Protocol

Probing Acoustic Communication during Fly Reproductive Behaviors

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During reproduction, male and female flies use wing vibration to generate different acoustic signals. Males produce a courtship song before copulation that is easily recognized by unilateral wing vibration. In copula, females produce a distinct sound pattern (copulation song) with both wings. Sexual rejection of immature virgins and aggressive encounters between males are also accompanied by sound pulses generated by wing flicks. Fly song has frequency ranges audible to the human ear and can be directly listened to after appropriate amplification. When displayed in an oscillogram, audio recordings can be mapped on wing-movement patterns and thus provide a fast and precise method to sample and quantify motor behaviors with high temporal resolution. After recording different fly sounds, their effect on behavior can be tested in playback experiments.

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

Drosophila flies

Fly food (standard, as used for rearing flies)

For example, Fly food recipes (2021).

Equipment

Audio recording equipment

For recording fly songs, we recommend using a custom-made recording unit with an array of pressure-gradient microphones as described by Arthur et al. (2013). In short, such a recording unit consists of fly chambers, microphones, and a data-acquisition and digitalization unit that is connected to a computer.

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Behavior boxes/behavioral room for experimenting

Perform all experiments in a behavior box or a dedicated room in a quiet environment with temperature and humidity control. Consider additional soundproofing of walls and doors when working in an area with a lot of ambient noise.

Blocks (flat-bottom, 96-well, 2-mL)

Use these for housing and aging many flies in isolation. Fill 96-well 2-mL flat-bottom blocks with 0.5 mL of food, cool, and cover with polymerase chain reaction (PCR) foil. Before use, punch air holes for each well and cut the foil along the walls separating the 12 rows with a scalpel or utility knife to allow each well to be filled individually. Store freshly prepared plates for up to 2 wk at 4°C.

Consumer camcorders (standard) or webcam equipment (optional, see Step 13)

Consumer speakers

For playing back fly songs, use simple consumer speakers covering the conventional frequency range. Place experimental flies in chambers covered by thin transparent acrylic nets facing the speakers (Fig. 1A) so that the sound can reach their antennal ear.

Courtship chambers for audio experiments

Fabricate these by three-dimensional printing or acrylic laser cutting. For audio experiments, use meshed chambers: Modify the chambers described in Protocol: ***Single-Pair Courtship and Competition Assays in*** **Drosophila** *(von Philipsborn et al. 2023). Cover one side of the chamber with thin transparent acrylic nets so that the sound can reach the microphone (for recording) or the flies' antennal ears (for playback).*

Fly aspirator

Use a fly aspirator (foot pump) for collecting and filling flies in chambers without anesthesia.

Fly pad with light CO_2 (optional; see Step 9)

Forceps (blunt)

Software for automated segmentation of pulse and sine song, such as freely available custom scripts developed in the Stern laboratory (Arthur et al. 2013), and computer

Spring scissors (fine; e.g., Fine Science Tools Vannas Spring Scissors with a 2- to 3-mm cutting edge or comparable)

Vials (as used for rearing flies; e.g., 80-mm × 25-mm plastic vials [Scientific Laboratory Supplies VIA6270], 80-mm × 25-mm plastic vials [VWR 734-2262P], or 25-mm × 28-mm polypropylene *Drosophila* breeding tubes [Semadeni 10405])

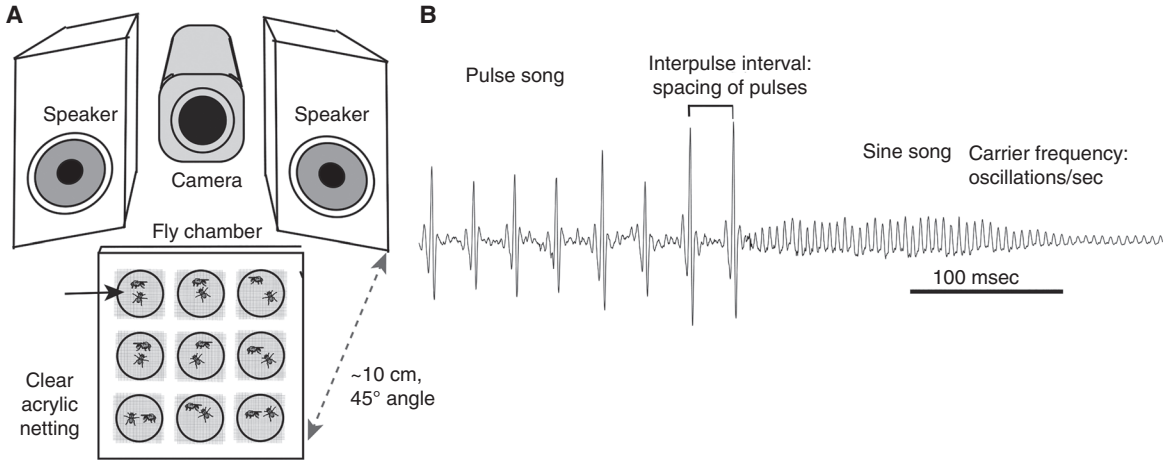

FIGURE 1. Playback setup and male courtship song. (*A*) A simple playback system with two speakers facing meshed courtship chambers. (*B*) *Drosophila* male courtship song with the most commonly analyzed parameters.

