Supporting File S1: Prep guide

Overview and Contents

This Supporting File is a comprehensive guide for prep staff, i.e. those who will prepare flies and classroom equipment for this Lesson Plan. We recommend that instructors also consult this document, even if they are not directly involved in the prep, in order to facilitate their coordination with the prep staff and to familiarize themselves with the supplies.

Altogether, this guide was written with the assumption that the reader does not have hands-on experience with *Drosophila*, and so we describe basic fly work in detail: equipment, some fly diet options, taking care of fly cultures, how to identify sexes and genotypes, and how to set up crosses. We tried to make instructions clear and complete, but hands-on experience is always best. Therefore, if you are new to flies, we suggest that you pay a short visit to a *Drosophila* research lab before starting this prep; even a half-day of making diet, sorting flies under a microscope, and setting up crosses should be informative. Although the Supporting File S5 slideshow is primarily intended for teaching purposes, parts of it (the fly images and demo video at the end) could be a helpful primer for prep staff, at least as an example of how to handle and score flies.

Those who are experienced with flies may wish to overlook the tutorial portions of this document and instead focus on what needs to be prepared and when—indeed, all prep staff will ultimately need this information. To address these priorities, we have compressed the essential prep information into the following tables and figure:

- **Table S1-3** (pp. 4-7) is a complete and detailed prep schedule.
- **Table S1-4** (pp. 10-11) lists which fly stocks to order.
- **Tables S1-3** (pp. 4-7) and **S1-6** (p. 25) describe which flies to collect and cross, and when.
- **Figure S1-7** (p. 34) and **Table S1-7** (pp. 35-36) list the equipment that we lay out in the classroom.

S1 is subdivided into 6 parts (A-F). To help you locate information of interest, **Table S1-1** (below) lists all content and associated page numbers.

<table>
<thead>
<tr>
<th>Table S1-1. Contents of Supporting File S1</th>
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</thead>
<tbody>
<tr>
<td><strong>Overview and Contents, 1</strong></td>
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</tbody>
</table>

**S1, Part A: Prep timetable, 3**
- **A1.** Controlling fly prep timing: a comparison of different *Drosophila* incubation temperatures, 3
  - **Table S1-2.** Comparison of different *Drosophila* incubation temperatures, 3
- **A2.** Planning your prep schedule, 4
  - Table S1-3. Recommended fly prep schedules for two standard *Drosophila* incubation temperatures, 4

**S1, Part B: Ordering stocks, 9**
- **B1.** When to get started, 9
- **B2.** Opening a new BDSC account, 9
- **B3.** Ordering stocks, 10
  - **Table S1-4.** Fly stocks to order for this Lesson Plan, 10
- **B4.** Payment for stocks, shipping times and seasonal considerations, 11
Table S1-1 (cont.)

S1, Part C: Fly equipment, handling, and diet, 13
- C1. Fly equipment, 13
- C2. Fly handling, 13
- C3. Fly diet, 14
  - C3-A. Yeast-cornmeal diet, 14
  - C3-B. Formula 4-24 Instant media (Carolina Biologicals, #173200), 16
  - C3-C. Nutri-Fly, Bloomington formulation (Genesee Scientific, #66-112), 16
  - Table S1-5. Comparative summary of diet properties, 16

S1, Part D: Maintaining and expanding fly stocks, 17
- D1. Diet selection, 17
- D2. Vials vs. bottles, 17
- D3. Incubation temperature, 18
  - Figure S1-1. Examples of “healthy” fly cultures at various stages of development, 18
- D4. Diet moisture content, 19
  - Figure S1-2. Monitoring and adjusting moisture content of diet, 19
- D5. Ambient humidity, 20
- D6. Transfer flies and larvae to fresh diet regularly, 20
- D7. Stock care: minimizing cross-contamination between stocks, controlling fungus/bacteria/mites, 20
  - D7-A. Minimizing cross-contamination between stocks, 20
  - D7-B. Controlling fungus and bacteria, 21
  - D7-C. Controlling mites, 22
  - Figure S1-3. Drosophila culture with a mite infestation, 22

S1, Part E: Setting up the prep crosses, 25
- E1. Crosses that prep staff will set up, 25
  - Table S1-6. Sexes and stocks of flies to collect for each type of prep cross, 25
- E2. Diet for collections and crosses, 25
- E3. The essential Drosophila biology to consider when collecting for crosses, 26
- E4. How to collect flies for the prep crosses, 26
  - E4-A. What to do within of few days before a collection, 26
  - E4-B. What to do the morning of a collection day, 27
  - E4-C. What to do the afternoon of a collection day, 28
  - Figure S1-4. Distinguishing female from male and younger/virgin from older/possibly mated Drosophila, 28
  - Figure S1-5. Comparisons of wild type vs. mutant cw, w, i, and Bar traits, 30
- E5. How to set up prep crosses, 32

S1, Part F: Classroom supply lists, 33
- Figure S1-6. Snapshot of our lab classroom, 33
- F1. Week 1 classroom supplies, 34
  - Figure S1-7. Snapshots of the Week 1 lab supplies, 34
  - Table S1-7. Week 1 classroom supplies, 35
- F2. Week 2 classroom supplies, 37
- F3. Week 3 classroom supplies, 37
- F4. Week 4 classroom supplies, 38
- F5. Alternative supplies, 38
  - F5-A. Fly stocks, 38
  - F5-B. Digital projector, screen, and dissecting scope with camera, 39
  - F5-C. Funnel and rubber stopper (Figure S1-7A, #5), 39
  - F5-D. Incubators, 40
  - F5-E. Anesthesia alternatives, 40
S1, Part A: Prep timetable

Here we provide key information to help you plan your prep itinerary: how fly incubation temperature affects overall prep timing, the major steps of the prep, which and how many fly cultures to generate for your students, when to prepare these cultures, and when to prep the classrooms.

A1. Controlling fly prep timing: a comparison of different Drosophila incubation temperatures

*Drosophila* strains can be reared at a wide range of temperatures (18-29°C), but temperature significantly affects overall culture health/durability as well as “generation time” (i.e. time between transfer of parental adults to fresh diet, and then emergence of significant numbers of F1 adults from that culture). **We recommend using the moderate 20-25°C range to start with** as staff become accustomed to implementing this module. However, lower and higher incubation temperatures do have their own advantages, and temperature does not significantly affect the penetrance or expressivity of the mutant phenotypes seen in this Lesson Plan. Therefore, low- or high-end incubation temperatures may be appropriate in some cases. Below is a discussion of the relative pros and cons of different incubation temperatures (and immediately following, Table S1-2 summarizes this discussion):

- **25°C** is probably the most standard incubation temperature for fly experiments. This is warmer than most lab interiors, so an incubator is required in most buildings. This temperature has a generation time of 10-14 days after adults have been transferred to fresh diet. Left alone, cultures tend to remain “healthy” for up to about 24 days, *i.e.* after 24 days, adult flies may die off at a significant rate, and fewer young adults may emerge to replenish the culture.

- If no incubator is available, **“room” temperature (20-22°C)** is very convenient and places lower stress on cultures, allowing them to remain healthy for longer than at 25°C (up to about 35 vs. 24 days). However, the lowered temperature extends generation time to 14-21 days.

- **18°C** has a generation time of 21-28 days and places low stress on the cultures (cultures can remain healthy for up to 42 days). This temperature will slow down the prep significantly, but it can be useful for staggering the timing of a generation or for maintaining the stocks during terms when this Lesson Plan is not offered.

- **26-29°C** can be useful if there is a time crunch for class prep, because this temperature range has a generation time of only 8-12 days. The high end of this temperature range can be desiccating and otherwise stressful to cultures, especially less healthy ones such as the Df strains (listed in Table S1-4). However, these drawbacks can be manageable if cultures are attended to carefully; Part D of this Supporting File suggests ways to care for stressed/unhealthy stocks and to expand cultures efficiently.

Table S1-2. Comparison of different Drosophila incubation temperatures

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Usually requires incubator?</th>
<th>Generation time (days)</th>
<th>Recommended time range before making a fresh culture from an older one (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>yes</td>
<td>10-14</td>
<td>16-24</td>
</tr>
<tr>
<td>20-22 (&quot;room&quot;)</td>
<td>no</td>
<td>14-21</td>
<td>21-35</td>
</tr>
<tr>
<td>18</td>
<td>yes</td>
<td>21-28</td>
<td>28-42</td>
</tr>
<tr>
<td>26-29</td>
<td>yes</td>
<td>8-12</td>
<td>12-20</td>
</tr>
</tbody>
</table>
A2. Planning your prep schedule

Table S1-3 (below) suggests prep schedules for relatively user-friendly, moderate incubation temperatures—if you wish to use lower or higher temperatures during any particular fly generation, consult Table S1-2 and adjust your prep schedule proportionately. The Steps and notes column of this table contains key information for prep staff, such as how many fly cultures or crosses to generate at crucial steps of the prep. The Informative Part(s) of S1 column references supporting details elsewhere in this document. (i)-(v) refer you to table footnotes on pp.6-7.

Table S1-3. Recommended fly prep schedules for two standard Drosophila incubation temperatures

<table>
<thead>
<tr>
<th>Phase</th>
<th>Timepoint in weeks before first Week 1 class, by incubation temperature</th>
<th>Steps and notes (i)(ii)</th>
<th>Informative Part(s) of S1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>20 - 22°C (&quot;room&quot;)</td>
<td></td>
</tr>
<tr>
<td>Obtain and expand fly stocks</td>
<td></td>
<td></td>
<td>1) Open BDSC account, order fly stocks.</td>
</tr>
<tr>
<td></td>
<td>16 - 0</td>
<td>25 - 0</td>
<td>2) Expand stocks once they arrive. See Steps 3 and 5 below for key expansion targets. (iii)</td>
</tr>
<tr>
<td>Crosses A1 and B1</td>
<td>6 - 4.5</td>
<td>7.5 - 6</td>
<td>3) Set up 16-24 fresh bottles of the Mutant stock and 4-6 fresh bottles of each of the WT stocks (for Step 4 Cross A1 and B1 collections).</td>
</tr>
<tr>
<td></td>
<td>4.5 - 2</td>
<td>5.5 - 3</td>
<td>4) From the bottles that were set up at Step 3, collect males and virgin females from each stock. Store in vials at room temperature, separated by sex and genotype. See Step 6 for target numbers. (iv)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5) Also set up 4-6 fresh bottles of Mutants, 2-3 fresh bottles of each WT stock, and 2-4 fresh vials of each Df and Dp stock (for Step 8 Df and Dp Cross collections and Step 12 prep of Parental vials).</td>
</tr>
<tr>
<td></td>
<td>2.5 - 2</td>
<td>3.5 - 3</td>
<td>6) Using flies collected at Step 4, set up Crosses A1 and B1:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• One Cross A1 or B1 vial per student, roughly equal distribution.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Cross A1: WT virgin females x Mutant males. Cross B1: Mutant virgin females x WT males. 12-20 virgin females and 7-15 males per cross vial.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Use a different WT strain for each lab section’s crosses.</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>• For spares, overshoot the number of crosses prepared for each section by 20-25%.</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>• Label each vial A1 or B1 and indicate its WT stock number.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7) 5-7 days after setting up the A1 and B1 Crosses (Step 6), remove their parental flies.</td>
</tr>
</tbody>
</table>

(Continued on next page.)
Table S1-3. (cont.)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Timepoint in weeks before</th>
<th>Steps and notes (i)(ii)</th>
<th>Informative Part(s) of S1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first Week 1 class, by</td>
<td>8) From bottles and vials that were set up at Step 5, collect Mutant males and virgin</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>incubation temperature</td>
<td>females, Df virgin females, and Dp males. Store in vials at room temperature, separated</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>by sex and genotype. See Step 9 for target numbers. (iv)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9) Using flies collected at Step 8, set up Df and Dp Crosses:</td>
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<tr>
<td></td>
<td></td>
<td>• One vial of a Df or a Dp cross per student—however, we share these crosses between</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>sections, so for the entire class, we only set up enough crosses for the number of</td>
<td></td>
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<td></td>
<td></td>
<td>students in our largest lab section, +30-50% for spares.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Dp crosses: 12-20 Mutant virgin females and 7-15 Dp males per cross vial. Label each</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>vial Dp and indicate the Dp stock number.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• Df crosses tend to be less healthy, so use more females if possible: 20-30 Df</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>virgin females and 7-15 Mutant males per cross vial. Label each vial Df and</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>indicate the Df stock number.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>20 - 22°C (“room”)</td>
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<tr>
<td>Df and Dp</td>
<td></td>
<td>3 - 1</td>
<td></td>
</tr>
<tr>
<td>Crosses</td>
<td>2 - 0</td>
<td>0.5 - 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 - 1</td>
<td>10) Set up 3–4 fresh stock bottles of Mutants and 1–2 fresh stock bottles of each WT</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>strain (for Parental vials during Week 3 prep, end of Step 12).</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11) 5–7 days after setting up Df and Dp Crosses (Step 9), remove their parental flies.</td>
<td></td>
</tr>
</tbody>
</table>

(Continued on next page.)
Table S1-3. (cont.)

