USA).

The Cas9 polyadenylated mRNA was made by digesting the MLM3613 plasmid [53] with the PmeI restriction enzyme (New England Biolabs, Ipswich, MA, USA), miniprep purifying the linearized plasmid, and *in vitro* transcribing the mRNA from the DNA fragment using the mMessage mMachine T7 ultra kit (Catalog #AM1345, Thermo Fisher Scientific). The mRNA transcript was purified using MEGAclean Transcription Clean-Up Kit (Catalog #AM1908, Life Technologies) and checked on the Agilent bioanalyzer (Agilent). MLM3613 was a gift from Keith Joung (Addgene plasmid #42251; <http://n2t.net/addgene:42251>; RRID: Addgene_42251).

The single stranded oligo nucleotide (ssODN) used to integrate *attP* recombination site sequences into exon 3 of *Ir8a* was synthesized by Integrated DNA Technologies (www.idtdna.com) using the 20 nmole ultramer service with standard desalting. The ssODN contains a 50bp *attP* site flanked by 75bp of homologous sequences to the target site on both sides:

GATTCTCGGTTCTGGATGCCTGACGGCAGTCGAATGTTACGATACAATTTGAA
CGTTTCGATTTGGACATCTGTCCTACGCCCCCAACTGAGAGAACTCAAAGG
TTACCCCAAGTTGGGGCACTACAACGGGTCTGCTGCAATACACCTTGGGACG
ATCGAGAAGGGTGATGTGGTACCGTTCGTGGGTCAGAAGATCAAA

(*attP* sequence in bold).

To generate the donor DNA to integrate into exon 2 of *IR8a*, the pSL1180:polyUBdsRED [54] was modified to include homologous sequences surrounding the CRISPR target site. The 796bp left arm and 1005bp right arms were amplified from Orlando strain *Ae. aegypti* genomic DNA using Novagen KOD polymerase (EMD Millipore, Temecula, CA, USA).

The following primers were used to amplify the exon: infusionIR8LA_1, infusionIR8LA_2, infusion_IR8RA1, and infusion_IR8RA2 (Table S1).

After the PCR fragments were amplified and purified with Qiaquick PCR purification kit (Catalog #28106, Qiagen, Hilgen, Germany), pSL 1180:polyUBdsRED was digested with both EcoRI and XhoI (New England Biolabs). The 3253bp fragment (pSL 1180 backbone) and 2321bp fragment (polyubiquitin:dsRED:SV40) were both gel purified. The left homologous arm PCR, polyubiquitin:dsRED fragment, the right homologous arm PCR, and psl1180 backbone were simultaneously assembled using a recombination based method, Infusion HD (Catalog #638909, Clontech). The resulting DNA was cut with the MluI restriction enzyme (New England Biolabs) and Sanger sequenced to confirm the proper integration of the fragments. The final plasmid DNA, pGT-Ir8a, was purified from *E. coli* using the Qiagen Endo-free Maxiprep DNA (Catalog #12362, Qiagen) isolation kit.

2.3.3. *Ir8a*^{dsRED} and *Ir8a*^{attP} mutant allele generation and detection

To generate stable germline mutations, CRISPR-Cas9 reagents were injected into the posterior end of the pre-blastoderm embryos. This stage allows nuclei that will become somatic and germline cells to be exposed to the CRISPR-Cas9 complex since the dividing nuclei have not yet undergone cytokinesis. Microinjection into *Ae. aegypti* pre-blastoderm embryos was performed at the Insect Transformation Facility at the University of Maryland. The microinjection mixes were prepared as follows: for *Ir8a*^{dsRED}, sgRNA (25 ng/μl), Cas9 mRNA (500 ng/μl), and donor plasmid (500 ng/μl) and, for *Ir8a*^{attP}, sgRNA (25 ng/μl), Cas9 mRNA (500 ng/μl), and ssODN (500 ng/μl) were diluted into nuclease-free H₂O.

A total of 1,069 and 509 embryos were injected to target exon 2 and exon 3, respectively.

Embryos were hatched 3 days after injection and reared to the adult stage as previously described [42]. A total of 50 G₀ females were sexed during pupation, allowed to mate freely with wild-type Orlando males, and females were blood-fed to generate G₁ progeny. G₀ females were put into oviposition vials to collect G₁ eggs. Thereafter, G₁ eggs were hatched and pupae were screened for *Ir8a*^{dsRED} insertion allele using a fluorescent microscope. Fluorescent G₁ individuals were sexed and reared to adulthood. Fluorescent females were outcrossed with wild-type animals for 5 generations and homozygosed. The dsRED fluorescent protein gene insertion was detected in the *Ir8a* locus by PCR. Mosquito genomic DNA was purified using DNeasy Blood & Tissue kits (Catalog #69504, Qiagen). PCR amplification was done using the following primers *Ir8a*^{dsRED}ForLA3 and Ir8aexon4rev3 (Table 1).

To further confirm site specific integration of the pGT-Ir8a donor DNA into *Ir8a* exon 2 and sequence the integration site, a 3483bp PCR product was amplified using Novagen KOD polymerase (Catalog #71086-5, EMD Millipore) with the forward primer (*Ir8a*^{dsRED}ForLA3) homologous to the first 20bp of *Ir8a* exon 2, which is also contained in the left arm of the donor plasmid, and the reverse primer (Ir8aexon4rev3) in *Ir8a* exon 4 outside the boundary of the right arm of the donor plasmid. This PCR product was then purified using NucleoSpin Gel and PCR cleanup kit (Catalog #740609, Machery-Nagel Inc., Bethlehem, PA, USA), cloned using the NEB PCR cloning kit (Catalog #E1202S, New England Biolabs) and Sanger sequenced using the SimpleSeq premixed Kit (Eurofins Genomics) with the following primers in Table S1.

Sequencing showed directed insertion into *Ir8a* exon 2 with no aberrant insertions or deletions.

Ir8a^{attP} mutant lines were screened for *attP* insertion and detected by fluorescent size-based genotyping each generation. Pupae were sexed and allowed to eclose in same-sex cages. Virgin G₂ females were allowed to mate freely with introduced wild-type males 24 hours after eclosion. Mosquitoes were then outcrossed to wild type in this manner for 5 generations. Single-pair crosses of G₅ siblings were set up and mosquitoes with the desired mutations were chosen for homozygosing. Females were blood-fed and placed in individual oviposition vials. After eggs were laid, females were sacrificed, and genomic DNA was extracted using the IBI Genomic DNA extraction kit (Catalog #IB47222, IBI Scientific, Peosta, IA, USA). A fluorescent amplicon was generated using Amplitaq Gold 360 PCR master mix (Catalog #4398881, Applied Biosystems, Foster City, CA, USA) for each female using the following primers: *Ir8aExon3for1* and *Ir8aExon3rev2* (Table 1). Samples were sent for capillary electrophoresis to the DNA Core Lab at Florida International University and were then genotyped using Peak Scanner software (v1.0, Applied Biosystems) to determine the fragment length.

To confirm the single stranded donor DNA insertion in the *Ir8a^{attP}* allele, PCR products were amplified from mosquitoes that contained the *Ir8a^{attP}* allele and were PCR purified using QIAEX II Gel Extraction Kit (Catalog #20051, Qiagen). After purification, the amplicons were ligated into the pCR2.1 TOPO TA vector (Catalog #K462040, Invitrogen, Carlsbad, CA, USA) and transformed with One Shot TOP10 chemically competent *E. coli* cells (Catalog #C607003, Invitrogen). Thereafter, colonies were picked and prepped using the QIAprep Spin Miniprep Kit (Catalog #27104, Qiagen).

The plasmid DNA was sequenced in the DNA Core facility lab at the Florida International University.

In order to determine where *Ir8a* is expressed in adult female mosquitoes, we dissected five different body parts including the whole body, head without antennae, antennae alone, head alone, and headless body from sugar-fed 7–10 days old wild-type *Ae. aegypti* of the Orlando strain. A total of 10 mosquitoes were dissected for each body part except for the antennae, where a total of 25 antennal pairs were used for the assay. In addition, we also dissected antennal tissues from *Ir8a^{dsRED/dsRED}*, *Ir8a^{attP/dsRED}*, *Ir8a^{dsRED/+}*, *Ir8a^{attP/+}*, and wild-type *Ae. aegypti* Orlando strain to determine if *Ir8a* mRNA expression was altered by the mutations we made. For this assay, a total of 10 mosquito heads were used for each genotype. Using a pair of forceps, samples were dissected under cold anesthesia into RNAlater stabilization solution (Catalog #AM7020, Invitrogen) or into a dry ice/ethanol bath. For each genotype or tissue type, at least three biological replicates were used.

2.3.4 *Ir8a* mRNA extraction and treatment

Mosquito tissues were suspended in a 1 ml solution containing 4 M guanidine thiocyanate (CAS: 593-84-0), 0.5% Sarkosyl (CAS: 137-16-6), Chloroform (CAS 67-66-3), and 0.1 M of 2-mercapthoethanol (CAS: 60-24-2). Thereafter, tissue samples were manually homogenized using RNase-free disposable pellet pestles (Catalog #12-141-364, Thermo Fisher Scientific). Phenol-chloroform phase separation was performed to separate the supernatant. This phase extraction process was repeated twice for the supernatant. RNA extraction was performed using the RNAid Kit supplied by MPBio (catalog #111007200).

Beads were washed twice using RNA Wash concentrate before eluting in 20µl DEPC-treated water. Sample concentration and quality were determined using NanoDrop 2000c (Thermo Fisher Scientific).

2.3.5 cDNA synthesis and qPCR

The RNA extracted was treated with Turbo DNA-free kit (Catalog #AM1907, Invitrogen) to remove any DNA contamination. RNA was diluted to 50 ng/ul for each sample. cDNA library was created with reverse transcriptase using the SuperScript II RT reagent kit (Catalog # 18080-051, Invitrogen) and primed using oligo dT. Each reaction comprises 200ng RNA to make up a final volume of 20µl. After cDNA synthesis, samples were treated with RNase cocktail enzyme mix (Catalog #AM2286, Thermo Fisher Scientific). cDNA samples were amplified using Amplitaq Gold 360 master mix (Catalog #4398901, Fisher Scientific) and primed by ribosomal protein L32 gene. Genomic contamination and PCR amplification was assessed by running the sample on an agarose gel electrophoresis.

Ae. aegypti Ir8a mRNA expression was quantified using RT-qPCR (Catalog #4345241, Thermo Fisher Scientific). Quantitative PCR was performed using TaqMan gene expression assay. The reaction consists of two sequence-specific PCR primers with a custom TaqMan probe (Catalog #4331348, Thermo Fisher Scientific) in 2X universal master mix. The TaqMan assay was performed with three technical replicates for each given biological replicate. The real-time PCR was performed using the TaqMan universal PCR master mix (Catalog #4324018, Thermo Fisher Scientific). The forward primer sequence (ATCAGTCCGATCGCTATGACAAG) and the reverse primer sequence (GGTTGTCAATACCTTTCGGCTTAC) were used as control primers (Table 1).

Raw data was analyzed using SDS v1.5.1 software with the detection threshold set at 0.2. Outliers with Ct values greater by 0.5 from the nearest technical replicate were discarded. The ribosomal protein L32 gene was used as endogenous control to normalize between cDNA samples. Relative fold change was calculated as previously described [55].

2.3.6 Behavior assays

Mosquito activity was monitored using Locomotor Activity Monitors (LAM 25; Trikinetics Inc., Waltham, MA, USA). Sugar-fed 5 to 7-day old female mosquitoes were singly aspirated into glass tubes (25 mm diameter, 125 mm long) directly from the cage without cold anesthesia. The glass tubes were sealed at both ends with cotton plugs. One of the cotton plugs was soaked in water and the other end was left dry. This was done in order to generate more activity in the tube. We hypothesized that mosquitoes would hygrotax to the wet end. The set up was maintained at 25-28°C and 75% relative humidity under a 14:10 light-dark cycle (lights on at 8 am). The movements of the mosquitoes triggered an infrared beam break. This was set to record at every second and tabulated into 1min bins. Bins with more than 60 beam breaks and trials that exceed 2000 beam breaks per day were excluded from the analysis. Activity count was recorded for 24 hours on the fourth full day of fasting.

The capillary feeder (CAFE) assay was adapted for mosquitoes with slight modifications from the feeding study carried out on fruit flies [45, 46]. Female mosquitoes aged 5 to 7 days were starved from sugar for 24 hours but had unlimited access to water. After fasting, five mosquitoes were transferred into each polystyrene vial (95mm long, 27mm wide) and sealed with a cotton plug. Thereafter, two calibrated 5 μ l capillary tubes (catalog #1904637, Blaubrand Intramark) were introduced into the vial.

