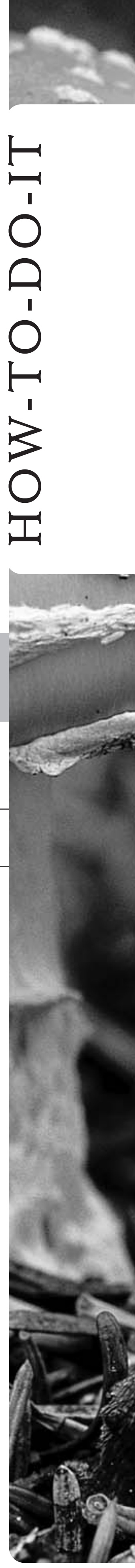
HOW-TO-DO-IT



THE Hornworm Assay: Useful in Mathematically-Based Biological Investigations

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Bioassays use the response of organisms as a measure of toxicity, for example in ecological studies of leaf toxicity and screening plant sources for possible medicines. Bioassays often use "naive" animals that have not had a chance to evolve responses to the suspected toxins.

The brine shrimp assay (Opler et al., 2002; Rice & Maness, 2004) has numerous applications in biological laboratory investigations. The response variable is the percentage of shrimp that survive when exposed to a certain concentration of the suspected toxin or extract containing it. This article describes another assay, in which the response variable is the weight gain of the organism.

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Manduca sexta is the sphinx moth, and its caterpillars are commonly known as tobacco or tomato hornworms. In the wild, the caterpillars eat leaves of the plant species in the nightshade family Solanaceae on which the eggs were laid. Caterpillars raised on tomato leaves will reject jimsonweed leaves, and caterpillars raised on jimsonweed leaves will reject tomato leaves (Rice, personal observation). In the laboratory, hornworms will accept some host plants outside of the nightshade family (DeBoer, 1993). Hornworms are therefore good assay organisms for leaf toxins other than those produced by plants in the Solanaceae (Schwartz & Snyder, 1985; Wrubel & Bernays, 1990).

Hornworms can also be raised on an artificial medium ("chow"), consisting of corn meal, soy flour, dry milk, yeast, and other additives and preservatives, which can be obtained in dried form ($20 per bag) from biological supply houses such as Carolina Biological Supply Company (Burlington, NC 27215). Hornworm caterpillars are readily available from biological supply houses. Carolina

Biological Supply Company charges $107 for 50 larvae, which includes the prepared chow and the stoppered vials for raising the larvae. The same kind of vials frequently used for rearing *Drosophila* may be used for raising hornworms. Although the caterpillars are commonly encountered in gardens, it is best to obtain a large number of approximately even-aged caterpillars.

Hornworms obtained from a biological supplier will have become accustomed to eating hornworm chow. Therefore the leaves or other suspected toxins should be incorporated into the chow. If the leaf material is not a large component of the chow, the caterpillars will continue to eat the chow.

The caterpillars can be individually weighed. The relative degree of toxicity can be expressed by the degree to which growth is inhibited relative to control caterpillars receiving pure chow.

The graphing and statistical analysis of hornworm growth curves can allow an integration of mathematics into the biology laboratory, consistent with the recommendations of the National Council on Education and the Disciplines (Steen, 2001).

Procedure

The following example uses leaves of post oak (*Quercus stellata*) obtained from a cross-timbers forest area near Durant, Oklahoma. Each hornworm consumed chow that contained leaf material obtained from one of three trees, on one of 13 sample dates. The hypothesis being tested was that leaves obtained from the trees early in the season (April-June) were more toxic than leaves obtained from the trees later in the season (July-October) (Rice et al., unpublished). This procedure can be applied to any other hypothesis involving comparisons of toxicity.

Preparation of Leaf Material

Because leaves were gathered at different times during the season, while the bioassay was performed at one time, the leaves had to be stored. Chemical composition of dried or stored leaves may change over time. In this investigation, we ground the leaf samples in liquid N_2 with a mortar and pestle that had not been contaminated by any other use, then stored the leaf material at -70˚C until use. The use of ground fresh leaves involves the least difficulty.

Preparation of Hornworm Treatments

Obtain hornworms from a biological supply company. They will generally be about 1 cm in length upon arrival. Stir the ground leaf material into the prepared chow. Stir thoroughly so that hornworms will not encounter and avoid clumps of undesirable leaf material. We incorporated 1% leaf weight per chow weight in some vials (low concentration) and 3% (high concentration) in others. Because hornworms are not extremely sensitive to plant compounds (Wrubel & Bernays, 1990), we found clearest results with the higher concentration.

