

Chapter 14

Herbivory and Anti-herbivory: Investigating the Relationship Between the Toxicity of Plant Chemical Extracts and Insect Damage to the Leaves

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Introduction

This investigation was developed for laboratories in a first-year introductory biology course for prospective science majors, and can also be appropriate for more advanced courses in ecology, evolution, or plant physiology. Our primary objectives were two-fold: 1) to model the integration of field and laboratory work in scientific research, and 2) to provide an open-ended, inquiry-based exercise for first-year students. The laboratory component of “Principles of Biology” meets weekly for 3 hours. We conduct the exercise over a three-week period; however, the investigation can be adapted for 1 or 2 weeks. E.g., if the field component (Part I) is omitted, leaves that are previously collected by the instructor can be furnished to students for the Part II extract preparation and bioassay. Alternatively, the third week, reserved for quantitative analysis of the results and computer graphing, can be omitted instead of, or in addition to, the field component (Part I).

The study of plant-animal interactions is an area of great interest in ecology (e.g. see volume 77 of *Ecology*, 1996), both from an evolutionary perspective and from a practical standpoint. Because an investigation of plant-herbivore interactions, in particular, uses knowledge and techniques from several subdisciplines - ecology, biochemistry, evolution, physiology, plant morphology - it provides a model for the integrated nature of research to beginning students. In addition, this field demonstrates direct connections to relevant, “newsworthy” issues such as our search for pharmaceutically useful chemicals from vanishing plant species, and the use of biological control methods in efforts to improve agricultural productivity. For further reading see Beck (1965), Futuyma (1983), and Jolivet (1992).

This lab will investigate the potential relationship between toxicity of crude chemical extracts prepared from leaves of a selected tree species and the level of insect herbivore damage sustained by those trees. Toxicity is gauged by a bioassay based upon survival of Brine Shrimp (*Artemia salina*) over a 24-hour period (McLaughlin 1991). Students are asked to generate their own hypotheses for this relationship. For example, students may propose that extracts from leaves showing heavy herbivore damage may prove to be less toxic than leaves that do not show as much damage; we could call this the “pre-attack barricade” hypothesis. Alternatively, students may propose the “post-attack response” hypothesis, that is, plants showing heavier insect damage will have higher levels of toxicity, presumably because they have increased the rate of production of allelochemicals *in response to* a prior onslaught by herbivores. A third possibility is that levels of toxicity are not related to the amount of herbivore damage observed in the leaves.

General Timetable

Week 1

Students are taken to a field investigation site, they examine and quantify the degree of insect herbivory on a selected tree species, and collect leaf material from 2 groups of trees (e.g. heavily-munched versus lightly-munched individuals). The leaves are re-refrigerated and used for the preparation of crude extracts in week 2. Time required: approximately 3 hours total (30 minutes for instructor's introduction at the field site, 60-120 minutes for collecting data, and the remainder for transport to and from the field site); data collection time can be adjusted up or down by modifying the number of trees students are required to sample.

Week 2

Students prepare crude extracts of chemicals from the leaves in both "herbivory" groups, prepare solutions of each extract at 3 different concentrations, and then count and add brine shrimp to each sample and to control solutions. After 24 hours, students return to the lab to count the numbers of surviving and non-surviving shrimp. Time required: one 3-hour lab period and an additional 30 minutes 24 hours later.

Week 3

Optional. We devote a 3-hour lab period to analyzing the results from this exercise. We teach the use of the Chi-squared "goodness-of-fit" test and we begin developing computer skills by constructing a data file and simple bar graphs using the SYSTAT software program.

Materials

Part I: Quantifying Variation in the Degree of Herbivory (Field Component)

For the entire class, we take a first-aid kit, a loud whistle, and insect repellent, if needed. Distribute one 9 × 13 inch plastic bag to each group of 4 students.

Each bag for every 4 students contains:

- Clipboard (1)
- Pencil (1)
- Table 1 data sheet (1)
- Figure 2 sheet, Appendix C (1)
- Grid, e.g. 2 squares/cm, but any size can be used, reproduced onto plastic overhead sheets (2)
- Piece of white posterboard, 8.5 x11 inches, (1)
- Bandana to be used as a blindfold (1)
- Waterproof marker (1)
- Self-sealing plastic bags, small (4)
- Flagging tape (1 roll or 10 - 20cm strips)
- Centimeter ruler or other measuring device (1)
- Map of field site (1)

Optional items for each bag, if insect herbivores are to be collected:

Scintillation vials (or equivalent screw-cap vials) half-filled with 70% ethanol (4)
Forceps (1)
Scissors (1)
Hand lens (1)
Insect field guide (1)
Index cards (2-4)

Part II: Bioassay for Toxicity of Leaf Extract from Plants Sustaining Different Levels of Insect Herbivory

For each classroom of 24 students:

Separatory funnels- 1 liter, containing Brine Shrimp (Appendix B) (2) equipped with airstones
Balance capable of weighing to .001g (1)
Plastic weigh boats, small (10)
Flask, 2-liter, containing 2% seawater (Appendix B) (2)
Sign-in sheet for 24-hour shrimp checks (1)
Methanol, 100% stock bottle in fume hood (1)
Pipette, 5 ml and bulb for methanol (1)

For each group of 4 students:

Mortars and pestles (2)
Air pump (One pump with multiple outlets or with a single outlet connected to multi-channel adapter available in aquarium supply stores; each outlet is connected to a 50 cm length of rubber tubing, which is connected to the top end of a 5-3/4 inch Pasteur pipet. The top of each pipet should be fitted with a small wad of sterile cotton to help filter the air.)
Pipets- 5 ml (10)
Pipet bulb, red (2)
Pasteur pipets (20)
Pipet bulbs, yellow latex (4)
Vial rack, Wheaton 30-50 vial capacity (1) or equivalent
Screw-cap vials, 17 x 60 mm (30) with one vial filled with dye to the 5 ml level
Waterproof glass markers (2)
Squeeze bottle containing 2% seawater (Appendix B), (1)
Pipetman- P1000 (1) with blue tips (16) *or* micropipettes-500 microliters (10) and plunger
Pipetman- P200 (1) with yellow tips (16) *or* micropipettes-50 microliters (10) and plunger
Pipetman- P20 (1) with yellow tips (16) *or* micropipettes- 5 microliter (10) and plunger
Petri dishes, glass, approximately 3 cm diameter (2)
Label tape, (1 roll)
Beakers - 50 ml (6)
Dissecting microscopes (2-4)
Scissors (1)

