# *Manduca sexta* Antibacterial Response: A Cell Biology Investigation

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## Overview

The antibacterial response of the moth *Manduca sexta* is used as a research model for a guided investigatory laboratory series in a sophomore-level cell biology course. Over a four week period, students examine differences in protein concentration, antibacterial activity, and protein composition between control (untreated) insects and those that have received an injection of killed-bacteria. This laboratory series emphasizes hypothesis testing, data collection, statistical and qualitative interpretation of data, integration of data sets measuring different aspects of the response, and comparison of student results with published literature. The exercise culminates in a student paper formatted as a formal research publication.

## Background: The Insect Antibacterial Response

Insects have several structural barriers that serve as passive protective mechanisms, preventing bacteria from gaining access to the body cavity; these include the exoskeleton and the peritrophic membrane. When these barriers are compromised and bacteria gain access to the hemocoel, the insect mounts a series of active responses, homologous in some respects to the vertebrate innate responses, aimed at eliminating microbial invaders (reviewed by Boman 1995; Cociancich *et al.*

1994; Dunn 1986; Gillespie *et al.* 1997). The initial response to large numbers of invading microorganisms is mediated by the hemocytes which eliminate bacteria from the circulation by phagocytosis and nodule[1] formation. Subsequently, there is an increase in antibacterial activity in the plasma fraction of the hemolymph. The fat body has been shown to be responsible for the bulk of the synthesis and secretion of these antibacterial proteins, although antibacterial agents are also released by the hemocytes. This secondary response is thought to kill any bacteria that have evaded the cellular responses and to provide some measure of protection during the time it takes to rebuild the hemocyte population depleted by nodule formation. The antibacterial peptides are the focus of this set of experiments.

The particulars of antibacterial responses differ among insect orders and life stages. The organism used for this set of experiments is the caterpillar stage tobacco hornworm, *Manduca sexta.* Three antibacterial proteins have been well characterized in *Manduca* larvae: lysozyme, the cecropin-like peptides, and the attacin-like proteins. Lysozyme and cecropin activities are a focus of the investigations for this laboratory exercise.

Lysozyme is an ~14 kDa enzyme that degrades the peptidoglycan layer of bacterial cell walls. It is most effective against Gram positive bacteria and is also thought to participate in the post-infection degradation of bacterial fragments released during the hemocytic responses. Lysozyme may be present in the hemolymph of untreated *M. sexta* larvae at low levels. The cecropin-like family of peptides (~4 kDa) have bactericidal activity against a broad spectrum of both Gram-positive and Gram-negative bacteria. The primary sequence and investigations of the secondary structure of the peptides indicate that the amino terminal one-third of the protein folds into an amphipathic helix; the remaining two-thirds of the protein

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[1]Nodules are formed by aggregation of hemocytes which also entrap bacteria in an extracellular matrix. This mass then undergoes melanization which is thought to kill the entrapped bacteria.

is very hydrophobic. Cecropins are thought to insert into bacterial cellular membranes, forming a pore that dissipates electrochemical ion gradients, resulting in the death of the targeted bacterium.

Previous studies (see Dunn 1986) characterizing the antibacterial response in *M. sexta* have been performed using axenically raised insects. Under these circumstances, the naive insect (supposedly never having encountered bacteria in its hemocoel) contains a low but constitutive level of lysozyme and no detectable cecropin activity. Upon treatment with bacteria or bacterial cell wall components, the levels of lysozyme and cecropin activity and protein become elevated over a period of 12-18 hours. The substantially elevated levels plateau over the next several days. Molecular studies have confirmed that an increase in the levels of mRNAs for these proteins is observed in the fat body and other tissues of the larvae (Mulnix and Dunn, 1994; Dickinson *et al.* 1988).

## Curricular Context

The course including this laboratory series has been at the sophomore level. Students in the course have had at least one introductory course in genetics, one in inorganic chemistry and one in organic chemistry. Students have had biology laboratory experience with micropipetting, bacterial transformation, *Drosophila* genetics, paper chromatography, and restriction fragment mapping of plasmid DNA. Many students have also taken the introductory ecology course which contains a student-designed research component. Recently, Earlham changed its academic calendar from trimesters to semesters; as a consequence, we have moved this set of laboratory experiences into the first year curriculum. My focus in this paper is on the course offered at the sophomore level.

## Goals of the Experience

Earlham has historically provided students with opportunities to participate in student-designed research project within the field biology/organismal courses in which they enroll. More recently, the department has begun to incorporate more open-ended, student-designed laboratories into the cellular/molecular areas of the curriculum. Our overall goal has been to incorporate progressively more independent laboratories from the introductory to upperlevel courses. This lab experience is provided early in the biology curriculum and provides a technical and conceptual foundation for work in more advanced courses.

