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Visual perception modulated by blood-fed status in *Aedes aegypti* mosquitoes.

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By

Michael Ramon

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To: Dr. Steven Oberbauer, Chairperson Department

of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Michael Ramon entitled "Visual perception modulated by blood-fed status in *Aedes aegypti*", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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ABSTRACT

Visual perception modulated by blood-fed status in *Aedes aegypti* mosquitoes.

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Mosquito-borne diseases are incredibly difficult to combat due to the vast array of biological tools the mosquito has for finding humans and obtaining a blood meal. Numerous modalities mediating mosquito attraction to hosts have been investigated including vision, olfaction, hygrosensation, thermosensation, audition and mechanosensation. Out of these senses, vision is known to mediate both short range and long range attraction, and therefore deserves further exploration (Bidlingmayer 1994). In this study, gravid and non-gravid mosquitoes were both tethered and placed in a virtual flight arena to examine behavior in response to a wide-field pattern and a bar pattern. Mosquitoes were also analyzed under a microscope to establish average counts for the ommatidia. After each behavior experiment, the different treatment groups were compared using a two tailed unpaired t-test. The results showed that blood-fed status alone is sufficient to suppress the response of mosquitoes to a visual stimuli ($p < 0.05$). Furthermore, the count of the ommatidia number per eye was found to be 317 ± 46 for females and 311 ± 70 for males. However, to know the accuracy of these counts different methods should be used in parallel since there is disagreement over the average amount of ommatidia in the literature ranging from 200 to 800 for each compound eye of the mosquito (Hu 2013; Singh et al. 2013).

By investigating these topics, we hope to gain a better understanding of mosquito behavior, particularly regarding the role of vision in navigation, host-seeking, and oviposition.

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INTRODUCTION

Organisms of all kingdoms have evolved complex sensorial systems that allow them to respond to diverse environmental stimuli. For animals, such responses are often displayed as behaviors resulting from the synergistic activation of multiple neural pathways (Zhen 2019). Guided by its somatosensory systems, organisms can avoid predation, locate food sources, and interact with other animals. Female mosquitoes are of interest to the field of vector borne disease due to their anthropophilic nature and disease vector capacity. Mosquitoes are a global health threat, with malaria alone claiming over 430,000 lives every year (Omodior et al., 2018). A simplified life cycle of a female mosquito from an egg through to its first gonadotrophic cycle is presented in Figure 1. The mosquito begins this cycle as an egg, and then proceeds through a series of aquatic developmental phases before eclosion, transitioning the mosquito from pupa to adult (1A). The adult mosquito then begins looking for nectar sources to build its nutrient reserves, and a mate (1B). After consuming a sufficient amount of nectar and mating, a female mosquito will search for a vertebrate host to obtain the nutrients required for egg development (1C). Once a mosquito has fully digested its blood meal, it is considered gravid and will begin to look for an oviposition site adjacent to a water source (1D). This cycle then continues with the eggs of the previous mosquito.

