Protocol

Single-Pair Courtship and Competition Assays in *Drosophila*

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Courtship in *Drosophila melanogaster* involves a series of innate, complex behaviors that allow male and female flies to exchange sensory information and assess the quality of a potential mate. Although highly robust and stereotypical, courtship behaviors can be modulated by internal state and experience. This protocol describes methods for designing and carrying out experiments that measure courtship performance in single-pair assays, in which a male is paired with a female, or in competitive assays, in which a male is presented with a female and with a male competitor. It also includes approaches for measuring female sexual receptivity during courtship.

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

Ethanol (70%)

Fly food (standard)

For example, Fly food recipes (2021).

Paint (two colors, preferentially H_2O-based and without alcoholic solvents)

Test the paint to be used beforehand to confirm that it does not induce any behavioral alterations.

RBS T230 (4%; Sigma-Aldrich) or Triton X-100 (10%) (see Step 13)

Equipment

Behavior boxes/behavioral room for experimenting

Rear and test the flies in an incubator with a light–dark cycle, set to 25°C with 50%–60% humidity, and perform all behavioral experiments in an incubator, a behavior box (Fig. 1A), or a dedicated room with light, temperature,

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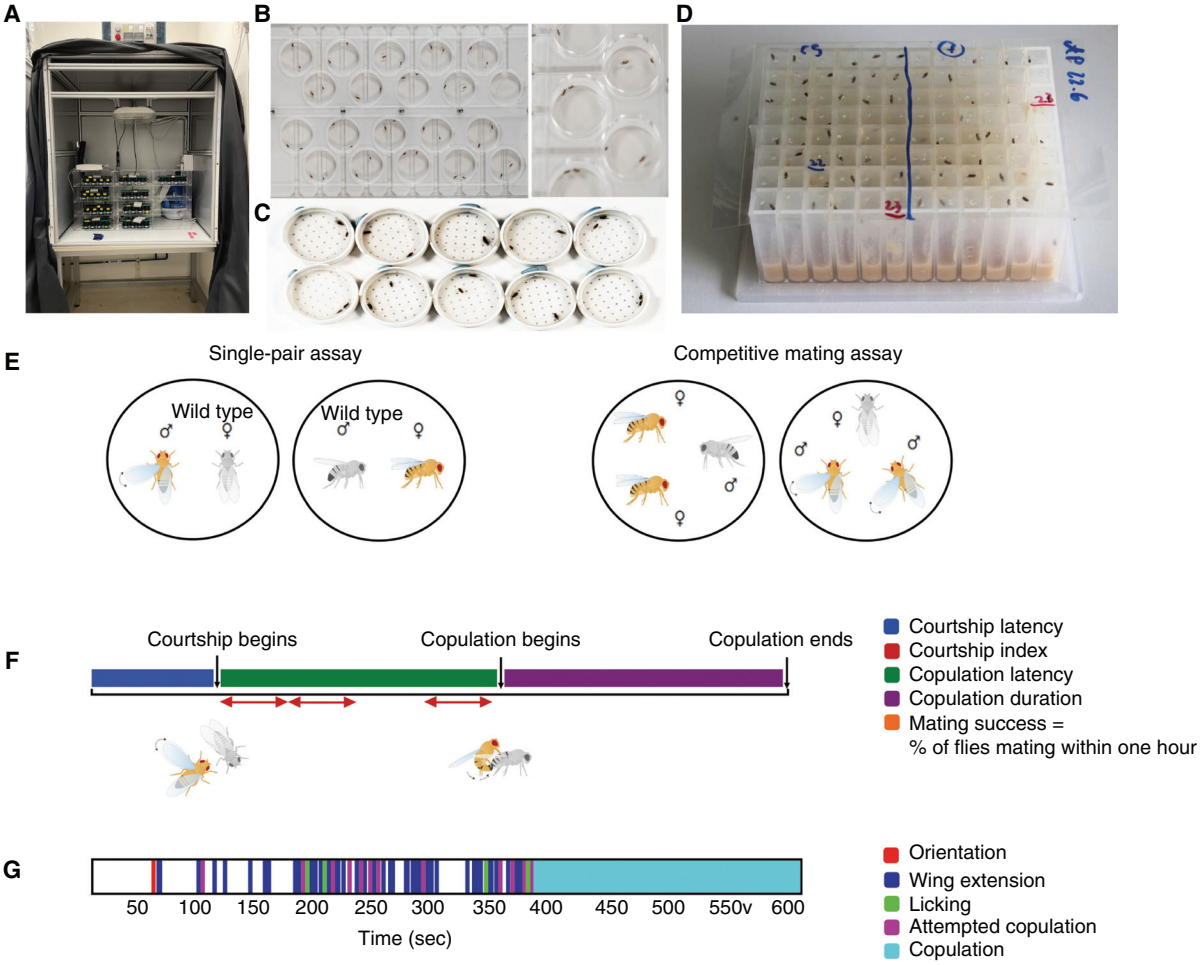