One goal of this particular laboratory series is to introduce students to a set of basic techniques, including micropipetting, standard curves, protein assays, enzymatic assays, zone of inhibition assays, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). These content goals are the vehicle by which we teach students about the process of doing science, including hypothesis generation and testing, basics of experimental design, data collection, data interpretation, statistical analysis, integration of data sets, reading and interpreting the research literature, and writing formal laboratory reports. Thus, a major goal is to provide a guided introduction to performing cellular/molecular research.

## Laboratory Logistics

The larvae are purchased from Carolina Biological Supply and are raised to the fifth instar according to the suppliers directions; experiments begin with the insects approximately in their first day of the fifth instar. Because it is difficult to get sufficient insects of the same age, the control and treated insects given to a student group are matched by weight. Approximately 24 hours before the first laboratory period, a set of insects is treated with formalin-killed *E. coli*.[2] The control insects

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[2]An overnight culture of *E. coli* were pelleted (3,000 x g, 20 min.) and resuspended in 0.37% formaldehyde in 0.85% NaCl and incubated at room temperature overnight. The formalin-killed

are not treated. Students work in groups of four. Each lab group receives both an untreated and a treated *M. sexta* larva which they bled during the first week of the laboratory series; hemolymph samples are stored in the freezer. The overview of the laboratory series is:

week one--hemolymph collection and protein assays;

week two--lysozyme and cecropin assays;

week 3--SDS-PAGE;

week 4--data analysis.

Prior to beginning this set of lab experiments, students are given basic background information on the antibacterial response in *M. sexta*. At the beginning of each laboratory period, students formulate a hypothesis regarding protein concentration, activity, or composition of the hemolymph from untreated and injected larvae.

## The Laboratory Exercises

In the first week, students perform protein assays using the Bradford method. Bradford reagent is purchased (BioRad) and used according to the supplier's directions. Each lab group generates a standard curve using known concentrations of bovine serum albumin (10-100 mg/ml). They then collect hemolymph from their two insects (untreated and treated) and perform Bradford reactions on these samples. The standard curve is used to estimate the concentration of total protein in the insect hemolymph samples.

Table 1 shows results from a class of six lab groups in a previous year.

### Table 1. Hemolymph Protein Concentration

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bacteria were then washed several times in 0.85% NaCl and resuspended to the desired concentration in 0.85% NaCl. $10^7$ to $10^9$ bacteria in 10 μl were injected into each larvae. See Dunn *et al.* 1987.

| UNTREATED (μg/μl) | TREATED (μg/μl) |
|---|---|
| 1.5 | 2.4 |
| 1.1 | 1.9 |
| 1.4 | 0.9 |
| 2.9 | 2.3 |
| 1.6 | 1.2 |
| 2.1 | 3.0 |
| mean +/- SD = 1.8 +/- 0.6 | mean +/- SD = 2.0 +/- 0.8 |
| student's unpaired t-test p = 0.57 | |

Hemolymph protein concentration was determined for untreated and bacteria-injected *M. sexta* larvae using the Bradford method. Triplicate assays were performed; the reported numbers are averages. This data represents one class of 6 groups (24 students) in one year.

In the several times that we have performed this laboratory, there has been no significant difference between the control and treatment groups, which is consistent with published reports (Dunn *et al.* 1987). The students typically hypothesize that a difference will be seen and can formulate an argument for either an expected increase or decrease. The fact that there is no statistically significant difference observed is typically somewhat problematic for them and generates a great deal of discussion. This is often a great teaching moment for some students who have come to believe that they must have done something wrong since the experimental results did not support their hypothesis.

In the second week, students assay their hemolymph samples, both untreated and treated, for two antibacterial activities: lysozyme enzymatic activity and cecropin bactericidal activity. The lysozyme activity is determined using a spectrophotometric assay[3] that measures a decrease in turbidity of a suspension of *Micrococcus lysodeikticus* cells. The rate of decrease is used to determine the

[3]9 mg of *Micrococcus lysodeikticus* cells were dissolved in 25 ml of 50 mM potassium phosphate buffer at pH 6.4. 1.94 ml of the cell suspension was used with 60 μl of undiluted hemolymph. The rate of decrease of absorbance was measured at 570nm over a period of 3 minutes. One EU = 0.01 absorbance units/min.

enzyme units in the hemolymph. Students perform these assays in triplicate during the lab period.

The cecropin activity is measured using a zone of inhibition assay[4]; several microliters of hemolymph are placed in a small well in an agar plate seeded with *E. coli* which is then incubated overnight. As cecropin peptides diffuse, the bacteria in the surrounding agar are killed. The remainder of the plate develops a cloudy appearance as the bacteria multiply. The diameter of the clear area surrounding the well is proportional to the amount of cecropin activity present in the hemolymph. Students load triplicate wells with undiluted hemolymph during the lab period and return briefly the following day to take diameter measurements.