The capillary tubes were filled with 10% sucrose to the 5 μ l mark. The assay lasted for 18 hours and feeding was estimated by recording the change in the sucrose volume. Control vials without mosquitoes were also set up to correct for water loss due to evaporation.

The fasting resistance assay was carried out accordingly as previously described [21]. A total of sixty male and sixty female mosquitoes from each of the five genotypes (*Ir8a^{dsRED/dsRED}*, *Ir8a^{attP/attP}*, *Ir8a^{dsRED/+}*, *Ir8a^{attP/+}* and wild-type) were tested for fasting resistance. Prior to the experiment, mosquitoes were fed on 10% sugar. Thereafter, each mosquito was aspirated into a fly vial (25 mm diameter, 95 mm long) containing 1 cotton ball soaked in 10 ml of distilled water and plugged with a cellulose acetate fly vial plug (Catalog # 49-101, Genesee Scientific, San Diego, CA, USA). In order to control for positional effects, vials were randomized for genotype and sex in the racks. Experiments took place in the behavior room at 25-28°C, 75% relative humidity under a 14:10 light-dark cycle (lights on at 8 am). To quantify fasting resistance, visual examination of the vials was carried out each day to observe for any movement. If no movement was noticed, the vial was tapped twice and visually inspected again. If the mosquito failed to move, it was scored as dead.

Electroantennogram (EAG) recordings were made using Ag-AgCl electrodes and glass capillaries filled with Ringer solution (8.0 g L⁻¹ NaCl 0.4 g L⁻¹ CaCl₂) connected to silver wire, which closed the electric circuit. Non-blood fed female mosquitoes (*Ae. aegypti*) were recorded at 3-4 days post-eclosion between 8am and 4pm. Mosquitoes were chilled for one minute before their bodies were secured between sticky tape and wax. The glass capillary connected to the indifferent electrode was placed within the eye of the mosquito.

The glass capillary connected to the recording electrode was connected to the tip of the antennae. The signals were passed through a high impedance amplifier (IDAC-4, Syntech 2004, Hilversum, Netherlands) and analyzed using a customized software package (Syntech EAG-Pro 4.6). Ten microliter aliquots of each chemical compound at a concentration of 1×10^{-3} mg/mL were added onto a pre-cut filter paper (Whatman No. 1, 20 mm), which was inserted into sterilized Pasteur pipette. The stimuli were delivered via an air stream at a flow rate of $1 \text{ L} \cdot \text{min}^{-1}$ with a puff (2 seconds duration) at 30 second intervals. Solvent control (Hexane) was tested at the beginning and end of each repetition. Each treatment contained 5 replicates.

The odor volatiles tested in the EAG experiment include: Geranylacetone (C.A.S. 689-67-8), 6-methyl-5-hepten-2-one (Sulcatone, C.A.S. 110-93-0), Linalool (C.A.S. 78-70-6), Dodecanal (C.A.S. 112-54-9), Lactic acid (C.A.S. 79-33-4) and were obtained from Sigma-Aldrich. Octanoic acid (C.A.S.124-07-2), Heptanoic acid (C.A.S. 111-14-8) and Butyric acid (C.A.S. 107-92-6) were supplied by ICN. Octanal (C.A.S. 124-13-0) and Nonanal (C.A.S. 124-19-6) were obtained from Chemicon:Acros. Limonene (C.A.S. 5989-27-5) and Nonanoic acid (C.A.S. 112-05-0) were supplied by Fluka. 2-ethyl hexanol (C.A.S. 104-76-7) was obtained from Lancaster; while 1-octen-3-ol (C.A.S. 3391-86-4) was supplied by Janssen Chimica.

Membrane-feeding assay was carried out as previously described with slight modifications [22]. A total of four 14 mm diameter glass-jacketed membrane feeders (#CG-1835-70, Chemglass) were connected in series using silicone tubing to a digital water bath (VWR international). The membrane feeder was prepared by stretching a thin layer of Parafilm M laboratory film (Catalog #PM996, Bemis) over the feeders.

Thereafter, a total of 1000 μ l of defibrinated whole sheep blood without any added ATP (Hemostat Laboratories) was transferred into each feeder. A dimension of 10 cm by 10 cm squares of nylon sleeves (Duane Reade), previously worn for 12 hours by a human subject, were perforated and stretched over the Parafilm and tied around the feeder. For each trial, 4 cups of mosquitoes (n=18-20) were set into their feeding positions in a room with regulated temperature (25°C) and relative humidity (75%). The assay was supplemented with CO₂ diffusion pads (8.0 cm by 11.5 cm; Tritech Research), placed at the edge of the cup, and set to release at a rate of 2500-2700ppm measured by a carbon dioxide monitor (Catalog # CO₂-100, Amprobe, Everett, WA). The blood in the membrane feeder was heated to 37°C to mimic human body temperature. For assays without heated blood, the temperature was maintained at room temperature (26°C). Feeding positions were alternated to control for position effects on the genotypes tested. The assay had duration of 15 minutes. Blood fed mosquitoes were visually scored and any mosquitoes that appeared not to have blood-fed were squashed between paper towels. If blood was found on the paper after squashing, the animal was scored as blood-fed.

A custom-built uniport olfactometer constructed by the Engineering Department at Florida International University was built to assess female mosquito attraction to human host stimuli. The uniport olfactometer is made of a large plexiglass tube (75cm long and 13cm wide) attached to a small cylindrical trap (13cm long and diameter is 5cm wide) which houses the mosquitoes before the experiment. The other end of the plexiglass tube is a hollow box (length is 25cm, breadth is 20cm and diameter is 13cm) connected to the stimulus chamber. Carbon-filtered, humidified air and CO₂ are able to mix with the odorants in the stimulus chamber to attract mosquitoes.

The CO₂ release rate in the stimulus chamber was measured by an acrylic flowmeter Model VFA-4-SSV (Dwyer Instruments Inc., IN, USA) set at 3 SCFH. The final concentration for CO₂ in the assay was maintained at 2500-2700ppm by a carbon dioxide monitor (Catalog # CO₂-100, Amprobe). Whereas, air flow rate was set at 21 standard cubic feet per hour by an air flowmeter (King Instruments CA, USA). The sealed design of the uniport, air filtration, and the positive pressure caused by the airflow in the apparatus isolated the assay from potential odors in the surrounding environment.

For each trial, approximately 20 female *Ae. aegypti* mosquitoes one-week post-eclosion were sorted under cold anesthesia (4°C) and placed in a small cylindrical trap. All females used in the assay had access to their mating partners but had not obtained a blood meal. Females were fasted with access to water for up to 24 hours prior to the assay. Pre-assay fasting and behavior experiments took place at 25°C and 70-80% relative humidity. In our experiments, we tested multiple genotypes including wild-type, heterozygous *Ir8a* mutant (*Ir8a^{dsRED}/+*), homozygous *Ir8a* mutant (*Ir8a^{dsRED/dsRED}*), heteroallelic *Ir8a* mutant (*Ir8a^{attP/dsRED}*), homozygous *orco* mutant (*orco^{16/16}*), heteroallelic *orco* mutant (*orco^{5/16}*), homozygous *Ir8a* and *orco* double mutants (*Ir8a^{dsRED}, orco¹⁶*), homozygous *Gr3* mutant (*Gr3^{cfp/cfp}*), heterozygous *Gr3* mutant (*Gr3^{cfp/+}*), and homozygous *Ir8a* and *Gr3* double mutants (*Ir8a^{dsRED}, Gr3^{cfp}*). Heteroallelic mutants were tested to control for background mutations that may have occurred independently in each line. Double mutants were tested to assess the epistatic interaction between the two genes.

To explore the behavior of these mosquito lines, starved mosquitoes were released from the small cylindrical trap and allowed to respond to the stimuli. After 8 mins, the number of mosquitoes attracted by host cues was counted. The 8 mins time point was determined empirically to produce consistently high responses. A blank trial with no odor stimulus was run to ascertain that the set-up was clean. Attraction greater than 20% indicates the presence of residual odor and the whole set up was cleaned up again with an odorless soap and allowed to air dry. Mosquitoes were scored as attracted if they were able to fly upwind through the tube into the attraction trap within the time frame. Mosquitoes that move out of the cylindrical trap within the assay period were scored as activated.

A total of 15 human subjects were recruited for the uniport assay. Each subject was tested twice to assess his or her attractiveness to mosquitoes in a uniport olfactometer as described above. All subjects were given informed consent before participating in the experiment and approved by the Florida International University Institutional Review Board. The volunteers were diverse in age (19 to 41 years), gender (Male=7, Female=8), and race (White [5], Hispanic [7], Asian [1], Black [2]). All of the human subjects were asked not to wear scented cosmetics, deodorants, or fragrances on the day of the assay. Any subject that did not follow our request was excluded from the study. Human subjects that adhered to this rule were asked to insert their forearm up to the elbow level into the stimulus chamber. The arm was inserted through a tight-fitted glove affixed to the olfactometer to prevent airflow between the assay and the room. Mosquitoes bit none of the human volunteers during the experiment.

The toe sections of women's knee-high pantyhose (Hanes Brands Inc., NC, USA) were cut off with scissors. A single volunteer subject was used for this assay to control for differential attractiveness. The subject wore the nylon sleeves on the arm and stretched it towards the armpit. This was worn overnight for 12 hours while the subject did not shower or apply any scented products. The nylon sleeves were later retrieved from both arms and immediately tested on the library of mutants already generated, as well as the wild type mosquitoes in the uniport olfactometer. New nylon sleeves perfumed with human odor were used for each day of testing without prior storage. Nylon sleeves that had not been previously worn were used as a negative control.

In order to test the response of wild-type and *Ir8a* mutant mosquitoes to lactic acid, we used the set up for the uniport olfactometer assay with slight modifications. Approximately 30 mosquitoes were used for each trial. A total of 4ml L-(+)- lactic acid solution (C.A.S. 79-33-4, catalog number 27714; 88%-92%, Sigma-Aldrich) was transferred by a pipette directly into the open lid of a polystyrene Petri dish (60mm diameter, 10mm height). The treatment was centrally placed in the stimulus port. For the control, the Petri dish was left blank. The number of mosquitoes attracted was recorded after 10 min. CO₂ concentration (2500ppm -2700ppm) was monitored by a carbon dioxide meter (Catalog # CO₂-100, Amprobe).

Carbon dioxide monitor (Amprobe CO₂- 100, Everett, WA) was used to measure CO₂ release rate in a custom-built uniport olfactometer. In this experiment, air flow rate was maintained at 21 standard cubic feet per hour (SCFH) as measured by an airflow meter (King Instruments CA, USA). The final CO₂ concentration in the assay was measured by placing the CO₂ monitor where the trapped mosquitoes were held.

For the assay with air alone, carbon-filtered humidified air was released into the stimulus port. For the assay with supplemented CO₂, an acrylic flowmeter Model VFA-4-SSV (Dwyer Instruments Inc., IN, USA) set at 3 SCFH was used to control CO₂ release rate into the stimulus chamber of the uniport. For the assay with a human arm, the arm of the subject was inserted through a tight-fitted glove affixed to the olfactometer to prevent airflow between the assay and the room. The sealed design of the uniport and the positive pressure caused by the airflow in the apparatus isolate the assay from potential odors in the surrounding environment. The stimuli (airflow, supplemented CO₂ and skin-emanated CO₂) traveled from the stimulus port into the large plexiglass tube (75cm long and 13cm wide) and exited via the aperture where the mosquitoes were released. The CO₂ final concentration was measured via the aperture where the mosquitoes were released.

A total of ten trials were carried out to measure the CO₂ release rate from the olfactometer. The device was turned on and allowed to calibrate to ambient CO₂ room concentration. For each measurement, the device was placed in the aperture and measurements were recorded when the digital recording was stabilized. The device recording was stable when the beep sound was consistent. After taking the recording, the device was removed from the aperture and allowed to calibrate to ambient room condition. This process was repeated consistently for the ten trials. Carbon dioxide release rates were measured in three different conditions: humidified air, humidified air and CO₂, and humidified air supplemented with CO₂ and human arm. Data was analyzed using one-way ANOVA. Conditions marked with different letters were significantly different according to post hoc Tukey's HSD test.