FIGURE 1. Equipment and experimental design for *Drosophila* courtship assays. (*A*) Behavioral box. (*B*) Courtship chambers fabricated from laser-cut acrylic. (*C*) Three-dimensional printed courtship chambers. (*D*) "Fly hotels": 96-well flat-bottom blocks covered with a punctured polymerase chain reaction (PCR) foil used for aging many flies in isolation. (*E*) Schematic of *Drosophila* assays to test courtship in pairs or in competition. (*F*) Behavioral parameters that quantify the courtship and mating sequence. (*G*) Example of an ethogram of a *Drosophila* male paired with a female fly. Colors represent the courtship steps displayed by the male over time.

and humidity control. This allows for a quiet and controlled environment and reduces variability between experiments.

Blocks (96-well plates, 2-mL, flat-bottom; QIAGEN 19585)

These can be used for housing and aging many flies in isolation (Fig. 1D). Fill each well with 0.5 mL of food, cool to room temperature, and cover with a 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.

CO_2 pad

Courtship chambers

Fabricate chambers by three-dimensional printing or acrylic laser cutting (Fig. 1B,C). The size and shape of the courtship chamber influence courtship initiation: The bigger the arena, the more time the male will take to find the female. Some researchers use round chambers 19 mm in diameter by 4 mm high (Cheriyamkunnel et al. 2021; Nojima et al. 2021), and others use ones 10 mm in diameter (Demir and Dickson 2005; Shohat-Ophir et al. 2012). For research about initial phases of courtship, bigger chambers can be used (even 12.7 cm in diameter) (Simon and Dickinson 2010). Detailed schemes for laser-cut chambers are presented in Supplemental Figures S1–S6. For scoring behavioral steps in the courtship process in detail or for automated tracking, it is advantageous to restrict vertical movement to prevent flies from walking on vertical chamber walls where they appear in side view from above. This can be achieved by designing chambers with sloped walls (Simon and Dickinson 2010;

Nojima et al. 2021). For decreased climbing on walls, coat the walls with siliconizing agents such as the fluoropolymer resin polytetrafluoroethylene (PTFE-30).

Fly aspirator (foot pump)

This can be used for selecting flies and transferring them to the behavioral setups.

Paintbrush (fine) or feather (see Step 2)

Software for measuring behavior (BORIS, Lifesong, and automated courtship tracking software [ctrax (Kabra et al. 2013; Robie et al. 2017), together with FixTRAX (Bentzur et al. 2021) or FlyTracker (Eyjolfsdottir et al. 2014)] and machine learning algorithms such as JAABA for automated and precise analysis of fly interactions [Kabra et al. 2013; Eyjolfsdottir et al. 2014]) and computer

Vials (plastic; 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])

Use vials for rearing flies and for experimenting.

Video recording equipment

Standard consumer camcorders or webcam equipment can be used for recording behavior. For recording over longer time spans (e.g., when observing remating), it can be useful if the camera has a time-lapse option. For tracking behavior, use a FLIR Firefly camera with an infrared pass filter or a comparable camera and record at a rate of least 30 frames per second to capture quick maneuvers. Place the courtship arenas in a rig to fix the position of the arenas. Backlight the arenas from below with an 880-nm near-infrared LED panel. To prevent buildup of heat, cool the backlight with coolant or with constant air circulation and a heat sink. For details, see, for example, Kabra et al. (2013).

METHOD

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

Collection and Rearing of Flies

Temperature and humidity have a strong effect on mating success in flies (Miwa et al. 2018). In addition, social conditions during development modify fly behavior in adulthood (Bentzur et al. 2021). Therefore, keep rearing and testing conditions constant.

1. Rear the experimental flies under controlled humidity and temperature conditions (typically, 25°C with 50%–60% relative humidity) on a 12-h light–dark cycle.

   See Troubleshooting.