Notes for the Instructor

1. Select a field site within a week before the scheduled lab; early fall is a good time since a season of herbivory will have preceded the investigation. You may wish to select a tree species likely to have toxic effects, e.g. Oaks (*Quercus spp.*), Sassafras (*Sassafras albidum*), or Black Cherry (*Prunus serotina*), or investigate some other species for which the results may be completely unknown, e.g. American Beech (*Fagus grandifolia*), Sugar Maple (*Acer saccharum*), or American Basswood (*Tilia americana*). Many comparisons are possible, e.g. contrast different species, or the same species between two seasons, or young versus older members of the same species, etc.
2. In the field, discuss the importance of consistency in a) definitions and categories for “munched” versus not, and what subset of the herbivory possibilities will be counted. Make sure all students can correctly identify the tree species you will use. Go over safety issues, such as boundaries, time to meet back at the meeting site, what to do if a student thinks s/he is lost, staying with partners, etc. Point out any biological hazards present at the site (e.g. poisonous snakes, insects, or plants). Identify any members of your group who may be highly allergic to bee or wasp stings.
3. We routinely have teaching assistants or instructors collect “extra” sets of heavily-munched and lightly-munched leaves as backups.
4. After the class data for Part I is compiled, it is instructive to demonstrate, or have the students conduct, a statistical t-test on the “percent munched” values for the heavily-munched versus lightly-munched groups. This will test whether the two groups of leaves (to be used later in the bioassay) are indeed statistically different groups (i.e. represent leaves from different populations). If they aren’t, then the toxicity comparison between lightly-munched and heavily-munched leaves is invalid.
5. Begin readying the Brine Shrimp tanks and supplies for Part II during the week you are conducting Part I (see Appendix B); it is a good idea to start several Brine Shrimp cultures staggered over several days so that you have plenty of shrimp available for lab sections that may meet on different days of the week.
6. During Part II, students are advised to use methanol in the fume hood only. They should also use gloves and safety glasses when handling methanol. We have one TA stationed at each fume hood while students are completing this step of the protocol. Advise your students not to breathe directly over vials containing methanol that are being evaporated with air lines.
7. It is advisable to have a TA or instructor staff the lab during the time when students must return for the 24-hour shrimp survival counts. We stock a section of the lab with small glass Petri dishes, squirt bottles containing plain seawater, Pasteur pipets, and dissecting microscopes. Post an extra copy of the protocol near the sign-in sheet. Clearly label where students are to deposit used shrimp and glassware along with a clean-up protocol.

Student Outline

Objectives

A. Concepts

1. To appreciate the ever-increasing importance of integrating “field” work with “lab” work in answering scientific questions.
2. To understand how to evaluate *variation* among individuals of the same species and the potential *consequences* of that variation.
3. To understand, through one in-depth example, the nature of the chemical “arm's race” between plants and herbivores, and to appreciate the consequences of this co-evolution to both parties involved, to the entire natural community of which these creatures form a part, and to humans through practical possibilities in medicine and agriculture.

B. Skills

1. To begin to learn field identification of a few common local organisms; using field guides and supplementary material for identification.
2. To learn how to state scientific questions and hypotheses and how to evaluate them.
3. To learn techniques such as pipetting, making dilutions, using a dissecting microscope, making careful counts of organisms, and running a common bioassay for toxicity.
4. To use the library to find references from scientific journals.
5. To develop teamwork while working with a group on a scientific investigation.
6. To learn to write some components of scientific papers.
7. To gain additional experience in using statistics (Chi-squared Test) to answer biological questions.

Background

This two-part lab will investigate the potential relationship between levels of insect herbivory in a native forest species (Part I) and the toxicity of defensive chemicals in those plants, as evidenced by a bioassay (Part II).

All higher plants contain **secondary substances**: these are chemicals that are not part of such major organic groupings as carbohydrates, proteins, fats and nucleic acids, that are of irregular occurrence (present in some plant species but not others), and that mostly have no known role in the metabolism of the plants in which they occur. Secondary substances have many different functions; of interest in this exercise is their role as chemical signals. Chemical signals act on different levels in the living world. Substances produced by one tissue that influence another tissue within the same organism are hormones. Those produced by one individual and influencing another individual of the same species are pheromones. Those active between different species are **allelochemicals** (Whittaker and Feeny 1971, Whittaker 1975).

Allelochemical interactions are a major realm of species adaptations that are normally invisible to us. Tannins, lignins, terpenes, alkaloids (such as caffeine), nicotine, strychnine and curare, and organic cyanides are just a few examples of the chemical arsenal that plants have evolved to defend themselves against animal consumers and pathogenic microbes (Jones and Firn 1991). One example is the way in which tannins, sequestered in vacuoles in the leaves of oaks and other plants, combine with leaf proteins and digestive enzymes in a herbivore's gut, thereby inhibiting protein digestion. Thus, tannins considerably slow the growth of caterpillars and other herbivores (Feeny 1969). In another study, Guglielmo et al. (1996) demonstrated that the cost to herbivorous Ruffed Grouse (*Bonasa umbellus*) of detoxification of coniferyl benzoate (found in the flower buds of quaking aspen, *Populus*

tremuloides) was significant; in addition, digestive efficiencies of grouse were reduced via dilution of utilizable nutrients by plant secondary metabolites. Of course, herbivores may counter such toxic effects through the evolutionary modification of their own physiology and biochemistry (Brattsen 1979, Tallamy 1986). Herbivore species that evolve detoxification mechanisms of secondary chemicals may be able to specialize on plant hosts that are poisonous to most other species.

There is increasing evidence that plant defenses may be induced by herbivore damage (Belsky 1986, Jones et al. 1993). That is, in response to wounds, toxic compounds may be produced - either in the area of the wound or systemically throughout the plant - that reduce subsequent herbivory; the timing of these responses is extremely variable (Ricklefs 1990, Bruin et al. 1995).

