Using the Wax Moth Larvae as a Model to Study the Immune System

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I. Science Background

The innate immune response of vertebrates is remarkably similar to the immune response in insects. One of the most common insect models for studying the innate immune response is the greater wax moth or honeycomb moth (*Galleria mellonella*). The larvae of *Galleria mellonella* (GML) are found in most regions of the world, from Europe to North America and Australia. Studies using the larvae have elucidated several positive correlations between virulence and host responses, and have proven to be a simple and reliable model system to study infections in this insect. Some of the microorganisms studied using GML include *Acinetobacter baumanii* (1,2), *Francisella tularensis* (3), *Pseudomonas aeruginosa* (4,5), *Yersinia pseudotuberculosis* (6), *Staphylococcus aureus* (7), *Streptococcus pyogenes* (8), *Streptococcus mutans* (9), *Enterococcus faecalis* (10,11), *Candida albicans* (12), and *Cryptococcus neoformans* (13). These studies have found functional similarities with innate immune responses to infection, expression of antimicrobial peptides, generation of reactive oxygen species, phagocytosis of invading microbes, and initiation of clotting cascades as observed in several other vertebrates and invertebrates (14–18). Therefore, the GML model is an ideal educational system to engage students in a practical and inquiry based understanding of immunity.

In the high school setting the use of GML offers an alternative experimental model to study immune responses that is both cost effective and technically simple for laboratory student instruction. Because of their small size (2-3 cm in length), the larvae do not need feeding, require little space, and are inexpensive to purchase. Furthermore, the techniques needed to work with this model are achieved after only limited instruction, and studies using this model are ethically more acceptable and permissible by most states than those which use a vertebrate model (14). The GML model also provides an efficient system to aid in the learning and understanding of the basic principles of the immune system in mammals. For example, the model can be used by students to obtain data about the insect's innate immunological response, some components of which parallel aspects of mammalian phagocytic and humoral responses. Furthermore, the GML model represents a low biohazard risk, and the larvae are easily kept in petri dishes with infected material made safe by autoclaving. As such, the use of GML in an educational setting clearly provides a unique opportunity for students to study the immune system. Furthermore, it provides a valuable alternative to design inquiry labs to study the immunological response to infection. For example, students can easily measure survival rates, response to toxicity levels, and chemical sensitivity. A number of investigations published in the literature show that there are various research experiments capable of providing useful and insightful information into the pathogenesis and response to bacteria, yeast, and toxic agents in GML (1,12).

Insects commonly respond to infectious organisms by the production and deposition of melanin pigments on the invading pathogen or parasite. This response is mediated by enzymatic and non-enzymatic reactions. Several studies have indicated that the phenoloxidase (PO) enzyme is significantly important for effective melanization by regulation through different cell types (hemocytes, plasmatocytes, and crystal cells) and an elaborate system of enzymatic steps (20). The phenoloxidase enzyme allows the insect to convert phenols to quinones, which subsequently polymerize to form melanin. Therefore, the circulating levels of PO are directly associated with the insect's innate response to infectious agents. By using PO, insects are able to respond against pathogens, such as bacteria (2), fungi (12), and even viral agents (19).

In the literature there are two classes of compounds that have been documented as substrates for insect POs: monophenols and polyphenols. Examples of monophenols include tyrosine or tyramine and catechol substrates, 1,2-dihydroxybenzene (catechol), 4-methyl catechol, DOPA, dopamine, *N*-acetyl dopamine (NADA), *N*-ß-alanyl dopamine (NBAD), *N*-acetylon epinephrine (NBANE), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzoic acid (20). Of these, the catechol DOPA has been most frequently used to assay PO activity experimentally. The activity of PO has been previously determined in the hemolymph of GML challenged with *E. coli* by measuring absorbance at 490 nm through a common assay (24), which is easy enough to be conducted by high school or college undergraduate students.

For the activity proposed here, the hemolymph of GML can be used to measure the PO activity response by the insect's effector cells. Since the PO response is similar to that of a cytosolic subunit of NADPH oxidase found in neutrophils, the measured hemolymphatic cellular response can be used by the teacher to illustrate similarities in responses observed during the phagocytic humoral response in mammals. For example, the hemocytic PO response of GML to kill bacterial and fungal cells can be correlated to similar mechanisms used by human neutrophils.

In this laboratory activity we propose to use an organophosphate insecticide as the inflicting agent. The most commonly presented hypothesis by the students may be one in which the insecticide will cause the death of the organism. However, the PO response is effective in protecting the GML and enhances its survival rate. Although the insecticide is an effective infectious agent that clearly initiates an immune response (PO activity), it does not cause death. Therefore, this kind of cellular hemocytic response will allow the students to investigate the survival rate of GML. Moreover, the response offers an opportunity to design an inquiry lab by modifying the protocol described here. The students, for example, may change treatments to observe variations of: 1) the survival rate of GML, and 2) the elicited production of PO in GML. In addition, advanced investigations could be added by measuring the elicited activity of superoxide in GML, the expression of required cytosolic enzyme, p47phox, for the production of superoxide in GML (26).