<table>
<thead>
<tr>
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<th>Timepoint in weeks before first Week 1 class, by incubation temperature</th>
<th>Steps and notes (i)(ii)</th>
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<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>20 - 22°C (“room”)</td>
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</tbody>
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Informative Part(s) of S1

| F |

Table S1-3 Footnotes:

(i) Information in this column applies to our typical class configuration: 65-120 students, split into 4-5 different lab sections, 12-24 students per section, each section meeting once a week at a separate time from the other sections, and each section using a different WT strain in their Crosses A1 and B1. Adjust numbers proportionately if you have a different class size or configuration. Table S1-4, and later Part F (F5-A, -E), discuss ways fly prep could be lightened while still giving students a good experience.
(ii) We refer to fly stocks and crosses by their general “types”. Stock types are: Mutant, WT, Df, and Dp. The individual stocks within each type are listed in Table S1-4. Cross types are: A1, B1, Df, and Dp. The parental genotypes and sexes we use for each cross type are described in Table S1-6.

(iii) “Expanding” a stock means to increase its number of healthy culture vials or bottles over generations, thereby increasing the number of flies available for the class. “Expansion targets” are the numbers of stock cultures we aim to have available by specific steps of the prep. Part D discusses the strategies we use to reach expansion targets efficiently.

(iv) During each of the two cross collection phases, we reliably gather more than enough flies to make all crosses after only 3-5 (preferably consecutive) days of collecting—this table blocks out much longer windows for both cross collections (2-2.5 weeks), but only to allow flexibility in scheduling the 3-5-day collection phase. See Part E for suggestions to maximize collection efficiency.

(v) F1 describes all Week 1 classroom equipment (Figure S1-7 and Table S1-7). After Week 1, prep is relatively light: some equipment and vials are swapped between subsequent weeks (F2-4), and we recommend that prep staff keep A2, B2, Df, and Dp Cross cultures healthy during Week 2 (see F2 for recommendations).
S1, Part B: Ordering stocks

All of this Lesson Plan’s fly stocks can be obtained from the Bloomington *Drosophila* Stock Center (BDSC, bdsc.indiana.edu). This part of the document describes when and how to open a BDSC account and order stocks.

B1. When to get started

If you are implementing this Lesson Plan for the first time, and especially if you are new to flies, open a BDSC account and order stocks no later than the applicable start time suggested in Table S1-3, Step 1 (generally 18-27 weeks before Week 1 of class). The reason for such an early start is to give you ample time to expand the stocks from a relatively small number of flies:

- Given that the BDSC must maintain thousands of different stocks, they cannot store excessive copies of any one stock. Therefore, the BDSC will only send you one vial of each stock that you order (i.e. they cannot supply a vial for each of your students).

- Vials received from the BDSC will not contain large numbers of flies, and sometimes the adults will not even survive the shipping process, although their progeny will almost always survive. Our suggested timeline provides a cushion for the rare case when a stock does not survive shipment and needs to be reordered.

In spite of these caveats, the fly prep is readily manageable, especially with the extra time cushion that we suggest. To help ensure a smooth prep, Table S1-3 (see Part A) lays out a full timeline, and Part D provides tips to efficiently expand the number of flies in your stocks.

If you wish to continue offering this Lesson Plan in future terms, there is a way to buy prep staff a lot of time and flexibility the next time around: Consider maintaining the fly stocks at your facility between terms, especially the WT and Mutant stocks because of their relatively high expansion targets (see Table S1-3, Step 14 for a suggested maintenance procedure). If a particular stock is lost, it can always be reordered from the BDSC.

B2. Opening a new BDSC account

You will need to open a BDSC account before ordering stocks for the first time. Read through the Ordering & Accounts section on the BDSC website for information about how to open an account and the available account options; the Stock Center provides several different types of accounts to accommodate your institution’s purchasing process.

While setting up your account, you will need to make a decision about your shipping service. You may choose to have your stocks sent by USPS, UPS, or by Fed Ex. (If you wish, you can change your account’s shipping service in the future by contacting the BDSC.) We have always opted for USPS shipping, which is cheaper but usually somewhat slower than UPS and Fed Ex, perhaps by as much as a few business days. USPS shipping cost is included in the BDSC invoice. UPS and Fed Ex shipping costs, on the other hand, are billed separate from the invoice to a UPS or Fed Ex account, so make sure your department has a UPS or Fed Ex account number before selecting either of these shipping options.
Initial setup of a BDSC account will take 1-2 business days, after which you will receive a Bloomington User Number ("BUN") that you will use when placing orders. After receiving a BUN, you may order stocks immediately and at any time in the future.

**B3. Ordering stocks**

Table S1-4 (below) lists the stocks that we used for this Lesson Plan. A BDSC webpage provides this same information in a format that should facilitate your ordering process ([https://bdsc.indiana.edu/stocks/misc/maplab1.html](https://bdsc.indiana.edu/stocks/misc/maplab1.html)). The Ordering & Accounts menu on the BDSC website contains links to the Order Form, as well as a description of pricing per stock ordered. If you are limited by budget, prep logistics, facilities, or a small class size, note that it is not essential to order all of the stocks in Table S1-4 because: (1) only about a third of the stocks are necessary to map cw to a small interval and then definitively to a single gene with your own data (see Table S1-4 footnotes for this “short list” of stocks), and (2) if you choose not to order some stocks, you could use Supplementary File S8 (Sample final dataset) to fill in the missing data.

**Table S1-4. Fly stocks to order for this Lesson Plan**

<table>
<thead>
<tr>
<th>Stock type</th>
<th>Genotype</th>
<th>Bloomington stock number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutant</strong></td>
<td><em>w</em> IP3K2&lt;sup&gt;vy-2&lt;/sup&gt; g&lt;sup&gt;2&lt;/sup&gt; f (&quot;w cw f&quot; in lab manual) (i)(ii)</td>
<td>84699</td>
</tr>
<tr>
<td><em><em>WT [P(w</em>) insertion strains]</em>* (iii)</td>
<td><em>w</em> P(w*)CG4004&lt;sup&gt;BG00856&lt;/sup&gt;</td>
<td>12442</td>
</tr>
<tr>
<td></td>
<td><em>w</em> P(w*)Brms&lt;sup&gt;TSG01329&lt;/sup&gt;</td>
<td>12470</td>
</tr>
<tr>
<td></td>
<td><em>w</em> P(w*)CG2540&lt;sup&gt;ST7715&lt;/sup&gt;</td>
<td>33328</td>
</tr>
<tr>
<td></td>
<td><em>w</em> P(w*)Tango&lt;sup&gt;G517&lt;/sup&gt;</td>
<td>32580</td>
</tr>
<tr>
<td></td>
<td><em>w</em> P(w*)Nup93-T&lt;sup&gt;G9998&lt;/sup&gt;</td>
<td>32628</td>
</tr>
<tr>
<td><strong>Deficiencies (&quot;Df&quot;)</strong></td>
<td>Df(1)Exel6245</td>
<td>7718 (iv)</td>
</tr>
<tr>
<td></td>
<td>Df(1)ED7170</td>
<td>8898</td>
</tr>
<tr>
<td></td>
<td>Df(1)ED7217</td>
<td>8952</td>
</tr>
<tr>
<td></td>
<td>Df(1)ED7165</td>
<td>9058</td>
</tr>
<tr>
<td></td>
<td>Df(1)BSC713</td>
<td>26565</td>
</tr>
<tr>
<td></td>
<td>Df(1)BSC766</td>
<td>26863</td>
</tr>
<tr>
<td></td>
<td>Df(1)BSC767</td>
<td>26864</td>
</tr>
<tr>
<td><strong>Duplications (&quot;Dp&quot;)</strong></td>
<td>Dp(1;3)DC263</td>
<td>30380</td>
</tr>
<tr>
<td></td>
<td>Dp(1;3)DC264</td>
<td>30381</td>
</tr>
<tr>
<td></td>
<td>Dp(1;3)DC265</td>
<td>30382</td>
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<tr>
<td></td>
<td>Dp(1;3)DC266</td>
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<td></td>
<td>Dp(1;3)DC267</td>
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<tr>
<td></td>
<td>Dp(1;3)DC273</td>
<td>30390</td>
</tr>
</tbody>
</table>

**Table S1-4 Footnotes:**

(i) Bloomington stock #84699 carries the IP3K2<sup>vy-2</sup> mutation that students will map. To simulate a primary research experience for the students, the lab manual (Supporting File S2) designates
B4. Payment for stocks, shipping times and seasonal considerations

Read through the BDSC website Ordering & Accounts menu for their current order processing and shipping schedules. At the time of this publication, the BDSC registers orders every Monday and Thursday around noon. After orders are registered, if you have a “Multi” account, stocks are prepaid and so processing is immediate. If you have a “Single” account, you will receive a price quote by email, and the order will be processed as soon as you make the payment, so pay promptly for prompt shipment. Stocks are generally mailed within 4-5 business days after payment. Shipping time will vary depending on your distance from the BDSC and whether you chose USPS, UPS, or Fed Ex as your shipping service. Table S1-3 of this document estimates up to 2 weeks from order to shipment arrival, which reflects our experience with a Single account, using USPS for domestic shipment. Table 1 of the main Lesson Plan document expands the range of expected shipment times to consider a broader array of institution locations and account types, but regardless of variations in shipping time, our prep timelines in Table 1 of the Lesson Plan and Table S1-3 of this document should provide more than enough time to complete your prep.

The Stock Center does an excellent job of sending healthy stocks throughout the year, so order stocks when needed. However, if you work in a region with particularly hot or cold seasons, consider timing your order to arrive before temperatures are at their most extreme to increase fly survivorship. For example, at our New England institution, we usually order stocks in late May/early June for an early October class, and generally avoid ordering stocks in January or July (although we have received healthy stocks during these months many times). If you are concerned about extreme


\[ IP3K2^{wy-2} \] as “cw” (crumpled wing)—a pseudonym not found in the literature—and the stock number is not given.

(ii) \( g^{2} \) (garnet) is a mutation that causes eyes to have a dull, brick-red color as opposed to the brighter red of wild type eyes. garnet is not discernible in the presence of the \( w \) mutation, and it only dulls \( P(w^{+}) \) eye colors slightly, which would not be feasible for students to score. Finally, scoring garnet would not increase the resolution of the three-point mapping experiment. Therefore, we chose to ignore garnet in this Lesson Plan.

(iii) We have always ordered at least four of these “WT” stocks. The first three of the five \( P \)- insertions we list map to the left of \( IP3K2 \) (“cw”) (12442, 12470, and 33328), and the last two map to its right (32580 and 32628). This in mind, if you want your students to map \( cw \) to a small chromosomal interval with completely their own three-point cross data, order at least one of the first three and one of the last two WT stocks listed, thereby flanking \( cw \). We also recommend that you order at least one more WT stock (and so at least 3 total). This will give students the opportunity to assess two different types of chromosomal intervals: one in which \( cw \) is located and at least one where it is not located.