Results of the lysozyme and cecropin assays are shown in Tables 2 and 3, respectively.

**Table 2. Lysozyme Activity**

| UNTREATED (EU/ml) | TREATED (EU/ml) |
|---|---|
| 21 | 41 |
| 27 | 107 |
| 20 | 51 |
| 22 | 85 |
| 25 | 98 |
| 23 | 93 |
| mean +/- SD = 23 +/- 3 | mean +/- SD = 79 +/- 27 |
| student's t-test p = 0.002 | |

Lysozyme activity of insect hemolymph from untreated and bacteria-injected *M. sexta* larvae. Spectrophotometric assays are used to determine the rate of decrease in turbidity of a bacterial suspension which is then converted to enzyme units/ml of hemolymph. Assays are run in triplicate.

**Table 3. Cecropin Activity**

[4]An overnight culture of bacteria *E. coli* strain D31 (available from *E. coli* Genetic Stock Center, Yale University phone: 203-423-9997) is used to start an approximately 5 hr culture of bacteria. A 1:10 dilution is made of the 5 hr culture; 100 ul. of the diluted culture is is mixed with 10 ml of Luria Agar at 50°C. The plates are stored at 4°C until used. 3 mm wells for hemolymph loading are punched in the plate prior to their use.

| UNTREATED (mm$^2$) | TREATED (mm$^2$) |
|---|---|
| 0.0 | 16.0 |
| 0.0 | 14.5 |
| 0.0 | 0.0 |
| 0.0 | 0.0 |
| 0.0 | 0.0 |
| 0.5 | 22.0 |
| mean +/- SD = 0.1 +/- 0.2 | mean +/- SD = 8.8 +/- 9.9 |
| student's unpaired t-test p = 0.08 | |

Cecropin activity of insect hemolymph from untreated and bacteria-injected *M. sexta* larvae. Activity was measured using a zone of inhibition assay. Assays were performed in triplicate.

In the several times that these experiments have been performed, the results have been variable. The above data indicates a statistically significant increase in lysozyme activity but not in cecropin activity. Some insects appear to respond with an increase in lysozyme activity and cecropin activity, while other insects appear to only experience an increase in lysozyme activity. These results are not consistent with data reported in the literature. Similar results have been obtained in one other year. In a third year, both the lysozyme and cecropin activities were elevated in all treated insects. Again, this is a surprising result and the students must give some thought to possible explanations.

In the third week of the laboratory series, students examine the protein composition of hemolymph from untreated and treated insects using SDS-PAGE. During the laboratory period, students are responsible for pouring the stacking gel, preparing their hemolymph samples, separating the proteins by electrophoresis and staining the gels. Teaching assistants or faculty prepare the separating gels prior to the beginning of lab and also return in the evening for destaining and drying of the gels. Students make qualitative comparisons between untreated and treated hemolymph samples, looking for differences in banding patterns and intensities of protein bands.

During the fourth and final week of the lab series, students perform a variety of data analyses. Prior to the class meeting, the group data for the protein concentration, lysozyme activity, and cecropin activity is pooled and distributed to the students. During the lab period, students are responsible for performing statistical analyses on the data sets. In addition, a significant amount of time is spent analyzing the results of the SDS-PAGE. Students first compare the pattern of proteins in the untreated and treated samples and identify up to four major differences (absence/presence of bands, differences in intensities of bands). They then generate a standard curve for estimating the molecular weights of those proteins. Pairs (or triples) are formed between students from different laboratory groups for writing the final laboratory report. Once a laboratory group has finished discussion of their results, the pairs form and compare their findings.

Another component of the in-class data analysis is a comparison of student results with several published papers provided to the students. Dunn *et al.* (1987) report results of protein concentration assays, elevation of lysozyme activity following bacterial injection, and a time-course for the response. Hughes *et al.* (1983) report the SDS-PAGE separation of hemolymph from untreated, saline-injected and bacteria-treated *M. sexta* larvae and pupae. This process places their results into a larger context and (hopefully) makes evident to the students some of the limitations of our experimental design,

The culmination of this lab series is a formal research report authored by a pair of students. Given that the students are working simultaneously on several additional assignments for this class, we have most recently focused on having the students write only a results and discussion section. The grading criteria for the papers include data presentation, data interpretation, synthesis across the data sets, comparison of their results with the published literature, and discussion of various

'problems' associated with the laboratory (e.g. sample size, lack of a saline-injected control).