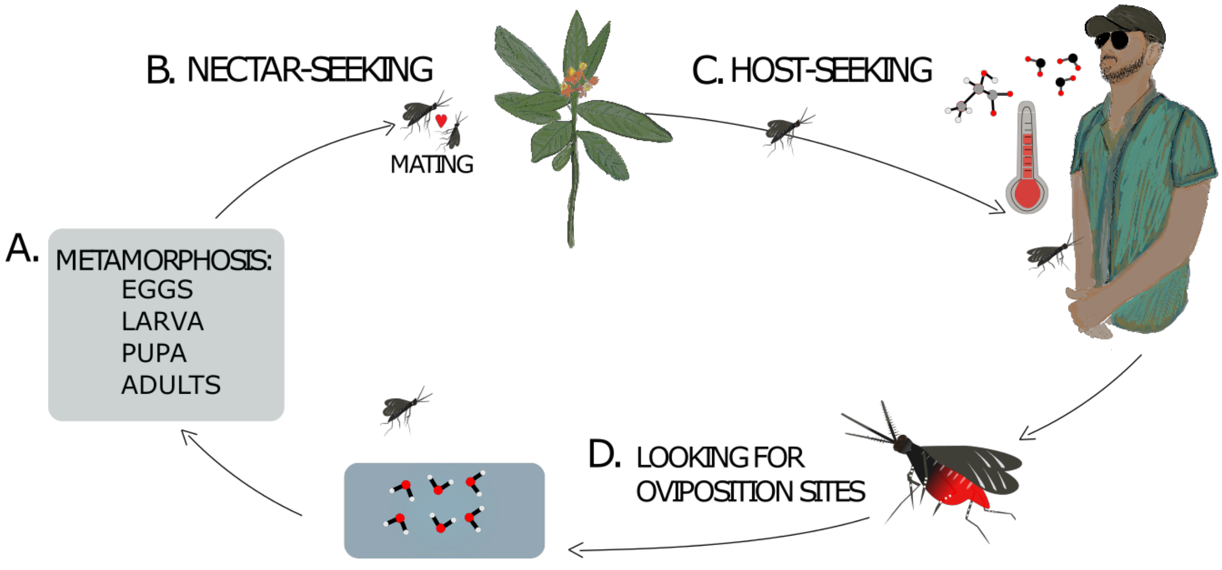


Figure 1. Feeding cycle of a female mosquito from an egg through to the first gonadotrophic cycle. 1**A.** Aquatic developmental phases of the mosquito. **1B-1D** Different cues that attract mosquitoes during adulthood.

The success of a female mosquito in finding humans stems from its ability to track different host cues. Vision, thermosensation, and olfaction, among other senses, aid mosquitoes in locating a vertebrate host to acquire a blood meal (Raji & DeGennaro 2017). Due to *Aedes aegypti* being a diurnal species, most of the activities will be taking place during daytime hours (Mcmeniman et al. 2014; Day 2016). This is an important consideration, as the time of activity influences the structure of the mosquito eye, as will be described in the paragraph below. Furthermore, because vision is important throughout the mosquito life cycle, but behavior is changing (Figure 1), we expect visual responses may be affected by factors such as blood-fed status.

Mosquito vision

Mosquito vision is important for long-range appetitive flight and short-range attractive flight (Bidlingmayer 1994). Understanding what causes this transition could elucidate how vision contributes to mosquito attraction to hosts. A large body of work has been done on the compound eye of *Aedes aegypti*, including determination of the average number of ommatidia, its structure (Singh et al. 2013), and the average interommatidial angles (Land et al. 1997). Each of these features has several associated benefits and tradeoffs. The number of ommatidia influences the ability of mosquitoes to acquire light from its surroundings. The greater the ommatidia number and diameter, the greater the amount of light acquisition. However, the sharpness of the image is inversely proportional to the interommatidial angle. Due to diameter size necessarily increasing interommatidial angles, organisms with compound eyes have evolved to favor either spatial sharpness or light gathering (Currea et al. 2018).

Structure also influences the efficiency of the eye in focusing light. Mosquito ommatidia take the form of a hollow cone, with a widened hexagonal distal end. This shape increases the angle over which the ommatidia can effectively absorb photons and avoid incident light spillage (Land et al. 1997; Singh et al. 2013). Additionally, mosquito ommatidia have an apposition configuration rather than superposition configuration. This means that the rhabdom inside the ommatidia where the light sensitive cells are located, is surrounded by a heavily pigmented sheath. This pigmentation prevents incident light from passing from one ommatidia into another. In the superposition configuration, the heavily pigmented sheath is absent, allowing photons to enter one ommatidia and stimulate the neighboring ommatidia. This phenomenon presents a tradeoff as apposition eyes tend to have better spatial sharpness, but superposition eyes tend to have better light acquisition (Greiner 2006). However, diurnal organisms with apposition structured eyes have adaptations that allow them to adjust to dark environments. Two known physiological adaptions are adjustments of eye shape through retinomotor activity and pigment migration. In the case of adjusting the shape of mosquito eyes, both larvae and adult mosquitoes are known to increase the volume of the rhabdom after prolonged periods in the dark (Brammer et al. 1978; white and lord 1975). This restructuring of the ommatidia increases the ability to perceive objects in dim light conditions. Pigment migration is the movement of the primary pigment cells surrounding the rhabdom, allowing incident light to stimulate the surrounding ommatidia. This amplifies the effect of photons entering an individual ommatidia. Studies also suggest that diurnal mosquitoes can neurologically amplify responses from different ommatidia (Land et al. 1999). The two main types of neuronal summation are temporal and spatial. In spatial summation the nerve responses of neighboring ommatidia are pooled to generate an amplified response. Temporal summation increases the integration time for photon increasing the overall brightness of the produced image. However, despite these adaptions, insects with superposition eyes tend to see better in dim light conditions (Warrant 2017).