(iv) For each offering of the class, we typically prep about 10-12 Df and Dp stocks combined. If you want your students to map \( cw \) to a single gene with their own complementation data, order at least the three stocks that are marked with this footnote (7718, 30384, and 30385) to cross to the Mutant stock. The reason for this is that \( IP3K2 \) is the only gene within the overlap between the associated deficiency and duplications, providing very strong evidence that \( IP3K2 \) is the \( cw \) locus [see Lesson Plan and Dean et al., 2015, G3 (Bethesda) 6(2): 299-310]. In addition, we recommend ordering at least one of the remaining Dp stocks and at least one of the complementing Df stocks (8898, 8952, 9058). This way, students will be able to use their own data to interpret complementation and noncomplementation from both Df and Dp crosses, providing a thorough learning experience. See Supporting File S8 (Sample final dataset) for a full set of expected results.
weather, package insulation can be requested free of charge if you contact the BDSC before your order is shipped. Also, you could reduce shipping time by upgrading your account’s shipping service to UPS or Fed Ex.
S1, Part C: *Fly equipment, handling, and diet*

After you receive flies from the BDSC, you will need to handle and culture the stocks in order to expand and eventually cross them. This section recommends prep equipment (C1), directs beginner staff to internal and external resources to help them learn how to handle *Drosophila* (C2), and describes fly diet options (C3).

**C1. Fly equipment**

To handle flies during the prep, we use the following supplies:

- **A single lab station with the fly-handling hardware described in F1** (*i.e.* one lab station with the hardware students are given during Week 1 of class). Table S1-7 in F1 lists manufacturers and specs for most equipment. In addition, the BDSC provides an extensive list of standard fly lab suppliers ([https://bdsc.indiana.edu/information/supplies.html](https://bdsc.indiana.edu/information/supplies.html)).

- Enough clean **fly vials, bottles, and plugs** for all of your stocks, collections, and crosses. (The BDSC link immediately above lists suppliers of these items as well.) Table S1-3 and D2 discuss how many vials and bottles we use at each major prep stage. We use glass vials and bottles, and autoclavable plugs, all of which we clean and reuse throughout the prep, and so for our class sizes of 65-120 students, a total supply of about 600 vials/vial plugs and 200 bottles/bottle plugs gave us a healthy cushion during peak prep season (Table S1-3, Steps 3-13). If you prefer to use disposable containers and plugs, you will need to order additional supplies to replenish those that you used earlier during the prep, so monitor your usage carefully to prepare for peak prep season.
  - Old cultures in disposable containers are simply frozen and then discarded. Old cultures in glass or autoclavable plastic containers are frozen, partially filled with water, autoclaved, brushed out in a sink equipped with a garbage disposal unit, sent through a dishwasher cycle, reautoclaved, and reused. Autoclavable plugs are autoclaved and reused (usually no washing necessary).

- **Diet ingredients** and any lab hardware needed to make the diet (see C3 for options).

**C2. Fly handling**

This section lists resources for those who have not worked with *Drosophila* before. If you are new to flies, we suggest the following reconnaissance before starting the prep:

- Read through Parts D and E of this document; these Parts provide detailed instructions and supporting images to help you keep cultures healthy, collect males and virgin females for crosses, recognize wild type vs. mutant traits, etc.

- Watch the fly handling demo video at the end of the Supporting File S5 slideshow.

- If possible, visit a fly research lab for at least a half day to: take stock of their equipment and procedures, make fly diet, learn to distinguish males from females and virgins from non-virgins, and help set up crosses.
• There are many excellent outside references for learning how to work with *Drosophila* in general. These include:
  
  o The BDSC website: Care & Information menu > Getting Started submenu (https://bdsc.indiana.edu/information/get-started.html).
  
  
  
  o Celeste Berg’s lab at the University of Washington provides a concise fly tutorial page with helpful images: http://depts.washington.edu/cberglab/wordpress/outreach/an-introduction-to-fruit-flies/

**C3. Fly diet**

Most of the stocks used in this Lesson are robust, and therefore should thrive on a variety of standard diets; the deficiency (Df) strains are the only exception to this, requiring some extra attentiveness (see below and Part D for suggestions). If you are experienced with flies and have a preferred diet formulation, by all means try it out at the start of the prep, and it will probably work fine for you. We have tested three diet options with particular care and compare and contrast them below (C3-A, -B, and -C). Also see Table S1-5 at the end of this Part for a comparative summary.

**C3-A. Yeast-cornmeal diet**

We used a modified version of standard “yeast-cornmeal” diet for most of our preps because it is what our lab uses for research [Dean et al., 2015, G3 (Bethesda) 6(2): 299-310] and, of all the diets we tried, stock health and fecundity were highest with this formulation. However, making this diet is somewhat more labor-intensive than the other two options that we list, and to generate a high number of progeny from cultures, we recommend using it within 2 days after making (see Steps 9-10 below). Labor and moderate perishability aside, this diet is affordable to make (about $7/L).

For every 100 ml water heated at the beginning, this recipe will make enough diet for approximately 12-14 vials or 2.5-3 bottles:

1) Turn on a hot plate equipped with a magnetic stirrer to a setting that will maintain the diet just at or near-boiling.

2) Add the desired volume of water (we use deionized) and a magnetic stir bar to a beaker. Heat the water to a low boil (we use a microwave for initial heating because it elevates temperature faster than the hot plate would). Stir water just at- or near-boiling on the hot plate.

3) For every 100 ml of water heated, add the following ingredients sequentially and slowly, with vigorous stirring to minimize clumping (especially with the agar):

   - 11 g dextrose (Fisher Scientific, #S25295B)
   - 1.5 g agar (Fisher Scientific, #S70213A)

Dextrose should dissolve easily, and then agar more slowly. Before proceeding, fully dissolve agar. To do so, you may need to increase heat slightly, increase stirring speed,
and/or microwave briefly. Once agar is dissolved (the solution will clarify but have a beige tint), add the remaining ingredients:

- 5 g Baker’s yeast (Red Star)—stir for several minutes before adding cornmeal
- 5.2 g commercial cornmeal (we use organic from Farmer Ground Flour, LLC, Trumansburg, NY)

Neither yeast nor cornmeal will fully dissolve, but thoroughly disperse these ingredients while maintaining the mixture at- or near-boiling (if necessary, briefly microwave to reheat).

(We store dextrose and agar at room temperature, and yeast and cornmeal are refrigerated until use.)

4) Take beaker off of heat. Pour diet into a tilt head electric food mixer bowl and mix at low- to medium speed. (If no mixer is available, manual stirring of diet with the beaker submerged in a cool water bath also works fine.) Cool diet down to 60°C while lining up empty vials or bottles to dispense diet into.

5) Once cooled below 60°C, continue to mix diet and, for every 100 ml of water that was originally heated, add the following preservatives:

- 750 µl of propionic acid (Sigma, #P1386), and
- 500 µl of 15% p-hydroxybenzoic acid methyl ester (also known as methyl paraben or “Tegosept”, Genesee Scientific, #20-258), dissolved in 95% ethanol.

(Propionic acid and dissolved methyl paraben can be stored indefinitely at room temperature before use.)

6) Pouring through a powder funnel that has a short, wide stem (e.g. Fisher Scientific #10-348A), dispense diet into vials or bottles to a depth of about 3-4 cm per container. Pour fairly quickly to minimize diet congealing before it is fully dispensed (this diet starts to become viscous when it cools below about 45°C).

7) Place the diet containers uncapped in a room free of stray flies (e.g. an office or lab that does not store Drosophila)—although uncapped, we sometimes cover the food containers with a cheesecloth for added protection. Let sit 1-3 hours to congeal diet and evaporate the condensation on the insides of the containers.

8) Add a pinch-sized portion of Baker’s yeast to each container (we use a lab spatula to drop the yeast in), then cap/plug containers.

9) Use yeast-cornmeal diet on the same day it was made or wrap the rack of containers in a plastic bag and refrigerate. This diet dries somewhat after 2 days of storage, so use within two days if expanding stocks (Part D) or setting up crosses (Part E), both of which require particularly healthy, hydrated cultures. But keep unused diet vials for at least two weeks, because older diet works fine to store adults during cross collections (Table S1-3, Steps 4 and 8), as well as to provide the Parental vials you will give students on Weeks 1 and 3 of class (Table S1-3, Step 12).

10) If stored in refrigerator before use, warm vials/bottles back up to near room temperature before placing flies into them. This can take 15-20 minutes, but if you are in a hurry, warm vials in your pocket (about 5 minutes)!

14/12/14
**C3-B. Formula 4-24 Instant media (Carolina Biologicals, #173200)**

This diet is somewhat more expensive than the previous option (just over $9 vs. $7/L), but convenient in that it has an indefinite shelf life in dry form and only requires a roughly equal volume of water to be prepared (no boiling or preservatives needed). However, adult fecundity was lower on Formula 4-24 than on the other diets that we have tried, and the Df stocks were relatively unhealthy on Formula 4-24 (but still survived). To help with these issues, we recommend supplementing each vial/bottle of hydrated diet with a small pinch of Baker’s yeast.

In addition, we have found that Formula 4-24 does not appear to be the most efficient “moisture buffer”: it is sensitive to desiccation and cannot absorb excessive moisture very well. Therefore, more so than with other diets, use Formula 4-24 media shortly after hydrating (no long-term storage after hydrating but before use), and check the cultures every few days for signs of drying or excessive moisture. If adjustments are needed, D4 describes tricks to adjust the moisture content of fly media.

Finally, the other two diets we discuss (see C3-A and C3-C) are agar-based and so stay relatively solid with handling, but Formula 4-24 media can sometimes fleck apart and make a small mess while students handle cultures. Monitoring moisture content can alleviate this issue. These caveats aside, the ease and convenience of Formula 4-24 media can be helpful if lab facilities, equipment, prep time, and/or staff are limited.

**C3-C. Nutri-Fly, Bloomington formulation (Genesee Scientific, #66-112)**

This diet is a commercially-available version of the diet used at BDSC, so it is well-tested over a huge array of stocks. It is reasonably priced, but the most expensive of the options that we tested (about $10/L). It is easy to make, although not as quickly made as Formula 4-24 media because you must dissolve Nutri-Fly ingredients in boiling water. After dissolving ingredients and cooling below 60°C, add propionic acid as a preservative (Sigma #P1386; see Nutri-Fly manufacturer’s instructions). Finally, as described in the yeast-cornmeal diet protocol (C3-A, Steps 6-8): pour diet into containers, congeal/dry, add pinches of yeast, and cap/plug containers.

Nutri-Fly is a nice option in that it keeps all stocks healthy, including the Df strains. Fly fecundity on Nutri-Fly is very good, but in our experience not quite as high as on the yeast-cornmeal diet. A definite advantage of Nutri-Fly media is that it keeps well after making: If you do not use it on the same day that it was made, wrap containers in a plastic bag and refrigerate, and the media should stay fresh and sufficiently hydrated for stock expansions (Part D) and crosses (Part E) for at least a week—possibly longer, according to colleagues who use this diet for their research, but we have not tested longer storage periods for this Lesson Plan. Nutri-Fly vials up to at least two weeks old, and probably older, are fine for storing adults during cross collections (Table S1-3, Steps 4 and 8) and for providing Parental flies to students in class (Table S1-3, Step 12). As with stored yeast-cornmeal diet (C3-A, Step 10), if you refrigerate vials/bottles of Nutri-Fly media, warm them back up to near-room temperature before placing flies into them (takes 20-30 minutes).