In this experimental activity, the disturbances introduced affect the dynamic homeostasis of GML, resulting in a measurable immune response. The physiological responses to the toxic substances will elicit measurable immunological responses to the toxins. Therefore, the student should be able to use representations and/or models to analyze quantitatively or qualitatively the effects of the disruptions to the dynamic homeostasis in the GML. The results should show an increased activity of the enzyme; which is 2-4 times higher than in the control groups.

Furthermore, to give this study a clear connection to real life applications, the students are also asked to conduct an independent project report on beekeeping. The study and control of GML is important for beekeeping; GML damage is the major biological constraint in the beekeeping industry. Therefore, understanding the use of the appropriate insecticide is a determining factor in controlling *Galleria mellonella*. Through this experimental procedure and research activity, the students could further enhance their understanding of the GML immune response by considering biological forms of controlling GML in beekeeping. For advanced studies, teachers and/or students could also investigate various articles which suggest that in addition to insecticides, a biological control of *Galleria mellonella* could be affected by using *Bacillus thuringiensis*, study the superoxide response, and the expression of p47phox.

II. Student Outcomes

The students will be able to perform a series of activities that will help them learn the principles behind innate and cell-mediated immunity. These principles will guide them to make predictions for a series of experiments in which the immunological response of insects to toxic agents will be demonstrated. These activities will allow the students to practice relevant scientific research and investigation skills to generate informational data. The generated data will then be used by students to exercise their critical and analytical thinking skills. Through their interpretation and analysis, the students will be able to engage in a more conducive discussion about immunological responses in biological systems and how the proper functioning of these systems maintains a homeostatic state.

This unit aligns with the following AP Biology Big Ideas and will have the following student outcomes:

A. More evoking and more accurate lessons about the dynamic homeostasis of a biological system as to how it is influenced by changes in the system's environment. More specifically, responses of the innate and adaptive immune systems (Big Idea 2.D).

B. Students will understand that all biological systems, from cells to ecosystems, are influenced by complex biotic and abiotic interactions.

C. Students will understand that homeostatic mechanisms reflect both continuity due to common ancestry and change due to evolution in different environments.

D. Students will have an opportunity to display more engaging discussions to address concepts relating to pro- and anti-inflammatory cytokines and their involvement in disease initiation and progression (Big Ideas 2.D.3, 2.D.4), providing an example of how biological systems are affected by disruptions to their dynamic homeostasis.

E. Students will have an opportunity to develop a better understanding of the adaptive immune concepts involved with production and function of polyclonal and monoclonal antibodies (Big Idea 3.D.2, 4.B).

F. Students will learn that mammals use specific immune responses triggered by natural or artificial agents that disrupt dynamic homeostasis. The mammalian immune system includes two types of specific responses: cell mediated and humoral. In the cell-mediated response, T cells, a type of lymphocytic white blood cell, targets extracellular and intracellular pathogens via recognition of surface antigens presented by antigen presenting cells. In the humoral response, B cells, a type of lymphocytic white blood cell, produce antibodies against specific antigens. Antigens are recognized by antibodies to the antigen. Antibodies are proteins produced by B cells, and each antibody is specific to a particular antigen. A second exposure to an antigen results in a rapid and enhanced immune response.

III. Learning Objectives

The observable measurements will assess:

1) The student's ability to perform well in identifying essential scientific cross cutting concepts. Students should accurately evaluate a reasonable cause and effect (i.e. PO measurements and insecticide concentrations). Through the appropriate understanding of the immune response they should make reasonable and defensible predictions when formulating a hypothesis and evaluating results. Students will also be able to apply the concepts to create justifiable explanations or analogies for the resulting data.

2) The student's ability to accurately execute scientific and engineering practices. Proficiency will be observed when the student independently designs and implements the experiment to explore and develop explanations for the immune response. The student's performance should display a clear and reasonable analysis and critique to determine the validity of their findings.

3) The student's ability to communicate their understanding of the concepts efficiently and accurately. Students demonstrate the ability to make connections between the results and the concepts learned about the humoral and cellular response in humans as modeled by the insect's response in the experiment. In addition, the student should be able to create or evaluate a viable solution to the issues currently faced by beekeepers. The student connections and evaluated solutions should directly relate to their hypothesis. For this level of education, an application of a wide range of vocabulary should reflect fluency and accuracy, and should be appropriately used. The students should use detailed relevant facts in immunology to show their understanding through accurate and precise descriptions, explanations and examples.

IV. Time Requirements

A total of ten periods is suggested. In calculating this time, the length of a single class period is assumed to be between 40-50 min each. Alternatively, a total of five block (90-120 min each) days would also be appropriate.