A robust consensus in our current understanding of mosquito vision is lacking in many aspects. Black colors may be highly attractive (Brett 1938; Sippell & Brown 1953; Hecht & Hernandez-Corzo 1963), and not attractive (Gilbert & Gouck, 1957) to mosquitoes. Some of these differences are attributed to the reflectivity of the paint mixture used to color the experimental visual targets (Muir et al. 1992), but these results may be an artifact of the physiological state of the mosquitoes tested. The current literature explaining why certain colored patterns are attractive to mosquitoes is also contentious.

Mosquitoes are known to steer toward long vertical black bars, a reflex called stripe fixation. The reason for this behavior is not entirely understood, but many landing behaviors have been associated with the stripe fixation reflex (Dickinson 2014). As a result, it is thought that mosquitoes may identify the bar as a landing zone. A picture of the vertical bar and the wide field pattern used in this study is presented in Figure 2.

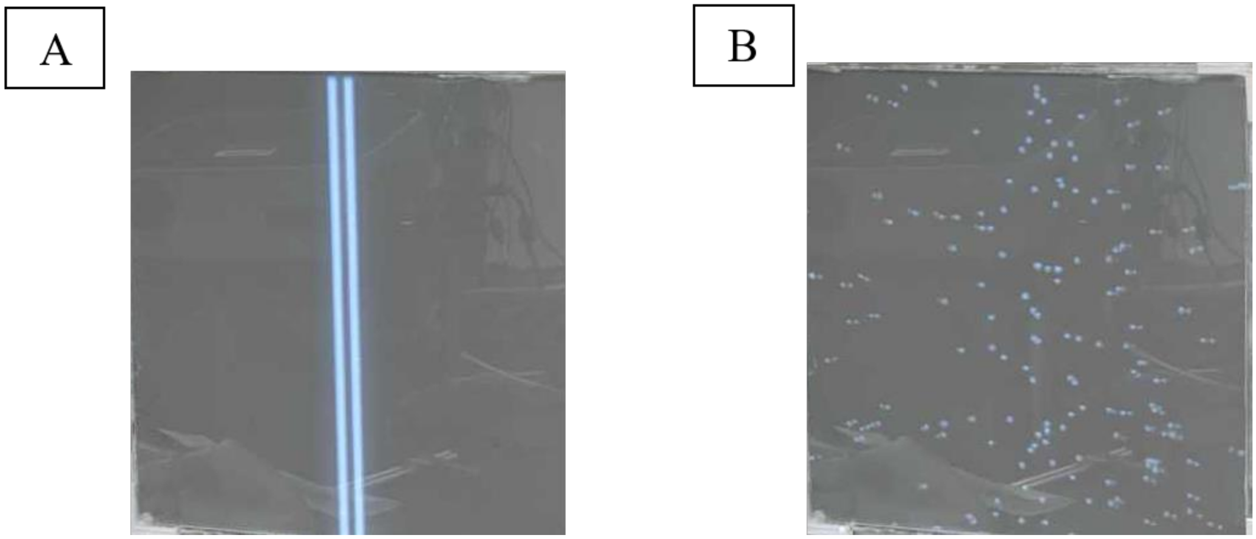
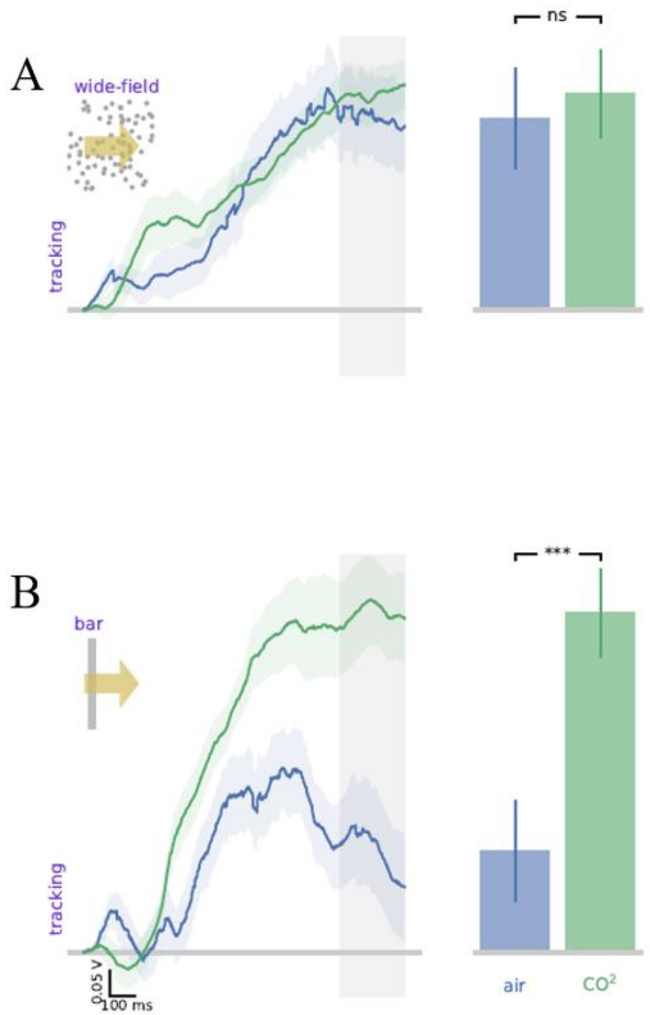