***Table S1-5. Comparative summary of diet properties***

<table>
<thead>
<tr>
<th>Diet</th>
<th>Adult fecundity</th>
<th>After making, use for cultures or crosses within...</th>
<th>After making, use for collections within...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast-cornmeal (C3-A)</td>
<td>very high</td>
<td>2 days</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Formula 4-24 (C3-B)</td>
<td>low/medium</td>
<td>immediately</td>
<td>immediately</td>
</tr>
<tr>
<td>Nutri-Fly (C3-C)</td>
<td>high</td>
<td>1 week</td>
<td>2 weeks</td>
</tr>
</tbody>
</table>

* Drawbacks aside, Formula 4-24 is convenient to make if small batches and few progeny are needed.
**S1, Part D: Maintaining and expanding fly stocks**

Prepping the flies for this Lesson Plan requires some advance planning. As explained in Part B, the BDSC can only send you one vial for each stock that you order, from which you will need to produce enough stocks and crosses for your entire class—Table S1-3 fully describes how many stocks and crosses to produce and when they will be needed. This is a significant effort, but quite manageable, even with a small prep staff: for example, our class sizes range between 65 and 120, and we have managed fine with one lab instructor prepping all diet and flies for this Lesson Plan and two technicians administering the other lab supplies while they prep another large class.

Two measures are key for a successful fly prep:

- Especially if flies or this module are new to you, **start prep early, 18 weeks or more before Week 1 of class** as recommended in Table S1-3 of this document. This will give you a comfortable margin for error as you develop your own prep logistics.

- Also, your prep will be much more efficient and flexible if you can **keep fly cultures healthy and reproductively active**. This part of the document recommends strategies to do so.

While maintaining, and especially while expanding stocks, we consider the following seven factors (D1-D7):

**D1. Diet selection**

Expanding stocks at a high rate will be the primary concern during your prep, and so during this time period, we suggest using yeast-rich diets that produce relatively large numbers of progeny, such as the yeast-cornmeal or Nutri-Fly diets that are described in Part C of this document (C3-A and C3-C). Most other standard diets should suffice, but we do not recommend using Formula 4-24 diet (C3-B) for stock expansions or crosses, because in our experience, it does not produce a large number of progeny. (However, Formula 4-24 it is very convenient to make in small batches, and so you might choose to try it out while maintaining stocks between terms.)

**D2. Vials vs. bottles**

As a general rule of thumb, we have found that a healthy stock can be established with 20-30 flies in a vial or 40-80 flies in a bottle. Using these guidelines and yeast-rich media, for example yeast-cornmeal (C3-A) or Nutri-fly (C3-C), **progeny from 1 stock vial can be used to set up at least 2-4 fresh stock vials or 1-2 bottles, and progeny from 1 stock bottle can be used to set up at least 2-4 fresh stock bottles or 4-8 vials.** At times, we have further enhanced these expansion rates by attending to factors described in D3-6 below.

Whether to prep with bottles or vials is largely a matter of preference and stock health. Vials are easy because you do not need a lot of flies to establish a healthy stock or cross within them, but if you can establish healthy cultures in bottles, they will yield a lot more progeny per unit, thereby accelerating your prep. **In our prep, we use bottles only to expand the WT and Mutant strains; all other cultures, and all crosses, are kept in vials:**

- Expand only the WT and Mutant stocks in **bottles** (stocks listed in Part B, Table S1-4).
• Expand the Df and Dp stocks in vials (these stocks also listed in Part B, Table S1-4).
• Set up the A1, B1, Df, and Dp crosses in vials (crosses described in Tables S1-3 and S1-6).
• On Week 1 of class, supply students with fresh vials of diet to set up one Cross A2 or B2 each. (We also recommend that staff set up some extra Cross A2 and B2 vials—see F2.)

All of these choices were deliberate: First, more flies are needed from the WT and Mutant stocks than from any other stocks, and these stocks are robust enough to easily thrive in bottles. On the other hand, the Df stocks are the least fecund and healthy of the strains used in this Lesson Plan, so we recommend keeping Df stocks and Df Crosses in vials only. Finally, Dp stocks, Dp Crosses, and Crosses A1 and B1 would progress nicely in bottles, but vials should produce more than enough flies for a wide range of class sizes, especially if our recommendations are followed (Table S1-3, Steps 6 and 9).

**D3. Incubation temperature**

As A1 and Table S1-2 discuss in detail, flies can be reared from 18-29\(^\circ\)C. Generation time is much faster at higher temperatures, but higher temperatures can also stress cultures, reducing viability and fecundity, so there is a trade-off. Moderate incubation temperatures (20-25\(^\circ\)C) thread the needle nicely between a reasonably short generation time and good culture health for a broad range of stocks (A1 and Table S1-3). For best results, be attentive to stock health, modulating incubation temperature back and forth within the 20-25\(^\circ\)C range as needed. When stocks first arrived from the BDSC, we left them out at “room” temperature (20-22\(^\circ\)C) for a few days to help them gain a foothold under low stress. From then on, we examined stocks once or more per generation to see if there were large numbers of eggs, larvae, and/or pupae (Figure S1-1, below). If a stock looked healthy, we incubated it at 25\(^\circ\)C or even more to obtain the next generation more quickly. If a stock looked unhealthy, we left it at 20-22\(^\circ\)C for a generation to help it recover, if necessary adjusting diet moisture as described in D4 below. Once a stock had a large number of healthy progeny, it was much less risky to transfer it to a higher temperature.

![Figure S1-1](image)

**Figure S1-1.** Examples of “healthy” fly cultures (i.e. stocks producing numerous healthy progeny) at various stages of development. (A) Surface of a small portion of diet from a healthy, 1-2-day old culture. Fecund adults have covered the food with eggs. Each egg is approximately 0.5 mm in length. At 25\(^\circ\)C, embryos hatch approximately 24 hours after egg laying. (B) Larvae in a somewhat older, healthy culture. If present, a large number of healthy larvae will “churn” the top of the diet 4-7 days after the stock was set up (timing varies with incubation temperature), giving the top of the food a custard-like appearance that is slightly darker and more homogeneous than the diet in lower layer. Many larvae might be seen, burrowing in the churned, top layer (example circled). Unhealthy stocks will not show significant larval churning at the top of the diet, and diet will often appear dry and flaky—see D4 and Figure S1-2 for troubleshooting suggestions. (C) Pupae from yet an older, healthy culture. Numerous pupae line the sides of the container above the food 7-14 days after a stock is established (again, timing varies with incubation temperature).
If you wish to implement this Lesson more than once, consider maintaining 2-3 vials or bottles of each stock at room temperature or lower until it is time to prep the next offering of your class (Table S1-3, Step 14). This will give you a nice head-start, and the low temperature will place little stress on the stocks, while also lowering the frequency at which you need to make fresh stocks between terms. If certain stocks die out or need replenishing, you can always reorder them from the BDSC.

**D4. Diet moisture content**

One of the best ways to maintain healthy fly stocks is to keep the diet sufficiently but not excessively hydrated. If the diet is too dry, the stock will be unhealthy, and if the diet is too wet and soupy, adults will become stuck and otherwise not be able to reproduce. Below, Figure S1-2 displays the spectrum and describes how to troubleshoot.

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**Figure S1-2.** Monitoring and adjusting moisture content of diet. Around 4-8 days after setting up a fresh stock or cross, check your culture vials/bottles for moisture content. **(A)** Diet that is too dry will be flaky at the top, crack throughout, and shrink from the sides of the container. To rehydrate this diet, draw water into a micropipettor tip or Pasteur pipette (we use deionized), bang culture vial/bottle on a mouse pad to knock adults down from the cap, quickly open cap and dispense water dropwise (usually 2-6 drops) until top of diet has a more smooth, custard-like consistency—moist as shown in **(B)**, but not sweating fluid or trapping an excessive number of adults like in **(C)**—and then reseal container before adults escape. **(B)** Optimal, custard-like moisture content for a larval culture. **(C)** If diet is too wet, it will be soupy and sweat clear fluid on its top. Moisture will spread up the side of the container. Adult flies can become immobilized in this environment and otherwise not be able to mate and lay many eggs. To remedy this situation, gently tap the culture on a mouse pad to knock the adults down from the top of the container, then working quickly: remove the culture cap, stuff an appropriately-sized piece of dry paper towel or Kimwipe into the container to mop up the excess fluid and give adult flies a safe perch, then reseal container. Another option is to add a pinch of dry Formula 4-24 media (C3-B) on top of the wet diet to absorb the excess moisture. In many cases, moist cultures may contain a high density of burrowing larvae. If this is the case (in fact, **B** and **C** both show examples of high-density cultures), you could increase stock expansion by transferring some of the larvae to a fresh culture vial/bottle as described in D6.
D5. Ambient humidity

If you are keeping stocks in an incubator, we recommend that you improvise a simple humidifier for the incubator chamber: Obtain a small casserole dish or food storage container (sandwich size should suffice). Nearly fill the container with water (we use deionized) and place container into the incubator. Use a short piece of paper towel to create a moisture wick: insert one end of the paper towel into the container’s water, and dangle the other end of the paper towel over the edge of the container, outside of the water. Monitor the water levels of the container every few days and refill as necessary. To avoid buildup of mold or mildew, wash the container and replace the water and wick about once a week.

This type of humidifier works nicely for a wide range of temperatures and incubator sizes. Rooms, however, are often too large to humidify in this way. Outside of an incubator (20-22ºC in the rooms of our building), we have kept stocks healthy without air humidification by adjusting diet moisture as described in D4 and in Figure S1-2. If you store your stocks in a small, enclosed room and your building interior is particularly warm or dry, you may wish to experiment with an electric room humidifier.

D6. Transfer flies and larvae to fresh diet regularly

If you have a culture that is thriving, you can greatly amplify its expansion by transferring animals to fresh diet frequently:

- **Make fresh stocks regularly.** If you have a relatively high number of adults in a stock, (say, >25 in a vial or >60 flies in a bottle), and they are laying many eggs, transfer these adults to fresh media every 4-6 days a couple of times.

- If they are healthy, **maintain the previous generations’ stock containers** after you have made fresh stocks from them. Keep these older stocks at room temperature to extend their durability. If additional adults emerge from the older stocks, use them to help make more new stocks or to supplement an existing, younger stock.

- **Transfer excess larvae to fresh diet.** If a culture is crowded with larvae (examples shown in Figure S1-1B and Figure S1-2B,C), scoop some excess larvae out of the top of the diet with a lab weighing spatula and smear them on top of fresh diet in other vials or bottles, then continue to incubate both the older and newer cultures. We have found that this will dramatically increase generational yield, perhaps due to decreased crowding and increased food availability for the larvae. (Of course, clean off spatula between transfers if transferring larvae from multiple different stocks.)

D7. Stock care: minimizing cross-contamination between stocks, controlling fungus/bacteria/mites

D7-A. Minimizing cross-contamination between stocks

Occasionally, flies from one stock will stray into another. An easy way to catch most cross-contaminations is to check the eye color/shape of the flies in each of your stocks: in this Lesson Plan, all Mutant flies should have white eyes; all WT and Dp flies should have yellow, orange, or red eyes; and all Df flies should have Bar eyes (Figure S1-5). Beyond checking eye color/shape and then the cv and f phenotypes under a scope regularly, discarding cross-contaminated vials/bottles,
and keeping all stocks tightly capped, there are two simple measures that minimize cross-contamination:

- **Do not mix stock clones.** Unless absolutely necessary, avoid mixing flies from different vials or bottles (i.e., different “clones”) of the same stock. For example, if you have two vials of a particular stock, do not mix the flies from these two vials before transferring them to fresh diet, but rather transfer the flies from one stock vial into fresh vials, then separately, transfer the flies from the other stock clone into different fresh vials. This precaution will contain cross-contamination within a single clone of a stock. If stock vials or bottles are found to be cross-contaminated with flies from another stock, discard them and use the non-contaminated backups for your prep.

- **Keep fly handling equipment clean.** After handling flies on the CO₂ pad, we often douse a paper towel with 70-95% ethanol and wipe down the pad, funnel, and brush before handling another culture. We also wash brushes and funnels if they become soiled with food, debris, etc.. These precautions remove residual larvae and eggs from the previous collection, minimizing cross-contamination.

**D7-B. Controlling fungus and bacteria**

Mold and bacterial infections can reduce the fitness of a fly culture, but we have found such infections to be very manageable:

- Follow the straightforward precautions described in D7-A: Do not mix flies from an infected container with flies from a clean container before making a fresh culture (i.e. keep clones of a stock separate). Be sure to clean fly handling equipment with 70-95% ethanol after handling a moldy or slimy stock.

