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Infrared Radiation Directs Host-Seeking Behavior Through TRPA1 in Aedes aegypti

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# Infrared Radiation Directs Host-Seeking Behavior Through TRPA1 in *Aedes aegypti*

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular, Cellular & Developmental Biology

by

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September 2020

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# Acknowledgements

When preparing to write this dissertation, I had the privilege to take inventory of those individuals who have touched my life, and in turn, made my work possible. Without this community of individuals, my accomplishments would have been impossible, and this work is dedicated to you.

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Major Field: Mosquito Sensory Biology

## Abstract

Infrared Radiation Directs Host-Seeking Behavior Through TRPA1 in *Aedes aegypti*

By Nicolas A. DeBeaubien

Female mosquitoes employ a diverse array of sensory cues when finding hosts for blood feeding. Among these cues, the mosquito's ability to detect body heat while host seeking has been appreciated for decades. This thermosensory system is largely understood to detect convective air currents around warm surfaces that activate thermosensory neurons in the mosquito antenna. Because these convective currents occur quite physically close to the warm surface, we chose to investigate whether mosquitoes can also sense infrared radiation emitted from hosts as well, which could function at a much greater distance. To do this we developed a novel behavioral paradigm that exposes mosquitoes to infrared cues and records their behavioral responses. Using this new paradigm, we found that mosquitoes detect infrared while exposed to other host cues and use this sense to navigate towards host objects. Furthermore, this sense is influenced by both the IR intensity of the source and environmental conditions. Lastly, we found that mosquitoes lacking the thermally activated ion channel TRPA1 completely lose their ability to detect thermal infrared. This work provides the first demonstration that IR is a cue used by mosquitoes for host seeking.

# Table of Contents

# Chapter 1

## A. Mosquitoes and Vector-Borne Disease

Despite their minuscule size, mosquitoes cause more human fatalities than any other animal. Mosquitoes are so deadly because of both their global distribution and their capacity for disease transmission. As an example, a single of these diseases, Dengue, poses a risk to over one-third of the world's population [1, 2]. Furthermore, the impact mosquitoes have on public health is exacerbated by mosquito populations being endemic to tropical and subtropical regions globally, often in regions of the developing world with limited public health infrastructure. In addition to fatalities caused by mosquito borne-illness, these diseases pose a burdensome opportunity cost for affected individuals, as well as impacting economies at large [3]. Finally, regions with endemic mosquito populations are shifting and growing due to global climate change, and thus the development of effective disease prevention and population control strategies is paramount for curtailing the threats that mosquitoes pose [4-6].

There are three major genera of interest when discussing mosquitoes and human disease: *Culex*, *Anopheles*, and *Aedes*. Each of these genera contain species that spread disease to humans. For example, the mosquito *Culex pipiens* is the major vector for West Nile Virus (WNV), and the mosquito *Anopheles gambiae* is a major vector of malaria. In addition to diversity for the diseases they transmit, different mosquito species also display behavioral differences, and therefore scientific investigation in each of these species is necessary to combat the cumulative effect of mosquitoes on global health.

The mosquito we study is the Yellow Fever mosquito, *Aedes aegypti*. In addition to yellow fever, *Aedes* mosquitoes are the major vector for Dengue, Zika, and Chikungunya. These mosquitoes are diurnal, that is, they are active during daylight hours. Therefore, physical barriers like bed nets which have been highly effective in the control of malaria transmission are ineffective against these mosquitoes. *Aedes aegypti* is a largely “urban” mosquito, living in close proximity to human populations. These mosquitoes are distributed globally throughout tropical and subtropical regions but are now also invasive in new areas previously unaffected by *Aedes,* like California [7].

The reason why mosquitoes are especially pernicious with respect to human health derives from their need to blood feed. Mosquitoes are not reservoirs for pathogens like some other animal-borne illnesses, but rather mosquitoes shuttle pathogens from affected to unaffected individuals through blood feeding. It should be appreciated that in the diversity of mosquito species, only some are particularly attuned for feeding on humans. So where does the need for this blood feeding derive? Female mosquitoes are the only ones that require blood feeding. This in part is due to their diet as adults which largely consists of nectar, therefore, most species of mosquitoes require a blood meal for proper egg development and reproduction [8].

Mosquitoes are dipteran insects, like the commonly-studied fruit fly, *Drosophila melanogaster*. These species are believed to be separated by over 300 million years of evolution, however, sequencing and characterization of their genomes have revealed considerable conservation [9-11]. This evolutionary relationship is also evidenced in their basic biology as well. For example, these species follow a similar life cycle, from eggs to larvae, to pupae, which then eclose into the most recognizable adult forms. A notable difference from fruit flies however is that the developmental stages prior to eclosion take place in an aquatic environment. Despite their differences, the movement of research techniques developed in the model system *Drosophila* into mosquitoes is a rapidly expanding field, hopefully leading to the development of both a wider understanding of their basic biology as well as novel and innovative vector control strategies.

Scientists have appreciated that mosquitoes can transmit disease for over a century, which has made these organisms a necessary area of study. However, because of previous technological constraints, much of the work done in mosquitoes in the intervening decades has been limited to characterizing their basic biology and behaviors. However, recently with the adaptation of CRISPR-Cas9 genome engineering in multiple mosquito species, researchers are now capable of investigating more complicated questions of mosquito biology at the molecular level [12-14].

We previously discussed the evolutionary divergence between *Drosophila* and *Aedes*, and in this time mosquitoes developed the necessary tools to effectively find hosts for blood feeding. Unsurprisingly, mosquitoes have become amazingly attuned to detecting stimuli associated with hosts, and furthermore, these stimuli, or host cues, have been known and studied for decades. Despite this historical appreciation, researchers are still now investigating some of the basic principles underlying these specific sensory mechanisms. We will detail these various host cues used by mosquitoes in the following section.

### B. Host Cues and Mosquito Sensory Systems

Previously we discussed the evolutionary divergence between *Drosophila* and *Aedes*, and during this evolutionary period, mosquitoes have developed unique adaptations for finding hosts for blood feeding. Furthermore, some of the signals that humans and animals produce that are attractive to mosquitoes have been known for decades [15]. Recently, due to the adaptation of CRISPR genome editing in multiple mosquito species, researchers are now capable of dissecting the molecular requirements for mosquitoes to sense these host cues. The cues that mosquitoes sense that are attractive are summarized in **Figure 1**.

Humans exhale $CO_2$ at a higher concentration relative to ambient atmospheric concentrations, and this elevated $CO_2$ concentration, in an open environment, then diffuses outwards. Thus, we create a $CO_2$ gradient surrounding ourselves, which mosquitoes can utilize for guided movement towards us. It should be noted that not all mosquitoes selectively feed on humans, but rather various kinds of mosquitoes target a diverse number of animals for blood feeding, and the emission of $CO_2$ is a largely consistent trait amongst all hosts. Therefore, this attraction to $CO_2$ is a fundamental cue that mosquitoes associate with hosts. In *Aedes aegypti*, sensing of $CO_2$ is mediated in part through the gustatory receptor 3 (Gr3) localized in the maxillary palp [16, 17]. The sensation of $CO_2$ is sufficient to increase flying behaviors in female mosquitoes, as well as affecting the directionality and characteristics of these flight patterns [18]. Additionally, exposure to $CO_2$ increases the sensitivity of mosquitoes to other host cues, like skin odor [19, 20].

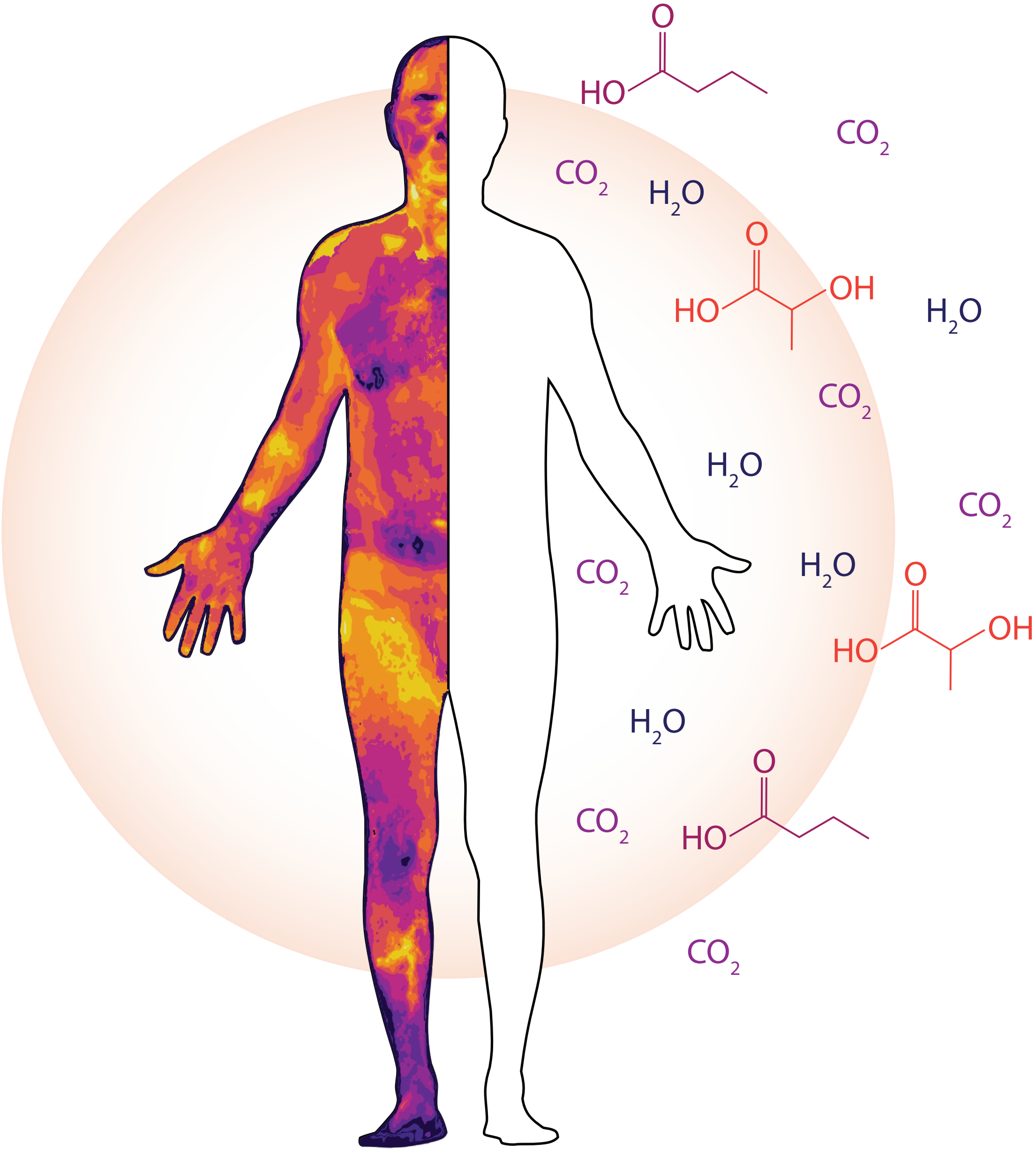

**Figure 1. Diverse Cues Enable Mosquitoes To Find Hosts.** Humans emit a number of cues that mosquitoes use for identification of targets. These cues include $CO_2$, volatile skin odors, moisture, and thermal cues.

As previously mentioned, mosquitoes also sense skin odors and compounds associated with hosts. The composition of odors emitted depends on host species, and there is even heterogeneity within species, like humans, for the amount and type of odors skin emits [21-23]. These attractive compounds consist of volatile organic compounds and non-volatile compounds found on skin. Some of these odorants can act synergistically with the aforementioned neurons that sense $CO_2$ [20]. Because both $CO_2$ and volatile odorants can diffuse far beyond the host location, these two stimuli are a fundamental component of host detection for the mosquito.

Mosquitoes also use visual cues to direct their host seeking behavior. It has been shown that when stimulated with $CO_2$, mosquitoes will fly towards a contrasting visual cue [24][. This vision-guided behavior is mediated through the mosquito compound eye. Work from our lab has demonstrated that loss of two light-receptor proteins, Op1 and Op2, together eliminates this visually directed movement while host seeking [25].

Another important cue for mosquito host seeking is temperature. Here we must distinguish between host-associated thermosensation and general thermosensory mechanisms. It has been demonstrated that insects possess a number of thermosensory mechanisms to enable them to find suitable thermal environments [26]. Furthermore, insects possess mechanisms for detection and escape from noxious thermal conditions [27]. We can consider these mechanisms of thermosensation as largely homeostatic, enabling insects to find desirable temperatures throughout their life cycle. Contrastingly, mosquitoes, and possibly other insects, possess thermosensory mechanisms that enable them to find thermal targets they associate as hosts for blood feeding.