2.3.7 Quantification and statistical analysis

Statistical analysis of our behavioral and quantitative PCR data sets was performed using the GraphPad Prism 7 software package (GraphPad Software, San Diego, CA, USA). Statistical analysis of our electrophysiological recordings (EAGs) was performed using GraphPad Prism 8 software package (GraphPad Software, San Diego, CA, USA). All details for statistical analysis including the statistical tests used, number of trials (n), number of animals, and how significance was determined can be found in the figure legends.

2.4 Results

2.4.1 Targeted mutagenesis of *Ae. aegypti* *Ir8a*, an antenna specific ionotropic receptor

Previous analysis of the neurotranscriptome of *Ae. aegypti* has suggested that *Ir8a* is expressed in the antenna and no other chemosensory tissues [24]. To confirm this result, we first asked if *Ir8a* expression can be detected in any other body tissues. Using quantitative RT-PCR analysis in wild-type mosquitoes, we compared the expression of the intact female to the head plus antenna, antenna, body minus the head, and head minus antenna. We show that *Ir8a* mRNA expression is enriched in the antenna and nearly undetectable in other tissues (Figure S1A). Given that olfaction is key for mosquitoes to host seek [6], and *Ir8a* acts as an obligate olfactory co-receptor that can complex with odor-tuned IRs that detect acids in *Drosophila* [45,48], we speculated that mutations in *Ae. aegypti* *Ir8a* should disrupt a distinct subset of IRs that are responsive to acidic volatiles in human odor.

To test this hypothesis, we generated targeted null mutations in the *Ae. aegypti* *Ir8a* gene using CRISPR/Cas9 RNA-guided gene editing [65]. We integrated two distinct donor DNAs to generate two independent alleles in the *Ir8a* locus at exon 2 and exon 3 (see methods). These two independent mutant alleles, *Ir8a*^{dsRED} (exon 2) and *Ir8a*^{attP} (exon 3), are predicted to produce truncated IR8a proteins that would eliminate *Ir8a* function. The sgRNA was designed to guide the Cas9 endonuclease to exon 2 of *Ir8a* and enable integration of the polyubiquitin promoter and dsRED fluorescent protein through homology dependent repair by way of a plasmid containing the promoter, fluorescent marker and SV40 terminator flanked by homologous DNA sequences surrounding the predicted cut site (Figure 3A). This insertion visually marked *Ir8a*^{dsRED} mutants with dsRED fluorescence and could be detected in the *Ir8a* locus by PCR using a primer that anneals to sequences in the donor construct homologous right arm in *Ir8a* exon 2 and a second primer that anneals outside the bounds of the donor construct in *Ir8a* exon 4 (Figure 3B & C, Table 1).

Sanger sequencing of the PCR amplicon showed site-directed integration of the donor plasmid into the *Ir8a* locus as expected (Table 1). To generate the *Ir8a*^{attP} allele, an sgRNA, *Cas9* mRNA, and a single stranded DNA oligo containing a 50 bp attP PhiC31 recombination site [66] flanked by 75 bp of homologous sequence surrounding the CRISPR binding site were injected into preblastoderm embryos. However, we observed a 17bp deletion in the CRISPR site and 2bp deletion in the attP site which may reduce PhiC31-mediated recombination frequency at this site. *Ir8a*^{attP} sequencing data suggests that it was a null mutation, as the predicted mRNA from the *Ir8a*^{attP} allele would contain 20 stop codons.

To reduce the possibility of off-target effects, both alleles were outcrossed to the wild-type Orlando laboratory strain for five generations and then homozygosed.

We next investigated if *Ir8a* mRNA is disrupted in *Ir8a* mutants. Using quantitative RT-PCR, *Ir8a* mRNA in the homozygous *Ir8a^{attP/attP}* mutants was nearly undetectable in both sexes (Figure S1B & C). This suggests that the mRNA is degraded by non-sense mediated decay and that the *Ir8a^{attP/attP}* mutant is an RNA null. However, the *Ir8a^{dsRED}* allele showed over-expression of *Ir8a* mRNA in male and female mutants (Figure S1B & C). The increase in *Ir8a* transcript levels observed in the *Ir8a^{dsRED}* allele is likely the result of the insertion of the polyubiquitin promoter dsRed fluorescent protein expression cassette into the locus. The predicted *Ir8a^{dsRED}* transcript cannot produce a full length IR8a protein. No behavioral phenotypic differences were found between the mosquitoes carrying these alleles suggesting that both *Ir8a^{attP}* and the *Ir8a^{dsRED}* alleles are null.

Figure 3. Mutagenesis of the *Ae. aegypti* *Ir8a* locus, and behavioral phenotypic analysis of *Ir8a* mutant response to lactic acid. (A) CRISPR sgRNAs designed to target the Cas9 nuclease to exon 2 and exon 3 of *Ir8a*. Integration location of the dsRED fluorescent marker and ssODN containing *attP* site into exon 2 and exon 3 of *Ir8a* respectively. (B) dsRED fluorescence in the *Ir8a*^{dsRED/dsRED} homozygous mutant (left) and wild-type (right) adult female mosquitoes. (C) PCR amplification from *Ir8a* exon 2 locus, note 1162bp amplicon from the wild-type *Ir8a* locus versus the larger 3483bp amplicon from *Ir8a*^{dsRED/dsRED} homozygous mutant female mosquitoes. (D) Uniport olfactometer assay designed to test behavioral response of *Ir8a* mutants to lactic acid. Mosquitoes that fail to leave the trap during the assay are scored as unactivated, whereas mosquitoes that leave the trap but remain in the main tunnel are scored as activated. Mosquitoes in the trap adjacent to the stimulus chamber are scored as attracted. (E) Percent female mosquitoes attracted to no odor (blank) and CO₂ alone (CO₂) as well as CO₂ and lactic acid in a uniport olfactometer. Lactic acid and CO₂ were co-presented to wild-type, *Ir8a*^{dsRED/dsRED}, and *Ir8a*^{attP/dsRED} female mosquitoes (right three panels). Trials with different letters are significantly different ($p < 0.0001$, $n=8-10$). Analysis was done using One-Way ANOVA with post hoc Tukey's HSD test. See also Figure S1 & S2, Table S1

A

B

Figure S1: Quantification of *Ir8a* mRNA expression. Related to Figure 3.

(A) Quantitative RT-PCR analysis of *Ir8a* mRNA expression in wild-type female mosquito tissues. Bar plots represent the mean and standard error. Samples marked with asterisks are significantly different from an intact female by Mann-Whitney U test ($P < 0.0001$). (B) Relative fold change in mRNA expression normalized to wild-type males ($p < 0.0001$) and (C) females *Ae. aegypti* mosquitoes ($p < 0.0001$). Bar plots represent the mean and standard error. Data was analyzed by Mann-Whitney U test. Genotypes marked with asterisks are significantly different from wild-type controls.

A concern with the application of CRISPR/Cas9 mutagenesis is off target mutations which may alter the function of other genes [67]. To rule out non-specific behavioral defects in the *Ir8a* mutants, we assessed their fitness [42,68,69]. Using a locomotor activity assay, we found no difference in activity in *Ir8a* mutants when compared to the wild-type and heterozygous controls in the number of times they move past an infrared beam (Figure S2A & B) [42]. We further investigated if *Ir8a* mutants may have feeding defects using the capillary feeder assay (CAFE) (Figure S2C) [68,69]. In this assay, mosquitoes were allowed to feed through a calibrated capillary tube containing 10% sucrose solution. We quantified sucrose consumption by recording the change in volume relative to control vials without mosquitoes. We recorded no difference in feeding in *Ir8a* mutants when compared to the wild-type and heterozygous controls after 18 hours of *ad libitum* feeding, (Figure S2D). We also evaluated the ability of the *Ir8a* mutants to survive fasting. This was tested by sugar-starving mosquitoes with access to water [42]. Our results suggest that our *Ir8a* mutant males and females can resist starvation as well as wild-type and heterozygous controls. (Figure S2E & F). Taken together, these studies attest to the fitness of *Ir8a* mutants as well as their suitability for further behavioral analysis.

2.4.2 IR8a pathway is required for sensing and responding behaviorally to acidic volatiles

Lactic acid has been previously identified to be a behaviorally active component of human sweat that has been used to lure mosquitoes into traps or attract mosquitoes in olfactometer assays, but olfactory receptors for the compound have not been identified [16,19,22,70].

Studies have shown that attraction to lactic acid in *Ae. aegypti*, *An. gambiae*, and *Culex quinquefasciatus* can be enhanced in the presence of CO₂ [43,53,71,72]. Neither lactic acid nor CO₂ is by itself a strong attractant [16]. However, both lactic acid and CO₂ can synergize when presented together to elicit attraction in wild-type mosquitoes. Using a uniport olfactometer with carbon-filtered airflow, we tested the responses of wild-type and *Ir8a* mutant female mosquitoes to filtered air, CO₂, as well as lactic acid and CO₂ (Figure 3D & E). In order to control for recessive background mutations that may have occurred in either of the two lines, we tested *Ir8a*^{dsRED/dsRED} and heteroallelic *Ir8a* mutants (*Ir8a*^{attP/dsRED}). The responses of *Ir8a*^{dsRED/dsRED} and *Ir8a*^{attP/dsRED} mutants to lactic acid and CO₂ were not significantly different from each other or the wild-type responses to CO₂ alone or filtered air (blank) (Figure 3E).

To confirm that *Ir8a* mutants have lost the ability to detect lactic acid, we examined the electrophysiological responses of female mosquitoes to a panel of odors. Unlike *Drosophila*, the olfactory receptor expression in mosquitoes has not been comprehensively mapped [73,74]. The location of IR8a expressing olfactory receptor neurons in the antenna is unknown making single sensillum recordings difficult to perform. To overcome this limitation, we used electroantennogram (EAG) measurements (Figure 4), which record the average signal output from the entire mosquito antenna for a given odor volatile [75].

The wild-type mosquitoes showed robust odor-evoked responses to all the odor panels tested (Figure 4A). However, *Ir8a*^{dsRED/dsRED} mutants are insensitive to the acidic components of human odor represented in the panel including lactic acid (Figure 4B). We observed a similar lack of olfactory sensitivity in *Ir8a*^{attP/dsRED} heteroallelic mutants (Figure 4C). As shown on the representative current traces (Figure 4D), *Ir8a*^{dsRED/dsRED} and *Ir8a*^{attP/dsRED} olfactory receptor neurons show weak responses to acidic volatiles similar to the solvent response of wild-type controls.

Our results show that *Ir8a* mutants have lost their behavioral and electrophysiological responses to lactic acid, and strongly suggests that IR8a is required to detect lactic acid. This was true of both the *Ir8a*^{dsRED} allele and the heteroallelic combination of the *Ir8a*^{dsRED} allele and the RNA-null *Ir8a*^{attP} allele, suggesting that both alleles retain no *Ir8a* gene function. In our study, *Ir8a* mutants are still able to detect other human volatile compounds including some alcohols, aldehydes and ketones suggesting that other olfactory pathways are not impaired in these mutants.

A **B** **C** **D**

Figure S2. Assessing locomotor activity, survival, and sugar-feeding behavior in *Ir8a* mutants. Related to Figure 1.

(A) Diagram of beam break assay to monitor mosquito locomotor activity. (B) Average daily locomotor activity of *Ir8a* mutants after 4 days of fasting measured by the number of infrared beam breaks (counts). On the dot plot, long lines represent the mean and short lines represent standard error. There were no statistical differences among genotypes ($p = 0.6224$, $n=12-13$). (C) Diagram of Capillary Feeder (CAFÉ) assay to quantify feeding behavior in mosquitoes. (D) Cumulative sucrose consumption after 18 hours of sugar feeding ($p=0.9411$, $n=25$). On the dot plot, long lines represent the mean and short lines represent standard error. Data was analyzed by one-way ANOVA, and genotypes marked with the same letters are not significantly different by post hoc Tukey's HSD test. (E) Percent survival of 300 females under sugar starvation (F) Survival of 300 males under sugar starvation. Data was analyzed using log rank test and Geahand-Wilcoxon test followed by pairwise log rank comparisons with Bonferroni correction (corrected significance threshold; $p<0.001$). Using this test, *Ir8a*^{attP/attP} males lived significantly longer than wild-type and *Ir8a*^{dsRED/+}. Whereas, *Ir8a*^{attP/attP} female mosquitoes lived significantly longer than wild-type, *Ir8a*^{dsRED/+}, and *Ir8a*^{dsRED/dsRED} mosquitoes. There was no difference for any other pair of curves.

