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An Enzyme Assay with Evolutionary Implications: You Are What You Eat! The Effect of Food Source on Activity of Esterases in Bean Beetles

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Here we present a multi-session, investigative enzyme lab using detoxification enzymes that have a potential role in the development of insecticide resistance in insect pests. Using the bean beetle (*Callosobruchus maculatus*) as a model organism, students will try to determine whether food source affects activity levels of detoxification esterase enzymes in bean beetles, which would have implications for the resistance of these pests to insecticides. The students will a) learn about the life cycle of the beetles, how to differentiate sexes, and how to culture the beetles, b) gain experience in designing a study using basic biochemical techniques such as standard curves and colorimetric assays, and collecting, analyzing, and reporting data for such a study, and c) discuss the interplay between natural selection, leading to the evolution of defensive chemicals in plants and detoxification enzymes in animals, with agroecology, leading to resistance to insecticides.

Keywords: biochemistry, inquiry-based learning, enzyme assay, detoxification enzymes, insecticide resistance, evolutionary arms race, bean beetle

Introduction

This lab exercise sequence is a multi-session, investigative enzyme lab using detoxification enzymes that may be involved in the development of insecticide resistance in insect pests. We use bean beetles (*Callosobruchus maculatus*) as the model organism. We are using a procedure previously described (Fermin *et al*., 2015) for obtaining a crude extract that can be used in enzyme assays. The focus of this lab exercise is to try to determine whether food source can affect activity levels of detoxification esterase enzymes in bean beetles, which would have implications for the resistance of these pests to insecticides. Thus the evolutionary arms race between insects and the plants on which they feed is linked to the agroecology of insecticide resistance.

The lab exercise as written is designed for three, 3-hour lab periods for students in our high-enrollment introductory biology. We standardize the enzyme assay data using protein concentration. A simpler way would be to standardize the enzyme assay data by weight of individual animals, as done by Gbaye *et al*. (2012), for example. At the end of this sequence, the students perform statistical analysis, including a t-test, on the class data, and they write their second formal lab report for the semester. Earlier in the semester the students learn about the life cycle of the beetles and how to culture them, they receive instruction in and practice using micropipettors, and they perform a 3-week lab exercise that includes designing and conducting a controlled experiment, and culminates in an introduction to descriptive and inferential statistical analysis including simple t-tests. For the lab exercise described here, it is helpful for the students to have received in class an introduction to the following topics: natural selection, cell-cell signaling, and enzymes.

For more advanced students this lab exercise can be modified to a longer-term project that requires more independent work including background research. Examples include investigating the effect of food source on other detoxification enzymes identified through a literature

review, optimizing pH and temperature conditions to study the various enzymes, and the potential detoxification of insecticides that contain ester bonds. There is published research related to these topics for other insect species that can inform the research the students may perform on bean beetles. Examples include Liang *et al*. (2007), Magaña *et al*. (2008), Wu *et al*. (2009), Yu (1984), and Yu (1999). Given the paucity of published research on insecticide resistance and the role of detoxification enzymes in bean beetles, the research students perform on bean beetles will produce novel data.

Most of the solutions can be made ahead of time and stored for later use. Thus the daily preparation time for each lab is generally minimal. Bean beetles are easily maintained in the laboratory provided a temperature-controlled incubator is available. Beck and Blumer (2011) provide extensive information with regards to culturing and maintaining the animals. To ensure adult beetles are available for the experiment, we have multiple cultures started at different times. This experiment relies on cultures of the same strain of beetles grown on different bean seeds for many generations. Therefore, if you are starting a bean beetle culture on a new species of legume seed, there will be a wait-time of at least one year for 10 generations to be reared on that seed species.

Student Outline

Objectives

- Describe how esterases are used in the animals' battle with plant chemical defenses and how this relates to insecticide resistance.
- Explain why bean beetles are a good system for studying the effect of food source on insecticide resistance.
- Design a study to investigate whether different food sources affect the activity levels of esterases in bean beetles.
- Collect, analyze, and present the data from such a study.
- Discuss the evolutionary and ecological significance of the findings.
- Discuss the limitations of the study and suggest future steps based on the results obtained.
- Explain how a colorimetric enzyme assay can be used to determine activity levels of an enzyme.
- Establish a standard curve and use a colorimetric assay to determine protein content in crude extracts.

Introduction

Bean beetles, in the genus *Callosobruchus*, are agricultural pests found in tropical and subtropical regions of Africa and Asia. They are pests of legume seeds (family Fabaceae) such as mung beans (*Vigna radiata*) and black-eyed peas (also called cowpeas, *Vigna unguiculata*). The adults do not require food or water and their short lifespan (1-2 weeks) is to mate and for the females to lay eggs on bean seeds. The larvae then feed on the bean embryo and endosperm and thus destroy the bean crop (Beck and Blumer, 2011). Hence, there is great interest in controlling or eliminating these pests and minimizing their effect on the bean harvest.

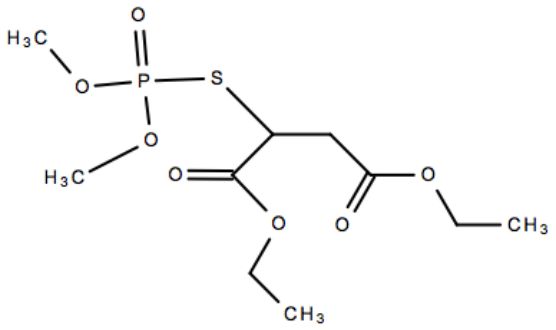


Figure 1. Malaoxon structure. Note the ester bonds.

Organophosphate insecticides such as malathion and its oxidation product malaoxon (Figure 1) have been used to control insect pests such as bean beetles. These insecticides work through their effect on the enzyme acetylcholinesterase (AChE). AChE is an important component of cell-cell signaling in the nervous system where it breaks down the neurotransmitter acetylcholine and helps to terminate the signal (see Figure 2). Inhibition of AChE interferes with this process. This is how the insecticide interferes with proper functioning of the nervous system in the insects and leads to their eventual death.

These insecticides have also been linked to anxiety in rodents (Sadeghi Hashjin *et al*., 2013) and suicide in humans (London *et al*., 2005), potential unintended consequence of use of these insecticides in nature. Agricultural runoff can bring these insecticides to rivers and other bodies of water, thus potentially subjecting aquatic organisms, such as various fish species, to harmful effects of these insecticides. For example, changes in behavior and morphological deformities have been reported in fish exposed to sublethal concentrations of these insecticides in the laboratory (Patil and David, 2010). For these reasons, there is interest in minimizing the amount of insecticide applied in the environment to control insect pests.