## Evaluation of the Labs

The lab series is a guided investigation, not a student-designed experiment. At the same time, the set of experiments is somewhat open-ended and interpreting the results is not always straight forward. Our experience has been that at the sophomore (and certainly the freshman) level, students have not yet had enough experience with the available techniques to be able to design and execute an experiment in cell/molecular biology in a period of three to four weeks. We also want to introduce students to additional techniques (DNA isolation, DNA sequencing, PCR) in the remainder of the semester and so are unwilling to use additional weeks in this course to allow students to design their own experiment. This laboratory series is thus a compromise among our competing goals of teaching students about the process of science, building their technical foundation for more advanced labs, giving students a experience with a broad variety of techniques, and providing students with a positive experience (i.e. they get interpretable results). Laboratories associated with upperlevel courses build on this foundation and incorporate additional opportunities for teaching science as a process.

We have not performed formal assessment of the outcomes but are, overall, pleased with the outcomes of the laboratory series. We are certainly asking students to participate in an activity that more closely resembles the process of science than the laboratories we used previously. The series has several particularly attractive pedagogical aspects, including that the student hypotheses are not always supported and that students are comparing their findings to the published literature. Both of these situations provide ample opportunity for discussion of data interpretation, experimental design, and role of statistical analyses.

Another benefit of the lab series is that it models that scientists usually ask several related questions about a particular system and use a variety of technique to explore a physiological response. Students must integrate their findings across several data sets. For instance, if they see an increase in lysozyme activity, do they also see an increase in lysozyme protein on the SDS-PAGE results? How can an increase in some protein activities be explained when the total protein concentration does not increase? This is a typical feature of sophisticated research but is not always possible to build into undergraduate exercises. The inclusion of the laboratory period for data analysis has been essential to pushing students to think critically, read the literature carefully, and to seriously consider the ambiguities that inevitably arise.

Our experience over the past several years is that insects obtained from Carolina Biological Supply Co. show a greater variability in their antibacterial responses than an axenically-reared research population of insects. It has also been unexpected to have some treated insects which exhibit an elevation of lysozyme activity but not cecropin activity. At present, I have no explanation for this observation.

Pitfalls of the laboratory exercises include the relatively large individual variability in response to treatment which sometime confounds the interpretation of data. The heavy reliance on the ability of students to micropipette is problematic for some but the exercises provide ample opportunity (and built-in feedback in the form of standard curves and triplicate assays) for improvement. Several of the exercises require students to return to the laboratory at other times to collect their data.

The most substantial change that we made over the three years in which we have used the laboratory was to include the fourth period for data analysis. In the first year we did these exercises, we asked students to interpret the results of the SDS-PAGE and read the articles on their own; their lack of experience in these areas

was reflected in misrepresentations and misinterpretations within their final papers. Subsequently, we have used a laboratory period and have provided a series of questions to guide the students through their SDS-PAGE analysis and comparison with the published literature. This extra time has also allowed us to help the students integrate the data from the several laboratory periods. Their final papers have been much improved.

## Initial Reflections on Moving the Lab to the Freshman Level

As mentioned above, a change in our academic calendar triggered a curricular change that moved this laboratory series from a second year Cell Biology course into a first year introductory course. To accommodate the fact that the students have less background in the content of both biology and chemistry, and less experience in the laboratory, we have added an exercise to the beginning of the laboratory series. For this exercise, students perform a spectral analysis on a solution of green food coloring to determine the wavelength of maximal absorption; they then construct a standard curve from which they determine the concentration of an unknown green solution. This exercise provides students with an introduction to micropipetting and the operation and theory of the spectrophotometer. In the final laboratory period, we have also been much more direct in helping the students interpret their results. Although the level of sophistication at which the students are able to interpret their data is lower, we feel the lab continues to meet the goals stated above.

## Future Directions

This set of experiments lays the foundation for a variety of potential independent projects, including:

- additional studies of the untreated insects supplied by Carolina Biological Supply company could be performed.

- the control group of insects for this experiment are untreated (no injection); an investigation of wounding would be possible using an injection of saline.
- a time course could be performed to examine the increase in antibacterial activity and to determine how long the elevated activities persists.
- different life stages (pupae and adult) of the organism could be examined.
- different species could be examined.
- individuals collected from the wild could be evaluated and compared with the results from the lab populations.
- other stress responses could be investigated using the same set of experimental techniques (e.g. response to cold, response to other microorganisms).
- a number of organism-level responses may also be observed, including diarrhea, reduced feeding, and general inactivity in response to the bacterial injection; these behavioral responses could be quantified.
- the insect fat body is relatively easy to culture and will secrete antibacterial proteins in the presence of bacterial inducers. A variety of experiments could be undertaken to further examine the cell biology of the response, for instance characterization of the signal transduction pathway.

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