Induction of Secondary Chemical Defenses

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Abstract: The production of chemical toxins is a common anti-herbivore defense. In some species, the synthesis or localization of defense chemicals is induced by leaf damage. Consequently, the feeding by an herbivore induces the prey plant to increase its defenses to make itself less vulnerable to future attack. In this study, we evaluate the inducible responses of tobacco, *Nicotiana alata*. Three questions are addressed: Do tobacco plants produce leaf toxins? Does herbivore damage induce increased leaf toxicity? Does physical damage alone yield the same response as herbivore damage? Leaf toxicity will be assessed using a simple brine shrimp bioassay.

Introduction

One of the most ubiquitous phenomena in the predator-prey interaction between plants and herbivores is the production of secondary chemical defenses by plants (Feeney 1992, Harborne 1993, Whittaker and Feeney 1971). Despite the importance of such defenses for our understanding of plant-herbivore interactions, there are very few experimental systems that permit undergraduate students to experimentally evaluate the responses of plants (but see Winnett-Murray, Hertel, and Murray 1997). This protocol presents a very tractable system in which both the plant (tobacco) and a natural herbivore (tobacco hornworm larvae) are readily available through commercial suppliers and rapid induction of chemical defenses can be artificially and naturally stimulated.

In this study, students use a bioassay to evaluate the toxicity of leaves from tobacco plants (*Nicotiana alata* or *N. tabacum*) and determine whether herbivore and herbivore-like damage will induce increased concentrations of toxic secondary compounds. A specialist herbivore on tobacco, larval tobacco hornworms, *Manduca sexta*, is used as a natural herbivore in the experiment (Villanueva 1998). We employ a Brine Shrimp Bioassay (Winnett-Murray, Hertel, and Murray 1997) to evaluate the toxicity of leaf extracts. The 24-hour brine shrimp nauplii bioassay provides a rapid measure of leaf toxicity that would not be possible using the natural herbivores of tobacco. However, tobacco

hornworms may be used for a bioassay with a real herbivore. In this study, three questions are addressed:

1. Do tobacco plants produce a toxic compound in their leaves?
2. Does herbivore damage to tobacco leaves induce the increased concentration of toxic compounds in other leaves on that plant?
3. Does physical damage cause the same responses by tobacco plants as herbivore damage?

This experiment is presented as a guided-inquiry protocol that is suitable for laboratory courses in ecology or in the ecology section of an introductory course. The protocol could easily be adapted for use with either majors or non-science majors. The protocol requires a minimum of two laboratory meetings, one week apart. The first week is for students to design experimental protocols and conduct damage treatments to plants, and the second week is for conducting leaf toxicity bioassays. The brine shrimp bioassay requires the collection of data after a 24-hour exposure period. This requires students to collect the bioassay results outside the normal laboratory meeting time in most courses. Collecting the brine shrimp bioassay results from one set of 15 vials takes 30-45 minutes.

The tobacco plants used in this study are ideally 15-30 cm tall. That size plant can be accomplished with growth in a greenhouse for one to two months (starting from plug seedlings) prior to your use date. Details on obtaining tobacco plants, preparing brine shrimp nauplii and purchasing tobacco hornworm larvae are presented in the Materials and Appendices.

Student Outline

Induction of Secondary Chemical Defenses

Objectives

1. Evaluate the presence of toxins in the leaves of tobacco plants (*Nicotiana*).

2. Perform an experiment to address the question: Does leaf damage, such as that caused by herbivores, induce an increase in secondary compound toxin concentation in tobacco?

3. Address the question: Is there a difference in the response of tobacco plants to physical damage alone and actual herbivore damage?

Introduction

At first glance, plant-herbivore interactions seem to be a highly unequal interaction between a mobile and responsive predator attacking an immobile and helpless prey plant. Yet, first impressions can be deceiving. Plants, in fact, are not helpless prey. Although they are sessil, most plant produce two types of defenses, physical and chemical. The timing of life history events, such as the production of flowers and fruits, can also be considered a form of defenses (for example, seed masting). Physical defenses include increased tissue toughness by means of cellulose and the production of defensive structures such as hairs, spines and thorns. Chemical defenses are part of an extremely diverse collection of compounds that are not part of the metabolic processes that plant require for their growth and maintenance in the absence of herbivores. Given the accessory nature of these chemicals, which include non-photosynthetic pigments and defensive chemicals, these compounds are termed secondary chemicals and secondary chemical defenses.