- **Frequent “flipping” of stocks** (i.e. transfer of flies to fresh media) helps to keep them clean.

- **Discard badly infected vials or bottles** unless you have no backups and need to salvage them.

- **Flip infected stocks “upside down”**. If you must salvage an infected culture, transfer adults only and no infected debris into the fresh culture—try the “upside down” flipping technique that we describe in D7-C.

- **Make sure diet, containers, and caps are not infected.** We tend to see fungal/bacterial infections within individual stocks as opposed to throughout our lab. If you see a more widespread infection, it may be a problem with your fly diet, containers, or caps. Most diet recipes require heating to dissolve agar, and so while mixing diet, make sure that it is heated to near-boiling after adding each ingredient (except for the preservatives at the end). Our favored diet formulation (C3-A) contains propionic acid and methyl paraben, two standard preservatives that have helped keep most infections at bay; make sure molten diet has cooled below 60°C before adding these preservatives. Make sure vials, bottles and caps are clean before using (all of ours are autoclavable).

- The **BDSC Care & Information menu > Fly Culture submenu** (https://bdsc.indiana.edu/information/fly-culture.html) has other helpful suggestions for controlling fungal and bacterial infections.
D7-C. Controlling mites

Mites can also infest stocks and negatively affect stock health. They are a more serious but less frequent threat to cultures than most mold or bacteria. (Twice over the past 16 years we have seen mites in some of our stocks, but successfully controlled both infestations.) Multiple mite species can infest fly stocks, but we believe both of our infestations were by the common species *Glycyphagus domesticus*, or perhaps one of its close relatives (Figure S1-3).

**Figure S1-3.** *Drosophila* culture with a mite infestation. (A) Adult mites (solid circle) and their eggs (dotted circle) can be difficult to see because they tend to cluster in crevices between pupal cases and around debris. (B) A clearer view of mite eggs between pupal cases to give a sense of scale (pupal cases are 3-4 mm long) (C) Adult mites (circle), we think of the common species *Glycyphagus domesticus*.

Mites disperse between cultures more insidiously than mold or bacteria, but mites are also a preventable or at least manageable problem if you take sensible precautions:

- **Discard infested cultures** unless you absolutely need to salvage them (salvage advice given at later points in this list). If you see mites or their eggs in a vial or bottle (both commonly are clustered around pupal cases), remove culture immediately and either autoclave it right away or freeze and then autoclave (the mites we encountered did not survive freezing). Check all your other stocks for mites and remove/freeze/autoclave as needed. Pour 70-95% ethanol on the counter area where the infested cultures were stored and wipe down with a paper towel. Wash vial/bottle racks that contacted infested containers (or even bake or autoclave racks if they are made of metal or glass). Wash your hands after handling infested cultures.

- **Check and quarantine newly-arrived stocks.** Mites tend to arrive within a stock that was shipped from elsewhere. Even if apparently mite-free, it is prudent to keep a newly-arrived stock at a separate shelf or room for 1-2 generations. After the quarantine, check for mites again before storing the new stock with established stocks.

- **Do not bring pets into the lab,** because they can carry mites. (For example, dogs can carry the mite species that we believe infested our fly stocks.)
• All of our suggested measures to prevent fly stock cross-contamination (D7-A) apply to controlling mite infestation: If possible, do not mix clones of the same stock when making fresh cultures, regularly wipe off equipment and work area with a paper towel soaked in 70-95% ethanol, and wash funnels and brushes.

• Produce fresh lab stocks regularly and discard especially old stocks, even if you do not notice mites. If you have mites, flip stocks shortly after the next generation of flies emerges—many mite species have a somewhat longer life cycle than that of the fly, especially at room temperature or lower, and so regular flipping could give flies the advantage. Discard older cultures unless they are still producing healthy flies that you need for the prep (see Table S1-2 for suggested timing).

• Especially during prep season, we often keep two complete sets of Mutant, WT, Df, and Dp stocks at separate locations, i.e. different shelves or incubators, perhaps in different rooms. We usually split up our Crosses in this way as well. This precaution can also be useful in the unlikely event of an incubator malfunction.

• Clean out mite-infested incubators right away. Remove stocks from incubator and discard all infested stocks—or indeed all stocks that were in the incubator if you do not need to salvage any. If you must keep the stocks that do not show mites, wipe the outside of their stock containers with a paper towel+70-95% ethanol and quarantine them outside of the incubator, away from your other stocks. Wipe incubator with paper towel+70-95% ethanol. If incubator shelves are removable, wash shelves thoroughly. Turn up the incubator to 40-45°C for a couple of days to help kill off leftover mites.

• A “moat” of soapy water seems to make a good mite barrier. During a mite infestation, we stood some mite-free cultures in a pan filled with water with dish soap added to break water surface tension. While a quirky measure, we have never had mites make the swim (Tim Lebestky, personal communication).

• Make sure your supply of vials, bottles, and caps are clean (ours are autoclavable). Some companies sell caps that can be effective mite barriers (e.g. Genesee Scientific, #49-102).

• If you do not have mite-free copies of a stock and absolutely need to salvage it: Anesthetize adult flies and check their bodies for mites or mite eggs. Establish multiple fresh copies of the stock from separate groups of mite-free adults, discard unused flies in a fly morgue, then clean off CO2 pad, counter, and brush with paper towel+70-95% ethanol. Check newer cultures for mites after a generation and keep those that are mite-free.

• Salvage with frequent and/or “upside down” flipping. Rather than inspect flies before salvaging, you might prefer to minimize handling of a mite-infested culture: flip infested stocks every couple of days for several times, then keep the last culture. As mentioned above, mites and their eggs tend to be clustered around Drosophila pupae, but we have less frequently found them on adult flies. This in mind, we have found it effective to transfer stocks “upside down”, with the older culture on the bottom and the fresh diet container inverted on top: With older culture right side up, bang the adults down, remove the culture cap, also remove a cap from a vial/bottle of fresh diet, and quickly invert the fresh vial/bottle on top of the infested culture, forming a tight seal between their openings. Many adult flies will walk up into the fresh diet, but no pupal cases, fly carcasses, or old diet—all of which are more likely to carry mites—will be transferred. After enough adults have moved into the fresh culture, bang the old culture again while keeping the new culture...
upside down, quickly recap both containers to prevent escapees, and freeze/autoclave older culture immediately thereafter.

- The BDSC Care & Information menu > Fly Culture submenu (https://bdsc.indiana.edu/information/fly-culture.html) and the University of Cambridge Department of Genetics Fly Facility (Working in the facility > Mites and how to control them, https://www.flyfacility.gen.cam.ac.uk/Flylab/mites) also give helpful mite control advice. The latter site even has an embedded video showing what is probably the same species that we observed (https://www.youtube.com/watch?v=PfSAAsrVANc#action=share). For a thorough discussion of mite biology and control, see Ashburner, M., Golic, K.G., Hawley, R.S. (2005). Drosophila: a laboratory handbook. Second edition. Cold Spring Harbor Laboratory Press. All three of these references agree with many of our recommendations, but some also suggest harsh chemical treatments to eradicate mites. We have not found these treatments to be desirable or necessary, but they can be kept in mind as a last resort.

- If the Lesson Plan is underway during a mite infestation, recall that Supporting File S8 is a complete backup dataset, so if a particular set of stocks or crosses becomes heavily infested, they could be discarded to help contain the problem, and S8 could be swapped in to cover any loss of data.
S1, Part E: Setting up the prep crosses

In this Part, we: (E1) describe the crosses that prep staff set up, (E2) recommend diet to use for collections and crosses, (E3) discuss *Drosophila* biology one should know to efficiently collect flies for crosses, (E4) describe how to collect and store flies for a cross (i.e. when to collect during the day, distinguishing sexes, and distinguishing virgins from non-virgins), and (E5) describe how to set up the actual crosses.

E1. Crosses that prep staff will set up

Over the course of the prep, staff will set up 4 types of crosses for the students: Crosses A1 and B1 for three-point mapping, and the Deficiency (Df), and Duplication (Dp) Crosses for complementation mapping. Table S1-6 (below) describes the parental stocks for each cross by their general categories, and the table’s footnotes (i)-(iii) refer you to more detailed information elsewhere in this document: the individual stocks to order within each parental stock category, how to collect the flies for the crosses, and when and how to set up each cross.

Table S1-6. Sexes and stocks of flies to collect for each type of prep cross.

<table>
<thead>
<tr>
<th>Cross type</th>
<th>Table S1-3 Step # to set up cross (ii)</th>
<th>Virgin females (iii)</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>6</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>B1</td>
<td>6</td>
<td>Mutant</td>
<td>WT</td>
</tr>
<tr>
<td>Df</td>
<td>9</td>
<td>Df</td>
<td>Mutant</td>
</tr>
<tr>
<td>Dp</td>
<td>9</td>
<td>Mutant</td>
<td>Dp</td>
</tr>
</tbody>
</table>

Table S1-6 Footnotes:

(i) This table groups stocks in terms of their broader categories: “WT”, “Mutant”, “Df”, and “Dp”. Table S1-4 of this document describes the individual stocks within each of these categories, and also designates the minimal list of stocks to prep that would enable your students to map *cw* to a single gene (and therefore designates which stocks to prioritize).

(ii) Ultimately, adult F1s from Crosses A1 and B1 will need to have emerged by Week 1 of class, and adult F1s from the Df and Dp Crosses will need to have emerged by Week 3 of class. This in mind, Steps 6 and 9 of Table S1-3 recommend timing for each prep cross according to incubation temperature and to when your classes will be held.

(iii) E3 explains why females need to be virgin for crosses, while males do not. E4 describes how to efficiently collect a large number of flies for the crosses, all while ensuring that the females are virgin.

E2. Diet for collections and crosses

As with stock expansions (D1), we recommend using a yeast-rich, agar-based diet for collections and crosses, e.g. yeast-cornmeal or Nutri-Fly, and not instant diet such as Formula 4-24 (diets described in C3). Yeast increases female fecundity; agar-based diets hold moisture longer than...
instant media; and while agar-based diets hold together, instant media can break apart, causing flies to become stuck as they are transferred into or banged out of a vial.

However, diet for collections, i.e. for storing adult sexes separately, does not need to be anywhere near as fresh as diet used for crosses, because collection vials are not used to culture larvae. For expansions and crosses, which do culture larvae, we recommend using yeast-cornmeal diet within two days (C3-A) and NutriFly within a week after making (C3-C), but for collections, we have used both diets after at least two weeks of refrigeration without problems (Table S1-5).

**E3. The essential *Drosophila* biology to consider when collecting for crosses**

- Most adult *Drosophila* emerge from their pupal cases during the morning hours (roughly 6AM-noon).
- An adult will not mate for at least 8 hours after emerging from its pupal case.
- Females can store sperm after mating. Therefore, when preparing a cross between two different genotypes, collect females that are certain to be virgins (i.e. females aged < 8 hours after adult emergence) and store them separately from males until the cross is initiated. This will ensure that all of their progeny result from the cross, and that none of their progeny result from pre-collection mating with male siblings from the same stock.
- On the other hand, males do not store (or lay) eggs after mating, so they need not be virgin when used for a cross.

With this biology in mind, Section E4 recommends a collection procedure.

**E4. How to collect flies for the prep crosses**

**E4-A. What to do within of few days before a collection**

As detailed in Table S1-3, staff will be preparing crosses in two phases:

- Crosses A1 and B1 (Steps 4 and 6 of Table S1-3)
  - Time the crosses so F1s emerge by Week 1 of the Lesson Plan.
  - At least one of either cross per student in your whole class (we overshoot by 20-25%).

- Df and Dp Crosses (Steps 8-9 of Table S1-3)
  - Time the crosses so F1s emerge by Week 3 of the Lesson Plan.
  - At least one of either cross per student in your largest lab section (we overshoot by 30-50%).

Within a few days of each cross collection phase, do the following prep:

1) Prepare a list of which and how many flies to collect for your crosses. Table S1-3 (Steps 6 and 9) recommends numbers of crosses to set up and numbers of flies per cross vial.

