Acoustic physiology in mosquitoes

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ABSTRACT

The acoustic physiology of mosquitoes is perhaps the most complex within the entire insect class. Past research has uncovered several of its — sometimes stunningly unconventional — principles, but many mysteries remain. Their solution necessitates a concerted transdisciplinary effort to successfully link the neuroanatomical and biophysical properties of mosquito flagellar ears to the behavioral ecology of entire mosquito populations. Neuroanatomically, mosquito ears can rival those of humans in both complexity and sheer size. The ~16,000 auditory hair cells within the human organ of Corti, for example, are matched by the ~16,000 auditory neurons in the Johnston's organ of a male *Anopheles* mosquito. Both human and mosquito ears receive very extensive efferent innervation, which modulates their function in ways that are as yet poorly understood. Different populations of neuronal and non-neuronal cell types divide the labor of the mosquito ear amongst themselves. Yet, what exactly this labor is, and how it is achieved, is at best vaguely known. For the majority of mosquitoes, biologically relevant sounds are inextricably linked to their flight tones. Either these flight tones are (directly) the sounds of interest or they contribute (indirectly) to the production of audible sound through a process called nonlinear distortion. Finally, male ears can generate tones themselves: the generation of an internal "phantom copy" of a female flight tone (or *self-sustained oscillation*) is believed to aid the male hearing process. We describe here four protocols that target the mosquitoes' auditory neuroanatomy, electrophysiology, and behavior to help shed light on some of these issues.

HEARING IS A KEY SENSE FOR MOSQUITOES

The sense of hearing is key to mosquito survival; without it, the mid-air copulation between male and female could not take place (Fig. 1a). Males identify—and locate—a female mating partner by listening out for her faint flight tones (Fig. 1b). Within the (acoustically and spatially) crowded environment of mosquito mating swarms (Downes 1969), where hundreds or thousands of males often compete for a few dozen females, this is a challenging sensory task. For a mosquito male, *reproductive fitness* might thus be approximated as *acoustic fitness*; the (positive) selection for genes that improve hearing, as well as the (negative) selection against genes that cause deafness, can be expected to have shaped the evolution of male mosquitoes.

Despite their crucial importance (without their sense of hearing, males could not execute the phonotactic chase of a female mating partner) and seeming simplicity (the only relevant sounds are the pure tone-like "flight tones"), the mechanisms of mosquito hearing have retained many of their secrets to this day. The assumption of simplicity has proven deceptive.

A BRIEF BREAKDOWN OF MOSQUITO HEARING

On the receiver side, the mosquitoes' flagellar ears convert airborne vibrations (such as those associated with a flight tone) into neuro-electrical signals, which are then propagated to the mosquito brain. The flagellar shaft is the actual sound receiver: it acts like an inverted pendulum that is set into motion by friction with the surrounding air (Albert and Kozlov 2016). The greater the velocity of the air particles, the larger the flagellar oscillation. The flagellum's plane of oscillation depends on the direction of the velocity vector, and thus the location of the sound source. To boost sound-induced motion, the flagellar receivers of male mosquitoes possess arrays of fibrillar extensions (or "fibrillae"), which increase the flagellum's effective acoustic surface. In Anopheline mosquitoes these fibrillae can also be collapsed. Mosquito ears have an exquisite sensitivity: in males, angular displacements of less than 1 mdeg (corresponding to flagellar tip displacements of less than 20 nm) (Su et al. 2018) are enough to excite the auditory neurons of the Johnston's organ (JO), the mosquitoes' "inner ear."

Mosquitoes, especially males, should thus be well adapted to detect the faint sounds produced by the wing beats of a nearby flying mate. However, testing the responses of their ears to naturally occurring flight tone frequencies tells a different story. Mosquito flight tones change with ambient temperatures (Villarreal et al. 2017). Under laboratory conditions (20-22°C), females emit flight tones around ~400 Hz and males around ~600 Hz. In both sexes, however, these tones fail to elicit responses in the auditory neurons of JO. Instead, the male JO shows a response plateau centered on frequencies of ~200 Hz [ranging from ~150 Hz to ~250Hz; see Somers et al. (2021) and highlighted areas in Fig. 1c]. No mosquito sound emissions in this frequency range have been reported. The solution to this puzzling mismatch is provided by an essential property of mosquito (and other) ears. As a direct consequence of their mode of operation, sound receivers display characteristic nonlinearities (e.g., gating compliances). The receivers' nonlinearities, in turn, introduce distortions into their response to external sound. Even when stimulated with two pure tones only (such as the mosquitoes' flight tones; see Fig. 2c, top), the receiver produces mathematically predictable distortion products (DPs) (Julicher et al. 2001), some of which will fall into the sensitivity range of the mosquitoes' auditory nerve (Fig. 1c, bottom). The nonlinear mixing of two — in themselves inaudible — flight tones thus can create an audible distortion tone within the mosquito ear (Gibson et al. 2010; Somers et al. 2021). DPs are, however, at least 2 orders of magnitude smaller than the original flight tones, and thus the exquisite displacement sensitivity of the flagellar ear is crucial to their detection.

AUDIBILITY IS A COMPLEX, MULTIVARIATE PROBLEM IN MOSQUITOES

Two likewise immediate, and far-reaching, conclusions can be drawn from the relations sketched out above. (i) The audibility of females is a key prerequisite for the males' pre-copulatory chase and thus for reproduction in mosquitoes in general; and (ii) audibility between mosquitoes is a nontrivial, inter-relational problem, as it does not simply arise from a match between an emitted signal (e.g., a female flight tone) and a receiver's properties (e.g., a male ear). Uniquely among all other reported hearing systems, audibility in mosquitoes also depends on sounds generated (and possibly controlled) by the receiver (Fig. 1d): for a male to be able to hear a given female, he must himself fly with the correct wing beat frequency (and thus the correct flight tone), which generates audible distortions when mixing nonlinearly with the female's flight tone in his own ear. Studies have started to shed light on these issues, exploring the mutual relation, and possible optimization, of male and female flight tones within the context of the mating swarm. However, many known (and most certainly many unknown) unknowns remain. The biggest mystery here is arguably the role of the self-sustained oscillations (SSOs) specific to male ears. SSOs represent self-generated flagellar oscillations, which closely mimic those induced by female flight tones. It has been speculated that SSOs act as internal amplifiers

of female flight tones, but their specific contributions, ecological relevance – and cellular origin (Fig. 1e) - are still unclear.