Figure 2. (2A) The vertical bar stimulus presented to mosquitoes during tracking experiments. (2B) The wide-field stimulus presented to mosquitoes during tracking experiments.

The direction of contrast polarity, and the degree of contrast between a vertical bar and the background increases the fidelity of mosquito tracking (Raji 2019; Liu and Vosshall 2019). The Wide-field pattern used in this study simulates self-motion; self-motion stimulates the oculomotor response through perceived side-slip (Chow et al. 2011). How these anatomical features affect behavior is not so clear, but the interpretation of the behavior could potentially benefit from a closer analysis of the mosquito eye. However, techniques for determining the number of ommatidia are time consuming and involve a lengthy tissue preparation, subsequent slicing, and microscope analysis. The goal of this project was to develop a relatively inexpensive and rapid way of determining the ommatidia number of the mosquito eye

Integration of visual stimuli, blood-fed status and carbon dioxide

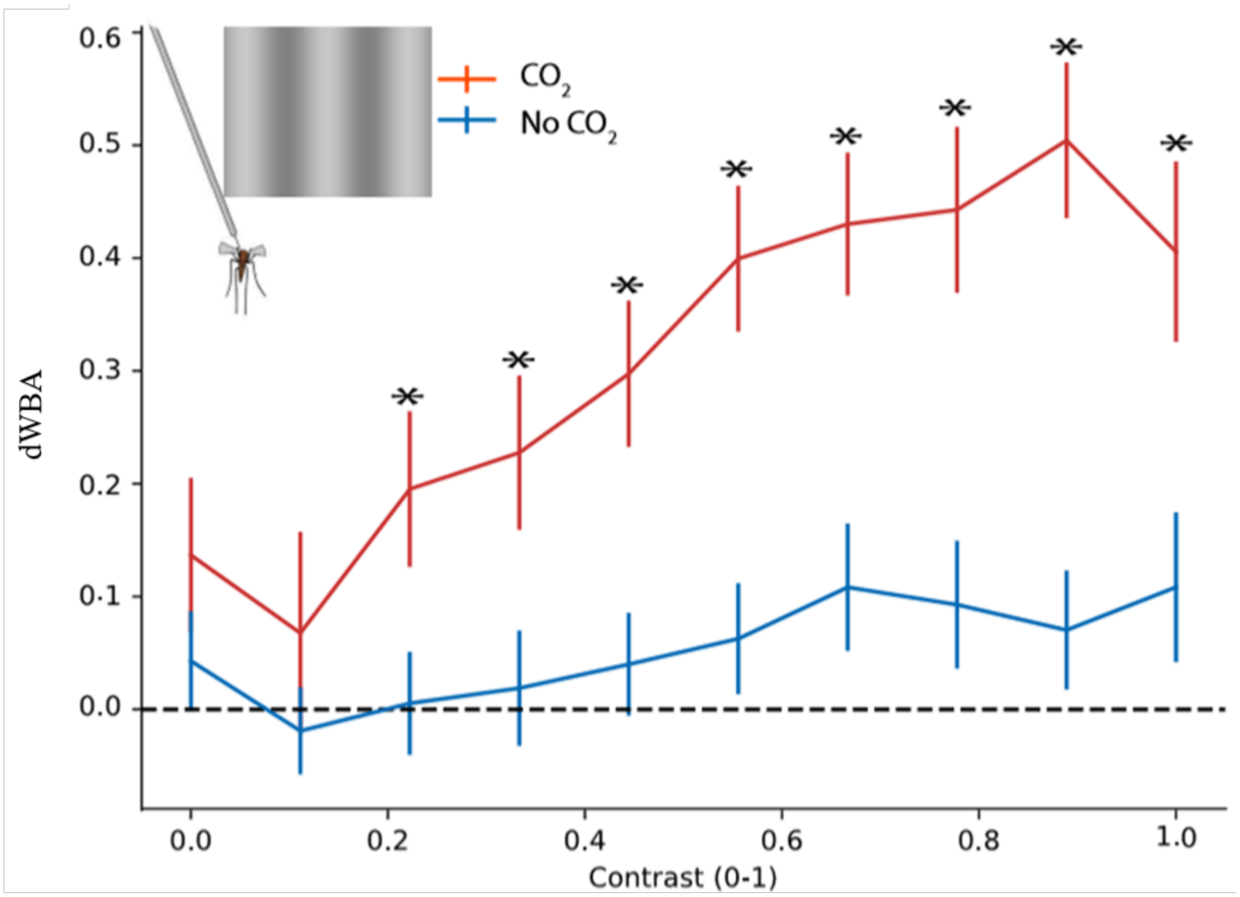
Carbon dioxide, a potent olfactory host cue (Mcmeniman et al. 2014), appears to increase mosquito tracking fidelity of a black vertical bar, but not of other visual cues tested (Vinauger 2019). Preliminary data support of these findings, suggests the presence of a CO_2 stimulus enhances female mosquito attention to a vertical bar (Figure 3 and 4, Raji 2019, submitted for publication). In contrast, a CO_2 stimulus failed to enhance visual tracking when the wide field pattern was presented (Figure 3). This suggests the bar may be representative of a host to a mosquito, since it synergizes with other host cues while other visual stimuli do not.