2. Collect male and female flies 0–6 h after eclosion (newly emerged sexually naive [virgin] males and virgin females) under light CO_2 anesthesia on a CO_2 pad. To sort flies under the microscope, use a fine paintbrush or a feather. Avoid exposing the flies for long periods (>2 min) to CO_2.

3. House flies in vials or 96-well blocks, keeping males and females separate before the defined start of the experiment. Fill and remove flies from vials or blocks using a fly aspirator. Place one fly per well in the block.
   - House virgin females in groups of five to 10 flies in vials with food.
   - House males singly or keep them in groups of two in vials with food.

     Some research groups place the males in bigger groups before testing them for behavior (e.g., in groups of 10). For housing many flies in isolation, use 96-well 2-mL flat-bottom blocks (Fig. 1D).

4. Age virgin males 4–7 d and virgin females 3–7 d under controlled temperature and humidity conditions until the experiment.

   Housing conditions and the age of the flies can vary depending on the aim of the experiment. We typically age males 5–7 d and females 3–5 d.

Testing

We recommended performing all behavioral experiments in a behavior box (Fig. 1A), an incubator, or a dedicated room with temperature and humidity control (typically 25°C with 50%–60% relative humidity). Test flies at roughly the same time each day to reduce circadian fluctuations. Courtship and mating can be either tested in a single-pair assay (Steps 5–7) or, alternatively, in a competitive assay (Steps 8–12).

Single-Pair Courtship and Mating Assay

The most-used method for measuring male courtship behaviors and female sexual receptivity in Drosophila *consists of singly pairing a male with a virgin wild-type female and recording and analyzing the behaviors for 10–20 min, depending on chamber size (Fig. 1E, left).*

5. Transfer one female into the courtship chamber using a fly aspirator.
6. Transfer one male into the courtship chamber using a fly aspirator. Avoid males with damaged wings.

   If the chamber has a removable slide separating the male from the female, keep the male and the female separated before the defined start of the experiment and allow them to acclimate to the chamber for 2–5 min before the start of the video recording.
7. Start video and record for 10 min or longer at 25°C with >50% humidity. When the experiment is over, proceed to Step 13.

   See Discussion.

Courtship and Mating in the Competitive Assay

To evaluate courtship in competitive mating assays, pair two males (test and control competitors) with one wild-type virgin female in a chamber (Fig. 1E, right; Demir and Dickson 2005).

8. Mark both test and control competitors randomly with different colors of paint (preferentially H_2O-based and without alcoholic solvents).
9. Let the flies recover for at least 24 h.
10. Place both males in a courtship chamber.
11. Place a wild-type virgin female in the chamber.
12. Record for up to 1 h.

    Although many investigators determine a "female preference" index, the measure of relative difference in mating success may also represent different male ability and persistence rather than any active choice from the female. The assay can also be performed with two females and one male to test for male preference/differential female receptivity.

    Proceed to Step 13.

Cleaning Courtship Chambers

Wash and clean courtship chambers between experiments to remove fly scents.

13. Rinse the arenas with H_2O several times and then place them in a container filled with 4% RBS or 10% Triton X-100, wash actively, and let them soak for 30 min or 1 h. Rinse the arenas with hot H_2O several times (at least two or three times). Spray the arenas with 70% ethanol and let them dry overnight.

    Do not use ethanol to clean the arenas, as it can damage acrylic material, and remnants of ethanol vapor inebriate flies.

Data Analysis

For evaluating courtship and mating in the single-pair assay, follow Steps 14–17. For evaluating courtship and mating in the competitive assay, follow Steps 18–21.

Single-Pair Courtship and Mating Assay

14. Watch the video and score the following behavioral parameters:
    i. Time to courtship initiation: time elapsed between introduction of flies and the first exhibition of any courtship behaviors displayed by the male toward the target female.

       See Troubleshooting.

    ii. Male courtship index: the proportion of time that the "courter" spends exhibiting courtship behaviors toward the "target" female over a 10-min window (Fig. 1F).

       Courtship behaviors are defined as following, circling, orientation, tapping, wing extension, quivering, licking, and abdominal curling/attempted copulation (Fig. 1F; Demir and Dickson 2005; Billeter et al. 2006; Fabre et al. 2012; Nojima et al. 2021; Ning et al. 2022).