Figure 4. IR8a olfactory receptor pathway is required for sensing acidic volatiles that are component of human odor. (A) Electroantennogram (EAG) responses of wild-type Orlando strain, (B) *Ir8a^{dsRED/dsRED}* mutants, and (C) heteroallelic *Ir8a^{dsRED/attP}* to volatiles that are components of human odor. (D) Representative EAG traces of the wild-type and *Ir8a* mutant mosquitoes. This figure is represented by a mean-max box and whisker plot. The ends of the box are the upper and lower quartiles. The median is marked by a horizontal line inside the box. The whiskers are the two lines outside the box that extend to the highest and lowest EAG response. The odors marked in red are acidic volatiles while the odors represented in blue include alcohols, ketones, aldehydes, monoterpenoid and alkene. EAG responses represented in orange dots are significantly different from solvent control. Responses labelled in grey are not significantly different from the solvent control. Statistical analysis was done using a mixed-effects model with Dunnett's multiple comparison test. Each column was compared with the mean of the solvent control column. EAG responses marked with asterisks indicate that it is significantly different from response to the control solvent (* = $p < 0.01$; ** = $p < 0.001$; *** = $p < 0.0001$; **** = $p < 0.00001$).

2.4.3. *Ae. aegypti* IR8a pathway responds to human odor cues during blood-feeding

Using a membrane blood-feeding assay (Figure 5A), we assessed the responses of *Ir8a* mutant and control female mosquitoes to heat, CO₂, and human odor cues, by determining the percentage of females that would blood-feed [43]. While CO₂ and human odor can activate and elicit mosquito attraction towards a blood source, the temperature of the meal is crucial for feeding to occur [76]. Experiments carried out with wild-type mosquitoes showed a robust feeding response when all cues were present, but feeding was reduced when human odor, CO₂, or heat was removed (Figure 5B).

In the presences of all three cues, we found that both the *Ir8a*^{dsRED/dsRED} and the heteroallelic *Ir8a*^{attP/dsRED} mutants exhibited reduced blood-feeding when compared to wild-type or heterozygous controls (Figure 5C). We attempted to determine the individual contributions of human odor, CO₂, and heat cues in the assay to the *Ir8a* mutant phenotype by eliminating one cue at a time. When only heated blood and CO₂ were used as attractants there was no significant difference among genotypes (Figure 5D). This suggests that *Ir8a* mutant females can respond similarly to these cues as wild-type females. When the CO₂ source was removed from the assay, the rate of blood-feeding was very low across all the genotypes (Figure 5E). Similarly, when the temperature of the blood was shifted from body temperature (37°C) to ambient temperature (26°C), the absence of the heat cue nearly eliminated blood-feeding across all genotypes (Figure 5F). The level of blood-feeding was very low in the absence of heated blood or CO₂, so it is difficult to discern genotypic differences. By comparing the results from assays that contain heated blood and CO₂ (Figure 5C & D), one can hypothesize that the defect found in *Ir8a* mutants is due to human odor detection.

Figure 5. *Ae. aegypti* IR8a pathway responds to human odor cues during blood-feeding (A) Illustration showing the membrane feeding assay to simulate female mosquito blood-feeding behavior. (B) Percent wild-type female mosquitoes responding to varying sensory cues ($n = 5$; $p < 0.0001$). (C) Percent female mosquitoes of indicated genotypes blood-feeding in the presences of CO₂, human odor and heat cues ($p < 0.0001$; $n = 7-10$). (D) Percent female mosquitoes of indicated genotypes blood-feeding in the presences of CO₂ and heat cues, without human odor ($n = 7$; $p = 0.468$). (E) Percent female mosquitoes of indicated genotypes blood-feeding in the presences of human odor and heat cues, without added CO₂ ($n = 5 - 6$; $p = 0.855$). (F) Percent female mosquitoes of indicated genotypes blood-feeding in the presences of human odor and CO₂ cues, without heat cue ($n = 6$; $p = 0.944$). For the dot plots in B-F, long lines represent the mean and short lines represent standard error. Statistical analysis was done using One-Way ANOVA with post hoc Tukey's HSD test. Genotypes marked with different letters are significantly different. Human odor was collected from subject 1. See Table 2.

2.4.4 *Ae. aegypti* IR8a pathway is required to detect humans and human odor

We subsequently investigated if the electrophysiological and behavioral defects recorded in *Ir8a* mutants could translate into impaired responses to human hosts. Using a uniport olfactometer with carbon-filtered airflow and added CO₂, we examined the attraction rate of olfactory receptor mutants to 15 different human subjects (Figure 6A, Table 1). Each subject was tested twice with wild-type (+/+), heterozygous (*Ir8a*^{dsRED/+}), *Ir8a* mutant (*Ir8a*^{dsRED/dsRED}), heteroallelic *Ir8a* mutant (*Ir8a*^{attP/dsRED}), *orco* mutant (*orco*^{16/16}), heteroallelic *orco* mutant (*orco*^{5/16}) and double mutant (*Ir8a*^{dsRED/dsRED}, *orco*^{16/16}) female mosquitoes. In these assays, wild-type mosquitoes and *orco* mutants showed robust attraction. In contrast, attraction to humans was significantly impaired in *Ir8a* mutants (Figure 6B). Differences in attraction were significant for both genotype and the 15 human subjects tested (two-way ANOVA, $P < 0.0001$ for genotype and $P = 0.0095$ for subject). The statistical difference in mosquito attraction to individual human subjects reflects the differences in attractive cues that emanate from each subject. Surprisingly, the additional loss of *orco* in the double mutants did not significantly enhance the *Ir8a* human detection defect in this assay (Figure 6B). This suggests the existence of other olfactory receptors that can respond to human odor since the double mutant still retains some attraction.

We next asked if the loss of attraction was dependent on olfactory cues from human hosts or on other cues emanating from live humans such as body heat, moisture, or visual cues. To test this, we excluded these other cues by trapping human odor on nylon sleeves previously worn on a human subject for 12 hours and exposing the scented sleeves to mosquitoes in our uniport assay (Figure 6D). In order to ascertain how long the

scented sleeves could continue to elicit attraction, we tested the responses of wild-type mosquitoes. Even after 7 trials, which take approximately 85 minutes to perform, we found no significant difference in attraction rate (Figure S3). Thus, a given scented nylon sleeve was never used for more than 7 consecutive trials. Nevertheless, we randomly tested all genotypes to control for any potential bias linked to when human odor was presented.

Robust attraction was recorded in the wild-type, heterozygous controls, and *orco* mutants when the human-scented sleeve was simultaneously presented with CO₂. Consistent with the study carried out with human subjects, *Ir8a* mutants show deficits in detecting nylon sleeves perfumed with human odor in these assays (Figure 6E). The weak attraction in the mutants cannot be explained by a defect in locomotion or overall fitness of the *Ir8a* mutants (Figure S2A-F). This suggests that impairment in sensitivity to *Ir8a*-dependent odor-ligands presented by humans is responsible for the behavioral defect seen in the uniport olfactometer assays with human subjects.

2.4.5 CO₂ differentially modulates the IR8a and Orco pathways

Several studies have shown that CO₂ can activate mosquito flight activity and facilitate host detection [77-81]. We asked if CO₂ activation could modulate the attraction rate of *Ir8a* mutants by testing the attraction of the mutants to a human subject without including added CO₂ in the assay. In contrast to uniport assays that included CO₂ (Figure 6B & E), we recorded a host-seeking deficit in *orco* mutants in the absence of added CO₂ that was not statistically different than *Ir8a* mutants (Figure 6C & F). This supports an earlier study which reported that CO₂ synergizes with human odor to rescue host-seeking defects in *orco* mutants [42]. Similar to what was observed with a human host when CO₂

is not included in the assay, both *Ir8a* and *orco* single mutants lose strong attraction to nylon sleeves perfumed with human odor compared to wild-type controls (Figure 6C & F). In contrast to *orco* mutants, *Ir8a* mutants' response to human odor was not changed by the presence or absence of CO₂ (Figure S4A). We found no difference in CO₂ levels in the uniport when airflow is co-presented with a human arm or not (Figure S4B). Passing airflow over a human arm in the presence of CO₂ did not increase CO₂ levels versus airflow and CO₂ alone (Figure S4B).

Similar to what was observed in figure C, attraction to human scented nylon sleeves is impaired in *Ir8a*^{dsRED}, *orco*¹⁶ double mutants when CO₂ is absent (Figure 6F). As the genetic interaction between *Ir8a* and *orco* is dependent on CO₂ sensation, we hypothesize that CO₂ can activate additional olfactory receptor pathways to integrate the response to host odor cues in the absence of the OR pathway. Unlike in *orco* mutants, the response of *Ir8a* mutants to human odor is not modulated by CO₂ (Figure 6C & S4A). This suggests that CO₂ sensitization and host odor detection by other olfactory receptor pathways is not sufficient to rescue the *Ir8a* host-seeking defect. To explore this difference, we tested the genetic interaction between *Ir8a* and *Gr3* mutants.

Figure 6. *Ae. aegypti* IR8a pathway is required to detect humans and human odor. (A) Illustration showing mosquito attraction to a human arm in a uniport olfactometer. (B) Dot plot of percent female mosquitoes of indicated genotypes attracted to 15 human subjects in the presence of CO₂. Each subject was tested twice. An analysis was done using two-way ANOVA to compare the mean difference in attraction between subjects ($n = 30$, $p < 0.0001$) and genotypes ($n = 30$, $p < 0.0095$). Human subjects (1-15) were differentiated on the plots by dot color in this figure (bottom panel). (C) Percent female mosquitoes of indicated genotypes attracted to a human arm in the absence of CO₂ ($p < 0.0001$; $n = 8 - 10$). (D) Illustration showing mosquito attraction to a human scented nylon sleeve in a uniport olfactometer. (E) Percent female mosquitoes of indicated genotypes attracted to human odor trapped on nylon sleeve in the presence of CO₂. Data was analyzed using one-way ANOVA ($n = 15$, $p = 0.0001$). (F) Percent female mosquitoes of indicated genotypes attracted to human odor trapped on nylon sleeve in the absence of CO₂ ($p = 0.0001$; $n = 15$). For the dot plots, long lines represent the mean and short lines represent standard error. All data above was analyzed using One-Way ANOVA. Genotypes marked with different letters are significantly different by post hoc Tukey's HSD test. Human subject 1 was used in the experiments in panels C, E, & F. See also Figure S3 & S4 as well as Table S2.

Figure S3: Time course experiment showing mosquito attraction to human-scented nylon sleeves. Related to Figure 4 & 5.

Percent wild-type mosquitoes attracted to human odor trapped on nylon sleeves (one-way ANOVA, $n=3$). The dot plot represents the mean and standard error. Genotypes marked with the same letters are not significantly different ($P=0.8576$) by post hoc Tukey's HSD test.

2.4.6 The loss of *Gr3* enhances the *Ir8a*-dependent host-seeking defect

The integration of host cues requires CO₂ and is dependent on *Gr3* in *Ae. aegypti* [43]. Similarly, *Drosophila Gr63a*, an orthologue of *Gr3* has been shown to mediate CO₂ detection as well as neurons that express Gr21a, which is co-expressed with Gr63a, are required for CO₂ detection to facilitate CO₂-dependent avoidance behavior [32,82-84]. The cpA neuron which expresses *Gr3* has been reported to respond to human odors beyond CO₂, but whether this requires *Gr3* in *Ae. aegypti* is unknown [35,36]. We investigated if *Ir8a* and *Gr3* act together to drive host attraction. Using our uniport assay with a human host and added CO₂, we found that host-seeking behavior is also impaired not only in the *Ir8a* mutants but also in the *Gr3* mutants (Figure 7A).

The loss of *Gr3* function causes a stronger reduction in host-seeking behavior than the loss of *Ir8a* function (Figure 7A & B). The loss of both *Ir8a* and *Gr3* causes a host-seeking defect similar to the loss of *Gr3* alone (Figure 7A & B). This suggests that *Gr3* may be necessary for *Ir8a* function. Unlike *orco* mutants or wild-type controls, the attraction rate of *Ir8a* mutants were not modified when CO₂ was excluded from olfactometer assays with human odor (Figure 7C & S4A). Therefore, CO₂ activation is not sufficient to rescue the *Ir8a* host-seeking phenotype. This suggests that detection of *Ir8a*-dependent ligands unlike *orco*-dependent ligands is a non-redundant component of mosquito host detection.