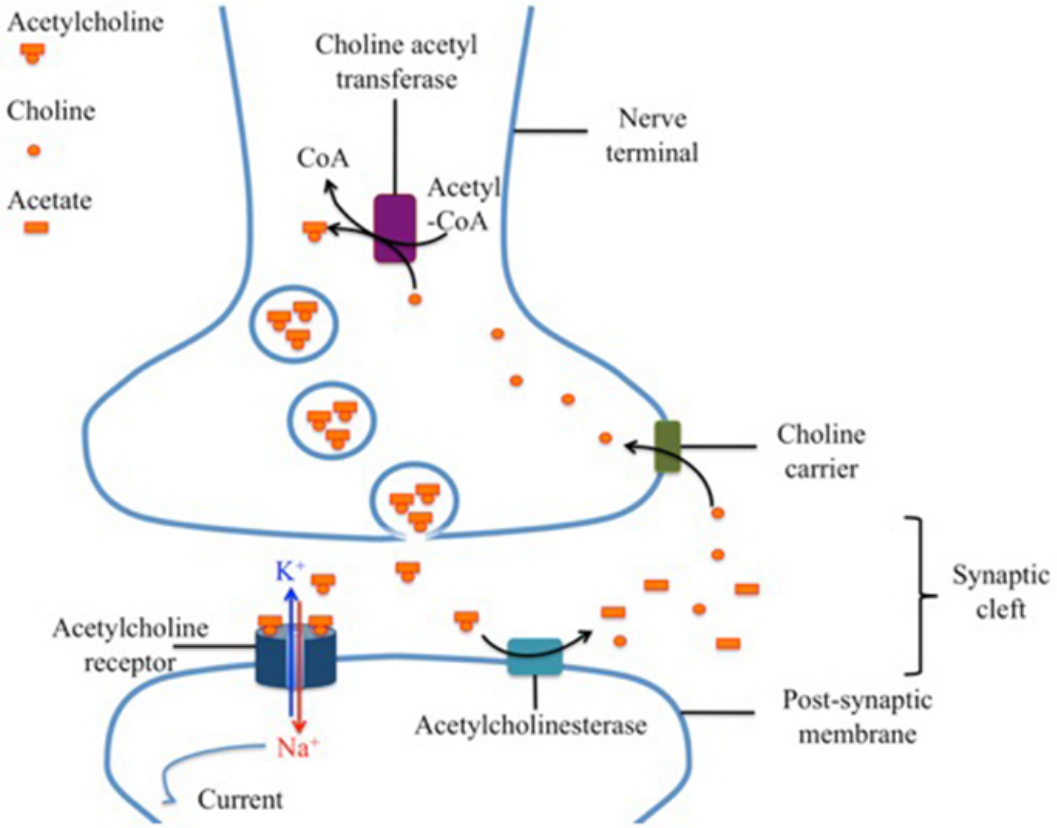


Figure 2. Synaptic signaling involving the neurotransmitter acetylcholine.

Resistance to these insecticides has been documented in various insects (Liang *et al*., 2007; Magaña *et al*., 2008; Wu *et al*., 2009; Reyes *et al*., 2011; Spencer *et al*., 1998; Walsh *et al*., 2001; Yu, 1984; 1999) and is an important problem in pest control. The host plants seem to have an effect on susceptibility of the insects to insecticides (Liang *et al*., 2007). One method of resistance is through the detoxification activity of esterase enzymes (Liang *et al*., 2007; Magaña *et al*., 2008; Wu *et al*., 2009), though this has not been reported for bean beetles so far. Presumably, the effect of the food source is due to the response of the insects to various defensive chemicals present in the various plants that serve as the food sources for the insects (Yu 1984; 1999). While plants have evolved chemical defenses, the insects have evolved mechanisms to detoxify the plant toxins. A side effect of this evolutionary arms race between plants and insects is that the detoxification strategies evolved in the insects may also allow them to detoxify the chemicals we use as insecticides to kill them (Devonshire, 1977)!

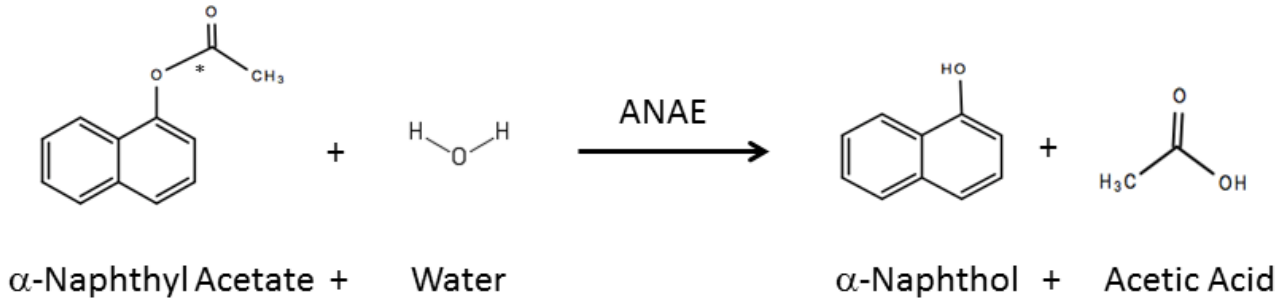
We are interested in the following question, with its evolutionary and ecological implications: will different food sources affect the level of activity of these detoxification enzymes in bean beetles? Different strains of bean beetles are available and some have been cultured on different legume seeds for many generations. We will focus on two detoxification enzymes, two different esterases that preferentially work on different substrates *in vitro*. You will use a crude protein extraction followed by colorimetric enzyme assays to determine the level of activity of each enzyme in bean beetles. You will compare the activity levels between bean beetles grown on two different legume seeds. These are important pieces of information that can help us to understand the evolutionary relationship between insects and their food sources as well as the potential for development of resistance in insect pests to insecticides and its ecological consequences.

Since each beetle will be a different size, and since different experimenters will not homogenize the sample tissues with similar levels of vigor, the concentration of each enzyme in the crude extracts will not be identical for each sample beetle. Therefore, you will need to account for such differences in the samples. One way to do this is to adjust the enzyme activity levels by total protein concentration in each extract. Protein concentration in the crude extracts can be determined using a colorimetric assay and known standards.

The enzyme assays

The enzyme assays we will use are based on the work done by Martínez Morcillo *et al.* (2013), Reyes *et al.* (2011), and Spencer *et al.* (1998). Basically we take advantage of the type of reaction carried out by each esterase by supplying different substrates to the crude extract *in vitro*. In the presence of the appropriate esterase enzyme, different substrates, α-naphthyl acetate or β-naphthyl acetate, will be hydrolyzed to produce different products, α-naphthol or β-naphthol, respectively (Figure 3). These products will interact with the dye provided (Fast Blue B Salt) and cause a change in absorption of light by the dye at a specific wavelength. We can thus measure the activity level of each enzyme by the change in absorbance as measured using a spectrophotometer.

a)



b)

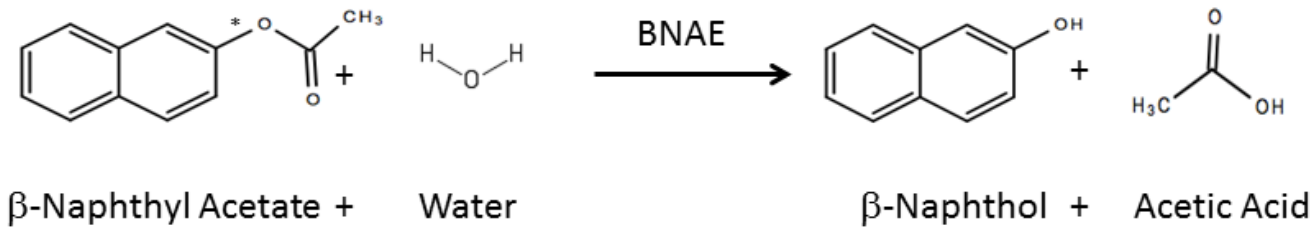


Figure 3. The reactions carried out by a) α-naphthyl acetate esterase (ANAE) and b) β-naphthyl acetate esterase (BNAE). The asterisks mark the ester bonds hydrolyzed by the enzymes.