Day 1: Learning the Basics of Immunology
Ask the students to follow instructions and complete the mission, read and take notes about the immune system on both of the following websites:

https://www.nobelprize.org/educational/medicine/immunity/about.html
http://missinglink.ucsf.edu/lm/immunology_module/prologue/objectives/obj02.html

Day 2: Research Access via Internet: The Immune Response in Mammals and Insects

http://www.asm.org/ccLibraryFiles/FILENAME/000000003514/znw01207000596.pdf
"Insect and mammalian innate immune responses are much alike." Kavanagh K,Reeves EP. (2007) Microbe. 2(12): 596-599.

http://biology.kenyon.edu/courses/biol470/insect%20immunity.pdf
"Insect immunity: evolutionary roots of the mammalian innate immune system." Vilmos P, Kurucz E, (1998) Immunol. Letters. 62: 59-66.

http://www.livescience.com/26579-immune-system.html
"Immune system: diseases, disorders, and function." Zimmermann KA. (2016) Live Science.

Day 3: Research Access via Internet: Invertebrate Immunity

http://www.jimmunol.org/content/179/11/7209.full
"Invertebrate immune systems–Specific, quasi-specific, or nonspecific?" (2007) Rowley AF, Powell AJ. Immunol. 179(11).

Day 4: LAB: Insecticide treatment for the survival data
Day 5: LAB: Preparation for assays
Day 6: LAB: Injection and Phenoloxidase assay
Day 7: LAB: Phenoloxidase assay
Day 8: Collaborative Groups: Data evaluation presentation/Class discussion
Day 9: Collaborative Groups: Continue survival data/Beekeeping research essay
Day 10: Collaborative Groups: Assessment

V. Teacher Preparation Previous to Experiment

Buffer solutions (2 hr)
Purchase Insecticides online
Purchase *G. mellonella* online
Prepare insect's food (if needed)
Pipet Tips Blue and Yellow
Microcentrifuge tubes

Phosphate Buffered Saline

1. Dissolve the following in 800ml distilled H_2O.
    - 8g of NaCl
    - 0.2g of KCl
    - 1.44g of Na_2HPO_4
    - 0.24g of KH_2PO_4
2. Adjust pH to 7.4 with HCl.

3. Adjust volume to 1L with additional distilled H_2O.
4. Sterilize by autoclaving.

People call this solution PBS 10mM, because the important component for buffer is Na_2HPO_4. The final ionic strengths are:
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4

Sodium Cacodylate Buffer 0.01 M --*ALWAYS handle in fume hood*--

THIS MUST BE MADE FRESH BEFORE PO MEASUREMENTS

NOTE: DO NOT ADD acid which may produce Arsenic gas

1) Dissolve 0.214 g Sodium Cacodylate in 80 ml of distilled water.
2) Add 0.05 g of Calcium Chloride to the solution
3) Bring the solution to a final 100 ml.

ALWAYS handle in fume cupboard and see the MSDS for safety precautions.

Insecticide
(ordered through Amazon 7 days in advance)

Demon Wp - Envelopes (4 of 9.5 g Packets) -1 Envelope

Sold by: Pest Control Pros

$10.45

Suggested Dilutions for the Insecticide Active compound Cypermethrin (Molar mass: 416.3 g/mol)
Create the concentrations of the insecticide from the original stock (4.73 µM, 9.46 µM,18.92 µM, 23.65 µM, 47.28 µM, 70.92 µM)

Insecticide (mg) per liter of Water

2 mg /1 L = 4.73 µM

4 mg / 1L = 9.46 µM

8 mg / 1L = 18.93 µM

10 mg / 1 L = 23.65 µM

20 mg / 1L = 47.28 µM

30 mg / 1L = 70.92 µM

***Galleria mellonella* Larvae**
Purchased from Speedy Worm

http://shop.speedyworm.com/wax-worms-p2.aspx

(Wax Worms) Price: $8.50 1 cup = 250 worms

Supplies

Materials (for a class size 30-35 students)

- *Galleria mellonella* larvae
- 48 Petri Dishes
- 8 Syringes of 10 mL
- 70% ethanol
- Insecticide Dilutions: Demon WP (cypermethrin)
- Distilled Water
- 84 Test tubes
- 8 Automatic Pipettes P20 and P1000
- 42 Automatic Pipette tips
- Incubator
- Scissors
- PBS Buffer
- Cacodylate Buffer
- Cotton Swabs
- Centrifuge
- ELISA reader spectrophotometer used at 490 nm wavelength

VI. A. Materials and Equipment

Suggested for each group of 4 students

Galleria mellonela Larvae (24) http://shop.speedyworm.com/wax-worms-p2.aspx
(Wax Worms) Price: $8.50 1 cup = 250 worms
Petri Dishes (4) labeled two sets of plates. Each set will be composed of one each for 3 larvae each for Control and three for treatment of selected insecticide. The other set will have the same number of larvae, but will be used for the phenoloxidase analysis.
Fisher Scientific S08184 United Scientific Supplies Disposable Petri Dishes $1.90 Pack of 10 or S33580A Plastic Petri Dishes $129 Case of 500
Insulin Syringe (1) to inject insecticide: 148291B Fisher Scientific BD Micro-Fine™ IV Insulin Syringes $40.19/100
Test tube (1): Fisher Scientific S32168 Kimble™ KIMAX™ Plastic Test Tubes $20.90 Pack of 250
Isopropyl Alcohol 4ml (70%) to disinfect the larvae prior to injection: Local store $1 a bottle
Cotton swabs to be used to apply the Ethanol on the larvae: Local store $1
Microcentrifuge tubes (12) to recover hemolymph Microcentrifuge Tubes
Fisher Scientific S348903 $27.05 Pack of 500