2) Make vials of diet for collections (see E2 for diet recommendations). For our classes of 65-120 students, about 40-60 vials provide more than enough diet for Step 4 or for Step 8 of Table S1-3. Use vials the same day or enclose the rack of vials in a plastic bag and refrigerate until needed. As mentioned in E2, we have used up to 2-week-old vials for storing adults during collections.
3) Set up fly collection stations for your prep staff (we have only needed one station)—F1 of this document lists standard fly station equipment (Figure S1-7 and Table S1-7).

4) If needed, review fly sexes and phenotypes with Figures S1-4 and S1-5 of this document and with Supporting File S5.

5) You will have set up stock bottles or vials to collect from (Table S1-3, Steps 3 and 5). 1-3 days before the next generation of adults starts emerging from these stock bottles/vials, remove the first-generation adults; either make fresh stocks with these adults or transfer them into a “fly morgue” (flask or bottle containing 50-70% ethanol with a funnel on top, shown in F1, Figure S1-7B). Emptying parentals from stocks reduces crowding, which provides more room for the next generation of emerging adults, facilitates removal of all adults during each collection, and makes virgin females easier to pick out.

E4-B. What to do the morning of a collection day [also see (i) in Figure S1-4 caption]

6) Each morning of the collection (6-10AM, the earlier the better): If vials of diet were made in advance and refrigerated, warm vials at room temperature for 15-20 minutes before placing flies into them—to save time, warm vials in your pocket for about 5 minutes.

Anesthetize flies on a CO₂ pad. Make sure you have removed all the emerged adults from the stock container. If any adults cannot be removed from the vial or bottle, use a lab weighing spatula or the handle of a paint brush to squish them into the fly diet; this ensures that all flies collected during the afternoon will be < 8 hours post-emergence, and therefore virgins. Gently brush the pile of anesthetized flies into a narrow line on the pad to help organize collections.

7) During these morning collections, gather any males and virgin females as needed for the crosses—Figure S1-4 compares virgins vs. older flies, and further details are listed below:

- Transfer any males needed for crosses into vials of diet (males can be either virgin or non-virgin). Roughly 60-80 flies can fit into a single standard fly vial without overcrowding. Label vials with the sex and genotype of the flies.

- Similarly, collect any virgin females needed for crosses: For the first few hours after emergence, adult fly cuticles are untanned and relatively soft and pliable, often with a meconium showing (e.g. Figure S1-4B and H, fly on left in each panel). Flies with this phenotype will not have mated, because they will have emerged much less than 8 hours ago. Therefore, during the morning collection, collect females only if they have an untanned, soft abdomen and store them in vials of diet separate from the males. Again, roughly 60-80 flies can fit in a vial—however, if you are inexperienced with flies or just wish to be extra careful, subdivide virgin females across more vials, perhaps 15-25 females per vial. This way, if a male or non-virgin female were mistakenly brushed into a “virgin female” vial, only a small number of females would need to be excluded from the cross (further discussed at Step 10). Label vials with the sex and genotype of the flies.

- Leave vials on their sides to allow flies to recover from CO₂ without becoming stuck in the diet. (Recovery time varies widely depending on the amount of time exposed to the anesthesia, but regardless, recovery should occur within 15 minutes.) Store at room temperature until needed for crosses.

- If you are collecting again in the afternoon, leave diet vials out at room temperature to keep them warm enough for immediate use.
8) **< 8 hours after the morning collection**, collect again as you did at Step 7, but this time, you may **collect all females (and males)**, almost regardless of their cuticle appearance. This is because if you collected from stocks—or at least emptied adults from stocks—in the morning, all flies that have emerged by the afternoon (< 8 hours later) should be virgin, even if their cuticles have darkened and hardened somewhat. There are rare exceptions due to error: very occasionally, a stock may contain a mated female that had accidentally not been emptied in the morning, and so, as a precaution, do not collect females that release an egg under anesthesia (Figure S1-4G) or that have a very tanned/hardened abdomen (Figure S1-4H, right; see (ii) in Figure S1-4 caption for further discussion).

Figure S1-4
(caption on next page)
**Figure S1-4 (previous page).** Distinguishing female from male and younger/virgin from older/possibly mated *Drosophila*. (A) Female (“♀”, left) and male (“♂”, right) *Drosophila*. Perhaps the most obvious difference between the sexes is their dorsal abdominal pigmentation. Although this difference is worth noting, flies tan with age, and so the sexes of young males or older females might be mis-scored using these criteria. The remaining panels of this figure depict the less obvious but more definitive sex traits. (B) Two female *Drosophila*. The younger fly (left) shows a relatively light-colored, soft abdomen and uninflated wings, indicating it is < 8 hours post-emergence (“PE”) from the pupal case. Older fly on right has a relatively tan, harder cuticle and inflated wings. Each arrow indicates the female genitalia (vagina, v), protruding dorsally from the abdomen. (C) Two male *Drosophila*. Again, younger fly on the left has an untanned, soft cuticle and its wings are not fully inflated, indicating it is < 8 hours post-emergence (“PE”). The fly on the right must be older because it has a relatively tan, harder cuticle and inflated wings. Arrows indicate the ventrally-located male genitalia (claspers, c), which will always be tan, even in very young males. (D) Legs of a male (left) and female (right). Males have sex combs (sc, dotted arrow), thick patches of bristles on the front pair of legs that are dark in color, even in very young males. (E) Ventral view of a male, clearly showing its sex combs (sc, dotted arrow) and claspers (c, solid arrow). (F, G, H) Several other views of female anatomy. (F) The dorsal, posterior abdomen of this female is fairly dark, somewhat overlapping with male pigmentation, but note the lighter pigmentation at the ventral, posterior tip of the female abdomen in comparison to males (compare F to E). Vagina (v, arrow) protrudes dorsally from abdomen. (G) Female with sex-specific abdominal stripes readily visible. Sometimes the CO$_2$ anesthesia will cause a female to release an egg from her ovipositor (o). Vagina (v) is more dorsal and also visible here. (H) Virgin (left) and likely non-virgin (right) females. In addition to the light-colored, soft abdominal cuticle of the female on the left, note the dark spot seen through the cuticle (arrow); this spot is the meconium (m), another positive indicator of a virgin fly that has recently emerged from the pupal case (< 8 hours PE). The much darker, more defined abdomen of the female on the right suggests a much older age (>> 8 hours PE). Ovipositor (o) is readily visible in the older female, and it has much less pigment than claspers (compare to E).

**Description of our cross collection strategy, referencing this figure’s images**

(i) **During the morning collections (E4-B, Steps 6-7):** Collect all males, but when collecting females, only collect those with a light, soft cuticle to ensure that they are virgins (e.g., females on left within panels B and H). Note that wings inflate within only an hour post-emergence, while abdomens remain untanned and soft for several hours longer. Therefore, looking for untanned, soft abdomens rather than uninflated wings will identify a broader swath of flies that are < 8 hours post-emergence.

(ii) **During the afternoon collections (E4-C, Step 8):** If you emptied adults in the morning, < 8 hours ago, all males and females seen in the afternoon should be virgins, and so collect essentially all flies needed, even if the abdomens have started to tan and harden somewhat. However, we would be inclined not to collect the female shown on the right of panel (H) or the female in (G). The female on the right of panel (H) has a particularly dark, shrunken, and “chiseled” abdomen, indicating it is probably at least several days post-emergence. As a precaution, we would exclude this female from a collection because she has probably mated already, but then we failed to remove her from the stock during earlier collections. We would also exclude the female in (G) from a virgin collection: Although virgin females do lay some unfertilized eggs, no female will lay eggs until 2 days post-emergence, so this female is undoubtedly old enough to have mated. All other females shown in this figure, even the somewhat tanned ones, look relatively young (lighter-colored/plump abdomens, cuticle not as defined, and no eggs released), and so we would be comfortable collecting them for crosses in an afternoon that follows a morning collection.
Figure S1-5 (caption on next page)
**Figure S1-5 (previous page).** Comparisons of wild type vs. mutant cw, w, f, and Bar traits. Prep staff can use this figure as a reference while they check Lesson Plan stocks for cross-contamination, and D7-A recommends ways to minimize and troubleshoot cross-contamination. In this figure, we refer to stocks by their general types: WT, Mutant, Df, and Dp. Individual stocks within each of these types are described in Table S1-4. (A) WT wings (left) are rather planar (as are Df and Dp wings), but Mutant wings (right) always have a buckle at a specific spot along their lateral margins (solid arrow), and often curl upwards at their distal ends (dotted arrow). This Lesson Plan calls the associated wing locus cw ("crumpled wing"), our pseudonym for the IP3K2 gene; we obfuscate the gene name to affect the two po types of WT strains can be yellow, orange, pale red, or red. (D) WT flies have long thoracic bristles that taper into sharp points (forked, or f). (E) Mutant flies have the forked (f) trait, where the thoracic bristles are often bent, broken, and gnarled, much as if they had been shaved with a dull razor (arrows). (Less frequently, f affects head bristles as well.) (F, F') Higher magnification views of forked" (F) and forked traits (F'). Of all bristles, the mutation affects the two posterior scutellar bristles (arrows) most frequently and severely. (G) Flies from the Mutant stock (and also the WT and Dp stocks) are actually wild type for Bar (Bar′), and therefore have round eyes (left). Flies from Df stocks will display the Bar mutant trait. Df females are homozygous for the Bar mutation, and so their eyes have a heart shape (bottom right). Df males are hemizygous for Bar, a more severe genotype, and so have a narrow, sliver-shaped eye (top right). Although not shown in this figure, note that Dp flies will appear more or less wild type for all traits (w′ and round eyes, normal wings and bristles).

9) Repeat daily collections (Steps 6-8) until you have all the flies you need for the prep crosses. In our experience, a typical collection regime has taken 3-5 (preferably consecutive) days in order to overshoot cross number targets as suggested (Table S1-3, Steps 6 and 9).

10) If you are experienced with flies and feel confident that you discerned males and virgin females correctly, collected flies may be used right away for crosses (move on to E5). However, if you are inexperienced with flies or just prefer to be extra careful, store collection vials for 4 days at room temperature and check them for fertilized eggs/hatched embryos before using for crosses. Consider the following biology for quality control:

- Adult females might mate as early as 8 hours after emergence, but they will not start to lay eggs until about 2 days later.

- Both mated and virgin females lay eggs, although mated females will usually lay more eggs sooner, and only mated females will produce eggs that hatch.

- Embryogenesis takes somewhat longer than 24 hours at room temperature.

This biology in mind, you can check your collection work as follows: Store collection vials for 4 days, then examine the bottom of the vial. If a "male" vial contains any eggs, even unhatched ones, there is at least one stray female in the vial. To salvage these males for a
cross, simply re-anesthetize the flies and remove the female(s). If a “female” vial contains hatched eggs, either the vial contains a stray male or at least one of the females in the vial is a non-virgin. The most conservative response would be to not use a vial of females containing hatched eggs. However, some mated females used for Cross A1 and the Df Crosses will not qualitatively affect the F1 phenotypes, or later the F2 phenotypes for Cross A2, and so for these crosses, we have “cheated” and used an occasional vial containing mated females—this workaround can be especially useful for the Df Crosses: they are unusually not as healthy, and so they benefit from as many parental females as possible.

E5. How to set up prep crosses

1) Table S1-5 describes which and how many Crosses A1 and B1 (Step 6), and Df and Dp Crosses (Step 9) to set up.

2) Make vials of diet for the crosses. Given that this diet will support active cultures, we recommend relatively fresh diet for crosses (details in Table S1-5, and later in E2). Table S1-3 (Steps 6 and 9) recommends the number of crosses to set up, and by extension, the amount of diet to make. If diet is not used the same day it is made, enclose the rack of vials in a plastic bag and refrigerate until needed. If refrigerated, warm vials at room temperature for at least 20 minutes before placing flies into them (or warm vials in pocket for 5 minutes before use).