The protein assay

The protein assay, based on Bradford (1976), is a colorimetric assay for measuring concentration of total solubilized protein. The assay relies on a specific dye, Coomassie brilliant blue G-250, which binds to proteins. This binding leads to a color change and a shift in light absorbance by the dye. A standard curve is established using known concentrations of a purified protein such as bovine serum albumin (BSA). Readings from unknown samples are compared against the standard curve to determine the total protein concentration in the unknowns.

Investigation: Will different food sources affect the level of activity of detoxification enzymes in bean beetles?

What are the class hypotheses with regards to the effects of food source on the activity of detoxification esterase enzymes extracted from bean beetles?

Alternative:

Null:

What are the variables?

Independent

Dependent

Standardized

What are the levels of treatment?

What are the replications and sample sizes?

What are the organisms being used for this study?

What is the prediction for the experiment?

Timing

- During lab 1 you will carry out Parts I and II of the procedure for beetles from one strain grown on one type of legume seed.
- During lab 2 you will carry out Parts I and II of the procedure for beetles from the same strain grown on a different type of legume seed.
- During lab 3 you will carry out Part III of the procedure for all beetle extracts and analyze your data.

Methods and Data Collection

Part A: Materials

- Bean beetles, *Callosobruchus maculatus*, one strain, grown on different legume seeds
- Small paint brushes
- Dissecting microscopes
- Lyophilized bovine serum albumin (BSA), reconstituted with deionized water, stock solution of 2.0 mg/mL BSA
- Bradford dye reagent
- Homogenizing buffer: 100 mM sodium phosphate pH 7.4 with 1% Triton X100
- α-Naphthyl Acetate (0.027 M stock)
- β-Naphthyl Acetate (0.027 M stock)
- Fast Blue B Salt Stain containing 10% SDS, made fresh by lab instructor
- Spectrophotometer
- Disposable, polystyrene, semi-micro 1.5 mL cuvettes
- Centrifuge
- Disposable pestles
- Vortexer
- 1.5 and 2.0 mL microcentrifuge tubes
- Ice bucket
- Micropipettors and tips
- Timers

Part B: Procedure

Lab 1:

I. Crude Extracts (Figure 4)

1. Wear gloves and goggles. There should be no exposed skin: covered legs and arms.
2. Use 4 beetles per group of 4 students during lab 1 (and 4 beetles per group during lab 2), if enough beetles are available. Make sure to determine the sex. Note the strain and the legume seed.
3. Take a clean 1.5-mL microcentrifuge tube. Label the tube by writing 1A on the cap and your group number on the side using a permanent marker.
4. Select one adult beetle. Put the animal in tube 1A. This is beetle #1 for your group.
5. Repeat steps 3 and 4 for beetles #2, 3, and 4 (tubes 2A, 3A, and 4A).
6. Use a micropipettor with the correct tip to add 500 µL (0.5 mL) of homogenizing buffer to each tube. Keep the tubes on ice.
7. Use the pestles provided to rapidly homogenize the tissues for 5 seconds. Use a separate clean pestle for each beetle. After you are done, you can rinse and dry the pestles and return them to the container for used pestles.
8. Add another 500 µL (0.5 mL) of homogenizing buffer to each tube. Close the tubes by snapping the caps shut. Invert 5 times to mix.
9. Put the tubes in the centrifuge. Make sure the centrifuge is balanced. Centrifuge for 5 minutes at maximum speed.
10. Label 2 clean microcentrifuge tubes: 1B and 1C (or 2B and 2C, etc.) on the cap and your group number on the side.
11. Transfer the supernatant from tube 1A into tube 1B (and from tube 2A into tube 2B, etc.). Avoid transferring the animal parts. Keep the tubes on ice.
12. Close the caps. Put the B tubes (1B, 2B, etc.) in the centrifuge. Make sure the centrifuge is balanced. Centrifuge for 5 minutes at maximum speed.
13. Transfer the supernatant from tube 1B into tube 1C (and from tube 2B into tube 2C, etc.). Avoid transferring the cloudy white layer from the top of the tubes 1B etc. You need 500 µL of solution in each tube. Keep the tubes on ice.
14. Close the tube caps. Use the vortexer to vortex tube 1C (and 2C, etc.) for 10 seconds for the solution to be mixed properly.
15. Centrifuge for 10 seconds to remove any bubbles. This is the crude extract you will use in parts II and III.

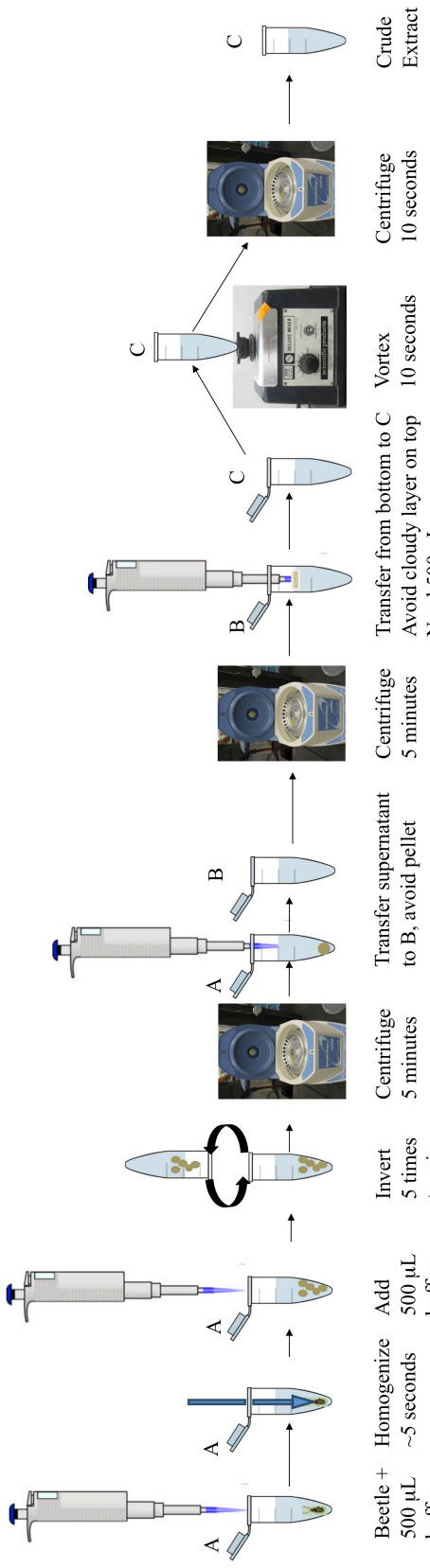


Figure 4. Procedure for crude protein extraction

II. Enzyme Assays (Figure 5a)

Your lab instructor will perform the following steps:

16. Fast Blue B Salt Stain: Mix 0.030 g Fast Blue B Salt (pre-measured) with 5 mL distilled water and vortex. Add 5 mL 10% SDS solution to the tube and invert 5 times to mix the solution. Aliquot 1.5 mL of the solution into microcentrifuge tubes for each group.
17. α-naphthyl acetate stock solution: Transfer 15 mL of homogenizing buffer to a large test tube. Transfer 240 µL of the α-naphthyl acetate stock solution to the test tube. Vortex for 20 seconds to mix. Label accordingly. Aliquot 1.5 mL of the solution into microcentrifuge tubes for each group. Label accordingly. This will be the α-naphthyl acetate solution each group will use in steps 26 and 29.
18. Repeat the above procedure (step 17) for β-naphthyl acetate. This will be the β-naphthyl acetate solution each group will use in steps 27 and 30.