These host-associated thermal cues play an important role in guided movement towards putative hosts. For example, mosquitoes have been shown to move towards warm objects while host seeking [28-31]. Furthermore, the sensation of host-associated thermal cues in the air is mediated through a pair of sensory neurons in the terminal segment of the mosquito antenna, in structures called large coeloconic sensilla [32-35]. Electrophysiological recordings of these neurons have demonstrated their sensitivity to warming and cooling while exposed to temperature-fluctuating air currents [32]. Loss of the thermally-activated channel TRPA1 in *Aedes* aegypti has been shown to reduce the avoidance of targets that surpass host temperatures, however, whether this mechanism is mediated through antennal neurons or contact-dependent sensory neurons in the mosquito tarsi is unclear [28]. Furthermore, loss of an ancestral cooling receptor, Ir21a, has been shown to affect mosquito thermosensation and taxis [36]. These studies were performed in conditions where mosquitoes experience conductive, convective, and radiative thermal cues, a distinction we will discuss in the next section, and therefore how these genes affect each of these mechanisms is still unclear.

The question of whether mosquitoes can sense infrared radiation, one specific mode of thermal energy transfer, has been studied and debated for decades, yet this question remains relatively open. In fact, some studies have argued that IR plays no role in thermal-based host finding in mosquitoes [30, 32]. However, these behavioral experiments were performed in the absence of other host cues, and perhaps fail to elicit IR-driven responses in mosquitoes under these conditions. Other animals that target warm-blooded hosts have been demonstrated to specifically integrate infrared radiation as a cue for host detection [37, 38]. For example, rattlesnakes possess a specialized IR-detection organ, the pit organ, that can detect infrared at a distance through TRPA1 [39, 40]. Furthermore, it has been demonstrated that tics can detect infrared cues associated with hosts as well, in some cases via specialized structures known as Haller’s organs [38, 41, 42]. Detection of infrared can be mediated both through specialized organs as previously mentioned, or as a general mechanism of thermosensation, and therefore, it stands to reason that mosquitoes should in one form or another be able to sense infrared. Whether mosquitoes can sense infrared in the host range and use this to direct their host-seeking behavior is the central focus of this study.

### C. Thermal Infrared and Heat Transfer

Mosquitoes employ a diverse array of sensory modalities to successfully find hosts for blood feeding. Many of these cues associated with hosts were identified decades ago, however, it is not until now that they are being characterized at the molecular level. One of these such cues is body heat. It has been known that body heat emitted from homeothermic animals is an attractive cue for mosquitoes [CITATION]. However, heat is in fact a complex stimulus, because the mechanism by which heat, or thermal energy, can be transferred is trifold: conduction, convection, or radiation (**Figure 2**).

The first of these modes, conduction, is arguably the most intuitive to anyone who has touched an object that is too hot and experienced a reflexive response to let it go. In a technical sense, conduction, is the mode through which thermal heat is transferred through physical contact of conductive materials. This mode of heat transfer can be simply replicated in the lab and studied. For example, work in *Drosophila* has characterized the thermal preference of larvae by exposing the larvae to a thermal gradient [43, 44]. To accomplish this, larvae are placed on an agarose pad that is differentially heated to generate a linear temperature gradient, and the larvae are allowed to freely move to regions of thermal preference. In this instance, thermal energy is being transferred from the agarose pad directly to the body wall of the fly larvae via physical contact. The temperature the larvae is experiencing is being directly conducted onto thermally activated neurons which allow the larvae to sense the thermal environment [45, 46]. The use of this

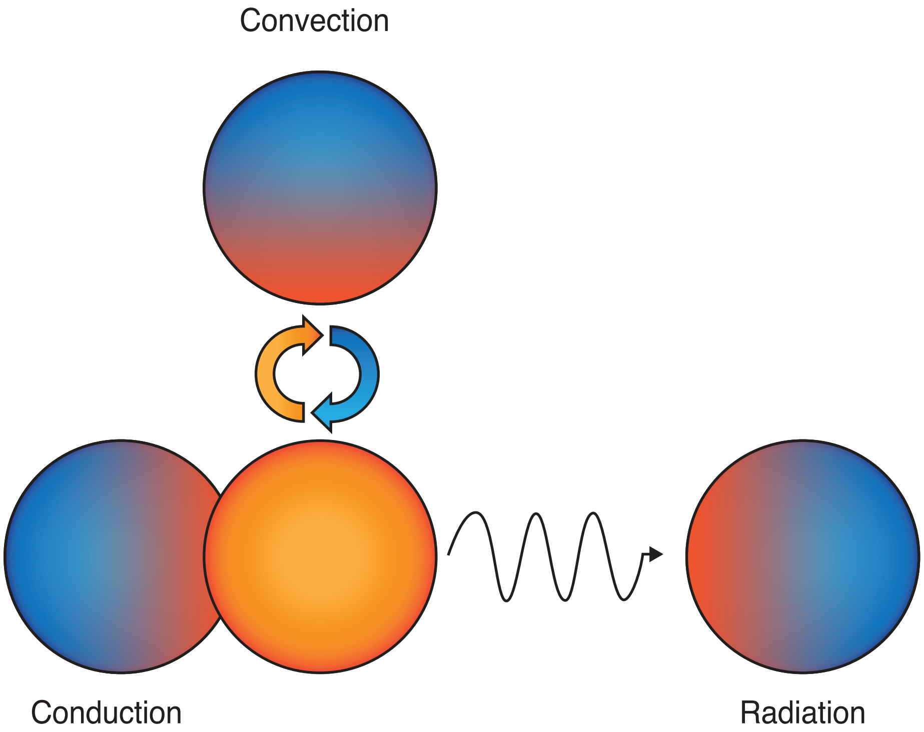

**Figure 2. Thermal energy is transferred through three modes.** The energy from a heat source can be exchanged via three mechanisms. Conduction transfers heat through a physical contact of conductive materials. Convection occurs in a fluid, like air, where warm particles rise and cold particles fall. Radiation in the infrared range is emitted from thermal objects and can be absorbed by surrounding material.

conductive-based assay allowed for the discovery of many thermally activated proteins which allow animals to sense the thermal environment.

Despite the power of these conduction-based assays, they fail to address the unique question we are posing, what components of heat are attractive to mosquitoes at a distance. To investigate this question, we must turn to the two remaining modes of heat transfer – convection and radiation.

Convection is the movement of thermal energy through a fluid such as air. The material in this case that carries the thermal energy being exchanged are the molecules in the air itself. Warmer currents of air rise, and cooler currents of air fall, forming a cyclical movement of thermal energy. This phenomenon can be more easily understood with the example of a two-story building, in which the warm air rises to the upper story, while cooler air will fall to the lower floor. The same is true of the small environment around our bodies. Air directly around our bodies is warmed by our skin, and this air moves upward as it is warmer than the surrounding environment. It has long been argued that these warm air currents alone are what stimulate the “thermal” preference in mosquito host seeking [30, 32, 47]. Work performed in mosquito and fruit fly has shown that these animals can detect temperature through this mechanism [48]. One question still remaining is that if in fact only convective air currents are attractive to mosquitoes, there is a physical limit to how far these are currents can travel, thus limiting this sense to only close proximity to the target. We propose that a sense that can function at the greatest distance of all these modes is the sense of radiation.

Radiative heating occurs through electromagnetic radiation in the infrared range. An example of infrared heating can be experienced while sitting near a bonfire, and feeling the warmth emitted from the flame. The physical experience the bystander is witnessing is not due to the hot air currents rising above the fire, nor through physical contact with the combusting material, but rather through radiative heat emitted from the source hitting their body. The radiation absorbed by the bystander causes them to feel the sensation of heat because, in fact, this absorption causes their skin to warm. Furthermore, through this example, radiation is the only means through which the bystander can experience this thermal cue at a distance.

So how does IR work? Emission of heat in the form of infrared radiation is an intrinsic property of material that has a temperature, that is, an object above absolute zero. Additionally, the waveform of the radiation is predictable based on the objects temperature and is described through Wien’s displacement law:

$$\lambda_{peak} = \frac{b}{T}$$

$$T = Absolute\ Temperature\ (K)$$

$$b = Wein's\ Displacement\ Constant$$

According to this relationship, the peak wavelength of infrared emitted from object near host temperature ranges (34 to 37 ºC) is approximately 10 µm. This wavelength falls far beyond the visual range, and therefore, this electromagnetic radiation is not absorbed and transduced through in the same way light is. Rather, the IR is absorbed by the material exposed to it depending on its physical properties, and this absorption of energy causes the temperature to increase.

The intensity of infrared emitted by a given object can also be predicted using Plank's Law:

$$E(\lambda, T) = \frac{2hc^2}{\lambda^5} \cdot \frac{1}{e^{\left(\frac{hc}{\lambda \kappa T}\right)} - 1}$$

$$h = Planck's\ constant$$

$$c = speed\ of\ light$$

$$\lambda = wavelength$$

$$\kappa = Boltzmann's\ constant$$

$$T = temperature\ (K)$$

According to this relationship, as the temperature of an object increases, the intensity of the infrared emitted also increases. Therefore, if mosquitoes do in fact sense infrared in the host range and use this information to guide their movement, they should possess a mechanism to discriminate the appropriate intensity of IR that is indicative of biological temperatures.

# Chapter 2

## A. Construction of an IR-based Attraction Assay

To specifically interrogate the question of whether mosquitoes can sense infrared radiation (IR) in the context of host seeking, we developed an assay suitable for addressing this question. We constructed the arena of the assay from custom cut acrylic panels machined generously by the UCSB Physics shop (**Figure 3A**). The arena was placed inside an incubator to maintain consistent environmental conditions while running experiments. All associated cables with this setup were run out of the incubator so that all experiments could be performed and recorded remotely while the incubator remained a closed system. One wall of the arena was cut with two recesses approximately 10 cm x 10 cm which function to house two Peltier plates. These Peltier plates were controlled using a USB interface and the surface temperature of the Peltier was measured using a thermistor and maintained at ± 0.01 ºC.

The walls of the arena were made white by application of white contact paper (Con-Tact) to both provide contrast with the mosquito bodies while imaging and to ensure the visual area of the mosquito was devoid of extraneous visual cues. The surfaces of the Peltier plates were also covered with this same material. One panel was left uncovered so to allow video recording. The Peltier plates were positioned in their recesses leaving a 1 cm gap between the surface of the Peltier and the interior surface of the arena. We then covered interior surface of the arena with four layers of Saran film, which functions as a convective barrier. Because the surface of the Peltier plates are not in contact with the Saran barrier, and air that is

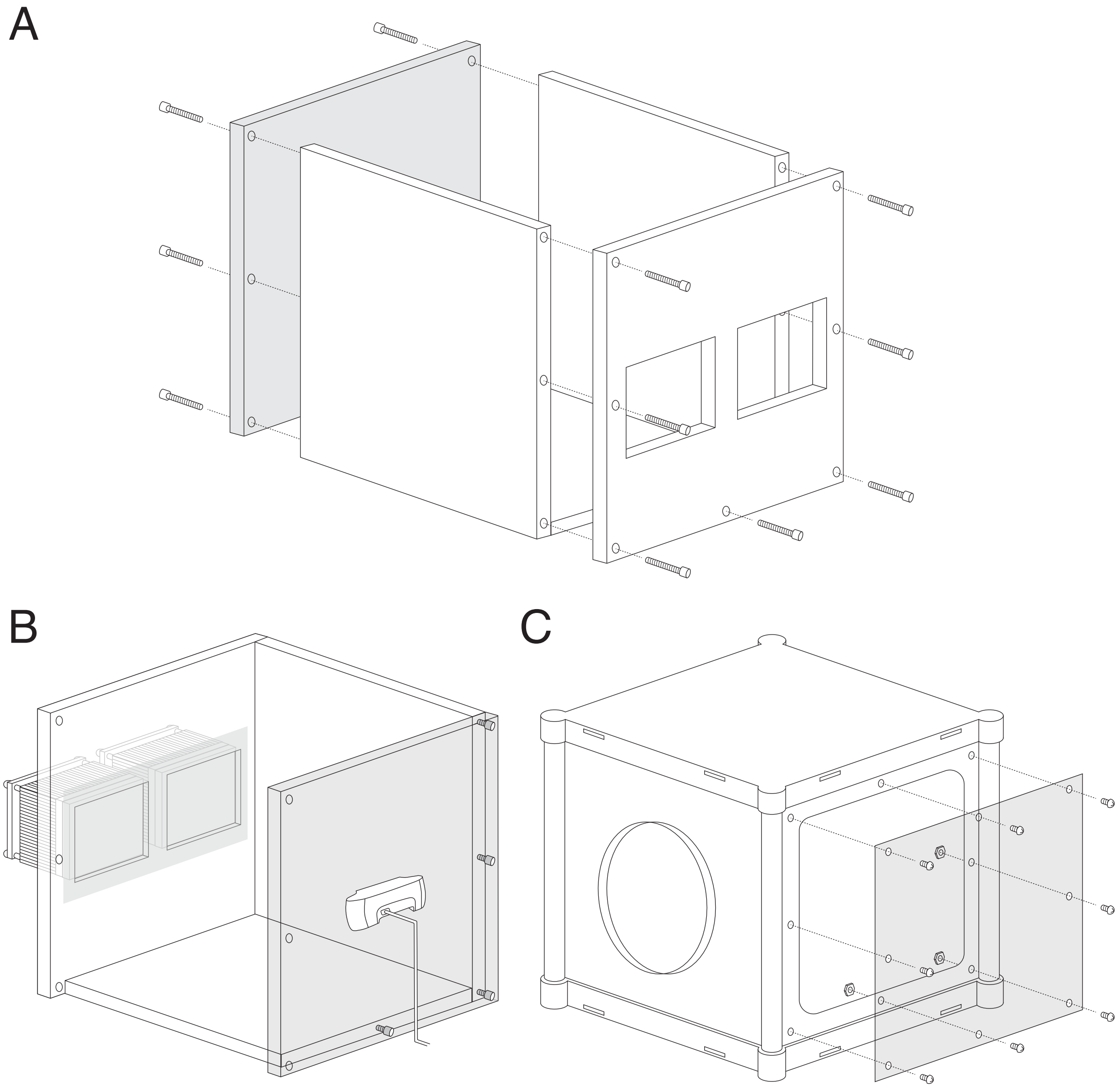

**Figure 3. Technical Drawings of Assay Components.** (A) Construction of the IR assay arena. Five panels of 1/2” acrylic were assembled as shown. One panel was cut with two 10 x 10 cm openings to house the peltier devices. All interior surfaces of the arena were covered in white contact paper, aside from the shaded panel, which was kept clear for video recording. (B) Final assembly of the IR assay arena with components (outer panel not shown for clarity). A webcam was mounted to the outside of the clear panel in a fixed position with respect to the Peltier surfaces. Two Peltier devices were mounted in the perviously described recesses. Surface of Peltier plates were were inserted 1 cm shy of flush with the interior face of the arena to provide an air gap between plates and convection barrier. (C) Construction of IR assay cages generated from a modified 30 x 30 x 30 cm mosquito rearing cage. Mosquito netting was removed from one side panel of the mosquito cage. A 1/6” panel of clear acrylic was attached to this panel as shown, to both re-enclose the cage and provide an unobstructed view for video recording.

warmed against the surface of the Peltier is blocked from reaching mosquitoes during the assay. We established that the region where the mosquito cage is positioned has the same air temperature on both the IR and control sides. Validation of the efficacy of this convective barrier is shown in **Figure 11B**.

