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Incorporating Evolutionary Medicine into an Introductory Biology Lab Program

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Since "nothing makes sense except in the light of evolution," we designed a multi-week lab unit that connects our students' interest in medicine and health care to evolutionary biology. Students design experiments to test an aspect of the antimicrobial hypothesis, which states that humans eat spices because this behavior is an adaptation that reduces infections and illness. Students explore the capacity of spices to affect the growth of various microbes;present their results; and integrate their findings with relevant published literature. This lab project is suitable for majors' introductory biology, microbiology, evolutionary biology, and non-majors' biology courses, with appropriate modifications.

Keywords: evolutionary medicine; antimicrobial; bacteria

Introduction

We redesigned our introductory biology program several years ago to address students' needs, interests, and preparation, and to fulfill our objective of enhancing the integration of the process of science into all aspects of the course. In the spring semester, the topics taught include introductory cell biology, genetics, and molecular genetics. We also revisit, regularly, the concepts of evolution that were taught during the fall semester. In lab, we implemented a new approach in which students engaged in scientific exploration and inquiry in a series of multi-week units. In these units, we emphasized experimental design; student teams planned and carried out research projects, which they presented to the class. Our challenge in designing these lab units was to develop feasible, meaningful projects. We also wished to reinforce evolutionary concepts in a way that would resonate with students. Our solution was to include the topic of evolutionary medicine in the lab program.

Evolutionary (or Darwinian) medicine seeks to provide ultimate, or evolutionary, explanations for disease and other physical issues or problems. This approach complements the medical perspective that attempts to explain proximate or mechanistic causes.

The project described here asks the question: why do humans eat spices? There are many possible answers but students explore the idea that spices have phytochemicals with antimicrobial properties. Consequently, eating spices decreases the risk of food-borne illness. The students' jobs are to figure out how to test this hypothesis; to design a series of experiments to do so; to implement the experiments; to analyze results; to integrate their findings with relevant primary literature; and to present their findings to the class.

Time Table

We devote three full weeks of lab for this unit and part of another week for presentations. Students are expected to come in outside of regularly scheduled lab times in order to collect data.

Week 1

Introduce topic; explain project and expectations; do pilot experiments; collect data; assign paper (Sherman, P.W. and J. Billing. 1999). Darwinian gastronomy: Why we use spices. *BioScience* 49 (6): 453-463.

Week 2

Design and implement experiment; collect data; analyze data; pose a follow up question or propose an additional experiment based on results.

Week 3

Implement next round of experiments; collect data; analyze data.

Week 4/5

Oral Presentations (these are scheduled for week 4 or 5 depending upon what other lab work is planned to get the next unit underway).

Student Outline

Evolutionary Medicine: Do Phytochemicals Affect the Growth of Microorganisms?

Introduction

Why Do We Get (or Feel) sick?

Why is heart disease a common health problem in the United States? A complete answer to this question requires two types of explanation: proximate and ultimate. Proximate explanations detail mechanisms. For example, heart disease is due to fat building up in blood vessels. Eventually, atherosclerotic plaques form and these can become so large that blood flow is completely blocked. If a coronary vessel is blocked, a heart attack results. Ultimate explanations answer *"why"*; they reveal the evolutionary basis of a phenomenon. Heart disease is the consequence of the evolution of the human body in an environment different from the one we live in now. More specifically, we evolved in an environment in which our diets were much higher in fiber and lower in fat. Moreover, our physical activity was much higher too. Heart disease is an outcome of the mismatch between us and our environment.

Medical explanations of disease usually focus on proximate causes but understanding ultimate causes is necessary for seeing the full picture. The field of evolutionary medicine looks at disease, and health, from an evolutionary perspective to explain why. This evolutionary approach does not replace proximate or mechanistic explanations but rather enriches them.

There are six categories of evolutionary explanations of disease:

1. *Defenses:* The body responds to illness defensively. These responses are not the actual disease but are efforts to minimize the damage and accelerate recovery. For example, coughing expels pathogenic organisms from the body, especially those captured in the mucus lining the interior of the lungs.

2. *Infections:* Microorganisms live in and on us in large numbers. In fact, the cells of our bodies are outnumbered 10 to 1 by these resident microorganisms. Humans (and other organisms) are in a continual state of resisting/fighting infection by pathogenic microorganisms while these invaders evolve ways to overcome our efforts. If we cannot keep pathogens in check, infection results. An overactive immune system, however, can produce other problems including allergies, asthma, and autoimmune disease.

