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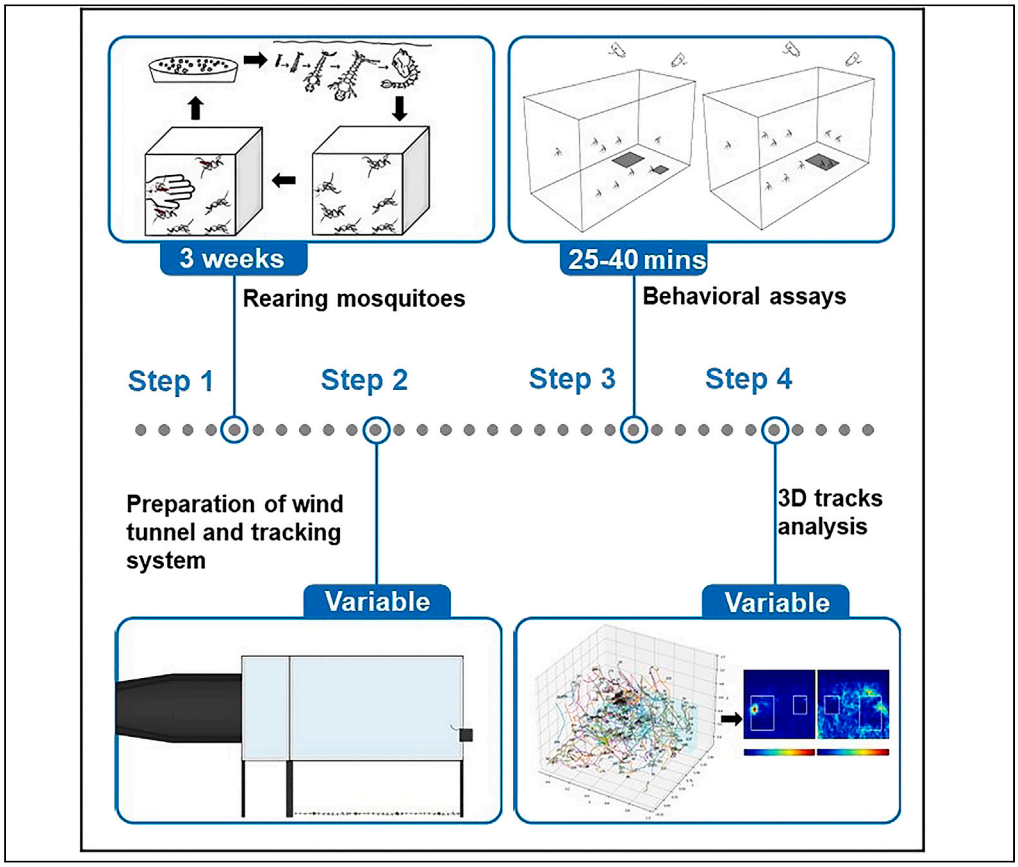
Protocol for rearing and using mosquitoes for flight path tracking and behavioral characterization in wind tunnel bioassays



Mosquito behavioral assays are an important component in vector research and control tool development. Here, we present a protocol for rearing *Anopheles* mosquitoes, performing host-seeking behavioral bioassays, and collecting 3D flight tracks in a large wind tunnel. We describe steps for setting up host-seeking landing assays, both as a non-choice and as a dual-choice assay, and analyzing flight tracks. This protocol can be applied in the research of several behavioral traits, including nectar seeking, resting, mating, and oviposition behavior.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for rearing *Anopheles* mosquitoes to be used in behavioral assays

Protocol for behavioral assays in a wind tunnel coupled with a 3D tracking system

Detailed workflow instructions for *Anopheles* host-seeking behavioral studies

Adaptable for other behavioral traits and other mosquito species

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Protocol for rearing and using mosquitoes for flight path tracking and behavioral characterization in wind tunnel bioassays

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SUMMARY

Mosquito behavioral assays are an important component in vector research and control tool development. Here, we present a protocol for rearing *Anopheles* mosquitoes, performing host-seeking behavioral bioassays, and collecting 3D flight tracks in a large wind tunnel. We describe steps for setting up host-seeking landing assays, both as a non-choice and as a dual-choice assay, and analyzing flight tracks. This protocol can be applied in the research of several behavioral traits, including nectar seeking, resting, mating, and oviposition behavior.

For complete details on the use and execution of this protocol, please refer to Carnaghi et al.[1]

BEFORE YOU BEGIN

Behavioral studies are a powerful tool that can be used to investigate how insect sensory systems function, what stimuli can be detected, and what, if any, reaction is produced at an organism level by the stimuli.[2] Animal behavior is however a very complex product of a continuum of responses to internal and external stimuli, thus care must be taken when selecting behavioral assays and conditions in which these are performed. Specifically, the behavior that is being observed may be only part of the entire sequence, and behavioral endpoints in particular, such as the number of individuals that land on a treatment or the number of eggs laid in a treatment, should be interpreted within the wider context of behavioral sequences. For example, a common practice used to measure insect 'attraction', defined as the response that draws the insect towards the source of the stimulus, is counting the number of insects that arrive at the source of the cue.[3,4] However, this endpoint result depends on a combination of several spatio-temporal responses to cues that drive the different stages of the host-seeking behavior. This sequence of behaviors, that ultimately leads to arrival, includes: initial activation, orientation, close-range approach, and landing. Attraction, as defined above, is therefore only one component within the behavioral sequence and the final number of insects arriving at the source cue might not accurately reflect the level of attraction at different distances from the cue nor the effect on other behaviors within the behavioral sequence.[5] The number of arriving insects is thus only a partial measure of attraction, a proxy that does not fully account for these other influential parameters. To understand the behaviors that ultimately contribute to an endpoint, and the effects of these on the endpoint, the methodology of behavioral assays should

be appropriately designed to control the variables of interest and to accurately separate and quantify specific behaviors at different spatio-temporal points.[5] Only by doing so can one gain an accurate understanding of the overall behavior of the insect and the role of different stimuli that affect behavioral components.[6] The protocol presented here helps to understand which variables are important to consider when studying close-range and landing behaviors of host-seeking *Anopheles coluzzii* mosquitoes, a principal vector of malaria. Here, we describe the specific steps to use to set up a behavioral assay in a large wind tunnel using *An. coluzzii* mosquitoes. This protocol is also applicable, with appropriate modifications, for the study in large wind tunnels of other behavioral traits, such as nectar seeking and feeding, resting, mating, and ovipositing. This protocol could also be used to test said behaviors in other mosquito species (e.g., *Aedes aegypti* or *Culex quinquefasciatus*) and in male individuals. Modifications should take into account the range of variables that drive each behavior, and how can each behavior be isolated and measured. The assay should also be carried out in a manner that allows for the behaviors to be expressed, e.g., carried out in the appropriate time of the insect light-cycle, under the appropriate environmental conditions.

In the field of insect vectors of human pathogens, gaining knowledge on their behavior has led to the development of new control tools and improvements of older established methods, thus helping towards the overarching goal of reducing the burden of vector-borne diseases.[7–11] This protocol provides detailed information that supports the development of new research on *Anopheles* mosquito behavior, an area critically needed for the current challenge of malaria control. Before beginning the behavioral bioassays, we recommend optimizing rearing conditions following our protocol to obtain a production of mosquitoes that present consistent behavioral traits. These features are important, since conditions experienced in the immature life stages of the mosquito can impact their behavioral and physiological traits.

Institutional permissions

Before initiating any of the systems described in this protocol, researchers should confirm they meet arthropod containment guidelines as defined by their institution.