Surrounding the perimeter of the Peltier housings we attached small perforated tubing, which was then attached to a valve which acted as a source of 5% $CO_2$ (AirGas) which was perfused into the arena during experiments. The conduits for this gas was run to outside the incubator so that perfusion of $CO_2$ could be controlled outside of the incubator while it remained closed.

We mounted a webcam (1280 x 720, 10 fps, Logitech) to the exterior of the arena positioned across from the Peltier surfaces (**Figure 3B**). By using a fixed position camera this ensured consistency across experiments to ease future video analysis.

To allow us to repeatedly measure the same group of animals, and to easily move them in and out of the arena, we modified mosquito cages (BugDorm) by replacing one vertical panel of the cage netting with a clear pane of acrylic (**Figure 3C**). This clear window was positioned opposing the surface of the cage to which we applied $CO_2$ and odor stimuli. This is a novel approach compared to other mosquito assays in which mosquitoes are freely released into the arena. This allowed us to test the same population of mosquitoes multiple times under the same experiment conditions, thus constituting technical replicates of one biological group.

### B. Video Processing

To increase the throughput of this study, we developed a set of custom video processing and object tracking programs in MATLAB to allow for automated and unbiased scoring of experiments. Because we used a fixed mounted camera for experiment recordings, we could develop optimized analysis parameters that could be used across all experiments. To do this we first ran raw experiment video through MATLAB's image processing toolbox to generate a binarized image for each frame (**Figure 4A**). Due to the high contrast between the dark bodies of the mosquitoes and the light background of the arena, we could successfully isolate mosquitoes using a thresholding function, followed by an erosion function to smooth object boundaries (**Figure 4B**). We will refer to these objects as blobs herein.

For each frame of experiment video, blob boundaries were used to calculate centroid position (the computed center of the object in two-dimensional space), and the total area of the object. When mosquitoes are standing or walking on the wall of the cage they have a stereotypical shape and size, which enables us to isolate them from erroneous objects (non-mosquito objects captured in the field of view following image processing) and flying mosquitoes which are captured as non-stereotypical blobs following image processing. A detailed demonstration of this isolation approach is shown in **Figure 7A**. Following this size exclusion step, centroid coordinates are recorded in a cell array, a data structure representing the two-dimensional position of all mosquitoes of interest for all video frames. These coordinates are then used for subsequent analysis.

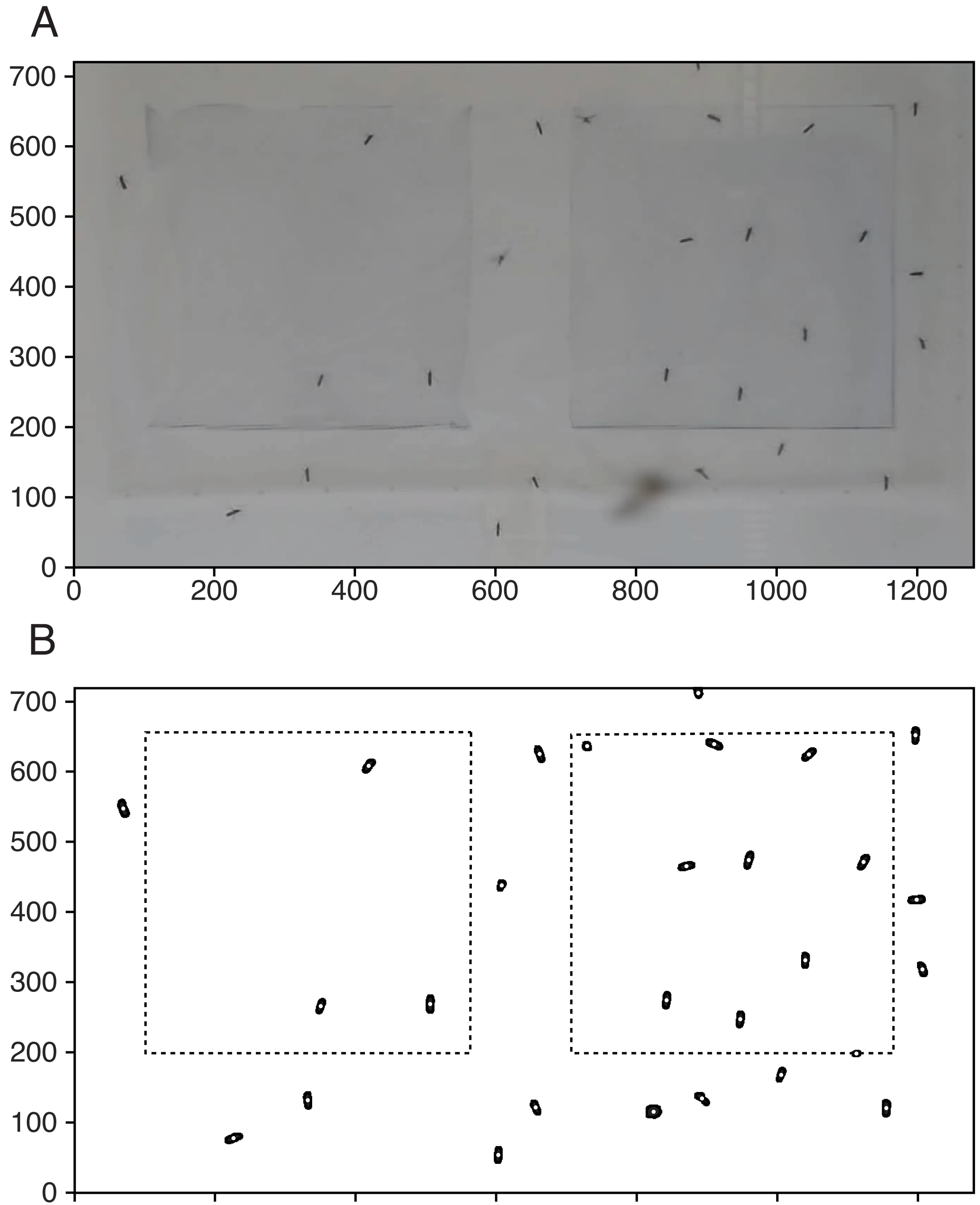

**Figure 4. Contrast-based Object Tracking.** (A) Video frame representative of typical experiment recordings. Mosquito bodies have high contrast with background of mosquito arena. Flying and landed mosquitoes are shown. (B) Video frame processed using MATLAB Image Processing Toolbox. Thresholded image isolates mosquioes of interest, and computes the centroid position of the mosquito (white dot). All units shown in pixels, video resolution 1280 x 720.

## C. Trajectory Reconstruction

As previously described, the coordinates of mosquitoes either stationary or walking on the wall of the cage are recorded for each frame of the experiment (**Figure 5A**). However, the relationship of recorded coordinates between frames at this point is unknown. Because we are interested in the behavior of mosquitoes over time, we must reconstruct trajectories of mosquitoes over time.

To do this we compared coordinate sets between a given frame (n) and the following frame (n+1). Because the interval of time between frames is short (10 fps) the number of recorded objects between frames is typically identical or very close. To determine which coordinates between frames referred to the same real object across time, we used a nearest neighbor function in MATLAB. This function takes each coordinate in a given frame, and determines the closest coordinate recorded in the next frame. The function then records this match, and additionally calculates the Cartesian plane distance between these points. This process is repeated for all frames of the experiment, and the reconstructed trajectories are recorded in a data structure, along with their interpoint distance.

As mentioned above, the number of points between frames is typically the same. However, as objects enter or leave the visual field, or are removed or added by the size exclusion function, the number of objects recorded between frames differ. The program we developed to reconstruct trajectories accommodates this by starting new trajectories for objects that appear in the visual field or ending trajectories for objects that leave the visual field (**Figure 5B**). The decision to begin

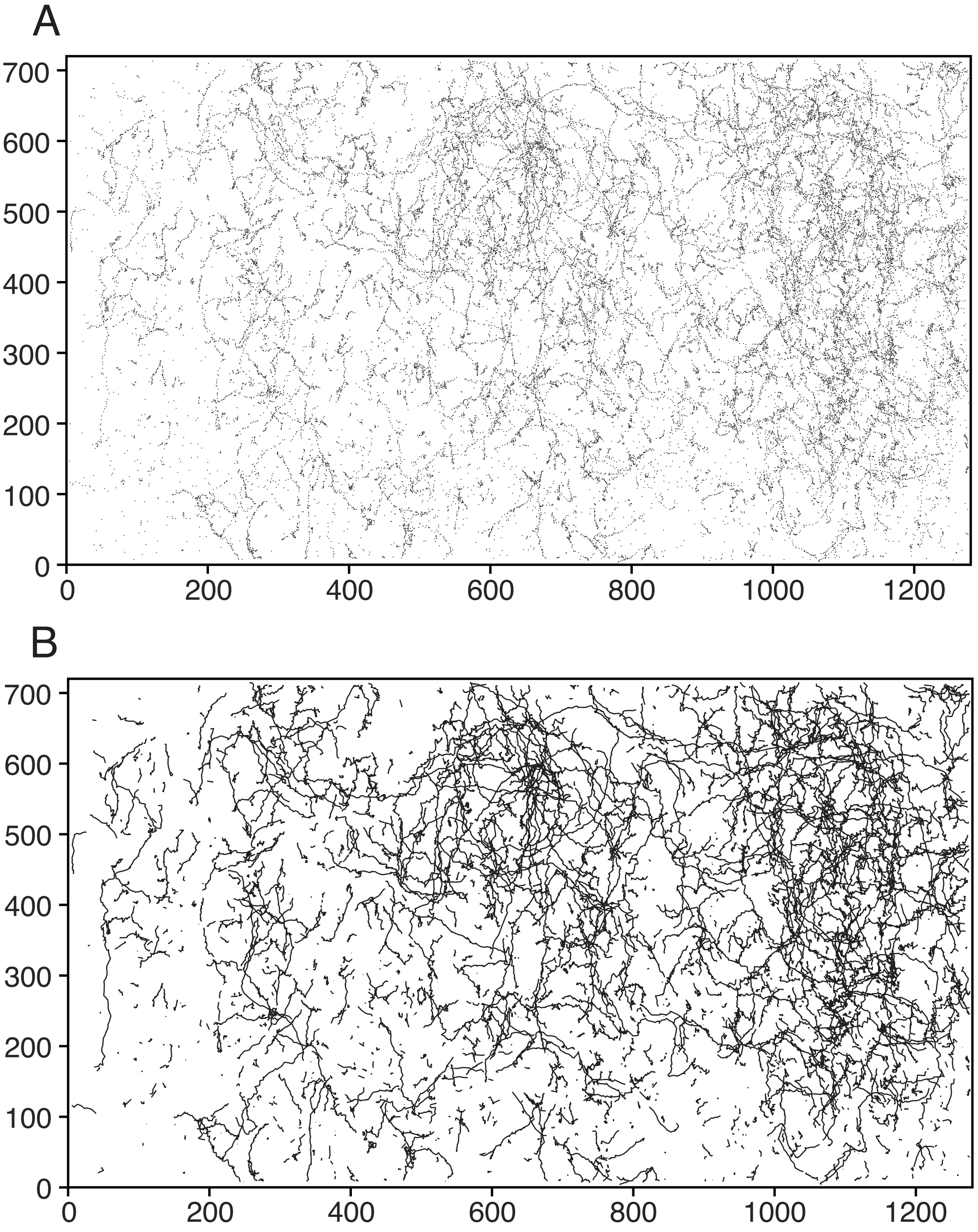

**Figure 5. Automated Video Tracking and Trajectory Reconstruction.** (A) Position of all recorded mosquitoes within a real, representative 5-minute experiment. Dots shown are the remaining data following size exclusion filter. (B) Reconstruction of all trajectories observed in A. Trajectories are filtered by both minimum and maximum velocity threshold to isolate walking mosquitoes from flying and stationary mosquitoes. All units shown in pixels, video resolution 1280 x 720.

or end trajectories is determined by the nearest neighbor function. If the nearest neighbor function determines the interpoint distance to be beyond an empirically determined parameter (**Figure 7B**) the trajectory is ended at that point. If a coordinate pair did not have a match in the previous frame, it is considered to be the start of a new trajectory.