       Courtship index is mainly defined by the orientation of the flies and the degree of following, wing extension, and attempted copulation, as tapping and licking are subtle behaviors and are more difficult to quantify. The observation times starts from the first instance of courtship behavior (first bout of courtship lasting >3–5 sec). Naive wild-type males should show a courtship index greater than ~0.7.

       See Troubleshooting.

    iii. Female sexual receptivity during courtship.

       Although the courtship index focuses mainly on male behavior, females show behaviors that signal sexual receptivity or rejection during courtship. Female-specific behaviors such as vaginal plate opening, partial ovipositor extrusion, and decreased locomotion indicate female receptivity before copulation (Rezaval et al. 2012, 2014; Aranha and Vasconcelos 2018; Mezzera et al. 2020; Wang et al. 2021). Rejection behaviors include kicking, full ovipositor extrusion, wing flicking, and decamping (Aranha and Vasconcelos 2018).

15. Measure the relevant parameters during a 3-min observation period (or until mating occurs) starting from courtship initiation. For example, measure vaginal plate opening as the number of vaginal plate openings performed by the female per minute during the observation period of courtship.
    i. Percentage of mating or sexual receptivity: number of flies that successfully copulate within a given time (generally 1 h, as a percentage) (Fig. 1F).

    ii. Latency to copulation: time elapsed until copulation occurs (Fig. 1F).

    iii. Copulation duration: time elapsed from initiation of copulation to its end (disengagement) (Fig. 1F).

       Many of the parameters outlined above can be measured in an automated way (Step 16) or using automated tracking software (Step 17).

       See Troubleshooting.

16. Analyze the recording and manually score different behavioral parameters over time (Fig. 1G). Use BORIS or Lifesong software (for instructions how to use, see Kabra et al. 2013; Robie et al. 2017) to simultaneously annotate different behaviors (Lin et al. 2005; Friard and Gamba 2016) to make ethograms and plot the probability that the fly is performing a given behavior over time.
17. Measure courtship using automated tracking software like ctrax (Kabra et al. 2013; Robie et al. 2017), together with FixTRAX (Bentzur et al. 2021) or FlyTracker (Eyjolfsdottir et al. 2014).

Courtship and Mating in the Competitive Assay

18. Watch the video and score the following behavioral parameters.
    i. Annotate the male who succeeded in copulation.

       See Troubleshooting.

    ii. Calculate the courtship index for each individual male until the first male copulates (see Step 14.ii).

       See Troubleshooting.

19. Calculate the percentage of copulations for each male genotype and/or condition or the so-called female preference index:

$$\frac{\text{copulations with males of genotype A} - \text{copulations with males of genotype B}}{\text{copulations with males of genotype A} + \text{copulations with males of genotype B}}.$$

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 1): Crosses are unhealthy and produce few progeny.
Solution: There are multiple potential solutions to this problem. Consider the following:

Keep *Drosophila* cultures hydrated. If the fly food is too dry, females will not lay eggs. Make sure to add H_2O drops into vials every ~2–3 d.

- Make sure that the *Drosophila* culture is mite-free. Follow this procedure to avoid this threat: http://www.flyfacility.gen.cam.ac.uk/Flylab/mites.
- Keep culture vials free of fungal and bacterial contamination.

Check the quality of food. Nutrition affects fly development, reproduction, and survival (Corrales-Carvajal et al. 2016; Gorter et al. 2016; Leitão-Gonçalves et al. 2017; Grangeteau et al. 2018). Variations in recipes could lead to behavioral differences. Add food (in particular, fresh yeast) into vials or behavioral chambers to increase the likelihood of laying eggs (Grosjean et al. 2011; Gorter et al. 2016).

- Discard and renew parents once every week or two to ensure young parents and viable eggs.

Problem (Steps 14.i and 14.ii): Wild-type flies have low courtship index or long latency to courtship.
Solution: Conducting experiments investigating a complex behavior such as courtship that is sensitive to internal and external factors requires consistency across experiments and attention to different details. Mature and healthy male flies usually initiate courtship within 30 sec in small courtship arenas and ~100 sec in larger arenas, and usually manage to copulate within 2–5 min. Several factors can affect courtship performance.

- Make sure that all flies are normally sized and did not originate from overpopulated cultures that give rise to small flies. Verify that wings are not damaged, as this may affect the male's ability to sing.
- Make sure that females are virgin and mature.

Make sure to test male flies at the optimal time of the day. Male flies court in the morning and dusk and are less active also in terms of courtship around noontime. Train the flies with daily cycles of 12 h.