Figure 7. The loss of *Gr3* enhances the *Ir8a*-dependent host-seeking defect. (A) Percent female mosquitoes of indicated genotypes attracted to a human arm in the presence of CO₂ ($p < 0.0001$; $n = 10 - 12$). (B) Percent female mosquitoes of indicated genotypes attracted to a human arm in the absence of CO₂ ($p < 0.0001$; $n = 9 - 11$). Analysis was done using one-way ANOVA by comparing mean attraction across all genotypes. Genotypes marked with different letters are significantly different by post hoc Tukey's HSD test. For the dot plots in A and B, long lines represent the mean and short lines represent standard error. (C) Percentage response of mosquitoes attracted to human in the presence and absence of CO₂ represented on a bar plot showing mean and standard error. Data compared in C is from figures 5A and 5B and analyzed by Two-Way ANOVA, grouped column statistics comparing *Ir8a* and *Gr3* mutants ($p < 0.001$). Genotypes marked with asterisks are significantly different. Human subject 1 was used in the experiments in this figure. See also Figure S4 and Table S2.

Figure S4: The attraction of *Ir8a* mutants to human odor is not modulated by the presence of CO₂. Related to Figure 6 & 7.

(A) Comparison of female mosquitoes attracted to human odor scented nylon sleeve in the presence and absence of CO₂. The bar plot represents the mean and standard error. Data compared is from figures 4C and 4F and analyzed by Two-way ANOVA, grouped column statistics comparing *Ir8a* and *orco* mutants. Genotypes marked with asterisk are significantly different ($P < 0.001$).

(B) Measurement of Carbon dioxide concentration in the uniport olfactometer at different conditions with amprobe-100. The presence of a human arm in the assay did not significantly increase the concentration of CO₂. The addition of CO₂ to the assay significantly increase the amount of CO₂ concentration detected. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.0001$, $n = 15$).

2.5 Discussion

Here we present evidence that strongly suggests that *Ir8a* is a crucial mediator of *Ae. aegypti* mosquito attraction to humans and human odor. *Ir8a* is necessary for the electrophysiological response of the mosquito antenna to acidic volatiles found in human odor including lactic acid. We present evidence that the *Ae. aegypti* IR8a pathway responds to human odor cues during blood-feeding as well as enables mosquito attraction to humans, human odor, and lactic acid. By varying CO₂ in our behavioral assays and testing the genetic interactions between *Ir8a* and *orco* as well as *Ir8a* and *Gr3*, we have connected the *Ir8a* pathway to the overall integration of host cues by mosquitoes. Taken together, our evidence supports the conclusion that the *Ir8a* pathway is a key participant in the multimodal integration of host odor cues by *Ae. aegypti* mosquitoes whose absence cannot be compensated for by other olfactory receptors.

Lactic acid and carboxylic acids are a major component of human sweat and may distinguish humans from other vertebrate hosts [52,53,85]. Lactic acid acts synergistically with certain volatile compounds to increase the attractiveness of either odor blends or human odor to mosquito vectors [52,53,70,72,86]. Our results support the conclusion that the detection of lactic acid and carboxylic acids by mosquitoes is necessary for robust attraction to humans. Further study will be required to determine if *Ir8a* mediates the decision to target human or other vertebrate hosts. In addition, the identification of IR8a as a co-receptor required for lactic acid detection allows for the recognition of the odor-tuned receptor(s) that are necessary to sense this important kairomone.

Our results have implications not only for IRs, but also for how sensory information from the OR and the CO₂-sensing GR pathways are integrated during mosquito host detection. Our experiments reveal that *Ir8a* and *Gr3* are both required for human host detection, but the genetic interaction of the two genes is non-additive. It could have been possible that the loss of both genes could eliminate mosquito host detection, but this was not the case. Double mutants lacking *Ir8a* and *Gr3* show a significant reduction in host detection when compared to *Ir8a* mutants alone (Figure 7A & B). However, the defects in the double mutant are not enhanced when compared to *Gr3* mutants with an intact *Ir8a* gene (Figure 7A & B). In addition, the phenotype of *Ir8a* mutants is not rescued by the presence of CO₂ (Figure 7C, S4A). This suggests that *Gr3* activation is necessary but not sufficient to promote *Ir8a*-dependent host attraction. *Gr3* activation by CO₂ or a select group of other odors [35,36] may make acidic volatiles in human odor salient to the mosquito, but *Ir8a* is still required to detect these odors.

Our evidence suggests that *Gr3* activation gates the responses of both the OR pathway and the IR8a pathway to promote host seeking, but how this interaction is achieved is not the same. Based on our results, the loss of *Ir8a* cannot be compensated for by using other olfactory receptors as in *orco* mutants. Our genetic interaction experiments support the conclusion that *Gr3* activation is required for IR8a pathway function, and CO₂-dependent gating of additional *Ir8a*-independent receptors can compensate for the loss of *orco* function. The identification of these receptors is imperative to understand how mosquitoes find their human hosts.

In this study, we presented the functional characterization of an ionotropic chemoreceptor family member in *Ae. aegypti* and provide insight into how olfactory receptor pathways can interact to mediate the detection of humans and their odor by mosquitoes. However, the understanding of how these sensory responses are integrated to facilitate host detection is far from complete. Determining if the integration of cues occurs within the antennal lobe or in higher olfactory processing centers in the brain will help reveal the dynamics of the innate neural circuits that enable mosquito host detection. Further study is necessary to uncover how mosquitoes sense their human hosts starting from the peripheral perception of cues, to the integration and processing of the information in the central nervous system, and finally, the motor circuit outputs that drive host-seeking behavior.

Primer name	Sequence
IR8aExon2 CRISPRF	GAAATTAATACGACTCACTATAGGGCGGACAAAAT GGCGTATGTTTTAGAGCTAGAAATAGC
IR8aExon3 CRISPRF	GAAATTAATACGACTCACTATAGGACATCTGTCTGA CGATAACGTTTTAGAGCTAGAAATAGC
sgRNArev	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGAT AACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCT CTAAAAC
infusionIR8LA_1	CCATGATTACGAATTCCGGGTGTTTGGTTCTCCAGA TTTG
infusionIR8LA_2	ATGGCCATTTCGAATTCATAGCATGCGATGTAAGTGC AGGTAC
infusion_IR8RA1	ATGTACAGAGCTCGAGCGGTATTCGACTACTACATT GTCTAC
infusion_IR8RA2	ACTAGTACTTCTCGAGAGTACCGCTTGGTCGGTTTG ATCTTC
<i>Ir8a</i> ^{dsRED} ForLA3	GTTGTTTCATGAACGTGAACAACCGG
Ir8aexon4rev3	CGTTTCCTGTAGGCCCAAGGG

Ir8adsRedForLA1	GAACGTGAACAACCGGAAGTACCT
Ir8a_polyU_For	GCGGCCCAAGTAAGCAGTG
Ir8adsRED_poly_re v2	CAGCAAGTGACGTCAACCCTTC
Ir8a_afterRA_rev	AACCTCGGTAGTTCCAACGCG
SV40For1	CTGCATTCTAGTTGTGGTTTGTCC
Ir8aExon3for1	6-FAM fluorescent modification- CGGATTCTCGGTTCTGGATG
Ir8aExon3rev2	CTCGGTAGTTCCAAGGCGAAAGTA
TaqMan Universal forward primer	ATCAGTCCGATCGCTATGACAAG
TaqMan Universal reverse primer	GGTTGTCAATACCTTTCGGCTTAC

Table 1: Table for oligonucleotides. Table showing the primers and their corresponding oligonucleotide sequences used in the experiments.

Individual ID	Age	Race/Ethnicity	Sex
Subject 1	28	Black/African	M
Subject 2	22	Black	M
Subject 3	22	White/Hispanic	F
Subject 4	28	White	M
Subject 5	23	Hispanic	M
Subject 6	22	White/Hispanic	F
Subject 7	26	Hispanic	F
Subject 8	25	White	M
Subject 9	21	Hispanic	F
Subject 10	21	White	F
Subject 11	41	White/Hispanic	M
Subject 12	20	Asian	F
Subject 13	24	Hispanic	M
Subject 14	19	White	F
Subject 15	21	White	F
*Subject 16	24	White/Hispanic	M
*Subject 17	22	White/Hispanic	M
*Subject 18	41	White	M

Table 2: Subject details used for the uniport olfactometer assay. Table showing the profile of the subjects used in the uniport olfactometer assay. Attraction to subject number 1 to 15 is shown in figure 4A. Subject number 1 was used for the remaining experiment with uniport assay to control for individual differences that exists in mosquito host attraction. Subject number 16 to 18 marked in asterisks did not complete the experiment or excluded due to attraction lower than 20%.

Table 3: Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
Geranylacetone	Sigma-Aldrich	C.A.S. 689-67-8
Methyl-5-hepten-2-one	Sigma-Aldrich	C.A.S. 110-93-0
Dodecanal	Sigma-Aldrich	C.A.S. 112-54-9
L-Lactic acid	Sigma-Aldrich	C.A.S. 79-33-4
Octanoic acid	ICN	C.A.S. 124-07-2
Heptanoic acid	ICN	C.A.S. 111-14-8
Butyric acid	ICN	C.A.S. 107-92-6
Octanal	Chemicon: Acros	C.A.S. 124-13-0
Nonanal	Chemicon: Acros	C.A.S. 124-19-6
Limonene	Fluka	C.A.S. 5989-27-5
Nonanoic acid	Fluka	C.A.S. 112-05-0
2-ethyl hexanol	Lancaster	C.A.S. 104-76-7
1-octen-3-ol	Janssen Chimica	C.A.S. 3391-86-4
Guanidine thiocyanate	Sigma-Aldrich	C.A.S. 593-84-0
Sarkosyl	Fisher Scientific	C.A.S. 137-16-6
Chloroform	Fisher Scientific	C.A.S. 67-66-3
Linalool	Sigma-Aldrich	C.A.S. 78-70-6
2-mercapthoethanol	Sigma-Aldrich	C.A.S: 60-24-2
Restriction enzymes		
PmeI	New England Biolabs	Catalog # R0560S
EcoRI	New England Biolabs	Catalog # R0101S
XhoI	New England Biolabs	Catalog # R0146S
MluI	New England Biolabs	Catalog # R1089S
Recombinant DNA		
pGT-Ir8a (used for donor plasmid)	This study	N/A
pMLM3613 (used for Cas9 mRNA)	[53]	Addgene # 42251
pSL1180:polyUBdsRED (donor plasmid)	[54]	Addgene # 49327
Oligonucleotides (primers)	Integrated DNA Technologies	See Table S1

Commercial Assays		
MEGAscript T7 Transcription Kit	Life Technologies	Catalog # AM1334
MEGAclear transcription clean-up kit	Life Technologies	Catalog # AM1908
mMachine mMachine T7 ultra kit	Thermo-Fisher Scientific	Catalog # AM1345
DNeasy blood & tissue kits	QIAGEN GmbH	Catalog # 69504
NucleoSpin gel and PCR cleanup kit	Machery-Nagel Inc.	Catalog # 740609
NEB PCR cloning kit	New England Biolabs	Catalog # E1202S
RNAlater stablization solution	Invitrogen	Catalog # AM7020
RNAid Kit	MPBio	Catalog # 111007200
TURBO DNA-free kit	Invitrogen	Catalog # AM1907
KOD polymerase	EMD Millipore	Catalog # 71086
Qiaquick PCR purification kit	Qiagen	Catalog # 28106
Infusion HD cloning kit	Clontech	Catalog # 638909
Qiagen Endo-free Maxiprep kit	Qiagen	Catalog #12362
SimpleSeq premixed kit	Eurofins	N/A
IBI Genomic DNA extraction kit	IBI scientific	Catalog # IB47222
Amplitaq Gold 360 PCR master mix	Applied Biosystems	Catalog # 4398881
QIAEX II Gel extraction kit	QIAGEN GmbH	Catalog # 20051
pCR2.1 TOPO TA vector	Invitrogen	Catalog # K462040
One Shot TOP10 cells	Invitrogen	Catalog # C607003
QIAprep Spin Miniprep kit	QIAGEN GmbH	Catalog # 27104
SuperScript II RT reagent kit	Invitrogen	Catalog # 18080-051
RNase cocktail enzyme mix	Thermo-Fisher Scientific	Catalog # AM2286
TaqMan 2X universal master mix	Thermo-Fisher Scientific	Catalog # 4324018
Custom TaqMan Ir8a probe	ThermoFisher Scientific	Catalog # 4331348
Experimental Models: Organisms/Strains		
<i>Ae. aegypti</i> : Orlando	Leslie Vosshall	N/A
<i>Ae. aegypti</i> : <i>Ir8a^{dsRED}</i>	This study	N/A
<i>Ae. aegypti</i> : <i>Ir8a^{attP}</i>	This study	N/A
<i>Ae. aegypti</i> : <i>Orco⁵</i>	BEI resources	NR-44377
<i>Ae. aegypti</i> : <i>Orco¹⁶</i>	BEI resources	NR-44378

<i>Ae. aegypti: Gr3^{cfp}</i>	BEI resources	NR-48760
Computer, Software and algorithms		
Syntech EAG-Pro 4.6	Custom-made	N/A
Peak Scanner software	Applied Biosystems	v1.0
Impedance amplifier	Syntech	IDAC-4
ZiFit	https://zifit.partners.org/ZiFit/	N/A
Real time PCR system	Applied Biosystems	7500
Locomotor activity monitor	Trikinetics Inc.	LAM 25
SnapGene & SnapGene Viewer	SnapGene	N/A
SDS software	Applied Biosystems	v1.4.1
GraphPad Prism	GraphPad	Prism v7 & v8
Others		
Tetramin tropical fish food	Tetra	Catalog # 16152
Capillary tubes	Blaubrand Intramark	Catalog # 1904637
Cellulose acetate fly vial plug	Genesee Scientific	Catalog # 49-101
CO ₂ diffusion pad	Tritech Research	Model # MINJ-DROS-FP
Membrane feeders	Chemglass	Catalog # CG-1835-70
Uniport olfactometer	Custom-made	N/A
Carbon dioxide monitor	Amprobe	Catalog # CO2-100
Acrylic flowmeter	Dwyer Instrument Inc.	Catalog # VFA-4-SSV
Suntan knee-high pantyhose	L'eggs brand, Hanes	Model # 39400
Disposable pellet pestles	Fisher Scientific	Catalog # 12-141-364

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Chapter 3: *Aedes aegypti* *Ir8a* mutant female mosquitoes show increased attraction to standing water

“Mosquitoes are like family. Annoying, but they carry your blood.”