Equipment for Entire Class

Incubator
Fisher Scientific Education Incubator Catalog No. S521081. See-through door allows for visual inspection without heat loss. $455.00 - $582.00
Microcentrifuge
Fisher Scientific Education S67601B Fisherbrand™ Standard Mini-Centrifuge $439
Note: The microcentrifuge will be used to extract the hemolymph. It should be spun for only 7 seconds at -touch- minimum speed. The gravitational force will make the hemolymph quickly flow down to the bottom of the microcentrifuge tube. The larva's body will be retained inside a pipet tip which is placed inside the microcentrifuge tube.
Pipette P20 to measure 10 µl of Insecticide and 10µl of hemolymph with Yellow tips (15)
Fisher Scientific Pipet S67826 $26.50/each OR Gilson™ PIPETMAN Neo Pipets F144564G $350 each
Fisher Scientific Pipet tip S98754A $52.50 pack of 1000

Pipette P100 to measure buffers and water used for dilutions with blue tips (15)
Fisher Scientific Pipet S67826 $26.50/each OR Gilson™ PIPETMAN Neo Pipets F144566 $350 each
Fisher Scientific Pipet tip S32252 $32.50 pack of 1000
Spectrophotometer
Fisher Scientific™ Educational Spectrophotometer S42669ND $995

VI. B. List of Materials and Equipment

Equipment	Consumables	Other	Data Tables
Incubator	Cacodylate Buffer	Glassware	Survival
Timer/Stopwatch	Insecticides		SOD Absorbance 560 nm
Glass Pestle	Ice		Phenoloxidase Activity 490 nm
Microcentrifuge	Distilled Water		
Refrigerator	Petri Dishes		
Freezer	Honey		
Cuvette	Glycerin		
Spectrophotometer	Dried Yeast		
	Wheat Flour		
	Skim Milk Powder		
	Polenta		
	Serological Pipet		
	Microsyringe		
	Microcentrifuge tubes		
	Pasteur Pipet		
	NaPO4 buffer, pH 7.0		
	Nitro blue tetrazolium (NBT)		
	50 mM sodium phosphate buffer (pH 7.8)		
	13 mM methionine		
	75 mM NBT		

	2 mM riboflavin		
	0.1 mM EDTA		
	15 W fluorescent lamps		
	L-dopa (4 mg/ml)		

VII. Student Prior Knowledge and Skills

Students should have a basic understanding of the immune system. In addition, they should be familiar with the bare basics of an immune response and how organisms respond to various treatments, such as vaccines.

Students Prior Knowledge should include
Know the Stages of insect development
The use of insecticides in real world situations
Know what distilled water is
Be able to use various scientific glassware
Conduct good observational skills
Understanding of the metric system
How to use a syringe
Familiarity with centrifuge tubes
Understanding how to use an automatic pipet
Understanding of the hemolymph
Understanding the principles of microcentrifugation
Understanding of concentration and Molarity
Know how to use a spectrophotometer
Know how to use an incubator

VIII. Daily Unit Plans

Day 1: Learning the basic concepts of immunology
Ask the students to follow instructions and complete the mission, read take notes about the immune system on both of the following websites:

Materials

https://www.nobelprize.org/educational/medicine/immunity/about.html
http://missinglink.ucsf.edu/lm/immunology_module/prologue/objectives/obj02.html

Concepts

AP-Biology Big Idea 3.D.2, 4.B

1. Students will have an opportunity to develop a better understanding of the adaptive immune concepts involved with production and function of polyclonal and monoclonal antibodies.
2. Students will learn that mammals use specific immune responses triggered by natural or artificial agents that disrupt dynamic homeostasis. The mammalian immune system includes two types of specific responses: cell mediated and humoral. In the cell-mediated response, cytotoxic T cells, a type of lymphocytic white blood cell, "target" intracellular pathogens when antigens are displayed on the outside of the cells. In the humoral response, B cells, a type of lymphocyte white blood cell, produce antibodies against specific antigens. Antigens are recognized by antibodies to the antigen. Antibodies are proteins produced by B cells.

Instructions

You have to work with a partner to correctly and sequentially organize a set of images that you will create to illustrate innate and adaptive immunity (cells, time, hours, and days). To support your understanding you will have an excerpt of the article *Old Meet New: The interaction Between Innate and Adaptive Immunity (30)*.

Day 2: To address how the immune system responds to infection and how a similar response may cause disease learn about the immune response in mammals and insects

Ask the students to read take notes about the immune system from all of the following websites:

Materials

http://www.asm.org/ccLibraryFiles/FILENAME/000000003514/znw01207000596.pdf
"Insect and mammalian innate immune responses are much alike." Kavanagh K. and Reeves E.P. (2007) Microbe. 2(12): 596-599.

http://biology.kenyon.edu/courses/biol470/insect%20immunity.pdf
"Insect immunity: evolutionary roots of the mammalian innate immune system." Vilmos P, Kurucz E, (1998) Immunol. Letters. 62: 59-66.