3. *Novel environments:* As already described using heart disease as an example, a mismatch between the body and the environment can result in illness.

4. *Genes:* Some genes contribute to positive physical and/or functional outcomes for organisms under certain circumstance but to negative ones under others. Thus, even though a gene may be associated with a disease, it may persist because it is advantageous sometimes. Sickle cell disease illustrates this phenomenon; an individual heterozygous for the sickle cell allele enjoys greater resistance to malaria than an individual lacking this allele. A person possessing two copies of the sickle cell allele will have sickle cell disease.

5. *Physical and Functional Compromises:* The early ancestors of humans were not bipedal. Whereas walking upright is an adaptive trait, it strains the lower back. The benefit of walking upright comes at the cost of an increased risk of back problems. Some diseases or health problems result from this type of compromise.

6. *Evolutionary Legacies:* During embryogenesis in vertebrates, the respiratory and digestive systems begin as a single tube that forms two tubes: the trachea and esophagus. Whereas the openings to both tubes remain next to each other,during swallowing the epiglottis covers the trachea, preventing the aspiration of food. We can conclude that an epiglottis-like structure arose, and its utility in preventing choking deaths increased fitness. The better the epiglottis, the lower the choking risk, the better the fitness. The modern epiglottis exists because of the evolutionary legacy of shared anterior openings to respiratory (trachea) and digestive (esophagus) systems. There are some features of our bodies that we are "stuck with" simply because they are the product of an evolutionary process; we were not engineered or designed.

Infections and the Antimicrobial Hypothesis

Evolutionary medicine provides an interesting and potentially useful perspective on health and disease. Yet simple experimental designs that test these ideas remain challenging. In the project you will undertake, we will explore the second category of evolutionary explanation: infection. More specifically, we will search for an evolutionary explanation for spice use in food preparation.

Spices and herbs are actually phytochemicals, or secondary metabolites, produced by plants. Secondary metabolites are chemicals, which while nonessential for life, nevertheless serve a variety of important functions including plant cell communication and plant defense against attack by herbivores, bacteria or fungi.

Perhaps humans take advantage of the phytochemicals that plants produce for their own protection when we use spices and herbs in food preparation. As described in your assigned reading, spice use in food occurs throughout the world in culturally and geographically distinctive patterns. One hypothesis proposes to explain spice use patterns as the result of the phytochemicals contained therein having antimicrobial activity. In other words, spices inhibit or stop the growth of potentially harmful microorganisms. In doing so, they reduce the risk of humans ingesting food-borne pathogens.

In the project described here, you will address this question directly by designing an experiment that will address some aspect of whether spices are indeed antimicrobial. A general protocol for setting up an experiment to test whether spices will affect bacterial growth is detailed below. You can use this description as a starting point for your own experimental design. Following this general protocol is a list of suggested avenues of inquiry for your individual experiment.

General Protocol

Each group will have the following materials available for use:

- 1, 2.0 mL liquid culture of *E. coli* in sterile 15 ml centrifuge tubes (Note: in follow up labs, other types of bacteria will be available).
- 1 test-tube rack
- 5 sterile small Petri dishes for soaking disks
- 20 Mueller-Hinton agar plates
- sterile water
- P-200 pipettor and box of sterile yellow tips
- balance (store in cabinets between labs)
- mortar and pestle for pulverizing spices
- various glassware
- antibiotic discs (penicillin and tetracycline in refrigerator)
- sterile blank filter paper discs in small bottle and an empty sterile Petri dish
- bacterial-waste can (lined with autoclave bag)
- metal forceps
- metal spatulas and weigh boats
- Bunsen burner with striker
- small beaker with 90-100% ethanol (~3 cm depth) covered with aluminum foil
- glass elbow or metal spreader
- 1-L beaker for fire control

NOTE: Use sterile techniques for this procedure.

