***DROSOPHILA* BEHAVIORAL GENETICS (2 week lab)**

The *foraging* gene (*for*) in *Drosophila* provides a rare example of a single gene that underlies a naturally occurring complex behavior. Two natural variants have been isolated from a wild population (Sokolowski, 1980). These natural variants have been selected such that we now have two strains that show different food-search strategies. Individuals with the rover allele (*forR*) move greater distances while feeding than do individuals that are homozygous for the sitter allele (*forS*). This difference is seen in both the larval and adult stages and is seen only in the presence of a food source.

Interestingly, allelic variation in the *foraging* gene is also known to be involved in attraction to a food source, but only in adult flies (Shaver, 1998). The pleiotropic nature of this gene is also demonstrated by its role in neuronal function such as habituation (Scheiner et al., 2004) and escape response (Engel et al., 2000). Furthermore, other loci in the genome (Perieera et al., 1995) have been shown to modify the allelic effects of the Rover and Sitter phenotypes, and additional genes in the same pathway can be mutated to affect foraging behavior (Riedl et al., 2005). Therefore, as appears to be the rule, the genetic regulation of a behavior is quite complex.

Many genes have been identified in *Drosophila* (and other organisms) that can be mutated to affect behavior (e.g. Sokolowski, 2001). However, the *foraging* locus was one of the first loci identified that underlies a naturally occurring variation in behavior. This raises the question as to how Natural Selection can maintain both phenotypes in a single population. If it is beneficial to be a rover in a particular environment, why do we still find sitters? Recently, it has been demonstrated that the Rover phenotype has a selective advantage under high population density, while Sitter is favored at low density (Fitzpatrick et al., 2007). This provided one of the few experimental demonstrations of **negative frequency-dependent selection**.

As you can see, despite the wealth of fruitful research regarding the behavioral affects of this one cGMP dependent protein kinase (Osborn et al., 1997), much remains to be discovered about the function, and evolution of its behavioral effects.

An abstract from a scientific conference reported that Rover males achieve copulation faster and copulate for longer than sitters (Pereira and Sokolowski, 1991). Those detailed behavioral data suggested that Rover males show longer bouts of wing vibrations and higher frequencies of licking than sitters. These behavioral differences likely contribute to their mating success. Without a high speed video recorder set on a microscope it will not be possible to accurately quantify duration of singing or the number of licking events. However, with the naked eye, we can quantify latency to initiate courtship, latency to first mating attempt, latency to successful mating, and the duration of copulation.

When researchers work with *Drosophila melanogaster,* they most often focus at the population level and rarely track individual differences. One might predict that individual variation in the activity of the *foraging* gene correlates with the observed intra-strain variation in foraging path length or mating behavior. While this is beyond the scope of this week's lab, it might make for an interesting independent project. How would you measure activity of the *foraging* gene?

For additional details see
http://flybase.org/data/allied-data/interactive-fly/hjmuller/foraging1.htm
Week 1 will be occupied with behavioral experiments in larvae
Week 2 will be occupied with molecular genotyping and behavioral assay of adults.

LAB SUPPLIES WEEK1:

Animals:

Drosophila melanogaster – These fly stocks were obtained from Seymour Benzer's lab at Caltech, though originally isolated in the lab of Marla Sokolowski. Larvae used in this experiment have been

Larvae - developmentally synchronized (~96 hours prior to class) by selecting 1st instar hatchlings that emerge within a 4 hour time frame. At this age, larvae should express the maximal differences in genetically based behavior (at 120 hours the larvae will leave the food in order to pupate and foraging behavior can no longer be observed (Sokolowski, et al., 1984).

Inexperienced adults - Each pair of students will be supplied with 3 virgin females and 3 inexperienced males from each strain. These animals were maintained in same sex populations for 4-5 days beginning within 2 hours of eclosion. They have been individually isolated for ~ 4 hours.

Equipment:

LAB

- Heat blocks set to 95° C
- Fly incubator set to 25° C
- 2 Dissection scopes with light sources and dissecting forceps.