METHOD

Carry out all steps in the following protocol at room temperature unless otherwise noted.

1. Prepare all equipment.

   Assembly and customization of the recording equipment may require some planning.

2. Test recording and/or playback setup: Try to record background noises at different locations and compare sound levels. For playback, use conventional audio file players that can loop shorter sound snippets.

   Audio recording and playback procedures can be part of one larger experiment or may be performed separately. When new to Drosophila *courtship experiments, we recommend that the experimenter be familiarized with single-pair courtship and mating assays (see Protocol: **Single-Pair Courtship and Competition Assays in** **Drosophila** [von Philipsborn et al. 2023]) before attempting audio recording and playback.*

   For audio playback only, perform Steps 3–7. Otherwise, proceed to Step 8.

Calibration of the Playback Setup

3. Collect virgin male and virgin female wild-type flies on the day of eclosion. Amputate the wings of some of the males by placing individual flies on a fly pad with light CO_2 anesthesia and using small spring scissors to cut the wings close to the wing hinge. Do not keep flies on the CO_2 pad for longer than 2 min. Hold flies gently in place with blunt forceps during the procedure. Allow recovery from wing amputation for at least 3 d.

   See Troubleshooting.

4. Using a fly aspirator (foot pump), fill males and females in meshed chambers at 25°C and 50%–70% relative humidity at an appropriate circadian time. Maintain these conditions for Steps 5 and 6.

   The time of 3 h after lights on and 3 h before lights off in a 12-h–12-h light–dark cycle gives the strongest courtship activity.

5. Confirm that, without any playback, wing-amputated males achieve no copulations or a very low copulation rate within 30 min.

6. Start playback of a looped recording of natural male song at previously estimated sound levels (Kerwin et al. 2020).

   As a rule of thumb, song clearly audible to the human ear, at the level of a loud conversation, is sufficient to stimulate flies placed at ~10 cm from a speaker. After 20–30 min, playback should markedly increase copulation rates, ideally to 60%–80%.

7. Compare playback copulation rates of wing-amputated males with copulation rates of intact males to estimate the efficiency of playback.

Fly Collection and Transfer

Perform all experiments in a behavior box or a dedicated room in a quiet environment with temperature and humidity control.

8. Using a fly aspirator (foot pump), collect experimental male and female flies 0–6 h after eclosion.

9. (Optional) On the day of eclosion after hardening of the wing veins, place individual flies on a fly pad with light CO_2 anesthesia and use small spring scissors to cut the wings close to the wing hinge. Hold flies gently in place with blunt forceps during the procedure. Allow recovery from wing amputation for at least 3 d.

   This procedure takes 1–2 min per fly.

   See Troubleshooting.

10. House flies in isolation at 25°C with 50%–70% relative humidity and a controlled light–dark cycle until the experiment in vials or blocks.
    - Keep virgin females in groups of three to 10 flies in vials.
    - House males singly. Using a fly aspirator (foot pump), add one fly per well to blocks.
11. Age virgin males 4–7 d and virgin females 3–7 d.

    Housing conditions and the age of the flies can vary depending on the aim of the experiment.
12. Using a fly aspirator, transfer flies into courtship chambers in a behavioral room ideally kept at 25°C with 50%–70% relative humidity shortly before the experiment.

    *Transfer flies with a fly aspirator (see Protocol: **Single-Pair Courtship and Competition Assays in** **Drosophila** [von Philipsborn et al. 2023]) to avoid adverse effects of CO_2 anesthesia on behavior. Pay attention to the circadian cycle of the experimental animals and perform the experiment at a defined time during the day. Courtship activity peaks during and 2–3 h after the lights are on and 2–3 h before the lights are off.*

    Proceed immediately to Step 13 to perform audio recording experiments or to Step 15 to perform audio playback with a pre-existing audio file.

Audio Recording

13. Start audio recording, with optional video recording using a camcorder or webcam.

    When recording male courtship song, ~2–4 min of song recording can sample several hundred song pulses from an individual male. Courtship song can be recorded with males courting mated or virgin females. When females are virgin, it is useful to video record when the pairs copulate and male courtship stops. When females are mated, wing amputation can be used to reduce confounding background sounds from rejection wing flicks.

    See Troubleshooting.
14. When recording female copulation song, record video or note when the pairs copulate.

