

Courtship Analysis

The most important aspect of courtship analysis is that you be as **CONSISTENT** as possible and not adjust any variables. Changes in variables can affect behavior

COLLECTION & AGING

Day 0: The night before beginning collections, remove all adult flies from the vials you will be collecting virgins from (this will make virgin collection much simpler)

Days 1-3: Collect virgins in the morning and late afternoon for 3 days. Whatever times you choose to collect should ideally be the same for each day and for all conditions examined. **Be as quick as possible during collections—extensive CO₂ is detrimental to flies.**

Males must be isolated in individual straws containing food (prepare straws and caps before you put the flies on the CO₂ pad; try to count the males in the vial prior to dumping on the CO₂ pad for an estimate of how many straws to add food to).

Females may be housed together (for up to 6 females). I also recommend using a new vial in the morning and afternoon collections of female virgins each day.

Collect as many flies as possible because some may die prior to courtship analysis.

Helpful advice on virgin collection: Collecting virgins involves looking for flies that have a dark spot in their abdomen called the meconium, which is visible for 2-3 hours from eclosion (i.e. emergence of the adult from the pupa). Females will not be receptive to males for up to 8 hours from eclosion so any fly that has a meconium can be safely considered a virgin. The most efficient way to collect virgins is to empty a vial of eclosing flies in the morning, selecting females with visible meconium, and returning in the evening. Any females in that vial will be virgins even if the meconium isn't visible, since less than 8 hours will have passed since your last collection.

Days 0-7: *Drosophila* used for behavioral analysis must be maintained in the incubator on a 12 hour light-dark cycle for the duration of their lives. **The door of the incubator must NOT be opened during the dark cycle.**

LOADING INTO THE COURTSHIP CHAMBER

Day 7: Use a clean courtship chamber (that has been cleaned in **DI water** for at least 48 hours (the scent of flies, soap, or ethanol can affect courtship))

Add a paper towel lightly moistened with tap water to the bottom of the chamber, then add the first plate with chamber wells (holes).

Add male flies to this first level, so that the males are in contact with the paper towels. To add males, turn the CO₂ pad on low, then quickly tap each male from each straw onto the pad. **It is important to minimize CO₂, so work as quickly as possible.** Quickly scan the flies using the microscope to make sure they do not have broken/torn wings or legs. Gently brush 12 flies onto the fly collector (the small piece of folded white cardboard), then gently brush one male into each well. Quickly cover with a clear plastic sheet, then add the next plate with chamber wells.

Repeat process for female flies. Again, move quickly to avoid prolonged CO₂ exposure..

Add top plate and attach binder clips to the plates to hold them together.

Follow these steps with a 3 hour incubation in dark to recover from CO₂ exposure. Place the chamber in a drawer with a bowl of water (to maintain humidity). Close the drawer and tape shut with a note warning people not to open the drawer. **If the drawer is opened in the 3 hour recovery period, you cannot move forward with the courtship assay.** Exposure to light during the recovery period will alter behavior; the flies are not supposed to see each other at all during the recovery period.

Note 1: If you are loading two courtship chambers and need to remove one from the drawer to film, you will need to turn off the lights in the lab and the hallway to avoid any light from entering the recovery space (the dark drawer) and affecting the second chamber..

Note 2: Every time you film, you should choose the same approximate times to add flies to the courtship chambers and film. (eg, add flies to the chambers between 9:00am-9:30am and film between 12:00pm-12:30pm). Circadian rhythms affect behavior.

FILMING

- Set up the filming station during the recovery period by attaching ring light and setting up the camera
- The correct focal plane should be understood prior to placing the courtship chamber under the camera (the time from removing the flies from the recovery space to filming should be minimized as much as possible)

- Place courtship chamber under the camera
- Turn ring light on
- Start recording (**before** removing the plastic separator)
- Remove plastic separator between chambers to introduce flies to one another
- Film for 10 minutes following introduction of flies to one another

CHAMBER WASHING

After the flies are used for courtship, leave them in the chamber until they die (I know, its sad).

Take apart the chamber and put all of the pieces in DI water to wash for 48 hours on a rocker, replacing the water often (2-3 times per day).

Only use DI water to clean the chambers.

Do not let the plates sit in water without rocking.

ANALYSIS

Upload the video file and name the file accordingly.

You will need to codify the naming system so that the person analyzing the videos will not know the genotype/exposure (**this is critical for minimizing experimental bias**—if you need help devising a system, just ask me and we can develop one together).

Observe the videos for 10 minutes or until successful copulation, noting the time at which each behavior occurs (latency), the total time engaged in courtship behavior until copulation (to calculate the courtship index), as well as the number of pairs that successfully perform a given behavior (frequency). Each behavior is listed below.

Use the courtship analysis excel file specific for a 10 minute courtship assay (we also have one for 15 minutes—do not use that one as it will yield incorrect statistical analyses).

Track the following behaviors using the excel spreadsheet::

- **Following** (male orients toward female and follows her)
- **Leg tapping** (male foreleg taps female directly on leg)
- **Wing extension** (male exhibits unilateral wing extension to 90 degrees)
- **Genital licking** (male licks female genitals)
- **Copulation attempt** (male curls abdomen under itself)
- **Successful copulation** (male successfully copulates with female—assay is over)

The “**courtship clock**” on the excel spreadsheet tracks the amount of time a male fly participates in these courtship behaviors. When a male is not participating in any of the behaviors listed above (eg, he stops and starts grooming himself), turn the courtship clock off.

Important Note: All courtship behaviors are "oriented toward the female". When the male orients away from her and engages in other behaviors (walking away, grooming, etc), stop the clock.

Therefore, if the male is not oriented toward the female, the courtship clock should be off. **Restart the clock with the next wing extension.**