EACH STUDENT PAIR

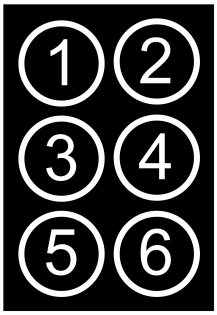
- 2 Agar petri dishes for storing larvae after wash
- 1 Larval foraging platform – Black Plexiglass with 6 individual test arenas
- 1 Squirt bottle with distilled water
- 2 Paint brushes for moving larvae from food to yeast and back
- 1 Squeegee
- 3 Empty Petri dishes for recording path length
- 1 Set of sharpie pens (6 different colors)
- 6 1.5 ml Eppendorf tubes for larval DNA prep
- 30 food tubes: 1.5 ml Eppendorf with 100μl fly food and small air-hole
- 200 μl pipettemen
- 1 stop watch

Reagents:

- Yeast paste 2:1 by weight with water made fresh that day.
- Squishing buffer (**SB**)
  - 10 mM Tris-Cl pH 8.2,
  - 1 mM EDTA, 25 mM NaCl,
  - 200 μg/ml Proteinase K, (diluted fresh from a frozen stock each day)

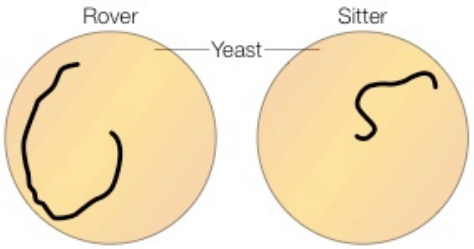
1: PROTOCOL WEEK 1

Two strains will be tested for larval foraging path length and latency to mate as adults.
Conduct the experiment "blind".
Test 18 individuals of each strain in the larval assay.
3 tested larvae of each strain will be used for DNA prep and genotyping.
15 larvae of each strain will be raised for adult test the following week.
Test 3 pairs of adults in the mating assay for each strain.



1A: LOCOMOTOR ASSAY

1) Collect 18 larvae of each strain (keep the strains separate) and rinse with H2O.
2) Store clean larvae in an agar filled Petri dish.
3) Label 6 petri dish lids for the 6 arena positions.
4) When your larvae are all collected, streak yeast paste evenly across the foraging platform (check that a track can be observed before setting up your whole experiment, yeast paste consistency may need to be adjusted).
5) Place an individual larva in the center of each arena.
6) Cover arenas with Petri dish lids and mark animal location
7) Start timer for 5 minutes – record start time in lab notebook.
8) Mark each animal's ending locations on Petri dish lids.
9) Trace larval path on Petri dish lids.
10) Save 3 larvae of each genotype in separate Eppendorf tubes for later genotyping.
11) Label each tube with necessary information for individual identification!
Strain#_Station_start-time-plate_position
REPEAT step 4 – 9 two more times for each strain to test at least 18 individuals of each strain (reuse lids but change pen color for each set of 6)
12) Save each tested larvae of both strains in individual tubes with food for adult test next week. Label each food and starvation tube with necessary information for individual identification! **Strain#_Station_start-time-plate_position**
(e.g. 1_A_13:15_2)
13) Place the flies in food tubes, and empty starvation tubes in your rack in the incubator.



1B: DATA ANALYSIS

14) Measure the path length by aligning a piece of string and then measuring the string. MAKE YOUR MEASUREMENTS IN **Millimeters**!
15) Drag the JMP document **Bio342_feeding_data_temp** from the courses server to your desktop.
16) Rename this files **Bio_342_feeding_data_INITIALS_THURS**(WED).
17) Enter your data - the automatic ID update will fill as you enter strain, station, start time, and position. Check that this ID matches that on your tube.
18) **Save** your data on the courses server in **fly-data.**
19) With your data set the behavior different for the two populations tested based on your own data?
    a) Calculate the mean path length, standard deviation, and coefficient of variation for each stock for your group. Put this information on the white board.
    b) Graph your own data.
    c) Determine if the two strains have **significantly** different for path lengths.
20) The class data will be combined for next week.