Each group will perform the following steps:

19. For each beetle, label 2 clean microcentrifuge tubes: 1D and 1E (2D and 2E, etc.) on the cap and your group number on the side.
20. Mix the solution in tube 1C (and 2C, etc.) before performing the next step.
21. Follow the steps below and Table 1 to set up the reaction tubes and the blanks for the enzyme assay.
22. Use a micropipettor to transfer 50 µL of the crude extract from tube 1C to tube 1D. Keep the tube on ice.
23. Use a micropipettor to transfer 50 µL of the crude extract from tube 1C to tube 1E. Keep the tube on ice.
24. Repeat for tubes 2C, 2D, and 2E, etc. Keep the tubes on ice.
25. Use a micropipettor and add 250 µL of homogenizing buffer to each of the D and E tubes. Vortex the tubes for 10 seconds.
26. Use a micropipettor to add 240 µL of the substrate α-naphthyl acetate solution to the D tubes (1D, 2D, etc.). Keep the tubes on ice.
27. Use a micropipettor to add 240 µL of the substrate β-naphthyl acetate solution to the E tubes (1E, 2E, etc.). Keep the tubes on ice.
28. Label two microcentrifuge tubes B-α and B-β. These will serve as the blanks for the spectrophotometer.
29. Prepare a Blank tube that includes the following; this is the blank B-α for the D tubes.
    - 300 µL of homogenizing buffer
    - 240 µL of the α-naphthyl acetate solution
30. Prepare a Blank tube that includes the following; this is the blank B-β for the E tubes.
    - 300 µL of homogenizing buffer
    - 240 µL of the β-naphthyl acetate solution
31. Invert all tubes 5 times to mix the solutions. Incubate the tubes in the dark at room temperature for 10 minutes.
32. After the incubation, add 110 µL of Fast Blue B Salt Stain solution to each of the tubes including the blanks.
33. Invert the tubes 5 times to mix the solutions. Incubate in the dark at room temperature for 20 minutes.

Table 1. Chemical components of the D, E, and blank tubes for the α-naphthyl acetate esterase (ANAE) and β-naphthyl acetate esterase (BNAE) enzyme assays. Add in the order from left to right.

Tube	Crude extract from tube C	Homogenizing buffer	α-naphthyl acetate	β-naphthyl acetate	Invert 5 times Incubate 10 min	Fast Blue B Salt Stain
D tubes	50 µL	250 µL	240 µL	-		110 µL
E tubes	50 µL	250 µL	-	240 µL		110 µL
B-α	-	300 µL	240 µL	-		110 µL
B-β	-	300 µL	-	240 µL		110 µL

34. Take 2 spectrophotometer cuvettes per beetle. Label them as 1D (and 2D, etc.), 1E (and 2E, etc.) plus your group number. Make sure to label them on the sides that do not interfere with light absorption; your lab instructor will show you how to do this.
35. Label two other spectrophotometer cuvettes: B-α and B-β and group number.

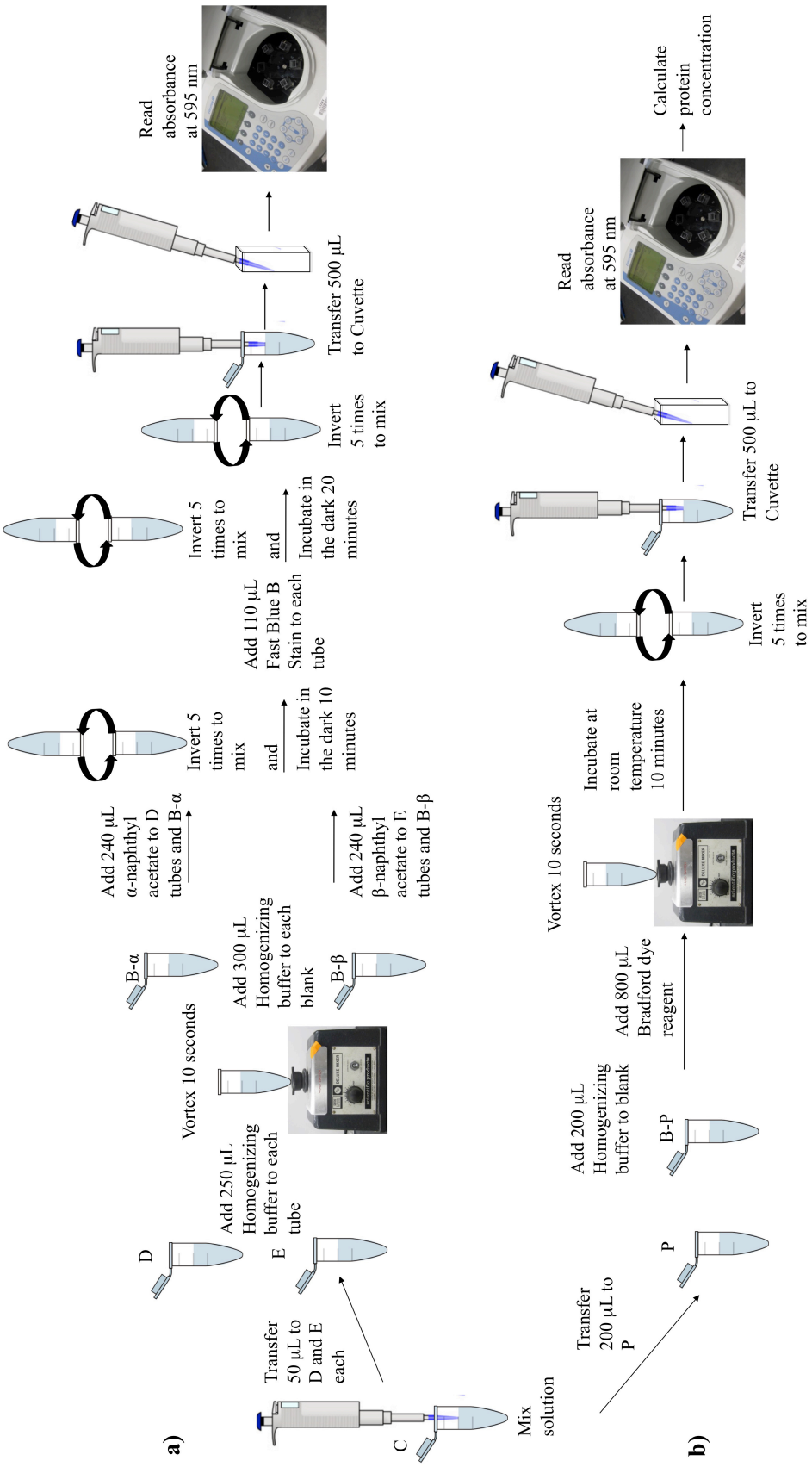


Figure 5.
a) The basic procedure for the esterase enzyme assays using the crude extract
b) The procedure for using the Bradford assay to determine the protein concentration in the crude extract

36. After incubation, invert each tube 5 times to mix the solution.
37. Use a micropipettor to transfer 500 μL of the solution from microcentrifuge tube 1D to the clean spectrophotometer cuvette 1D. Do this very gently, and touch the tip of the pipette to the side of the cuvette to avoid forming any bubbles. Discard the tip. Repeat for tubes 2D, 3D, and 4D.
38. Use a micropipettor to transfer 500 μL of the solution from microcentrifuge tube 1E to the clean spectrophotometer cuvette 1E. Discard the tip. Repeat for tubes 2E, 3E, and 4E.
39. Use a micropipettor to transfer 500 μL of the solution from microcentrifuge tube B-α to the clean spectrophotometer cuvette labeled B-α. Repeat for B-β.
40. For each cuvette measure the absorbance at 595 nm using the spectrophotometer provided. Make sure to use the correct blank tubes for the D and E tubes. Record the absorbance readings at 595 nm in Table 2.
41. Place tubes 1C, 2C, etc. in the freezer for later use. Make sure the tubes are properly labeled with your group name.
42. Your instructor will tell you how to discard your tubes and chemicals.