### D. Assay Conditions

It is known that low percentage (~4-5% as opposed to 0.04% in environmental air) stimulates host seeking in females. Additionally, among the repertoire of attractants females use for host seeking, volatile skin olfactants tune females towards hosts. Therefore, with the addition of IR we sought to test what permutations of stimulus combinations evoked the strongest response. We propose that experiment conditions that stimulate the highest fraction of the population to host seek will generate a larger number of points for analysis, and thus be less prone to wide variances due to chance.

To determine which combinations of stimuli evoked the strongest response, we measured the overall host seeking activity in a 5-minute experiment period under various conditions (**Figure 6**). We define “host seeking” behavior as mosquitoes that land on the wall of the cage in the visual field, and that subsequently move about this region, probing through the netting of the cage. This type of behavior enables an animal to find a vein for a blood meal when it lands on a host. Furthermore, we measure these mosquitoes as those that travel a minimum distance between frames (videos recorded at 10 fps). As an intuitive metric, we define “host seeking activity” as the average number of mosquitoes observed host seeking per frame. Thus, a host seeking activity of 5 indicates, on average there are 5 mosquitoes exhibiting host seeking behaviors.

We found that no stimuli, $CO_2$ alone, and IR alone evoked a near-zero response. This is consistent with previous publications that have argued against the function of IR in host seeking [30]. It has been shown that mosquitoes are stimulated

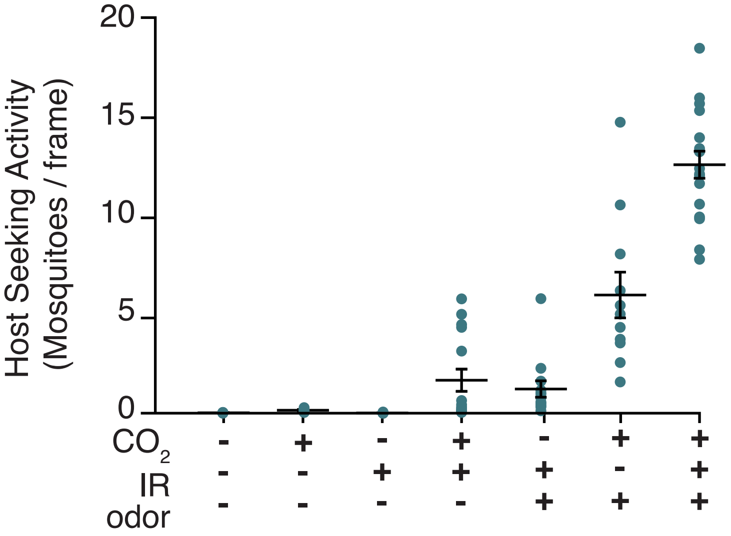

**Figure 6. Increasing Host Seeking Rate with stimuli.** Host seeking response evoked by combinations of stimuli. Mean of biological and technical replicates, error bar = SEM.

by elevated CO2 concentrations relative to atmospheric levels, at a distance [49-52]. However, in this study we are tightly defining our behavior of interest, and therefore any elevation in flying behaviors, landing behaviors, etc. are not represented in this data. We then found that the response to IR with $CO_2$, and IR with odor were similar, and mosquitoes began to display probing behaviors.

In the presence of $CO_2$ and odor, the response was elevated compared to conditions previously discussed. We should note, that this increased reaction to these stimuli could be, in part, due to the movement of $CO_2$ acting as a vehicle for the movement of volatile skin odorants. That is, as $CO_2$ is perfused into the cage, this movement of gas brings with it higher concentrations of odorants deeper into the cage. Additionally, the increase in this response could be, in part, also due to underlying sensory integration in the mosquito, which activates a stronger response than these stimuli *per se*. Lastly, we observed the strongest overall host seeking response in the presence of all three stimuli, indicating this condition should be used for all further experiments. Here we demonstrate that mosquitoes integrate multiple sensory cues to initiate probing behaviors. The standard conditions used for IR-assays throughout this paper are 28°C ambient temperature, 34°C IR-source, with 5% (v/v) perfused $CO_2$ and skin odorants.

### E. Optimization of Mosquito Tracking

Mosquitoes which are actively host seeking extend their proboscises through the netting of the mosquito cage in attempts to make contact with skin. Additionally, as these mosquitoes fail to make contact with skin, they explore the two-dimensional space in efforts to make contact. Therefore, mosquitoes that have landed on the wall of the cage within the visual field represent two distinct behavioral populations, stationary and host seeking. We chose to only focus those mosquitoes which were actively host seeking and use these data for further analysis. Additionally, these mosquitoes must also be separated from flying mosquitoes that appear in the visual field.

The first method for selectively identifying mosquitoes that are on the wall of the cage was to filter the incoming data based on object size. To determine the object size most representative of landed mosquitoes, we analyzed all recorded object sizes recorded during a 5-minute experiment (**Figure 7A**). We then inspected the distribution of object sizes and found two overlapping distributions (dashed lines indicating distributions were added manually for emphasis). The first distribution, which was small with respect to both object size and its overall number of observations, is closely correlated to flying mosquitoes. The reason that flying objects appear as smaller objects following image processing is that they appear more diffuse on video. The second distribution, larger with respect to both object size and overall number of observations, captures the mosquitoes of interest, that is, mosquitoes that are landed on the wall of the cage. To selectively capture these mosquitoes, we filtered the object size parameter used in our MATLAB script to

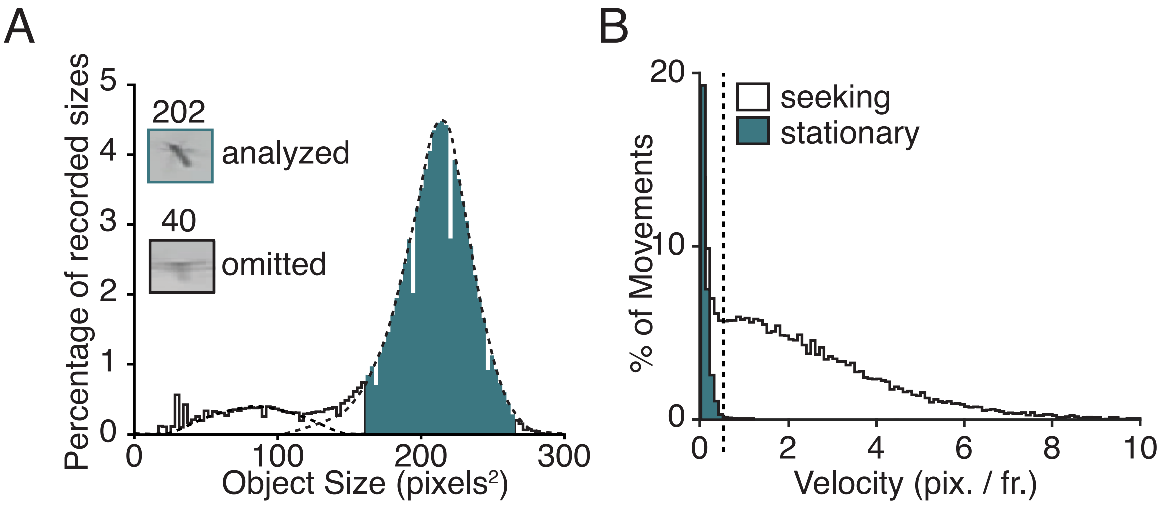

**Figure 7. Isolation of host-seeking mosquitoes.** (A) Distribution of object sizes from real experiment data. Representative images of flying and landed mosquitoes shown with recorded object size (pixels$^2$). Dashed line added manually to show overlapping distributions. (B) Distribution of velocities recorded in a composite data set of known walking and stationary mosquitoes (pix. = pixels, fr. = frame). Dashed line indicating 0.5 ppf (pixels per frame) minimum velocity threshold.

include those between 170 and 270 pixels$^2$. To determine the error rate of this parameter, we generated a random sample of objects falling within these parameters, and manually scored the corresponding original images and found a false identification rate of 1% (10/1000 samples, data not included). As we will discuss, we use additional filters for selectively identifying mosquitoes of interest, and therefore, this was an acceptable rate. This parameter was used in video analysis throughout this study. We should also note that all parameters defined in this study may be adjusted for future applications of this program.

Next, we sought to define a speed parameter that successfully captured walking mosquitoes apart from stationary and erroneously included flying mosquitoes (**Figure 7B**). By observing the real trajectories of walking mosquitoes, an upper limit of 10 pixels/frame retained nearly all data relating to walking mosquitoes, while excluding faster moving objects, likely observations of flying mosquitoes. Despite using the terminology "stationary", non-moving mosquitoes are rarely calculated to be at the same centroid position between frames. This is due to small image fluctuations during video recording, in part attributable to signal noise, which cause the computed centroid position to fluctuate, known as "object jitter". Thus, to successfully separate these two behaviorally distinct objects we must set a minimum velocity threshold for walking mosquitoes.

To address this question, we generated a composite data set of manually identified traces that were representative of *stationary* and *walking* populations. These trajectories were manually extracted from real experiment recordings so that they were as representative as possible (**Figure 8A**). When

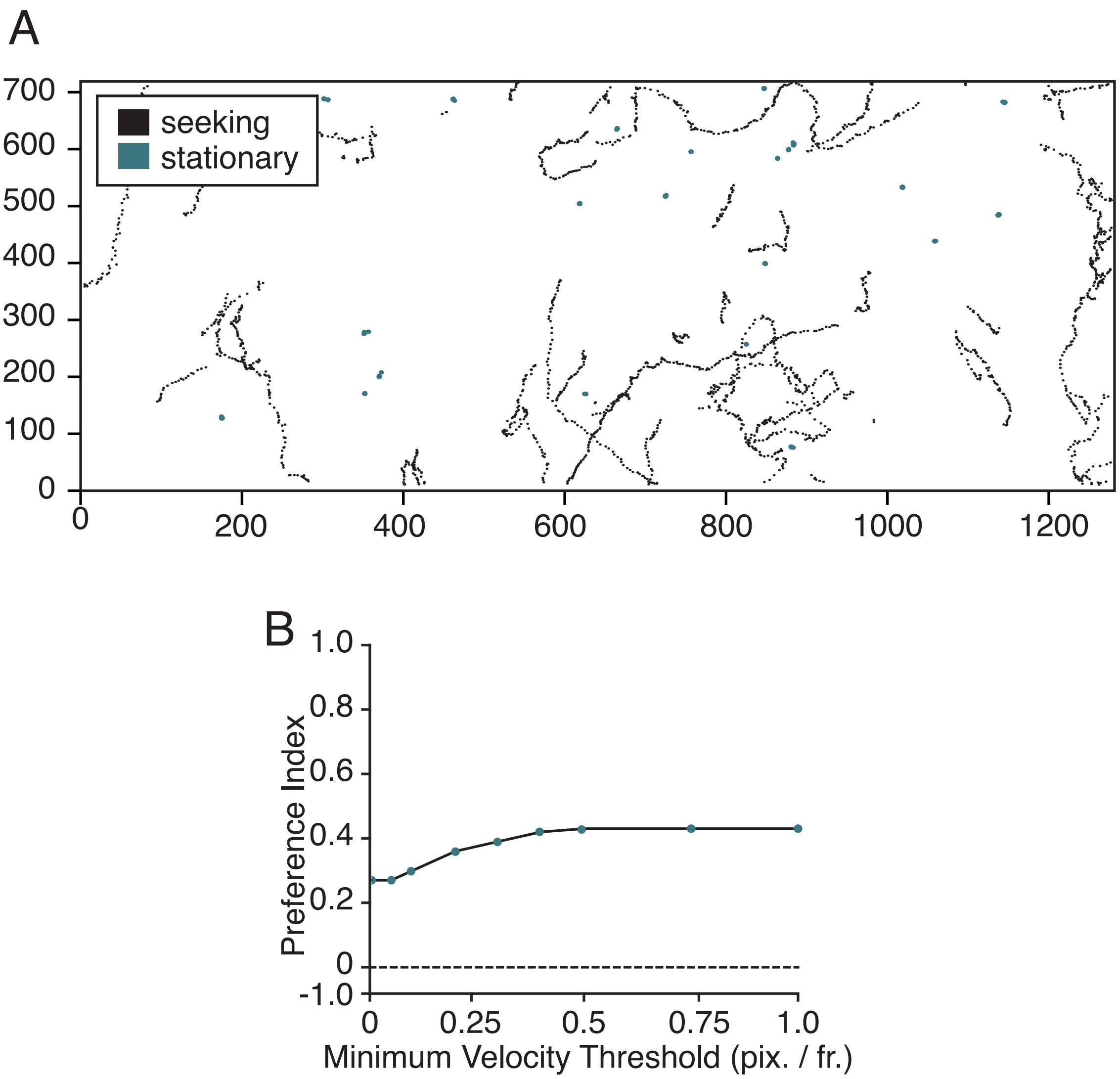

**Figure 8. Inclusion of stationary mosquitoes skews analysis.** (A) A composite data set of known walking and stationary mosqutoes generated from manually selecting real experiment data. (B) Calculated preference index of a composite data set of known walking and stationary mosquitoes at various minimum velocity thresholds.

looking at the distribution of velocities we see that the *object jitter* ranges from approximately 0 to 0.5 pixels/frame, which overlaps with a percentage of velocities seen in seeking objects (**Figure 6B**). While mosquitoes are seeking, their movements are discontinuous, and therefore, this overlap may represent stopping points, turning points, etc. during a seeking bout. However, because of this ambiguity, we choose to set a minimum velocity threshold of 0.5 pixels/frame to indicate *seeking* behavior. To underscore the importance of this threshold and to demonstrate how stationary mosquitoes can impact the scoring of assays we used this composite data set as a representative experiment sample, and ran analysis using a range of minimum velocity thresholds and recorded to the resulting calculated preference index (**Figure 7B**). We saw that in this representative data set, failure to exclude stationary mosquitoes may heavily skew the resulting preference index. Furthermore, increasing beyond a threshold of 0.5 pixels / frame, there was no effect on the final preference index. We use this empirically determined threshold throughout this study and may be modified for future applications depending on experiment setup or design.

