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Fluorescing flies: examining protein targeting and insect reproductive behavior using Green Fluorescent Protein.

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Biologists gain insights into protein function by examining protein localization, expression, and movement in cells and tissues. The discovery of green fluorescent protein (GFP) and its development as a protein probe (reviewed in Hazelrigg, 1998) have made it possible for biologists to study protein function in living organisms including animals such as the fruit fly *Drosophila melanogaster*. We describe a two-hour lab activity for students in an introductory biology class in which students use green fluorescent protein (GFP) to explore male seminal fluid protein transfer to females and female re-mating behavior in fruit flies. Students are given female flies pre-mated to GFP males, and female flies that had not previously mated. Students will determine that pre-mated flies are refractory to additional mating. Normally, unmated flies mate readily, but 95% of pre-mated flies do not mate (reviewed in Wolfner, 1997). Specific objectives we cover in this activity include: observing fruit fly mating behaviors, learning how proteins are tagged, and making and testing predictions regarding how previous mating experience will affect a female fly's subsequent mating behaviors. These are important topics in the fields of animal behavior, genetics, and developmental and reproductive biology. The direction of the discussion can be tailored to fit the area of expertise of the instructor, for example by emphasizing the behavioral aspects of the observations, or the fluorescence microscopy, or transgenic technology, or evolutionary questions. The lab activity can also be easily adapted for more advanced students. The lab activity can be run by a single instructor, although it works best if two people (two instructors or an instructor and an assistant) are available to run it.

The activity centers around the use of male flies possessing a transgene that generates GFP fused to the male seminal fluid protein Acp26Aa (Lung and Wolfner, 1999). The fusion protein is produced only in the tissue that makes seminal proteins. We mate the transgenic males to virgin females, and then use the protein's fluorescence as a tool to investigate insect reproductive behavior. Since students can tell whether a female has mated with a GFP male by whether or not her reproductive tract contains fluorescing GFP-tagged protein, they can test whether female fruit flies that have mated once show a change in their willingness to mate again.

The guiding questions in this activity are: 1) does previous female mating experience affect subsequent male-female interactions in fruit flies? and 2) how can we determine whether or not a female fly has previously mated? Prior to starting the lab activity, we introduce the topic by discussing the occurrence and frequency of female multiple-mating in insects and other animals (Krebs and Davies, 1997). We also explain the role of male seminal proteins in regular mating behaviors (reviewed in Wolfner, 1997) and, briefly, how protein tags are useful to follow seminal fluid proteins once transferred to females. We describe fly mating behavior (Greenspan, 1995, provides useful images, and background

for this). Finally, additional background on GFP is discussed during the time when the students are observing the flies (see below).

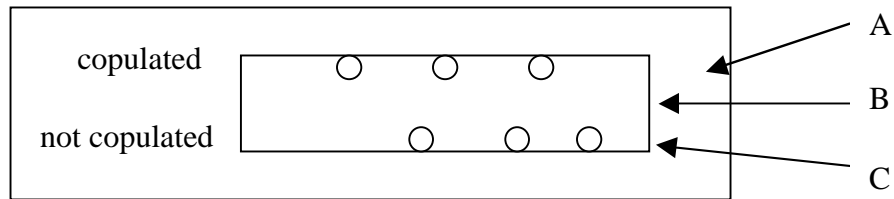
Students work in pairs to set up the fly matings. Each student pair receives 6 vials each containing a single *D. melanogaster* female from the Oregon-R strain. The vials are coded (code known to the instructors only) to correspond to the female's prior mating status. Half the vials contain a single unmated (virgin) female flies. The other half of the vials contain single females that were previously mated a single time with males whose seminal fluid protein, accessory gland protein (Acp)26Aa, was tagged with GFP (Lung and Wolfner, 1999). It is important that these females be mated no more than 60 min before the start of the activity to ensure that the Acp26Aa-GFP fusion protein will still be detected in the female reproductive tract during the activity. By longer times, the protein will have been expelled from the female's reproductive tract. Student pairs also receive 6 vials each containing one male from the Oregon R strain. [Having extra vials of males and females on hand is useful in case the students encounter difficulty during the transfers; this occurs only rarely.]