FROM BIOPHYSICS TO VECTOR CONTROL

From what we do know, direct lines can be drawn from the in-depth study of mosquito auditory biology to vector control efforts. First, hearing is a key sensory modality that guides male reproductive behavior within the mating swarm. The mating swarm has been identified as a prime target for future vector control efforts (Sawadogo et al. 2017), and current programs aiming at the release of genetically modified mosquitoes have almost exclusively concentrated on males (as the non-biting sex). A better quantitative understanding of mosquito hearing — and audibility — thus carries the dual promise of (i) providing novel molecular targets and mechanistic routes for mosquito population control efforts and — perhaps even more importantly — (ii) supporting ongoing release programs through improved predictive models of expected reproductive interactions (and compatibility) between populations of genetically modified and native mosquitoes (Su et al. 2020). One simple way that this could be achieved is to "acoustically match" mutant and wild-type mosquitoes in order to optimize their respective mating interactions. This discussion and the three associated protocols have been written in an effort to promote auditory research in mosquitoes on the cellular (see Protocol: **Immunohistochemical staining of the mosquito ear**<protXXXXXX> [Andres & Albert 2022]), biophysical (see Protocol: **Electrophysiological measurements of compound action potential responses from the antennal nerve in response to stimulation**<protXXXXXX> [Su & Albert 2022], and behavioral (see Protocol: **Recording and extraction of mosquito flight tones** [<protXXXXXX> [Georgiades & Albert 2022] and Protocol: **Mosquito phonotaxis assay** [<protXXXXXX> [Georgiades & Albert 2022]) levels

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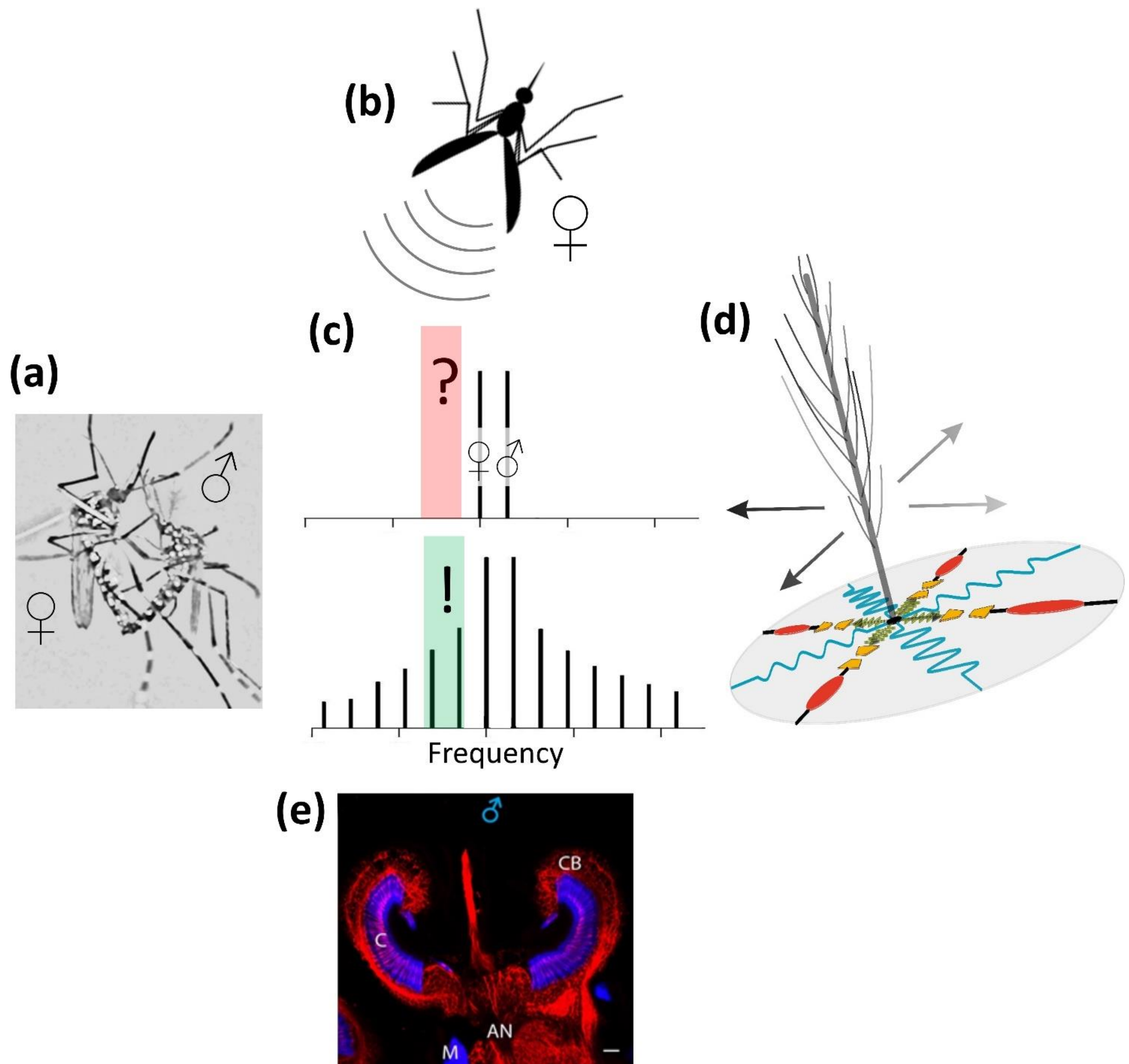

Figure 1. The acoustic ecology of mosquitoes. (a) Mosquitoes copulate mid-flight, following a pre-copulatory pursuit (often taking place in large mating swarms) during which the male chases the female. (b) Males use the females' flight tones to identify females within the swarm and to track them during the phonotactic chase. (c) Top, the mosquitoes' flight tones (bars) are nearly inaudible to both sexes, as they fall outside the mosquitoes' hearing range (red highlighted area); bottom, when male and female flight tones mix nonlinearly within the mosquitoes' ears, however, they produce audible distortion tones (additional bars), which fall partly within the mosquito hearing range (green highlighted area). (d) Audibility thus arises from an interaction between (male and female) flight tones and the mosquito flagellar ear (see schematic drawing). Understanding the biophysical operation of the mosquito flagellar ear is key to understanding mosquito hearing and auditory behavior. (e) Within the Johnston's organ (JO), different populations of ciliated neurons (neurons red, cilia blue), and non-neuronal cell types, coalesce to mediate, and modulate, the process of mosquito hearing. The antennal nerve

carries both afferent and efferent nerve fibers, which transmit, or modulate, sensory signals, respectively. CB, cell body; C, cilia; AN, antennal nerve; M, muscle.

Protocol 1

Immunohistochemical staining of the mosquito ear

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Immunohistochemistry has played a major role in improving our understanding of the anatomy and function of the nervous system. The use of fluorescent dyes that label different antigens reveals how biological tissues are built and how interactions between cells take place. Obtaining this information is particularly important in the case of the mosquito ear given its highly complex anatomy. This protocol describes an immunohistochemical technique to stain the mosquito ear. The first steps of the procedure include the embedding of the tissue in albumin-gelatin and its sectioning into thin slices to allow antibody penetration. The immunohistochemical procedure can be exploited to detect protein expression and localization by using antibodies specifically raised against the protein of interest or that recognize epitope tags fused to proteins using genome editing methods.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

PBS

Albumin-gelatin mounting medium<R>

Blocking solution: 5% bovine albumin serum, 2% normal goat serum, and 0.3% Triton X-100 in PBS

The solution is aliquoted in 1-ml tubes and stored at -20°C until used.