- Make sure that the environment is quiet with no loud sounds like construction work.
- Make sure that temperature and humidity are optimal (temperature, 25°C; humidity, ~60%).

Check the quality of food. Nutrition affects fly development, reproduction, and survival (Corrales-Carvajal et al. 2016; Gorter et al. 2016; Leitão-Gonçalves et al. 2017; Grangeteau et al. 2018). Variations in recipes could lead to behavioral differences. Adding food (in particular, fresh yeast) into vials or behavioral chambers increases the likelihood of laying eggs and promotes male courtship, female sexual receptivity, and successful mating (Grosjean et al. 2011; Gorter et al. 2016).

Anesthesia via CO_2 can affect fly behavior. Reduce fly-collection time as possible and allow recovery time. Refrain from using CO_2 after the initial collection. Transfer flies to courtship chambers with a fly aspirator.

- Make sure that flies are not contaminated with bacteria, fungi, or mites (see Troubleshooting for Step 1).

- In the case of transgenic flies, those with light eye colors are usually less successful in courtship; possible solutions to this issue are discussed in Protocol: **Measurement of Courtship Behavior in *Drosophila melanogaster*** (Ejima and Griffith 2007).

Genetic background can affect courtship performance. To control for genetic background, follow the instructions outlined in Protocol: **Measurement of Courtship Behavior in *Drosophila melanogaster*** (Ejima and Griffith 2007). Compare flies from similar genetic backgrounds (backcross all genotypes to appropriate genetic background for at least five generations).

Problem (Steps 14, 15, 18, and 19): Results are not reproducible or robust.
Solution: There are multiple potential solutions to this problem. Consider the following:

- Make sure to compare all genotypes at the same time.

Make sure to test the flies at the same time of the day. Male flies court in the morning and dusk and are less active also in terms of courtship around noontime. Train the flies with daily cycles of 12 h.

- Use a power test to calculate the number of flies required for an experiment. Repeat all groups in parallel at least three times on different days.

DISCUSSION

An effective way for creating virgin female courtship targets to test alongside experimental males consists of using a heat-shock-induced, conditional lethal proapoptotic transgene to kill males in a population of flies ("virginator" lines). Specifically, flies bearing the proapoptotic gene *hid* on the Y chromosome are heat-shocked for 2 h at 37°C by immersing a vial into an H_2O bath during larval stages, which induces *hid* expression and male lethality. A detailed procedure has been described (Boutros et al. 2017).

The role of different sensory modalities during courtship can be assessed following the approaches described in an earlier protocol (see Protocol: **Measurement of Courtship Behavior in *Drosophila melanogaster*** [Ejima and Griffith 2007]).

To test the contribution of vision to courtship, flies can be tested under dark or dim red light conditions. In addition, the fly's eyes can be covered with black acrylic paint (Saleem et al. 2014). Note that flies with the *white* gene mutated undergo retinal degeneration (Ferreiro et al. 2018). Mini *white*$^+$ constructs used in standard transgenesis that lead to orange eye color are often not sufficient to restore full vision. We therefore recommend using experimental flies with *white*$^+$ background (wild-type red eye color) or controlling for background in an appropriate way.

To evaluate the role of olfactory cues during courtship, the antennae can be removed or glued before experimentation. *Orco* mutant flies are anosmic to most odors (Larsson et al. 2004). To test the role of olfaction and gustation independently, flies can be separated by a mesh (Rezaval et al. 2016). Audition and olfaction can be simultaneously abolished by removing the aristae and the third antennal segment (Ebbs and Amrein 2007).

To block gustation, the fly's labella or front legs can be removed (Saleem et al. 2014).

Deaf flies can be generated by removing aristae (Murthy 2010). This procedure is difficult and the funiculus on the third antennal segment might still transmit loud sound; therefore, hearing (mechanosensory) mutants should be used for further validation. Male courtship song can be abolished by amputating the male wings close to the hinge with fine spring scissors. Additional manipulations include visual, olfactory, gustatory, and mechanosensory mutants (McKay et al. 1995; Larsson et al. 2004; Billeter et al. 2009; Krstic et al. 2009; Liu et al. 2012).

Before testing physically or genetically manipulated flies, we recommend establishing the described assay with wild-type stocks or control flies that are known to court and mate at high rates. Handling flies can require some manual skill and attention to detail that is normally quickly acquired by the experimenter after performing the assay several times.

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