Teaching About Behavior with the Tobacco Hornworm

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Overcrowding of a species is often its undoing. Competition for limited resources, spread of disease, and behavioral changes lead to death or severe stress that ultimately reduces the population. This important biological principle is not easily demonstrated in the classroom since a reasonably large population of easily viewed organisms must be maintained. In our work with the tobacco hornworm, *Manduca sexta*, we noted curious behavioral changes in overcrowded larvae that may be used to demonstrate both the intermediate as well as the long-term effects of overpopulation. These responses include increased mobility and aggressiveness that delay development and reduce fecundity. From a classroom standpoint, the behavioral changes can easily be monitored in a few minutes and, importantly, offer the student a chance to observe, develop hypotheses and make systematic observations to better understand basic principles of behavior. The relatively large size of the larva, its surface-feeding behavior, and its sessile nature make student observations simple.

The Model

The tobacco hornworm has been a model for physiological, biochemical and molecular research for more than 30 years. It is a holometabolous insect that displays four life stages: the egg, the larva, the pupa and the adult. The adult female, a relatively large, night-flying moth, usually lays one egg per plant. The female lays eggs on plants of the solanaceous family, including tobacco, tomato, potato, pepper and nightshade. After 3 to 5 days of embryonic development, the larva emerges by chewing its way out of the egg. The newly emerged larva is 2 to 3 mm long with a very characteristic black "horn" on the posterior end. Often this horn, which gives the insect its common name, is longer than the body of the newly emerged caterpillar. The pale green larva begins to feed on leaves soon after emergence and will continue to feed intermittently for several days. It then quits feeding and spins a pad of silk which is deposited on the leaf. The larva then climbs onto this pad, hooks its crochets (terminal claws on the legs) into the silk, and begins the process of molting. Meanwhile, a new, highly folded exoskeleton has already been laid down under the old exoskeleton. After about 24 hours, the old exoskeleton is split and the larva crawls out, complete with a new exoskeleton that will grow and expand as the insect feeds. Depending upon resource availability and temperature, the insect will repeat the molting process another three times during the next two to three weeks before undergoing the molt to the pupal stage. Detailed information about the life cycle and biology has been presented elsewhere (Hodges 1971; Goodman et al. 1985); for classroom rearing of the insect, see the manual on the website at http://manduca.entomology.wisc.edu.

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Materials & Methods

Typically, *M. sexta* larvae are reared on commercially available diets. We have developed an alternative diet composed of ingredients that are readily available in a large supermarket. Diet preparation requires only a kitchen blender and a microwave oven. The finished diet, having the consistency of tofu, can be easily sliced into any shape or size and the quality of the diet can be modified by adding or subtracting various chemical components. The diet consists of:

- 1 cup (100 g) of non-toasted wheat germ (Bobs Red Mill, Milwaukie, OR)
- 1/3 cup (25 g) of nonfat dry milk (Sanalac, Fullerton, CA)
- 4 tablespoons of agar (generic)
- 1 teaspoon pure raw linseed oil (non-boiled, Sunnyside Corp., Wheeling, IL)
- 1/2 tablespoon nutritional flake yeast (generic)
- 1 vitamin C tablet (1000 mg) (generic)
- 2 vitamin B tablets (generic)
- 2 multivitamin tablets (generic)
- 1 tablespoon of table sugar (generic)
- 2.5 cups water

Place vitamin tablets in blender and reduce to a powder. To this powder, add the wheat germ, powdered milk and sugar, and blend until the dry components are well-mixed. Remove the dry mix from the blender and add 2.5 cups of boiling water. While mixing at low speed, add the agar. Be careful to replace the lid on the blender before turning it on. Blend for one minute and then add the dry mix and continue to mix. Add the linseed oil and increase blender speed. You may need to manually blend the diet while the blender is running. After blending for about 5 minutes, add the nutritional yeast flakes and continue blending for another minute. Components in the yeast are heat labile, thus yeast is added as late as possible. Once the diet is thoroughly mixed, pour it into a plastic tray that has a sealable airtight lid. The diet will solidify and remain usable for about 7 to 10 days if kept refrigerated.