Raji 2019, submitted for publication

Figure 3. Differences in tracking between CO_2-treated and filtered air-treated mosquitoes in response to visual stimuli. **A.** Difference in wide-field tracking of both groups. **B.** Difference in bar tracking of both groups. Results are from a dissertation submitted for publication (Raji 2019).

The synergism between relative contrast and CO_2 was examined (Figure 4). Tracking fidelity between the groups became significantly different as the relative contrast was increased. This results suggests that mosquitoes may be more attentive to different levels of contrast when other host cues are present, and that contrast may be representative of a potential host cue to a mosquito.



Raji 2019, submitted for publication

Figure 4. Differences in wing beat amplitude (dWBA) in response to different levels of relative contrast in the presence CO_2 or filtered air treatments. Results are from a dissertation submitted for publication (Raji 2019).

Interestingly, calcium imaging has shown that olfactory cues activate neurons in the optic lobe (Vinauger et al. 2019), showing a clear sensory integration between vision and olfaction. This further supports the hypothesis that vision and olfaction generate and amplified response when presented together.

Blood-fed status suppresses the response of gravid mosquitoes to host cues, due to abdominal distension and humoral inhibition (Farjana & Tuno 2013; Thahsin et al., 2013). However, whether attraction will persist when the host cues are reduced to a visual stimulus only and a difference in blood-fed status is still an open question. Recent studies have been unable to find a difference between the responses of blood- and saline-fed mosquitos to black vertical bars (Zhen & Vosshall 2019). However, the conditions under which the mosquitoes were reared were not ideal. This question will be revisited in this study with *ad libitum* access of mosquitoes to a 10% sucrose solution rather than starving the mosquitoes for a period of time. Furthermore, a waiting period of 72 hours rather than 48 or 96 hours will be used, since 3 days is the point at which mosquitoes are expected to have finished digesting a blood meal in tropical climates (Day et al., 2016). Based on the work presented in Figure 3 and 4 a vertical black bar is thought to represent a visual host cue for mosquitoes, thus it was hypothesized that responses to this stimulus would be suppressed in gravid mosquitoes. At a later stage, this study will expand upon the available information on integration of smell, blood-fed status and vision in mosquitos by studying how blood-fed mosquitoes respond to CO_2 cues in conjunction with a vertical bar.