1C: DNA PREP FOR GENOTYPING

PCR Genotype individual larvae at the *foraging* locus

You will use a polymorphism (R390C) that leads to a change in the amino acid encoded by the *for*^{R} and *for*^{s} alleles. “R” (Arginine) is the amino-acid encoded by *for*^{R}, “390” is the amino acid position in the *for*-T1 protein sequence , and “C” (Cysteine) is the amino acid encoded by *for*^{s}. At the nucleotide level this is a C to T change between *for*^{R} and *for*^{s,} respectively. The functional significance of this polymorphism is currently unknown, however, it segregates in *for*^{R} and *for*^{s} stocks and provides a suitable marker for these laboratory experiments as it introduces a new restriction site. Polymerase chain reactions will be conducted using genomic DNA extracted from a single larva.

Each student pair will genotype three individual flies from each of the two stocks

DNA PREPARATION FROM SINGLE LARVA PROTOCOL:

1. Place one fly in a 0.5 ml tube and mash the fly for 5 - 10 seconds with a pipette tip containing 50 µl of SB, without expelling any liquid (sufficient liquid escapes from the tip).
2. Then expel the remaining SB.
3. Incubate at 25-37^{o}C (or room temp.) for 20-30 minutes.
4. Inactivate the Proteinase K by heating to 95^{o}C for 1-2 minutes.
5. Store samples at 4^{o}C degrees for next week.

PCR Genotype individual larvae at the *foraging* locus
LAB SUPPLIES WEEK 2:

Equipment:

LAB
PCR machine
Gel Doc system
Gloves

EACH STUDENT PAIR
minifuge
Tube rack
PCR tubes
200 μl, 20 μl and 2μl pipettemen
gel box to run 7 PCR reactions (precast gels)
power supply for gel box

Reagents:

PCR primers at 5μM
PCR buffer, 2mM dNTPs, Taq enzyme.
DNA size standards
Gel loading buffer
Gel Staining and Destaining Buffer

Each student pair will genotype three individual flies from each of the two stocks **IN YOUR LAB NOTEBOOKS** – demonstrate that you understand the process of PCR amplification and Restriction enzyme digest as it relates to this genotyping experiment. If you are not clear on this technique after reading this entire protocol ask for an explanation in class.

2A: PCR Set up.

1) Number tubes 1 – 7 (6 DNA samples and one negative control) and your initials
2) Prepare a "mastermix" containing reagents a-f in sufficient volume for 7 reactions of 50 μl each. Record your mastermix recipe.
   a) 39.3 μL ddH_2O
   b) 5 μL reaction 10X Taq buffer
   c) 1 μL dNTPs (Promega, each 10 mM),
   d) 2 μL R390C sense and antisense primer combination (5 μmol each/mL)
   e) 0.2 μL Taq polymerase
   f) 2.5 μL crude genomic DNA prep
3) Aliquot 47.5 μl of your PCR mastermix into each of your 7 tubes.
   (if you do not have enough, make note of this and short your negative control. Think about why you didn't have enough)
4) Add 2.5 μl of DNA sample to each tube.
   Record in your notebook which sample is in which tube.

5) Mix the reactions well and spin fluid to the bottom of the tube.
6) When 3 groups are ready start the program on the thermocylcer.
7) (verify that the program matches what is written below)

The PCR primers are :
R390C_S: CTGGTGGATCCGAATTTCAT and
R390C_AS: GGAGATACCCAATGCCCTCT.
They were ordered from Integrated DNA Technologies Aug 10th 2006 and have been stored at -20 °C in a concentrated (100mM) stock solution until now

The thermocycler will be programmed for: (check this before pressing start)

- ☐ 94 °C for 4 min,
- ☐ 40 cycles of: 94 °C for 15 s,
- ☐ 59 °C for 15 s,
- ☐ 72 °C for 30 s,
- ☐ followed by a final step of 72 °C for 7 min.

WHILE YOUR PCR REACTION RUNS, SKIP AHEAD TO THE ADULT MATING BEHAVIOR, <u>SECTION 2E</u>.

2B: Restriction Enzyme Digest

The recognition site for the enzyme *Hin*P1 is G|CGC which will cleave the *for*^{R} PCR product to produce two bands (286 bp and 57 bp) where as the *for*^{s} PCR product will not be cleaved (the first C is replaced with T leaving a band of 343 bp).