Commercial sources of diet are available but these dry mixes still require some preparation. Sources of commercial diet are: Carolina Biological, Dry Hornworm Medium, Catalog Number

WW-14-3905, (800) 334-5551; ICN Biochemicals, Gypsy Moth Wheat Germ Diet, Catalog Number 960292, (800) 845-0530; Southland Products, Tobacco Hornworm Diet, (501) 265-3747. *M. sexta* eggs are available at a nominal cost from Carolina Biological, Catalog Number WW-3880, (800) 334-5551. Although the exercises presented here do not require the student or instructor to rear the insect for more than 2 or 3 days past egg emergence, information on rearing is available on our web site.

Classroom

After chewing their way out of the egg, larvae migrate to diet blocks and begin feeding. Using normal rearing practices, sufficient diet is provided to avoid overcrowding; however, when the insects are forced to feed in a limited area, their behavior changes dramatically. We have used this as a model to study both population dynamics and behavior. By simply placing differing numbers of unhatched eggs on diet blocks, the newly emerged insects can be crowded into any area that the students may wish to observe. Daily observations on population numbers, emergence times of individuals and their fate, and size of the remaining individuals provide a valuable lesson in population dynamics and resource availability.

From a behavioral standpoint, manipulation of resources and population number offers a unique opportunity for the student to explore the ramifications of population density and behavioral response. Newly emerged larvae can be manipulated with a pair of fine forceps by grasping the horn and moving the insect. Although the horn contains nerves and a small part of the circulatory system, its hardened exoskeleton makes it an excellent handle for safely moving the insect from one area to another. The advantage of using newly emerged larvae is that students can develop hypotheses, design behavioral experiments, and then rapidly create the environment by moving the larvae to the experimental arena.

Uncrowded tobacco hornworms display several behavioral characteristics that can be observed and categorized by the student. To begin observations, larvae should be transferred to diet cubes in plastic petri dishes (Figure 1). Once the insects are transferred, the petri dish top is replaced to prevent air currents from disturbing the insects. Although the behavior can be easily monitored with the unaided eye, a camscope, dissecting microscope, or hand lens will aid the process. A stopwatch or timer is also useful. Students should be given sufficient time to become familiar with feeding and other types of behavior characteristic of uncrowded larvae. Students are asked to closely observe, record and then categorize the behaviors in verbal terms that can convey the idea to other students. Once a list of behavioral acts is agreed upon, the students can begin to develop questions about the behavioral characteristics they observed.

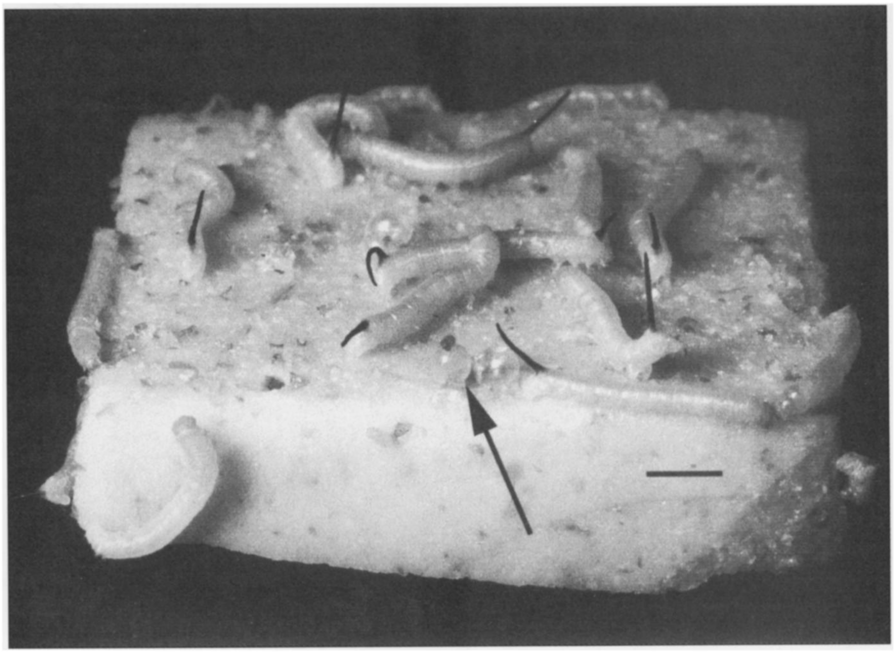

Figure 1. Eggs and newly emerged larvae of *Manduca sexta* on artificial diet. The blue-green egg (1.5 mm in diameter) near the edge of the diet cube is several days from emergence. Larvae emerge from eggs by chewing through the egg shell. During the first period of larval development, the insects change from pale yellow (larva at bottom left) to the characteristic blue-green color of larvae on top of the diet. The "horn," a brownish black structure protruding from the posterior end of the insect, can be used as a handle for moving the insect. The arrow points to an unhatched egg. The bar beneath the arrow represents 2 mm.