In the first part of this exercise, we will evaluate the differences in the level of insect herbivory on the leaves of a selected plant species; after assessing insect damage, two sets of leaves will be collected: those from trees sustaining heavy insect damage (“heavily-munched” leaves) and those from trees sustaining lower levels of insect damage (“lightly-munched” leaves). In the second part, we will screen our two sets of leaves (heavily-munched versus lightly-munched) using a “benchtop” bioassay, the Brine Shrimp Bioassay (McLaughlin 1991). Brine shrimp (*Artemia salina*) larvae, called nauplii, have been used for over 30 years in toxicologic studies. Many researchers are now using brine shrimp as a pre-screen for plant extracts because they provide a quick, inexpensive, and desirable alternative to testing on larger animals. It is known that a positive correlation exists between brine shrimp lethality and 9KB (human nasopharyngeal carcinoma) cytotoxicity; brine shrimp are therefore used in many prescreens for potential anti-tumor activity. In addition, brine shrimp bioassays effectively predict pesticidal activities (a property pertinent to our own study) and respond to a broad range of chemically and pharmacologically diverse compounds (McLaughlin 1991).

Bioassays such as this are used for a variety of purposes - in general they allow researchers to screen for plant chemicals that show biological activity -- that is, that show a targeted biological effect (e.g. effects on survival, growth, or reproduction) on selected research organisms. For example, botanists and biochemists are currently using bioassays to screen unstudied plants for potentially useful, naturally-occurring drugs. In particular, the rapid rates of destruction of tropical rainforests and new research suggesting potential sources of medicinally beneficial chemicals in the tropics have precipitated increased funding for botanical research supported by the National Institutes of Health and several major U.S. pharmaceutical companies. The U.S. National Cancer Institute has also launched an intensive effort in “chemical prospecting”, especially in the tropics. This agency alone is screening some 10,000 substances/year for activity against cancer cells and HIV (the virus that causes AIDS), as well as chemicals useful in the fight against heart disease and many other health problems (Wilson, 1989).

While it is unlikely (but not impossible) that we will discover an unidentified cancer-fighting chemical in the plants investigated, it is important for us to keep in mind that the reason most of those chemicals exist in the first place is the result of co-evolution between plants and herbivores. The more we know about “who” makes those chemicals, *why* they are made, *how* they are made, and the consequences of allelochemicals to both parties involved, the more we will know about the evolutionary process and about potential practical uses of such chemical evolutionary products for our own benefit.

Part I: Quantifying Variation in the Degree of Herbivory on a Selected Tree Species

1. Upon arrival at your field investigation site, you should look for, and examine, different kinds of insect damage on the leaves of a tree species assigned by your instructor. Compare the kinds of damage you find with diagrams from a source such as Hogan (1994; Appendix C: Signs of Animals Eating Plants).

Is the amount of insect damage sustained the same on different plants? The same on different leaves or portions of the same plant? A great deal of *variation* exists in the level of herbivory, often even on the scale of an individual plant. Although there are *many* possible and interacting factors that could contribute to this variation, this investigation will focus on one possibility - toxicity of the plant to insect herbivores.

2. After discussing the problem with your group, develop an hypothesis to explain the relationship between variation in toxicity of plant chemicals and differences in insect damage between different plants. Then, discuss what you would *measure*, and how, *in order to test this hypothesis*. Be ready to discuss this as a class.
3. After a brief class discussion about the protocol for assessing herbivore damage, your class will decide on categorical names to include as evidence of herbivory (Fig. 2, Appendix C). During data collection, you will count only these types of herbivory. Your group will be assigned to sample trees from one area of the forest. Do not overlap with other groups. For each tree you sample:
 - a. Select 5 leaves *at random*, using the blindfold. Do not remove these leaves.
 - b. Sandwich one leaf at a time between the plastic grid and the white posterboard backing; then count the *total* number of grid sections that contain any part of the leaf.
 - c. Count the total number of grid sections that contain any part of a leaf that shows any one of the selected categories of herbivore damage.
 - d. Repeat steps b. and c. for the 5 randomly selected leaves.
 - e. Write the *total* values for b. and c. for all 5 leaves in Table 1, including the kind of herbivory observed, and any notes on actual herbivores found.
 - f. Your instructor may ask you to identify potential herbivores in the field, using a field guide or an insect key, or s/he may instruct you to collect labelled samples for later identification in the laboratory. In either case, you should make an attempt to match up potential herbivores with the kinds of damage observed on the leaves. Use the last column on Table 1 for your observations.
 - g. Tie a small strip of flagging tape to the tree and label it with the corresponding number from column 1 of Table 1.
 - h. Repeat steps a through f for the number of trees assigned by your instructor.
 - i. Complete Table 1 by calculating the “percent leaf squares munched” by dividing column 3 by column 2 for each plant. Circle the plant with the highest level of herbivore damage (as indicated by the 5-leaf sample) and the plant with the least amount of herbivore damage.
 - j. Return to each of those two trees separately. From each, *randomly* (using blindfold) collect 3 leaves and place in a plastic bag. Insert a label which contains your names, date, lab time, instructor and whether the leaves are from the “heavily-munched” plant or the “lightly munched plant”. Do not destroy more of the plant than is absolutely necessary to obtain the 3 leaves. Make sure your plastic bag is sealed to avoid desiccating the leaves. Remove the flagging tape from the plant.
 - k. Remove the flagging tape from *all* of the plants you surveyed, and return the flagging strips to your storage bag.
 - l. Your instructor will show you where to store leaves after you return to the lab; the leaves will be kept refrigerated until you are ready to prepare extracts for the bioassay.

Table 14.1. Herbivory field data sheet.

Date: _____
 Location: _____
 Group Members: _____
 Plant Species: _____

Plant #	# of Leaf Sections	# of Leaf Sections	% of Leaf Sections Munched	Possible ID of Munchers
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

Complete the following questions to familiarize yourself with the kinds of information that should be included in the initial portions of a scientific paper: introduction, study area, and methods. In Part II, you will complete additional parts of an outline for a scientific paper (results and discussion).