At the start of the experiment, students transfer individual males into each of the females' vials and observe the flies' subsequent behavioral interactions. (These behaviors can be observed without microscopy.) *Drosophila* males perform a series of stereotyped courtship behaviors towards females before copulation occurs (see Greenspan, 1995, for review). The male approaches the female and orients towards her, tapping her with his foreleg. He follows her, vibrating his wing to produce a courtship song. Then, he goes towards her posterior and licks her genitalia. Finally, he attempts to copulate by mounting her. In response to male courtship virgin females initially run around, but will mate readily. Females that have previously mated will, instead, actively reject male courtship by fluttering their wings, depressing or elevating their abdomen, running away, kicking and extruding their ovipositor. Students are encouraged to record both male and female behaviors for the next 40 minutes and note all pairs that copulate.

During the 40 min observation period, students receive additional instruction. While one member of each student pair watches the flies' behavior and records observations, the other member of the pair attends a ~10 min discussion that introduces GFP, and how animals are engineered make GFP-tagged proteins (*Drosophila* transgenics is briefly covered in chapter 21 of Hartwell *et al.*, 2000). The instructor also explains how protein labeling allows scientists to mark and follow both proteins and cells, and discusses some of the advantages and limitations of GFP tagging compared with both conventional antibody staining and other existing fluorescent probes (see Hazelrigg, 1998). When the first group's discussion is over, the students in the pair reverse activities: the second student receives instruction about GFP while his/her partner observes the flies and records the data.

At the end of the 40 minute observation period, students group their flies depending upon whether or not the flies copulated during the observation period. Students then chill the flies on ice to arrest further activity, permitting the flies to be mounted on a slide for microscope-observation. A glass slide (A on figure below) with a piece of double-sided tape along its length (B) is used to hold the flies in place. Chilled females from each group (copulated or not copulated) are placed ventral side up in two rows on the tape (C). Their wings are pressed against the tape with blunt forceps. The slides are kept on ice to keep the flies immobilized. Students are encouraged to predict, before making their observations, which females (copulated, non-copulated) will show GFP-fluorescence. Students observe each female fly's abdomen under a stereomicroscope with a fluorescence attachment (we used Olympus SZX9 and SZX-RFL2, respectively). Alternatively, abdomens can be observed using a low power objective on a compound fluorescence microscope equipped with GFP filters. Females that didn't mate during the observation period will generally have a discrete region of bright green fluorescence at the base of their abdomen, due to the GFP-fusion protein received in the previous mating. Most females that mated

during the observation period should lack this localized GFP-fluorescence. [There is background fluorescence in the abdomens of all female flies, but it is diffuse and yellowish; the GFP signal is greener and localized to the tip of the abdomen. It is helpful to have available some positive (GFP-fluorescing) and negative (non GFP-fluorescing) control flies for the students to observe.] The intensity of the observed GFP signal varies among females, so occasionally it may be more difficult to tell whether or not a female had previously mated.



After students have observed their flies, they meet together to discuss the results. If all went as expected, the group will conclude that female flies that didn't mate during the observation period usually had a discrete region of GFP-fluorescence at the base of their abdomens and that females that mated did not have such fluorescence. Thus, previously-mated females did not re-mate during this period. [A few flies may not follow this paradigm. Not all virgin female flies mate in this short time period (though the large majority do), and a small proportion of the females that previously mated with GFP-males may not have had observable GFP-fluorescence in their abdomens. This variability in female response can be useful to seed discussion about the causes of variation in the amount of seminal fluid protein produced or transferred by different males, and possible variation in the female responses to males.] Students also can discuss their observations of fly mating behaviors and relate them to the conclusions. The lab session can then conclude with a more general discussion. For example, we point out that the observed changes in female behaviors are caused by proteins transferred by the male during mating (review in Wolfner, 1997). From this, we discuss female mate choice, female multiple mating, sperm storage, and sperm competition (reviewed in Birkhead, 1995; Gowaty, 1994). We suggest that some of the observed male seminal proteins might serve as a mechanism to avoid sperm competition.

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