Mosquito rearing

Timing: Approximately three weeks to obtain adult mosquitoes five days post-emergence

Methodologies for mosquito rearing and colony maintenance have a direct impact on the behavioral and physiological traits that are selected generation after generation.[12] For example, larval rearing conditions have been proven to affect the immune responses,[13] and the fitness and body size[14] of adult mosquitoes. Similarly, the conditions in which mosquitoes are kept once they emerge can influence their biological traits, and thus their responsiveness to experimental conditions. Thus, when planning behavioral assays, it is important to ensure that mosquitoes used in the experiments are reared under consistent conditions. Particular attention should be given to factors such as the ambient temperature, ambient humidity, and light cycle in which the colonies are kept, the feeding source, the mating status, and age of the mosquitoes used in the experiment.

It is also important to highlight that given the strong alterations to biological traits that result from rearing and colony maintenance conditions,[12] and the subsequent possible differences that arise between colonized mosquitoes and wild mosquitoes,[15] care must be taken when comparing results of experiments that use different mosquito strains or populations.

Note that the protocol presented below for the rearing of mosquitoes is specific for *Anopheles gambiae s.l.* species, but with adequate adjustments (e.g., changing the food source) it could be adopted to rear other mosquito species.

For general protocols on laboratory sanitation, general maintenance, and mosquito colony management we recommend following instructions detailed elsewhere.[16]

Throughout the rearing process, keep mosquitoes at 25 ± 2°C, with a relative humidity between 65%–80%, and with a light period of 12:12 h LD cycle.

1. Prepare isotonic water solution as per steps outlined in materials and equipment set up.
2. Prepare larvae tray.
   a. Place approximately 1.5 L of isotonic water solution in a clean container. Containers should have a surface area of at least ~ 400 cm^2 to allow larvae to have sufficient surface access.
   b. Cover the internal walls of the larvae tray with a band of filter paper approximately 5 cm high, so that ~ 2.5 cm of the filter paper is submerged in water and the remaining 2.5 cm are above water surface.
3. Transfer approximately 500 eggs from oviposition dishes to larvae tray by squirting water with a pipette onto the oviposition dish and letting it drip onto the larvae tray.
4. Gently blow on the water surface to push eggs toward the edges of the container, where they adhere to the filter paper.

   Note: Although pushing the eggs toward the edges of the container is not essential, it will make step 6b easier.

5. Add food source to larval trays ad libitum.
   a. Add powdered organic baby rice and fish flakes following indications on Table 1.
   b. Add rice powder and fish flakes when preparing new larval trays (day 1 and day 5 of the cycle, see step 6), and solely fish flakes on all remaining days.

   Note: Adjust the quantity indicated on Table 1 depending on larval stage, mortality, pupation, and food residue in water (i.e., if water presents a heavy presence of food residue from the day before reduce or pause the quantity of food administered that day).

   ⚠ CRITICAL: It is important not to overfeed larvae as this will lead to high mortality rates.

6. Larvae maintenance.
   a. Check larval tray every day to assess water conditions. Food must be present at all times but must not be excessive. If excessive food is present on the floor of the larval tray, remove it by drawing it into a disposable plastic pipette and discard.

   Note: If the water surface presents a film of fat, gently skim the surface with a paper towel to remove the film. This tends to occur when the food source contains a high percentage of fat, e.g., when other food sources are used.

Table 1. Quantity of food to be provided during larval growth per larva

Days since eggs were first transferred in larval tray	Weight of fish flakes	Weight of organic baby rice powder	Notes
Day 1	0.05 mg	0.1 mg	Preparation of larval trays
Day 2	0 mg	0 mg	
Day 3	0.1 mg	0 mg	
Day 4	0.1 mg	0 mg	
Day 5	0.2 mg	1 mg	Preparation of new larval tray and separation of larvae in groups of approx. 100 per tray.
Day 6	0.75 mg	0 mg	
Day 7 and subsequent days until larvae have pupated	1 mg	0 mg	

It is recommended to calculate this quantity depending on the number of larvae present in each tray. Prior to administering the food, visually inspect the water to determine whether the nutrient level in the water needs adjustments.

b. After 24 h from the egg transfer, remove unhatched eggs and place them into a newly prepared larval tray.
c. Once the eggs are in the new tray, mechanical stimulation can be performed to promote hatching, e.g., vigorously squirt water on top of the eggs using a disposable pipette, causing them to be submerged in water. Add food as per step 5.
d. Five days after having put the eggs in the larvae tray prepare new trays as per step 2 and separate larvae into groups of approx. 100 individuals per new larvae trays.

Note: Larvae can be separated by scooping them using a small sieve or can be individually picked up by using a plastic pipette. Larvae are counted manually using a hand tally counter.

Note: Step 6d is not essential, but highly recommended in order to provide the optimal conditions for larval growth, and therefore to obtain adults with adequate body size, as this parameter strictly relies on larvae density and surface water availability.

Note: Changing the water helps maintaining the correct level of nutrients in the water, thus preventing accumulation of toxic levels of food detritus and hazardous bacteria in the water.

7. Collection of pupae (approximately one week after eggs hatch).
   a. Separate pupae daily using disposable plastic pipettes and place them in plastic cups containing approx. 100 mL of isotonic water.
   b. Place the pupae dishes into 30 cm × 30 cm × 30 cm adult insect cages.

Note: For behavioral experimental purposes, keep records of emergence date for each batch of mosquitoes and remove non-emerged pupae from the cage in order to keep track of the exact adult age of each mosquito batch. This will be important as different behavioral traits are age dependent.

8. Maintenance of adults and egg production.
   a. Mosquito density per cage may vary depending on the number of mosquitoes required for each experiment, but it may never exceed approx. 400 adults per cage to avoid overcrowding.

Note: By maintaining male and female mosquitoes in the same cage, mosquitoes will naturally mate. For experiments using unmated mosquitoes, separate pupae according to sex, place the two groups in two separate cages and 12 h after emergence check thoroughly all individuals in the cage to confirm the correct sex assignation. If needed, remove adults that were incorrectly assigned to a cage. Remove the pupae dish, and if it still contains pupae, place it in a new cage. This will ensure that cages only contain individuals of a specific sex and, in cases where pupae were incorrectly separated, not enough time was given to the adult form to perform mating, thus making sure that all individuals in the cage are unmated.

Note: As male mosquitoes tend to emerge before their female siblings, the first batch of emerged adults is mostly composed of male individuals.

   b. Place one sugar feeder per adult cage and replace it every five to seven days or when visibly contaminated with mold, which can occur when there is poor ventilation in the room. A sugar feeder consists of a plastic container filled with sucrose solution (see materials and equipment for instructions on the preparation of sucrose solution). To prepare the container (Figure 1):
      i. In the lid of a plastic container (approx. 10 cm diameter and 7 cm height) cut a hole of about 2 cm diameter.
      ii. Cut a 20 × 4 cm strip of lint and place a large ball of cotton wool (approx. 2–3 cm diameter) in the center.
      iii. Dip the lint-covered cotton wool ball into the sucrose solution.

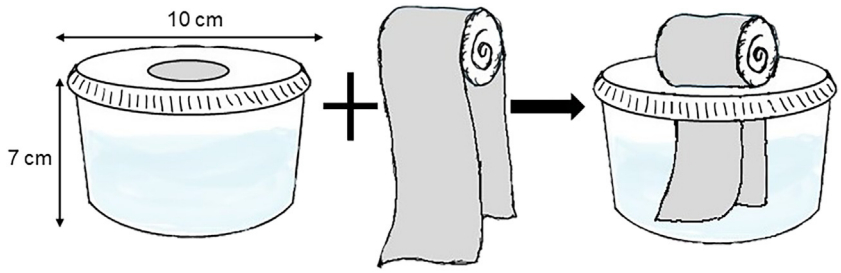

Figure 1. Steps to create a sugar feeder include cutting a hole in the plastic container lid, creating a lint-covered cotton wool ball, and placing the lint strips into the hole of the lid whilst keeping the soaked cotton wool ball on the outside of the lid

iv. Insert the lint strips into the hole of the lid, so that the strips are submerged into the solution, whilst the cotton ball remains on top of the lid.
v. Place the lid onto the container.

