Chapter 7

Using Ant and Butterfly Pollination to Involve Students in Scientific Exploration

Mary Puterbaugh Mulcahy and Mary Blaine Prince

Allegheny Institute of Natural History
Biology Program
Division of Natural Sciences
University of Pittsburgh at Bradford
300 Campus Dr.
Bradford, PA 16701
(814)362-0259
mnp1@exchange.ubp.pitt.edu

Mary Puterbaugh Mulcahy is an assistant professor of biology at the University of Pittsburgh at Bradford. She teaches courses in ecology, evolution, and plant-animal interactions among others. Her claim to fame is the discovery of an ant-pollinated plant above tree line in the Rocky Mountains. Her current research interests are in plant-animal interactions between bryophytes and small invertebrates. She and her students are presently investigating a relationship between an epiphytic liverwort species and Bdelloid rotifers.

Mary Blaine Prince received her B.S. in Biology from Madison College and her M.S. in Biology from Edinboro University. At Pitt-Bradford, she teaches lecture and laboratory portions of introductory biology for majors and nonmajors as well as an environment and ecology course for nonmajors.

Reprinted From: Puterbaugh Mulcahy, M. and M. Blaine Prince. 2004. Using ant and butterfly pollination to involve students in scientific exploration. Pages 135-157, *in* Tested studies for laboratory teaching, Volume 25 (M. A. O'Donnell, Editor). Proceedings of the 25th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 414 pages.

Contents

Introduction to Ant-Exercise

This exercise involves students in the scientific process and introduces them to pollination biology. Students generate a list of plant species divided by their major pollinators. Students are allowed to make observations about the relative numbers of plants whose flowers are visited by bees, birds, flies, and butterflies and the relative few that are reported to be visited and pollinated by ants. Students observe that reports of ant-pollinated plants are much rarer than plants pollinated by other organisms. The general format of this lab is as follows:

OBSERVATION: Reports of plants pollinated by ants are rare.
The instructor may simply tell students this observation or I have found two ways to have students make this observation themselves. One involves a database of the flower visitors to an entire Illinois prairie community available online (see Appendix B). A second way involves searching scientific journal databases to see how many papers are published on ant-pollination versus other types of pollination.

QUESTION: Why is ant-pollination rare?

HYPOTHESES: Student-Generated
This lab exercise is investigative, and I have tried to design it so that the students take charge of much of the conceptual work. So, try to let students come up with these on their own. Guide the students by helping them make their hypotheses as specific as possible. The more specific the hypothesis, the easier it is to test.

PREDICTIONS AND TESTS: Student-Generated

Once students have listed some hypotheses, you can assign individual students or groups of students to try to gather data to test their hypotheses. Ants are generally available for students to collect and manipulate, and consequently, students can obtain data fairly quickly. I often use this as the first lab of an upper level college course. Enough data can be generated for students to write-up this exercises as their first laboratory report and the scientific literature on this subject is relatively easy to access and to understand. Alternatively, you can have students present their data to the entire class rather than write a lab report. This has worked well in a non-majors course.

FOLLOWUP EXPERIMENTS: Professor-Guided

There is one hypothesis in particular for the rarity of ant pollination that students are unlikely to generate on their own. This is the hypothesis that glandular secretions on the ants' bodies kill or damage pollen (proposed originally by Andrew Beatty, an Australian ecologist, and his colleagues). We will demonstrate one way to test this hypothesis. These data are nice to have if the students are going to compare or discuss their results in the context of other published scientific data on the topic.

Materials for Ant-Exercise

While the list of materials for this lab seems long, I regard this exercise as a low preparation lab. Once you have made basic fuchsin gel you usually will not have to make it ever again. The only fresh solution you need is the pollen germination media. If you are missing a few of the items listed, you can often make-do without. However, if you intend to do the pollen germination, it is absolutely essential that you have fresh pollen (and I would encourage you to test germination the day before the students).

Overview of all materials

Items for catching & keeping ants

- Jar to keep ants in
- Flat dish (such as pie plate) for students to sort through dirt to find ants
- Epi-tubes (any size okay; 1-ml is good) for students to put the ants they catch into
- A vacuum collector to catch ants (optional; directions in appendix)

Items to enable analysis of pollen

- Fresh flowers with fresh pollen! I usually use wild jewelweed flowers (*Impatiens capensis*), but I have also used store-bought *Impatiens* and *Petunias.* Scott (1995) has several other recommendations including: common wild flowers *Melilotus officinalis* (yellow clover) and *M. alba* (white clover), Tradescantia ("spiderwort"), *Gibasis geniculata* (the "bridal veil" plant), "sweet pea," available in florists. I strongly recommend testing for pollen germination the day before the students begin the lab.
- One dissecting and one compound microscope per pair of students
- One pair of forceps per student or per pair of students
- Microscope slides (depression slides with one or more indentations are nice)
- Tips cut off of the bulb of plastic pipettes (note these will be used to trap ants on slides with pollen, they may not work well for very small ants, such as Argentine ants)
- Coverslips (preferably glass)
- wooden clothes pins (to allow students to hold slides over flame without burning themselves)

- 70% ethanol to rinse pollen off of forceps (handy to have in squirt bottle)
- Alcohol lamps containing alcohol (1 per every 5-10 students is good); alternatively, you can use several hot-plates on low or medium low heat.
- Tally counters for keeping track of the number of numbers of pollen grains
- A package of plastic pipettes; you need to cut the tip of the bulb off of each pipette. You will use these to trap an ant on a slide (see Fig. 2 in the student outline section). [If you can find an alternative cap of similar size and transparency, try them instead!]

Items for 1000 ml pollen germination media (from Scott, 1995)

- Latex/vinyl gloves for students to wear
- Protective goggles for students to wear
- Volumetric flask (1 L) or other container to hold pollen germination media (or smaller if you make less)
- sucrose (100 g; table sugar from the grocery store)
- boric acid, H_3BO_3 (0.1 g)
- calcium nitrate, $Ca(NO_3)_2.4H_2O$ (0.3 g)
- distilled water (1 L or so)

Items for 1 petri plate of basic fuchsin gel (probably more than you'll need) (modified from Beattie, 1971; also see Kearnes and Inouye, 1993)

- latex/vinyl gloves to wear (just for the one or few individuals making the gel)
- protective goggles to wear (just for the one or few individuals making the gel)
- glass Pyrex or Kimax covered petri dish
- hot plate
- one packet of Knox gelatin (approximately 7 g/packet)
- distilled water (at least 25 ml)
- glycerin (glycerol) (at least 35 ml)
- crystalline basic fuchsin stain (pararosanilin)/color of claret (less than 0.1 g)
- (1 g crystalline phenol may be added as preservative, we omit this)

Items for field work

- lab notebooks/clipboards
- stop watch or watch with seconds hand
- fluorescent dusts for marking ants; two companies that make vast quantities of there are:
  Radiant Color Company *(www.radiantcolor.com)* - Series R-103-G
  Day-Glo Color Company *(www.dayglo.com)* - product Arc Yellow (orange) A-16
  (these companies will sometimes provide free samples)
- toothpicks for applying dust to ants
- (optional) Refractometer for measuring sugar concentration of nectars

Notes for the Instructor: Ant-Exercise

Break Students into Groups

Other ways exist to organize this laboratory, but I usually start the lab by having the student come up with the hypotheses. (The hypotheses that my students and I have come up with are in the Appendix.) After breaking students into groups, I assign them or let them choose one or a few hypotheses to try to test. For most of the hypotheses, I try not to tell students how to address the hypotheses, and leave it up to the students to think creatively. However, here are a few examples for the benefit of the instructor. For example, I might have one group focus on the hypothesis that pollen does not attach to the body of ants and that ants groom off pollen. I might have a second group focus on whether ants visit flowers in the first place and whether they are too small to contact the reproductive parts of plants, and a third group focus on whether the foraging behavior of ants (in particular how quickly and distantly they move) might preclude them from cross pollinating.