### F. Minimum Conditions for Analysis

As with many behavioral paradigms, criteria must be established to determine whether experiments meet a minimum allowable threshold for analysis. For example, some aspects of this assay are derived from the *Drosophila* binary choice assay, in which, a minimum fraction of flies must eat the colored die for the assay to be used. For our IR assay, the scoring method is different, and thus, a threshold for analysis cannot be used in which the number of overall responding mosquitoes is the metric. Our assay measures the overall response of mosquitoes in the assay by tabulating the number of observations or frames in which objects correspond to trajectories of seeking mosquitoes. This cumulative value is then divided by the number of frames in the experiment recording (10 fps, 5-min recordings) to provide a more intuitive metric for overall response. This metric represents the number of mosquitoes on average that are actively seeking during an experiment. As shown above (**Figure 6**) this value can vary greatly under experimental conditions. Additionally, other factors may impact the observed response like circadian rhythm, metabolic state, or age. We have controlled for these factors, however, we still see a variance in the overall response rate, even between technical replicates of the same biological group, hence, the development of an empirical criterion for inclusion or exclusion of data is necessary.

### G. Modeling the Effect of Response Rate on Assay Variance

When developing and optimizing this assay, our logic held that higher overall response rates would require more data points for analysis, and therefore should be a better representation of the overall features of the population being studied. Additionally, we held that at a certain response rate, the effect of random variance in the data would reach an acceptable level. To provide some evidence for the effect that small response rates has on experimental outcomes, we developed a model to simulate the distribution of outcomes that could be expected at certain response rates.

Parameters of the model were informed by experiment data, like walking bout length, walking speed, and walking orientation. We developed a model by taking the dimensions of the visual field used in our assay (1280 x 720 pixels) as well as zone coordinates taken from real experiments to generate a fictive area with which we could populate mosquitoes. We have shown that walking mosquitoes in our assay have an interframe velocity from 0 to 10 pixels (**Figure 9A**). To mimic this walking behavior, mosquitoes that are placed in the model travel on a random walk and each frame can move between 0 to 9 pixels (this upper limit is defined by the limits of displacement in the x- and y-dimension). Furthermore, this range captures the bulk of experimentally overserved velocities (**Figure 7B**). To approximate the shape of mosquito walking trajectories we analyzed the directionality of all real traces from a 5-minute experiment. To do this we calculated the angle, $\theta$, between the x-axis and the displacement vector of the movement. By analyzing the distribution of these headings, we found that upward movement

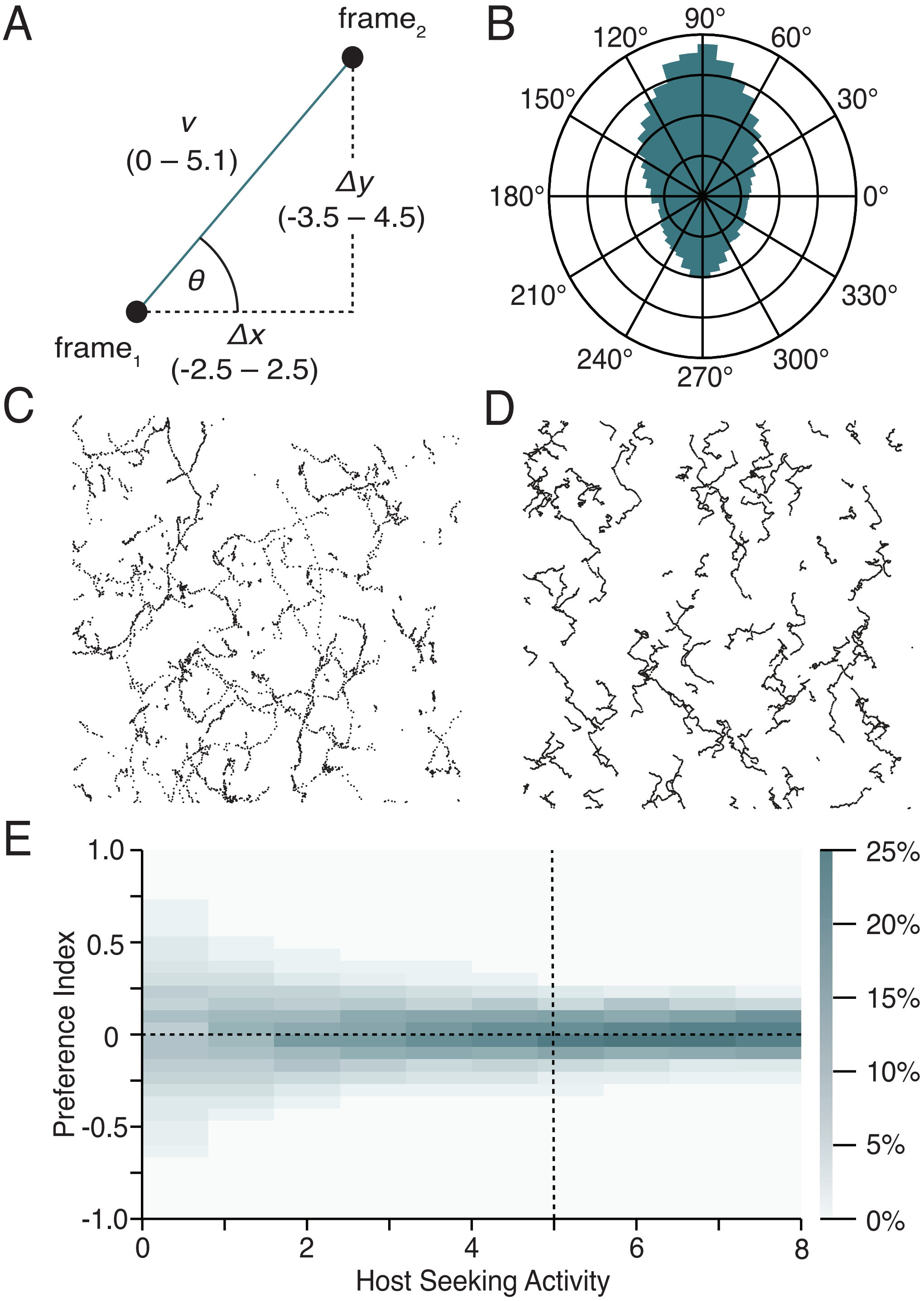

**Figure 9. Development of Scoring Threshold.** (A) Movement paramameters of the model. Ranges of displacement informed by real experiment data. Displacements selected randomly from a normal distribution in the ranges shown. The overall displacement is consisent with real experiment range values. (B) Distribution of angular movements observed in a 5-minute experiment. (C) Representative traces of trajectories observed in real experiments (whole view not displayed). (D) Representative traces generated by the model (whole view not displayed). (E) Distribution of model outcome PIs at varied host seeking activitlies. Number of mosquitoes in the model ranged from 1 to 10, and was run at 1,000 iterations. Output mosquito traces were then analyzed like real data and host seeking activity and PI were recorded.

(positive movements in the y-axis) were favored by the mosquito, which is evocative of the *Drosophila* negative-geotaxis behavior (**Figure 9B**). Importantly, the assay from which we drew these data had a neutral preference index (P.I. ≈ 0.0), and commensurately we saw no bias in direction in the distribution of headings with respect to the x-axis. Lastly, the greatest represented headings in this distribution were upward, as compared to lateral or downward, indicating an overall bias for upward displacements. Together, these data informed the mosquito movement in our model, in which the code that generates the random walk between frames biases upward movement in the y-direction, and additionally biases the magnitude of displacement in the y-direction to contribute more to the displacement vector.

To show how real traces from experiments (**Figure 9C**) compare to these fictive traces, a random sample of these traces are shown (**Figure 9D**).

To model the behavior of mosquitoes in our assay under neutral conditions, that is with equal distribution of stimuli, we placed mosquitoes in this fictive field and allowed for 30 sec of random walk. After this, a new group of mosquitoes were populated in the field and then allowed to walk randomly. A total of ten groups were added per iteration of the model, and then then the distribution of points recorded were fed into the preference index calculation and recorded.

We then examined the distribution of model outcomes by analyzing the variance observed across all model iterations. Because mosquitoes are randomly populated in the fictive field, the resulting average preference index for all model iterations should approximate 0 (**Figure 9E**). We then looked at the standard deviation that resulted at each of these response levels and found there to be a non-linear decrease in the overall variance as response rates increased. Intuitively, at low levels of mosquito host seeking, the variance attributable to chance was relatively high, and therefore inclusion of these data points could contribute significant variance in this study. According to the results of this model the decrease observed in result variance as overall activity increases is non-linear, and at high levels of mosquito response there becomes smaller and smaller benefit with respect to the reduction in outcome variance. Furthermore, setting a threshold for acceptable experiments at such a high level would require the use of many more animals, result in the exclusion of much more experiments, and be quite burdensome. For all of these reasons we have identified a Host Seeking Activity index of 5 to be an ideal threshold that experiments must meet for inclusion in this study. We believe that at this level, experimental outcomes should reliably approximate the population mean, and across replicates produce results with suitable variance for the power of this study.

### H. Scoring

Following trajectory reconstruction, and isolation of trajectories associated with host seeking, the tracking program calculates the distribution of all remaining coordinates to determine the overall preference index for that experiment. To do this, the program computes which coordinates fall within the boundaries of the IR-associated zone or the reference zone. The overall preference index for the 5-minute experiments is calculated as:

$$Preference\ Index\ (P.I.) = \frac{Counts_{zone\ 2} - Counts_{zone\ 1}}{Total\ Counts_{zone\ 2+zone\ 1}}$$

Aside from the preference index, the program scores the overall host seeking score by dividing the total number of host-seeking observations by the total number of frames (3000 frames for a 5-min experiment recording). This value indicates the average number of host seeking mosquitoes in an experiment at any given time. This value is then used to evaluate the quality of the data as described in the response rate modeling section.

These two metrics constitute the bulk of the analysis in this study. However, video recording and analysis is a powerful tool to capture other features present in an experiment. As shown in the section *Correlations Show IR-Sensation Occurs In Flight*, the program we developed can also capture features like average time the mosquito spends in the zone per bout, the average bout distance travelled, and the total number of bouts per zone.

# Chapter 3

## A. Female mosquitoes sense infrared to direct host seeking

To investigate if female mosquitoes detect the infrared (IR) component of heat we constructed a behavioral assay wherein host-seeking mosquitoes land in either IR-positive or IR-negative zones. Five-minute recordings of these assays were analyzed using a suite of MATLAB scripts to generate an overall preference index (P.I.). To ensure the air temperature in these zones remains unaffected throughout the experiment we measured using a shielded temperature probe, such that it measured the ambient temperature without receiving incident IR from the Peltier device (**Figure 10A**). Importantly, the ambient air temperature 1 cm directly opposing the Peltier sources remained unchanged within a range of Peltier settings of 34-37 ºC. The measured temperature recordings represent an ambient air temperature range because of the nature of the incubator used in these experiments (~2 min. cycles ± 1.5 ºC).