3) After checking collection vials to make sure males and virgin females were collected properly (E4, Step 10), anesthetize flies for a given cross on a CO2 pad. Brush 12-20 virgin females and 7-15 males of the appropriate genotypes into each vial. Label vials with their cross type (A1, B1, Df, or Dp) and distinguishing WT/Df/Dp parental genotypes or stock numbers (listed in Table S1-4) so that students will know what they are scoring, for examples: Cross A1 (12442), Cross B1 (33328), Df Cross (7718), or Dp Cross (30380). (There is only one Mutant strain and students will know the cross schemes, so it is not necessary to indicate the Mutant stock on vials.) Leave vials on their sides to allow flies to recover from the CO2 without becoming stuck in the diet. Recovery time varies widely depending on the amount of time exposed to anesthesia, but regardless, recovery should occur within 15 minutes.

4) After flies recover, stand up vials in a rack so that days later, larval feeding will not cause food to ooze out of place and trap adults. Place rack in incubator if you are using one. As discussed in D3-D6, monitor the health and progress of each cross and if necessary, adjust temperature, diet moisture, etc..

5) 5-7 days after setting up a cross, empty adult flies from the vials into a “fly morgue” (F1, Figure S1-7B). If any adults cannot be removed from the vial or bottle, use a lab weighing spatula or the handle of a fly brush to squish them into the fly diet—this ensures that all flies examined by the students will be F1s.

6) If any cross vials are particularly full of larvae, you might wish to make spares: as discussed in D6, scoop out some larvae from the diet with a lab weighing spatula and transfer them to fresh vials of diet.

7) If crosses are timed as suggested in Table S1-3 (Steps 6 and 9), Cross A1 and B1 F1 adults should emerge by the Week 1 class, and Df and Dp Cross F1 adults should emerge by the Week 3 class. Distribute Cross A1 and B1 vials to student stations shortly before the Week 1 class (F1), and distribute Df and Dp Cross vials shortly before the Week 3 class (F3).
Classes for this Lesson Plan span over 4 weeks. In this Part, we list our particular classroom supplies for each of these weeks (F1-4). Finally, in consideration of different facilities, time availability, budgets, etc., we discuss some room for supply flexibility in F5.

The bulk of our classroom prep comes before Week 1: We distribute most of this Lesson Plan’s supplies several days before the first Week 1 class section meets (F1, Figure S1-7 and Table S1-7), then leave nearly all of them in place through the end of Week 3—only the fly vials and CO₂ tanks are replaced regularly during Weeks 1 and 3. We clear out all of the lab supplies after the last Week 3 section, providing room for students to work on laptops during Week 4 (F4). Week 4 is a solely computer-based exercise, so it can be held in any classroom with internet access.

As mentioned earlier in this document, our total class sizes range from 65-120, and we subdivide the class into 4-5 lab sections that each meet at a different time (and usually a different day) but in the same lab room. Adjust your supply list accordingly if you have a different class size or section number, or if sections meet in multiple rooms simultaneously.

Regarding infrastructure and amenities, our lab room contains (Figure S1-6):

- 3 benches; each bench has eight stations, and so we can comfortably fit up to 24 students in a section.
- Each station has an electrical outlet (for scopes and recharging a laptop), and also an air spigot (for connection to a CO₂ tank).
- Just outside of the lab, two closets each contain tubing that connects with the lab’s air spigot system: one closet’s tubing connects to the air spigots of two benches (16 stations), while the other closet’s tubing connects to the spigots at the third bench (8 stations). We store up to three CO₂ tanks in each closet, with one tank per closet connected to the air system at any given time.
- An incubator for rearing student fly crosses.
- Two small dry erase boards at the front of the lab for announcements, data collection, etc. (one board fixed to wall, one mobile).
- A digital projector and projector screen. Projector has wiring to connect to the instructor’s laptop (for prelab lecture) as well as to a dissecting scope camera.
- A dissecting scope equipped with a digital camera. Camera is connected to the digital projector during the fly handling demo, allowing all students to simultaneously view the flies that the instructor is handling under the scope.
- Internet access for the Week 4 exercise.

Of course, not all of these features are crucial, and there are straightforward workarounds if your lab is not equipped with most any of these items—see F5 for some suggestions.
F1. Week 1 classroom supplies

Figure S1-7. Snapshots of the Week 1 lab supplies that we lay out (A) at each student station, (B) at each bench of 8 stations, and (C-F) for the entire lab classroom. The numbers that mark each supply refer you to associated information (specs, catalog numbers, etc.) in Table S1-7, which starts on the next page.

Numbered supplies: (A) (1) Dissecting scope, (2) Parentals fly vial, (3) Cross A1 or B1 fly vial, (4) fresh diet vial, (5) funnel with fitted rubber stopper, (6) CO₂ pad with gas tubing, (7) two plastic rings, (8) fly brush, mouse pad, and Sharpie. (B) (9) Fly morgue with mouse pad, (10) fly trap. (C, C’) (11) Incubator (C shows its exterior, C’ shows the interior shelving and class section labels). (D) (12) CO₂ regulator with heating unit, (13) CO₂ tank. (E) (14) Pipe heating cable. (F) (15) Wrench for changing CO₂ tanks, (16) CO₂ gaskets. Flies and vials (panel A, #2-4) will need to be replaced between sections and CO₂ tanks will be depleted and then replaced as needed (panel D, #13), but all the other equipment shown here can stay in place through Week 3.
Table S1-7. Week 1 classroom supplies.

<table>
<thead>
<tr>
<th>Supplies to provide...</th>
<th>[Panel], [#] in Figure S1-7</th>
<th>Item</th>
<th>Specs, other notes</th>
<th>Example of supplier and model</th>
</tr>
</thead>
<tbody>
<tr>
<td>...at each student’s station</td>
<td>A, 1</td>
<td>Dissecting scope</td>
<td>Equipped with an overhead light and adjustable magnification (roughly 8X-40X).</td>
<td>Zeiss, Stemi 305</td>
</tr>
<tr>
<td></td>
<td>A, 2</td>
<td>Parentals fly vial</td>
<td>Fresh mixture of adult Mutant and WT flies. See Table S1-3, Step 12 for prep description.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>A, 3</td>
<td>Cross A1 or B1 fly vial</td>
<td>A1 and B1 are reciprocal crosses between Mutant and WT strains. See Table S1-3, Step 6; Table S1-6; and E5 for prep description.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>A, 4</td>
<td>Fresh vial of fly diet</td>
<td>Part C lists some diet options. Attach a blank piece of labelling tape to the vial for students to label their Cross A2/B2.</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>A, 5</td>
<td>Powder funnel with fitted rubber stopper</td>
<td>Cushions CO₂ pad while knocking flies onto it. Optional item because not essential for students and stopper type currently has limited availability—see F5-C for workarounds. Funnel: polypropylene, top diameter=65 mm, height=67 mm, stem length=22 mm, stem diameter=16 mm Stopper: size 11 with one 15 mm diameter hole drilled in center.</td>
<td>Premade pads and component materials are available: Genesee Scientific, Lab Scientific, and some other suppliers are listed on the BDSC website. Funnel: Fisher Scientific, #10-348A Stopper: see F5-C</td>
</tr>
<tr>
<td></td>
<td>A, 6</td>
<td>CO₂ pad</td>
<td>We use a standard 12.5 cm x 7.5 cm x 32 mm thick sheet of UHMW porous polyethylene attached to the top of a 2 cm deep plastic base. Metal adaptor on one side of base connects to Tygon tubing (#E-3603), which connects to a station’s air spigot.</td>
<td>Rings were cut from hard plastic tubing in our machine shop, but other material with similar dimensions should work fine (e.g. from a hardware, arts-and-crafts, or dollar store).</td>
</tr>
<tr>
<td></td>
<td>A, 7</td>
<td>2 plastic rings</td>
<td>1.5 cm diameter, 0.5 cm thick. Used for sectioning of a small number of reference flies on the CO₂ pad.</td>
<td>Fly brushes from Carolina Biologicals (#173094) have somewhat different dimensions from the model displayed, but they work nicely and are affordable.</td>
</tr>
<tr>
<td></td>
<td>A, 8</td>
<td>Fly brush</td>
<td>Fine brush with bristles 1 cm long and 2 mm wide if all bundled together. Handle 16.5 cm long. Model shown appears to have been discontinued, but other brushes of similar dimensions should be effective.</td>
<td>Office, hardware, or outdoor supplier.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thick mouse pad</td>
<td>For cushioning as students bang fly vials on the counter. Roughly 18.5 cm x 11.5 cm and 0.5-1 cm thick. Any cushioning material of similar dimensions should do (e.g. a cut of high-density EVA foam from a sleeping bag mat).</td>
<td>Office, hardware, or outdoor supplier.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sharpie</td>
<td>Extra fine or ultrafine point, black.</td>
<td>Any standard office supplier.</td>
</tr>
</tbody>
</table>

(Continued on next page.)
### Table S1-7. (cont.)

<table>
<thead>
<tr>
<th>Supplies to provide…</th>
<th>[Panel], [#] in Figure S1-7</th>
<th>Item</th>
<th>Specs, other notes</th>
<th>Example of supplier and model</th>
</tr>
</thead>
<tbody>
<tr>
<td>...for each bench of 8 stations</td>
<td>B, 9</td>
<td>Fly morgue with mouse pad or similar cushioning material</td>
<td>Flask or bottle partially filled with 50-70% ethanol, capped with a funnel (use rubber stopper on bottom of funnel if flask/bottle is fragile). Mouse pad (see previous page, #8 for specs) is for cushioning as students bang flies into the morgue. When morgue is full, dump its contents down sink while running water.</td>
<td></td>
</tr>
<tr>
<td>...for the entire classroom</td>
<td>B, 10</td>
<td>Fly trap</td>
<td>Flask or bottle containing fruit and/or vinegar. Cap with funnel, rubber stopper optional. Attracts and contains stray flies. Tape over the top of funnel and label with warning to prevent students from accidentally dumping flies into the trap instead of the morgue (notice red tape on top of trap funnel in Figure S1-7B). Freeze and then remove contents after use.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C’, 11</td>
<td>Incubator (if not available, see F5-D for workarounds)</td>
<td>Set at around 25°C so that Crosses A2 and B2 produce a good number of progeny by Week 3. Students are instructed to remove F1 flies from their cross vial 5-7 days after their Week 1 class (<a href="#">Supporting File S2, Week 2 Procedure</a>). Prep staff monitor and adjust the moisture of diet in all cross vials and if possible, make some spares from excess larvae as described in D3-D6 and F2.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D, 12</td>
<td>CO₂ regulator with heating unit</td>
<td>Controls airflow and prevents gas from freezing tubing. Attach to a CO₂ tank that is being used. Plug in before opening the tank. CGA 320, fitted with a Profax 120 V AC, 100-watt CO₂ heater.</td>
<td>Consult with your CO₂ tank provider for regulator compatibility. (We use an older Airco 806 9235 model.)</td>
</tr>
<tr>
<td></td>
<td>D, 13</td>
<td>CO₂ tank (see F5-E for alternatives)</td>
<td>Non-syphon, 50 lb. carbon dioxide gas tank. Tygon tubing (#E-3603) connects our tanks to the room’s air distribution system. For our class sizes, we hook up two tanks at a time. During each Week 1 and Week 3 section, we use up roughly 1.5 tanks for a total of about 12 tanks per Lesson Plan offering.</td>
<td>Noble Gas Solutions, item CDK.</td>
</tr>
<tr>
<td></td>
<td>E, 14</td>
<td>Pipe heating cable</td>
<td>An extra measure to protect against freezing. Wrap around the tubing proximal to the regulator as shown in Figure S1-7E. We have found this helpful for the tank supplying 16 stations, but not necessary for the other tank, which supplies only 8 stations.</td>
<td>Hardware supplier.</td>
</tr>
<tr>
<td></td>
<td>F, 15</td>
<td>Wrench</td>
<td>Used to attach and detach CO₂ regulators. Check dimensions to fit on your CO₂ regulator (a 1-1/8” wrench works well on our standard regulators).</td>
<td>Hardware supplier.</td>
</tr>
<tr>
<td></td>
<td>F, 16</td>
<td>CO₂ gaskets</td>
<td>Used to help seal the juncture between the CO₂ tank and regulator.</td>
<td>Through your CO₂ supplier.</td>
</tr>
</tbody>
</table>
F2. Week 2 classroom supplies

No class this week. Instead, as described in the Lab Manual (Supporting File S2), students come into lab 5-7 days after their Week 1 class section met, empty the adult flies from their Cross A2 or B2 into a fly “morgue” (Figure S1-7B and Table S1-7, #9), then replace their Cross vial where it was stored.