PCR products will be digested at 37 ^oC for 20 minutes in a 30 μL reaction

1) Prepare a "mastermix" by combining reagents a,b,c in sufficient volume for 7 reactions. (the recipe should be written in your lab notebook!)
a) 16.5 μL ddH_2O.
b) 3 μL NEBuffer 2 (New England Biolabs) (labled "2")
c) .5 μL *Hin*P1 (New England Biolabs, R0124L)
2) 10 μL PCR product
3) Aliquot 20 μl of the cocktail into each of 7 tubes.
4) Add 10 μl of the PCR reactions to the corresponding Restriction Digest reactions.
5) Mix the reactions well and spin fluid to the bottom of the tube.
6) Incubate the tubes at 37 degrees C for 20 minutes using the PCR machines
7) set to constant temperature.

While this reaction is running, complete the adult behavioral testing, or take a coffee break.

2C: Genotype Analysis

Both the digested PCR products and the original PCR products will be visualized using a 2% agarose gel run at 115 V for ~20 min.

1) In a new tube, combine 10 μl of the Digest reaction with 4 μl of the gel loading buffer (5X GLM light).
2) Load each into a single well on the gel (**note: it makes sense to run each uncut sample next to the corresponding cut sample rather than running all uncut next to each other and cut next to each other**).
3) Record the order of sample loading in your lab notebook.
4) Use one lane on the gel for size standards 100 bp ladder (10 μl).
5) Connect the gel box to the power supply ("DNA runs to red") and turn on the power adjusting the voltage to 115 V.
6) Run ~ 20 minutes (stop before 1st blue dye runs off)***** WEAR GLOVES FOR THESE STEPS *** Even though we are not using Ethidium Bromide (which intercalates DNA) these gel boxes have been used with this carcinogen.****

Before imaging, the gel must be stained in staining solution for 10 minutes and rinsed through the rinse solutions. ***********DO NOT LOOK AT THE UV LIGHT ***********

Image your gel using the Kodak system in the 2nd floor common area, print a hard copy for each lab notebook. (use Quick Print) *****DO NOT WEAR GLOVES AT THE COMPUTER.*******

1) If you want to save an electronic version of your image use "take picture" and export the image as a jpeg. (in your lab book record the file name and location that you have stored this data).
2) DISPOSE OF THE GEL as solid hazardous waste by putting it into a Ziploc bag along with any gloves that were used to handle the staining solution and gels after staining.
3) Put the gel image in your lab notebook. Label the gel image and also summarize results (did the PCR fail, did the Enzyme digest) for each lane on the gel.
4) Combine these genotyping results with the behavioral results from last week and by adding this to your data file.
5) Write your conclusions and summary in the lab notebook.
6) If your genotyping fails to identify the stocks clearly, include a statement of the possible reasons for the failed genotyping experiment.

ADULT MATING BEHAVIOR
LAB SUPPLIES WEEK2:

Animals:

Drosophila melanogaster adults – in individually labeled tubes from last week. These flies should be transferred to starvation tubes with clipped wings 1 hour before lab.

Strain 1 - (all metamorphosed since last week)
Strain 2 - (all metamorphosed since last week)
(The behavioral experiments will be run blind)

Equipment:

LAB:
Dissecting microscopes (~3)

STUDENT PAIRS:
1.5 ml tube "mating chamber" (15)
stop watch
jeweler's loupe
sharpie

2D: BACKGROUND:

Our current protocol was developed as an independent project by Burrill and Shrestha in 2008. Two students working as a team discovered that under Reed lab conditions the flies require slightly longer than reported in the published protocol (Ejima and Griffith, 2007). They also noted the need for undisturbed observation. By testing flies that had been transferred under cold anesthesia, and CO2 anesthesia onto either non-nutritive agar or standard fly food media, these students demonstrated that neither the mode of anesthesia, nor the isolation chamber characteristics, prevented mating. Sample size was not sufficient to determine a difference between the conditions, nor between the strains. Nonetheless, the efforts of these two students have produced a viable protocol with which to assay strain difference in mating behavior in a lab-class setting.