Part I Questions

A. INTRODUCTION

1. What is the question that you are investigating?
2. Why is this problem scientifically interesting? That is, why do we want to know the answer to this question?
3. What is your working hypothesis? State your hypothesis and back it up with biological reasons for why you think so.
4. What other work has been done in this area that has influenced the initiation of this study, its predictions, and possible outcomes? Find 4 relevant articles from the primary scientific literature and list those references in the *correct* format below. Suggestions for useful journals:

Annual Review of Entomology, Annual Review of Ecology and Evolution, Nature, Science, Ecology, Phytochemistry, Trends in Ecology and Evolution, Journal of Chemical Ecology, Journal of Plant Physiology, Oecologia, American Journal of Botany, Oikos, Recent Advances in Phytochemistry, American Naturalist.

- a.
- b.
- c.
- d.

B. METHODS (Part I).

1. Study Area: The exact location and a general description of the habitat including dominant vegetation and/or physical characteristics of the study area are typically given for field investigations.
2. Date of study. Time of day and weather conditions are also given if the investigator believes that these factors may play a role in the outcome of the study.
3. Species used - Always give the scientific name(s).
4. What exactly did you do?. Be sure to define any methods of measurement such as “heavily-munched” and “lightly-munched” or “heavy herbivory” and “light herbivory”. You must tell the reader exactly what criteria were used in assigning categories. Give exact sample sizes for all groups. List important variables that were *controlled* (e.g. date leaves collected; species from which they came, etc.).

Part II: A Bioassay for Estimating the Toxicity of Leaf Extracts from Plants Sustaining Different Levels of Herbivory

Protocol for Brine Shrimp Bioassay—Day “0”

1. Divide your group of 4 students into two teams. One team will run the assay for “heavily munched” (**H**) and the other team will run it for “lightly munched” (**L**). Each team (of 2) will follow all of the procedures below, separately. Use separate data sheets to record results for **H** and **L**.
2. Obtain 11, 2-dram vials and a small amount of your plant specimen (either H or L) for preparing an extract. Label the vials 1-11 and EITHER H or L.

We will run two different kinds of control treatments in this experiment. The *water control* (vial #1) will allow us to determine if there are significant effects of the plant chemicals as compared with “normal” survival of brine shrimp over a 24 hr. period. Why is this necessary? Do we expect all brine shrimp placed in seawater in small vials to survive? In addition, vial #2 is a *methanol control*; it will allow us to gauge the effect of one portion of our protocol (the dissolving of plant material in methanol) independently from the effects of plant material. This is necessary because methanol is likely to be toxic to brine shrimp; if not all of the methanol is removed during the evaporation procedure, then an additional variable affecting brine shrimp survival has been introduced. Results from which 2 vials will be compared to test whether or not we have introduced a “methanol effect” in brine shrimp survival? Why then are both control treatments required?

Mark vial #1 as the water control, vial #2 as the methanol control, vials 3-5 with 10 micrograms/ml, vials 6-8 with 100 micrograms/ml and vials 9-11 with 1000 micrograms/ml. These are the 3 *final* concentrations of the plant extract you will test.

3. One team member (a) should prepare the plant extract while the other team member (b) should prepare airlines.

Team Member (a): Carefully dry a mortar and pestle. Remove all traces of animal material from your plant specimen and place the plant specimen in the bottom of the mortar. Grind it *thoroughly* for *no less than 5 minutes*. Weigh out 40 milligrams (not micrograms or grams) of the plant material on an electronic balance and then transfer the 40 mg sample to a small beaker or vial. Take the sample to the *fume hood*. You will find the methanol at the hood because **it is highly flammable and toxic**. *It is important to avoid contact with your skin and eyes. Remind yourself of the location of the eyewash stations, just in case.* Methanol is being used as a general solvent for the chemicals present in the plant tissue. Carefully add 4 ml of 100% methanol to the plant material. (What is the concentration of your extract at this point? $40 \text{ mg/ml} = 10 \text{ mg/ml}$). Allow the material to dissolve in the methanol for 5 minutes.

Team Member (b): Ready several airlines for evaporating the methanol from samples you will prepare later. You will want a gentle stream of air coming out of each of the glass pipettes attached to the airlines. Test for “gentleness” by trying the air stream in a beaker of tap water FIRST.

The remainder of the protocol is carried out by both team members:

4. Obtain 3 micropipettes for measuring 500 microliters, 50 microliters, and 5 microliters, respectively. Familiarize yourself with the use of the micropipettors by practicing with small aliquots of water.
5. When the 10 mg/ml plant extract has dissolved in the methanol for 5 minutes, add 5 microliters of the mixture to EACH of the 3 vials labelled 3, 4, and 5, add 50 microliters to EACH of the 3 vials labelled 6, 7, and 8, and add 500 microliters to EACH of the 3 tubes labelled 9, 10, and 11. Do not add any extract to water control tube #1. To methanol control tube #2, add 50 microliters of plain methanol (not the plant extract mixture).
6. Evaporate the methanol from vials 2-11, **starting with the vials containing the most methanol**. To do this, insert the tip of the glass pipette/airline into the bottom of the vial and gently bubble air through the sample until ALL liquid vanishes. This should take NO LONGER than 20 minutes for the vials containing the greatest volume. Be ready with the next vial each time an airline is freed up. If you bubble too vigorously and lose some of your extract, you will need to prepare a new vial to replace it - why?

Table 14.2. Calculating the final concentration of plant material in $\mu\text{g/ml}$ for each sample vial.

Vial Number	Amount of 10 $\mu\text{g/ml}$ Extract Used Per Vial	μg of Plant Material in Each Vial	Final Volume of Seawater	Final Concentration of Plant Material in $\mu\text{g/ml}$
1	0	0	5 ml	0
2	No plant extract; 50 μl of methanol	0	5 ml	0
3, 4, 5	5 μl	50 μg	5 ml	10 $\mu\text{g/ml}$
6, 7, 8	50 μl		5 ml	100 $\mu\text{g/ml}$
9, 10, 11	500 μl		5 ml	1000 $\mu\text{g/ml}$

7. While the first few samples are evaporating, complete Table 2 to calculate the amount of *plant material*, in *micrograms* that is in each vial, and then, the *final concentration* that will be present in each vial, in *micrograms/ml*.