Concepts

AP-Biology Big Ideas 2.D.3, 2.D.4

Students will have an opportunity to address concepts relating to pro- and anti-inflammatory cytokines and their involvement in disease initiation and progression as an example of how biological systems are affected by disruptions to their dynamic homeostasis.

Instructions

Interactively use the internet to understand how a normal immune response is conducted and how the immune system may cause disease.
Your assignment:
1. Take Cornell notes.
2. Complete the table where you list the main ideas of innate and adaptive immunity.
3. Write a compare and contrast essay addressing the following prompt: Homeostasis is key for the survival of all organisms. Although the immune system plays a major role in maintaining a homeostatic state in a healthy organism, the same immune system can cause a disease state. Explain and describe how this can be possible by comparing and contrasting the healthy and self-destructive immune system's roles (autoimmune disorders).

Some useful sites to conduct your research investigation:

http://missinglink.ucsf.edu/lm/immunology_module/prologue/objectives/obj05.html
http://www.hhmi.org/biointeractive/cells-immune-system
https://www.youtube.com/watch?v=MI-BLaj5nFk

Day 3: The immunity of invertebrate immunity
Ask the students to read take notes about the immune system from all of the following websites:

Materials

http://www.jimmunol.org/content/179/11/7209.full
"Invertebrate immune systems–Specific, quasi-specific, or nonspecific?" (2007) Rowley AF, Powell AJ. Immunol. 179(11).

(Suggested to conduct the activity over a weekend)

Outcomes
Concepts: AP-Biology Big Ideas 2.D.4, 4.A.4
Plants and Animals – including insects – have a variety of chemical defenses against infections that affect dynamic homeostasis.

Instructions
Your assignment includes:
Read the handout titled: Immunity in insects-innate immunity.
Read the article and Watch the video: The Insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogens. Take good notes as you will be conducting an experimental inquiry laboratory using this insect to study its immune response to insecticides.

Day 4: Initial planning and treatment of larvae with insecticide

Concepts: Big Ideas 2.D.3, 2.D.4 as an example of how biological systems are affected by disruptions to their dynamic homeostasis.

Students will select the insecticide concentrations to be used in the experiment-teacher may assign these, prepare & label equipment, treat larvae and began the larvae incubation.

1. Label your two sets of petri dishes. The first set is for the survival rate data. The second set is for the PO study data. Both sets should be labeled as control and treatments.
2. Clean the larvae with a cotton swab immersed with ethanol.
3. Hold the larvae gently and prepare to inject in the lower half of their body.
4. Using a pre-loaded syringe with water, inject the larvae with 10 microliters of water for control and place them on a petri dish after injection.
5. Using an insecticide pre-loaded syringe inject the larvae with 10 microliters with the selected concentration of the insecticide for treatment groups, and place them on a petri dish after injection.
6. Between the uses of different concentrations make sure to rinse the syringe with 75% ethanol.
7. Set the incubator at 28°C.
8. After all samples have been injected, place the petri dishes in the incubator at 28°C.
9. Be sure the temperature is consistent throughout the experiment.
10. Take daily notes on the survival rate.

Day 5: Collect survival data and preparation for PO

1. Upon arrival to the class, take daily notes on the survival rate on both sets of plates.
2. Before hemolymph extraction starts, prepare the following set of tips inside a microcentrifuge tube per each hemolymph extraction. Cut off 3 mm in length of a blue pipette tip. Also, cut 2 mm of a yellow pipe tip. Place the yellow tip inside the blue tip and place both inside a microcentrifuge tube.

3. Prepare the PO analysis buffer mixture in a microcentrifuge tube that contains 150 µl of distilled water, 20 µl of PBS Buffer, 20 µl cacodylate.
4. Each day after the first 24 hours one larva from the second set of plates will be used for the PO analysis. One larva from each plate, both control and treatment groups, will be used to extract the hemolymph.
5. Use scissors to slightly cut the larvae from the end side of its lower half of its body.
6. Insert the larva into the yellow pipette tip, which is inside the blue tip, within the microcentrifuge tube.
7. Make sure the larvae are fully inserted inside the tip with the fissure to the bottom.
8. Start the centrifuge for a maximum of seven seconds. If spun any longer the organs will flow down in the test tube along with the hemolymph. The extracted hemolymph should be at the bottom of the microcentrifuge tube; it should look clear yellow.
9. Transfer 10 µl of hemolymph into the previously prepared PO analysis buffer mixture microcentrifuge tube.
10. Mix the sample.
11. Sample out 150 µl of the PO analysis mixture into a cuvette and read absorbance at 490 nm.
12. Record the absorbance measured on a data table.

Days 6 and 7: Continue the collection of survival data and PO measurements.