1. Turn over a Mueller-Hinton plate so that the lid-side is down. Do not open the plate. Divide the plate into four approximately equal sized areas using a Sharpie.
2. Label the bottom of the plate with your lab section and group's initials. Your lab instructor will instruct you on how to label the four quadrats.
3. To prepare a test disc, weigh out a specific amount of your spice as appropriate for your experiment, and add it to a mortar. Add an appropriate volume of sterile water. Grind the mixture with a pestle.
4. Soak blank filter paper discs in this mixture to impregnate them with the chemicals present in the spice.
5. Alternatively, some materials can be tested by placing them directly on the nutrient agar in the appropriate section.
6. Resuspend the bacterial culture by vortexing or tapping the sides of the tube.
7. Transfer 100 µl of the bacterial suspension onto the Mueller-Hinton Agar.
8. Discard the used pipet tip in the waste bin lined with an autoclave bag.
9. Spread the bacterial suspension with a sterilized glass or metal elbow evenly over the entire agar surface. Aim to inoculate the bacteria uniformly so that a homogenous "lawn" of bacteria grows on the plate.
10. After the microbial suspension has dried on the agar turn the plate right side up, open the dish, and place a paper disc:
    a. soaked in sterile water on one section of the agar (negative control),
    b. containing the antibiotic penicillin on another section (positive control),
    c. containing the antibiotic tetracycline on a third section (positive control),
    d. soaked in the test substance in the fourth section (experimental treatment).

11. If you have not yet labeled the four sections on the bottom of the Petri dish, do this now without inverting the Petri dish. If you flip the plate over, the discs may lose contact with the agar.
12. Incubate the cultures overnight at the appropriate temperature for your experiment (35°C for *E. coli*). Examine cultures at 24, 48, and 72 hours. At each observation, record whether there is a zone of growth inhibition around any of the discs, and if so, record the inhibition zone diameter in millimeters.

Week 1:

1. Read "*Darwinian Gastronomy: Why we Use Spices*" by Paul W. Sherman and Jennifer Billing (1999).
2. Begin the data analysis by calculating the average inhibition zone diameter in mm for each treatment and the standard error of the mean. Prepare a properly formatted data table for these results. Summarize results in *no more than 2-3 sentences*. In a separate paragraph, interpret results.
3. Based upon the reading and preliminary results, decide upon a direction to take for your own experiment. Articulate the specific question(s) you are asking and state your hypotheses and predictions. Explain the rationale for each particular hypothesis and prediction.
4. Design an experiment to address your question. Make a flow chart of the specific steps you will take for your particular investigation. Be sure to include replicates and appropriate controls.

Suggested Avenues for Inquiry

- Test different spices
- Test dried versus fresh spices
- Test spices in combination
- Test various types of bacteria
- Test spices in combination with other cooking ingredients
- Determine whether heating spices influences their antimicrobial abilities
- Test various concentrations of a spice including the amount found in a typical recipe
- Compare the effects of spices characteristic of different ethnic cuisines
- Test incubation temperature
- Test the effect of humidity
- Compare the effects of spices used to prepare meat dishes versus those used for vegetable dishes
- Your question

Week 2:

1. Finalize independent research projects and run first set of experiments. Collect data at 24, 48, and 72 hrs.
2. Calculate averages and standard errors of the mean. Prepare a properly formatted data table.
3. Find at least two primary or secondary peer-reviewed references related to your specific project. Print out the first and last page of each. Provide full citations using proper scientific reference format. In two or three sentences, explain how each reference is relevant to your experiment.

Week 3:

Discuss last week's results with your group members and instructor. Design and set up next round of experiments. Collect data at 24, 48 and 72 hrs.

Possible Extensions

- See the list of suggested avenues of inquiry to identify any that might be logical extensions of your experimental work
- Determine whether particular spices delay or prevent food spoilage
- If you observe inhibition of microbial growth, determine whether the cells have stopped growing or if they have been killed
- Your idea

Final Assignments: Group Oral Report and Individual Written Results and Discussion

Overview

You should present your project in a unified format that focuses on the main question or set of related questions you pursued. In most cases this will involve presenting results from the second and third set of experiments. The first week of this unit involved becoming familiar with procedures and running a pilot study. Depending upon your group's focus, these initial data may or may not be relevant to your chosen line of inquiry.

Oral Presentation

Each person in the group must participate. Please note that it may also be helpful to review the guidelines for a written summary to see what type of information belongs in each major section of the presentation. This includes: Introduction, Procedure, Results, Discussion and Conclusion. Emphasize visuals (pictures, graphs, diagrams) and **minimize** text. Use bullet statements not narratives.