When mosquitoes were assayed under neutral conditions (both Peltier devices turned off), the resulting P.I. of 0.02 indicates that the assay is balanced with no inherent preference for any zone observed (**Figure 10A**). When the Peltier device was turned on (34 ºC) the wildtype mosquitoes preferred the IR Source (P.I. = 0.58). To further demonstrate that the IR emitted from the Peltier surface was specifically driving this preference, we obscured the IR source behind a sheet of aluminum foil (low IR transmission). This reduction in IR intensity as revealed through infrared thermography (**Figure 10A**) caused a commensurate reduction in the overall preference for the IR+ zone (P.I. = 0.28). To further reduce the intensity of IR we

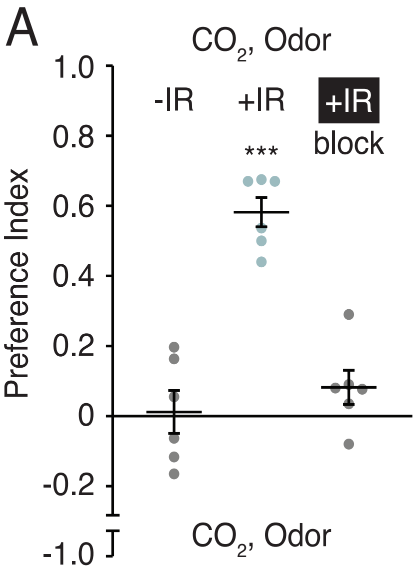

**Figure 10. Female Mosquitoes Sense IR While Host Seeking.** (A) Female mosquitoes show no preference in the absence of additional IR stimulus. When IR is added, the preference index observed significantly favors the IR zone. When transmission of IR to the mosquitoes is physically blocked, the preference returns to neutral. Data analyzed using One-way ANOVA followed by a multiple comparison test (*** $P < 0.001$).

blocked the incident radiation using a combination of acrylic, foil, and foam (Fig. 1B) and saw a further reduction in the overall preference for the IR+ zone. Together these data show that female mosquitoes may sense infrared radiation from a thermal body in the absence of convective or conductive inputs and use this stimulus to direct host seeking. This represents the first demonstration that IR is a sensory cue that *Aedes aegypi* use in combination with other cues for host seeking.

### B. Infrared preference is intensity-dependent

According to the Stefan-Boltzmann equation, the intensity of IR emitted from a thermal body is intrinsically linked to its temperature. Therefore, increasingly hot objects will emit a greater intensity of IR. To determine if an intensity threshold exists with respect to IR-directed host seeking, we compared the relative attractiveness of various IR-intensities with a reference intensity of ~28 ºC (**Figure 11A**). When relatively low intensities were investigated (25 ºC vs. 28 ºC), there was no clear preference for either zone. This result indicates that despite the intensity of IR emitted from the 28 ºC zone being greater than that of 25 ºC, the resulting intensity falls below a critical threshold to influence host seeing behavior. We next tested whether slightly warmer temperatures could elicit preference in these mosquitoes by testing 31 ºC vs. 28 ºC (**Figure 11A**). IR-intensity at this temperature was sufficient to elicit slight preference for the IR-zone (P.I. = 0.13), and was greatest at 34 ºC (P.I. = 0.58). Additionally, there was no further increase in preference above 34 ºC (37 ºC, P.I. = 0.51), indicating a non-linear relationship between IR intensity and preference observed (**Figure 11A**). In all, these data demonstrate an IR-specific detection of thermal objects within a biologically significant range (31-37 ºC). Moreover, we found that IR detection peaked when the IR source was at a temperature (34 ºC), which is most typical of skin temperature.

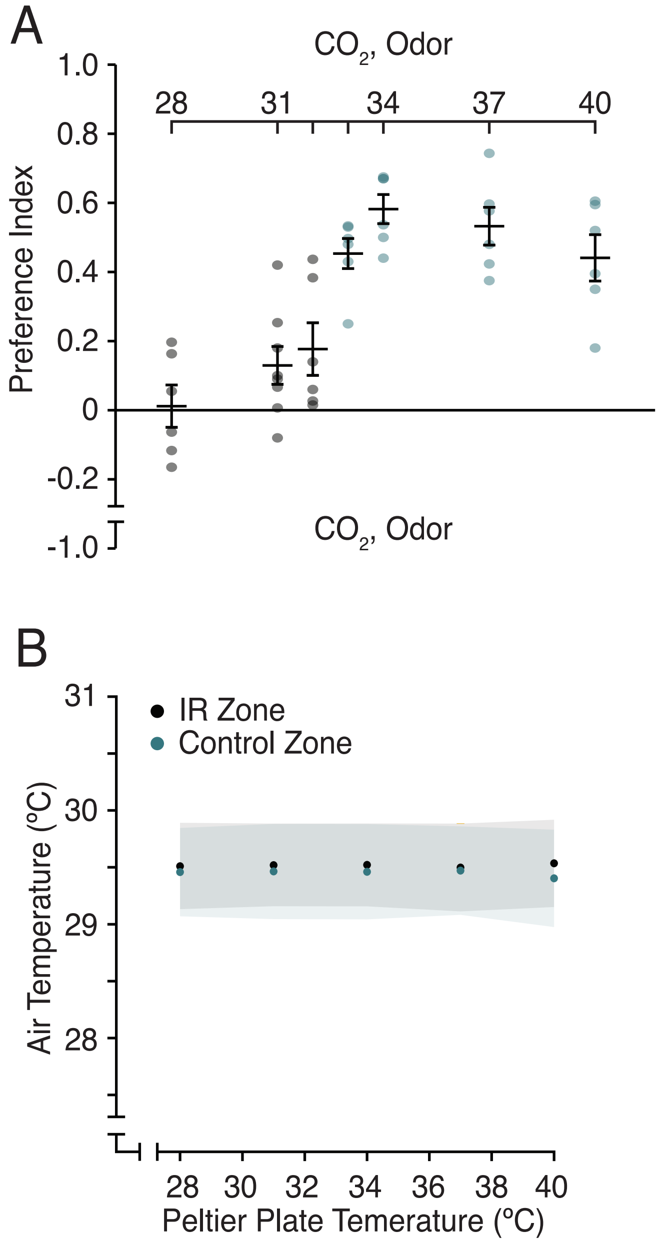

**Figure 11. Mosquito IR preference is intensity-specific.** (A) The recorded preference index under differing IR source intensities. The intensity of IR was modified by setting the Peltier plate to the indicated temperature (degrees Celsius). (B) Air temperature measurements taken 4 cm away from the surface of the arena housing the Peltier plate, taken in front of the IR source plate (black) and the reference plate (blue). Data was collected for 5 minutes at 10 second intervals and fit to a sine curve model of temperature fluctuaiton. Shaded regions indicate the minima and maxima of the temperature curves.

### C. Infrared discrimination depends on ambient conditions

For female mosquitoes to successfully find hosts for blood feeding, they must integrate multiple senses to discriminate between host and non-host objects. Therefore, if IR-sensation plays a role in host discrimination, the emission of IR from the thermal host must contrast with the environmental landscape. To study this question, we set the Peltier IR source to 34 ºC as it previously elicited the strongest preference response. We then performed choice assays under increasingly hot conditions to distinguish if the preference for 34 ºC was intrinsic or relied on comparative discrimination between two zones. To increase the ambient thermal profile of the arena, we increased the temperature setting of the incubator which housed our arena. To then identify the IR profile of the reference zone we used IR thermography to measure each surface (**Figure 12A**). Notably, due to the convective heating of the incubator, the measured temperature of the reference zones fell below the incubator set temperature (37.0 ºC set, 34.1 ºC observed). Despite these differences, the preference response of mosquitoes was still measured over a range of ambient conditions (28 ºC to 37 ºC). Within this range, the preference for the IR+ zone was strongly linearly correlated with the magnitude of temperature difference between the two zones, (**Figure 12B**, $R^2 = 0.99$).

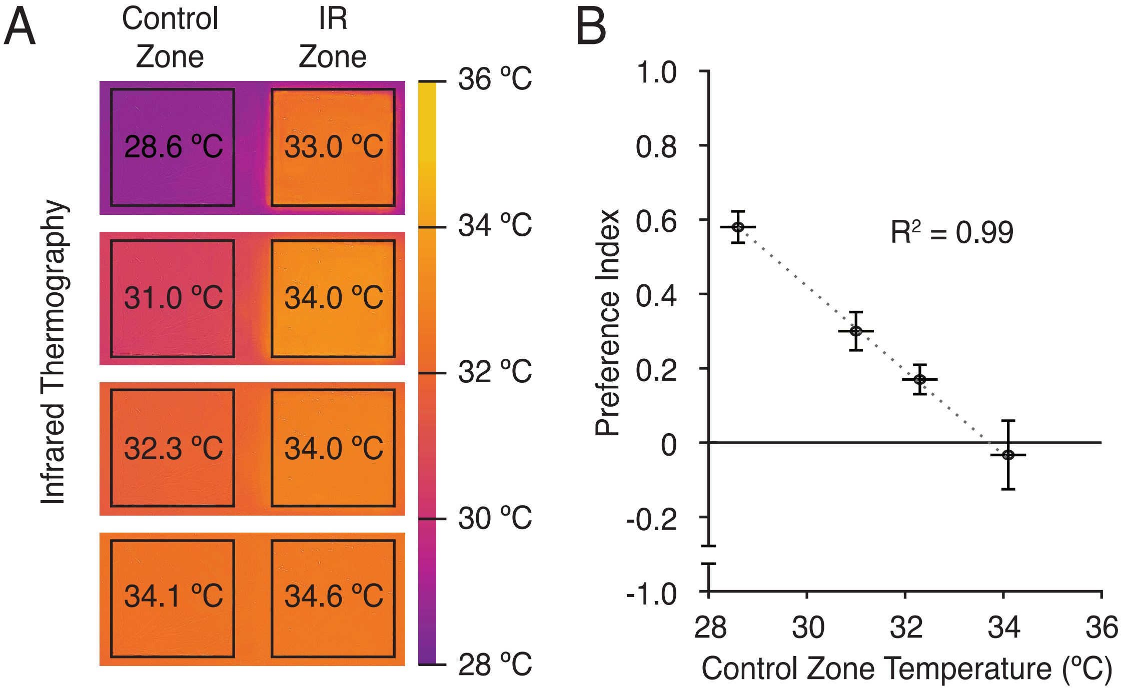

**Figure 12. Warmer environments eliminate the preference for IR.** (A) The ambient temperature of the experiment setup was manipulated by changing the temperature setting of the incubator arena housing. After the system reached equilibrium and without opening the incubator, apparent surface temperatures (IR emission intensities) were measured using infrared thermography (FLIR One). (B) Observed preference index of mosquitoes host seeking in the four conditions shown in A.

### D. The cation channel TRPA1 is required for IR-sensation

Previous studies have investigated the molecular components for thermosensation in the host range for mosquitoes (CITATION). However, presently no studies have identified behavioral responses elicited by elimination of these molecules within host range temperatures. To further characterize the molecular components required for IR-sensation, we proposed a model that incident IR is somehow translated to thermal signals that turn on thermally-activated channels in the mosquito. Building on evidence in *Drosophila* and mosquito studies, we screened available candidate genes for thermally-activated channels (**Figure 13A**). In tandem with previous results showing strong wildtype preference in Liverpool (LVP) wildtype mosquitoes, we tested an additional wildtype strain (Orlando, ORL) for IR-sensation. Similarly, this line showed robust response to IR (P.I. = 0.63). These results show that IR-sensation is conserved across distinct wild isolates of *Aedes aegypti*.

The gene candidates we aimed to test included the family of transient receptor potential (TRP) channels, opsins, as well as a gustatory receptor (GR) gene homolog shown to be thermally activated in *Drosophila*. Firstly, elimination of Gr19a ($Gr19a^{DsRed}$), showed no effect on IR-directed preference (Figure 4A). This result together with previous studies may suggest that despite its sequence conservation, Gr19a may not play a thermotaxis-related role in *Aedes aegypti*. Furthermore, the *Aedes* homolog of the *Drosophila* thermally-activated TRP channel Painless, AAEL006538 (herein referred to as $pain^1$), did not affect the response of female mosquitoes to IR.

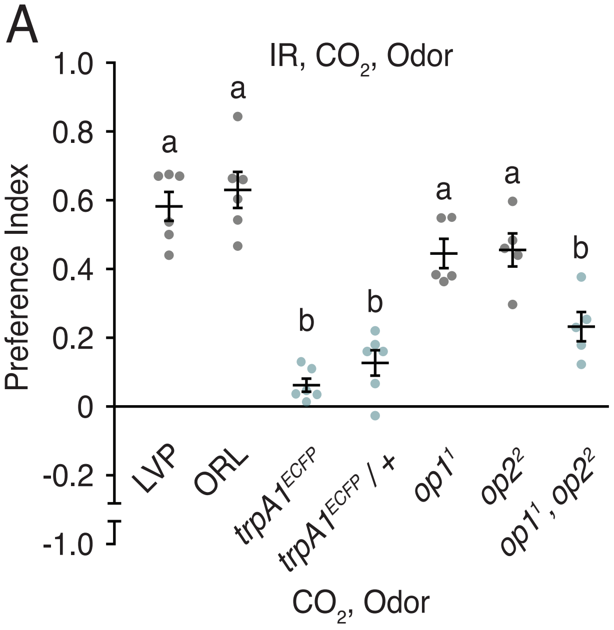

**Figure 13. IR sensation is affected by loss of TRPA1, Op1, and Op2.** (A) Measurements of preference for an infrared source set at 34°C. Two independent wildype strains Liverpool (LVP) and Orlando (ORL) show consisent attraction to the infrared source. Loss of TRPA1 eliminates the preference for infrared. Statistical significance determined by One-way ANOVA followed by a multiple comparison test. Groups a and b are statistically significant ($P < 0.001$).