Thousands of secondary chemicals have been identified in plants and many have clearly demonstrated defensive functions (anti-herbivore, anti-microbial or anti-fungal activity) (Feeney 1992, Harborne 1993, Whittaker and Feeney 1971). These chemicals include nitrogen compounds, terpenoids, and phenolics, and include chemicals that are important in human affairs. Compounds traditionally used as spices are often anti-microbials (Billings and Sherman 1998). The pleasure we get from consuming the plant products coffee, tea and chocolate is provided by a nitrogen compound, caffeine (an alkaloid), which is produced by plants to poison their herbivores. Cocaine, morphine and nicotine are in this same class of secondary chemicals.

The complexity of many secondary chemical defense compounds and the use of limiting nutrients (particularly nitrogen) in many of these compounds has long suggested that chemical defenses are costly for plants to produce and maintain (Karban and Baldwin 1997). Such defense expenses could be minimized if plants could produce expensive chemical defenses only when they were needed (Baldwin 1998). Experimental evidence for rapidly inducible chemical defenses, producing or increasing chemical defenses in response to an initial herbivore attack, is very clear in a wide variety of plants (Karban and Baldwin 1997). The interactions between herbivores and plants occur in both

ecological (the life span of a given organism) and evolutionary time. The production of an effective chemical defense may be overcome by a specialist herbivore that selectively feeds on the least defended parts of a plant, or evolves detoxification mechanisms that permit it to overcome a particular defensive chemical. The occurrence this kind of plant-herbivore arms race and the resulting co-evolution of chemical defenses and herbivore specializations has produced some of the most unusual and interesting plant-animal interactions (Futuyma and Keese 1992). Some specialist herbivores even use the toxins of their host plant for their own protection (Harborne 1993, Sotka, Wares and Hay 2003).

In this study, we will use a bioassay to evaluate the toxicity of leaves from tobacco plants (*Nicotiana alata* or *N. tabacum*) and determine whether herbivore and herbivore-like damage will induce an increase in toxic secondary compounds. A specialist herbivore on tobacco, larval tobacco hornworms, *Manduca sexta*, will be used as natural herbivores in your experiment (Villanueva 1998). We will employ a Brine Shrimp Bioassay (Winnett-Murray, Hertel, and Murray 1997) to evaluate the toxicity of leaf extracts. Brine shrimp (*Artemia salina*) larvae, or nauplii, are commonly used in toxicological studies as a humane and inexpensive proxy for vertebrate animals. The 24-hour brine shrimp nauplii bioassay will provide a fairly rapid measure of leaf toxicity that would not be possible using the natural herbivores of tobacco. However, tobacco hornworms could be used in a more natural bioassay. How might tobacco hornworms be used to evaluate the toxicity of the tobacco plants in this study? Design a protocol for a tobacco hornworm bioassay.

We will address three questions in this study:

1. Do tobacco plants produce a toxic compound in their leaves?

2. Does herbivore damage to tobacco leaves induce the increased concentration of toxic compounds in other leaves on that plant?

3. Does physical damage cause the same responses by tobacco plants as herbivore damage?

Methods and Materials

Pretreatment

At least one week prior to preparing the leaf extracts, each group will perform pre-treatments on three tobacco plants. Label one plant "undamaged", a second " insect damaged", and a third “physically damaged”. The undamaged leaf should receive no manipulation. Treat the plants as described in below (Table 1). Physical damage can be produced in a repeatable manner by holding a piece of cardboard under a leaf while firmly rolling a fabric marking wheel twice over the upper surface of that leaf (Baldwin 1998). This method will leave a track of puncture wounds with 4.5 holes per cm of leaf surface. One week after pre-treatment, harvest one leaf from each plant (but not a leaf that was directly damaged) and prepare as described in Leaf Extract Preparation below.

Table 1. Pre-treatment of Tobacco Plants

Damage Treatment	Method
Undamaged	Do not damage any leaves.
Physical Damage	Make two fabric wheel tracks on each of four leaves.
Insect Damage	Put one tobacco hornworm larva on the plant for 24 hours.

Behavioral Responses by Tobacco Hornworms

In class, you will design an experimental protocol for evaluating the behavioral responses of tobacco hornworm larvae to leaves from control plants compared to damaged plants. What will you actual measure and how will you measure it? If you weigh the leaves prior to presenting them to the hornworm larvae, you also can evaluate the 24-hour consumption of leaves by the larvae. How will you measure 24-hour leaf consumption?