Table 2. Measured absorbance values for reaction by α-naphthyl acetate esterase (ANAE) and β-naphthyl acetate esterase (BNAE) (samples D and E, respectively) for beetles grown on _____ seeds.
Note Beetle Strain: _____

Beetle #	Sex	Protein Content (μg)	Absorbance for D tubes (AU)	ANAE activity (AU/μg protein)	Absorbance for E tubes (AU)	BNAE activity (AU/μg protein)
1						
2						
3						
4						

Lab 2:

43. During lab 2, repeat Parts I and II for 4 beetles of the same strain grown on a different legume seed. Make sure to label the tubes accordingly for beetles #5-8. Record the data in Table 3.

Table 3. Measured absorbance values for reaction by α-naphthyl acetate esterase (ANAE) and β-naphthyl acetate esterase (BNAE) (samples D and E, respectively) for beetles grown on _____ seeds.
Note Beetle Strain: _____

Beetle #	Sex	Protein Content (μg)	Absorbance for D tubes (AU)	ANAE activity (AU/μg protein)	Absorbance for E tubes (AU)	BNAE activity (AU/μg protein)
5						
6						
7						
8						

Lab 3:

III. Protein Concentration

Using the crude extract (Figure 5b)

44. Retrieve tubes 1C, 2C, etc. for all 8 beetles from labs 1 and 2 from the freezer. Thaw the solutions.
45. Label a clean 1.5-mL microcentrifuge tube for each beetle (1P, 2P, etc.). Label another microcentrifuge tube as B-P for Blank.
46. Mix the crude extract solution in tube 1C (and 2C, etc.).
47. For each beetle, transfer 200 µL of the crude extract from the C tubes to the microcentrifuge tube you labeled above (1C to 1P, 2C to 2P, etc.).
48. To the Blank tube (B-P) add 200 µL of homogenizing buffer.
49. To each tube add 800 µL of pre-filtered Bradford dye reagent.
50. Vortex the tubes for 10 seconds.
51. Incubate the tubes at room temperature for 10 minutes.
52. Take 1 spectrophotometer cuvette per beetle. Label them as 1P (and 2P, etc.), plus your group number. Make sure to label them on the sides that do not interfere with light absorption; your lab instructor will show you how to do this. Label another spectrophotometer cuvette for the blank B-P.
53. Mix the solution in each microcentrifuge tube by inverting 5 times. Transfer 500 µL of the solution from each tube to the corresponding cuvette.
54. Measure the absorbance at 595 nm. Record the absorbance values in Table 4.

Table 4. Measured absorbance values at 595 nm and calculated protein concentrations for different beetles.

Beetle #	Absorbance (AU)	Protein Concentration (µg/mL)

Setting up the standard curve

55. Using the BSA stock solution (2 mg/mL = 2000 µg/mL), prepare different concentrations of the protein standard. The dilutions should be 2×, 5×, 10×, 20×, and 40×. Calculate and record the final concentrations in Table 5. Check your calculations with your instructor.
56. Label one microcentrifuge tube 2×. Using a micropipettor with the correct tip, add 250 µL of BSA stock solution to the labeled tube. Add 250 µL homogenizing buffer. Vortex for 10 seconds. This is a 2× dilution:
    250 µL stock / 500 µL total volume = 2× dilution
57. Label one microcentrifuge tube 5×. Using a micropipettor with the correct tip, add 200 µL of solution from the 2× tube to the 5× tube. Add 300 µL homogenizing buffer. Vortex for 10 seconds. This is a 2.5× dilution of the previous solution and makes a 5× dilution of the BSA stock (2.5× times 2×):
    200 µL solution / 500 µL total volume = 2.5× dilution
58. Label another microcentrifuge tube 10×. Using a micropipettor with the correct tip, add 250 µL of solution from the 5× tube to the 10× tube. Add 250 µL homogenizing buffer. Vortex for 10 seconds. This is a 2× dilution of the previous solution and makes a 10× dilution of the BSA stock (2× times 5×):
    250 µL solution / 500 µL total volume = 2× dilution
59. Follow the procedure in step 58 to make the other dilutions for the standard curve.
60. Label 5 microcentrifuge tubes for the BSA standard curve (C-2, C-5, C-10, etc.). Transfer 200 µL of each concentration of BSA from the tubes above (2×, 5×, etc.) to its correspondingly labeled microcentrifuge tube.

61. Label another microcentrifuge tube as blank (C-B). Add 200 µL of homogenizing buffer to the blank tube.
62. Add 800 µL of filtered Bradford dye reagent to each microcentrifuge tube containing the BSA standards (C-2, C-5, C-10, etc.) or the buffer (C-B).
63. Vortex the tubes for 10 seconds.
64. Incubate the tubes at room temperature for 10 minutes.
65. Label spectrophotometer cuvettes for the blank and different concentrations of BSA.
66. Invert each tube 5 times to mix the contents. Transfer 500 µL of solution from each tube to the corresponding spectrophotometer cuvette.
67. Measure the absorbance for each tube at 595 nm using a spectrophotometer as you did previously for the crude extracts. Record the absorbance readings in Table 5.

Table 5. Measured absorbance values at 595 nm for different concentrations of BSA.

Dilution Factor	BSA Concentration (µg/mL)	Absorbance (AU)
2×		
5×		
10×		
20×		
40×		

68. Your instructor will tell you how to discard your tubes and chemicals.
69. **Note** that if the absorbance readings using the Bradford reagent for your crude extracts are **outside the range** of the standard curve, then you will need to add more concentrations to your standard curve. Ask your instructor for help.

Data Analysis

70. Use the data from Table 5 and make a scatter plot in Microsoft Excel. Add a trend line to get the best-fit line to the data. Decide whether a linear or a logarithmic trend line is a better fit. **Include the graph in your lab report**.
71. The equation for the best-fit line can be used in determining the concentration of soluble protein in the unknown samples. Write the equation for the line in the space below. Rewrite the equation for finding protein concentration.