The *Aedes* TRPA channel TRPA1 has been shown to be thermally-activated in heterologous expression systems [53]. To test if Aedes TRPA1 is required for IR-directed host seeking, we tested trpA1$^{ECPF}$ mosquitoes in the IR-Preference assay. The *trpA1*$^{ECFP}$ mutants showed a significant decrease in their IR preference (P.I. = 0.22). This allele was generated in the ORL background, therefore effects from the strain lineage on IR preference is unlikely as ORL mosquitoes behave normally in this paradigm (Fig. 4A). Since TRPA1 is thermally activated with a threshold of 32 ºC, these data support the model that IR radiation is detected by heating up local tissue, leading to thermal activation of TRPA1 [53].

Interestingly, we assayed the IR preference of heterozygous trpA1$^{ECFP}$/+ mosquitoes and observed a similar reduction in IR response to that of homozygous trpA1$^{ECFP}$ mosquitoes. This result may be due in part to the nature of the TRPA1$^{ECFP}$ allele. Insertion of the fluorescent reporter transgene and disruption of the endogenous trpA1 CDS occurs downstream of the coding region for transmembrane region 5 (TM5) in a loop region known as the pore loop. Truncated expression of this protein may contribute to malformation of TRPA1 homomultimers *in vivo*, which would eliminate its channel ability.

### E. The *Aedes* opsin genes op1 and op2 function in IR-sensation

Opsins are canonical light-sensors in animals. In addition, our lab has uncovered light-independent functions for Drosophila opsins in bitter taste, thermotaxis, and suppression of sugar attraction by cool temperatures [43, 54, 55]. Because of their known function in *Drosophila* temperature sensation, we screened the available mutant alleles of opsins. Moreover, based on RNAseq data, both of these opsin RNAs appear to be expressed in antennae, which have roles in thermosensation [56]. The opsins op1 (AAEL006498) and op2 (AAEL006529) displayed slight reductions in their IR preferences, however, these results were not statistically significant (**Figure 13A**). Due to their sequence similarity and expression in antennae, we generated a recombinant double mutant ($op1^1$, $op2^2$). We found these double-mutant mosquitoes showed a significant decrease in their preference for IR as compared to wildtype, but not as sever a defect as seen in the $trpA1^{ECFP}$ mosquitoes. These results may indicate a redundant role of these opsins as upstream signaling molecules involved in IR sensation.

### F. Correlations Show IR-Sensation Occurs in Flight

In our paradigm, the preference for one zone over the other, or lack thereof, is determined by the number of observations in each zone. As such a shift in preference index occurs when the number of observations on one zone exceeds the other, however, the behavioral explanation for this shift is not immediately apparent.

There are some explanations that are non-exclusive – that mosquitoes are tending to stay in that region for a longer time, mosquitoes are traveling longer distances per bout and thus exiting the zone more frequently, and that mosquitoes are simply landing in this zone more frequently.

To determine whether mosquitoes are on average spending more time in a given zone and are therefore causing the preference index to shift to that region we looked at the correlation between preference index and the difference in average track time (ATT) between zones (**Figure 14A**). We found no strong correlation that mosquitoes occupy zones for a longer duration when the population is preferring that zone ($R^2 = 0.37$). This result argues that the mosquitoes are not choosing to occupy a given zone based on cues they are experiencing there. One concern is that the surface of the cage is absorbing the incident IR emitted from the cage, and subsequently mosquitoes who randomly encounter this warm surface are spurred to stay there longer through conductive heat inputs they are receiving. This is not the case. The material of the cage is open weave and therefore has a relatively small surface area for IR absorption, and that based on this correlation conductive cues would cause mosquitoes to stay longer once they have landed, which is not the case.

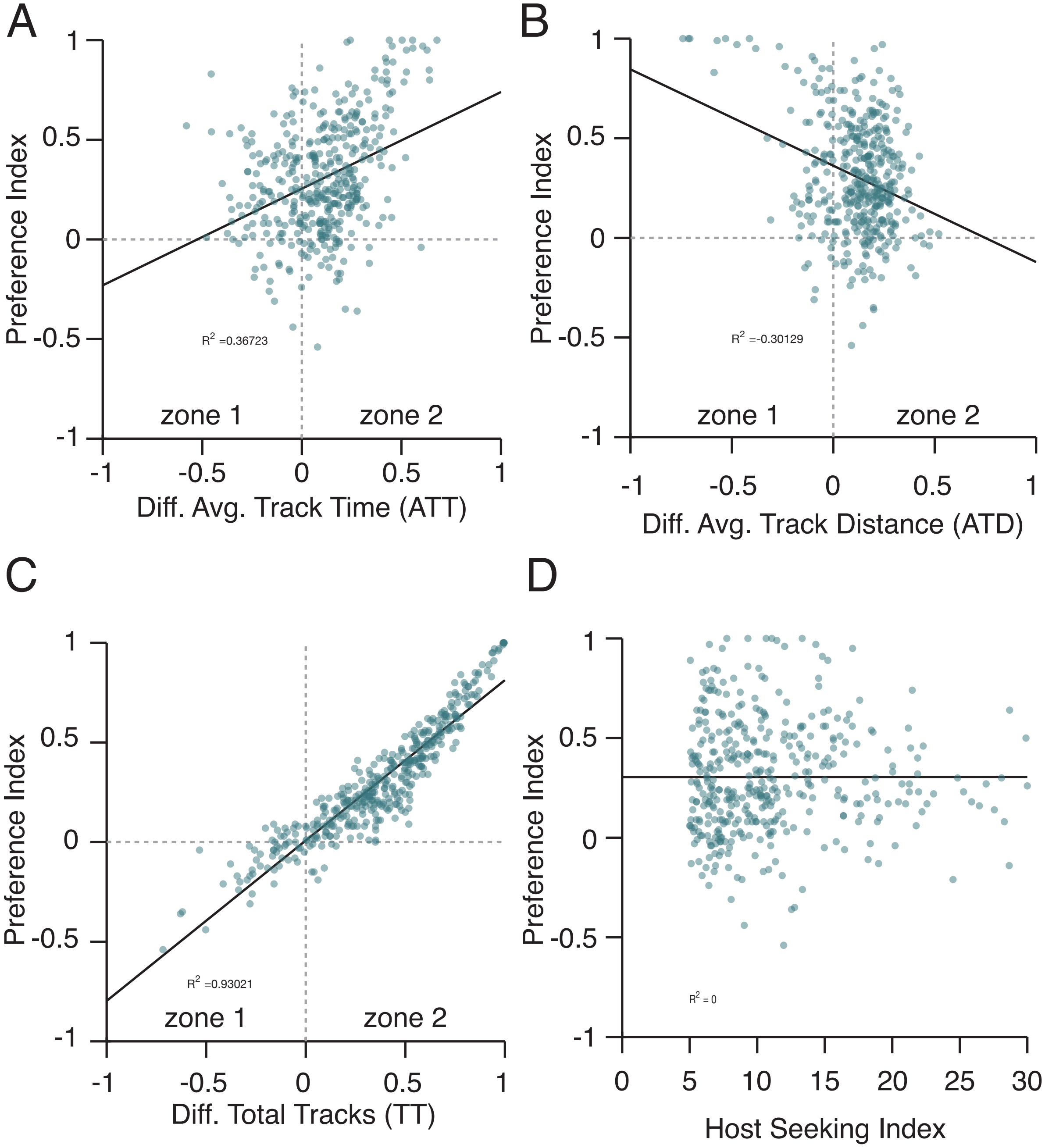

**Figure 14. Change in Preference Index is Correlated with Mosquito Landings.** Data from 410 unique experiments, including various IR and genotype conditions, to determine what behaviors correlate with shift in PI. (A) Correlation of preference index and the normalized differential in average track time (ATT) in each zone $(ATT_{zone2} – ATT_{zone1})/max(ATT_{zone2}\ ATT_{zone1})$, $R^2 = 0.36$. (B) Correlation of preference index and normalized differential in average track distance (ATD) in each zone $(ATD_{zone2} – ATD_{zone1})/max(ATD_{zone2}\ ATD_{zone1})$, $R^2 = –0.30$. (C) Correlation of preference index and the normalized differential in total tracks (TT) in each zone $(TT_{zone2} – TT_{zone1})/max(TT_{zone2}\ TT_{zone1})$, $R^2 = 0.93$. (D) Correlation between preference index and

We additionally looked at whether the mosquitoes on any given side tend to travel longer distances per bout, which could cause them to leave the zones of interest at a greater frequency. We again found no strong correlative evidence that this is the case (**Figure 14B**, $R^2$ = -0.30).

We then looked at the correlation between normalized number of total tracks observed in each zone and the resulting preference index, we found a strong correlation (**Figure 14C**, $R^2$ = 0.93). This result suggests that when the preference index favors one zone over the other, this is due to a greater number of mosquitoes that are host seeking in that region. In order for this to be the case, mosquitoes would need to integrate sensory cues prior to landing on the wall of the arena and subsequently to choose to land in that region. We demonstrated that the ambient air temperature in either region is consistent under various IR conditions, and therefore in flight the mosquitoes are most likely integrating the IR they are sensing and choosing to land in that area.

We lastly looked at whether preference index was correlated with the overall host seeking activity for a given experiment (**Figure 14D**). We found no correlation between these metrics in the data. This result suggests that changes in preference index to not occur from an extraneous explanation when the mosquitoes are either hypo- or hyperactive.

## Chapter 4

### A. Integration of IR Sensation with Other Host Cues

Our work has shown that detection of host temperature objects via IR sensation works best against a contrasting environment with cooler ambient temperatures. This would then argue that when in environments that approach the temperature of host skin (~34 C) perhaps other sensory cues become more salient for host detection. For example, as the air temperature in the environment increases, perhaps this also increases the volatility of organic compounds on skin, thus increasing the output of attractive odorants in the environment. Conversely, in much cooler environments the reverse may be true in which output of volatile organic compounds (VOCs) from the skin may be decreased. However, the efficacy of IR-based contrast detection may be boosted in these conditions.

To experimentally determine the effects that ambient temperature has on IR and odor detection simultaneously may be rather difficult. However, in work not included in this study have found that our assay can also be accurately used to determine preference when animals are given a non-uniform odor attractant. Using this experiment design, we could assay how the preference for skin odor is affected at various ambient temperatures. By examining the effects that temperature has on odor-based taxis in host seeking in comparison to our results showing its effect on IR-based detection, we could begin to understand how these two sensory modalities intersect and allow mosquitoes to find hosts in diverse environments.

### B. Understanding the limits of IR Sensation

Having shown that mosquitoes detect infrared and integrate this information into host seeking behavior, we wish to further understand how this sensation functions at greater distances. With any stimulus there must be a physical limitation to when it is no longer sensed or salient to the animal. In this study, our experimental paradigm used an arena in which mosquitoes can be at a maximum distance of ~34 cm from the emitting IR source. However, in this paradigm mosquitoes are allowed to fly as close as 4cm away from the emitting surface, and therefore mosquitoes only sense this stimulus as they fly nearer the emitting surface. In the current behavioral setup it is difficult to determine the minimum distance at which the sense begins to guide host seeking behavior.

The mosquito body has a finite surface area, some of which may be associated with the IR sensory organ, and therefore as the mosquito is placed farther and farther away from the IR emitting source, the intensity decreases as an inverse square function of the distance. Therefore, there should be a physical distance from the source where the source is no longer detectable by the mosquito. In order to empirically determine this limit, one could move the IR emitting source (Peltier plate used in this assay) incrementally further away and record the corresponding preference index of these mosquitoes. Presumably, the preference would decrease as the distance of the surface was increased, eventually reaching a limit of detection. Despite the simplicity of this approach, the experiment paradigm as is may not be best suited for this strategy. Firstly, the assay relies on the observed preference between two 10 cm x 10 cm zones which are relatively close to one another, and therefore if the emitting surface were moved farther away, the observed loss of preference may simply be a function of the more dispersed IR spread over both of the zones. Along these lines, perhaps an assay in which opposing surfaces of the cage differentially exposed to an IR source would enable such a distance-based approach. Secondly, as constructed, the Peltier surfaces are housed within the arena itself and as such moving these further away from the mosquito cage cannot be done. For these reasons, physically moving the Peltier emitting surface farther away from the mosquitoes may not be the most amenable approach within this experimental paradigm.

An alternative strategy for determining the limits of IR detection is to serially reduce the surface area of the emitting surface and measure the observed preference index. As previously mentioned, the emitting surface used in these experiments is a 10 cm x 10 cm Peltier plate heated usually to 34 ºC. To mimic the effect of the emitting surface being farther away, we could decrease the surface area using a series of filters with smaller and smaller apertures. In this version of the assay, the preference for these zones would be measured as normal, and presumably the preference would decrease as the apertures became smaller and smaller. By looking at the relationship between surface area and preference index, once could gather more information of the distance-limits of IR detection.