Repeat the procedure described on day 5.

Day 8:

1. Upon arrival to the class, take daily notes on the survival rate plates. The PO plates should have already been used on days 5, 6, and 7.
2. Concepts: AP Biology Big Idea 3.D.2, 4.B
   Students would have an opportunity to develop a better understanding of the adaptive immune concepts involved with production and function of polyclonal and monoclonal antibodies.

Day 9:

1. Upon arrival to the class, take daily notes on the survival rate plates. This is the last day to collect survival rate data.
2. Graphing and analysis of data should be continued.

Day 10: Teacher leads class data evaluation and class discussions and/or independent work.

Independent Assignment Beekeeping Research Essay Prompt: Utilize the results obtained from this experiment and the concepts of immunology to inform the beekeeping industry about the appropriate use of insecticide to control pests.

Teacher Discussion Guidelines:
Based on the data recorded for the survival rate, the insecticide used in the experiment (Cypermethrin) does not kill off the larvae. The control larvae died within the same amount of days as the larvae that were injected with the insecticide. Therefore, the insecticide seems to be ineffective. The data for PO shows a proportional response to the amount of cypermethrin. The insecticide seems to induce a sufficient PO response which provides and sustains a protective effect against the insecticide.

Possible Test/Quiz questions: 1) What is the difference between a specific and a non-specific immune response? 2) What is the difference in humoral versus cell-mediated immunity? 3) What is an effector response and how is it connected to immunological memory? 4) Describe two different types of lymphocytes. 5) How are antigens and antibodies connected? 6) Describe antibody-antigen specificity.

IX. Summative Assessment

Based on 2007 AP® BIOLOGY FREE-RESPONSE QUESTIONS (Form B)
BIOLOGY
SECTION II
Directions: Answer all questions.
Answers must be in essay form. Outline form is not acceptable. Labeled diagrams may be used to supplement discussion, but in no case will a diagram alone suffice. It is important that you read each question completely before you begin to write. Write all your answers on the pages following the questions in the goldenrod booklet.

1. Without adaptive behaviors, animals would not survive.
(a) Describe the common physiological responses to infection in mammals. Explain how these adaptive physiological responses increase the relative fitness for an individual's survival.
(b) Compare and contrast the immune system of vertebrates and invertebrates.

2. The defenses of the human body to the entry and establishment of a pathogen (disease-causing organism) can be divided into nonspecific responses and specific responses.
(a) Explain at least how THREE types of nonspecific defenses can prevent the entry and/or establishment of a pathogen in a person's body.
(b) Discuss how the immune system responds to an initial pathogenic exposure, and how this initial exposure can lead to a quicker response following a second exposure to the same pathogen.
(c) Write an essay based on your research investigation to gather informative notes, citations and quotes to support the following claim: "Autoimmune conditions may arise from an overactive immune response that actually attacks itself."

3. Utilize the results obtained from this experiment and the concepts of immunology to inform the beekeeping industry about the appropriate use of insecticide to control pests.

Student Section

I. Introduction and Rationale:

In this experiment you will be infecting wax moth's larvae with the insecticide. During the process they will not be fed. The amount of insecticide injected will be correlated to the survival ratio of the organism. In addition, the hemolymph of the larvae will be extracted. By doing this we will be able to study the larva's immune system response to the insecticide.

Why is it relevant?

The study and control of GML is important for beekeeping; GML damage is the major biological constraint in the beekeeping industry. Beekeepers are attempting to produce a sufficient amount of honey; however, the moth *Galleria mellonella* is preventing this from taking place. The *Galleria mellonella* enters the beehives and consumes the honey and wax. Currently, there is no single efficient way to control this kind of pest. One probable solution may involve the use of insecticides developed to help the beekeeping industry.

Introduction to the experiment

Galleria mellonella is a moth (wax moth) that has been a huge issue for beekeepers because the moths ingest all of the wax and honey that is created within the hives. Larvae are a commencing stage that leads to the development of the wax moth. By discovering what concentration of an insecticide kills the larvae, a probable solution will be

developed to assist the beekeepers in controlling pests. In order to do so, it is essential to comprehend concepts of immunology. *Galleria mellonella* contain a complex and essential network of immune cells which play a crucial role in sustaining health throughout the body. In insects, Phenoloxidase is an enzyme that aids in the response to infection, hemolymph coagulation, cellular phagocytosis, oxidase-based melanization and some other examples of responses of the immune system to defend the body against any harm. For instance, insecticides are a substance that the immune system would recognize as foreign and leading to an immune response. Therefore, in this experiment you will study the larva's immune response to an insecticide.