    Proceed to Step 16.

Audio Playback

Recorded or artificially generated song can be played back to flies to evaluate their behavioral effect and to test the response of male or female flies to auditory stimuli (Li et al. 2018; Deutsch et al. 2019; Ishikawa et al. 2019; Kerwin et al. 2020). Various behaviors can be tested in response to song playback, and the exact procedure and data evaluation depend on the individual experimental design. Male–female pairs can be examined for copulation and courtship, groups of male flies can be examined for chaining, and single flies can be examined for locomotion.

15. Start playback, and video record the behavior of interest. Use either silence or white noise playback as a control.

    *Data analysis of audio playback experiments strongly depends on the behavior recorded. For example, perform and evaluate single-pair courtship assays as described in Protocol: **Single-Pair Courtship and Competition Assays in** **Drosophila** (von Philipsborn et al. 2023) while playing back an audio file.*

    For analysis of the audio file used in playback, proceed to Step 16.

    See Troubleshooting.

Data Analysis

16. Analyze the acquired wave files using software for automated segmentation of pulse and sine songs, such as freely available custom scripts developed in the Stern laboratory (Arthur et al. 2013).

    See Troubleshooting.
17. Confirm the segmentation results by visual inspection of oscillograms or listening to the audio files after amplification of the signal.

    Pulse and sine songs are well within the frequency range detectable by the human ear and are easily discernible after short practice.

18. Quantify songs per minute of precopulatory courtship rather than songs per total time of pairing of the couple when flies copulate in the assay.
19. Quantify the following behavioral parameters (Fig. 1B):
    i. Amount of pulse song: Measure in pulses/minute of courtship time or the fraction of time spent singing pulse trains.
    ii. Amount of sine song: Measure as the fraction of time spent singing sine.
    iii. The ratio between pulse and sine songs
    iv. Carrier frequencies of pulse and sine songs
    v. The interpulse interval: the median or mean spacing of pulses in a train of pulses.

    The interpulse interval will be influenced by the definition of a pulse train, i.e., the maximum distance at which two subsequent pulses are still considered to be part of one train (often defined as 80 msec or 150 msec), and the minimum train length (i.e., the minimum number of pulses making up a train, often defined as two or three).

    vi. Pulse cyclicity: Count the number of wing beats used to generate a single pulse and the *x*-axis crossings of the oscillogram during the pulse event or derive pulse cyclicity from pulse carrier frequency and pulse duration (i.e., multiply pulse carrier frequency by pulse duration).
    vii. Pulse train length: Take the mean or median number of pulses in a train.

    Most studies of male courtship song focus on the above-listed basic parameters (von Philipsborn et al. 2011; Arthur et al. 2013; von Philipsborn et al. 2014; O'Sullivan et al. 2018), but other features of the song, such as amplitude modulations (Brüggemeier et al. 2018), can also be analyzed.

TROUBLESHOOTING

Problem (Steps 3 and 9): Wing-amputated flies die or show general impairments.

Solution: Do not amputate the wings of very young flies when their cuticles are not yet fully hardened. If a big drop of liquid exits the wing veins upon removing the wing, the fly is probably too young. Pay attention not to harm the fly with sharp scissor tips. Practice amputation before performing it on the experimental flies.

Problem (Steps 3 and 9): Wing-amputated flies still produce sounds.

Solution: Carefully remove the whole wing; even small parts of the wing blade can produce sounds at lower volumes than sounds from intact wings, as flies typically continue to move their wing stumps.

Problem (Steps 13 and 15): Flies do not court.

Solution: Make sure that flies are in good condition and tested at controlled temperature, humidity, time of day, and age, as described in the Troubleshooting section in Protocol: **Single-Pair Courtship and Competition Assays in *Drosophila*** (von Philipsborn et al. 2023).

Problem (Steps 13, 15, and 16): Recordings contain noise and no or low signal.

Solution: This problem has multiple potential solutions. Consider the following.

- Identify the source of noise by carefully listening (noncourtship activity of flies; external sources such as human voices, doors, etc.; system-internal background).
- Make sure that all microphones are wired up properly and grounded and that the courtship chambers are properly placed close to the microphones.
- Consider electrical shielding and/or filtering of the recording.

Problem (Step 15): Playback has no effect on flies.
Solution: This problem has multiple potential solutions. Consider the following.

- Vary playback amplitude and relative positions of speakers and fly chambers.
- Test different audio files, starting with longer courtship song recordings containing both pulse and sine elements.

DISCUSSION

In the majority of studies involving audio recordings, the focus is on male courtship song, a well-characterized signal (Bennet-Clark and Ewing 1968). However, flies also use other types of acoustic signals—for example, during aggression, rejection, or copulation (Swain and von Philipsborn 2021). This protocol is suitable for recording all sounds produced by fly wing vibration.

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