Formaldehyde, 4% in PBS with 1% Triton X-100

The solution is divided into aliquots in 1-ml tubes and stored at -20°C until used.

Formaldehyde, 6% in PBS

The solution can be kept in the refrigerator for 1-2 months. To post-fix the albumin-gelatin blocks, the solution will be poured into a 50-ml Falcon tube where blocks will be placed. The solution can be reused 3-4 times.

Methanol

PBS-T

PBS buffer with 0.3% Triton X-100 is used throughout the protocol as indicated. **[Author: You define PBS-T as containing 0.3% Triton X-100 here, but 1% in the listing for 4% formaldehyde: please either make that consistent or avoid the term "PBS-T" in the formaldehyde recipe.]**

Mounting medium

Equipment

Cell culture plate, 24 well

Cold chamber

Disposable glass Pasteur pipettes and rubber pipette bulb

P200 micropipette, with a 200 µl non-filter tip
Embedding silicon or rubber molds
Filter paper
Forceps
Fridge
Hot plate with magnetic stirrer
Microcentrifuge tubes, 1.5 mL
Micro-dissecting tweezers, preferably Dumont #55
Microscope slides and cover slips, 50 mm long
Orbital shaker
Parafilm
Polyethylene Pasteur pipettes, fine tip
Polypropylene tubes, 50 mL
Razors
Use single-edge steel razor blades for higher security.
Rotating shaker
Suitable for 1.5-mL microcentrifuge tubes.
Stereomicroscope
Vibratome
Water bath

METHOD

We recommend using young mosquitoes (3-5 days old). Some conditions (fixation and antibody incubation times) may require modification depending on the antibodies used. Although the times for most incubations and washes are relatively flexible, it is important to be accurate with fixation times.

Head Fixation and Vibratome Sectioning

1. Remove mosquito heads from the bodies and fix them in 1 ml of 4% formaldehyde in PBS-T for 1 hour at room temperature. Place the samples in a rotator shaker.
   To facilitate the diffusion of the fixative in the mosquito head, remove the mosquito proboscis. Formaldehyde is toxic, so it is essential to work under a fume hood.
2. While the samples are being fixed, heat the frozen albumin gelatin aliquot in a water bath to 42°C.
   It can take 30-45 min for the albumin-gelatin medium to reach this temperature. Keep a thermometer in the albumin-gelatin and monitor the temperature carefully, as an incorrect temperature can ruin the embedding process (it curdles the albumin-gelatin).
3. After fixing the samples for 1 hour in the rotator, proceed to embed them in the silicon molds filled with albumin-gelatin.
   i. Before embedding, place the materials required (tweezers, glass Pasteur pipettes and molds) on a hot plate to warm them up.
   The albumin-gelatin medium in the Falcon tube should be kept in the water bath during sample embedding to avoid cooling and solidifying.
   ii. Take a P200 micropipette, with a 200 µl non-filter tip and cut the end of the tip. Use the micropipette to collect 3 heads from the fixative, and place them on a filter paper to dry them and remove any residual fixative.
   iii. Use the glass Pasteur pipette to carefully pour albumin-gelatin into the molds, avoiding the formation of bubbles. Use tweezers to collect the heads and place them on the surface of the albumin-gelatin. Work under a stereomicroscope for better

precision. Gently push the heads under the surface, so that they are completely covered by the albumin-gelatin but still stay superficial. Use tweezers to orient them in a frontal plane (Fig. 1A).
iv. Let the molds chill for 5 minutes in a fridge before the post-fixation step.
Any remaining albumin-gelatin can be frozen and reused later.

4. Place the molds in 50-ml Falcon tubes containing 6% formaldehyde and allow them to post-fix overnight at 4°C.
5. Take the molds out of the fixative, briefly dry them on a filter paper, and place them for 10 minutes in a 50-ml Falcon tube containing 20 ml of methanol.
Methanol is neurotoxic, so work should be conducted under a hood. Molds can be dabbed onto filter paper to remove residual fixative.
6. Take the molds out of the methanol, briefly dry them on a filter paper, and place them in 50-ml Falcon tubes containing 20 ml of PBS.
Molds can be sectioned immediately or kept for 1-2 hours in PBS at room temperature.
7. Use a vibratome to create 40-μm sections of the embedded heads.

Different vibratomes can be used for this step. We recommend using a Leica VT1000S.
i. Fill the buffer tray with PBS.
ii. To section the albumin-gelatin block, cut it in a trapezoidal shape and cut sections in a frontal plane (Fig. 1B).
iii. Place the sections in a well of a 24-well cell culture plate filled with 1 ml of PBS.
We recommend collecting sections from two blocks in the same well.

Immunostaining

8. Rinse and wash the sections for 10 minutes on an orbital shaker in 1 ml of PBS-T at room temperature. Repeat three times.
PBS-T contains 0.3% Triton X-100. “Rinse” means adding PBS-T to the cell culture plate well and immediately removing it. “Wash” means adding the PBS-T and allowing it to sit for 5 minutes. Avoid bubble formation. To remove the solutions, use fine-tip polyethylene Pasteur pipettes.
9. Incubate the samples on an orbital shaker in 0.5-1 ml of blocking solution for one hour at room temperature.
Incubation time can be increased up to 3 hours. We generally use a blocking solution containing bovine and goat serum. However, be aware that this will prevent the use of any anti-goat secondary antibody. If it is necessary to use an anti-goat secondary antibody, adapt the blocking solution by omitting the goat serum.
10. Incubate the samples on an orbital shaker in 0.3-0.5 ml of primary antibody diluted in blocking solution at 4°C overnight. Use Parafilm to seal the culture plate.
The selection of antibodies to be used depends on the proteins to be detected. Several primary antibodies can be used together if they are raised in different animals and can be recognized by secondary antibodies conjugated to different fluorochromes. It is important to plan the combination of primary and secondary antibodies in advance to ensure that they work together. If a primary antibody conjugated to a fluorochrome is used, add it directly in step 5. If only a primary antibody conjugated to a fluorochrome is used (without any other primary antibody), steps 3-5 can be skipped.