You will use the adult flies that you have previously tested for larval path length (minus those used for genotyping). These flies will have eclosed ~ 3 days before your lab. Because they were housed individually, the females will be virgins and the males should be eager to mate.

Courtship Description.

Male *Drosophila* initiate courtship behaviors in response to various sensory inputs the most important of which is vision. Once a male finds a female, he starts courting with orientation behavior accompanied by slight shaking of both wings or body wagging. Males are also able to find the female using olfactory and mechanosensation cues. Following orientation, the male taps the female's body with his foreleg to obtain information about sex and species. Detection of female sex pheromones via the gustatory organs on his foreleg will prompt

singing via wing vibration. The female will choose to accept or reject the male based upon the quality of his song, which he fine-tunes in response to her reaction. After, or during wing vibration, the male extends his proboscis and licks the female's genitalia and attempts to mate by grabbing the female body and curling the tip of his abdomen. Only if the female is receptive will she spread her wings to allow the male to mount. Successful copulation usually requires several courtship attempts. Copulation will continue for 10 to 20 min while the male ejaculates seminal fluid into the female. During lab in week two, we will see a short movie of these behaviors.

COURTSHIP MEASURES (* indicates behavior we will measure today)

courtship index - **CI** broadly represents the overall courtship enthusiasm of the male.
It is equal to the total duration of the male's performance of any steps of courtship as a fraction of the observation period. CI for a wild-type male ranges from 0.3 to 0.9 depending on the conditions.

wing extension index - WEI is equal to the total duration of wing vibration calculated as a fraction of observation period. While WEI corresponds to a portion of CI they do not always show a linear correlation.

Courtship latency* is the time lag between initial pairing and initiation of the first courtship orientation behavior.

Mating latency* is the time lag between initial pairing and the male's first mating attempt.

Copulation latency* is the time lag between initial pairing and successful copulation. This usually requires multiple copulation attempts.

Number of Copulation Attempts is simply the number of a times a male attempts to mount before he is successful (each bout of mounting that is separated by another courtship behavior (singing, chasing, licking) is counted as a separate attempt.

Mating success* is a measure of the number of pairs that achieve successful copulation during a certain period.

Copulation duration* is the time between the male's successful mating success and when he dismounts.

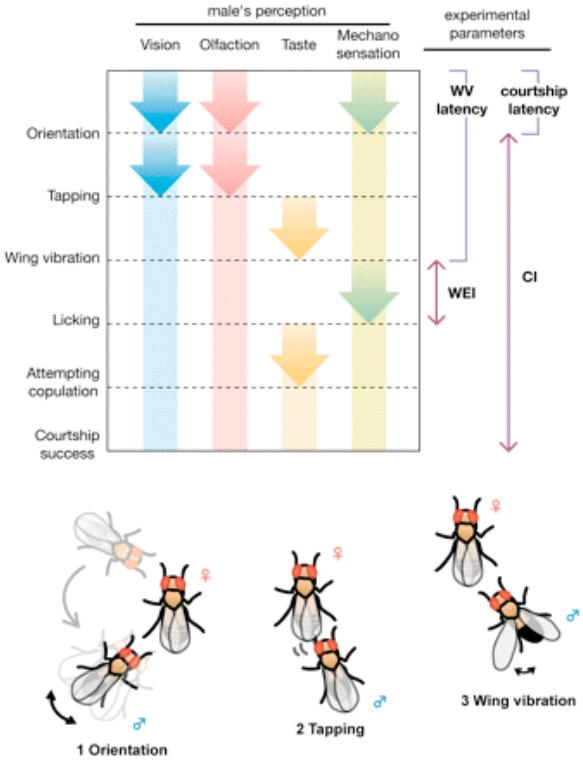


Figure 1. The stimulatory sensory inputs that progress courtship behavior in male *Drosophila*. Arrows indicate a strong contribution to courtship drive

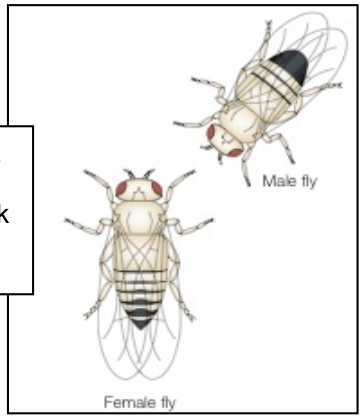


Figure 2. The last 2-3 segments of a males abdomen are dark. The female abdomen is tan, but not black and has a more pointed shape.