Place one worm in each vial and close it with a stopper. Keep the vials under uniform room temperature conditions: We recommend a day-night cycle of illumination. Your sample size will depend upon the hypothesis you are testing: We began with 146, which included treatment and control individuals. You should expect some mortality: By Day 5 of the experiment, 23% of our hornworms had died.

Measurements of Worm Response

In a brine shrimp assay, percent survival is the response variable. A hornworm assay will probably not contain enough individuals to permit valid calculations of percent survival. Instead, we used weights of individual worms as the response variable.

The worms should be weighed when first exposed to the treatments, and every few days thereafter. Before weighing the worm, rinse away wastes and chow that may cling to its body with de-ionized water; also remove solid wastes from the vial. Gently dry the worm with a paper towel before weighing. We began our treatments when the worms averaged between one-half and one gram (Day 1), then weighed them on Days 5, 7, 13, and 15. They grew rapidly, some worms increasing their weight forty-fold during that time. Weighings can be discontinued when each worm reaches a maximum weight.

Observation of Results

We noticed that control hornworms had a bright blue-green color, and produced light-colored solid wastes. The worms that ate chow into which oak leaf material was incorporated had a yellowish appearance and produced darker wastes. It was apparent that the oak leaf toxins were having a physiological effect upon the hornworms.

A simple scatterplot of worm weight as a function of time can be used to analyze growth. Figure 1 presents data for worm growth when fed leaves from early in the season vs. late in the season (results shown only for the high concentration). Clearly, the worms exposed to early-season leaves grew more slowly than the worms exposed to late-season leaves. This agrees with the results of a brine shrimp assay (Rice et al., unpublished) that was performed on the same leaves.

Figure 1.
Changes in hornworm weight (mg) from first day of exposure to post oak leaf material (Day 1) until they attained maximum weight (about Day 15). Cubic equations are fit through data. Late-season leaf material, incorporated into worm chow, inhibited worm growth less than early-season leaf material.

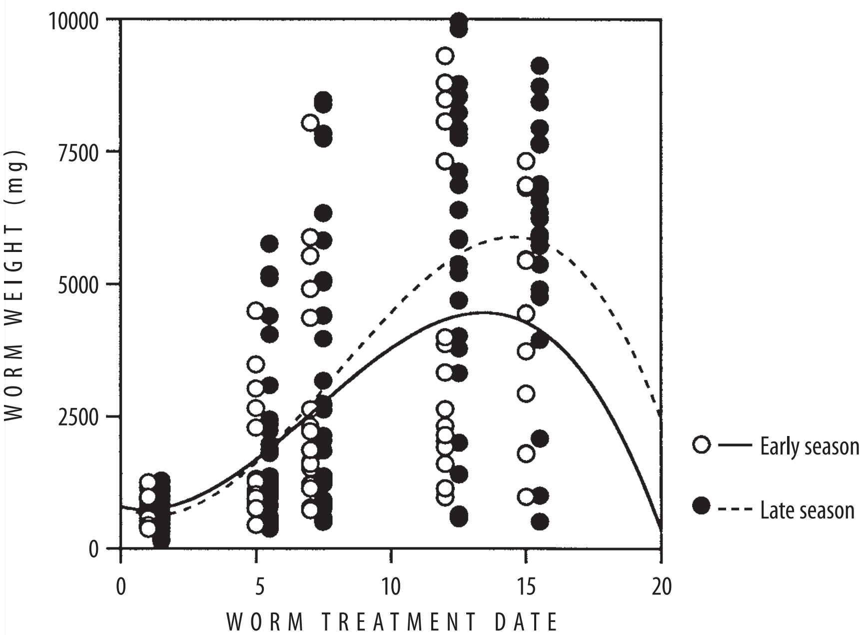

Table 1.
Maximum weights (Day 13) of hornworms from cubic equations (mg).

	Exposed to Early Season Leaves	Exposed to Late Season Leaves	Controls
Mean	4816	5803	8178
Upper 95% confidence limit	4485	5503	7584
Lower 95% confidence limit	5147	6103	8772

Analysis of Results

For classes with limited mathematical experience, a simple comparison of means can be performed. The worms exposed to late-season leaves grew to an average weight of 5.68 grams; the control worms grew to an average of 7.39 grams. Therefore it appears that the oak leaves contained some toxins that inhibited the growth of the hornworms. To test the statistical reliability of this conclusion, a *t*-test indicated that these two means were different at $p = 0.045$. This means that there was only a 4.5% probability that these differences occurred by chance. Scientists use 5% ($p = 0.05$) as a cutoff for believability or "significance."