Lastly, as previously mentioned, as the emitting object moves further and further away from the observer, the intensity decreases as an inverse square function of the distance. Additionally, according to the Stefan-Boltzmann law, the intensity of IR emitted by a black body radiator is tied to its absolute temperature, that is to say, cooler objects emit less IR. As shown in **Figure 11A**, we performed experiments in which the preference demonstrated by mosquitoes was recorded while using varying intensities of IR by varying the temperature of the Peltier surface. In these results we observed that the shift in mosquito preference began over 31 ºC. Because the emissivity of the Peltier can be determined, as well as the intensity of the IR emitted (according to its temperature) this could be used to approximate a minimum intensity to which mosquitoes can detect.

In all, further understanding how IR sensation functions at a distance is an important question, and one we wish to further investigate. However, because of the previously mentioned concerns, experimentally determining these limits requires an elegant experimental design.

### C. Understanding the Role of TRPA1 in IR Sensation

Our work thus far supports the idea that TRPA1 is required for mosquito sensation of IR in a host seeking context. We found that mosquitoes that lack TRPA1 (*trpA1*$^{ECFP}$) lose their preference for the IR target in our assay. To further support these findings we wish to generate an additional loss of function allele for trpA1 (*trpA1*$^{LexA}$) which will also serve as a transcriptional reporter via expression of the inserted LexA transcription factor. This line may be crossed with *LexAOP* reporter lines we have generated in our lab to visualize the cellular localization of TRPA1 expression. Aside from confirming these results via the use of additional alleles, we tested trpA1$^{ECFP}$ mosquitoes using our experimental setup, however, in this case we only added human odor to one side of the cage with uniform $CO_2$, and in the absence of additional IR (data not shown). We found that in this condition, the preference index recorded was extremely high for the odor zone (~0.95). These results are important as they demonstrate that mosquitoes lacking functional TRPA1 protein do not have a general olfactory deficiency, nor do they have a general locomotor or coordination defect which prevents them from direction movement towards stimuli. Together these results support our claim that TRPA1 is required for IR sensation.

As previously mentioned, we wish to visualize the cellular localization of TRPA1 in *Aedes aegypti*. Previous reports have shown that TRPA1 is localized to sensory neurons in the mosquito antenna in *Anopheles gambiae*, which putatively enable the mosquito to sense the ambient air temperature [57]. Furthermore, this TRPA1 localization is near, if not in, thermosensory neurons which have been characterized through electrophysiological approaches [32]. In order to demonstrate the expression pattern of TRPA1 in *Aedes aegypti* we have tried traditional immunostaining approaches using antibodies generated against *Ae. aegypti* TRPA1, however, these efforts have been largely unsuccessful. An additional approach for visualization we intend to try is an in situ-based method known as RNA-Scope, which amplifies the signal of probes bound to target transcripts. Lastly, as previously mentioned, we intend to generate transcriptional reporter lines ($TRPA1^{LexA}$) which are in progress.

Our current model for how TRPA1 functions in IR sensation requires activation of this channel in thermosensory neurons in the mosquito antenna. To test this model, we plan to remove sections of the antenna and then test how the mosquito preference for IR is affected. In preliminary experiments (not included) we have found that removal of half the length of each antenna does not eliminate the mosquitoes response to olfactory cues nor does it eliminate their probing response which is indicative of host seeking under our experiment conditions. However, the overall host seeking activity of these mosquitoes is affected by removal of antenna portions. This may be due to secondary effects caused by physical removal of the antenna, or may be directly due to removal of specific neurons in the distal end of the mosquito antenna. Despite this, we are now working to overcome these concerns and to test and analyze the effect on IR preference that occurs when the distal antenna is removed.

Previously we showed that mosquitoes that are heterozygous for the $TRPA1^{ECFP}$ allele also show a defect in IR sensation. This result is quite interesting given there was no previously reported heterozygous phenotype when mosquitoes were assayed in a different experimental paradigm[28]. The differences in aims between these two experimental setups could explain this discrepancy given that this study is solely looking at the effect that IR alone has on mosquito host seeking. We suggest two possible explanations for this heterozygous phenotype in our paradigm, that one functional copy of the *trpA1* gene is insufficient to elicit a wildtype response, an example of haploinsufficiency, or rather that the specific allele *trpA1*$^{ECFP}$ functions as a dominant negative. The *trpA1*$^{ECFP}$ allele was generated via insertion of sequence containing a transgenesis marker in exon 16 of the TRPA1 gene. Furthermore, this insertion site is positioned near the end of the *trpA1* gene and disrupts the coding region between transmembrane loops 5 and 6 of the TRPA1 protein. We suggest that perhaps this allele results in transcription and expression of a truncated form of the TRPA1 protein, which in turn functions as a dominant negative when expressed in trans to the wildtype sequence. This is because TRPA1 associates into a homotetramer in order to form a functional membrane channel. We propose that inclusion of a single monomer of truncated TRPA1 may block proper channel formation and thus reduce the overall amount of functional TRPA1 channels.

The hypothesis that *trpA1*$^{ECFP}$/+ is haploinsufficient is rather difficult to determine empirically, therefore we aim to test the dominant negative hypothesis first. We plan to test whether co-expression of wildtype TRPA1 with the putative truncated form present in *trpa1*$^{ECFP}$ animals in cell culture affects channel function using patch clamp recordings.

It is predicted that the *Aedes trpA1* gene expresses five different isoforms, of which some have been characterized in cell culture experiments [53]. We wish to understand which specific isoform(s) are required for IR sensation. We plan to first characterize which isoforms can be detected in the female antenna via RT-PCR using isoform specific primer pairs. Because of the gene structure and splicing pattern of *trpa1*, we cannot make mutants for a single specific isoform and thus must find an alternative approach to determine which specific isoform is required for IR sensation. One possible alternative is isoform specific rescue using *LexAOP-TRPA1-A/B/C/D* transgenic lines that express one specific isoform under the *LexAOP* transcriptional regulatory sequence. As previously mentioned, if efforts to generate a functional *trpA1-LexA* reporter line are successful, this driver line in combination with the isoform specific rescue lines could demonstrate which isoform(s) functionally rescue IR sensation. These efforts are underway.

### D. Understanding the Role of Opsins in IR Sensation

As previously mentioned, we found an intermediate defect in IR detection in the *op1, op2* double-mutant mosquito. These results are consistent with unrelated work emerging from our group looking at the role of these opsins in vision-based host seeking behavior, in which the strongest phenotype was observed in the double mutant as well [25]. This would suggest a highly redundant role of these two opsins. Despite having a vision-based host seeking defect, we should note that in our behavioral paradigm there is no visual contrast between the IR and reference zones, and therefore any difference in preference observed in our assay would not be attributable to this visual defect. Rather we believe that the function of these opsins may be consistent with known roles in thermosensation that have emerged from our lab [43, 55].

Work performed in *Drosophila* has found that opsins function upstream of TRP channels and, for example, in the visual phototransduction cascade activate TRP channels via secondary messenger molecules once they are photoconverted. We believe that this transduction cascade may in fact be consistent in sensory transduction in non-visual contexts. Work from our lab has demonstrated that opsins play a role in thermosensation in *Drosophila* larvae, enabling the animals to sense very small differences in temperature in the comfortable range through signal amplification [43]. Furthermore, in *Drosophila* taste, our lab has found that at low concentrations of certain bitter compounds such as aristolochic acid, flies lacking specific opsins show a phenotype. The opsins then couple to an amplification cascade that culminated with activation of TRPA1 [54]. Thus, low levels of aristolochic acid activate opsins, which indirectly activate TRPA. However, the opsins are dispensable for sensing high levels of aristolochic acid that are sufficient to directly activate TRPA1. Our model is that opsins function as amplifiers of low signals upstream of TRP channels, while TRP channels can also be directly activated by high levels of the same stimulus as well.

We would like to test whether this model is consistent with the function of op1,op2 and TRPA1 in IR sensation. We have previously shown that at an IR intensity generated from a 34 ºC source there is complete loss of IR preference in the TRPA1 mutant. Furthermore, under these same conditions, the opsin double mutants we examined showed an intermediate phenotype. Therefore, we wish to test the preference index observed in these double mutant mosquitoes in our assay when the source intensity it modified similar to the experiments we performed in wildtype mosquitoes. Perhaps at lower IR intensities (below 34 ºC) the phenotype would become more severe due to the decreased signal intensity. Conversely, perhaps at higher signal intensities, direct activation of the wildtype TRPA1 present in op1,op2 double mutant mosquitoes would cause the preference index to shift to that seen in the wildtype mosquito. Should these results occur, this would be consistent with the model that these opsins in the context of IR function as low signal amplifiers upstream of direct activation of TRPA1, allowing mosquitoes to detect sub-activation threshold levels of IR. Similarly, as the mosquito approaches the host, perhaps then the incident IR on the mosquito would directly activate TRPA1.

Another focus we aim to complete is visualizing the expression of both op1 and op2. As previously mentioned, we also wish to visualize the expression of

TRPA1. In co-labeling experiments we propose to find co-expression of op1, op2, and TRPA1 in the same sensory neurons, however, if this is not the case this could argue that an alternative mechanism is responsible for the function of these opsins in IR sensation.

In order to confirm the phenotype, we also plan to test the behavioral responses of additional op1 and op2 alleles that our lab has generated, as well as a second double mutant line. Should the phenotype be consistent with our previous observations, these data would strongly suggest that these opsins play a critical role in IR sensation.

### E. Electrophysiology of IR Sensation

As previously mentioned, our working model of how IR functions in host seeking is that incident IR that hits the mosquito antenna is converted to thermal energy in the endolymph surrounding thermosensory sensilla. The thermosensory sensilla we suspect play a role in IR sensation are the large coeloconic sensilla on the terminal segment of the mosquito antenna. Previous studies have performed electrophysiological recordings from antennae using warm air to activate these neurons, and record action potentials generated by these thermosensory neurons [32]. Because the authors of that study used a convective air source to heat these neurons, it is largely suspected that only convective cues allow for mosquitoes to detect ambient thermal cues. However, at a fundamental level there is no difference with respect to the underlying thermosensory neuron whether this heat is collected from radiative or convective means. If the surrounding endolymph is heated beyond the activation threshold of thermosensory channels, presumably TRPA1, then the neurons will be activated.

We plan to test in our lab whether these neurons may be directly activated with exposure to IR alone. A main argument that we would like to make in this study is that in fact mosquitoes should be capable of detecting all three forms of heat transfer, just as humans and most animals do. To accomplish this, however, we need to demonstrate that these neurons can be directly and solely activated by IR, and to do so we will perform sensillar recordings from the terminal segment of the mosquito antenna. Additionally, if we can successfully evoke action potentials in these neurons using IR alone, we additionally have the mutant alleles at our disposal to examine at a cellular level the effect these mutants have on thermal activation of these neurons. We would presume that IR activation of these neurons would be lost in TRPA1 mutants, and furthermore, the firing rate or activation threshold may also be affected in op1, op2 double mutants, should these opsins be expressed in these specific neurons.

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# Appendix

## Methods

### Animals and Stocks

*Aedes aegypti* mosquitoes were reared in the laboratory at 28°C / 80% humidity and kept under 12L:12D light conditions. Larvae were reared on fish food (Tetramin Tropical Granules) until pupae emerged. Adult mosquitoes were allowed to eclose into 30 cm x 30 cm x 30 cm mosquito cages (Bugdorm) and maintained on 10% sucrose solution (w/v).

Wildtype mosquitoes used were derived from either the Liverpool or Orlando strains where indicated. The $trpA1^{ECFP}$ and $Gr19a^{DsRed}$ lines were generously provided from the Leslie Vosshall Lab (Rockefeller University). All additional lines used were generated in our lab.

Generation of $Op1^1$ and $Op2^2$ transgenic were generated by Yinpeng Zhang [25].

### IR Choice Assay

One to three-week old mated female mosquitoes were manually aspirated from mixed-sex cages without anesthetization and placed in mosquito cages (30 cm x 30 cm x 30 cm). To allow for clear video recording we replaced one side of these cages with a panel of clear acrylic (McMaster-Carr, 1/16”). Mosquitoes were allowed to acclimate to the experiment cages for a minimum of 18 hours prior to study and were consistently maintained on 10% sucrose.

The IR choice arena was custom fabricated (UCSB Physics Machine Shop). The arena housed two 10 cm x 10 cm Peltier devices. For all experiments, one Peltier was left off to function as a reference zone for comparison. The “IR” source Peltier was set to the indicated temperature (USB Controller thermocouple). The IR Choice arena was placed inside an incubator maintained at 28 °C.

Immediately prior to behavior experiments, the zone directly opposing the Peltier surfaces was treated with human odor by uniformly rubbing a used latex glove over the surface (the gloves of the same investigator were used in all reported experiments). Behavior recordings began immediately following placement of the test cage within the IR choice arena. The cage was perfused with 5% $CO_2$ (v/v) gas mixture (AirGas) for 30 seconds, then 10 seconds every minute of recording. Behavior experiments were run for a minimum of five minutes. Each biological replicate (80 adult female mosquitoes) was assayed a minimum of three times.