Write your hypotheses for the question: does physical damage or herbivore damage induce an increased chemical defense by tobacco plants? For each hypothesis, write your prediction for the behavior of tobacco hornworm larvae presented with leaves of control plants compared to damaged plants. Write your predictions for the 24-hour consumption of leaves from control plants compared to damaged plants.

Leaf Extract Preparation

The extract preparation protocol and the brine shrimp bioassay we will use are based on the methods developed by Winnett-Murray, Hertel, and Murray (1997). Using an analytical balance, weigh 40mg (0.04g) of fresh leaf from each source (pretreatment: undamaged, physical damage and insect damage). Grind each 40mg leaf sample (separately) in a glass mortar and pestle and a small pinch of sand. Transfer the 40mg of ground material to a vial and add 4ml of 100% methanol. **Use care handling methanol, it is highly flammable and toxic. Safety glasses and gloves must be worn for your safety.** Be careful not to contaminate between source treatments. Use only clean and dry mortar

and pestles. Let methanol-leaf mixture sit for 5 minutes. The concentration of each extract is now 10mg/ml (40mg in 4ml of solvent).

Prepare a series of 15 vials (Table 2) to evaluate the toxicity of each leaf extract. It is critical that your vials are clearly marked indicating both the source of the extract (Damage Treatments: undamaged, physically damaged, or insect damaged leaf) and the bioassay treatment (Table 2). Testing one leaf extract requires a total of 15 vials, so testing three damage treatments (three extracts) will require three complete sets of 15 vials each. Vial racks are available to hold your vials.

Table 2. Bioassay treatments (after Winnett-Murray, Hertel, and Murray 1997)

Bioassay Treatment (Vial Label)	Quantity of 10mg/ml extract	Plant material in extract (µg)	Final volume of 1% NaCl	Final concentration of plant material µg/ml
Water control	0	0	5ml	0
Methanol control	No extract but 50µL methanol	0	5ml	0
Low Concentration	5µL	50µg	5ml	10µg/ml
Medium Concentration	50µL	500µg	5ml	100µg/ml
High Concentration	500µL	5000µg	5ml	1000µg/ml

Sets of micropipettes are pre-set to measure 500µL, 50µL, and 5µL volumes. If you are unsure how to use the micropipettes, ask and practice pipetteting water before working with your methanol extract.

After the leaf material has been extracted in methanol for 5 minutes, pipette 5µL of extract to each of the 3 vials labeled "low", 50µL in each of the 3 vials labeled "medium", and 500µL in each of the 3 vials labeled "high". The 3 vials labeled "methanol control" should each receive 50µL of 100% methanol (not extract).

Evaporation manifolds have been placed in and near the fume hood. Carefully adjust the air flow so evaporation of vial contents will occur, but vial contents are not blown out of the vial (and into your face). Start by evaporating the vials with the greatest volumes. Change the glass pipette on the evaporation manifold between each vial. Be sure that vials are completely dry at the end of evaporation.

An example calculation of the final concentrations of plant material in each vial is shown below for the Low Concentration treatment (Table 2):

$$5\mu\text{L}\left[\frac{10\text{mg(plant tissue)}}{\text{ml}}\right] = 0.005\text{ml}\left[\frac{10\text{mg}}{\text{ml}}\right] = 0.05\text{mg}$$

$$0.05\text{mg(plant tissue)} \;=\; 0.05\text{mg}\left[\frac{1000\mu\text{g}}{\text{mg}}\right] = 50\mu\text{g}$$

Brine Shrimp Survival

Be sure that all methanol has evaporated from vials prior to preparing the brine shrimp bioassay. Add 1ml of 1% sodium chloride (NaCl) to each vial, tighten vial cap, and shake vigorously to put material into solution.

Using a dissection microscope, carefully isolate 15 groups of 10 vigorously swimming brine shrimp nauplii in small volumes of 1% NaCl. Use glass Pasteur pipettes to isolate and count the nauplii. To each of your 15 vials, transfer 10 vigorously swimming brine shrimp nauplii. Do not transfer dead animals or unhatched "eggs". After you transfer the animals to a vial, examine the vial to be sure there are 10 nauplii present.

Bring the volume of each vial up to 5ml with 1% NaCl. Use a vial containing a measured volume of 5ml as a measurement standard. Screw the cap **loosely** on each vial so the nauplii do not sufficate. Racks of vials should be kept at room temperature (**not** on a window sill or in direct sunlight) for the next 24 hours.