However, when we compared the worms exposed to early-season leaves with the worms exposed to late-season leaves, the *t*-test results were not significant ($p = 0.751$). The worms will always have a large range of initial weights because of differences in time of hatching. A *t*-test does not take differences in initial worm weight into account. Therefore a lower-level biology class should use the hornworm bioassay only for investigations that show big differences, e.g. between control worms and worms exposed to leaf material. A fine scale of comparison, e.g. between early-season and late-season leaves, requires more sophisticated analysis.

For classes with more mathematical experience, further analysis is instructive. A statistical package (in this case, JMP (SAS Institute Inc., 1995) can fit curves through the data and provide formulas. We used cubic equations that contained x, x^2, and x^3 as independent variables. We also included initial worm weight as a covariate, which is necessary because the worms will differ from one another in weight when they arrive, and the differences may be magnified as the worms grow.

Other computer packages such as Mathematica (http://www.mathsource.com) can calculate the first and second derivatives of the cubic formulas. When the first derivative equals zero, the slope is zero, which may occur at two places on the growth curve: first, a transient dip near the beginning (which is an artifact of curve-fitting and can be ignored), and second, when the worms reach their maximum weight. When the second derivative equals zero, the slope has reached its maximum, which indicates the date on which the worms are experiencing their maximum growth rate. The classes do not need to actually know how to calculate

Table 2.
Means & analysis of variance of hornworm weights on day of maximum growth rate (Day 7) from cubic equations.

Mean, worms exposed to:	
Early Season leaves (mg)	2606
Late Season leaves (mg)	3282
Leaf date effect from ANOVA[a]	$p = 0.0007$
Dosage effect from ANOVA	$p = 0.833$ ns[b]
Initial weight [dosage] from ANOVA[c]	$p < 0.0001$

[a]ANOVA = analysis of variance
[b]ns = not statistically significant
[c]Initial worm weight nested within dosage

derivatives; this activity will allow them to practice with the *concepts* of first and second derivative.

The dates of maximum weight (at which f' [x] = 0) were Day 13 for the worms that ate leaf material from early in the season, and Day 14 for the worms that ate leaf material from later in the season. We obtained the weights on Day 13 from the cubic equations (Table 1). Worms that ate leaf material reached a lower weight than the control worms (which weighed 8.2 grams). Worms that ate leaf material from earlier in the season grew less (4.8 grams) than worms that ate leaf material from later in the season (5.8 grams) (Table 1). The non-overlapping confidence intervals demonstrate that all of these differences are statistically significant. The means here presented are not the same as the raw means presented earlier, as we obtained these from the cubic equations.

The dates of maximum growth rate (f'' [x] = 0) were Day 7 for both sets of experimental worms. Worms that ate leaf material from earlier in the season weighed less (2.6 grams) than worms that ate leaf material from later in the season (3.3 grams) (Table 2). An analysis of variance indicated that the difference between early and late leaves was significant; that the dosage level (3% vs. 1% of chow weight) had no significant effect; and that the initial worm weight (Day 1) significantly affected worm weights on Day 7 (Table 2). This analysis of variance uses Type III sums of squares, in which the significance of each factor is evaluated *after* the other factors have been included in the model. This means that the worms that ate leaves from early in the season grew less than the worms that ate leaves from later in the season, even when adjustment has already been made for the initial worm weights in the analysis.

Because of repeated observations (each surviving worm is weighed on each date), proper statistical analysis requires that the initial worm weight be nested within the dosage effect in the analysis of variance (SAS Institute Inc., 1995).

Advantages & Disadvantages

Because of the great variability in worm weights, and the amount of care needed to maintain them, the hornworm assay is less useful in ecological and toxicological research than is the brine shrimp assay. Like the brine shrimp assay, the hornworm assay allows students to learn about experimental design and hypothesis testing.

However, the hornworm assay has a number of educational advantages in the student laboratory.

- It allows students to have direct contact with the caterpillars. Young children can raise caterpillars just to observe them, but older students (high school and college) need to have a better reason than this to raise caterpillars. This project gives them a hypothesis to test while raising caterpillars.
- It requires the students to work in groups and take responsibility to care for the caterpillars.
- The physiological effects of leaf toxins can be directly observed in the color of the worm hemolymph and wastes, as well as in the analysis of weight gain.
- Most laboratory activities lend themselves to mathematical analysis, e.g. *t*-tests and analyses of variance. Because the response variable in this activity is worm weight, cubic growth curves can be analyzed for date of maximum weight and date of maximum growth rate, using simple calculus. This activity allows more integration of mathematical skills into a biology laboratory than do many other lab activities.

Acknowledgments

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