MATERIALS AND METHODS

Insect rearing

The 58 mosquitoes used in this study were from the *Aedes aegypti* Orlando laboratory strain. At every developmental stage, mosquitoes were housed in an insectary at 75% relative humidity, 25-28° C, and under a 14:10 light-dark cycle (lights on at 8 am). Mosquito eggs were hatched in deoxygenated, deionized water with one dissolved tablet of Tetramin tropical fish food (Tetra, Melle, Germany). Larvae were fed for a week, up until the point of pupation. Pupae were moved to BugDorm-1 cages, (Bugdorm, Taiwan) where they eclosed. Adult mosquitoes were given *ad libitum* access to a 10% sucrose solution, except when being blood-fed.

Blood-feeding

Four days after eclosing, female mosquitoes were fed defibrinated sheep blood (Fisher Scientific, Waltham, MA) through an artificial membrane feeder (CG -1835-70 Chemglass) in groups of 10-50 mosquitoes per cage. Each feeder was covered with a stretched Parafilm layer and supplied three milliliters of blood, and 0.25 mg of ATP (Acros Organics, Geel, Belgium). The feeder was kept at 37 °C with running warm water. As a control, 10 to 26 mosquitoes were sorted out of the cage prior to blood feeding and placed in a separate cage.

Mosquito preparation for flight arena experiments

A week after eclosing, adult mosquitoes were cold-anesthetized for fewer than five minutes and then glued (Bondic, Witmer, NY) by the dorsal thorax to a tungsten rod. The mosquitoes were then allowed 15-60 minutes to recover, and a piece of a Kimwipe was placed over their legs to allow them to rest. The tether rods were then placed inside a custom-built virtual flight arena capable of delivering both olfactory and visual cues. Mosquitoes were centered over a photosensor inside the arena which detects the shadow cast by each wing. The differences in the amplitude of the shadow indicate changes in flight patterns. For example, a small shadow cast by the left wing, and a relatively larger shadow cast by the right wing would indicate an attempted turn-to-the-left motion.

In the flight arena, mosquitoes were surrounded by a Perspex cube covered with a black projector screen. First-surface mirrors were positioned at a 45-degree angle from the sides of the cube to allow images to be projected onto the arena and transition smoothly across it. The projector was positioned to illuminate all five sides of the cube, covering about 10.47 steradians of the visual field and was refreshed at a rate of 360 frames per second. During each experiment, all mosquito responses were recorded as an array in a numeric binary file. All mosquitoes were tested only once in a behavioral assay to ensure the studied responses were innate.

Data analysis

After completing a trial, the mean difference in the left and right wing beat amplitude was calculated for each of the stimuli presented by taking the mean amplitude of the right wing and subtracting it from the mean amplitude of the left wing. The result is a measure of the difference in wing beat amplitude which is proportional to the yaw torque. This value should be positive for attempted right turns, negative for attempted left turns, and zero when the mosquito is not attempting to turn. However, the wing beat amplitude was sign corrected based on the direction the stimulus was moving to make positive differences in the wing beat amplitude representative of tracking. The total tracking response was calculated for each treatment and compared between treatment groups using a two tailed unpaired t-test. All analysis was done using the python IDE Spyder.

Mosquito eye microscopy

To estimate the extent to which mosquitoes can perceive environmental stimuli, the mosquito eye was examined under a Zeiss Axioscope 5 microscope with a ProgRes CF CCD camera attachment. Each eye was photographed at different height levels to obtain multiple ommatidial layers. Each layer was separated by a depth of 0.0126 millimeters. A 3D focus stack was then created by combining all the layers together using a custom Python script. From this image, the number of ommatidia in the mosquito eye was determined by automated counting of the individual ommatidia that appeared in the high-resolution 3D focus stack using another custom Python script.

RESULTS

Effects of blood satiation on behavior toward a visual stimulus

To test the effects of blood-fed status, two groups of mosquitoes of the Orlando strain were tested with the only difference being that the gravid group was blood-fed 72 hours prior to experimentation. The average tracking is plotted in Figure 4 as a solid line, and the range of responses is represented as the shadow around each line. The large vertical shadow represents the region compared for significant differences. The bar was a long vertical black strip, and the wide field was a random cloud of lit pixels. The difference in the tracking between the two treatment groups was significantly different for the bar stimulus ($p < 0.05$). These results suggest that physiology alone is enough to cause a shift in attraction to a host cue.