For the size question, the first group may go measure ants and measure flower morphology to see whether an ant can contact both stigma and anther simultaneously (of course even if ants can not, they could still pollinate.) To address the question of whether pollen sticks to ants, students can take anthers and dust ants with them and then bring them into the lab to determine if any pollen stuck to the ant. By examining a dusted ant over time, they can test whether ants groom off the pollen. The third group can measure distances that they see ants move, and how fast they see ants move. They can measure the distances between flowers to see if the distances ants move are further or shorter than the distances between most flowers. The third group can also see whether ants tend to go from a single food source to their nest and back, and if the ants appear move from food source to food source as might be necessary for cross pollination. Hopefully, your students will come up with even better and more creative ideas that those suggested here! Note: *Data can be gathered even if students do not find any ants on flowers! My students usually do find ants on flowers of goldenrod and Queen Anne's Lace.*

Avoid Fire Ants

FOR SAFETY REASONS, FIRE ANTS SHOULD BE AVOIDED BY STUDENTS. Fire ants refer to several species of highly aggressive ants that are introduced to the United States. Fire ants have a painful sting, and a small number of people are highly allergic to the venom. Worker fire ants range in size from 1.5 to 4 mm and are reddish brown with a darker abdomen, and nest in mounds. The range of the ants is primarily in the southern US including the states of Texas, Florida, Oklahoma, Arkansas, Georgia, Alabama, Mississippi, South and North Carolina, Tennessee, California. Louisiana, New Mexico, and Nevada. Some of these states have their own web sites on the fire ant problem. More general web sites with information on and pictures of fire ants are at the following address (this sites will also link you with other sites):
<http://www.ceris.purdue.edu/napis/pests/ifa/index.html#sites>

Collecting Ants in Case of Bad Weather

Field exercises on insects are great, but bad weather can cause pollinators to become inactive. We suggest that a couple of days before the laboratory and when the weather is good, go out with a spade and collect some "emergency" ants. We suggest NOT using these ants unless the weather is such on the date of the laboratory exercise that the students are unable to get their own ants. To collect ants: dig up some ants with their dirt (you don't need a queen so this should be an easy task),

and stick them in a jar with holes in the lid (so the ants get some air and will still be alive the day you do the exercise - but the holes need to be smaller than the ants or they will escape!). Leave at least a couple inches between the jar lid and the dirt level (you can leave more room). You'll need to get enough ants for each pair of students to have about three or four ants each. You can use *any* type of ant you find; though it seems most relevant to collect flower-visiting ants. Medium-sized ants are easier to catch and work with than very tiny ants (such as Argentine ants).

Pollen Germination Media

Make a fresh batch of the pollen germination media given by Rodney Scott in the 16th ABLE Proceedings. The recipe is reprinted in Appendix B.

Pollen Staining Gel

The pollen staining gel needs to be made at least several hours before lab, and can be made several weeks sooner. One batch can last many years, depending on how many students need to use it. I make this and do NOT ask students to make it. The recipe is in the appendix. Students should wear gloves when handling the gel (and so should you).

Data on Other Insects

This laboratory exercise could be expanded to include other insects. Some data on other insects can be found in Herrera (1987).

Ant Vacuum

Students may find it hard to collect ants. The instructions for creating an ant vacuum are included in Appendix B (Fig. 7).

Ant Diagram

A diagram of an ant indicating the approximate location of the metaplural gland is found in Appendix A (Fig. 6).

Student Outline

If you sit and watch a single patch of flowers for an extended period of time, you will notice that flowers of one plant species often get visited by a diverse array of insects. Flowers of one plant species may be visited by more than one species of bee or may even be visited by insects of more distantly related taxa: beetles, flies, and bees. One question that interests pollination biologists is whether all those insects that visit flowers are really pollinators. Other intriguing questions are, "What features of an insect make it more likely to be a pollinator?" and "What characteristics make a pollinator most effective at pollinating?" Because many of our native and endangered plant species depend on insect pollination to reproduce, these questions are important to conservation biologists. Farmers are interested in these questions because some of our crops are insect pollinated (radishes, pumpkins, fruits trees, etc.). These questions fascinate evolutionary biologists because there exist over 100,000 flowering plant species that appear to have arisen in the last 2 million years, and animal-mediated pollination may have played a role in speciation.

Today, we will consider these questions in the context of one particular plant-animal interaction: ants that visit flowers, and flowers that are visited by ants. Two eminent ant biologists, Bert Hölldobler and E. O. Wilson (1990: 1), have written that "about one-third of the entire animal biomass of the Amazonian *terra firme* rain forest is composed of ants and termites, with each hectare of soil containing in excess of 8 million ants and 1 million termites.... Although comparable biomass measurements have not yet been made elsewhere, it is our subjective impression that the eusocial insects, ants foremost among them, are comparably abundant in most other principal habitats around the world." Even though ants are so abundant, fewer than 20 plant species have been conclusively documented by biologists as ant pollinated (Table 1). In contrast, many thousands of plant species are pollinated by other insects such as bees and wasps and flies. Why do you suppose that ant pollination is so scarce? Numerous hypotheses have been proposed to explain the global rarity of ant pollination, but no general conclusions have yet been reached.

Table 1. Some ant-pollinated species (not a complete list!).

Species	Common Name	Location Studied	Source
Alyssum purpureum	none given	Mediterranean	Gómez et al., 1996
Arenaria tetraquetra	none given	Mediterranean	Gómez et al., 1996
Diamorpha smallii	Small's stonecrop	North & South Carolina	Wyatt and Stoneburner, 1981
Hormathophylla spinosa	none given	Mediterranean	Gómez and Zamora, 1992
Paronychia pulvinata	alpine nailwort	alpine Colorado	Puterbaugh, 1998
Polyganum cascadense	Cascade knotweed	Western Cascades of Oregon	Hickman, 1974
Retama sphaerocarpa	none given	Mediterranean	Gómez et al., 1996
Sedum anglicum	none given	Mediterranean	Gómez et al., 1996

One hypothesis that was proposed by several Australian researchers is that ants are rarely pollinators because they secrete substances from their bodies that kill the pollen (Beattie et al., 1985, 1984). One explanation for why ants secrete such substances is that ants live close to soil and may be exposed to a lot of bacteria and fungi; glandular secretions may inhibit unhealthy growth of bacteria or fungi on the body of the ant. One way to test the hypothesis that the ants secrete pollen-killing substances is to compare pollen that has not been exposed to ants with pollen that has been exposed to ants. If the hypothesis is valid, pollen exposed to ants should have a lower germination rate than pollen that is not exposed to ants.