After 24 hours, count all brine shrimp nauplii in each vial. Record the number alive and the number dead. Be sure to account for all 10 individuals that went into each vial, animals may get stuck to the vial when it is emptied.

Data Analysis

The analysis of behavioral data will depend on the type of data collected. If you recorded the amount of time each tobacco hornworm spent on control leaves compared to damaged leaves, a t-test will be the most appropriate statistical analysis. Analysis of the 24-hour leaf consumption experiment may also be performed with a t-test (evaluating the change in mass of control and damaged leaves during a 24 hour period).

The brine shrimp bioassay data will be analyzed using an analysis of variance (ANOVA) to compare the brine shrimp survival at different concentrations of extract (for a given plant treatment) and to compare brine shrimp survival between plant treatments (at a given extract concentration).

After everyone has entered their data, you will be provided with a complete data file (Induction Raw Data) containing the counts (that you entered) of brine shrimp that were alive and dead after 24 hours exposure to each treatment. Start by calculating the mean number of brine shrimp alive for the three vials (at each concentration) that were from the same individual plant source. Different plants are the independent cases in this study.

Keep in mind that the null hypothesis for this test is no relationship between the concentrations or treatments being compared and the number of brine shrimp alive after 24 hours. A p-value of less than 0.05 means you can reject the null hypothesis of no difference between the treatments being compared.

Laboratory Safety and Clean-up

Please dispose of glass pipettes properly as directed by your instructor. Any broken glass should be placed in a glass disposal container. Waste methanol, including excess leaf extracts, must be poured in a methanol waste bottle as directed by your instructor.

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Notes to the Instructor

Damage Treatments

We have not determined the minimum length of time required to obtain a tobacco plant response to leaf damage, but one-week is sufficient to get biologically meaningful induction. Nicotine is actually synthesized and stored in the roots, so induction involves both new synthesis and translocation of nicotine from the roots to the leaves (Baldwin 1998). The physical damage with a fabric-marking wheel is fully described in the student outline. Keeping a tobacco hornworm larvae confined to a given tobacco plant for 24-hours is best accomplished with an insect cage (Appendix A). A screen insect cage will not only keep the tobacco hornworm confined, but will ensure that no more than one larva feeds on a given plant. If you use older (and larger) larvae or if the tobacco plants are small, biologically meaningful damage by tobacco hornworm larvae may be accomplished in a much shorter period of time than 24-hours. The purpose is not to defoliate the plants but to induce a response in intact leaves.

Counting and Transferring Brine Shrimp Nauplii

Isolating, counting, and transferring brine shrimp nauplii is not difficult but good technique can make this task much easier. Transfer a pipette-full of nauplii from the stock culture (from the top or middle of the beaker, but not the bottom where unhatched cysts will have dropped), to a 35mm Petri dish or watch glass. Place this dish under a dissection microscope and place an empty (isolation) dish of the same size nearby. Have students hold a glass Pasteur pipette in the palm of the hand, so they can squeeze the bulb in the palm. Squeeze the bulb, then looking through the microscope, move the tip of the pipette into the dish of nauplii, placing the tip opening next to a swimming nauplius. Gently and very slight releasing of pressure on the pipette bulb will suck the nauplius into the pipette tip. Don't pull it all the way up! Keep the nauplius in the pipette tip and squeeze it out in a small drop in the nearby isolation dish. Repeat until ten nauplii are in the isolation dish. Check the isolation dish to ensure that there are ten nauplii present. Remove all the nauplii as a group with a glass Pasteur pipette and transfer to one of the experiment vials. After the transfer is made, inspect both the vial and the pipette to ensure that all ten nauplii were transferred successfully. This entire process must be repeated 15 times to prepare a single bioassay, so it really helps if students are comfortably seated for microscope use, and the microscope oculars are properly adjusted for both focus and inter-ocular distance.

Previous Results

The data reported below were collected by M. Denton in an experiment performed on 15 plants, five in each herbivory treatment group. The values shown are the mean number of brine shrimp nauplii alive after 24 hours for the three replicate vials in each treatment.