Note: In rooms with strong ventilation, the sucrose solution could evaporate within five days. Thus, we recommend assessing the status of the feeder every two days to ensure mosquitoes have optimal feeding opportunities.

c. Blood feeding: at the start of the scotophase, offer to five- to ten-day-old adult females a blood meal either using an artificial feeder with animal blood (e.g., horse blood or sheep blood) or using a human meal (i.e., by inserting an arm into the cage).

Note: To maximize egg production, present two blood meals for two non-consecutive days with a day of rest in between.

Note: When rearing anthropophilic species it may be advantageous to rear female mosquitoes by feeding them on human blood from a live host by inserting an arm into the cage. This is done to offer the whole compliment of host-associated stimuli and thereby maintain as much as possible in a laboratory colony the anthropophagic behavioral trait and anthropophilic preferences that these animals express in wild populations. On the other hand, when rearing zoophilic species, or when human blood cannot be sourced, a blood meal using animal blood in a membrane feeding system is a suitable alternative.

d. Insert oviposition dishes into adult cages. Oviposition dishes consist of a Petri dish of ~ 9 cm diameter containing a moist filter paper placed on an isotonic water-soaked cotton wool layer.
e. Once the eggs are laid, recover them from the cage and follow step 3 to continue rearing a new generation of mosquitoes.

⚠ CRITICAL: Oviposition dishes need to be kept moist at all times. If the filter paper dries, eggs will no longer be viable.

Note: We suggest inserting the oviposition dishes in the cage 12 h after a blood feeding event to prevent them from contamination deriving from the mosquitoes' excrements which result from the blood digestion. This step is not crucial, and dishes can be placed in the cage immediately after the blood feeding event.

Wind tunnel and 3D tracking system preparation

◷ Timing: Variable

When studying the behavior of an animal, it is important to replicate in the experimental set up the conditions that the animal would normally experience to obtain observations that reflect more faithfully their natural behaviors. For mosquitoes, this encompasses providing appropriate illumination, ambient temperature and humidity, and conducting the experiments at a suitable time with respect to the animal photocycle and the circadian periodicity of behaviors of interest. It is also important to provide an adequate area in which the animal is to be studied. Small arenas pose external constraints on mosquito behaviors and therefore might influence their overall response to presented stimuli. Thus, it is important to ensure that mosquitoes have adequate space to freely execute flight maneuvers.[5] We recommend conducting the testing in wind tunnels that provide sufficient space for the behavior in question to be executed. When using male mosquitoes, experimenters should exercise caution to not introduce sounds in the range of ~ 200–700 Hz (deriving for example, from the fans or atomizing humidifiers) in the arena, as these could inadvertently trigger a behavioral response in male mosquitoes.[17]

To underpin the precise behavioral steps elicited by a set of cues it is necessary to analyze movement-by-movement the response that the animal exhibited. The implementation of a 3D tracking system can therefore substantially augment the information obtained in a behavioral assay beyond crude endpoints, and can provide insights into the complete sequence of behaviors that take place,[1,10] information that otherwise could be lost, and misleading conclusions could be extrapolated without it.

Note: The section below describes the generic set up for the 3D tracking system used in our laboratory to track mosquito flight behavior.[1,8] However, it is important to note that there are several 3D tracking systems available, and each system may have different tracking paradigms. For an exhaustive review on the available tracking tools, the advantages and disadvantages of each different tool, and the different aspects that need to be taken into account when setting up a tracking system, we suggest reading Spitzen and Takken's review.[18] We recommend always following the bespoken instructions provided by the different 3D tracking software providers.

9. Set up a wind tunnel arena. The wind tunnel arena has to be equipped with the capability of maintaining a stable environmental temperature of 25 ± 2°C and humidity of 65 ± 5% RH.

Note: Dimensions depend on the behavior that is tested, and on the available equipment. For host-seeking behaviors we recommend setting up a space of at least 1 m^3.

Note: For 3D tracking purposes, the walls, roof, and floor of the arena should be made of a uniform, pale color material, such as anti-static clear acrylic plastic sheets. This allows cameras to have a clear view of the area inside the arena.

10. Place visual cues in the wind tunnel to allow insects to navigate using optomotor responses.

Note: Visual cues can be presented in several ways, e.g., checkerboard pattern projected on the floor of the arena,[19] or square tiles (~ 9 cm per side) of a color that contrast against the background which can be placed randomly on the floor,[1] e.g., black tiles placed on a white background.

Note: It is important to ensure that the tiles or the projected pattern do not interfere with the tracking system. Tiles should be made of infrared (IR) transmitting material.

11. Set up the airflow in the arena by having a system of impelling and extractor fans.

Note: Air should be purified by passage through activated charcoal filters, warmed and humidified to equate the environmental conditions of the arena. Air flow should be laminar and with a wind speed of less than 0.2 m s^{-1} as indicated in previous studies.[1,8,20–22]

12. Set up the light source to be used by the insects during the assay.

Note: The intensity of the light used depends on the mosquito species and the type of behavior to be examined. For *Anopheles* mosquitoes and host-seeking behavior we recommend a low illumination level at approx. 1.16 W m^{-2} between 420 and 680 nm, which is comparable to the light level mosquitoes experience in the field under natural starlight illumination.[8]

13. Set up 3D tracking system package (i.e., the 3D tracking software and the post-tracking analysis software).
    a. Create a diffuse and evenly distributed IR background illumination around the wind tunnel.

⚠ CRITICAL: Ensure that the are no blind spots of IR illumination in the field view of interest. If the IR illumination is patchy, the 3D tracking system will not be able to detect mosquitoes in dark areas where the illumination is insufficient. Likewise, the system will not accurately detect mosquitoes flying on top of areas that are too bright. The level of IR illumination should be consistent across the whole area that is intended to be analyzed.

Note: Different tracking systems may require different illumination settings. We recommend following instructions provided by the supplier of the 3D tracking system package, and where possible, take advantage of the bespoke lighting solutions offered by some software providers.

    b. Set up the high-resolution analog cameras so that their field of view covers the entirety of the area of interest. The cameras should be equipped with IR filters that only permit the passage of light > 800 nm, thus ensuring that the only light visible for the cameras is the evenly distributed IR illumination.

Note: Equipping the cameras with IR filters is particularly important as *Anopheles* mosquitoes exhibit host-seeking behaviors at low intensities of visible light, rendering it difficult for the cameras to capture mosquito silhouettes via visible light only. Thus, the IR illumination allows for the insects to be well lit for the cameras, whilst also providing a background illumination which is invisible to mosquitoes.[2] The filters ensure that fluctuations of visible light do not impact the image recorded by the cameras.

    c. Set up the 3D tracking software on a computer following manufacturer instructions.

Note: Depending on the 3D tracking software the requirements for the computer to be used may differ.

    d. Set up the post-tracking analysis software by following instructions from the 3D tracking system package or by choosing one of the available automated and custom-made programs freely available online.[18]

Odor source preparation

Timing: 24 h

For host-seeking behavioral assays it is common to activate female mosquitoes by using olfactory stimuli. The closer the resemblance there is between the odor stimuli provided and the odor signature of the mosquito's natural host, the more the behaviors observed will reflect mosquito natural responses to host odor. As such, a variety of different odor sources can be used, depending on the mosquito species being studied. We recommend using a combination of carbon dioxide and host skin odor.