Sample calculation for final concentration (Vial #3):

$$5\mu\text{l} \times \frac{10 \mu\text{g plant tissue}}{\text{ml}} = 0.005 \text{ ml} \times \frac{10 \mu\text{g}}{\text{mg}} = 0.05 \text{ mg}$$

$$0.05 \text{ mg of plant material} = 0.05 \text{ mg} \times \frac{1000 \mu\text{g}}{\text{mg}} = 50 \mu\text{g of plant material}$$

8. When a sample vial has been evaporated, add 1 ml of *plain* sea water (2% sea salts) to it.
9. Then add 10 **healthy, live** brine shrimp to each of the 11 vials, as follows:
- Obtain about 50 ml of seawater containing brine shrimp in a beaker and bring it to your table. Ready a dissecting microscope for counting shrimp. Use Pasteur pipets to *carefully* drop several small aliquots of seawater into a small petri dish. Count the shrimp under a dissecting scope, removing groups of shrimp in droplets to add up to 10. This takes a little trial-and-error, so be patient.
 - Place each group of 10 shrimp in one of the 11 vials, and be sure to add 1-2 ml of **plain** seawater with them.
 - Do not contaminate your vials with dead brine shrimp or eggs, because the assessment of toxicity is based upon survival as the dependent variable!
 - When you think you are finished with each vial, *double-check* by holding the vial under the dissecting scope to make sure that it contains no eggs, no dead shrimp, and 10 live shrimp.
 - Leave the screw cap off OR placed on the vial loosely because brine shrimp need oxygen, just like you do!
 - Locate the 2 dram vial at your table with a mark at the 5 ml level. Using this vial as a “ruler”, carefully add PLAIN seawater to EACH of the 11 vials, so that each contains a final volume of 5 ml. *Do this as carefully as you can; if you go over the mark, you will need to start that vial over because the concentration will not be accurate.*
10. Record Day 0 information and all of the headings on Table 3. Label the vial rack belonging to your group with your group name, the date, and your lab section (day and time). Ask your instructor or TA where to store the rack.

Protocol for Brine Shrimp Survival Counts - Day “1”

Return to the lab 24 hours after beginning the cultures to count the shrimp in all 11 vials. You will need your copy of Table 3 and these instructions.

- Count each vial *one at a time*, as follows: Pour the contents into a small watch dish. Rinse the vial once with a small squirt of plain sea water, and add the rinse to the watch dish. Count numbers of live versus dead shrimp *separately* and record in Table 3. Presumably live + dead should add up to 10, right? If they don't, follow these steps:
 - Rinse the vial with seawater again; perhaps you didn't get all of them. Check the vial itself by holding under the microscope - are there any bodies caught under the rim or elsewhere?
 - Dead shrimp fall apart quickly; perhaps you have recorded two halves of a body as 2 bodies - check and make sure.
 - If you are *sure* that your total of live + dead shrimp exceeds 10, then line through the number indicated in column 3 for Day 0 and write the correct number. *Do not erase the old number.*

- d. Do the same thing if you are *sure* the total is less than 10, but make sure you have completed step 1.a. first.
2. Sign in on the sign-in sheet.
3. Look over Table 3 to make sure it is complete, including the last column.
4. Pour out your shrimp vials in the area designated, rinse each vial thoroughly and place the vials and racks in the area labelled for used glassware. Thoroughly clean the watch dishes you used and replace where you found them.
5. Turn in your completed Table 3 to your instructor for compilation.

Table 14.3. Brine Shrimp (*Artemia salina*) bioassay data sheet.

Group Members: _____

Herbivory Level (Heavy or Light): _____

Lab Day, Time and Instructor: _____

Vial #	Final Conc. µg/ml	# Live Shrimp Day 0	# Live Shrimp Day 1	# Dead Shrimp Day 1	% Survival
1	0-water control				
2	0-methanol control				
3					
4					
5					
6					
7					
8					
9					
10					
11					

Part II Questions.

Results

1. Summarize what your group learned from Part I. How much variation was there in the levels of herbivore damage on plants of this species? What kind of damage did the herbivores do to the

plants? What were the identities of the most likely culprits? Was there a significant difference in the “percent leaf damage” in the groups of leaves selected for the “highly-munched” group versus the “lightly-munched group”? If there is not a significant difference between the level of herbivory estimated for these two groups, would it be wise to continue with Part II? Explain.

2. Attach a copy of the compiled class results and a copy of the original data sheets your group completed.
3. Construct a bar graph showing **Percent survival** of Brine Shrimp in the 5 treatments (2 controls treatments and 3 extract concentrations).
4. Complete a X^2 test for EACH of the 5 comparisons between heavily munched and lightly munched leaves. *In addition, you will need to complete a Chi-squared test for water control versus methanol control - why?* Attach one X^2 sheet (Table 4) for each of those 6 analyses. Include also the completed Chi-squared summary sheet (Table 5).

Table 14.4. X^2 table for Brine Shrimp (*Artemia salina*) bioassay data.

Test Treatment:

Null Hypothesis Tested by X^2 :

Working Hypothesis:

Observed Values			
Independent Variable	# Surviving	# Not Surviving	Row Totals
Extract from heavily munched leaves (H)			
Extract from lightly munched leaves (L)			
Column Totals			N = Grand Total

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

To compute expected values (E) for each Observed value (O), $E = \frac{\text{Column total} \times \text{row total}}{\text{Grand total } (N)}$

5. Did we expect a X^2 test to show a significant difference between H and L for the two control groups? Why or why not? What can we conclude about the rest of our results if we have a significant difference between H and L for either of these tests?
6. Write a summary of the patterns in your results, as indicated by your graph and the X^2 results.

Table 14.5. Summary of χ^2 test of equal survival rates of Brine Shrimp (*Artemia salina*) in various concentrations of plant extract.