Absorbance (AU) =

Protein Concentration (µg/mL) =

72. Based on the equation for the standard curve you generated, solve for the protein concentrations in the unknown beetle samples given the absorbance values you measured above (see Table 4). Record the protein concentrations in Table 4.
73. Each tube used in the enzyme assays contained 50 µL of crude extract. Multiply the protein concentration (µg/mL) for each beetle by the volume of crude extract used (50 µL = 0.050 mL) to calculate protein content (µg) for each tube. Record these values in Tables 2 and 3.
74. Correct the enzyme activity by dividing the absorbance values (AU) by the amount of protein in each tube (µg protein). Record in Tables 2 and 3.
75. Use the **class data** to determine the following for beetles grown on each seed type:
    - The mean and standard deviation for ANAE activity corrected for protein content. Use the corrected absorbance values. Record in Table 6.
    - The mean and standard deviation for BNAE activity corrected for protein content. Use the corrected absorbance values. Record in Table 7.
76. Make a column graph of the means ± s for the ANAE (α-naphthyl acetate esterase) enzyme activity for the beetles grown on different legume seeds. Include in your lab report. Do the means look different?
77. Make a column graph of the means ± s for the BNAE (β-naphthyl acetate esterase) enzyme activity for the beetles grown on different legume seeds. Include in your lab report. Do the means look different?

Table 6. The ANAE (α-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed		
Sample size		
Class Mean (AU/μg protein)		
Class Standard deviation (AU/μg protein)		

Table 7. The BNAE (β-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed		
Sample size		
Class Mean (AU/μg protein)		
Class Standard deviation (AU/μg protein)		

78. Perform a t-test to determine whether the ANAE (α-naphthyl acetate esterase) activity levels are different between the two seed types. Record the results of your t-test in Table 8 below. Is this a one-tailed or two-tailed test? What does the t-test tell you? How confident are you about your conclusion? Include in your lab report.

Table 8. The results of the t-test for the comparison of ANAE (α-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	
t-critical for 95% confidence level	
|t-calculated|	
Confidence level	

79. Perform a t-test to determine whether the BNAE (β-naphthyl acetate esterase) activity levels are different between the two seed types. Record the results of your t-test in Table 9 below. Is this a one-tailed or two-tailed test? What does the t-test tell you? How confident are you about your conclusion? Include in your lab report.

Table 9. The results of the t-test for the comparison of BNAE (β-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	
t-critical for 95% confidence level	
|t-calculated|	
Confidence level	

80. For each strain of beetles, are there differences between the two sexes? How would you determine that?

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Materials

List of Materials for a Class of 24 Students for each of lab periods 1 and 2

Common supplies for a class of 24

15-ml Falcon Tubes (for diluting reagents)	3
Spectrophotometer (1 per two groups of 4 students each)	3
Kimwipes™	6 boxes
Waste solution container (100 mL or larger)	1
Bio hazardous waste disposal bag and container	1
Container for used pestles	1
Microcentrifuges (10,000-14,000 rpm) (1 per two groups of 4 students each)	3
1.5 mL Microcentrifuge tubes (for distributing reagents to groups)	18
Homogenizing buffer (for diluting reagents)	30 mL
Ice bucket	1
Live bean beetles grown on different legume seeds	24

At each station for a group of 4 students

p-100-1000 micropipettors	2
p-20-200 micropipettors	2
Pipet tips Yellow; 1 to 200µL	1 Pack
Pipet tips Blue; 101 to 1000µL	1 Pack
Waste container for used tips	1
Disposable pestles	4
Fine-point permanent markers, different colors	2
Small paint brushes	2
Ice bucket	1
Dissecting microscope	1
100 mm Petri dish	1
Microcentrifuge tube rack	3
1.5 mL Microcentrifuge tubes	22
Vortexer	1
Disposable, semi-micro 1.5 mL cuvettes	10
Adult bean beetles	4

Solutions per group of 4 students

7 mL Homogenizing Buffer	1
1.5 mL α-Naphthyl Acetate (freshly diluted)	1
1.5 mL β-Naphthyl Acetate (freshly diluted)	1
1.5 mL Fast Blue B Salt Stain containing 10% SDS, freshly made by lab instructor	1

List of Materials for a Class of 24 Students for lab period 3

Common supplies for a class of 24

Spectrophotometer (1 per two groups of 4 students each)	3
Kimwipes™	6 boxes
Waste solution container (100 mL or larger)	1
Bio hazardous waste disposal bag	1
Microcentrifuges (10,000-14,000 rpm) (1 per two groups of 4 students each)	3
Ice bucket	1

At each station for a group of 4 students

p-100-1000 micropipettors	2
p-20-200 micropipettors	2
Pipet tips Yellow; 1 to 200µL	1 Pack
Pipet tips Blue; 101 to 1000µL	1 Pack
Waste container for used tips	1
Fine-point permanent markers, different colors	2
Ice bucket	1
Microcentrifuge tube rack	3
1.5 mL Microcentrifuge tubes	22
Vortexer	1
Disposable, semi-micro 1.5 mL cuvettes	15

Solutions per group of 4 students

300 µL BSA stock solution	1
4 mL Homogenizing Buffer	1
14 mL Bradford reagent	1

Notes for the Instructor

This laboratory exercise was designed for an Introductory Biology class. Our class typically has 300+ students with multiple lab sections with 20 students per lab. By the time this set of experiments begins our students have done multiple case studies of parsing experiments, and have performed a 3-week lab sequence in which they have designed experiments, carried out the experiments, analyzed the data with descriptive and inferential statistics (simple t-tests), and written a lab report with the option to rewrite.

Introducing the Students to the Lab

We introduce the beetles to the students during the microscopy lab (early in the semester). The students learn about the life cycle of the beetles, observe them under the dissecting scope, and learn to distinguish males and females. The students start their own cultures following the recommendations from the bean beetles handbook (Beck and Blumer, 2011).

Later in the semester, after the students have reviewed basics of enzymes in class, the investigations are carried out in lab. Alternatively, one could use the lab as a means of introducing these topics.

We focus on the effect of food source on the activity levels of two esterases. We start out with one strain that has been growing on mung beans for many generations (more than 20) compared to the same strain grown on adzuki beans or cowpeas. The experiment could be designed to involve more independent work by allowing the students to pick a focus for further studies: different detoxification enzymes identified through literature searches, optimizing pH and temperature conditions to study the various enzymes, the potential detoxification of insecticides such as malathion (and its oxidation product malaoxon) that contain ester bonds, differences among the different strains of beetle available (a couple of strains are available from Larry Blumer at Morehouse College, other strains may be available from labs at Kenyon College and University of Kentucky), age dependent changes in esterase activity levels in post-emergence adults, and differences between larvae that are actively feeding on the beans and adults that do not usually eat, to mention a few possibilities.

The lab exercises could be paired with discussion of different types of scientific literature focusing on the effects of organophosphate insecticides and mechanisms conferring resistance to insecticides. Examples include:

- Chapters in "Silent Spring" (Carson, 1962).
- London *et al.* (2005) a review article focusing on the potential link between organophosphate insecticides and suicide.
- Liang *et al.* (2007) a primary research article showing effects of host plants on insecticide susceptibility and carboxylesterase activity in two insect species.
- Yu (1999) a primary research article that focuses on another detoxification enzyme and how it is affected by allelochemicals.
- Guo *et al*. (2012) a primary research article that focuses on the effect of plant secondary metabolites on changes in gene expression in bean beetles, including genes for detoxification enzymes.

Pre-Laboratory Preparation of Material

You need to have a large population of adult beetles for each investigation. We have multiple cultures started at different times in our incubator so that adults are available for labs 1 and 2. Depending on when students start their own cultures in the semester, they may be able to use adults from their own cultures as well. With our incubator, 5 weeks is the correct time interval to have adults. You should try growing the cultures in your lab in your incubator to get an idea of what the time interval between adult generations will be for you.

