Protocol: XO vs XY Male Mate Choice Assay with PCR Validation

Materials

- Virgin *D. affinis* flies (aged 5-7 days) from the following lines:
    - **M40 males** (XO line virgins)
    - **Dark males** (XY line virgins)
    - **Dark females** (virgins)
- Standard fly malt food vials
- CO_2 anesthetization setup or cold pad
- Aspirators for transferring flies
- Microscope
- 1.5 mL microcentrifuge tubes (DNA extraction tubes)
- DNA extraction reagents and kit
- PCR reagents and thermocycler
- Y-specific primers

Methods

1. Fly Preparation

1. Day 1:
    a. Collect virgin flies from each line and age them for 5-7 days to ensure sexual maturity.
    b. Keep males and females separate until assays begin.
    c. Assume that you will do 20-30 flies per trial at first, but this could increase when you are comfortable
2. Day 4:
    a. Place on new food

2. Mate Choice Assay

1. Day 6 (early):
    a. This is the day before the mating trial
    b. Use CO2 to anesthetize flies - try to use light CO2 and make this brief
    c. Place 2 males (M40 and dark) in a single malt food vial (scored and with yeast)
2. Day 7 (early - mating is probably most likely shortly after lights on):
    a. Use an aspirator to move one virgin female to the vial with the two males

    b. Repeat for all vials, but do this quickly and watch for matings in the vials you already set up
3. Observe flies until mating occurs or for 2 hours
4. Once a mating pair is observed:
    a. **Remove the non-mating male** immediately and place it into a labeled **"loser" tube**.
    b. Transfer the **mating male and female pair** under a microscope to confirm identity.
        i. Record the winner by eye color: mark "1" for the winning eye color (sepia or red) and "0" for the losing eye color.
    c. Place the winning male into a labeled **"winner" tube** (1.5 mL DNA extraction tube).
5. Repeat for all vials until all pairs have been tested.
6. You can use one tube for all losers and one tube for all winners.
7. Record how long it took for mating to occur

Mating Date	Female_ID	Time Started	Time Mated	Genome(red)	Dark(sepia)
9/24/25	1			1	0
9/24/25	2			1	0
9/30/25	1			0	1

If the pilot experiment shows a clear signal, we will conduct future trials using only Dark backgrounds, determining the mated partner with the following method:

3. DNA Extraction

1. Extract genomic DNA from each **"winner"** containing the winning male
2. Use single-fly squish-prep DNA extraction protocol.
3. Store extracted DNA at −20 °C until PCR.

4. PCR Identification

1. Set up PCR reactions using the extracted DNA.
2. Use **Y-specific primers (hp1b)** to test for the presence of the Y chromosome.
    a. If the Y primers are negative for both males, use another primer (white?) to be sure you have good DNA
3. Run PCR products on an agarose gel and visualize bands under UV.

5. Data Recording

- Set up a Google Spreadsheet with the following columns:

Mating Date	Female_ID	Time Started	Time Mated	Extraction Date	PCR Date	Winner ID	Loser ID
9/24/25	1			9/27/25	9/30/25	XY	XO
9/24/25	2			9/27/25	9/30/25	XO	XY
9/30/25	1			10/1/25	10/4/25	XY	XO

- For the Winner and Loser ID:
    - **Band present** → Male was **Dark (XY)**.
    - **No band** → Male was **M40 (XO)**.

6. Data Analysis

1. To begin, check whether all females have one of each XO and XY males
    a. If not, censor that female from the analysis
2. The simplest analysis is a Chi-square test to see if the preference differs from 50/50