The purpose of today's lab is to test the hypothesis that ant secretions kill pollen. Additionally, you are expected to come up with other plausible hypotheses as to why ant pollination is scarce. You will be given an opportunity to creatively design experiments and collect data to evaluate your hypotheses. As you work through this laboratory exercise, notice that we are focussing on a specific question, "Why is ant pollination rare?" but keep in mind that our question falls into a much bigger context, "What characteristics have been important in the evolution of insect pollination?" and "What characteristics of behavior or morphology influence the effectiveness of a pollinator?" We will not answer these two bigger questions today, but we will contribute to a growing body of data gathered by many researchers throughout the world that collectively help to give insight into these larger questions. Scientists frequently use this approach to focus their research: find a small question that is one that can feasibly be investigated, but choose a question that will help in a small way to answer a larger, more important question.

Questions

Please answer following questions 1-4 before coming to lab:

1. What conditions are necessary for an animal to pollinate a plant?

2. Is every insect or animal that visits a flower necessarily a pollinator? Explain.

3. Hölldobler and Wilson have written that, “about one-third of the entire animal biomass of the Amazonian *terra firme* rain forest is composed of ants and termites, with each hectare of soil containing in excess of 8 million ants and 1 million termites.... Although comparable biomass measurements have not yet been made elsewhere, it is our subjective impression that the eusocial insects, ants foremost among them, are comparably abundant in most other principal habitats around the world.” Even though ants are so abundant, fewer than 20 plant species have been conclusively documented by biologists as ant pollinated! Yet many thousands of plant species are pollinated by other insects such as bees and wasps and flies. Why do you suppose that ant pollination is so scarce? Why aren't there more ant-pollinated plant species? List as many hypotheses as you can for why ant pollination is rare.

1. *Ants secrete antibiotic substances from their bodies. These secretions kill the pollen that comes into contact with the ants and result in ants being less likely to be pollinators than other insects that do not secrete such substances.*

2.

3

4.

5.

Feel free to add more!

4. Are any pair of the hypotheses you listed above mutually exclusive? [In other words, if one of the above hypotheses is correct does that mean that another hypothesis must be incorrect? If two hypotheses can both be correct simultaneously then they are not mutually exclusive.]

5. For the first half of the laboratory period, your task is to work with your student group to use your creativity and intelligence to collect data that will evaluate the hypotheses you have been assigned. [In other words, there are no directions on how to do this – you must figure it out yourself.] Below is a short list of equipment available to you. For the second half of the laboratory period, you should pair up with one other student to follow the directions provided on how test the hypothesis that ants secrete pollen-killing substances.

Equipment Available to You:

- *Ant vacuum*: The ant vacuum will help you collect ants from outside.
- *Notepads and clipboards*: One of the most important parts of designing a field experiment is to set up a consistent data sheet that will ensure that if more than one person works to address the same hypothesis, you will collect similar data. A sample data sheet is provided in the materials section, but you are encouraged to design your own data sheets.
- *Ethanol in squirt bottle*: For rinsing forceps etc. to clean off any pollen.
- *Fluorescent powder*: With a toothpick, you may be able to mark ants. To do this, pick up a small amount powder with the toothpick and gently dab a foraging ant on the rear end. Be careful, if you touch the ant too hard, she may run away. Catching the ant first with the vacuum, marking and releasing it, is generally not advisable since the ant often will not return to foraging.
- *Basic fuchsin gel*: Whole ants that have been caught can be squashed in this gel on microscope slide. The gel makes it easier to find pollen because the pollen stains red. You can also take a small bit of unmelted gel, dab it against an insect, and then melt and examine the gel alone (without the insect) to see if contains pollen.
  To use the gel, remove a small blob of basic fuchsin gel and place it on a glass microscope slide. By a small amount, we mean about an amount that is about the size of the square below (in area) and a few millimeters thick (Figure 1).

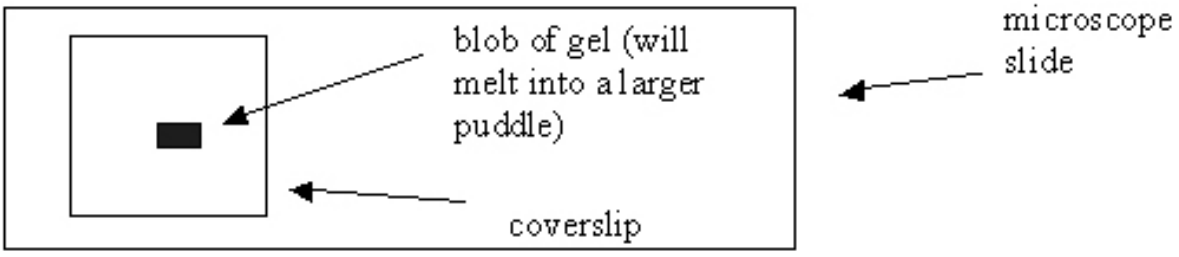


Figure 1. Diagram of microscope slide with basic fuchsin gel.

Holding one end of a slide with a clothespin, gently move the other end back and forth over the flame of a alcohol lamp or rest the side of the slide with the fuschin gel on the edge of a hot plate. Do not put the slide near the wick of the lamp, but hold it a centimeter or more above the wick. After moving it over the flame a few times, you may want to hold it away from the flame or hot plate

and watch as the heat transfers from below the slide to the top of the slide and the gel begins to melt. Try not to boil the gel. Once the gel becomes a small melted puddle, grasp an ant and place it in the gel and relatively quickly, add the coverslip to the slide. The larger the ant, the more difficult it is to lay the coverslip flat. You may wish to break the ant up into different parts and stain each separately. In addition to the method just described, the blob of gel can be rubbed against an insect and then examined for pollen. However, be sure that the gel is really pollen free before touching an insect (what control could you use to show this?).

- *Refractometer*: (may not be available) The amount of sugar in a solution (such as nectar) can be determined with this instrument.
- *Diagram of ant*
- *Stop watch*
- *Your eyes and ears*: Information can be gathered just by watching and paying attention, but design your data sheets well so that you are sure to know what to look for and to keep good records of what you see.

Directions for testing hypothesis #1:
Antibiotic secretions from the bodies of ants reduce the viability of pollen carried by ants
Every student should attempt to test this hypothesis, and the data will be summarized at the end of the lab.