No Damage						
	mean number of brine shrimp alive after 24 hours					
	Plant 1	2	3	4	5	mean
Water Control	7.67	8.33	7.67	6.33	6.67	7.33
Methanol Control	7.33	7.67	7.67	6.00	6.67	7.07
Extract [Low] 5μL	7.00	5.67	6.67	4.33	5.33	5.80
Extract [Medium] 50μL	6.33	6.33	5.67	3.67	5.33	5.47
Extract [High] 500μL	5.00	5.67	5.33	4.00	4.67	4.93

Physical Damage						
	mean number of brine shrimp alive after 24 hours					
	Plant 1	2	3	4	5	mean
Water Control	7.33	7.33	7.00	7.00	7.33	7.20
Methanol Control	6.67	7.00	6.67	7.00	6.67	6.80
Extract [Low] 5μL	4.67	5.67	4.67	5.33	4.67	5.00
Extract [Medium] 50μL	4.33	3.00	3.67	4.33	3.67	3.80
Extract [High] 500μL	2.67	2.00	2.33	2.67	2.33	2.40

Insect Damage						
	mean number of brine shrimp alive after 24 hours					
	Plant 1	2	3	4	5	mean
Water Control	6.33	7.33	6.67	6.67	6.67	6.73
Methanol Control	6.33	7.33	6.33	7.00	6.33	6.66
Extract [Low] 5μL	5.33	5.67	4.67	4.67	5.00	5.07
Extract [Medium] 50μL	4.00	5.00	4.00	5.33	4.00	4.47
Extract [High] 500μL	2.33	3.00	2.67	3.33	3.33	2.93

Comparisons performed within a Damage Treatment (Figure 1) reveal whether there is a difference between the controls (is there a toxic residue in the methanol), whether there is a difference between the extracts and the controls (do the plants produce a toxin), and whether there is a difference between the different concentrations of extract (do the plants produce a toxin and does it have a dose dependent negative effect on brine shrimp survival). Comparisons between Damage Treatments (Figure 2) should be made within a given extract concentration to determine whether the damaged plants exhibited an induced increase in leaf toxicity.

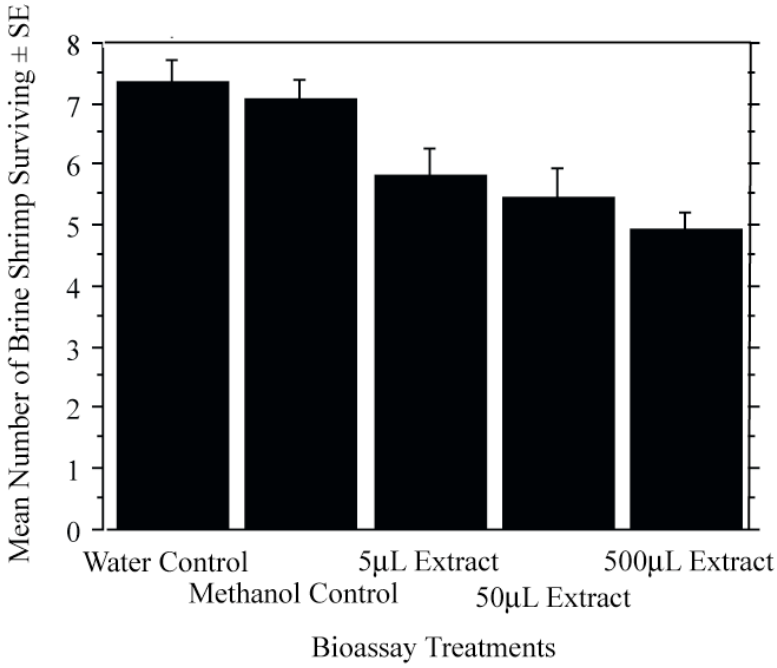

Figure 1. Comparision between bioassay treatments. The bars indicate the mean number of brine shrimp nauplii that survived for 24 hours in each replicate vial starting with ten animals in each replicate. All the data represent means for five plants in the No Damage Treatment with three replicate vials for each bioassay treatment. The Low, Medium, and High extract treatments used extract from the same five plants. There is a highly significant difference between the survival of brine shrimp among the five bioassay treatments (ANOVA df=4, F=6.83, $p<0.001$). Sheffe post-hoc tests indicate no significant difference between the water control and the methanol control, suggesting that there is no toxic residue in the methanol. The survival of brine shrimp in the high concentration of extract was significantly less than that in either controls ($p<0.025$ for both comparisons) suggesting that the undamaged plants do produce a toxin.