14. Set up the carbon dioxide delivery system.

a. Connect the pure carbon dioxide canister to a stimulus controller (pulser) and set the outflow to be delivered in a pulsed manner (8 s on and 7 s off), with a flow rate of approx. 5 L min^{-1} and a final concentration of ~ 4.5%.
b. To better resemble human breath, humidify the carbon dioxide enriched air by pumping it through deionized water in a Dreschel bottle. The output tube of the Dreschel bottle is to be positioned where the experimenter wants the odor source delivery platform to be placed during the assays.

15. Set up the skin odor delivery system.
    a. Prior to the commencement of the experiment, designate a volunteer that will wear a pair of 15 denier sheer knee-high 100% polyamide nylon socks, where the foot odor will be collected.

Note: Prior to using the nylon socks, wash them with fragrance-free washing detergent at 60°C and allow them to air-dry before wearing them.

    b. One hour prior to wearing the socks, the volunteer should wash their feet with water and fragrance-free soap. The volunteer should wear the socks for 24 consecutive hours. Whilst wearing the socks the volunteer should, where possible, wear the same footwear for all odor collections.
    c. After wearing the socks, place them at the odor source delivery platform, ready to be used in the experiment.

Note: When not in use during experiments, the socks should be kept sealed in a resealable plastic bag at −20°C to minimize variation in odor composition.

    d. Replace the socks with a new pair of freshly worn socks every week.
    e. Throughout the duration of the experiment, the volunteer should abstain from smoking, consuming alcohol or spicy food, using perfumes or perfume soaps and washing detergents as these substances might alter the skin odor collected.

Preparation of the treatments to be tested

Timing: 3 h

In the case of studies that focus on landing or close-range host-seeking behaviors, prepare suitable targets that mimic the host cues that are to be investigated.

It is important to note that physical stimuli are often intrinsically bound to the properties of other stimuli. For example, thermal stimuli strongly depend on the intrinsic properties of the material used to construct the target, which in turn can also affect other properties, such as the visibility and dimension of the target. Likewise, the orientation of a target, i.e., whether this is positioned horizontally or vertically, can also affect the area size in which the thermal plume is generated, and therefore, can affect how mosquitoes detect the target. Similarly, the evaporation rate on a target surface of water vapor is strongly associated with the target surface temperature. These are just a few examples of how different stimuli are interlinked between each other. It is clear that experimental methods for decoupling the effect of the different stimuli should be developed if the aim is to understand the specific behavioral effect of each stimulus.

Note: For the study of other behavioral responses, prepare suitable cues that would elicit the behaviors of interest.

Note: If using a 3D tracking system, ensure that the material employed in the construction of the target does not obstruct the cameras and allows IR light to go through, i.e., use IR transmitting material.

16. Create a target of the desired shape and size by using an IR transmitting black acrylic sheet.
17. Position the thermal dispensing device (i.e., the part of the target that presents the thermal cue) on top of the acrylic sheet. The thermal dispensing device is composed of a water-filled transparent bag, or multiple bags, that can be warmed to the desired temperature.[1]
    a. Custom-make the transparent plastic bags according to the size and shape of the target by sealing three sides of the bag with a heating source.

Note: The dimensions of the plastic bag should be adjusted depending on the type of stimulus required (e.g., if the target is to be presented with the entirety of its surface heated or unheated, create a plastic bag that covers the entire surface of the acrylic sheet; if a target is to present only half of its surface as heated, create two plastic bags sized according to half of the acrylic sheet surface).

    b. Fill the bags with an adjusted volume of water so that the final thickness is equal for all bags.
    c. Remove air bubbles and seal the remaining side using a heating source.

Note: Once made, the plastic bags can be re-used for multiple experiments. It is recommended to check the integrity of the bag at the commencement of every assay and replace it if it is leaking.

    d. If the target is to present a thermal stimulus, warm the sealed bag(s) by placing them in a water bath so that the entire bag reaches the desired temperature. If the target does not include a thermal stimulus, skip to step 17e.
    e. Place the custom-made sealed bag(s) on top of the black acrylic sheet.
    f. Cover the target with one layer of transparent adhesive film.

Note: The adhesive film will capture landing mosquitoes as they adhere upon touching the surface of the film.

    g. Change the layer of transparent adhesive film at the end of every assay.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Defibrinated animal blood	TCS Biosciences	Depending on the animal, for horse blood: HB034, for sheep blood: SB054.
Chemicals, peptides, and recombinant proteins		
Aquarium salt	Tropic Marin	Pro-Reef
Organic baby rice	Aptamil	4–6+ months
Fish food	TetraMin	Tropical flakes
Experimental models: Organisms/strains		
Anopheles coluzzii	Institut de Recherche en Sciences de la Sante, Burkina Faso	N/A
Software and algorithms		
TrackIt3D (version 3.0)	SciTrackS GmbH	N/A
Track Analysis (custom-made program)	This paper	https://zenodo.org/records/10092390
Other		
Rearing cages for adults	BugDorm	4M3030
Transparent plastic bags	Polybags	Clear Polythene Bags
Fragrance-free washing detergent	Surcare	N/A
Fragrance-free soap	Childs Farm	Baby Wash Unfragranced
IR-transmitting black acrylic sheets for targets and optomotor squares	Southern Acrylics	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IR LEDs	JC	N/A
Analog cameras	Basler	acA2440 – 75 mm
Camera lenses	Fujinon	HF6XA 5M
IR filters for camera lenses	MidOpt	LP830 band pass
Transparent adhesive film (for the creation of the target)	Barrettine	FICSFILM
Artificial blood feeders starter pack comprising power unit, feeders, reservoirs.	Hemotek	SP6W1-3
Stimulus controller (pulser)	Syntech	CS-05
Nylon socks	ASDA	N/A

MATERIALS AND EQUIPMENT

Short recipes

Isotonic water solution: dissolve 10 g of aquarium salt in 10 L of deionized water. This solution can be stored in closed containers at ambient temperature for up to two weeks. Do not use this solution if noticeable algae contamination occurs.

Sucrose solution: dissolve 10 g of sucrose in 100 mL of deionized water. This solution can be kept in closed containers refrigerated at $\sim 4^{\circ}C$ for up to one week.

Equipment

For the behavioral studies we used a large wind tunnel with a flight arena which was kept at $25 \pm 2^{\circ}C$ and $65 \pm 10\%$ and has the following dimensions: 1.2 m wide × 1.2 m high × 2 m long. The walls of the arena are made of white opaque Perspex while the roof panel is made of transparent Perspex, thus allowing the cameras positioned above the roof to have a clear view of the inside of the arena (Figure 2). The flowing air was drawn from outside the building by an impelling fan, it was purified by activated charcoal filters, humidified and heated to match conditions kept in the flight arena, and it was pushed through a cotton screen to create a laminar airflow. At the downwind end of the wind tunnel an extractor fan pulled air out of the laboratory, thus creating a constant flow of air of 0.2 m s^{-1} from the upwind end to the downwind end and providing negative pressure to the wind tunnel and flight arena. To provide an evenly diffused IR illumination the wind tunnel structure was encased in white sheets, and the IR LEDs were set up on each corner of the flight arena on the floor and on the outside walls and were angled away from the arena and towards the white sheets (Figure 2). Thus, the IR light that arrived on the arena consisted of the reflection that was projected on the white sheets, ensuring an even and diffuse illumination.

Note: Between experiments or replicates clean the wind tunnel surfaces by wiping them with deionized water, then wipe them with 70% ethanol and leave to air dry. All fabric components of the wind tunnel (i.e., nettings and brushed cotton screen) are to be washed at $60^{\circ}C$ with a fragrance-free detergent.