As listed in Appendix A, some of the solutions can be made ahead of time and stored at room temperature or in the refrigerator. Some have to be made fresh at the beginning of lab, or have to be diluted at the beginning of lab.

Challenges with Introductory Students in the Lab

We have tried this project in our introductory biology class (Bio 10100 at City College of New York) for three semesters. The biggest challenges have been:

- Pipetting correct amounts of solutions using micropipettors.
- Cross-contamination of solutions.
- Making sure the solutions that are divided between microcentrifuge tubes or spectrophotometer cuvettes were uniformly mixed and there were no solid objects floating in the solutions that would interfere with absorption measurements (problems with disturbing the pellets after centrifugation).
- Making sure the lab instructors review the background information and data with the students to put the experiment into context. Despite having weekly prep sessions, the variability among the lab instructors is unavoidable.

We have restricted the labs to comparison of two food sources: either mung beans and adzuki beans, or mung beans and cowpeas. Therefore, the data lend themselves to simple t-tests. Depending on the level of the course in which this exercise is used, multiple comparisons and more advanced statistical analysis can be used. We introduce descriptive statistics and t-tests early in the semester and

by the time of this lab sequence the students have already performed t-tests and written lab reports that include the results of the t-tests. Sample data are presented in Appendix B.

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Fardad Firooznia received his BS in Biology from Yale University and his PhD in plant physiology from Cornell University. He has been an instructor at The City College of New York since 2010, where he teaches large introductory biology courses as well as upper level seminar courses in ethnobotany, ecophysiology, and plant physiology.

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Avajé Jackson was born and raised in St. Catherine, Jamaica, but now resides in New York City. She is an alumna of The City College of New York with a BS

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Appendix A: Solutions and Supplies

Recipe for Making Stock Solutions

- BSA stock solution 2.0 mg/mL

Reconstitute lyophilized bovine serum albumin (BSA) with deionized water. Store at 4°C.

- Homogenizing Buffer

Stock Solution A = 200 mM NaH_2PO_4 (0.2 M Sodium Phosphate Monobasic)
Dissolve 13.8 g NaH_2PO_4 in 400 mL deionized water, then add deionized water to bring the volume to 500 mL. Autoclave and store at room temperature.

Stock Solution B = 200 mM Na_2HPO_4 (0.2 M Sodium Phosphate Dibasic)
Dissolve 26.81g Na_2HPO_4 in 400 mL deionized water, then add deionized water to bring the volume to 500 mL. Autoclave and store at room temperature.

Homogenizing Buffer, 100 mM sodium phosphate pH 7.4, 1% Triton X100; To make 500 mL:
Add 47.5 mL stock solution A and 202.5 mL stock solution B to 250 mL deionized water. Check pH and Add 0.5 mL Triton X100. Stir the mixture for 20 minutes. Store for up to 3 months at 4°C.

- Bradford reagent

Available commercially. Or, to make your own:
Dissolve 100 mg Coomassie brilliant blue G-250 dye in 50 mL 95% ethanol, add 100 mL 85% (w/v) phosphoric acid. Once the dye has completely dissolved, dilute to 1 L with deionized water. Filter through Whatman #1 paper just before use.

- α-Naphthyl Acetate stock solution (0.027 M)

Dissolve 0.025 g of α-naphthyl acetate in 5 mL of acetone, resulting in a concentration of 0.027 M.

- β-Naphthyl Acetate stock solution (0.027 M)

Dissolve 0.025 g of β-naphthyl acetate in 5 mL of acetone, resulting in a concentration of 0.027 M.

- Fast Blue B Salt Stain containing 10% SDS, made fresh right before use

Mix 0.030 g Fast Blue salt with 5 mL of distilled water and vortex the mixture. Add five mL of 10% SDS solution to the tube and invert 5 times to mix the solution.

- 10% SDS

Dissolve 10 g of SDS in 80 mL of water, and then add water to bring the volume to100 mL.

General Supplies Shopping List

- α-Naphthyl Acetate (1-Naphthyl Acetate) available from Sigma Aldrich (catalog number N8505)

- β-Naphthyl Acetate (2-Naphthyl Acetate) available from Sigma Aldrich (catalog number N6875)

- Fast Blue B Salt available from Sigma Aldrich (catalog number D9805)

- Fisherbrand™ Disposable Pellet Pestles 12-141-364

To reuse: wash them thoroughly and subject them to UV light for 10 min.

- Disposable, polystyrene, semi-micro 1.5 mL cuvettes available from several companies. For example:

Bio-Rad (product number 2239955)
Fisher Scientific (catalog number 14-955-127)

Appendix B: Sample Data

Sample data from one group of four students:

Table 10. Measured absorbance values for reaction by α-naphthyl acetate esterase (ANAE) and β-naphthyl acetate esterase (BNAE) (samples D and E, respectively) for beetles grown on Adzuki bean seeds. Note Beetle Strain: LB

Beetle #	Sex	Protein Content (μg)	Absorbance for D tubes (AU)	ANAE activity (AU/μg protein)	Absorbance for E tubes (AU)	BNAE activity (AU/μg protein)
1	F	7.95	0.473	0.0595	0.168	0.0211
2	F	5.66	0.408	0.0721	0.135	0.0239
3	M	4.01	0.487	0.121	0.131	0.0327
4	M	4.96	0.565	0.114	0.149	0.0300

Table 11. Measured absorbance values for reaction by α-naphthyl acetate esterase (ANAE) and β-naphthyl acetate esterase (BNAE) (samples D and E, respectively) for beetles grown on Mung bean seeds. Note Beetle Strain: LB

Beetle #	Sex	Protein Content (μg)	Absorbance for D tubes (AU)	ANAE activity (AU/μg protein)	Absorbance for E tubes (AU)	BNAE activity (AU/μg protein)
5	M	3.76	0.348	0.0926	0.097	0.0258
6	M	4.62	0.815	0.176	0.224	0.0485
7	F	5.15	0.971	0.189	0.265	0.0515
8	F	7.42	1.721	0.232	0.419	0.0565

Table 12. Measured absorbance values at 595 nm and calculated protein concentrations for different beetles.

Beetle #	Absorbance (AU)	Protein Concentration (μg/mL)
1	1.114	159.05
2	0.957	113.13
3	0.799	80.29
4	0.896	99.10
5	0.769	75.23
6	0.864	92.45
7	0.914	103.05
8	1.082	148.38

Table 13. Measured absorbance values at 595 nm for different concentrations of BSA.