2E: ADULT MATING PROTOCOL

1. Before you start this section, read the Background above.
2. Retrieve your flies from the incubator.
3. With the aid of a dissecting scope or a loupe determine the sex of each individual and label their tubes.
4. Obtain a mating observation score sheet.
5. Note the time of day on the score sheet.
6. You will establish up to 5 observation chambers at once.
7. You will do that at least twice, once for each strain.
8. Start a timer in count-up mode.
9. Gently tap a male to the bottom of his tube.
10. While he is disoriented, snap open the lid of his tube and place the opening flush against the opening of an empty eppindorf tube.
11. Use the same technique to introduce a female from the same strain to the male in the empty tube.
12. Snap the lid shut.
13. Set this tube upside down on the score sheet in its test position.
14. Note the time displayed on the timer as "start time" for that pair and record whether this pair is strain one or strain 2.
15. Do not disturb the tube!
16. Repeat steps 7-12 for an additional 4 mating pairs.
17. Watch all 5 pairs simultaneously
    This technique is behavior sampling with continuous recording.
18. When one of the males performs one of the behaviors listed below, record the time on the observation sheet in the space provided for that courting pair.
19. Record the time for initiation of courtship by the male.
20. Record the time for the first mating attempt.
21. Record the time for the successful mating.
22. Record the time that copulation is terminated.
23. Continue recording for up to one hour or until all pairs have successfully mated.
24. Establish additional pairs if you have flies remaining.
25. Convert your data into seconds (rather than minutes and seconds).
26. Attach these data sheets to your lab notebook.
27. From the courses server, copy the blank data template Bio342_mating_temp to your desktop.
28. Open the data template in JMP and enter your data.
29. Saved that files as Bio342_mating_yourInitials and put a copy on the courses.

2: EVALUATED: (hand this in the week after the 2nd week of the fly lab also attach a copy to your lab notebook)

write up a 1 page summary including

-Brief introduction that includes the hypotheses to be tested.
-Statistical analysis of class' larval data set
    Include a graphical representation of the data (with figure legend)
    and a statistical methods statement
-Statistical analysis of class' adult mating data set
    Include a graphical representation of the data (with figure legend)
    and a statistical methods statement
-Statement regarding your own PCR genotyping results
-A conclusion that summarized the conclusions that can (or cannot be made) with the larval and adult datasets in light of the PCR results.

Possible independent projects:

Is there a circadian effect on food-search strategy?

Does temperature affect food-search strategy similarly for both rover and sitter phenotypes?

Is foraging gene expression level correlated with individual variation of path length within either Rover or Sitter genotype.

Determine whether the latency to courtship is affected primarily by the genotype of the male or by that of the female.

Sensory inputs to the test male may be manipulated using the following methods:

i. To eliminate visual input, use dim-red light conditions.

This manipulation is useful when investigating chemosensory mechanisms because visual cues are very strong courtship stimuli and they often mask subtle changes in other modalities (Joiner and Griffith 2000).

ii. To investigate the visual contribution to courtship, use a pheromone-less decoy, whose cuticular hydrocarbons (sex pheromones) have been washed off with hexane, as a courtship target.

iii. To block active movements by the female, use anesthesia or decapitation. Although a *Drosophila* female doesn't demonstrate an active courtship display, her reaction to male courtship (acceptance or rejection) affects the performance of the courting male. Using a motionless target not only reduces mechano-sensory input to a male, but also prevents copulation, allowing the investigator to have consistent observation periods for each experiment.

see Ejima and Griffith (2007) for more detailed courtship observation and measurement protocols.

References: (many of these are available on the lab website for week 5)

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