Anonymous

3.1 Abstract

The detection of water sources is crucial for insects such as mosquitoes to avoid desiccation and survive. In addition, mosquitoes use humidity cues to successfully navigate the environment in search for a suitable oviposition site. Previous studies on *Drosophila* have implicated some members of the ionotropic receptor family in humidity sensing. Here, we investigate if an *IR8a* co-receptor mediates water detection in *Aedes aegypti* mosquitoes. Using a simple behavioral assay, we examined the attraction of *Ir8a* mutant mosquitoes to standing water. The *Ir8a* mutant mosquitoes were able discriminate between traps containing water and those without as well as wild type and heterozygous control females. Surprisingly, the female mutants were more robustly drawn to standing water than control mosquitoes. Further investigation revealed that the strong behavioral attraction to water is not mediated by a metabolic need or an activity defect.

3.2 Introduction

The ability to sense water in the environment (hygrosensation) has been previously studied in different insects [1-5]. The availability of water impacts insect longevity, fitness and geographic distribution [6]. Although insects are covered by chitinous exoskeleton, they constantly experience water loss via the cuticle and through their open respiratory systems [7]. Insects with large surface area-to-volume ratios such

as mosquitoes must figure out a way to replenish water loss and maintain internal osmotic balance. Water vapor emanating from oviposition sites have been shown to elicit pre-oviposition behavior in *Anopheles gambiae* [3]. Anthropophilic mosquitoes do not only rely on heat, odor and visual cues to find their human hosts [8], the detection of hygrosensory cues has also been proposed to be important during host-seeking [9]. Functional mapping of the pathways that mediate water-seeking behavior could inform how mosquitoes monitor and adjust its hydration state to maintain optimum physiological homeostasis or seek oviposition sites. Understanding the molecular basis of mosquito water-seeking behavior could lead to novel approaches for controlling mosquito populations and manipulating gravid female attraction to water-baited traps.

Insects possess two distinct systems for detecting water sources: the gustatory system, which is tuned to sensing liquid water [10,11], and the hygrosensory system required for detecting water vapor [4,5]. In *Drosophila*, behavioral response to liquid water was disrupted by ablating the *ppk28* gene function which labels the gustatory water sensory neurons [11]. The gustatory system was activated only after the sensory neurons had made direct contact with liquid water. The observation draws attention to the hygrosensory system that detects water vapor from a distance. The TRP channels, *nan* and *wtrw*, previously identified in *Drosophila* mediate two contrasting behavioral responses to dry air and moist air respectively [12]. Studies have shown the existence of water sensitive receptors expressed in the coeloconic sensilla of the *Drosophila* antennae [13]. Some ionotropic receptors (IRs) including IR25a, IR93a, IR68a and IR40a which are expressed in these sensilla have been reported to mediate humidity sensing in *Drosophila* [4,5,14].

3.3 Methods and Results

We recently reported that the *Ir8a* gene (AAEL002922) is key for yellow fever mosquitoes to detect acids in human sweat [15]. Here, we asked if the IR8a pathway also drives water-seeking behavior. To test this, we presented two ramekins (3.8cm height by 5cm width) housed in a trap (16cm height, 9cm width, 6cm diameter) and set at an angle 45° opposite and placed 3.7cm apart from each other inside a rearing cage (30cm height, 28cm diameter, 15cm diameter). One of the ramekins contained 25ml deionized water whereas the other was left blank (Figure 8A-D). A total of 50 mosquitoes aged 7-10 days old, previously starved on water for 24hr were introduced into the cage. The assay lasted for 15hrs (27°C, 40% RH) under a 14:10 light-dark cycle (lights on at 8 am).

Thereafter, mosquitoes were cold anesthetized at 4°C for 30 mins. The number of mosquitoes inside each trap was visually scored. In order to control for possible position effect, the ramekins containing water was swapped after each trial. Surprisingly, *Ir8a* mutant female mosquitoes were more strongly attracted to the water trap than the wild-type and heterozygous controls (Fig. 9A). The behavior is sexually dimorphic as it was observed in the *Ir8a* mutant females but not males (Fig. 9B). All the genotypes tested including males and females show strong preference for water trap over the blank one (Fig. 9C-D). The observation suggests that *Ir8a* is not required to find standing water, but rather it influences the intensity of the response to water.

Figure 8: Material designs for the water trap assay. (A) Illustrations showing the dimensions of a mosquito cage, (B) trap, and (C). ramekin used for the water trap assay (D) Illustration showing ramekin housed in a trap and set at an angle 45° opposite each other and 3.7cm apart. One of the ramekins contained 25ml deionized water whereas the other was left blank.

Figure 9: *Ir8a* mutant female mosquitoes are more attracted to water (A) Response of female mosquitoes and (B) male mosquitoes to water over a period of 15 hours. Genotypes varied in their response to water. Females (one-way ANOVA, $p < 0.0001$, $n = 12-14$). Males (one-way ANOVA $p = 0.0023$, $n = 12-14$). (C) Figure showing female (one-way ANOVA, $p = 0.213$, $n = 12-14$) and (D) male mosquitoes' preference towards water source (one-way ANOVA, $p = 0.055$, $n = 12-14$). Genotypes marked with different letters are significantly different by post hoc Tukey's HSD test. On the violin plot, the central line represents the median. The shape of the kernel represents the density of the population. Wider sections of the violin plot represent a higher probability that members of the population will fall within the section.

We asked if the strong attraction to water recorded in *Ir8a* mutant female is due to physiological need for hydration or carbohydrates. Using the capillary feeder assay (CAFE) as previously described [16], we quantified the amount of water ingested after 2hrs, and compared to the wild-type and heterozygous controls. Interestingly, we found no significant difference in the total volume of water ingested by *Ir8a* mutants when compared to the wild type and heterozygous controls (Fig. 10A). We then wondered if *Ir8a* mutant females are more attracted to the water source because they are looking for a sugar meal. A water solution containing 10% sucrose was presented to the mosquitoes using the CAFE assay. After 4hrs of *ad libitum* feeding, we could not record any feeding difference between the wild-type and *Ir8a* mutant females (Fig. 10B) similar to what has been previously reported for 18 hours of feeding [15]. Taken together, these findings suggest that the increased attraction to water seen in *Ir8a* mutants cannot be explained by thirst or lack of carbohydrate reserves.

We next investigated if *Ir8a* mutant females have increased activity in humid environments. We previously reported that the IR8a pathway does not regulate mosquito activity [15]. As reported, the glass tube was plugged with a dry and a water-saturated cotton at opposite ends to create a humidity gradient. We reasoned that water saturating both cottons would simulate a wet environment that favors the *Ir8a* mutants, and we could record higher infrared beam breaks triggered by the water-seeking behavior of the mutants. Using a locomotor activity assay adapted to mosquitoes (Fig. 10C) [17], we found no difference in activity in the *Ir8a* mutants when compared to the wild-type and heterozygous controls (Fig. 10D).

Figure 10: *Ir8a* mutant female mosquitoes feed normally on water and sugar, and show normal activity. (A) Water ingestion quantified after 2hrs (one-way ANOVA $p = 0.3525$, $n = 45$) of drinking. (B) Sucrose consumption recorded after 4hrs of feeding, (one-way ANOVA $p = 0.7772$, $n = 25$). (C) Illustration of the beam break assay. The red line represent the infrared beam triggered by mosquito movement in the glass tube. An activity count is recorded when a mosquito moves past the beam. (D) Average daily locomotor activity after 4 days of fasting on water, measured by the number of infrared beam breaks (counts). There were no statistical differences among genotypes ($p = 0.9320$, $n=12$). On the dot plots, long lines represent the mean and short lines represent standard error. Data was analyzed by one-way ANOVA, and genotypes marked with the same letters are not significantly different by post hoc Tukey's HSD test.

3.4 Conclusion

Taken together, the robust water-seeking behavior recorded in the *Ir8a* mutant female mosquitoes cannot be explained by a physiological need for water or sugar. Also, the mutants show no locomotor activity difference as inferred from the activity assay. We propose that the strong attraction to standing water could be mediated by chemosensation. A possibility is that *Ir8a* mutant females might be compensating for the loss of intact olfactory sensory modality by priming the sensory system towards vital resources key for survival. This evidence supports the claim that the loss of one sensory input may cause enhanced sensitivity to a completely different sense [18]. Another hypothesis is that *Ir8a* could be part of a neural circuit that represses water sensing when female mosquitoes are hydrated. *Ir8a* may also be important for finding a suitable habitat that is less humid. Future study is needed to uncover the relative contribution of the *Ae. aegypti* IR8a pathway in water-seeking behavior.

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Chapter 4: Carbon dioxide mediates increased visual attention in tethered

Aedes aegypti and *Drosophila melanogaster*

“A mosquito can fly, but a fly cannot mosquito.”

Anonymous

4.1 Abstract

Insects strongly rely on visual processing during flight to steer towards targets, avoid obstacles, and make corrections when wind or other factors introduce perturbations. Given that *Aedes aegypti* and *Drosophila melanogaster* are distantly related dipterans, we reasoned that both insects would possess diverse visuomotor reflexes. Using a tethered flight experiment, we investigated the interaction between olfactory and visual stimuli on the control of wing kinematics in the yellow fever mosquitoes and fruit flies. When given a choice between star field and a bar in the absence of positive olfactory cues, vinegar flies show strong preference for wide-field motion over object tracking. However, mosquitoes show no preference for both visual cues. Supplementing the assay with CO₂ elicits robust tracking response in both dipterans. Using reverse genetics, we provide evidence that the CO₂ sensory pathway is necessary for *Ae. aegypti* mosquitoes to effectively track visual information. CO₂-insensitive *Gr3* mutant mosquitoes cannot associate CO₂ with visual cues. In addition, CO₂ rescues weak sensitivity to visual contrasts in mosquitoes but slight effect was recorded in the vinegar fly. Taken together, we provide evidence that CO₂ enhance attention to visual cues in the two dipterans.

4.2 Introduction

Studies in many insects demonstrate that visual feedback is a key to flight control (Grimaldi, and Engel, 2005). It is the primary means by which they maintain altitude, avoid obstacles, and approach targets (Gotz, 1968). Olfaction complements vision by allowing insects to locate resources and avoid hazards that are obscured or too far off to be seen, such as steering towards food or away from predators (Murlis et al., 1992). Much less understood is the interaction between vision and olfaction but integrating the two cues is important for effective navigation (Vinauger et al., 2019; van Breugel et al., 2015; Duistermars and Frye, 2008), allowing neural circuits to activate behaviors in a context-specific manner.