Written Assignment.

Each ***individual*** is expected to write the following in a paragraph format:

- State your hypotheses and predictions for each question(s) asked over weeks two and three (and possibly week one, if relevant).
- State the reason(s) for pursuing this line of research. For example, draw on your pilot study results and the article by Sherman and Billing (1999). Always cite your sources.
- Results section: This should include properly labeled data tables and figures as well as the narrative.
- Discussion section.
- Works cited.

The grading criteria for this unit will be:

1. How well you followed directions.
2. Overall quality of oral presentation
3. Overall quality of written presentation
4. Whether the research pursued was relevant to the assigned topic.
5. Whether results are compared to published research

Literature Cited

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Notes to the Instructor

Preparation Instructions

Recipes (5 lab sections; five lab teams per section; four students per team):

1. **~1500-1750 sterile Mueller-Hinton agar plates** (500-580 plates/week X 3 weeks). Follow directions on side of container and see information below for how to prepare agar and pour plates.
2. **170, 16 ml test tubes with 5 ml sterile Mueller- Hinton broth.** Prepare 850 ml broth first in large flask and mix/heat to dissolve. When solution "clears", distribute 5 ml broth into each test tube and place metal cap on top. Place tubes in autoclavable test tube racks; affix autoclave tape on to front of rack and autoclave (liquid cycle, 20 minutes).
3. **25 small bottles with sterile water.** Choose bottles from media glass supply. Fill half way with deionized water. Place label (deionized water) on each bottle. LOOSELY place screw cap on each bottle. Autoclave on liquid cycle for 15 minutes. Once bottles have cooled, tighten lids.

Bacterial Culturing Information: Use Sterile Technique

Several of the following bacteria will be available and need to be sub-cultured 24-48 hrs before each lab:

Escherichia coli(Gram -)
Bacillus cereus (Gram +)
Bacillus coagulans(Gram +),
Bacillus subtilis (Gram +),
Enterobacter aerogenes (Gram -) (does best at cooler temperature than other species; 30°C is ideal)
Pseudomonas fluorescens (Gram -).

Note: *E. coli* will be used exclusively the first week and the other species will be available for projects during weeks 2 and 3.

From Solid Media

Using a sterile loop or toothpick (if from plate) transfer a small quantity of bacteria to a sterile tube containing ~5 ml sterile nutrient broth. Place in the shaking incubator for 24-48 hrs. Store tubes in test tube rack at room temperature until needed in lab.

From Liquid Culture

Mix culture by vortexing or gentling swirling the tube/flask. Transfer about 100 µl of bacterial suspension into a sterile tube containing ~5 ml sterile MH broth. Place in the shaking incubator for 24-48 hrs. Store tubes in test tube rack at room temperature until needed in lab.

Available Spices and Related Materials

Allspice, ground and whole; anise seed; dry basil; bay leaves; caraway seeds; cardamom seeds; celery seeds; chives; cilantro; cinnamon, ground and stick; cloves, ground and whole; coriander, ground and seed; cumin, ground and seed; dill seed and weed; fennel seed; garlic, powder, granules, and fresh; ginger, ground and fresh; juniper berry; lemon grass; mace, ground; mint; mustard, ground and seed; nutmeg, ground and whole; onion; oregano, ground; pepper, white ground; pepper, black ground; pepper, habanero flakes; pepper, cayenne ground; pepper,red chili flakes; pepper, whole chipotle; pepper, whole Habanero; pepper, whole jalapeno; peppercorn, black; peppercorn, Szechuan; poppy seed; rosemary, leaves; sage, leaves and ground; salt; tarragon leaves; thyme, leaves and ground; turmeric ,ground; vanilla, beans and extract; vanilla,artificial; Cajun seasonings mix; chili blend mix; five spice mix; Italian seasonings mix; lemon and pepper mix; wasabi powder; coffee; tea black and green; citric acid; lemon juice; limes; vinegar,white; fructose; honey; various cooking oils.

Evolutionary Medicine and the Antimicrobial Hypothesis: Some Helpful Reading

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About the Author

Donna Bozzone received her B.S. in biology from Manhattan College and her masters and Ph.D., also in biology, from Princeton University. After a post-doctoral position at the Worcester Foundation for Experimental Biology, she joined the faculty of Saint Michael's College where she is a Professor of Biology.

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