Hypotheses Building

Once a set of behavioral characteristics has been observed, discussed and agreed upon, the students are encouraged to brainstorm and discuss experimental design concerning overcrowding experiments. For example, what is aggressive behavior in insects? (See Figure 2.) Is territory involved? Are edges of the diet block preferred and should that be taken into account? Are horizontal surfaces more preferred than vertical surfaces and how would the experiment be designed to answer that? If surface is important, is there a way to minimize its impact on the experiment? How many insects should be placed on the diet block to elicit a particular behavior?

We have found that the discussion phase of the exercise is very important as it leads to analytical thinking and forces students to evaluate the validity of experimentally derived information. Ideally, the ideas should come from the students, but if the discussion proves difficult, ask them to define an optimal "*Manduca* space," i.e. the closest packing of the larvae that leads to no aggressive behavior. Discussion about the effect of preferred feeding sites (edges, horizontal or vertical surfaces) on available space could be used to stimulate the more advanced teams. Once hypotheses have been generated and experimental design developed within the group, students can begin their observations again.

Although student-generated hypotheses and experiments are suggested here, the instructor may wish to take a more structured approach and suggest the question. For example, what is the effect of crowding on feeding? At least two alternative hypotheses should be formulated: Hypothesis 1 states that the rate of feeding is measurably reduced by crowding, while Hypothesis 2 states that crowding will have no effect on feeding. A reasonable design for each student group might consist of an uncrowded treatment of 4 larvae on a single 1 x 1 x 0.2 cm

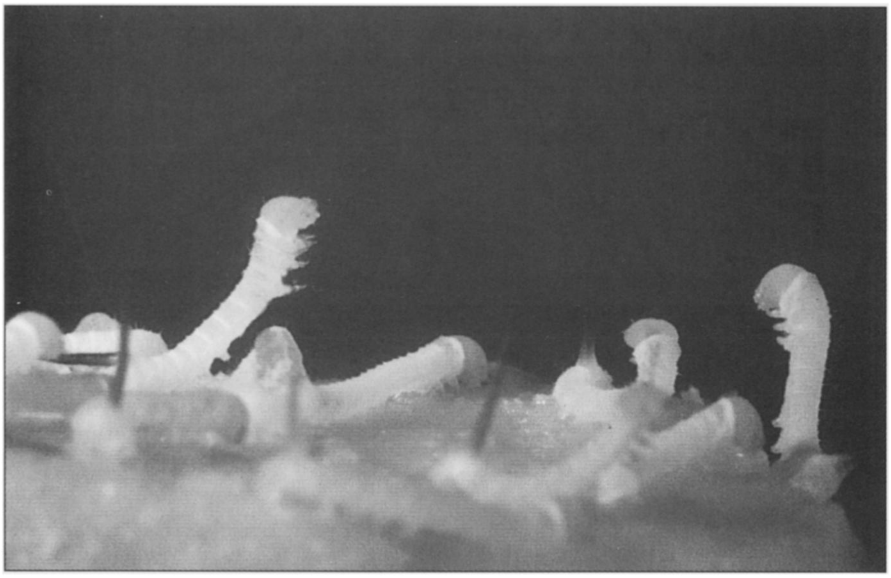

Figure 2. Newly emerged larvae displaying aggressive behavior. The larva to the left has just reared up in response to contact with a nearby larva. It does not show the curled posture of the insect seen at the right. If the disturbance persists, the larva may take the very characteristic "sphinx" posture seen in the larva on the right.

block of diet and a crowded treatment of 4 larvae on a 0.5 x 0.5 x 0.2 cm block. A thin diet block (0.2 cm) will force larvae to either feed on the horizontal surface of the diet or crawl off. After carefully transferring the larvae to the test diet cubes, give them at least a few minutes to recover from the disturbance. Students then "scan sample" the feeding behavior, which involves the observer moving from one larva to the next and recording for each whether it is feeding or not. The best results are achieved if one student calls out the observations and another acts as recorder. Scan sampling all 8 larvae in this way should take no more than 1 minute. Sample size can be increased by having student groups pool their 8 scans with those of other groups, by setting up more blocks in each treatment, or by taking repeated scans of the same sets of larvae. If the latter approach is used, sufficient time should be allowed between scans to ensure that successive events are independent.