Flying to a target successfully requires first setting a heading that steers towards a target, then making corrections when perturbed by wind (Combes and Dudley, 2009; Ravi et al., 2013). Although natural scenes contain countless intermediate visual stimuli, and neural pathways are now known to include neurons that process motion in complex ways (Bahl et al., 2013, Fenk et al., 2014, Theobald et al., 2008), at least some neurons respond preferentially to small moving targets, while others respond to wide moving flow fields (O'Carroll, 1993). As a result, both small visual features, such as vertical bars, and wide visual fields, such as the optic-flow produced by self-motion, can elicit robust behavioral responses in flight (Maimon et al., 2008; Krapp et al., 2012). The smaller targets can be prey, predators, mates, flowers, or nearly any other small image that an insect might want to approach or avoid. The wide fields on the other hand, fill large swaths of the visual field, and when all the images flow in a characteristic way, provide a strong cue of self-motion (Krapp et al., 2000; Taylor and Krapp 2000; Borst et al., 2010).

But even combined with more sophisticated visual processing (Theobald et al., 2010), these strategies can be insufficient to detect vital features if they are far off, visually obstructed, or obscured in the visual clutter—especially when viewed by low-resolution insect eyes (Land and Nilsson et al., 2012).

Olfaction provides a means for insects to find less visible resources, as odors can signal the presence of food, mates, or predators. However, the plumes that carry odors are structurally complex and quickly varying, so by themselves they are a poor directional cue (Murlis et al., 1992). Instead, odors can operate together with visual cues by modifying responses to targets and optic flow, and hence influencing flight paths (Chow and Frye, 2008). These flight-mediated responses enable olfactory navigation (Chow et al., 2011). Odor and visual cues can also elicit synergistic behaviors that insects will not perform without both cues combined. For example, moths will not track odor plumes reliably without simultaneous presentation of visual cues (Raguso and Willis, 2005), fruit flies alter the gain of their wide-field steering responses when they smell attractive odors (Chow et al., 2011), and CO₂ causes mosquitoes to initiate a search for visual objects in a wind tunnel (van Breugel et al., 2015). Here, we investigate the interaction between olfactory and visual stimuli on the motor control of wing kinematics in *Ae. aegypti* and *D. melanogaster*.

4.3 Materials and Methods

4.3.1 Insect rearing and preparation

The *Ae. aegypti* mosquitoes were reared and maintained at 25-28°C, 75% relative humidity under a 14:10 light-dark cycle (lights on at 08:00h). Non-blood fed wild-type female Orlando strain and *Gr3* mutant mosquito lines aged 5-7 days old were used in the study. Mosquitoes were fed *ad libitum* on sugar and were not starved before the experiment. Female *D. melanogaster* were raised on a standard food medium, maintained on a 16h: 8h light: dark cycle and collected 4 – 6 days post eclosion. Mosquitoes and flies were cold anesthetized and glued by the dorsal thorax to a rigid tungsten rod. We allowed them to rest and recover by placing a small piece of tissue paper on their legs, which stops their wings beating. The insects are then affixed into the center of the flight arena, still suspended by the rod, at which point they initiate tethered flight.

4.3.2 Experimental equipment

A custom-built flight arena delivered simultaneous visual and olfactory stimulation to rigidly tethered flying mosquitoes and flies. The arena is a Perspex cube with 200 mm edges, covered with back projection screen material and first-surface mirrors affixed at 45 degrees to the sides with the back left open for access (Fig 11A). A projector illuminates five sides, covering 10.47 steradians of the visual field. The projector images a three-dimensional scene onto each face at 360 frames per second, perspective-corrected for the center of the cube.

4.3.3 Measuring steering responses

Within the arena, mosquitoes and flies were positioned between an infrared light above, and a pair of photodiodes below. The shadow of each wing on the photodiode

measures the amplitude for every stroke, recorded as voltage (Fig. 11B). Flies have higher wing beat amplitude and lower wing beat frequency compared to mosquitoes, which we accommodated by adjusting the gain and gate width of the wing beat analyzer. *Drosophila* responds to visual cues by modulating the relative amplitudes of right and left wing beats (Gotz, 1968), giving an indication of steering effort (Frye and Dickinson, 2004).

4.3.4 Olfactory Stimulus

For assays supplemented with olfactory cues such as air and CO₂, the gas flow rate was measured by an acrylic flowmeter Model VFA-4-SSV (Dwyer Instruments Inc., IN, USA) set to release the air or carbon dioxide at 0.5 SCFH via the four forward facing corners of the arena. The final concentration of CO₂ leaving the arena was maintained between 25000ppm – 29000ppm measured by a CO₂ meter (Catalog # CO2-100, Amprobe, Everett, WA).

4.3.5 Visual Stimulus

Object tracking was tested with moving vertical bars projected near the frontal visual field, and wide-field tracking was tested with moving star fields projected in the front and periphery, both in open-loop. Tracking tests were interspersed with 3s closed-loop bar fixation, where an insect can stabilize a bar with its wing beat responses (Fig. 11C-D). Insects that failed to hold a stable bar, or beat wings throughout an experiment, were eliminated from further analysis. For the sinusoidal grating experiment, the visual information contains 10 levels of light intensities and the scene can move either left or right.

Figure 11. An arena for simultaneous delivery of visual and olfactory stimuli (A) Computer controls a high-speed projector, and an odor delivery manifold, to coordinate the experimental delivery of perspective-corrected, panoramic images with airborne odor delivery from four directions. Right image shows the inside of the flight arena. (B) Turning attempts manifest in differential wing beat amplitudes (bottom trace), which are captured as infrared shadows cast onto a pair of photodiodes below the insect, producing a record of steering effort with each wing beat. (C) Representative traces showing the tracking behavior of a flying tethered mosquito to a bar (left) and star field (right). Representative traces showing the tracking behavior of a flying tethered fruit fly to a bar (left) and star field (right).

4.4 Results and Discussion

4.4.1 Object and wide-field tracking differ in *Ae. aegypti* and *D. melanogaster*

Mosquitoes and fruit flies are distantly related dipterans (Severson et al., 2004) with distinct feeding behaviors. By presenting bars and star field, we asked if different visual cues could elicit distinct tracking responses in both insects. We used eight types of visual information consisting of a star field moving either left or right; a bar moving either left or right; a bar and star field simultaneously moving together; and a bar and star field simultaneously displayed but moving in opposite directions. The order of tests was randomized to avoid hysteresis.

Interestingly, we found marked differences in the tracking behavior of the two dipterans. In visual test condition where bar and point fields move in opposite directions, mosquitoes show no preference for either ($p = 0.632$). On the contrary, fruit flies show a strong preference for the wide field over a moving bar when both cues move in opposite direction ($p = 0.0016$). The preference implies that *Drosophila* prefers to track wide field motion that fills large swaths of the visual field over a single object. Since fruit flies are cosmopolitan and generalist species, a visual field presenting a wide scene may be indicative of food or mates. In addition, when the bar and point fields move in the same direction, the responses recorded in *Ae. aegypti* is summative of the steering efforts when either bar or star field was presented. Since object tracking is enhanced when the wide field moves in the same direction, mosquitoes may also pay attention to the background of a potential host while tracking. However, no summative response is recorded in the flies (Fig. 12A). *Drosophila* showed a robust tracking of all the visual information displayed, whereas mosquitoes show very weak responses.

Since mosquitoes integrate several cues to detect their human hosts (Raji and DeGennaro, 2017), visual information alone may not be a strong cue for finding resources.

4.4.2 CO₂ increases attention to visual cues in *Ae. aegypti* and *D. melanogaster*

Earlier studies have reported that mosquitoes can associate vision with olfactory cues and heat to find a target (van Breugel et al., 2015). We reasoned that supplementing the tethering arena with positive olfactory cues might enhance mosquitoes' attention to fixate on visual stimuli. We delivered CO₂ into the arena at the rate of 0.5 Square per Cubic Feet per Hour (SCFH) to maintain a final concentration that fluctuated between 2500-2900 ppm. For an assay without additional CO₂, we replaced with dry air set at the same flow rate.

When CO₂ was added, a moving bar became more salient to mosquitoes ($p = 0.015$) but not the wide field ($p = 0.9657$, Fig. 12B). Since CO₂ is a very important cue that sensitizes mosquito host detection system (Raji et al., 2019; McMenniman et al., 2014), it is likely that the tethered mosquitoes take the moving bar for a vertebrate host. A possibility is that CO₂ primes mosquitoes' host detection system to seek potential hosts. As a search strategy to find resources, insects assign subjective value to inanimate objects (Robie et al., 2010). Since visual representation of the star field does not closely relate to a host, this may explain why tracking response to the wide-field motion was not robustly enhanced. Mosquitoes' response in trials where the bar and point-field move in the same direction was significantly more than when CO₂ was not added to the assay ($p < 0.001$). This strengthens the role of CO₂ in enhancing visual tracking behavior in *Ae. aegypti*.

Figure 12. Carbon dioxide increases attention to visual cues in *Ae. aegypti* and *D. melanogaster* (A) Response of *Ae. aegypti* mosquitoes (n=24) and *D. melanogaster* (n=14) to visual cues in the absence of positive olfactory cues. (B) Response of *Ae. aegypti* mosquitoes to visual cues in the presence (n= 21) and absence (n = 18) of CO₂. (C) Response of *D. melanogaster* to visual cues in the presence (n = 15) and absence (n= 12) of CO₂. (D) Response of *Gr3 Ae. aegypti* mutant mosquitoes to visual cues in the presence (n = 24) and absence (n = 15) of CO₂. Visual cues tested are illustrated below each graph. Figures marked with asterisks are significantly different when analyzed by paired t-test.

Figure 13. Carbon dioxide enhances sensitivity to visual contrast in *Ae. aegypti* and *D. melanogaster*. (A) Mosquitoes' response to sinusoidal grating in the presence (n= 17) and absence (n = 10) of CO₂. Fruit flies' response to sinusoidal grating in the presence (n = 22) and absence (n = 12) of CO₂. Figures marked with asterisks are significantly different when analyzed by paired t-test.

In tethered *D. melanogaster*, intermittent release of CO₂ robustly enhanced visual response to both wide field and object in motion (Fig. 12C). Suggesting that the detection of CO₂ cue is not limited to blood-sucking insects. Generalist insect species such as fruit flies may rely on CO₂ sensory modality to find food sources. Interestingly, when the direction of the bar contradicts the star fields, fruit flies show a very strong preference towards the star fields independent of the presence of CO₂. In contrast, overall tracking response (Δ WBA) of mosquitoes to the opposing visual cues is not significantly different from the baseline ($P > 0.05$). Supplementing the arena with CO₂ is not sufficient to rescue object tracking when the direction contrasts the wide field. This suggests a possibility that mosquitoes rely on the background motion of a potential host to effectively fixate.

The presence of CO₂ has been shown to increase mosquitoes' attraction to a human arm in a uniport olfactometer tunnel (Raji et al., 2019). This was recorded in the wild-type Orlando strain but not in the CO₂-insensitive *Gr3* mutant mosquitoes. We reasoned that the addition of CO₂ would make no difference in the response of *Gr3* mutant mosquitoes to a moving bar. The recent advancement in gene editing allowed creating loss-of-function mutation and study gene of interest. Thus, we tested *Gr3* mutant lines to visual cues in the presence and absence of added CO₂. Based on our data, neither object ($p = 0.967$) nor point field motion tracking ($p = 0.898$) is enhanced when the assay was supplemented with CO₂. It is worth noting that visual tracking is greatly impaired in the *Ae. aegypti* *Gr3* mutants as inferred from the wing beat amplitude (Fig. 12D). This finding provides evidence that CO₂ gates response to visual cues in *Ae. aegypti* mosquitoes. Unlike *Gr3* mutant mosquitoes that cannot associate CO₂ plumes with visual cues during a tethered flight (Fig. 12D), the disruption of the canonical CO₂ pathway in

fruit flies does not abolish in-flight attraction to CO₂ (Wasserman et al., 2013). These findings suggest that differences may exist in the visual-olfactory integration of the two dipterans.

Surprisingly, CO₂ flow rate induce differential behavioral responses in the two insects. The ability of the two dipterans to fixate is severely impaired when the CO₂ flow rate was increased to 3SCFH. This suggests that a CO₂ concentration threshold exists beyond which tracking behavior is impacted. Even at low CO₂ consistent flow rate (0.5SCFH), fruit flies showed poor tracking performance compared to when the gas was released intermittently. However, the consistent CO₂ flow rate (0.5SCFH) elicits robust mosquito tracking activity (Fig. 12B). A possibility is that fruit flies have relatively smaller body size, which makes the wing kinematics more liable to perturbation by elevated airflow. It also remains unclear whether fruit flies are more susceptible to CO₂-induced anesthesia. Studies have shown that CO₂ can impact flight behavior in fruit flies (Bartholomew et al., 2015) and cause disorientation in mosquitoes (Turner et al., 2011). The gas release rate was corrected for *D. melanogaster* in our experiments by transiently releasing CO₂ gas every 30 sec and set it to flow for 1 sec at 0.5SCFH rate.