No additional lab equipment is necessary for Week 2, and then all Week 1 supplies except for the fly vials (i.e. everything but #2-4 in Figure S1-7A and Table S1-7) are reused on Week 3. Therefore, if you do not have a different lesson running in the same room during Week 2, leave all Week 1 supplies except fly vials in place for Week 3 (F3).

During Week 2, we recommend that prep staff check and maintain the health of the A2, B2, Df, and Dp Crosses, using Part D of this document as a reference:

- Remember to remove the parental flies from the Df and Dp Crosses before the next generation of adults emerges (Table S1-3, Step 11). (We do not ask students to do this.)

- If an incubator is used, humidify its interior (D5).

- If a cross culture looks dry (D4, Figure S1-2A), add drops of water to the diet.

- If a cross culture is too wet (D4, Figure S1-2C), add a small piece of paper towel to the vial.

- If some cultures look particularly full of larvae (Figure S1-2B), use a lab weighing spatula to transfer excess larvae to fresh vials of diet (D6). Label new cross vials according to the source of their larvae and keep them as spares for students who do not have enough flies to score on Week 3.

- During some terms, we have told students that they did not need to come into lab during Week 2, and then prep staff took care of emptying all F1 adults (a fast process). Although this disengaged students somewhat, it made culture maintenance easier for the prep staff.

- Another strategy to generate spare A2/B2 cultures: right after each Week 1 class section, prep staff could gather spare Cross A1 and B1 vials and use them to set up extra Crosses A2 and B2. These extras could be stored and monitored alongside all of the students’ Cross A2 and B2 vials.

F3. Week 3 classroom supplies

Same equipment as Week 1, except for the following modifications:

- Remove the Week 1 fly cultures and diet vials (Figure S1-7A and Table S1-7, #2-4).

- Give each student the Cross A2 or B2 vial that they set up during their Week 1 class (Supporting File S2).

- Also give each student a Df or Dp Cross vial that was set up by the prep staff (Table S1-3, Step 9). Make sure each Df and Dp Cross is labelled with its Df or Dp stock number (Table S1-4) so that students will know what they are scoring. We share these vials between sections, and so we ask students to return flies to their vials after scoring them.
• Table S1-3, end of Step 12, suggests that you **make fresh Parental vials for the Week 3 prep**. Given that the vast majority of the Cross A2 and B2 progeny will resemble either the WT or the Mutant Parental flies (i.e. most progeny will either have all-mutant or all-wild type traits), and that students will have had fly training on Week 1 and been given images of each phenotype, we have not found this extra step to be crucial and have left it out some terms. Nevertheless, Parental flies can be a useful reference for students because of the certainty: eye color is easy to score, and 100% of white-eyed Parental flies will display mutant cw and f traits, and 100% of yellow/orange/red-eyed Parental flies will display wild type cw^+ and f^+ traits. Therefore, if possible, we recommend preparing fresh Parental vials right before Week 3.

• **Have a computer in the lab displaying your class’ datasheet (Supporting File S7)** so that students can enter their data into a common file. After all Week 3 sections have collected data, curate and send their data file to the entire class. Supporting File S8 is an example of a final dataset—it can be used to supplement your class data if necessary.

**F4. Week 4 classroom supplies**

Students will work through a computer-based exercise this week. **Each student will only need their Laboratory Manual (Supporting File S2), their own or a borrowed computer (either a PC or Mac, but we like the mobility of laptops to facilitate student collaboration), and web access.** For web browsers, we recommend Safari or Firefox but not Chrome because in our experience, Chrome had difficulties interacting with GBrowse graphics.

We hold Week 4 class in the same lab we used for Weeks 1-3, clearing supplies from the stations so that students will have space to move about and confer with each other while working on their laptops. However, no flies or lab equipment are necessary this week, and so instructors could hold class elsewhere if they wish—anywhere with sufficient space, computers, and a good internet connection.

**F5. Alternative supplies**

We gave this Lesson Plan a modular design to accommodate a wide range of learning goals, available prep/class times, budgets, class sizes, and lab facilities. Here we address instructor concerns, and also consider prep staff purview, suggesting alternatives for specific equipment in the supply list (Figure S1-7 and Table S1-7) that may be expensive or impractical to acquire or prepare.

**F5-A. Fly stocks**

If instruction and prep are carried out by different people, we particularly recommend that they **communicate about the fly stocks that are ordered and prepped**. Although students will benefit from collecting at least some of their own data, remember that a complete backup dataset is available to use as needed (Supporting File S8). Therefore, if class time, prep time, prep resources, student number, and/or class sections are limited, there are many ways to reduce the fly prep while still giving students a solid learning experience, including:

• Order all stock types, but somewhat fewer stocks of each type—more specifically, still order the Mutant stock, but then order only a subset of stocks of the WT, Df, and Dp types. If you do so, we suggest you still order the stocks that will enable the students, with their own
data, to positively identify the cw \((IP3K2)\) locus (these stocks are indicated and discussed in Table S1-4).

- Order many fewer stocks of only one type—for example, you could prep only one WT stock for your A1/B1/A2/B2 Crosses without at all changing the students’ experience of observing flies in the lab. Data from excluded WT stocks could then be obtained from Supporting File S8.

- Prepare only a subset of the cross types for class, for example only the Df and Dp Crosses. Students could score these crosses within only a week, and instructors could supplement their data with Cross A2 and B2 results from Supporting File S8. Care would need to be taken to ensure that students understood the crosses that they did not actually score.

- In a more advanced class, students could help set up their own crosses. One possible arrangement could be that prep staff empty stock bottles/vials early in the morning, then students collect virgins during an afternoon class (collection procedure described in E4).

Regardless of your chosen reconfiguration, provide Parental flies (Table S1-3, Step 12) to help students learn how to discern fly sexes and WT vs. Mutant phenotypes before they collect data.

**F5-B. Digital projector, screen, and dissecting scope with camera**

On Week 1 (and sometimes Week 3), we use a digital projector and screen to give a Prelaboratory Lecture (Supporting File S5), then connect the projector to a dissecting scope camera to demo fly handling, thus allowing all students to observe what is under the instructor’s scope simultaneously. These are definitely useful teaching tools, but they are not essential for the class. In lieu of these tools:

- The Laboratory Manual (Supporting File S2) contains useful images of \textit{Drosophila} traits for the students to consult.

- Even without projecting the slideshow, images from the Prelaboratory Lecture Slideshow (Supporting File S5) could be printed and given to students for them to refer to while they examine their fly cultures.

- Before we acquired a dissecting scope with an attached camera, instructors would gather the students around a single lab station and conduct the fly handling demo without the aid of a projector (demo link shown at end of Supporting File S5). Combined with the slideshow images, this earlier approach was certainly adequate. Our recent scope-to-projector addition did add a significant “wow factor” and nice opportunities for teacher-student interaction, but it was not essential for student learning of fly phenotypes.

**F5-C. Funnel and rubber stopper (Figure S1-7A, #5)**

Size 11, solid rubber stoppers are widely available, as are rubber stoppers with 3-5 mm holes, but ones with the recommended 15 mm hole are apparently difficult to find new (we had older ones in our supply cabinet). Solid rubber is difficult to drill, and 3-5 mm holes are too narrow to dispense flies through to the CO\(_2\) pad efficiently.

However, as mentioned in Table S1-7, the funnel and rubber stopper are optional—they are meant to minimize the chance of damage to CO\(_2\) pads as flies are knocked onto the pads. This in mind, there are several easy workarounds if you do not have an old stash of the right stoppers:
• Cut a square of thick cork board and drill a roughly 14-15 mm hole into it with a cork borer. Insert funnel stem into hole.

• Wrap a strip of packaging foam or bubble wrap tightly around the base of the funnel without blocking the stem opening, and tape in place.

• The funnel and stopper could be excluded from the supply list completely. In this case, students would invert their fly vials directly on their CO₂ pads and tap flies out. (See the demo video at the end of Supporting File S5—imagine tapping flies onto the CO₂ pad as demonstrated, but without a funnel and with the inverted vial directly touching the CO₂ pad.) The only drawback to this approach is that students will need to tap the vials a little more gently to avoid damaging their CO₂ pads or flies.

F5-D. Incubators

If you do not have access to incubators, you probably will be rearing Crosses A2 and B2 at room temperature (20-22°C) instead of at 25°C. Generation time at room temperature is 14-21 days as opposed to 10-14 days at 25°C (Table S1-2), and so if no incubators are available there are two good solutions:

• Postpone Week 3 class one week. Hold the “Week 3” class on Week 4 to allow a full 21 days after Week 1 for adequate F2 generation time.

• Prep staff could set up all crosses a week earlier than recommended. Set up Crosses A1 and B1, and then the Df and Dp Crosses, a week earlier than detailed in Table S1-3 (Steps 6 and 9). They would also set up A2 and B2 for the students a week before the Week 1 class, which would make progeny available for the actual Week 3 classes. After collecting F1s from the Cross A1 and B1 vials, original A1/B1 crosses would be kept at room temperature, allowing additional F1s to emerge before the Week 1 class without F2s having time to emerge.

F5-E. Anesthesia alternatives

If your lab room is not equipped with an air distribution system, there are several different workaround options:

• **Directly connect CO₂ tanks to the CO₂ pads on each bench.** Strap a CO₂ tank with a heated regulator to the end of each bench. Connect each regulator to tubing that runs down the middle of its bench, then connect CO₂ pads to the main tube via T-fittings (e.g. Genesee Scientific #59-123).

• In some of our smaller class sections, we have successfully used an affordable liquid anesthetic called FlyNap (Carolina Biologicals #173010). It can be messy, but it can also knock out flies for a longer time than CO₂ (at least 50 minutes, according to manufacturer), allowing students to examine their flies on a piece of paper without further anesthesia. Recall that students will anesthetize the progeny of Crosses A1 and B1, and then will need to keep the progeny healthy enough to set up Crosses A2 and B2. They will not have to set up any other crosses, but many other vials that they examine will be reused between sections, so students will need to anesthetize all their flies without compromising their health. In our experience, this Lesson Plan’s fly strains remain viable and fertile after FlyNap treatment, but under- or overdosing is possible, so experiment with FlyNap before class to ensure that you can demo its effective use to the students. **Regardless of whether your**
students use FlyNap, we strongly recommend that staff use CO$_2$ anesthesia throughout the prep because CO$_2$ is the easiest, safest, and most efficient option for handling and expanding large numbers of fly cultures.

- **We do not recommend using ether** as an anesthesia because it is difficult to avoid overdosing the flies. Also, the fumes can be an irritant to many people.

- **Freeze flies before class.** Prep staff could set up all crosses for students, then before class, they would dump flies that students will examine into empty vials/bottles, cap, then store in a frost-free freezer (flies should completely freeze after only 30 minutes). Morphology and color are preserved for at least three weeks. Keep flies frozen until immediately before students will examine them—previously frozen flies will dry out and shrivel if left out at room temperature for more than several hours.

- **“Pickle” flies** that students will examine **with 70% ethanol at room temperature.** Morphology and color are well-preserved for at least 90 days, but a drawback is that neck connectives become fragile, and so if jostled, fly heads can break off of their bodies. To minimize this issue, store flies in 50 ml Falcon tubes that are completely filled with the 70% ethanol and minimize tube jostling. Immediately before each class section, gently dispense fly/ethanol suspensions into small petri dishes so that students can examine flies under their microscopes (Alfredo Zuniga and Jason Duncan, personal communication).