This may be a good point from which to introduce the use of statistics in the evaluation of data. For example, if 32 larvae are scanned 4 times and the data are pooled for the 16 larvae in each treatment, the results might be compiled as shown in Table 1. Hypothesis 2, which states that crowding has no effect on feeding, would predict that equal numbers of feeding events should be seen under both treatments. In the case of the data given in Table 1, the chi square test tells us that the observed values do not deviate enough from the expected values for us to reject Hypothesis 2.

Another structured exercise might focus on the question of whether larvae seek an environment in which they maximize rate of nutrient intake. If larvae are crowded, do they sort themselves out in the available space to maximize growth rate? Hypotheses suggested here relate to the marginal value theorem (Charnov 1976). For example, if 10 larvae on a 2 cubic centimeter diet block are given a second block of the same size a few millimeters away, will 5 larvae eventually end up on each block? If so, is the dispersal facilitated by aggressive interactions among the crowded larvae? Students can test whether larval feeding rates on the two diet blocks are the same or higher than they were under the crowded conditions.

Nutrient quality plays an important role in insect development and reproduction. If crowded larvae are given a choice between two blocks of diet identical in size but differing in nutrient content, will they sort themselves out accordingly so that the rate of nutrient ingestion is the same on each diet block? In other words, will larval behavior change when the insect is provided with a richer, more nutritious diet?

The experiments outlined above are designed to be conducted in three sessions; a brainstorming/hypothesis-building session combined with observations, a second session focusing on implementation of the experiments, and a third session for data reduction and presentation of results. Longer term experiments can be designed to demonstrate the physiological effect of overcrowding. For example, if 10 newly emerged larvae were placed on a diet block, how many would survive to the first molt? Does overcrowding lead to cannibalism?

One of the fascinating questions arising from these studies centers on recognition of crowding. Despite their apparently simple appearance, larvae are equipped with sensory organs that detect touch, taste/smell, light, temperature and humidity. Establishing the senses involved in overcrowding will challenge students to devise creative methods of testing their hypotheses. Teams of students may be assigned a sensory type to test. For example, in the case of touch, we have cut bristles from a test tube brush and implanted them into the diet block to mimic various levels of crowding. A single, newly emerged larva is placed between the bristles and observed. Students can then compare the reaction and "*Manduca* space" of this "pseudo-crowded" larva to one that is surrounded by live insects. Are the reactions the same? Does the behavior suggest that neural input is coming from a single type of sense organ? One team might examine the idea of limiting population density by chemical signals. Some insect species secrete signaling compounds onto the food. In some cases, the signals may recruit other insects of the same species; conversely, some compounds may repel insects to keep the density low. This response can be tested by feeding a number of insects on a block of diet. After a given period of time, the larvae are removed, and a solitary insect is placed on the same block and its behavior compared

Table 1. Sample results of a test of the influence of crowding on feeding behavior.

		No. of feeding events	
Treatment	*No. of scans*	*Observed*	*Expected*
Crowded	64	32	37
Uncrowded	64	42	37
Total	128	74	74

to an insect feeding on a non-conditioned diet block.

Another team might explore the idea that the insects see their neighbors and thus display aggressive behavior. It is generally believed that most insect species do not respond to visual cues in the red region of the light spectrum. Students can watch insects under a red light or red transparency in a dimly lit room. Since the larvae are described as nocturnal feeders, their levels of activity generally increase and their levels of aggression may change. This can be compared with insects that are held under white light. Finally, the students might study the effect of temperature on aggression. Small heating pads can be placed under the observation dish and the temperature elevated to a preselected level. Rates of contact and body movement can be compared to solitary insects at the same temperature. Moreover, the rates of movement as a function of temperature can be examined.

The experiments described here represent an entry point for discussing ecological and behavioral principles. Although the concepts are in many ways self-evident, modeling them using living organisms reinforces the interrelationships between resources and the evolutionary success of the species. The simple nature of the experiments focuses attention not on technique, but on questions of biological significance. The fascinating and complex behavior of insects stimulates a natural curiosity that compels the student to ask that most basic of questions, "What if. . .?" What more could we ask for?

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