4.4.3 CO₂-induced sensitivity to visual contrasts is markedly enhanced in *Ae.*

aegypti* mosquitoes than *D. melanogaster

We next investigated the visual acuity of mosquitoes and fruit flies to bright scenes on a dark background set to move at a constant motion but varying contrast. It is known that vision becomes blurry as the contrast between an object and its background decreases (O'Carroll and Wiederman, 2014). We hypothesized that the tracking behavior of the two dipterans will correlate with increased contrast and dependent on CO₂. Contrary to what

we observed in the fruit flies, mosquitoes show a very weak response to increased contrast in the absence of CO₂ (Fig. 13A). However, visual response in fruit flies correlates with increased contrast, until it reached a saturation point where sensitivity begins to plummet (Fig. 13B).

Given that CO₂ robustly enhanced object tracking in *Ae. aegypti* (Fig. 12B) and *D. melanogaster* (Fig. 12C), we reasoned that the addition of CO₂ plumes to the assay would increase contrast sensitivity. Interestingly, we recorded a very robust response by mosquitoes to the visual presentations when CO₂ was added (Fig. 13A). Similarly, the addition of CO₂ increases visual attention in the fruit flies. However, the response from fruit flies (Fig. 13B) was not as dramatic compared to what was observed in mosquitoes (Fig. 13A). Other behavioral studies in these dipterans also showed that they integrate visual and olfactory cues to effectively respond in a complex environment (van Breugel et al., 2015; Frye et al., 2004). Mosquitoes appear to be less interested in visual cues and would rely on CO₂ sensitization to associate a subjective value to an inanimate object within their visual field. We observed a saturation point to visual contrast in both insects, suggesting that visual acuity can be lost when the visual contrast is too high. Even at a very low visual contrast, visual tracking was greatly impaired. Study has shown that the ability to discriminate the finest detail between bright objects on a black background can be lost due to the unreliability of the photon catch (Barow, 1964).

These results implicate the CO₂-sensing pathway in visual tracking of information. Recent study also supports our finding that olfactory and visual cues integrate as to enhance tracking behavior in yellow fever mosquito (Vinauger, et al 2019). But another group reported a different result that CO₂ does not enhance visual

tracking in *Ae. aegypti* (Liu and Vosshall et al., 2019). The method of CO₂ delivery and the shape of visual cues presented could explain the different results. Study has shown that mosquitoes use visual cues to associate odor plumes with heat, but this conclusion is not supported by genetic evidence. Using the *Gr3* mutant mosquitoes, we provide the evidence that visual tracking in yellow fever mosquito is dependent on CO₂ detection.

A comparative study of spatial and temporal acuity in the two dipterans would be a step forward to understanding how insects' innate behavior can modulate visual perception. Given that mosquito host seeking behavior is state dependent, it is unknown whether gravid blood-fed female mosquitoes would prefer wide field motion, which could indicate an oviposition site. Future study is needed to determine if stages of mosquito gonotrophic cycle can influence visual and olfactory processing. The neurophysiological basis of olfactory driven behavior has only begun to be understood (Lutz et al., 2017), however relatively less is known about visual-olfactory integration in the insect brain. Addressing these research gaps would enhance our understanding of how CO₂ modulates visuomotor reflexes in insects.

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Chapter 5: Conclusions and Future Directions

“Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.”

Sydney Brenner

5.1 Conclusions

Mosquitoes must bite to transmit diseases to humans. This observation stresses the importance of understanding how mosquitoes find their human hosts. The specific genes and neural circuits mosquitoes use when host-seeking are largely unknown, but it is clear that olfaction plays an essential role at a far distance, whereas visual cues are critical at close range. We present evidence that strongly suggests that *Ir8a* is a crucial mediator of mosquito attraction to humans and human odor. *Ir8a* is necessary for the electrophysiological response of the mosquito antenna to acidic volatiles found in human odor including lactic acid. It is also shown that *Ir8a* enables mosquito attraction to humans, human odor, and lactic acid. By varying CO₂ in our behavioral assays and testing the genetic interactions between *Ir8a* and *orco* as well as *Ir8a* and *Gr3*, the *Ir8a* pathway to the overall integration of human host cues by mosquitoes have been connected. Taken together, our evidence supports the conclusion that the *Ir8a* pathway is a key participant in the multimodal integration of host odor cues by *Ae. aegypti* mosquitoes whose absence cannot be compensated for by other olfactory receptors.

This study represents the first genetic analysis of the IR8a co-receptor in *Ae. aegypti* and builds upon knowledge gained from studies in other Dipterans. In *Drosophila*, RNAi mediated knockdown of *Ir8a* and mutants lacking *Ir8a* showed physiological and behavioral defects in sensing acidic volatiles (Ai et al., 2013).

Drosophila Ir8a mutants show humidity preference similar to wild-type controls suggesting that IR8a is not involved in humidity detection in this species (Enjin et al., 2016; Knecht et al., 2016). IRs other than IR8a have been reported to mediate taste (Ahn et al., 2017; Chen and Amrein, 2017; Hussain et al., 2016; Zhang et al., 2013), circadian rhythm (Chen et al., 2015), and thermosensation (Enjin et al., 2016; Knecht et al., 2016). Our evidence suggests that IR8a is not required for heat or CO₂ detection as *Ir8a* mutants respond similarly to wild-type controls to these stimuli. It is likely that IR8a is a co-receptor specific for the olfactory detection of chemical stimuli in insects. Although the general principles of IR function are likely conserved in insects particularly amongst co-receptors like IR8a, there are likely to be species-specific differences. Given the crucial role played by *Ir8a* in host seeking, it is important to understand the mosquito odor-tuned IRs that require the *Ir8a* co-receptor and connect these IRs with their odor-ligands. These could be interesting molecular targets for repellent design.

We have shown that *Ae. aegypti* mosquitoes lacking the *Ir8a* co-receptor cannot detect acidic human odor volatiles. *Ir8a* mutants lose odor-evoked responses to lactic acid and all carboxylic acid compounds represented in the odor panel tested in this study. Lactic acid and carboxylic acids are a major component of human sweat and may distinguish humans from other vertebrate hosts (Cork and Park, 1996; Dekker et al., 2002; Steib et al., 2001). Lactic acid acts synergistically with certain volatile compounds to increase the attractiveness of either odor blends or human odor to mosquito vectors (Allan et al., 2010; Dekker et al., 2002; Okumu et al., 2010; Smallegange et al., 2005; Steib et al., 2001). We show that *Ir8a* is also necessary for female *Ae. aegypti* mosquitoes to behaviorally respond to lactic acid and human odor when tested in olfactometer assays.

5.2 Genetic pathways for mosquito host seeking

Host odor detection involves the interaction of multiple olfactory receptor pathways. The IR8a pathway is non-redundant and specialized to respond to acidic human odor volatiles, while the ORs sense a more diverse class of compounds including ketones, alcohols, and esters, not represented in the IR8a chemical space. GR3 is necessary for the detection of CO₂. The epistasis analysis of the *Ir8a*, *orco* and *Gr3* genes informed our understanding for the role of carbon dioxide in the sensitization of *Ae. aegypti* mosquitoes to human odor. The CO₂ activation of the GR3 pathway permits the downstream activation and synergistic interaction of the IR8a, the OR pathway, and other olfactory receptors to promote host odor detection (Figure 14).

Since host detection is not completely abolished in any of the single or double mutants we tested and the *orco* mutant phenotype can be eliminated by the addition of CO₂, we predict that other olfactory receptors could be crucial for host odor detection. Candidate genes include *Ir25a* and *Ir76b* which sense amines, but it remains unclear which olfactory receptors are attuned to other chemical compounds present in human odor such as ammonia. In the tethered flight experiment, we provide evidence that the GR3 pathway is crucial for mosquito to integrate CO₂ with visual cues. The possible pathway with which GR3 interacts with other olfactory receptors to enhance host detection has been clearly illustrated (Fig. 14).

Figure 14. Model illustrating the genetic interactions between the olfactory receptor pathways during mosquito host detection.

5.3 Future directions

Our results have implications not only for IRs but how the OR pathway (Orco and the odor-tuned ORs) and olfactory CO₂-sensing GRs (GR1, GR2, and GR3) are integrated during mosquito host detection. The genetic evidence we present provides insight into how olfactory receptor pathways can genetically interact to mediate the detection of humans and their odor. However, the understanding of how these sensory responses are integrated to facilitate host detection is far from complete. Determining if the integration of cues occurs within the antennal lobe or in the mosquito brain will help reveal the dynamics of the innate neural circuits that enable mosquito host detection. Further study is necessary to uncover how mosquitoes sense their human hosts starting from the peripheral perception of cues, to the integration and processing of the information in the central nervous system, and finally, the motor circuit outputs that drive host-seeking behavior.

Molecular approaches that identify chemical compounds that hyper-activate olfactory receptor neurons in mosquitoes have been successfully established to provide a basis for developing next generation repellents (Chen and Luetje, 2012; Jones et al., 2011; 2012). This study implicates IR8a, Orco, and GR3 as promising targets to control mosquito-borne illnesses. High throughput screening assays can be used to identify chemical compounds that modulate the function of these receptor genes (Rinker et al., 2012). This can be used to design attractants and repellents to control mosquito behavior that are more effective than DEET (DeGennaro, 2015). Chemical compounds that activate these receptors can be used to lure mosquitoes into an odor-baited trap.

Alternatively, humans can be made more difficult to detect using chemical compounds that inhibit these receptors. However, host-seeking behavior is ablated but not totally abolished in all the mutants screened in the study. It is worthy of note that *Ir25a* and *Ir76b*, which remain intact in the mutants lacking the *Ir8a*- and *orco*-dependent olfactory receptor pathways could be responsible for the residual host-seeking seen in these double mutants. A recent study has shown that *An. gambiae Ir25a* and *Ir76b* receptors are tuned to amines (Pitts et al., 2017), a class of chemical compounds found in human skin emanations (Bernier et al., 2000). *Ir25a* is also crucial for sensing other cues that facilitate human hosts detection such as moisture and temperature (Enjin et al., 2016; Knecht et al., 2016; 2017). Future work is needed to identify the complete list of the olfactory receptors that are necessary for mosquito host seeking to determine how many molecular targets will need to be screened.

The functional characterization of the ionotropic chemoreceptor family in *Ae. aegypti* is an important step towards understanding olfactory-driven behavior in an insect that continues to negatively impact human health in large areas of the world (Leta et al., 2018). It is expected that the evidence provided in this study will be applicable to other mosquito vectors. Current vector control strategies have been insufficient to stop the spread of arboviruses or eliminate malaria (Enayati and Hemingway, 2010; Fernandes et al., 2018). The use of insecticide treated bed nets and indoors residual spraying have been effective in reducing malaria cases by half since 2000, but resistance to insecticides is widespread and growing. Bed nets are also ineffective against day-biting mosquitoes such as *Ae. aegypti*. Thus, it is imperative to develop alternative vector control strategies.

Our investigation on the role of IR8a also led us to uncovering its role in mediating strong attraction to water. Mosquitoes use humidity cues to successfully navigate the environment in search for a suitable oviposition site and also to rehydrate when thirsty. We propose that *Ir8a* may be important for finding a suitable habitat that is less humid. Future study is needed to uncover the relative contribution of the *Ae. aegypti* IR8a pathway in water-seeking behavior. A comprehensive understanding of genes like *Ir8a* that enable mosquito attraction to humans and water may provide molecular targets to generate new behavioral control reagents that could alter their sensory system from detecting a oviposition site. It could inform our knowledge on finding repellents to prevent mosquito bites as well as attractants to improve mosquito surveillance and population reduction strategies.

Having explored the aspect of visual and olfactory integration in *Ae. aegypti*, we learned that CO₂ can make mosquitoes pilot a fictive course and enhance their attention to an inanimate visual representation. A comparative study of spatial and temporal acuity in the mosquitoes would be a step forward to understanding how behavior can modulate visual perception. Given that mosquito host seeking behavior is state dependent, it is unknown whether gravid blood-fed female mosquitoes would prefer wide field motion, which could indicate an oviposition site. Future study is needed to determine if stages of mosquito gonotrophic cycle can influence visual and olfactory processing. The neurophysiological basis of olfactory driven behavior has only begun to be understood (Lutz et al., 2017), however relatively less is known about visual-olfactory integration in the insect brain. Addressing these research gaps would enhance our understanding of how CO₂ modulates visuomotor reflexes in insects.

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