11. Rinse and wash the samples on an orbital shaker for 15 minutes in 1 ml of PBS-T at room temperature. Repeat four times.
12. Incubate the samples on an orbital shaker in the secondary antibody diluted in blocking solution for 1 hour at room temperature. Cover the samples to keep them in the dark.
    Incubation time can be increased up to 3 hours. If a primary antibody conjugated to a fluorochrome is used, add it in this step. From this point on, exposure of samples to light should be minimized to avoid photobleaching. Aluminum foil or a cardboard microtube storage box can be used to cover the culture plate.
13. Rinse and wash the samples on an orbital shaker for 15 minutes in 1 ml of PBS-T at room temperature. Repeat four times.
    Keep in the dark.
14. Rinse and wash on an orbital shaker the samples once for 10 minutes in 1 ml of PBS at room temperature.
    Keep in the dark. This step is performed to avoid any autofluorescence caused by Triton X-100.
15. Mount the sections on a slide and seal them for confocal imaging as follows (Fig. 1C).
    i. Cut the tip of a 200-μl tip and transfer 30 μl of mounting medium to the slide.
    ii. Use a wooden stick to collect the sections and carefully place them in the mounting medium. Using tweezers, carefully arrange the sections in the slide (5-7 per slide).
    Mounting medium can be added if the sections stick to each other and cannot be separated.
    iii. Carefully use a 50-mm-long cover slip to cover the sections, trying to avoid the formation of bubbles. Place a small, moderately heavy object on top of the cover slip and remove any mounting medium seeping out of the cover slip. Seal the preparation using nail polish.

    We recommend using an anti-fading mounting medium such as Vectashield or glycerol with DABCO.

    When imaging the preparation under a confocal microscope, we recommend using a slice thickness of 1 μm.

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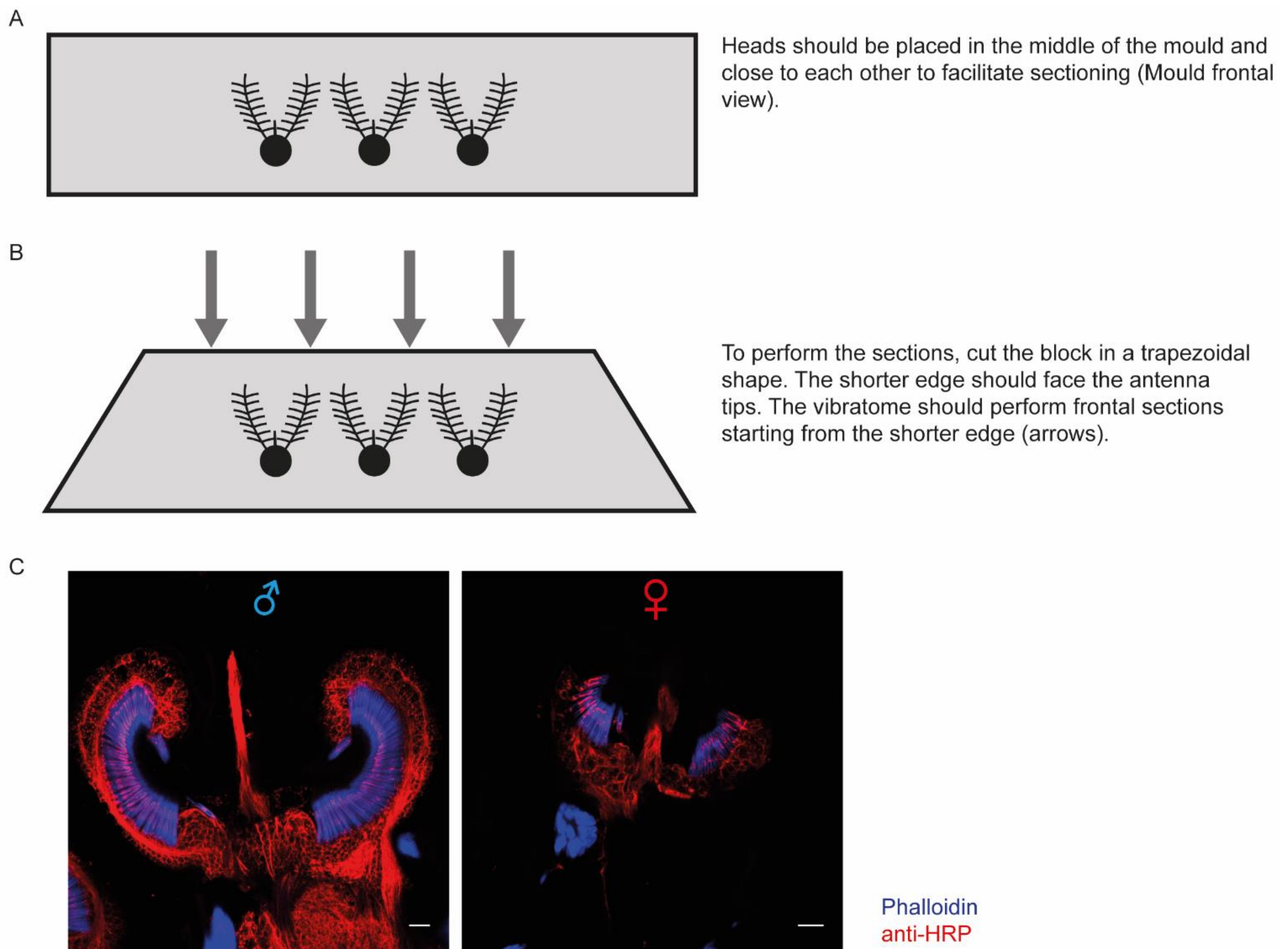

Figure 1. Immunostaining of the mosquito ear. (A) Position of the mosquito heads in the mold filled with albumin-gelatin. (B) The hardened block should be cut into a trapezoidal shape for vibratome sectioning. (C) Example of male (left) and female (right) mosquito ear immunostaining showing labelling with a neuronal marker, anti-HRP, and phalloidin, which binds to actin and labels the auditory cilia (Su et al. 2018).

Protocol 2

Electrophysiological measurements of compound action potential responses from the antennal nerve in response to stimulation

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Electrophysiological recordings taken from the antennal nerve can provide essential information on the general auditory condition of the mosquito tested. Furthermore, electrophysiological recordings provide detailed information on what types of stimulation induce the largest nerve responses. When these are utilized in conjunction with a vibrometer to measure the corresponding movement of the antennal ear during stimulation, a comprehensive overview of hearing function can be obtained. This protocol can be applied to male and female adults from any mosquito strain, and can be scaled relative to available resources.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Equipment

Blue-light-cured glue (e.g. DEHP Flowable Composite Syringe; article number 530017; Henry Schein Dental, UK)

Environmental incubator

The mosquitoes' circadian state and environmental conditions at the time of the experiment must be controlled for. Incubators that allow environmental parameters (light, temperature, humidity) to be controlled are recommended for entraining mosquitoes (e.g. I-30VL, Percival Scientific, Iowa, US). Common entrainment settings are 12 h:12 h light-dark (LD) cycles at constant temperature (ranging from 22 to 28°C) and 60–80% relative humidity.

Forceps, regular (for mosquito transfer) and inverted (for glue application)

Ice

Mosquitoes can survive on ice for at least one hour.

Laser Doppler vibrometer/interferometer (optional)

Though the use of a vibrometer/interferometer is not strictly necessary for obtaining high-quality electrophysiology data, these measurement tools can provide extremely valuable information regarding the actual movement of the antennal ear during stimulus presentation. This allows careful calibration of ear movement in response to a range of stimulus amplitudes, and can allow confirmation of hearing function.