Treatment	Your Computed χ^2	“Critical” Value of χ^2 at $P=.05$, $df=1$	Is Your $P < .05$?	Equal Survival of H & L?	Accept Null Hyp?
Water control		3.84			
Methanol control					
Water versus methanol control					
10 $\mu\text{g/ml}$					
100 $\mu\text{g/ml}$					
1000 $\mu\text{g/ml}$					

Discussion

1. Does the evidence from this study support your working hypothesis (Part I.A.) or not? Explain.
2. What is the relationship between *concentration* of plant extract and percent shrimp survival (if any) and what is the biological significance of this?
3. Are there significant differences in the toxicity levels of heavily munched versus lightly munched plants? If not, how would you explain this biologically? If there are differences, how would you explain that biologically? What might be the mechanism accounting for such differences (in other words, what could be the proximate cause of the variation in amount of allelochemicals)?
4. If there are significant differences in toxicity to herbivores among individuals belonging to the same species, what are the consequences to plants of this variation? What might be the evolutionary response of insect species which feed upon those plants, given that some plants are highly toxic and others are not?
5. Alternatively, what other phenomena may have accounted for the data you collected? That is, even if the data support the hypothesis, is your hypothesis the **ONLY** explanation for what we measured, or are there still other possibilities? If so, name some.
6. Regardless of what our results were, they should probably be viewed as preliminary because we are using a brine shrimp “model” rather than the actual herbivores. In addition, we are using extracts that probably contain numerous chemicals, not just one. If you were a researcher interested in pursuing a “lead” uncovered by our class results, what would you select as your **NEXT** investigation? Describe your experimental protocol briefly, but in enough detail to demonstrate your understanding of experimental design.
7. If producing anti-herbivore chemicals is beneficial to plants, then why don't all plants produce lots of them all the time? (In other words, think about what the **COSTS** might be to the plants of producing such chemicals).

8. Refer to Bruin, Sabelis, and Dicke (1995) or other published literature. Perhaps not all plants produce anti-herbivore chemicals all the time; if they don't, explain how this study indicates that they might be able to “know” WHEN to produce allelochemicals.

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Literature Cited

- Beck, S.D. 1965. Resistance of plants to insects. *Annual Review of Entomology* 10: 207-232.
- Belsky, A.J. 1986. Does herbivory benefit plants? A review of the evidence. *American Naturalist* 127: 870-892.
- Brattsen, L.B. 1979. Biochemical defense mechanisms in herbivores against plant allelochemicals. Pages 200-270 *in* Rosenthal, D.A. and D.H. Janzen, editors. *Herbivores: Their Interaction with Secondary Plant Metabolites*. Academic Press, New York, New York, 718 pages.
{part of a book}
- Bruin, J., M.W. Sabelis and M. Dicke. 1995. Do plants tap SOS signals from their infested neighbors? *Trends in Ecology and Evolution* 10: 167-170.
- Feeny, P.P. 1969. Inhibitory effect of oak leaf tannins on the hydrolysis of proteins by trypsin. *Phytochemistry* 8: 2119-2126.
- Futuyma, D.J. 1983. Evolutionary interactions among herbivorous insects and plants. Pages 207-231 *in* D.J. Futuyma and M. Slatkin, eds. *Coevolution*. Sinauer, Sunderland, Massachusetts, 555 pages. {part of a book}
- Guglielmo, C.G., W.H. Karasov, and W.J. Jakubas. 1996. Nutritional costs of a plant secondary metabolite explain selective foraging by Ruffed Grouse. *Ecology* 77: 1103-1115.
- Hogan, K. 1994. *Eco-inquiry: a guide to ecological learning experiences for the upper elementary/middle grades*. Institute of Ecosystem Studies/Kendall Hunt Publishing Co., Dubuque, Iowa. 392 pages. {book}
- Jolivet, P. 1992. *Insects and Plants: Parallel evolution and adaptations*. Second edition. St. Lucie Press, Florida, 192 pages. {book}
- Jones, C.G. and R.D. Firn. 1991. On the evolution of plant secondary chemical diversity. *Philosophical Transactions of the Royal Society of London*, B. 333: 273-280.
- Jones, C.G., R.F. Hopper, J.S. Coleman, and V.A. Krischik. 1993. Control of systemically induced herbivore resistance by plant vascular architecture. *Oecologia* 93: 452-456.
- McLaughlin, J.L. 1991. Bench-top bioassays for the discovery of bioactive compounds in higher plants. *Brenesia* 34: 1-14.
- Ricklefs, R.E. 1990. *Ecology*. Third edition. W.H. Freeman Company, New York. 896 pp.

- Tallamy, D.W. 1986. Behavioral adaptations in insects to plant allelochemicals. Pages 273-300 in L.B. Brattsen and S. Ahmad, editors. Molecular aspects of insect-plant associations. Plenum Press, New York, New York, 346 pages. {part of a book}
- Truchan, L.C., and I. Deyrup-Olsen. 1993. Experimental design and testing: Hatching and development in brine shrimp. Pages 1-16, in Tested studies for laboratory teaching, Volume 7/8 (C.A. Goldman and P.L. Hauta, Editors). Proceedings of the 7th and 8th Workshop/Conferences of the Association for Biology Laboratory Education (ABLE), 187 pages.
- Whittaker, R.H. 1975. Communities and ecosystems. Second edition. MacMillan Publishing Company, New York. 385 pages.
- Whittaker, R.H. and P.P. Feeney. 1971. Allelochemicals: chemical interactions between species. *Science* 171: 757-770.
- Wilson, E.O. 1989. Threats to Biodiversity. *Scientific American* 261: 108-116.

APPENDIX A
Sample Results for Tables

Answers for Table 2: The correct value for vials 6, 7, and 8 is 500 μg of plant material in each vial, and the correct value for vials 9, 10, and 11 is 5000 μg of plant material in each vial.

Table 14.7. Brine Shrimp (*Artemia salina*) bioassay data sheet: a sample data set for the effect of Sassafras (*Sassafras albidum*) on brine shrimp survival

Vial #	Final Conc. $\mu\text{g/ml}$	# Live Shrimp Day 0	# Live Shrimp Day 1	# Dead Shrimp Day 1	% Survival
1	0-water control	10	9	1	90
2	0-methanol control	10	8	2	80
3	10	10	10	0	100
4	10	10	8	2	80
5	10	10	6	4	60
6	100	10	8	2	80
7	100	10	6	4	60
8	100	10	9	1	90
9	1000	10	1	9	10
10	1000	10	0	10	0
11	1000	10	1	9	10

Table 14.8. χ^2 table for brine shrimp bioassay data: comparison of Brine Shrimp (*Artemia salina*) survival in water versus methanol controls.