1. You will be working in pairs. One student will be responsible for following germination of the pollen that has not been exposed to ants (NO ANT TMT) and his/her partner will be responsible for following germination of the pollen that has been exposed to ants (ANT TMT). Flip a coin to randomly determine who will do each treatment.
2. Each student will need one depression slide and one plastic pipette bulb tip.
3. Using the forceps, the students should jointly attempt to remove anthers from the living flowers in the lab. The students should smear pollen in the depression on each slide and into the pipette bulb tip. Preferably, both students in a pair should make use of pollen from the same anthers. The reason for this is to control for the fact that sometimes the pollen in a particular anther is simply completely unviable (or only partially viable) to begin with. This ensures that the differences in germination between one student's slide and their partner's slide are not due to the accidental chance that one student obtained a dud anther and the other student didn't. If you are using *Impatiens* flowers, your instructor may need to help you identify the pollen.
4. Next, get your plastic pipette bulb tips ready. These tips will be placed over the pollen in the well. The student who is doing the ANT treatment should obtain an ant and trap it under the tip so that the ant is crawling around on the slide (Fig. 2). Cover the NO ANT treatment with the pipette tip as well as a control. This ensures that the pollen is treated identically in both treatments
5. Let the ant run around on the pollen for 10-15 minutes. Very small ants (such as Argentine ants) will probably climb up into the plastic pipette bulb. If possible, knock the ant down to the slide again or even try holding the entire setup upside down to see if the ant will return to the slide. If you placed enough pollen in the bulb tip, your ant should still contact some of

the pollen, but you should record whether you think the ant contacted the pollen and consider this when evaluating your results.

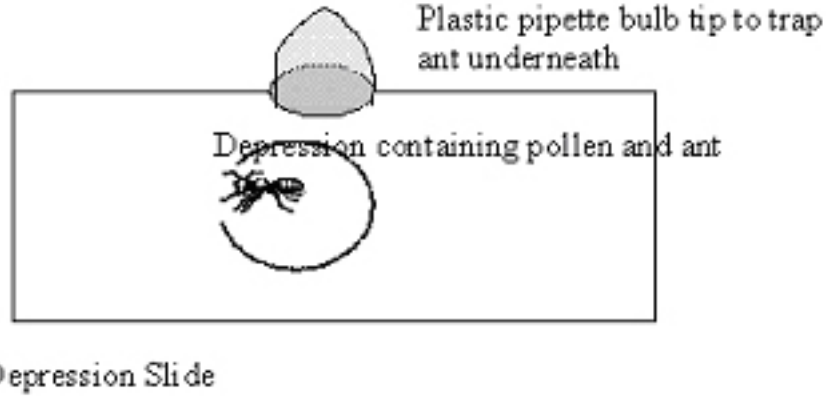


Figure 2. Slide with pipette bulb tip and ant.

6. Remove the ant and pipette tip. You may want to stain the ant in fuschin gel to determine if any pollen is sticking to it. See instructions in the "Equipment available to you" section.
7. For both the ANT and NO ANT treatments, squirt about 4-8 drops of pollen germination media onto the slide. If the ant spent most of the time in the pipette bulb tip, put the pollen germination media into the cap instead and then pour the media and pollen onto a fresh depression slide. Whether you use pollen from the slide or from the bulb tip, you should treat the NO ANT treatment identically. There is no need to add a coverslip.
8. Immediately place the slide on the microscope stage, and lock it into place if possible. Focus on the slide on low power and then go to the highest power, being cautious not to allow the objective to dip into the solution (your instructor may be able to help you determine the maximum power that your microscope will permit for this purpose before you begin).
9. While looking through the microscope, locate an area on the slide that has ample pollen but where the pollen isn't too clumped together. Count the total number of grains and also count the number of germinated grains. The easiest way to do this is to have a tally counter in each hand. For the very first count, there should be no germinated grains.
10. Repeat the above, three more times over the next 15 minutes to 1 hour. The amount of time between each count and the total time will depend on the plant species used. *Impatiens* pollen germinates more quickly than *Petunia* pollen does. It is important not to let the germination media evaporate entirely from the slide during the hour. Add additional media for the next half an hour as necessary. However, try not to add so much media that it overfills the depression well or the edge of the flat slide. Your instructor may suggest that you put a cover slip on the slide to avoid desiccation.
11. When the data has been collected, you can perform a *chi*-square test on you and your partner's data. If you wish to test the entire class's data, you could choose a paired *t*-test or you could just lump all the ANT data and lump all the NO ANT treatments and do one chi-square test for the class.

Table 2. Laboratory observations on pollen germination.

Cell culture well:	Time 0 (0 minutes)		Time 1 (___ minutes)		Time 2 (___ minutes)	
	Total Grains	**Germinated Grains**	**Total Grains**	**Germinated Grains**	**Total Grains**	**Germinated Grains**
Without an ant (control)						
With an ant (treatment)						

Total Grains = germinated + ungerminated grains

Table 3. Summary of the reading taken at Time 2.

Cell culture well:	Proportion Germinated = Germinated Grains /Total Grains
Without an ant (control)	
With an ant (treatment)	

Keep this data to share with the rest of the class.

Note: Beattie et al. (1984) suggested that the metaplural gland is particularly responsible for secreting substances that reduce pollen viability. Not every ant species has functional metaplural glands. Determining whether the ant species you are using has or does not have functional metaplural glands is unfortunately beyond the scope of this lab. If you find no difference between the pollen that contacted ants versus the pollen that did not contact ants, you might consider using this as a topic of conversation.

Literature Cited for Ant-Exercise

Beattie, A. J. 1971. A technique for the study of insect-borne pollen. Pan-Pacific Entomologist 47: 82.

*_____, C. L. Turnbull, T. Hough, S. Jobson, and R. B. Knox. 1985. The vulnerability of pollen and fungal spores to ant secretions: evidence and some evolutionary implications. American Journal of Botany 74: 606-614.

*_____, C. L. Turnbull, R. B. Knox, and E. Williams. 1984. Ant inhibition of pollen function: a possible reason why ant pollination is rare. American Journal of Botany 71: 421-426.

*Gomez, J. M. and R. Zamora. 1992. Pollination by ants: consequences of the quantitative effects on a mutualistic system. Oecologia 91: 410-418.

*_____, R. Zamora, J. A. Hódar, and D. García. 1996. Experimental study of pollination by ants in Mediterranean high mountain and arid habitats. Oecologia 105: 236-242.

*Haber, W. A., G. W. Frankie, H. G. Baker, I. Baker, and S. Koptur. 1981. Ants like flower nectar. Biotropica 13: 211-214.

Herrera, C. M. 1987. Components of pollinator "quality": comparative analysis of a diverse insect assemblage. Oikos 50: 79-90.

*Hickman, J. C. 1974. Pollination by ants: a low-energy system. Science 184: 1290-1292.

Hölldobler, B. and E. O. Wilson. 1990. The Ants. Belknap Press, Cambridge, MA, 732 pages.

*Janzen, D. 1977. Why don't ants visit flowers? Biotropica 9:252.