Microscope

Mosquito gluing and electrode insertion into the base of the ear both need to be done under a dissection microscope.

Micromanipulators
Micromanipulators are necessary to hold the rod onto which the mosquito is glued, to hold the two electrodes that will be inserted into the mosquito, and to mount the stimulation source (actuators or speaker).
Plastic rod ~5 cm in length
Recording software
The Spike2 software offers a high-end option for electrophysiology recordings; free alternatives are available however. Likewise, the VibSoft software offered by Polytec can be utilized for vibrometer recordings.
Stimulation source: electrostatic actuators (preferred) or speaker
Although speakers can provide a range of stimuli to the mounted mosquito, the use of electrostatic actuators provides a number of benefits (not least because required speaker volumes can be inconveniently loud for researchers). Actuators are straightforward and inexpensive to make (for details, see Albert et al. 2007; Duong and Kim 2015; Weinberger et al. 2017; Su et al. 2018).
Temperature-controlled room
Many mosquito behaviors and physiological performances are strongly influenced by changes in temperature, including mosquito antennal frequency tuning. It is vital that recordings of hearing function be made under strict temperature control to ensure that the results obtained are comparable across experiments.
Tungsten electrodes
*Electrodes can be reused by sharpening/cleaning through electrochemical etching in between recordings (Duong and Kim 2015).***[AUTHOR: What does "(1)" refer to - a reference? Please clarify.]** *Two electrodes are required per recording: one unbiased/reference electrode inserted in the abdomen, and one biased/recording electrode inserted near the antennal nerve.*

METHOD

In principle, the hearing function of female and male mosquitoes of any genotype can be tested. Rearing conditions for individual mosquito lines are dependent on the respective lines and local safety protocols.

Preparation and Entrainment of Mosquitoes

1. Three days before the start of the experiment, transfer mosquitoes from the group of interest into light- and temperature-controlled incubators.
2. Entrain mosquitoes to desired entrainment pattern for three days.
    12 h:12 h light-dark (LD) cycles at constant temperature are suggested as a standard entrainment pattern.
3. Ensure that the circadian time in the incubator matches desired experimental time.
    A circadian/experimental time matching that of dusk is suggested.

Mounting of Mosquitoes

4. Remove mosquitoes from incubator and sedate by placing on ice.**[AUTHOR: Is this just "sedate by placing on ice" (that sedates them), or is there another step?]**
5. Apply glue to the tip of the plastic rod, and carefully transfer the mosquito onto the glue using forceps.
    It is essential that glue not be spread onto the mosquito ears, as this may inhibit flagellar movements. Only the mosquito body below the head should be placed into the glue at this stage.

6. Carefully apply further glue to the body and head so that only the antennal ear can move freely.

Spread glue across each part of abdomen and thorax to prevent kicking and general movement. Then apply glue to the mouthparts and neck to keep the head in a stable position. Take care to leave the respiratory openings (spiracles) on thorax and abdomen as open as possible.

7. Direct the blue light source at the glue surrounding the mosquito and hold for ~5-10 s to cure the glue.

Insertion of Electrodes

8. Place rod in micromanipulator so that the mosquito head is clearly visible under a microscope.

9. Position micromanipulators holding electrodes so that the unbiased electrode can be placed into the mosquito abdomen and the biased/recording electrode can penetrate the cuticle between the mosquito Johnston's organ (JO) and head.

10. Insert electrodes gently, starting with the unbiased/reference electrode, so that the minimum amount of electrode enters the mosquito at first.

The male JO is substantially larger than the female JO, which makes electrode insertion typically far easier for males than for females. JO size also varies between species, with Anopheles females having perhaps the smallest JO size amongst human-biting species.

Stimulus Provision and Measurement

11. Place actuators/speaker directly in front of the mosquito flagellar tip.

12. Start recording, and provide stimulus

The stimulus could be a sweep of pure tones covering a distinct relevant frequency range (e.g. 1–1000 Hz) at different amplitudes.

13. Record multiple repeats of stimulus (at least 10 per amplitude).

Measurement of Flagellar Movement (Optional)

14. If access to a vibrometer/interferometer is available, focus the laser towards the tip of the mosquito flagellum.

For females, focus on the base of the third flagellomere from the tip; for males, on the base of the second.

15. Record the movement of the flagellum both before and during stimulus provision.

Data Analysis

16. Antennal nerve responses can be analyzed for multiple key components, but perhaps the most important are the maximum compound action potential (CAP) amplitudes and the stimulus frequency at which these occur.

Male and female mosquitoes have well-defined sexual dimorphisms in hearing function — specifically, the peak male action potential response should occur for stimuli between ~250 and 350 Hz, whilst female peak responses occur around ~150 Hz. Male action potential responses are typically greater than those of females (due to the higher number of neurons).

17. First, create averages of nerve responses from the multiple repeats conducted per mosquito.

18. Then smooth the resulting data to reduce the influence of noise on peak extraction.

19. The peak response amplitude can then be identified, in addition to the frequency which generated this peak.

20. For analysis of flagellar movements, a harmonic oscillator function can be fit to raw data that enables the extraction of other key parameters (such as the flagellar best frequency and tuning sharpness).

TROUBLESHOOTING

Pay particular attention to the location of glue application on the mosquito — no glue should be applied to the ear that will be tested, as this could alter flagellar movement. The application of too much glue can also shorten mosquito life expectancy significantly.

Electrodes should be inserted as gently as possible, as deep insertion can cause critical damage. Pay attention to correct position of electrode and laser beam (see **Fig. 1** for details).

Action potential magnitude increases nonlinearly with the stimulus amplitude. Carefully measure the minimum stimulus required to evoke the smallest measurable action potential.