Test Treatment: Water versus Methanol Control

Null Hypothesis Tested by χ^2 : Control treatment type has no effect on shrimp survival

Working Hypothesis: Control treatment type has no effect on shrimp survival

Observed Values

Independent Variable	# Surviving	# Not Surviving	Row Totals
Extract from heavily munched leaves (H)	O = 73 E = 72	O = 15 E = 16	88
Extract from lightly munched leaves (L)	O = 72 E = 73	O = 18 E = 17	90
Column Totals	145	33	N = Grand Total 178

To compute expected values (E) for each Observed value (O),
$$E = \frac{\text{Column total} \times \text{row total}}{\text{Grand total } (N)}$$

$$X^2 = \frac{(73 - 72)^2}{72} + \frac{(15 - 16)^2}{16} + \frac{(72 - 73)^2}{73} + \frac{(18 - 17)^2}{17} = 0.15$$

Table 14.9. Summary of χ^2 test of equal survival rates of Brine Shrimp (*Artemia salina*) in various concentrations of Sugar Maple (*Acer saccharum*) extract: sample data set.

Treatment	Your Computed X^2	“Critical” value of X^2 at $P=.05$, $df=1$	Is your $P < .05$?	Equal Survival of H & L?	Accept Null Hyp?
Water control	1.25	3.84	Yes	Yes	Yes
Methanol control	2.03	3.84	Yes	Yes	Yes
Water versus Methanol Control	0.15	3.84	Yes	Yes	Yes
10 $\mu\text{g/ml}$	0.07	3/94	Yes	Yes	Yes
100 $\mu\text{g/ml}$	1.61	3.84	Yes	Yes	Yes
1000 $\mu\text{g/ml}$	8.69	3.84	No	No	No

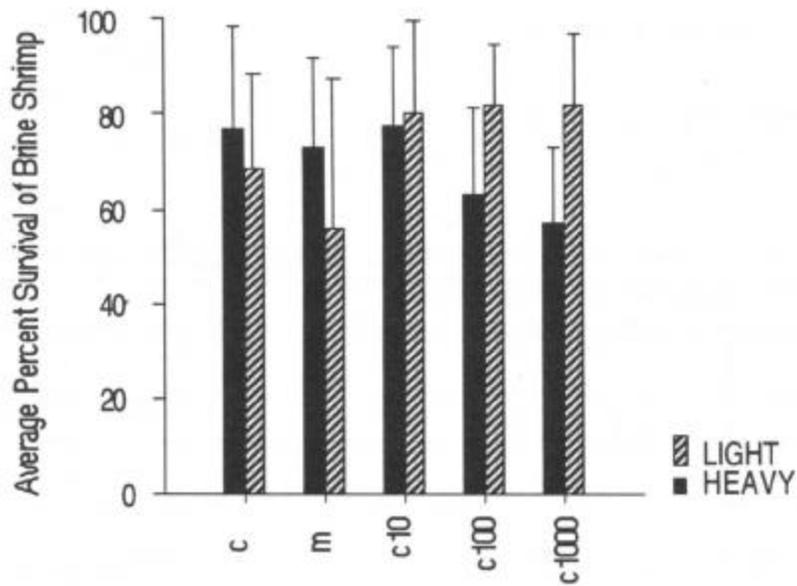


Figure 14.1: Sample results: The effect of American Beech (*Fagus grandifolia*) leaf extract on the survival of Brine Shrimp (*Artemia salina*). Light=Lightly-munched leaf samples (mean= 18.6% of leaf surface showing herbivore damage, s.d. = 9.5, n = 66); Heavy=Heavily-munched leaf samples (mean = 68.1% of leaf surface showing herbivore damage, s.d. = 17.6, n=66). The treatments are: c = water control, m = methanol control, and leaf extracts at concentrations of c10 = 10 $\mu\text{g/ml}$, c100 = 100 $\mu\text{g/ml}$, and c1000 = 1000 $\mu\text{g/ml}$, respectively.

APPENDIX B

Instructions for Obtaining Supplies and Rearing Brine Shrimp

1. Pipettors and micropipettes: If you do not have access to Pipetmen or similar pipettors, you can use capillary pipets for the 5 μ l and 50 μ l measurements. They are available from Carolina, Fisher, and other suppliers:

5 μ l capillary pipet	Carolina #K3-21-4512	Extra plungers K3-21-4526
	Fisher #21-175-A	Extra plungers not available
50 μ l capillary pipet	Carolina #K3-21-4517	Extra plungers K3-21-4538
	Fisher #21-175-E	Extra plungers not available
2. Vials: Vials are 17 x 60 mm 2 dram capacity. Fisher #03-339-21D; Carolina #K3-71-5122.
3. Air lines: If you have air lines available in your lab, use these for evaporating the methanol. They work faster than the aquarium pumps. To either, attach tubing and gang valves to make several lines from one air source. Attach Pasteur pipets (with cotton plugging) to the tubing. These pipets are put into the vials and can be changed as often as necessary. Aquarium pumps, tubing, and gang valves can be purchased wherever fish tank supplies are sold.
4. Brine Shrimp preparation: An excellent resource for culturing brine shrimp, with diagrams of developmental stages is Truchan and Deyrup-Olsen (1993). Brine shrimp eggs, also labeled “*Artemia* Cysts” are available from stores selling marine aquarium supplies or from Carolina Biological Supply. Carolina sells them as “eggs” (Catalog #K3-L610). ABLE member Art Littlepage recommends the pet store variety; they are less expensive and hatched faster when we tried them.