Kearnes, C. and D. W. Inouye. 1993. Techniques for Pollination Biologists. University Press of Colorado, Niwot, CO, 583 pages.

Peakall, R., S. N. Handel, and A. J. Beattie. 1991. The evidence for, and importance of, ant pollination. In C. R. Huxley and D. F. Cutler, [eds.], Ant-Plant Interactions, pp. 421- 429, Oxford University Press, Oxford, 601 pages.

Puterbaugh, M. N. 1998. The roles of ants as flower visitors: experimental analysis in three alpine species. Oikos 83: 36-46.

Scott, R. 1995. Pollen tube formation and the central dogma of biology. Pages 121-134, *in* Tested studies for laboratory teaching, Vol. 16 (C. A. Goldman, editor). Proceedings of the 16th Workshop/Conference of the Assocation for Biology Laboratory Education (ABLE), 273 pages.

Wyatt, R. and A. Stoneburner. 1981. Patterns of ant-mediated pollen dispersal in *Diamorpha smallii* (Crassulaceae). Systematic Botany 6: 1-7.

*Articles marked with an asterisk are very good articles to give students to read and to use as references for a writing up a laboratory report.

Introduction to Butterfly-Exercise

This exercise is a test of whether Painted-Lady Butterflies can learn to associate certain colors with a sugar-water reward. The exercise follows the scientific method and reinforces experimental design. Students enjoy the exercise because they raise the butterflies from larvae and construct artificial flowers to test learning.

Materials for Butterfly-Exercise

- Carolina or Wards replacement Painted Larvae butterfly kits (Wards: 87 W 5654; Carolina: ER-14-3992 or WW-14-3992 (online)) * The "replacement" kits come with larvae food, and are completely adequate if you have your own butterfly cage. There is no need to purchase the "original" kit.
- A tack or something to poke holes in the lids of the larvae containers (sometimes the supply company already does this)
- At least three butterfly cages (a simple way to make this is to buy a sturdy see-through mess laundry bag and to drape it over a legal sized hanging office filing rack; you can make better and bigger ones with a little carpentry & bridal veil). See Fig. 3. For a sample picture of a better design, see: <*http://www.georgetown.edu/departments/biology/faculty/weissm/#Butterfly%20Learning*>

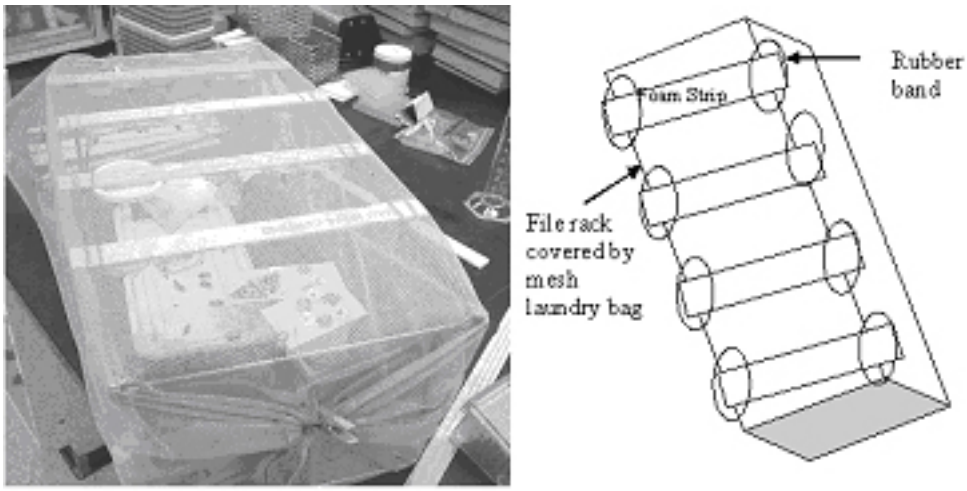


Figure 3. Easily constructed butterfly cage.

- One or two pieces of foam board (20" x 30" x 3/16") approximately 5 mm thick that you can easily put tacks in and that the tacks won't fall out (an alternative is paint stirrers)
- A exacto knife or razor blade to cut the foam board into strips
- Rubber bands to secure the foam strips to inside the cage
- Tacks to hold the larvae to the foam strips
- Brightly colored construction paper
- Scissors
- One or several packets of cheese cloth
- Several PVC t-joints from the hardware store. The T needs to be perfectly symmetrical & preferably the tubing needs to be 1≈ inches or larger

- 20 artificial flowers of one color and 20 artificial flowers of another color. I made these with the small medicine cups/vials that came in the Carolina and Wards kit (after being washed). I make a round bull's eye circle for the flower with a hole in the middle out of construction paper. I cut a small hole in the cleaned lid of the vial/cup. I wrapped and twisted a piece of cheese cloth to make a wick. I stuck this through the hole in the lid and put the construction paper bullseye on the lid. I then put the lid on the vial (Fig. 4).

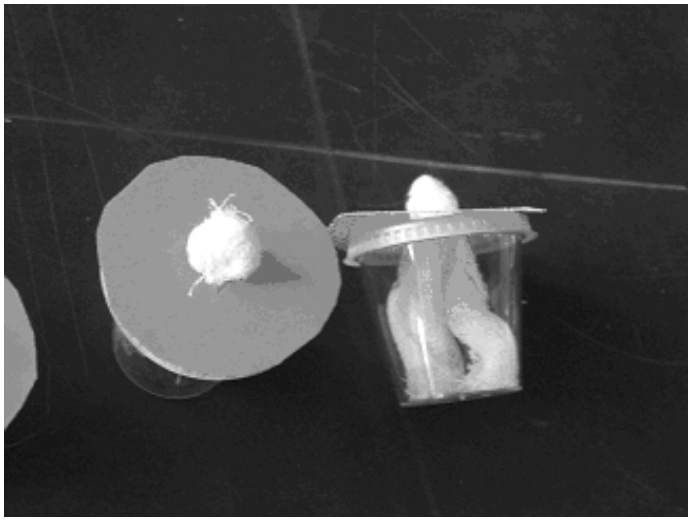


Figure 4. One possible construction of the artificial flowers.

Instructor Notes for Butterfly-Exercise

This exercise will work, but the methods are still less than ideal. My primary complaint with my own methods is that the testing apparatus (the t-tube) probably gives very unrealistic results. Butterflies are usually choosing while flying (Weiss, personal communication), not while being shoved into a small dark hole. This is something to discuss with the class. You may also decide to try testing them in another way. The reason for using my methods for testing is that they force the butterfly to make a choice very quickly, and avoid a lot of frustration watching butterflies without them making any choice. Teachers should expect that they may get low butterfly hatching rates. Further, our results so far suggest that the butterflies do not learn, though they may show a slight innate preference to yellow (Weiss, personal communication). This is in contrast to studies done on other butterflies (actually, this fact makes it a more challenging & in my mind interesting lab for students!). Don't let yourself be confined by my methods on making a butterfly cage, my artificial flowers, or my t-tube testing apparatus. You and your students may well be able to come up with a better design!