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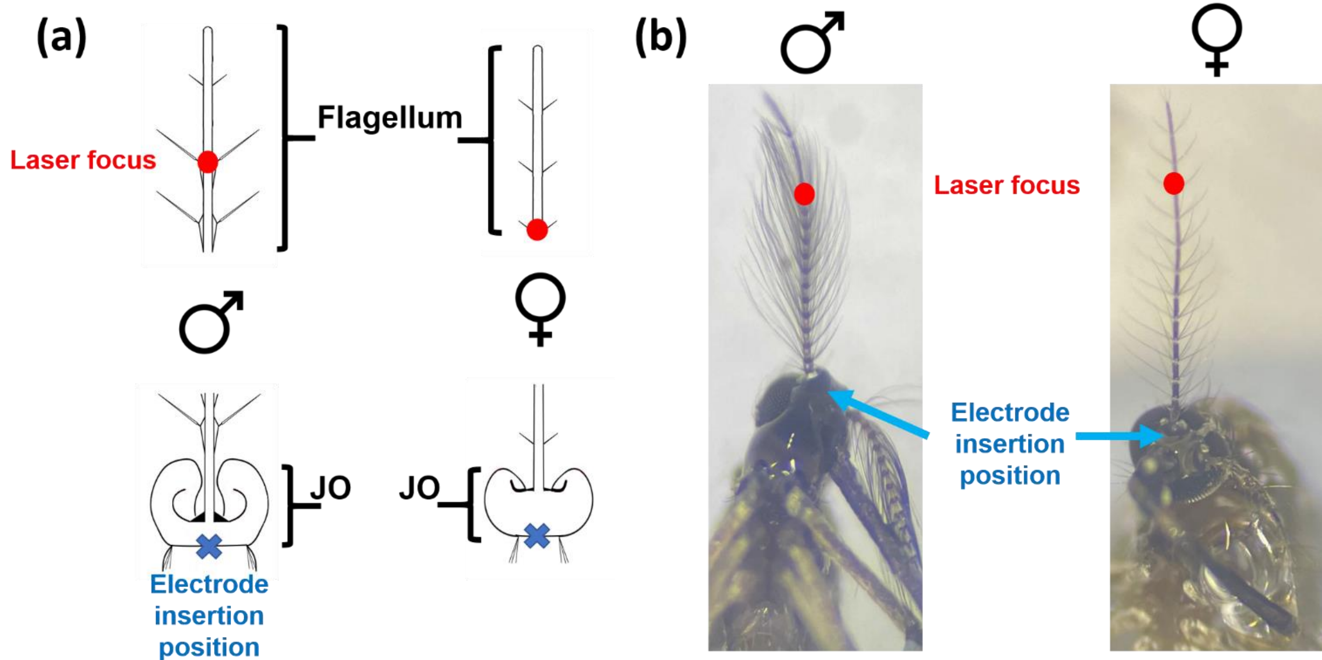

Figure 1. Anatomical differences between male and female ears and resulting effects on electrode insertion position and laser focus point, (**a**) schematic drawing, (**b**) photographs of real anatomy. Male (♂) and female (♀) ears are comprised of a JO ('inner ear') and a flagellum ('sound receiver'). Though the electrode insertion position (marked as blue cross) is the same for both sexes i.e. at the base of the pedicel, the focal position of the laser (marked as red point) is slightly different due to dimorphisms in flagellar anatomy. For males, the laser should be focused at the base of the second flagellomere from the tip, whilst for females, the third flagellomere should act as the focal point.

Protocol 3

Recording and extraction of mosquito flight tones

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Despite the artificial conditions, flight tone recordings taken from tethered mosquitoes can provide valuable information on the acoustic signals produced by male and female mosquitoes. Although auditory responsiveness appears to be largely (and possibly exclusively) restricted to males, the flight tones of both sexes have sensory-ecological relevance, as it is the mixing of the two tones that produces audibility in males and thereby facilitates reproduction. Flight tone information can supplement parallel phonotaxis experiments to provide an improved picture of the acoustic space mosquitoes inhabit.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Equipment

Blue light source

Blue-light-cured glue

Most light-cured dental glues would be suitable (e.g. DEHP Flowable Composite Syringe; article number 530017; Henry Schein Dental, UK).

Environmental incubator

Due to its multiple effects on neural function and behavior, the mosquitoes' circadian state at the time of the experiment must be controlled for. This also applies to environmental conditions, which could alter the circadian state during the experiment. Incubators that allow environmental parameters (light, temperature, humidity) to be controlled are recommended for entraining mosquitoes (e.g., I-30VL, Percival Scientific, Iowa, US). Common entrainment settings are 12 h:12 h light-dark (LD) cycles at constant temperature (ranging from 22 to 28°C) and 60–80% relative humidity. Ice blocks

Microscope (optional)

Microphone holding device

Particle velocity microphone

Microphones sensitive to the air particle velocity changes associated with the mosquitoes' wing beat should be used (e.g., pressure gradient microphone NR-3158, Knowles Acoustics, Itasca, US). Prior calibration of microphones is preferable for insect wing beat recordings as it makes it possible to extract both spectral (flight tone frequency) and magnitude (air particle velocity) information.

Red-light source (optional)

Temperature-controlled room

Mosquito wing beat frequency is sensitive to temperature fluctuations. Before the experiment, adjust the room (or setup) temperature to the value under study. If the effect of temperature is not being investigated, experimental temperatures should be kept at constant value (within the range 22–28°C, with 22°C being the most widely used laboratory temperature).
Temperature logger
It is strongly recommended that ambient temperature fluctuations be tracked with a temperature logger throughout the experiment for future reference and analysis.
Tethered mosquito holding device
Tungsten wire
Tweezers

METHOD

To control for the confounding effects of ageing on behavioral performance, all mosquitoes recorded should be of similar age, and averaging across age groups (unless statistically verified) should be avoided. We recommend using four- to seven-day old mosquitoes; these have already assumed their final body size but can be expected not to suffer from age-dependent declines of flight performance (*Wishart and Riordan, 1959).*

The mating status of all experimental mosquitoes should be registered and controlled for. Unless the mating status is itself the object of investigation, we recommend separating all mosquitoes by sex during the pupal stage and keeping them separate and virgin throughout adulthood prior to the experiment.

Microphone and Mosquito Holder Set-up

1. Place the particle velocity microphone and mosquito holding devices onto a stable surface.
    Recording microphones should be placed at a fixed distance of 2-4 cm between microphone surface and mosquito and oriented such that their sensitive side (if they have one, as is the case for the particle velocity microphone NR-3158) **[Author: The meaning of the preceding parenthetical phrase is unclear - please revise for clarity. Do you mean that some microphones, e.g., the NR-3158 model, have sensitive sides and others don't?]** *faces the mosquito. If more than one mosquito is to be recorded (e.g., same- or opposite-sex pairs) simultaneously, a separate microphone should be used for each. In this case, it is important to ensure that each microphone is sufficiently distant from mosquitoes other than the one it is meant to record from to prevent signal cross-talk (experimental validation is required).*

Mounting of Mosquitoes

2. Transfer the mosquitoes from the incubator into a small compartment (e.g., glass vial), and place in the recording room along with an ice block.
3. Cut a small piece of tungsten wire and leave to the side.
4. Expel a small portion of glue inside a small container, or on a petri dish or small plastic lid .**[AUTHOR: Do you mean "add a small portion of glue to a container? Or do you mean you are placing this on the outside of a container - if so, what type/size of container?]**
5. Place a piece of tissue on the ice block, and unload the mosquitoes from the compartment on top of this tissue.
    The tissue protects mosquitoes from damage via direct contact with the ice block. Wait a few seconds until the mosquitoes are anesthetized before removing the glass vial. Return

excess mosquitoes to the compartment using tweezers, carefully holding them by their legs.

6. With the tweezers, grab the small piece of tungsten wire and dip it in the glue.
7. Attach the wire to the mosquito's thorax and hold it steady while applying blue light to the glue to cure it (typical curing time ~10-20 s).