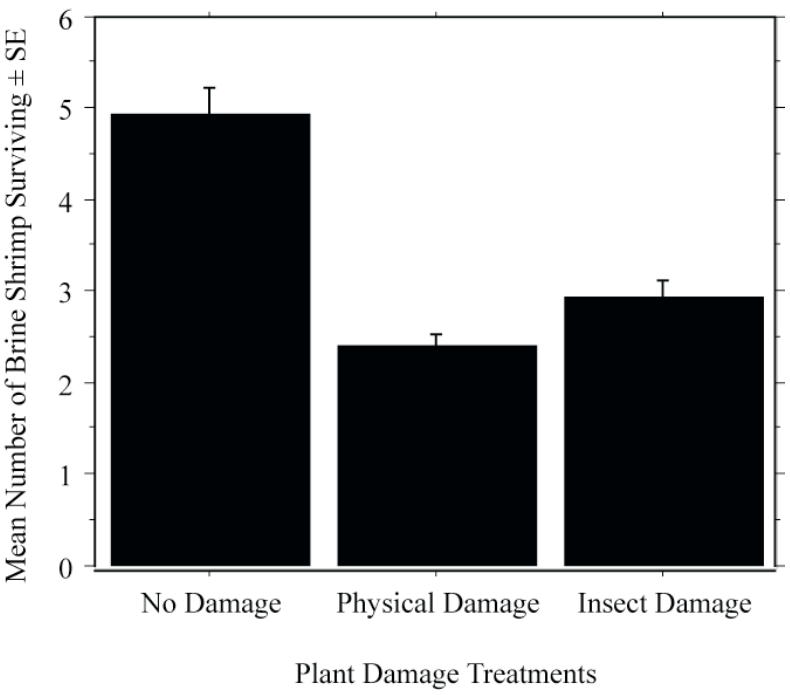

Figure 2. Comparisons between Damage Treatments. The bars indicate the mean number of brine shrimp nauplii that survived for 24 hours in each replicate vial starting with ten animals in each replicate. The data represent means for five plants in each Damage Treatment with three replicate vials per plant. The comparisons are for the high concentration of extract (500µL) which yielded the most striking and significant differences (ANOVA df=2, F=39.5, $p<0.0001$). Sheffe post-hoc tests indicate that differences between physical damage and insect damage are not significant. These results indicate that both physical damage and insect damage have a significant inducing effect on leaf toxicity seven days after the occurrence of damage.

Materials

Materials for a class of 24 students, working in groups of 3:

- Dissection microscopes: 24 needed, one per student to isolate and count brine shrimp nauplii.
- Analytical balance to weigh 40mg quantities. Use aluminum foil to make small weigh boats.
- Plants: Each group needs three plants so 24 individually potted flowering tobacco (*Nicotiana alata*) are needed. They can be purchased at a garden shop ($3.00 per potted plant) or grown in advance from seed (*Nicotiana* seeds available from W. Atlee Burpee and Co. for 1.00 - $4.00 per packet of 50-100 seeds). If started from seed, start several months (two or more months) prior to use date. Commerical leaf tobacco (*N. tabacum*) may be used as a substitute. *Nicotiana* plugs also can be purchased (Park Wholesale 800 845-3366). Plugs are sold in trays of 125 and should be ordered one-month prior to the desired delivery date. Transplant plugs to 3" pots and grow for one-month prior to use in this experiment.
- Tobacco hornworm larvae: Only eight larvae are needed to create the insect damage treatment. Live larvae can be purchased from Carolina Biological (Tobacco hornworm larvae, living, pk 12, #14-3882, $34.75 + shipping in 2005). See notes on maintaining larvae until use in the experiment. If you have the class design and conduct the hornworm behavioral experiment you will need a minimum of 12 larvae the week after the damage treatment is conducted.
- Brine shrimp nauplii: A total of 3600 newly hatched nauplii will be needed for a class of 24 Brine shrimp eggs can be purchased from Carolina Biological (Brine shrimp eggs, 1 oz bottle, 14-2240, $6.45). See notes below on techniques for successful hatching in large numbers.
- plastic aquarium gang valves with 6 outlets: 24 units needed
- rubber tubing 24" long to supply air gang valve aquarium airline tubing cut in 6" lengths and fit onto gang valves
- short disposable glass Pasteur pipettes 700 needed and 24 latex bulbs
- graduated pipettes (1ml and 5 ml) with pipette helpers: Need 4 pipette helpers and 4 graduated pipettes of each size.
- 50ml beakers (24) for transferring 1% NaCl from supply bottle to bench for distribution in bioassay vials
- small plastic Petri dishes for isolating and counting brine shrimp nauplii: 35 x 10mm (Falcon 351008) 200 should be more than enough if both lids and dishes are used to hold nauplii being counted for the start of the bioassay and are reused after animals are transferred to vials. These same dishes will be needed to count the nauplii at the end of the bioassay. Plastic tissue culture plates may be used instead of individual Petri dishes to temporarily hold the counted nauplii (and to save on supply funds). Twelve-well plates would be appropriate for this task.
- large plastic Petri dishes: 150 x 25mm for observing behavior of tobacco hornworms and for conducting 24-hour leaf consumption study.
- small scissors and single edge razors 24 needed
- marking pens to write on glass vials and Petri dishes (12 needed)
- stopwatches or countdown timers (12 needed)