Note: To minimize contamination of human skin odor onto wind tunnel equipment, it is recommended to always wear clean surgical gloves when touching the equipment.[21]

Software

To track mosquitoes in this study we used the software TrackIt3D (version 3.0).

Alternatives: There is a wide range of software that allows 3D tracking of moving objects.[18] Any software that can track multiple objects that move at the same time and of the approximate size of a mosquito can be used.

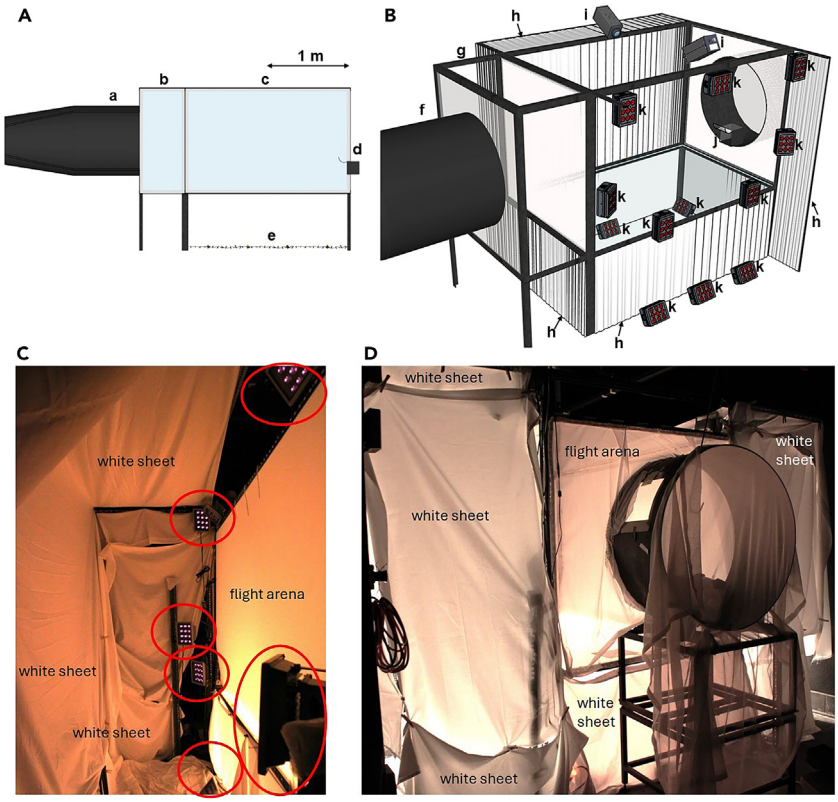

Figure 2. Wind tunnel and flight arena schematic

(A) Simplified schematic of the lateral view of the experimental area with its different components and scale bar indicated on the top right: (a) air conveying tube, (b) odor delivery chamber, (c) flight arena, (d) release cage, (e) set of lighting array on the floor that provide the light used by the insects during the assay. Note that the air conveyed into the wind tunnel is purified by passage through activated charcoal filters, is warmed and humidified to specific experimental conditions, and is pushed through a screen of brushed cotton to create a laminar air flow.

(B) View of the experimental area with the components for the 3D tracking system: (f) air conveying tube, (g) odor delivery chamber, (h) white sheets encasing the wind tunnel, (i) cameras connected to the 3D tracking system, (j) release cage, (k) IR LEDs on the floor underneath the wind tunnel and on the side of the flight arena. Note that all IR LEDs were angled away from the flight arena and were pointing towards the white sheets. Note that to show the IR LEDs the white sheets on the right side of the wind tunnel have been removed from the schematic.

(C) Photo of the set up outside of the flight arena to show the IR LED, which are indicated by the red circles. The flight arena can be seen on the right side, encased in white sheets which can be seen on the top, left, and bottom side. The IR LED are angled away from the arena and shine the light towards the white sheets which reflect the light back on the arena. This creates a diffuse and evenly distributed IR lighting effect. Note that for illustrative purposes the light level in the photo is brighter compared to light levels used during experiments, which resembled moon-light levels.

(D) Photo of the set up outside of the flight arena, taken from the downwind side looking upwind. The white sheets and flight arena are indicated.

For the post-tracking analysis, we used the post-tracking analysis software available with the 3D tracking system package and a custom-made program, freely available on Zenodo (see key resources table). This program was also used for data visualization, specifically for the creation of 3D plots of flight tracks in the arena and of heat maps that indicated the average value of a parameters (e.g., number of flight track recorded) in different parts of the arena. Instructions on how to run the program and the type of analysis available are presented in the files on Zenodo.

Alternatives: The post-tracking analysis can be done by any program that is able to read the output of the 3D tracking software and filter erroneous data points, interpolates missing coordinates, and provide the means to analyze flight parameters. Typically, tracking data is

output in the form of a spreadsheet that lists the objects recorded and their associated coordinates in three dimensional axes of x, y, and z, with a time stamp for each coordinate.

STEP-BY-STEP METHOD DETAILS

Selection of mosquitoes and habituation

Timing: 5 h

Prior to the commencement of each assay, mosquitoes should be selected, starved to increase their propensity to seek food, and left in dark conditions to allow the ommatidia to adapt to low light levels.[23] The explanation on how to do so is described in the steps outlined below.

Note: When planning the time of the day in which to conduct the experiment, take into account the internal circadian light cycle of the mosquitoes and ensure to match this with the specific behavior examined and the light conditions required for this behavior to be exhibited. For example, for experiments studying host-seeking behavior in *Anopheles* mosquitoes, conduct the experiments during the period of time that corresponds to the scotophase, as this behavior peaks during crepuscular and nocturnal periods.

1. Five hours prior to the commencement of the assay, select the female mosquitoes that will be used in the assay.

   Note: In our studies we released 25 mosquitoes in every assay, however, this number can be adjusted depending on the size of the arena, the tracking capacity of the 3D tracking system, and the specific requirements of each behavioral response.

   Note: For analysis purposes, we recommend using the same number of mosquitoes during all replicates and assays.

   Note: The age of the mosquito can be adjusted depending on the type of assay and on the behavior to be assessed. For host-seeking behavior, select adult female mosquitoes between five- and ten-days post-emergence.

   Note: Selected females for host-seeking behavior should be expected to be mated (as they have been kept several days post-emergence in cages together with male individuals) but should not have been offered a blood meal.

2. Separate the selected mosquitoes in a cage with no access to water or sucrose solution for 4 h (i.e., starve the mosquitoes to promote them to be responsive to host-cues).
3. One hour prior to the commencement of the assay, using a mouth-aspirator, gently transfer the selected mosquitoes to a release vial.

   Note: The release vials can be made from any type of tubes (>∼ 4 cm diameter, >∼ 10 cm height) that is open at both ends to allow air to flow through, but where the openings are covered by netting to prevent the mosquitoes from escaping.

4. Wrap the release vials containing mosquitoes in blackout fabric, to keep them in darkness.

Conducting a two-choice behavioral experiment in a large wind tunnel

Timing: 25 min–40 min

This experiment can be carried out to determine behavioral responses to two different treatments and determine mosquito overall preference, if any, for either treatment. Treatments can be composed of either individual cues or by a combination of multiple different cues. The final outcome of the behavioral response has to be a variable that can be quantified by simple counting (e.g., number of mosquitoes that landed on or approached a target, number of eggs oviposited in a specific substrate). When conducted in synchrony with 3D tracking recording, this experiment provides information on mosquito close-range behavioral responses.

Alternatives: By applying the required changes in the experiment duration and illumination settings, and by adjusting the set of cues provided, this assay could be performed to assess other behavioral traits (e.g., oviposition preferences for different substrates).