Either you or your students may prepare the larvae for pupation and hatching. The Wards or Carolina supply replacement kit come with most of the supplies you need including vials to hatch the pupae and food to feed them, and you should read and follow the directions that come with the kit. The food comes in one or two large vials of media. These large vials are actually quite good for rearing small numbers of the larvae however the kits will tell you to use the much smaller vials (medicine cups) provided. If you use the smaller vials, make absolutely certain that you fill only the bottom ≈ of the vial with media. Otherwise the pupae will hang in the left over media and may not

do well. However, you also do not want to have the larvae run out of food before they are ready to pupate! Regardless, I have found that if you follow the directions in the kit, you end up putting slightly too much food in vials. Make sure that the lids have pin holes in them so that the larvae get air. In the most recent kit that I purchased, the instructions called for putting Kimwipes between the cup and the lid of the vial. I found that the Kimwipes fell apart, and that I had better success when I simply allowed the larvae to pupate on the lid of the vial. In other words, after putting a layer of media in the small vial, just put one larva in each of the small vials and then put the cap on. You should only put one larva per small vial, but if you have larger vials available, you can put more larvae per vial. For instance, if you use a size more like the size that the media comes in, you can put 5 or 6 larvae per jar. If you have left over media and larvae and you have used up all the little vials, it is a great solution to put them in the larger vial that the media came in.

The larvae will take a week or two to pupate. Depending on how large the larvae were when you received them, you may only have to wait a few days for the first larva to pupate. (If the larvae are very tiny, you should actually wait a few days and let them get larger before moving them to the smaller vials.) Place the larvae in an area out of direct sunlight where they will not be disturbed. Do not expect more than a 10-20% survival rate of larvae to butterflies, though you may get lucky, and I have found that my hatching success improved with experience. Regardless, prepare the students not to expect all larvae to pupate and hatch. Humidity may be important and you may wish to spray your cage with a squirt bottle occasionally.

Once they have fully pupated and hopefully attached themselves to the lids of the vials, you can remove the lid and tack it to the foam strips in your butterfly cage. Hatch all the butterflies in the same cage (the Hatching Cage). If any of the pupae are not hanging or fall off, simply place them gently on a paper towel at the bottom of the butterfly cage. The butterflies will live about two weeks. You may want to put plain water in the cage as well to ensure the butterflies have enough to drink.

Ideally, each butterfly should be tested independently. However, if you have inadequate butterflies, you can test the butterflies repeatedly. Initially, I had thought it would be ideal to simply place a grid of red and yellow squares (the same paper used to make the flowers) on the floor of the Testing Cage and simply record where each butterfly chooses to land. This may work if the butterflies are in bright sunlight. However, I had trouble getting the butterflies to land anywhere on the grid, so the student outline is for a t-tube choice chamber.

Each student or pair of students can be responsible for testing one or more butterflies especially if you have multiple t-tubes (I bought my t-tubes for less than a dollar a piece at the hardware store). I put one yellow (empty, no sugar water) and one red flower (empty, no sugar water) in two 150 ml glass beakers. I then laid each glass beaker (on its side) on either end of the t-tube. For the central opening to the t-tube, I constructed a small stiff opaque (black) circular “door” that I partially taped.

Sample data are provided in Appendix D

Student Outline to Butterfly-Exercise

What features of an animal enhance the potential of that species to be a pollinator? One characteristic that can make a difference is whether an insect visits flowers indiscriminate of plant species or whether the pollinators are "faithful" and transfer pollen among flowers of the same species. A flower visitor that moves pollen from one plant species to flowers of another plant species will be unlikely to provide any benefit to either plant species. What determines whether a flower visitor will be faithful? Either the visitor must have an innate preference for a particular flower species or it must learn to prefer a particular flower species. Martha Weiss, a researcher at Georgetown University, has shown evidence of both of these: some butterflies have preferences for certain colors, and furthermore, those preferences can change depending on experience. In other words, butterflies can learn to be faithful to certain plant cues. The innate preferences and learning ability of butterflies help to make them a better pollinator than if they did not have these abilities. (Can you think of several reasons why this statement is so?) Data on the preferences and learning abilities of butterflies are scarce, and little has been published on this topic with regards to the Painted Lady Butterfly (*Vanessa cardui*). In this exercise, we will investigate whether Painted Lady Butterflies have a flower color preference, and whether the butterflies can be trained to prefer certain colors.

Instructions for Rearing Larvae

1. Each larvae needs to be placed in the small plastic medicine cups with a cap on top. First, wash your hands to try to remove unnecessary bacteria. Then, fill the cup about one-quarter full with the media provided. This media contains some of the plant matter that the larvae would eat in the wild. Try not to overfill the cup or the left over media will rot and the pupae will hang in the media and die. After you put the media in the cup, very gently move a single larva into the cup with a paintbrush. Make sure the lid has several small "breathing" holes in the top (if not, make these with a tack or pin). Place the cap tightly on top of the cup.
2. Wait a week or two for them to pupate. Depending on how large the larvae were when you received them, you may only have to wait a few days for the first larva to pupate. Place the larvae in an area out of direct sunlight where they will not be disturbed. Do not expect more than a 10-20% survival rate of larvae to butterflies. (Expect that you will get many fewer butterflies than you have larvae to start with, though you may get lucky!)
3. Once they have fully pupated and hopefully attached themselves to the lids of the vials, you can remove the lid and tack it to the foam strips in your butterfly cage. Hatch all the butterflies in the same cage (the Hatching Cage) provided by your instructor. If any of the pupae are not hanging or fall off, simply place them gently on a paper towel at the bottom of the butterfly cage.
4. Once the butterflies have hatched, give them time to dry their wings (a day). Randomly assign each butterfly to one of the other two cages (the "Yellow" and the "Red" cage, or whatever two colors of flowers you are using). To randomly assign butterflies, count the butterflies, and number the same number of small pieces of paper as there are butterflies. Place the numbers in a grab bag, and then blindly select half the numbers for the Yellow Cage and half the numbers for the Red Cage. Then each butterfly caught should be numbered sequentially and placed in their appropriate cage. Butterflies can be moved using a small fish tank mesh net (with handle). You

can also catch them gently in your hands. A small loss of powder from the butterflies' wings will not hurt the butterflies. They can also be handled gently by the thorax and legs.

Training Your Butterflies

1. Make your artificial flowers. Take the small cups/vials with lids. Make a round bull's eye circle for the flower with a hole in the middle out of construction paper. Cut a small hole in the cleaned lid of the vial. Wrap and twist a piece of cheese cloth to make a wick. Stick this through the hole in the lid and put the construction paper bull's-eye on the lid as shown in Fig. 4. Place ten flowers of one color and ten flowers of the other color in both cages. For example, if your two colors are red and yellow, place 10 red and 10 yellow in both the Red Cage and the Yellow Cage. In the Red Cage, fill the red flowers with 10% sugar water and leave the yellow flowers empty. In the Yellow Cage, fill the yellow flowers with the sugar water and leave the red flowers empty.
2. Over the next several to 10 days, replace the sugar water in the appropriate flowers as necessary. You are now "training" the butterflies. The butterflies will typically live two weeks.