- Fabric marking wheels: one per group or 8 total needed. Serrated tracing wheel Dritz #745 are the correct size and are available at fabric and sewing supply stores (Jo-Ann Fabrics $3.00 each).
- 2 dram screw cap vials: each group will need 50 vials so a total of 400 vials would be needed for 8 groups. An alternative to using individual vials are 12-well plastic tissue culture plates. Each well would replace one vial so two plates would be needed for each run a 15-vial bioassay. Each group would need six plates and a total of 48 plates would be needed for 12 groups.
- vial racks: 24 to hold 2 dram vials
- glass mortars and pestles: 24 needed, 2 oz size is ideal
- micropipetters pre-set to measure 500μL, 50μL, and 5μL volumes and plastic pipette tips

  Students can share these micropipetters, so having one set for every four groups will work. A class of 24 in 8 groups would need two sets of these micropipetters.
- methanol (100%) dispensed in small bottles in fume hood. Each group needs 12 ml so the total volume needed is approximately 100ml. Since the methanol used in the mortar and pestle often evaporates quickly, be prepared to supply more than this volume. It may be possible (as suggested by an ABLE reviewer) to replace the methanol extraction with a hot-water extraction of the tobacco leaves. If such a preparation were successful, it would also eliminate the evaporation procedures and streamline the bioassay protocol.
- 1% NaCl solution in deionized water: The water used to make this solution must be aquarium quality free of chlorine and metal ions that would be toxic to the brine shrimp nauplii. The volume needed for each group will be 225ml so the entire class of 8 groups would need approximately 2L. Prepare 4-5L since a minimum of 2L will be used to hatch the brine shrimp nauplii (Appendix B) prior to the day the bioassay will be started.

Acknowledgments

We thank Dr. Mark Hay, Georgia Institute of Technology, for suggesting tobacco as a model system for inducible defenses.

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About the Authors

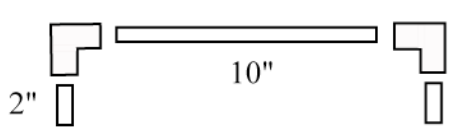
Larry Blumer earned his graduate and undergraduate degrees from the University of Michigan, and he is currently Associate Professor of Biology and Director of Environmental Studies at Morehouse College. His teaching interests are in the areas of Ecology, Environmental Biology, and Evolution and Behavior. His research interests are in the same fields, and include recent studies on the neurobiology of mate choice in fishes and insects and the neuroendocrine correlates of social stress in fishes.

Marius Denton earned a BS in Biology from Morehouse College in 2006. A native of West Memphis, AR, he is presently working as a laboratory assistant at Morehouse and making graduate school plans.

Lea Brooks is the research coordinator in the Blumer lab. She attended Xavier University in New Orleans and has worked at Morehouse for the past five years. In addition to supervising undergraduates conducting research projects and testing new laboratory teaching protocols, she assists in the preparation of laboratories materials for introductory biology and ecology courses. Prior to coming to Atlanta, she worked for Project Kaleidoscope, Washington, DC.