Note: To perform a choice experiment, all combinations of the two types of treatments that are to be tested should be performed. Thus, for two treatments (i.e., for a two-choice test) three types of assays need to be completed: treatment A presented along with an identical treatment A, treatment B presented along with an identical treatment B, and treatment A presented along with treatment B. Note that depending on the research question, one of the treatments presented may be a control treatment.

Note: Ensure to control for the effect of testing sequence and the daily variation bias by testing assay types in a quasi-randomized order between and within days. For more details, refer to problem 1 in the troubleshooting section.

5. Turn on the wind tunnel fans and set the environmental conditions of the flight arena.
6. At the upwind end of the flight arena, present simultaneously two different sets of cues.

Note: For example, the two targets can be of different dimensions, or two targets with different superficial temperatures.

   a. Space the two targets so that there is sufficient space in between them so that one set of cues does not mask the other set of cues.

Note: In our case, we placed two landing targets with at least 20 cm of space in between them.

   b. To avoid position effect bias, maintain symmetry in the setup, by for example, keeping the targets at the same distance from the lateral arena walls. Consult problem 2 in the troubleshooting section if results indicate position bias.
7. Turn on the illumination source for the experiment, including the IR lights, and turn off any other light source.
8. Turn on the computer that runs the 3D tracking software and open the 3D tracking application.
9. Transfer the mosquitoes from the release vial to the release cage and leave them in the cage for approximately 10 min to habituate to wind tunnel conditions.
10. At the end of the habituation period, commence the assay.
    a. Position the socks in the odor release platform and initiate the carbon dioxide stimulus.
    b. Initiate the 3D tracking recording.
    c. Open the release cage.

Note: We recommend having a system that allows for the experimenter to open the release cage remotely and in a gentle manner, to avoid introducing human odor into the arena and avoid mechanically agitating the test mosquitoes.

    d. Leave the room.

11. At the end of the assay (duration of which can vary, for host-seeking behavior we suggest conducting assays for 15 min–30 min) return to the room and end the assay.
    a. Close the release cage.
    b. Stop the 3D tracking recording and save the file with a code name that references the assay conducted.
    c. For experiments studying landing behavior, count the number of mosquitoes recovered in each part of the wind tunnel as follows:
        i. Count the number of mosquitoes on each of the treatments presented, i.e., count the number of mosquitoes caught on each of the targets.

    Note: This number will correspond to the number of mosquitoes responding to the treatment by landing.

        ii. Count the number of mosquitoes in the release cage.

    Note: This number will correspond to the number of mosquitoes that were not considered activated. If the proportion of mosquitoes found in the release cage at the end of the assay is high, it could mean that mosquitoes failed to respond to the cues provided. For possible solutions on how to solve this, consult problem 3 in the troubleshooting section.

        iii. Count the number of mosquitoes in the flight arena.

    Note: This number will correspond to those mosquitoes that were activated but did not land on the targets.

    Note: For studies investigating other behavioral traits, record the result of the chosen finite variable, e.g., count the number of eggs oviposited in each treatment.

    d. Interrupt the odor stimulus and store the socks if necessary.
12. Once the assay terminates, report the results in a database, clean the wind tunnel and leave it to air out for 10 min with no odor stimulus present before setting a new assay if needed.

Conducting a no-choice behavioral assay in a large wind tunnel

Timing: 25 min–40 min

This experiment can be carried out to determine mosquito behavioral responses to a treatment. Treatments can be composed of either individual cues or by a combination of multiple different cues. The final outcome of the behavioral response has to be a variable that can be quantified by simple counting (e.g., number of mosquitoes that landed on a target, number of eggs oviposited in a specific substrate). When conducted in synchrony with 3D tracking recording, this experiment also provides information on mosquito close-range behavioral responses.

To prevent testing sequence bias, consult problem 1 in the troubleshooting section.

Alternatives: By applying the required changes in the experiment duration and illumination settings, and by adjusting the set of cues provided, this assay could be performed to assess other behavioral traits (e.g., oviposition preferences for different substrates).

13. Turn on the wind tunnel fans and set the environmental conditions of the fight arena.
14. Position the set of cues at the end of the flight arena, in the center between the two lateral walls.
15. Follow steps 5–12 of the previous section with the only difference that in step 11c-i, the count will only be done for the number of mosquitoes found on the sole treatment provided.

Analyzing 3D tracks obtained in behavioral assays

Timing: Variable

This section explains how to analyze the data acquired from the 3D tracking system in order to obtain flight parameters that can be used to describe behavioral responses. Careful evaluation of the recorded tracks is needed before formulating any meaningful behavioral interpretation.[18]

Note: The steps described below correspond to the steps taken when analyzing the data acquired from TrackIt3D. The process will be similar irrespective of what software is used to record the flight tracks, as long as the software provides the results in a spreadsheet containing the positional coordinates of the mosquitoes (i.e., the tree Euclidean dimensions X, Y, Z), the mosquito ID code given by the program, and the time stamp for each coordinate.

16. Filter erroneous data points. This can be done using the custom-made program which is freely available on Zenodo (see key resources table) or by using the post-tracking program of the 3D tracking system package. Consult problem 4 in the troubleshooting section for solutions on how to minimize erroneous recordings.
    a. Exclude points recorded beyond the arena limit (i.e., artefacts).

Note: This can be done by visually inspecting the superimposed image of the flight trajectories obtained in each assay and the 3D space of the flight arena, i.e., the volume tracked. Flight trajectories that fall beyond the arena limit should be excluded.

    b. Exclude tracks that have fewer than 25 consecutive coordinates.
    c. Exclude tracks that last less than 0.5 s.
    d. Exclude tracks that had a total displacement in all three axes of less than 1 cm.
    e. Exclude tracks that present an abnormal flight speed, basing the filter on previously reported parameters.

Note: We recommend visually inspecting suspicious tracks (e.g., tracks with abnormal flight parameters) to determine their inclusion or exclusion.

Note: Erroneous data points should be rare. Obtaining numerous erroneous entries can be an indication of a malfunction in the recording system, for which re-calibration might be advisable.

17. Run the data files through the post-tracking program to improve the quality of the recorded data.
    a. Smooth tracks points using the ad hoc spline function, created based on mosquito flight parameters.[18]

Note: This step is not essential but is a common function included in many post-tracking programs that are included in the 3D tracking system package. We recommend following instructions provided by the 3D tracking system package to obtain the best results.

Note: Smoothing should be kept to a minimum to avoid over-manipulation and should be reported precisely. Smoothing should aim at reducing irregularities that are a product of sampling or tracking errors without removing irregularities that are intrinsic to the ground truth tracking.

    b. Interpolate missing points by estimating missing coordinates.