Basic vocabulary in immunology

These are some important terms that will come up frequently in immunology:

- Immunity is defined as resistance to infectious disease.
- Immune system is the collection of cells and tissues that protects the body from infection.
- Immune response is the coordinated reaction of the cells of the immune system to a pathogen.
- Antigen is a general term that applies to molecules that bind to antibodies or T cell receptors. Antigens come in many forms. For example, small molecules in the environment and a huge array of bacterial and viral surface proteins might all act as antigens. Many times, you will see the terms antigen and microbe used interchangeably, since most antigens are derived from larger pieces of a microbe.
- Lymphocytes are cells found in the blood, lymphoid tissues and most organs of the body that express receptors for specific antigens and mediate immune responses. The lymphocytes that we will talk the most about are B cells and T cells. (B cell = B lymphocyte; T cell = T lymphocyte)
- Effector cells are produced when B and T cells become activated; they divide and mature and do the job of fighting the microbe.
- Plasma cells are mature B cells; they secrete antibodies, which are glycoprotein molecules that bind antigens with high affinity and help eliminate those antigens.
- Effector T cells are mature T cells that either assist ("help") leukocytes to kill ingested microbes or directly kill infected cells.
- Humoral immunity is the type of adaptive immunity that is mediated by antibodies produced by plasma cells. Humoral immunity is the main mechanism for defending against extracellular microbes and their toxins.
- Cell-mediated immunity is the type of adaptive immunity mediated by T lymphocytes; cell-mediated immunity is the main defense mechanism against microbes that survive within phagocytes (i.e. the bacteria that causes Tuberculosis) or that infect the cytosol of non-phagocytic cells (i.e. many viruses).
- Cytokines are secreted proteins that work as mediators of immune and inflammatory reactions. Cytokines provide a mechanism for cells of the immune system to "talk" to one another to coordinate a response. Interleukin is another term for a cytokine that acts on other leukocytes.
- Dendritic cells are antigen-presenting cells. Their main function is to process antigen material and "present it" on the cell surface to the T cells. They act as messengers between the innate and the adaptive immune systems.

Proper Use of Equipment

Incubator:

1) Plug in and switch on incubator.
2) Mount thermometer where designated.
3) Set to correct temperature.

Syringes:

1) Consist of two sections: plunger and barrel. The liquid can be extracted by pulling the plunger.
2) The barrel contains the measurements of amount of liquid required. To release the liquid, push the plunger.

Pipette:

1) Input the amount of volume needed by adjusting it in the caliber pipette line.
2) Apply pipette tip.
3) To obtain the specified liquid, press down the pipetting plunger to the first stop with the thumb.
4) To extract the liquid, hold the tip against the side of the receiving container.
5) Push down the pipetting plunger to the first stop and then to the second stop to release all the liquid.

Centrifuge:

1) When setting the test tubes, make sure they are balanced.
2) Make sure to balance the rotor by placing equal number of tubes on opposite sides of the rotor.
3) After setting the test tube, close lid and begin to spin.

Spectrophotometer:

1) Turn it on.
2) Go to settings to adjust the wavelengths to needed amount.
3) Organize plate layout.
4) Eject plate.
5) Insert substance into the plate.
6) Close plate.
7) Press start button to initiate readings.

Precautions and Safety

- Be careful with insecticide.
- DON'T inject yourself/someone with Syringe.
- Don't get insecticide into your eyes.
- Use Goggles.
- Don't consume Larvae.

II. Materials

- Petri Dishes
- Insecticide
- Syringes
- Automatic Pipette
- Pipette Tips
- Buffers
- Test Tubes

III. Lab Procedure (Protocol modified from J Vis Exp. (2012) 70: 4392.)

A. Insect selection for treatment

1) Select larvae which are 2-3 cm long and 180-250 mg in weight 24 hr. before treatment.
2) Put them into an empty box to starve them.
3) Remove the nascent silk cocoon around the larvae.

B. Insecticide treatment

The insects will be treated with an insecticide previously selected by you or your teacher**.** The concentration values to be used should aim to have a lethal concentration of 10% (LC10) and 50% (LC50) of individuals (based on previous data). This is the section in the lab that requires student's inquiry and experimental design.

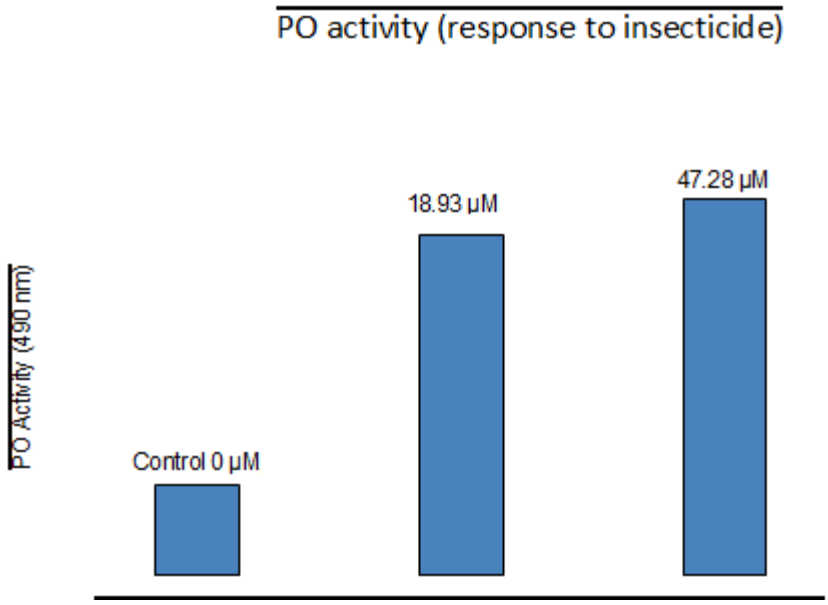
1) Larvae will be injected once.
2) At least one group will use an organophosphate insecticide.
3) The control will consist of the same kind of injection, but with distilled water.
4) Wax moth larvae will be kept in groups of individuals in 90-mm Petri dishes at 28°C.
5) The insect's survival rate will be recorded over the period of 10 days.
6) To study immunity parameters in the insects, 24 h after the initial exposure to insecticide concentration larvae will be tested to determined activities of phenoloxidase.