   Blue light can be damaging/straining to the eyes; avoid the direction of application during the glue-curing step. The identification of an appropriate position on the mosquito's thorax for attaching the wire should be done under a microscope, if one is available, to facilitate visibility. When attaching the wire to the mosquito, take care to avoid blocking its wings with the wire (or the glue) or damaging or getting glue on the two flagellar protrusions (external compartment of the mosquito ear, located on its head).
8. Transfer the wire-attached-mosquito to the holding apparatus and leave for a minute to recover.

Prompting and Recording Flight

9. Start the recording.

   To ensure sufficient resolution, set the sampling frequency reasonably high (e.g. ≥10 kHz). Higher sampling rates allow the application of running averages to the time series data prior to the analysis and thereby noise removal.
10. Activate the tarsal reflex to promote wing flapping, by cutting a small piece of cotton wool and transferring it, using tweezers, underneath the mosquito; while still holding the cotton wool, wait for the mosquito to place its legs on the cotton wool and then swiftly remove it. This will activate the tarsal reflex, which provokes the mosquito to initiate flight.

    Alternatively, to trigger wing flapping, try blowing a gentle puff of air at the mosquito's back.
11. Aim to record continuous flight over at least 10 seconds.

    This will facilitate subsequent necessary data processing steps such as cropping of the flight recording. If the mosquito stops flapping its wings before the 10-second mark, repeat the aforementioned flight-triggering procedures a few times before resorting to a new animal. Recorded flight lengths of different mosquitoes that you plan to compare should not vary greatly — stop recording before flight cessation by the animal, if necessary.

Extracting Wingbeat Frequency from Recordings

12. Discard the data for the first and last 2 seconds of flight prior to the start of data analysis.

    This step ensures that steep gradients of wingbeat frequency due to flight initiation and cessation are not included in the analysis.
13. Apply a bandpass filter to the cropped data with a low-pass cutoff point at 1 kHz and high-pass cutoff point at 200 Hz.

    Cutoff values assume the recorded mosquito's wingbeat frequency falls well within the 200-1000 Hz frequency range; modify accordingly if necessary.
14. Apply a sliding fast Fourier transform (FFT) with 200-ms window (corresponding to 2000 data points, given a 10-kHz sampling rate) over the recording, and shift the FFT window by 100-ms increments for every sliding step until the end of the recording length is reached (*Figure 1*).

    The 50% overlap between successive FFTs enhances the visibility of events occurring faster than the processing duration of samples in the FFT window.
15. For each step (and thus each FFT), extract and store f_0, the value in the frequency domain (x-value), which gives the maximum FFT amplitude (y-value) (*Figure 1*).

16. Append the extracted frequencies together to obtain the wingbeat frequencies of the mosquito over the processed flight recording.
17. Utilize values according to experimental goals.
    Suggestion: For phonotactic experiments, assign the mean (if data is normally distributed) or median (if not normally distributed) of the extracted frequencies as the mosquito's wingbeat frequency.

TROUBLESHOOTING

Some disease-transmitting species (e.g., *Anopheles gambiae*) are prone to inactivity throughout most hours of the day, with the exception of short circadian time slots. If it is consistently difficult to stimulate flapping in the animals, the animals' circadian entrainment (and thereby their subjective time of the day) or the environmental conditions of the experimental room (which can influence the circadian state) may play a role in this. Ensure that (a) the animals' entrainment is known, (b) the experiments are conducted at a time when the animal is expected to be active based on their entrainment, and (c) the light conditions in the experimental room are aligned with those expected by the animals' given their circadian state. Use a red-light source to conduct experiments in the dark, if necessary.

DISCUSSION

This protocol considers a fast Fourier transform (FFT) procedure for estimation of wingbeat frequencies from tethered flight recordings. The procedure can be coded and processed using a programming language of personal preference (e.g., Python, R, MATLAB). The steps outlined here provide a 5-Hz frequency resolution for the wingbeat frequency estimates, assuming a sampling rate of 10 kHz for the recordings. Frequency resolution F_{res} is given by:

$$F_{\mathrm{res}} = \frac{F_{\mathrm{sampling}}}{N_{\mathrm{data}}}$$

where F_{sampling} is the sampling frequency and N_{data} is the number of data points for any given FFT window.

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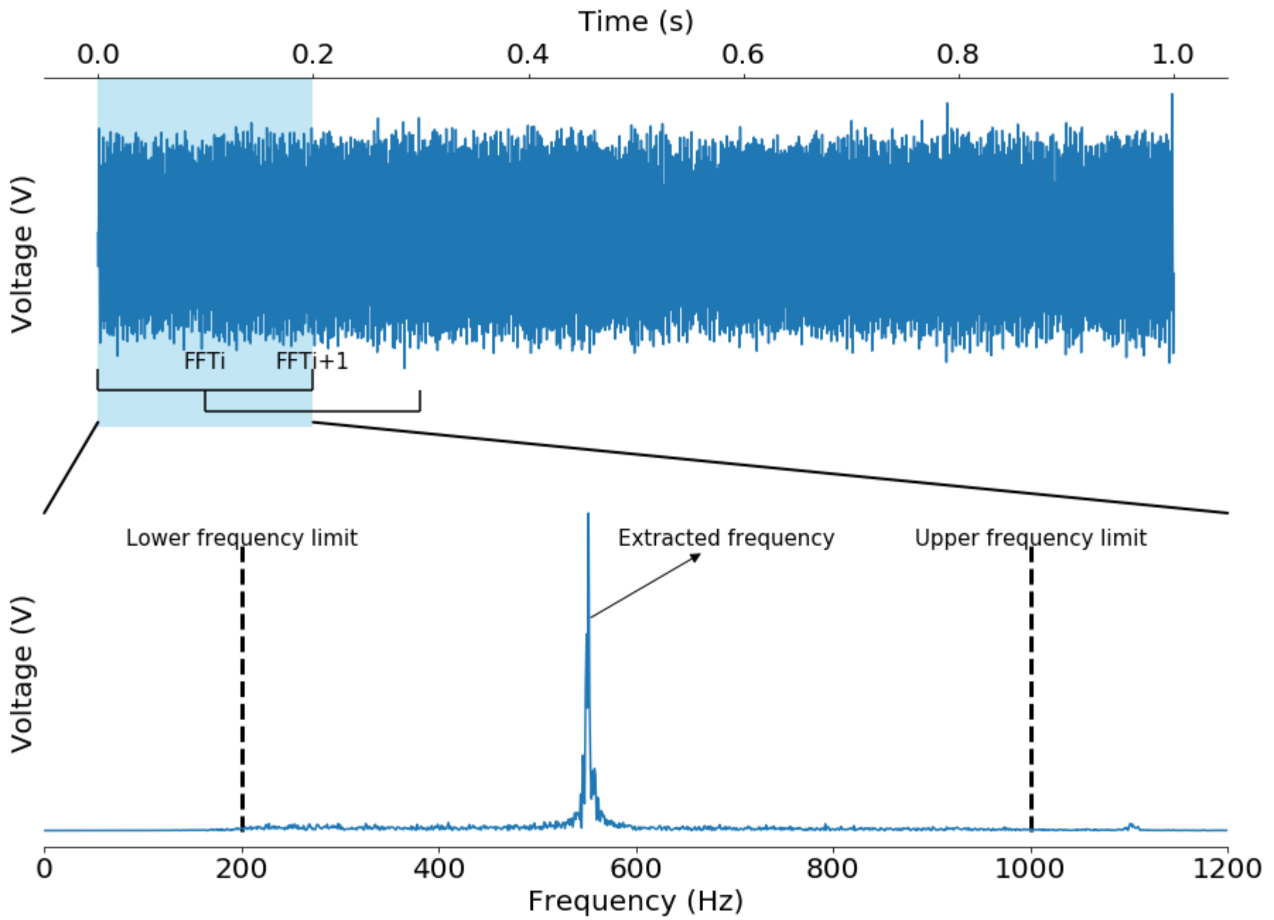