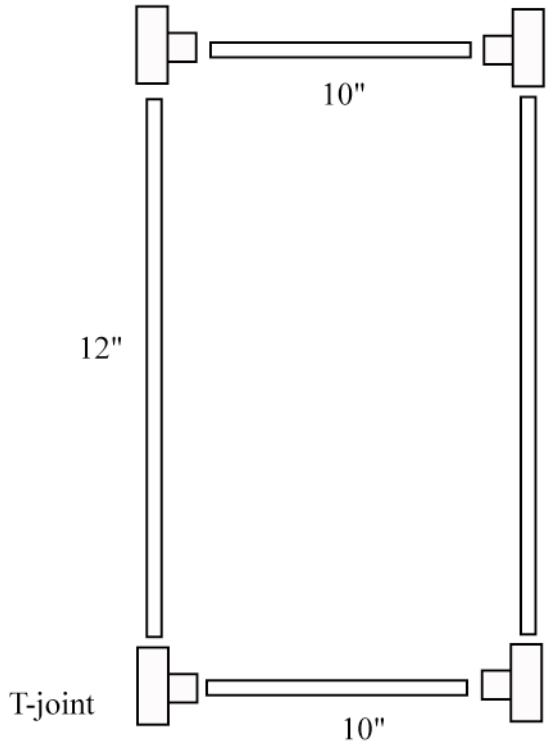
Appendix A: Insect Cage Design

Side strechers (right and left) at top and bottom (4 pieces needed)



Front and back frames (two needed)

Finished dimensions:
20" tall x 12" x 12"



PVC pipe is press-fit to the joints, no glue or sealant is necessary. Cut pipe with a PVC cutting knife-plyers.

Parts list for each cage:
PVC pipe 1/2" schedule 40 160" total as follows (two 10' lengths will leave a 6' scrap):
- 10" - 8 pieces
- 2" - 4 pieces
- 16" - 4 pieces

PVC 1/2" 90° elbows - 8 pieces (purchase in bags of 10)
PVC 1/2" T-joints - 8 pieces (purchase in bags of 10)
fiberglass screening to cover (20" x 48" - one piece, 20" x 12" - two pieces)
silicone caulk to glue screening to frame
velcro strips to make insect proof door at top (44" total)

Appendix B: Preparing Brine Shrimp Nauplii

Brine shrimp nauplii can be readily hatched from desiccation resistant eggs by placing them in 1% NaCl solution that is vigorous aerated. Each student group will need 450 nauplii so a class of 8 groups would need 3600. With reasonable care, those numbers can be easily provided if prepared as follows:

3-days prior to the start of the bioassay: fill two 1L glass beakers each with 500 - 750ml 1% NaCl water. Add approximately 1/8 teaspoon brine shrimp eggs to each beaker. Place an airstone in the bottom of each beaker and aerate vigorous so the brine shrimp eggs do not remain settled on the bottom of the beaker. Cover loosely with saran to minimize evaporation and spash. Keep at room temperature 25°C.

2-days prior to the start of the bioassay: fill two more 1L glass beakers each with 500 - 750ml 1% NaCl water. Add approximately 1/8 teaspoon brine shrimp eggs to each beaker. Place an airstone in the bottom of each beaker and aerate vigorous so the brine shrimp eggs do not remain settled on the bottom of the beaker. Cover loosely with saran to minimize evaporation and spash. Keep at room temperature 25°C.

Staging two beakers on each day will ensure that there are sufficient brine shrimp nauplii on the day that students will start the bioassay. Dry brine shrimp eggs can be purchased from Carolina Biological (Brine shrimp eggs, 1 oz bottle, 14-2240, $6.45) and hatch very reliably. Keeping unused eggs at room temperature improves hatching success. We have equally good success with liquid de-shelled brine shrimp eggs from Carolina (Brine shrimp eggs without shells, 30g dropper bottle, 14-2250, $9.20).

Appendix C: Tobacco Hornworm Larvae

Tobacco hornworm larvae may be purchased live at an early larval stage from Carolina Biological (Tobacco hornworm larvae, living, pk 12, #14-3882, $34.75 + shipping in 2005). Purchasing larvae rather than eggs ensures that you have larvae on the date you need them and the older instars (instars 2 and older) are much easier to handle without damaging the insects than the first instar. Since the hornworms are used in this experiment to simply consume leaf biomass for 24 hours, the age of the larvae is not a critical issue if all the larvae used in one experiment are approximately the same age. Place your order with Carolina Biological so you will use the larvae in the experimental pre-treatment within one week of receiving the animals. Live larvae are shipped as a group in a plastic cup containing food media. Plastic culture tubes containing solid media are shipped with live larvae orders. Holding the larvae at 25°C is fine. Leave the larvae in the cup until they are 2cm long. If you are not ready to use them at that size, transfer individual larvae to plastic tubes containing medium (one larva per tube). Fungal growth and excessive moisture are serious problems in these culture tubes but the problem can be almost completely controlled by capping each tube with plastic window screen mesh instead of the foam plugs supplied by Carolina Biological. Store tubes on their side.