C. Recording insect mortality images for Kaplan-Meier graph*

1) The larval mortality will be checked regularly every 24 hr. for a seven day period. Dead larvae are inert and usually turn black.
2) The standardized pathology assay looks at a stage where the larvae stop feeding and the coloration begins to darken and turn eventually black. This stage usually lasts around 5 days.
3) Mortality data will be graphed.
4) Online support at http://eurekastatistics.com/
5) Instructions can be found at: https://www.medcalc.org/manual/kaplan-meier.php
6) Free Online Calculator or survival probability: http://www.hutchon.net/kaplan-meier.htm

D. Phenoloxidase activity assay

1) An aliquot of 10 µl of insecticide will be inoculated to larvae (148291B Fisher Scientific BD Micro-Fine™ IV Insulin Syringes).
2) The hemolymph will be collected for each individual larva after 30 min to 1 hour after injection.
3) To obtain the hemolymph specially designed tubes will be microfuged at 400 g for a maximum of 10 sec to gently precipitate the hemocytes and have a cell-free plasma.
4) A total of 10 µl of plasma will be removed with a pipette and diluted with an equal volume of cacodylate buffer.
5) Obtain the plasma and the intracellular cytosol (40) as presented above and either fresh or by thawing frozen samples place samples on ice.
6) Just before running assays prepare a fresh solution of L-dopa (4 mg/ml).
7) Pre-warm your cuvettes for the spectrophotometer (or Plate Reader) to 30^{o}C (room temperature 24^{o}C works fine too).
8) While samples are still on ice add 150ul distilled water, 20 µl PBS, 10 µl of hemolymph/cacodylate buffer and last, add 20ul of L-dopa (4 mg/ml).
9) Transfer sample to the cuvette and read at 490 nm the absorbance of samples for a total time of 60 min.
10) Read the sample as many times as possible to create several points over the 60 min time period.



IV. Data Collection

Data should be collected for survival rate. Count the individual surviving larvae in each plate. The counting should be completed daily for up to 10 days or up to when all larvae have died. The data collected for PO activity will be the absorbance at 490 nm for each individual larvae sacrificed.

V. Discussion/Analysis

1) What is the difference between a specific and a non-specific immune response? 2) What is the difference in humoral versus cell-mediated immunity? 3) What is an effector response and how is it connected to immunological memory? 4) Describe two different types of lymphocytes. 5) How are antigens and antibodies connected? 6) Describe antibody-antigen specificity.

Teacher Notes for Discussion of Results

The GM larvae model is useful to facilitate student's learning by generating results that can be easily correlated to the mammalian model (31). In both systems the innate immune response is mediated by cellular components. In the GM insect model the effector cells are hemocytes, while in the mammalian model the effector cells are phagocytic cells (32). Both systems require a cascade (33) of events initiated by the offender (insecticide, bacteria, etc.) and proceed quickly in matter of minutes (34). In the invertebrate GM model the cellular components are hemocytes produced in the lymph glands, while in the mammalian model they are phagocytic cells produced in bone marrow. In the GM invertebrate systems these cells initiate a cascade of events that lead to the activation of the enzyme phenoloxidase that signals the production of melanin (35). This latter molecule encapsulates the offender agent, and thereby isolates it and renders it ineffective (36). Similarly, in the mammalian system macrophage cells mediate a cascade of events through reactive oxygens (ROIs) and nitrogen intermediates (RNIs) to induce, in this case, an oxidative damage (37). Furthermore, the ROIs and RNIs induce a similar encapsulation to melanin by the generation of a granuloma. Although intrinsically the melanization and granuloma are different, they both offer an educational perspective to comparatively illustrate and stress the similarities and to contrast the differences of both systems. To stress the similarities, the process of phagocytosis can be illustrated by comparing the granulocyte's phagocytosis (34, 38) in the GM model to the neutrophilic phagocytosis in the mammalian system. To illustrate the contrasting aspects of both systems students could be introduced to the complexity of the granuloma formation, the variety of phagocytic cells (macrophages, neutrophils and dendritic cells) and granulocytes (eosinophils, basophiles, mast cells, natural killer cells); as contrasted to the limited number of hemocytes like in aegypti adult females (granulocytes, oenocytoids and prohemocytes) (39).

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