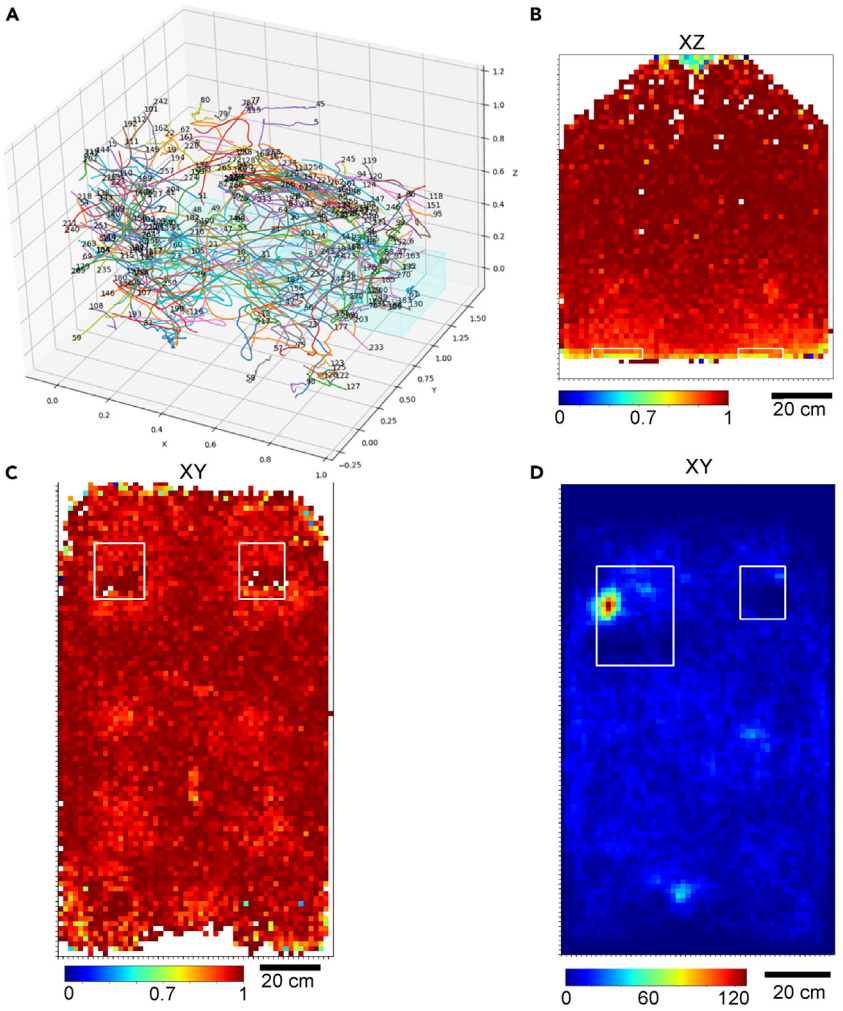

Figure 3. Examples of possible outcomes of the 3D tracks analysis

(A) Example of all the filtered tracks recorded by the tracking system in an assay. Areas of interest, here represented in light blue, were chosen depending on the type of targets used.

(B and C) Example of heatmaps of two different 2D planes showing the mean tortuosity index of tracks recorded during an assay; lower values of tortuosity index indicate more convoluted tracks.

(D) Example of heatmap showing the density of tracks recorded in different areas of the flight arena during an assay. For all heatmaps, white rectangles indicate the position where the targets were placed. Each image shows a 2D plane of two axes as indicated at the top of the image. At the bottom of the plane the color scale indicates the values associated with each color, and the scale bar is on the bottom right.

Note: Interpolation of points should be kept to a minimum and only between points that are at relatively close distance to each other. This distance should be clearly defined when presenting the results, for example, one could choose to interpolate up to five consecutive missing points before considering the two tracks as separate entities.[1]

18. Designate areas of interest within the flight arena.
    a. Determine which areas of the flight arena contain the set of cues that are being tested.
    b. Create areas of interest that encompass the areas with the set of cues plus a buffer area around the cues.

Note: The creation of areas of interest is not essential as one can analyze the totality of tracks recorded in each assay. However, as each assay generates a vast number of tracks (Figure 3), it

Table 2. Flight parameters that can be calculated for each track

Parameter	Equation for calculation	Unit
3D linear distance of a segment (straight line between the initial and final positions)	$\sqrt{(x_n - x_i)^2+(y_n - y_i)^2+(z_n - z_i)^2}$	m
Total 3D distance of a track	$\sum_{k=i}^{n-1}\sqrt{(x_{k+1} - x_k)^2+(y_{k+1} - y_k)^2+(z_{k+1} - z_k)^2}$	m
Total track time	$t_n - t_i$	s
3D linear speed	$\frac{\sum_{k=i}^{n-1}\sqrt{(x_{k+1} - x_k)^2+(y_{k+1} - y_k)^2+(z_{k+1} - z_k)^2}}{t_n - t_i}$	m s^{-1}
Tortuosity index	$\frac{\sqrt{(x_n - x_i)^2+(y_n - y_i)^2+(z_n - z_i)^2}}{\sum_{k=i}^{n-1}\sqrt{(x_{k+1} - x_k)^2+(y_{k+1} - y_k)^2+(z_{k+1} - z_k)^2}}$	Not applicable (dimensionless)
3D linear acceleration	$\frac{\sum_{k=i}^{n-1}\sqrt{(u_{k+1} - u_k)^2+(v_{k+1} - v_k)^2+(w_{k+1} - w_k)^2}}{t_n - t_i}$	m s^{-2}
Angular speed	$\frac{1}{n-i}\sum_{k=i}^{n-1}\frac{\cos^{-1}\left(\frac{u_{k+1}u_k+v_{k+1}v_k+w_{k+1}w_k}{\sqrt{(u_k{}^2+v_k{}^2+w_k{}^2)(u_{k+1}{}^2+v_{k+1}{}^2+w_{k+1}{}^2)}}\right)}{t_{k+1} - t_k}$	rad s^{-1}

Note that 'i' and 'n' are the initial and final time indexes respectively of either the complete or part of the track, i.e., track segment. Note that for the 3D linear acceleration and the angular speed it is first necessary to calculate the linear speed for a track segment at time index k. The indexes u_k, v_k, and w_k refer to the linear speed at time index k on axis X, Y, and Z, respectively.

is common practice to focus the analysis on those tracks within the areas of interest. Caution should be exercised in setting areas of interest so as to avoid excluding areas where behaviors of interest may occur. A general interpretation of all tracks recorded in each assay can be carried out via visual evaluation of heatmaps, and 3D representations of tracks (Figure 3).

19. For each replicate, using the custom-made program, determine the track parameters of the tracks that entered the areas of interest as indicated in Table 2.

Note: The 3D linear speed and acceleration are calculated for each track as a whole and do not take into consideration the variation that can occur in different segments of the same track, where segments are intended as consecutive parts of a track. Alternatively, one can compute the instantaneous 3D linear speeds or accelerations on each segment of a track and calculate the mean or median of that parameter of all segments of the track.

20. To aid in the interpretation of the 3D tracking results, create heatmaps, histograms, and 3D representations of the tracks (Figure 3) using the custom-made program or any other program that can produce these figures.
21. Analyze results obtained in step 19 with appropriate statistical tests.

EXPECTED OUTCOMES

Following this protocol, we carried out multiple assays aimed at investigating the close-range and landing behavior of *An. coluzzii* mosquitoes when offered targets that presented different combinations of three host-associated cues. Assays following this protocol will generate two types of results: raw counts of the finite variable measured (e.g., number of mosquitoes landing, number of eggs recovered in oviposition dish), and the 3D flight tracks which can be used to calculate a number of parameters that quantify flight behaviors, such as the number of mosquito visits to specific areas of the flight arena, time to first visit and visit duration, the tortuosity of flight tracks, and the moment-to-moment acceleration, linear speed and angular speed of the flight trajectories.