Figure 1. Sketch of the paradigm for the wingbeat frequency extraction algorithm. An FFT sliding window scans though the recording; the dominant frequency is extracted with each sliding step (modified from Su et al. 2020).

Protocol 4

Mosquito phonotaxis assay

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Phonotaxis experiments can provide information on the spectrum of sounds relevant to mosquito acoustic behaviors. It is widely known that males of disease-transmitting species are attracted to tones with frequencies resembling the wingbeat frequencies of their conspecific females. Thus, phonotaxis experiments can be coupled with wingbeat frequency measurements to inform the development of vector control tools such as acoustic traps and lures.

MATERIALS

Equipment

Decibel meter (optional)

Environmental incubator

Due to its effects on neural function and behavior, the mosquitoes' circadian state at the time of the experiment must be controlled for. This also applies to environmental conditions, which could alter the circadian state during the experiment. Incubators that allow environmental parameters (light, temperature, humidity) to be controlled are recommended for entraining mosquitoes (e.g. I-30VL, Percival Scientific, Iowa, US). Common entrainment settings are 12 h:12 h light-dark (LD) cycles at constant temperature (ranging from 22 to 28°C) and 60–80% relative humidity.

Insect cage

An insect cage with sides of woven mesh is used for phonotaxis stimulation of mosquitoes (e.g. BD42222F, BugDorm). For 20-30 mosquitoes, we recommend using a cage with dimensions 25cmx25cmx25cm.

Loudspeaker

Particle velocity microphone

To be used for evaluating the frequency content and loudness of the stimuli (e.g., pressure gradient microphone NR-3158, Knowles Acoustics, Itasca, US).

Red light source (optional)

Temperature-controlled room

Mosquito wing beat frequency is sensitive to temperature fluctuations. Before the experiment, increase or decrease the room temperature to the value under study. If the effect of temperature is not being investigated, room temperatures should be kept at a constant value (within the range 22–28°C, with 22°C being the most widely used laboratory temperature).

METHOD

Many disease-transmitting mosquito species, such as Anopheles gambiae, mate in reproductive swarms that are under circadian regulation. Such species exhibit phonotaxis only during their preferred mating time period, which could be, under natural conditions, at sunset or during darkness (and possibly suppressed by light). For this reason, the mosquitoes' circadian time (i.e., their "subjective" time relative to a prior entrainment) and the illumination conditions of the experimental room must be considered. A red-light source (~620-650 nm) can be used to conduct the phonotaxis experiments under scotopic conditions or effective darkness.

Speaker Set-up and Experimental Calibration

1. Set the sound speaker on the surface where the recordings are to take place.
2. Place an empty mosquito (insect) cage so that one of its meshy sides makes contact with the speaker.
   Mark the region where the speaker makes contact with the cage with a marker. This region constitutes the area that counts as indicating positive phonotaxis, if approached by mosquitoes during sound stimulation.
3. Mark the position of the cage and speaker on the surface where they sit for reference in subsequent phonotaxis experiments.
4. Append a particle velocity microphone to the cage on the side that is most distant from the speaker.
5. Ensure tones emitted from the speaker are sufficiently pure and audible to the mosquitoes.
   Play the tones of interest via the speaker and record with the microphone. Fourier analysis can then be applied to the recording to ensure that the right frequencies were being produced. To get an estimate for the intensity of each tone and ensure similarity of loudness across tones, calculate the root mean square amplitude of the signals recorded for each tone and compare across tones. Alternatively, use a decibel meter. As a reference point, mosquitoes can be expected to respond to tones with a sound amplitude of ≥60 dB SPL when assessed within a 25 cm × 25 cm × 25 cm cage volume.

Phonotaxis Experiment

6. Transfer 20–30 mosquitoes to the insect cage and place the cage back to the marked spot where calibration was conducted.
7. Control for mosquitoes being attracted to cues other than sound.
   At the beginning of the experiment, when you position the cage and speaker in contact with each other, count how many mosquitoes are attracted to the marked area of contact for 1 minute during which no sound is being emitted.
8. Present the tones to the mosquitoes in the desired pattern.
   Suggested protocol for a single experimental replicate: Randomize the order of tone presentation, then present the first tone for 1 minute, followed by 1 minute of silence, followed by the next tone, and repeat until the sequence of tones has reached its end. Repeat for 3 runs (each with a newly randomized sequence of tones).
9. Count the mosquitoes approaching the area of positive phonotaxis during stimulation with each tone.
   The counting can be done by eye. Count mosquitoes which make contact with the area of positive phonotaxis during stimulation.

TROUBLESHOOTING

If you observe a large variance in responses to the same tones across experiments conducted on different days, we recommend looking for and controlling for confounding factors. The usual suspects, both of which influence the animals' wingbeat frequencies, are the ambient temperature of the room (Villarreal et al. 2017) and the animals' circadian state (Somers et al. 2021). Note that changes in the mosquitoes' own wing beats will change the flight tones they can hear.

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Recipes

Albumin-gelatin embedding medium

2. Carefully dissolve 24.2 g albumin (Sigma A5253) in 66 ml distilled water. Use a magnetic stirrer to slowly dissolve the albumin in the water, and add the albumin gradually (over 1 hour) to avoid the formation of clumps or foam. Try to dissolve any clumps using a spoon. After albumin has been dissolved, stir gently for another 30 minutes and discard any foam that forms.
3. Warm 25 ml distilled water to 55°C and add 5.7 g gelatin (Sigma G2500). Place the solution on the magnetic stirrer, keep it warm, and gently mix until gelatin has been completely dissolved.
4. Add the dissolved gelatin to the albumin solution while slowly stirring on the magnetic stirrer for approximately 5 minutes.

Divide into 15-ml aliquots in 50-ml Falcon tubes and freeze at -20°C until used.