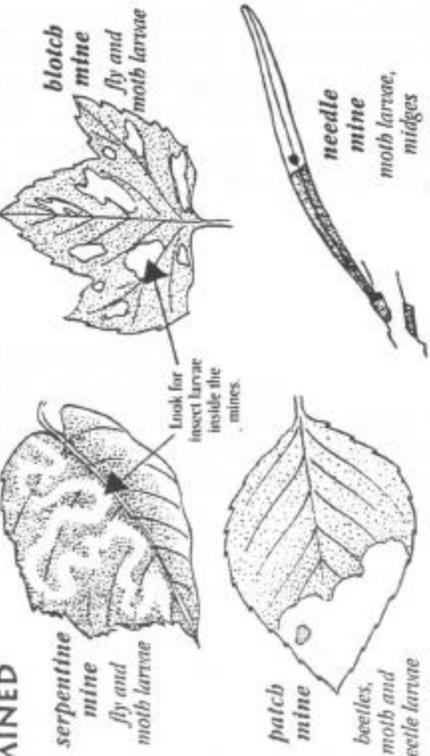
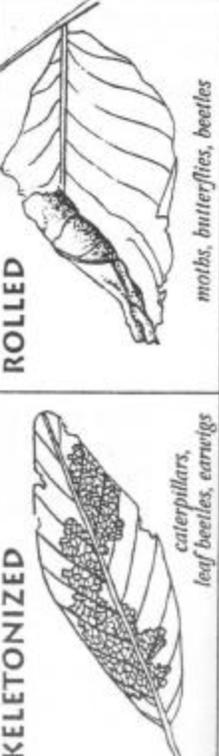
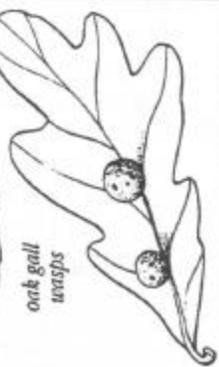
For our labs, we raise the brine shrimp in sea water made from a purchased salt mix such as “Instant Ocean” (Carolina #K3 67-1436) or “Forty Fathoms” (Carolina #K3 67-1442). We use a 2% sea salts solution aged for 24 hours before the addition of shrimp or eggs. You can raise brine shrimp in a small 5 gallon aquarium with an air stone for circulation. Art Littlepage recommends raising them in a 1-liter separatory funnel with an airline and pipet for circulation. This works very well. You can *remove* the airline shortly before lab time. This allows the eggs to rise to the surface and separate from the shrimp. Some eggs may sink but these can be decanted from the funnel before removing the shrimp. We start cultures 72, 48, and 24 hours before lab, so that there are adequate backups. The shrimp usually hatch within 24 hours.

APPENDIX C

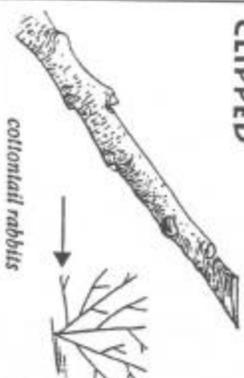
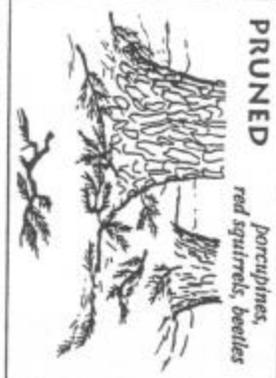
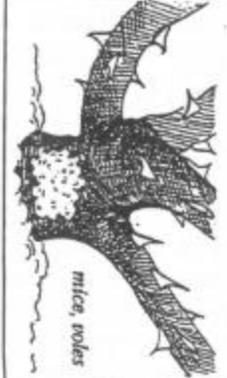
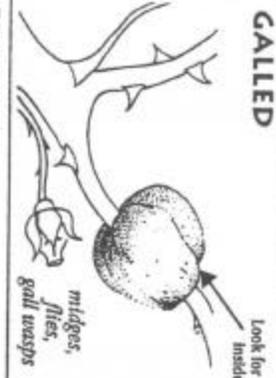
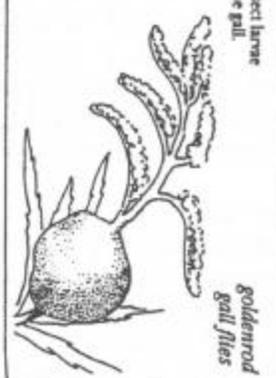
Signs of Animals Eating Plants

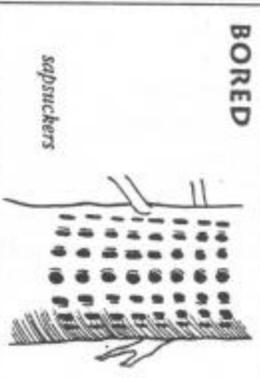
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SIGNS OF ANIMALS EATING PLANTS

LEAVES		SEEDS, NUTS, AND FRUITS	
<p>CHEWED</p>  <p><i>grouse, snowshoe hare</i></p> <p><i>leaf beetles, caterpillars, leafcutter bees</i></p> <p><i>caterpillars, beetle larvae</i></p> <p><i>grasshoppers, caterpillars</i></p>	<p>GALLED</p>  <p><i>mites</i></p> <p><i>gall midges, aphids, mites</i></p>	<p>MINED</p>  <p><i>serpentine mine</i> <i>fly and moth larvae</i></p> <p><i>blotch mine</i> <i>fly and moth larvae</i></p> <p><i>needle mine</i> <i>moth larvae, midges</i></p> <p><i>patch mine</i> <i>beetles, moth and beetle larvae</i></p> <p>Look for insect larvae inside the mines.</p>	<p>CHEWED</p>  <p><i>bangeroo rats, orioles, coyotes</i></p> <p><i>red and gray squirrels, chipmunks, jumping mice, white-footed mice, deer mice</i></p>
<p>SKELETONIZED</p>  <p><i>caterpillars, leaf beetles, earwigs</i></p>	<p>ROLLED</p>  <p><i>moths, butterflies, beetles</i></p>	 <p><i>white-footed mice, deer mice, fox, deer, opossum, woodchucks</i></p>	

SIGNS OF ANIMALS EATING PLANTS *continued*

STEMS AND TWIGS	
<p>CLIPPED</p>  <p>cottontail rabbits</p>	<p>CHEWED</p>  <p>deer</p>
<p>PRUNED</p>  <p>porcupines, red squirrels, beetles</p>	<p>GNAWED</p>  <p>mice, voles</p>
<p>CALLED</p>  <p>Look for insect larvae inside the gall.</p> <p>midges, flies, gall wasps</p>	<p>GIRDLED</p>  <p>twig pruner beetles</p>
<p>FROTHED</p>  <p>spittlebugs</p>	

TRUNKS, LIMBS, AND LOGS	
<p>BARK STRIPPED</p>  <p>woodchucks, squirrels</p>	<p>CHEWED</p>  <p>porcupines</p>
<p>BORED</p>  <p>sawflies</p>  <p>bark beetles</p>	<p>CHEWED</p>  <p>cottontail rabbits</p>  <p>cottontails, jackrabbits, ground squirrels, tortoises, woodrats, pocket mice</p>
<p>BORED</p>  <p>rabbits, hares</p>  <p>deer</p>	