Testing Your Butterflies

1. Prepare your t-tube for testing (Fig. 5). An empty and unused artificial flower of one color should be put on one side the t-tube and the other color on the other side. A coin should be flipped to determine which side will get which color. The butterflies are very responsive to light so if possible, you want to set up the choice chamber so that both sides are getting equal amounts of light (and one is not pointing toward a window for example).

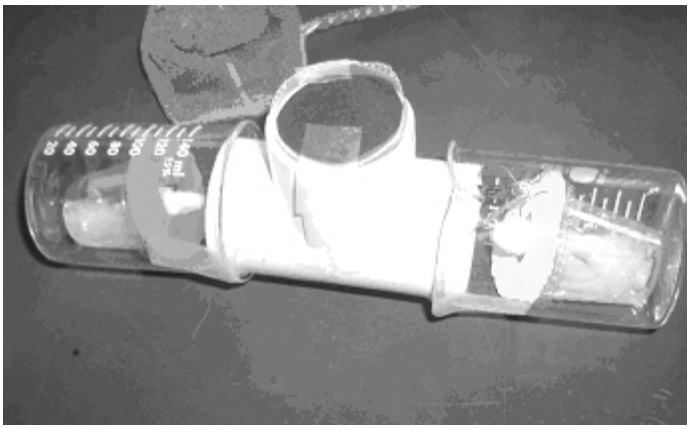


Figure 5. One possible arrangement for t-tube testing chamber.

2. Take a butterfly and gently push it into the central opening, and wait for the butterfly to choose a flower. Summarize the entire class data in the table below.

Butterflies were trained to what color?	In the choice test, how many butterflies chose Color 1?	In the choice test, how many butterflies chose Color 2?
Trained to Expect Reward with Color 1		
Trained to Expect Reward with Color 2		

3. Use a *chi*-square test to test whether the butterflies trained to expect a reward from Color 1 showed a preference. Do a separate test to see if butterflies trained to expect a reward with Color 2 showed a preference.

   Ho: There is no association between color and butterfly choice.
   Ha: There is an association between color and butterfly choice.

$$X^2 = \sum \frac{(O-E)^2}{E}$$

   df=1,
   critical *chi*-square = 3.84

Acknowledgements

I came up with the idea for the lab after reading literature by Martha Weiss. I also received considerable advice via email from Dr. Martha Weiss and her students Doug Blackison and Erin Wilson. This lab would not be here without their input!

Literature Cited for Butterfly-Exercise

Kelber A. 1996. Colour learning in the hawkmoth Macroglossum stellatarum. Journal of Experimental Biology 199 (5): 1127-1131.

Lewis, A.C. 1986. Memory constraints and flower choice in *Peris rapae*. Science 232: 863-865.

Weiss, M.R. 2000. Brainy butterflies; insects are willing to learn. Natural History 109: 38-41.

Weiss, M.R. 1997. Innate colour preferences and flexible colour learning in the pipevine swallowtail, *Battus philenor* (Papilionidae). Animal Behaviour 53: 1043-1052.

Weiss, M.R. 1995. Floral color change: a widespread functional convergence. American Journal of Botany 82:167-185

Weiss, M.R. 1991. Floral colour changes as cues for pollinators. Nature 354: 227-229

Appendix A: Data Sheets for Ant-Exercise

Sample Data Sheet

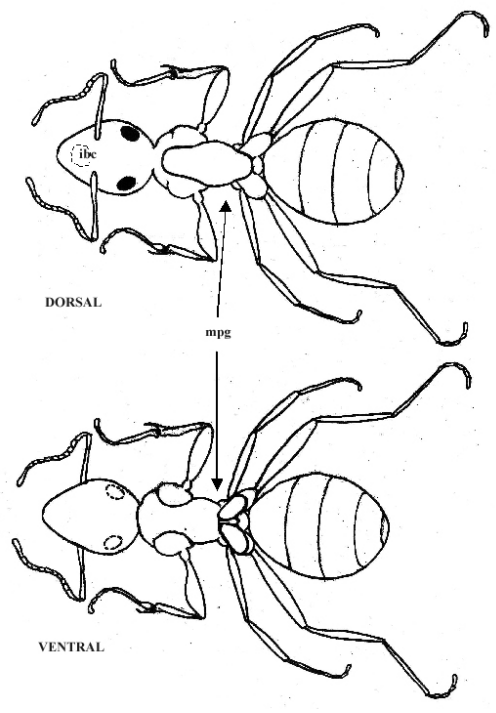
Field Observations

Total Number of Ants Found on Flowers:_____

Total Number of Species of Plants with Ants on Flowers:_____

ANT	DESCRIPTION OF PLANT	TIME SPENT WATCHING ANT (MINUTES)	NOTES ON ANT BEHAVIOR (Did ant move among flowers? What did ant appear to be doing? Maximum distance moved between flowers?)
example: #1	orange-flowered jewel weed	5 minutes	stayed in flower entire time; appears to be collecting nectar
example: #2	yellow composite	10 minutes	stayed on flower for 3 min. - crawled down stem and didn't return to a flower
etc.			

Figure 6. Diagram of ant. You might consider indicating where on the ant you have observed pollen grains. You may notice an abundance of pollen in the infrabuccal chamber (*ibc*). Take note of how close the pollen that you observe on the ant is to the approximate location of the metapleural gland (*mpg*). The closer to the gland, the more likely the pollen will contact the secretions.



Appendix B: Recipes for Ant-Exercise

Recipes

Pollen Germination Media

100 g of sucrose, 0.1 g of H_3BO_3, and 0.3 g of $Ca(NO_3)_2.4H_2O$ made to 1,000 ml with distilled water (weigh carefully)

We use plain old table sugar for the sucrose. Rather than putting a preservative into the media, we have the students make it fresh the day of the lab (or we make it ourselves the day before) and dispose of left-overs. The media can be kept in the refrigerator for short periods of time (a week). However, it is a tasty media and well-liked by microorganisms so it will go bad quickly if you don't add a preservative or don't use it quickly. I do not filter the media (I just shake well!). If you have students make the media themselves, ask them to think about what the sugar is for and why it is so important to weigh carefully. [...takes energy for little pollen grains to grow tubes many times their size & the osmotic pressure of the solution needs to be right or the pollen grains will burst or shrink...]

Pollen Staining Gel

The pollen staining gel needs to be made several hours before lab. One batch can last many years, depending on how many students need to use it. We make this ourselves and do NOT ask students to make it. The recipe is in the appendix. Students should wear gloves when handling the gel (and so should you).