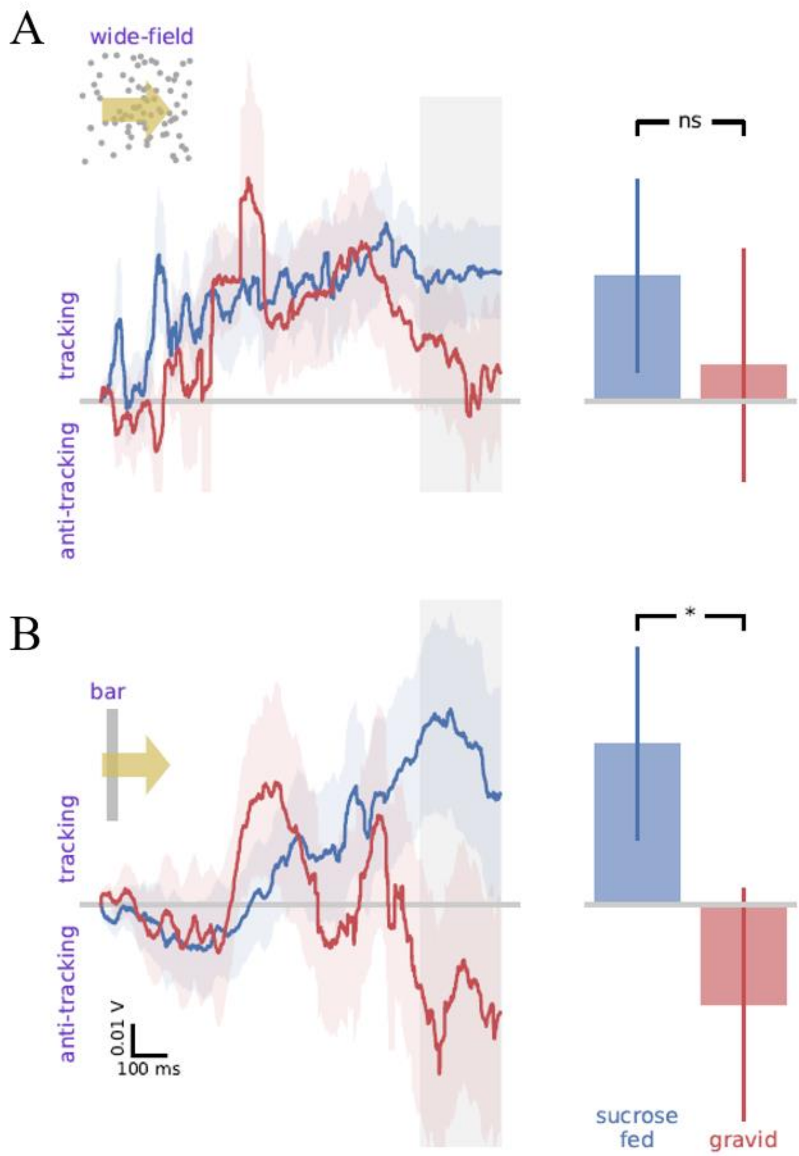


Figure 4. Visual tracking (dWBA) between gravid (n = 26) and non-gravid (n = 25) mosquitoes in response to visual stimuli. The solid line represents the average response, and the shadow surrounding the line represents the range of response for a particular treatment. The large vertical shadow represents the region compared for significance. **A**. Difference in wide-field tracking (dWBA) of both groups. **B.** Difference in bar tracking (dWBA) of both groups. Asterisks represent a significant difference between the two treatment groups using a two tailed t-test ($p < 0.05$).

Analysis of mosquito eye anatomy

Figure 5A shows one of the 3D focus stacks generated for a female mosquito. Due to a lower than optimal number of photos being used, the resolution of the corners of the image was poor, and there are slight alterations in the color of the eye. After being generated, the focus stack was fit to a manually created outline of the eye and counted using another custom Python script (Figure 5B). This approach is useful for quality assurance and allows manual counts to be made if any ommatidia are missed by the software. The results of the automated ommatidia counts are recorded in Table 1.