In a first study, we presented mosquitoes with a single target at a time (no-choice assay), and by counting the number of mosquitoes recovered on the target surface at the end of each assay, we were able to quantify the individual and the combined effect of three common host-associated cues: thermal, visual,

and olfactory.[22] We found that specific combinations of the host-associated cues, specifically the thermal and odor cue together, and the thermal, odor, and visual cue together, interacted synergistically to increase landings.[22] In a second set of no-choice assays, we tested mosquito landing responses to a single black target where the area of the target that was heated differed in each assay.[1] All assays were conducted in presence of host odor and in conjunction with the recording of mosquito flight tracks, examples of outcomes from the 3D tracking system are presented in Figure 3. In this study we were able to conclude that landing rate is a response directly proportional to the size of the surface area that presents a thermal cue, and results from the 3D track recording demonstrated that non-heated targets remained highly attractive to mosquitoes in terms of number of visits and time spent hovering above the targets. Interestingly, the mosquitoes did not land on unheated targets, which suggests that the thermal cue is a decisive factor that triggers initiation of a landing responses, whereas attraction towards the target is modulated by the thermal and visual inputs.[1] We also carried out two-choice assays to determine mosquito preferences in their short-range and landing behavior for targets of different sizes and targets placed in two different spatial orientations, positioned either vertically or horizontally.[1] Thanks to the combination of both the count of mosquitoes recovered from landing on the targets and analysis of the flight track data, we were able to conclude that larger targets outcompeted smaller targets in terms of landing numbers, but smaller targets remained highly attractive as were repetitively visited by mosquitoes. In all cases we found that the use of the 3D tracking system complements the information obtained by simple count assays, and aids in forming a more comprehensive picture that more accurately describes the behavioral responses of the insects and explains the relative importance of different sensory modalities.

LIMITATIONS

Conducting behavioral studies in a wind tunnel offers control over the conditions in which the experiments are carried out but constrains mosquitoes to an artificial environment within a limited physical area. Animals in the wild are constantly presented with a multitude of stimuli and information,[24] whilst laboratory studies offer a rather simplistic environment. In order to reach their hosts, mosquitoes in the wild must navigate complex and dynamic environments that present numerous barriers, confounding elements, and multiple stimuli. Thus, in the wild, mosquito responses might deviate from predictions obtained in laboratory settings. To better understand mosquito responses to real-world scenarios, semi-field or field-base studies should be carried out to corroborate information obtained during laboratory studies.

An inherent complication of conducting behavioral assays where multiple mosquitoes are released simultaneously is that it is not possible to discern which tracks belonged to which mosquitoes, as tracking multiple objects increases the likelihood of recording discontinuous tracks. To be able to attribute with absolute certainty all flight tracks from an assay to a specific mosquito, the assay must be carried out with one individual at a time.

TROUBLESHOOTING

Problem 1

The order of the sequence in which different assays or treatments are tested can influence the overall outcome. This problem can affect a two-choice and a no-choice behavioral assay.

Potential solution

To control for testing sequence bias, or daily variations, test the assay types or treatments in a pseudo-randomized order between and within days. Ideally, if possible, each day run a control assay and all other treatment assays in a randomized order.

Problem 2

In two-choice experiments, the position of the targets within the flight arena may interfere with the final results (step 6). This is particularly important if the wind tunnel structure is not 100% symmetrical, or when some parts of the wind tunnel are touched by the user or used more frequently than

other parts, as this will lead to the build-up of human odor on specific areas of the wind tunnel or the experimental room.

Potential solutions

- To control for positional bias, alternate the position of each target or set of cues in each replicate.
- Only touch the wind tunnel surfaces with gloved hands. Avoid remaining in the experimental room for prolonged periods of time in between assays.
- Regularly clean the wind tunnel after a small set of assays.

Problem 3

Several exogenous and endogenous factors can influence mosquitoes' activity levels, which in turns can affect their propensity to become activated in search for a host from which to take a blood meal (step 11c-ii).

Potential solutions

To ensure that mosquitoes are prone to exhibit a specific behavior, provide the conditions needed for said behavior. Ensure that the behavior is being examined.

- In the appropriate timing of their circadian cycle associated with the behavior of interest.
- In the appropriate environmental conditions (including adequate humidity and temperature in the chamber, and suitable lighting and air speeds).

Furthermore, ensure that.

- The mosquitoes used are of an appropriate age.
- The mosquitoes used are in the appropriate physiological state.

For host-seeking and landing behavior, if mosquitoes are failed to be activated, aside from all the factors listed above we suggest to.

- Prolong the starvation period to prime them for food-seeking.
- Prolong the dark habituation period to ensure acclimation.
- Provide and test an established positive control, for example by using a freshly worn socks every day or a protected human hand, or testing a synthetic olfactory attractant that produces a known behavioral response.

Problem 4

Several factors can cause the tracking system to record short tracks or erroneous tracks (step 16), which in turn may cause bias in the tracks analysis or might create misleading results.

Potential solutions

In general, short tracks originate when the 3D tracking program does not keep track of the mosquitoes for long periods of time. This could happen when mosquitoes enter areas that are not within the field of view of the cameras and therefore the program loses their position. It could also happen when mosquitoes fly over dark patches (for example, fly over a non-IR transmitting object) and the cameras are not able to differentiate the mosquito silhouette from the dark patch. Occasionally, depending on the computing power of the computer used, and on the settings of the 3D tracking program, a track could also be abruptly cut when a mosquito intercepts the trajectory of another mosquito, as the program is not able to reconstruct which track belongs to which mosquito and thus interrupts both tracks. Erroneous tracks may be recorded if the 3D tracking program is saturated (i.e., it is unable to compute all the tracks being simultaneously recorded) or could arise as a result of vibrations within the tracking area causing very small movements of the arena itself or minor fluctuations in IR illumination.[18]

To avoid these complications, we recommend.

- Keep the IR illumination stable and avoid changing the position of the IR LEDs after calibration.
- Prevent mosquitoes from flying out of sight by using barriers (e.g., nets) to create a contained area for mosquitoes to fly in.
- Wherever possible, use solely IR transmitting material, so that the material does not appear dark when illuminated with IR light.
- Use a computer with adequate computational power. The required specification should be clearly indicated by the 3D tracking software of choice.
- Where possible, reduce the number of mosquitoes released in the arena at any one time to reduce the probability of track interruption.
- Do not move the cameras from their original position after the system has been calibrated. Recalibrate the system every time the cameras or the wind tunnel are moved or are suspected to have been moved, even if this is by a few millimeters.

Problem 5

An unevenly diffused IR background lighting will create blind spots or areas in which the cameras will not be able to detect the silhouette of the mosquitoes, either because the area is not sufficiently illuminated or because the illumination in the area is too bright.

Potential solutions

To prevent blind spots created by the IR illumination:

- Where possible (i.e., for smaller arenas), use many low luminous intensity IR LEDs instead of a few high luminous LEDs. If the area is too large to be illuminated by low power IR LEDs and high-power IR LEDs need to be used, a cover made of pale fabric can be placed on top of the IR LEDs to dim and diffuse the light output.
- Avoid directing the IR LEDs toward the arena. Direct the IR LEDs toward a white surface so that the arena is illuminated by secondary reflection instead of direct illumination. This will also help in creating diffuse lighting.
- Open the 3D tracking program or camera software to adjust the camera settings (exposure time, gain, brightness) so that all areas appear homogeneously illuminated.
- Where possible, take advantage of the bespoke lighting solutions offered by some software providers.
- Consider reversing IR light conditions by having an IR-absorbent background and IR lights on the side of the cameras, so that the IR lights face the same direction faced by the cameras. In this way, the lights will illuminate the mosquitoes which will appear bright mosquitoes over the dark background.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Manuela Carnaghi (manuela.carnaghi@greenwich.ac.uk).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Manuela Carnaghi (manuela.carnaghi@greenwich.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code generated during this study is available at Zenodo: https://zenodo.org/records/10092390.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.C., R.J.H., S.R.B., and F.M.H.; methodology, M.C., F.M., J.J., L.F., and F.M.H.; software, M.C. and F.M.; investigation, M.C.; data curation, M.C. and F.M.; formal analysis, M.C., F.M., J.J., L.F., and F.M.H.; visualization, M.C. and F.M.; supervision, R.J.H., S.R.B., and F.M.H.; funding acquisition, R.J.H. and F.M.H.; writing – original draft, M.C.; writing – review and editing, M.C., J.J., L.F., and F.M.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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