Sprinkle one packet of Knox gelatin onto the surface of 25 ml distilled water in a glass Pyrex or Kimax petri dish (or in a 600 ml beaker). Allow gelatin to absorb water (~ 1 min.). Place covered dish on hot plate at a setting of 4 (~ 160^{o}C) just until mixture clears. Carefully remove the dish from the hot plate and gently stir in 22 ml glycerin and 0.005-0.01 g crystalline basic fuchsin (also 0.1g phenol if desired). Beattie (1971) suggests the standard gel be the color of claret. Re-cover the dish and allow to cool at room temperature until the gel is solidified. Seal with parafilm, invert the dish, and refrigerate for long-term storage (until it molds).

Ant Vacuum

An ant collector can be made with a vial and a two-holed stopper; two tubes are inserted into the stopper (Figure 7). A little cloth mesh is stuck into the vial end of one of glass tubes; students point tube at an ant and suck on the mesh-containing tube.

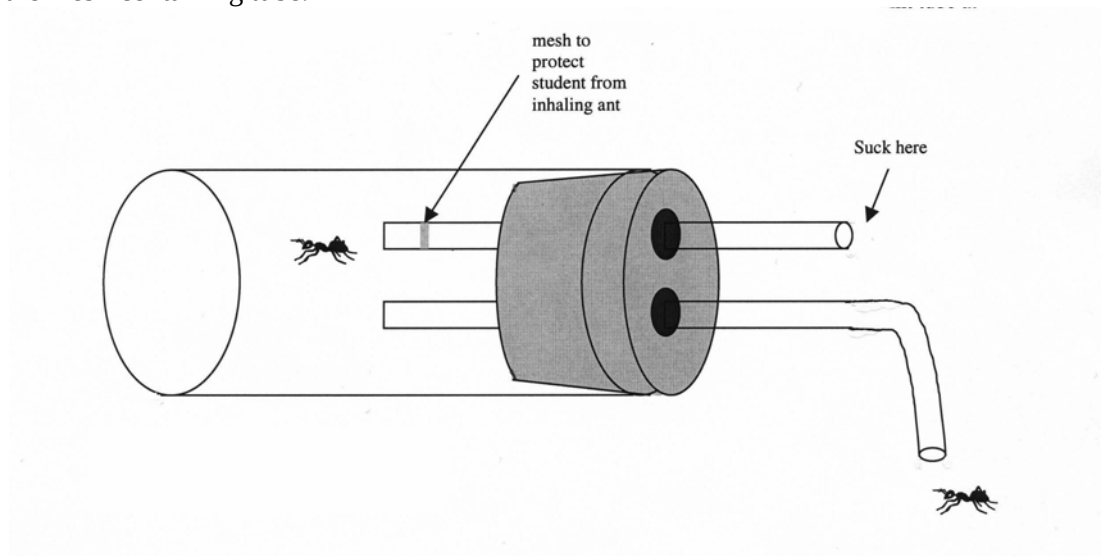


Figure 7. Ant vacuum.

Appendix C: Data and Hypotheses for Ant-Exercise

Some additional hypotheses that students may come up with for why reports of ant pollination is rare:

1. Researchers have not looked hard enough for ant pollination, and it is actually more common.
2. Ants do not visit flowers often enough.
3. Ants do not move sufficient distances quickly enough to cross-pollinate flowers.
4. Ants are too small and do not contact the reproductive parts of most flowers.
5. Ants do not seek nectar but instead are chewing insects that destroy rather than pollinate flowers when they do visit flowers.
6. Ant bodies are smooth and pollen does not stick to the body of ants.
7. Ants groom very frequently so any pollen is removed from ants' bodies by their grooming behavior.
8. The foraging pattern of ants is such that they usually go from one flower to their nest and rarely from one flower to another, reducing the likelihood of cross pollination.

Sample Data & Sample Lab for Ant-Exercise
Null Hypothesis: Pollen germination rate is not associated with ant presence.
Alternative Hypothesis: Pollen germination rate is associated with ant presence.
Alpha = 0.05

Treatment	**# Ungerminated Grains**	**#Germinated Grains**	**Total**
ANT	58 (143*88/204 = 61.7)	85 (143*116/204 = 81.3)	143
NO ANT	30 (61*88/204 = 26.3)	31 (61*116/204 = 34.7)	61
TOTAL	88	116	204

Observed values with expected values in parentheses.

To calculate the expected frequency (O= observed value, E= expected value):

Chi-square = $$X^2 = \sum \frac{(O-E)^2}{E} = \frac{(58-61.7)^2}{61.7} + \frac{(85-81.3)^2}{81.3} + \frac{(30-26.3)^2}{26.3} + \frac{(31-34.7)^2}{34.7}$$

Chi-Square = 1.305
df = 1
Critical value 3.84

Since our value is less than the critical value, we can not reject the null hypothesis.
(How to calculate the expected values: Nrow*Ncolumn/Ntotal)

Data on whether ants visit flowers: <*http://www.shout.net/~jhilty/*>

In 1929, Charles Roberston published a book called: Flowers and Insects. In this monograph, he attempted to list every single flower visitor than he observed visiting all of the plant species in an Illinois prairie. His work is one of the very few complete pictures of pollination (or at least flower visitation) in an ecosystem. This database has been put online in a format that students can easily access. You can assign students to research sets of species from this database to determine the number of species of animals that visit their flowers. The process of going through this dataset really changes the students' view of pollination.

Flower-Visiting Insects of Slender False Foxglove

- *Agalinis tenuifolia* (Slender False Foxglove)
  (Bees collect pollen or suck nectar; beetles gnaw on the flowers; flies feed on stray pollen & are non-pollinating; Halictid bees collect stray pollen & are non-pollinating; butterflies suck nectar & are non-pollinating)

- Bees (long-tongued)
  Apidae (Apinae): *Apis mellifera fq*; Apidae (Bombini): *Bombus fraternus, Bombus griseocallis, Bombus impatiens fq, Bombus pensylvanica fq;* Anthophoridae (Ceratinini): *Ceratina dupla dupla;* Megachilidae (Megachilini): *Megachile brevis brevis fq cpt, Megachile latimanus, Megachile mendica*

- Bees (short-tongued)
  Halictidae (Halictinae): *Augochlorella striata, Lasioglossum versatus;* Andrenidae (Panurginae): *Calliopsis andreniformis fq icp, Heterosarus parvus (Mch)*

- Flies
  Syrphidae: *Eupeodes americanus*

- Butterflies
  Pieridae: *Colias philodice, Pieris rapae*

- Beetles
  Meloidae: *Pyrota mylabrina*

Appendix D: Sample Data for Butterfly-Exercise

Pink-Trained Butterflies:

Null Hypothesis: The butterflies have no preference for a particular color flower.
Alternative Hypothesis: The butterflies have a preference for a particular color flower.
11 chose Pink
13 chose Yellow
Total = 24
Expected (12 yellow, 12 red)

$$X^2 = \sum \frac{(O-E)^2}{E} = \frac{(11-12)^2}{12} + \frac{(13-12)^2}{12} = 0.683$$

df=1,
critical *chi*-square = 3.84
Since the test *chi*-square is less than the critical, the null hypothesis can not be rejected.