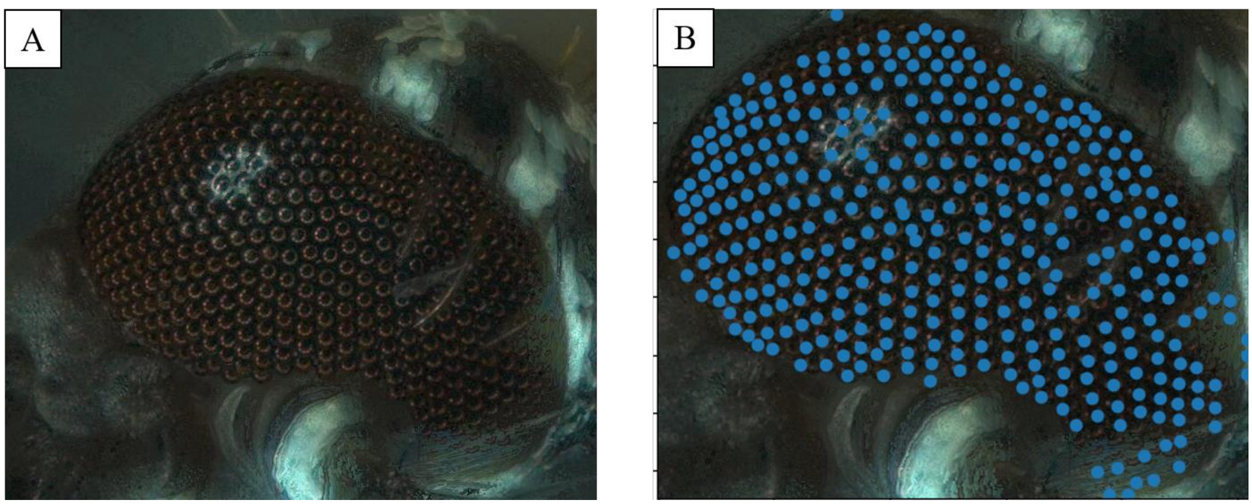


Figure 5. A. 3D focus stack made by layering 27 photos taken by a confocal microscope with a resolution of 0.0126 millimeters between each layer. **B.** Automated counting of the ommatidia in the focus stack. Each ommatidium is marked by a blue dot to show that it has been detected.

Table 1 enumerates the differences between ommatidia number in male and female mosquitoes with its corresponding standard deviation. No significant difference was found between sexes using this method ($p > 0.05$).

Table 1. Ommatidia counts of the 3D focus stacks for both males and female mosquitoes.

Sex	Females	Males
Ommatidia counts	317 ± 46	311 ± 70
N	3	4

DISCUSSION

Mosquito host-seeking behavior is complex and multifaceted. However, tethering technology has provided an efficient approach to determining how vision is used during navigation of mosquitoes at different life stages. Furthermore, the effects of blood–fed status on the response of female mosquitoes to visual cues was studied, along with a new system to quantify the anatomical features of the mosquito eye.

Integration between blood-fed status and vision

It was found that digesting a blood meal is enough to significantly suppress the attention of female mosquitoes to a bar ($p < 0.05$). This is the first evidence to this writer's knowledge of blood-fed status alone suppressing the response of a mosquito to a visual cue in a tethered mosquito assay. Since mosquito behavioral responses are strongly increased when multiple host cues are present (Raji and DeGennaro 2019), it is expected that synergism between vision and other host cues might be further repressed in gravid mosquitoes when compared with non-gravid mosquitoes. To investigate this, olfactory cues such as CO_2, human sweat, or lactic acid will be presented in combination with a visual stimulus. It is hypothesize that non-gravid mosquitoes will have an amplified response to the bar, in contrast with gravid females. On the other hand, olfactory cues such as the earthy organic smell of Geosmin are expected to be appealing to mosquitoes navigating toward a water source for oviposition (Melo et al. 2020). Therefore, reactions to a visual stimulus may be amplified in gravid mosquitoes when both Geosmin and a visual stimulus are combined. These experiments may elucidate a connection among blood-fed status, olfactory cues, and vision.

Quantifying mosquito eye anatomy using microscopy

The results of the ommatidia eye counts were 317 ± 46 in females on average and 317 ± 46 for males. Currently the literature is unclear on the precise average amount of ommatidia, and reported values range from 200 - 800(Hu 2013; Singh et al. 2013). To determine the accuracy of the method used to quantify ommatidia in this study, a comparison of multiple methods of ommatidia counting will be done in parallel at a later stage. However, it appears there is an issue with artifacts appearing in the image resulting from the photo stack, and occasional missed counts. This is especially true near the corners and edges of the eye of the mosquito. Therefore, the parameters of this experiment, including a smaller difference in depth of the images taken and their quality may need to be optimized before this method can provide useful information to the field.

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