Dilution Factor	BSA Concentration (μg/mL)	Absorbance (AU)
2×	1000	1.830
5×	400	1.645
10×	200	1.299
20×	100	0.991
40×	50	0.436

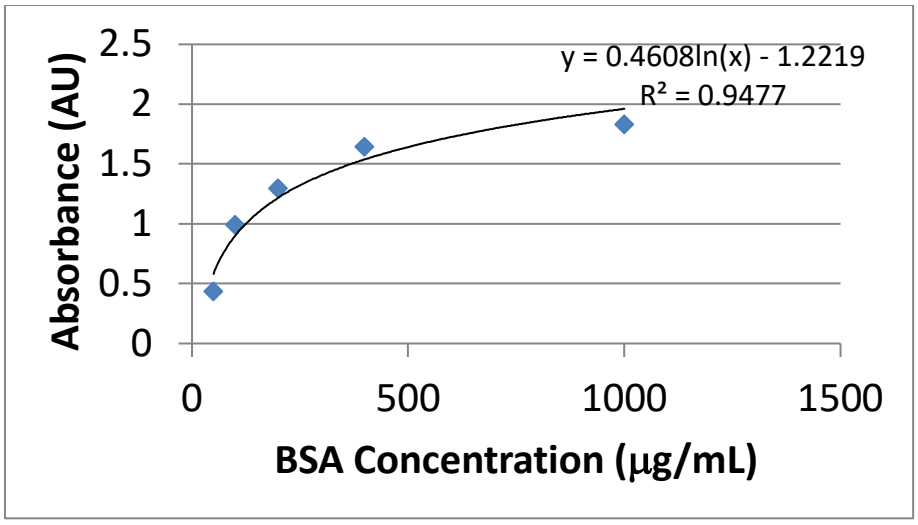

Figure 6. Standard curve for protein concentration using the Bradford assay.

Sample data from one lab section with six groups:
Note: several outliers were removed from the data analysis. The hypothesis for this class was two-tailed = the food source will lead to a difference.

Table 14. The ANAE (α-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed	Adzuki bean	Mung bean
Sample size	26	29
Class Mean (AU/µg protein)	0.155	0.068
Class Standard deviation (AU/µg protein)	0.147	0.066

Table 15. The BNAE (β-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed	Adzuki bean	Mung bean
Sample size	25	33
Class Mean (AU/µg protein)	0.061	0.025
Class Standard deviation (AU/µg protein)	0.088	0.040

Table 16. The results of the t-test for the comparison of ANAE (α-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	53
t-critical for 95% confidence level	1.6741 for one-tailed test 2.0057 for two-tailed test
|t-calculated|	2.88
Confidence level	>99.5% for one-tailed test >99% for two-tailed test

Table 17. The results of the t-test for the comparison of BNAE (β-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	56
t-critical for 95% confidence level	1.6725 for one-tailed test 2.0025 for two-tailed test
|t-calculated|	2.01
Confidence level	>97.5%, <99% for one-tailed test >95%, <98% for two-tailed test

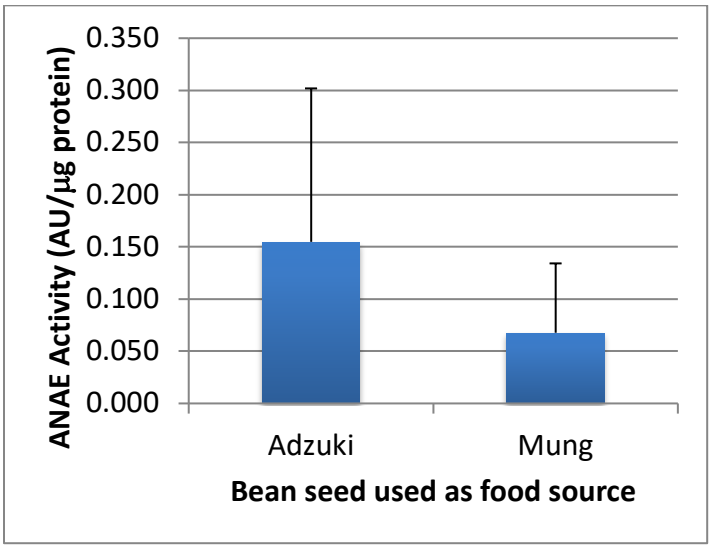

Figure 7. The effect of food source on which beetles were grown on the activity level of ANAE (α-naphthyl acetate esterase) enzyme. Columns represent means, and error bars represent standard deviations. Sample size was 26 for Adzuki beans and 29 for Mung beans. The means are statistically different at 95% confidence level.

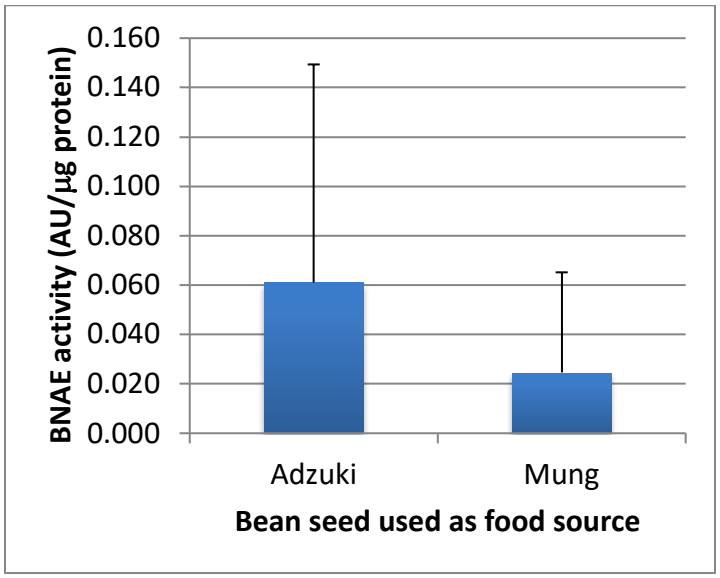

Figure 8. The effect of food source on which beetles were grown on the activity level of BNAE (β-naphthyl acetate esterase) enzyme. Columns represent means, and error bars represent standard deviations. Sample size was 25 for Adzuki beans and 33 for Mung beans. The means are statistically different at 95% confidence level.

Sample data from a second lab section with five groups:
Note: The hypothesis for this class was two-tailed = the food source will lead to a difference.

Table 18. The ANAE (α-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed	Cowpea	Mung bean
Sample size	20	20
Class Mean (AU/μg protein)	0.0762	0.187
Class Standard deviation (AU/μg protein)	0.0907	0.285

Table 19. The BNAE (β-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed	Cowpea	Mung bean
Sample size	20	20
Class Mean (AU/μg protein)	0.0192	0.0342
Class Standard deviation (AU/μg protein)	0.0222	0.0302

Table 20. The results of the t-test for the comparison of ANAE (α-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	38
t-critical for 95% confidence level	1.6859 for one-tailed test 2.0244 for two-tailed test
|t-calculated|	1.66
Confidence level	<95% for one-tailed test <90%% for two-tailed test

Table 21. The results of the t-test for the comparison of BNAE (β-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	38
t-critical for 95% confidence level	1.6859 for one-tailed test 2.0244 for two-tailed test
|t-calculated|	1.793
Confidence level	>95%, <97.5% for one-tailed test <95% for two-tailed test

Sample data from a third lab section with five groups:
Note: One outlier was removed from the data analysis. The hypothesis for this class was two-tailed = the food source will lead to a difference.

Table 22. The ANAE (α-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed	Cowpea	Mung bean
Sample size	18	19
Class Mean (AU/μg protein)	0.132	0.314
Class Standard deviation (AU/μg protein)	0.126	0.316

Table 23. The BNAE (β-naphthyl acetate esterase) enzyme activity for the beetles grown on two different legume seeds. The values are corrected for protein content.

Legume seed	Cowpea	Mung bean
Sample size	11	16
Class Mean (AU/μg protein)	0.0487	0.106
Class Standard deviation (AU/μg protein)	0.0590	0.0898

Table 24. The results of the t-test for the comparison of ANAE (α-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	35
t-critical for 95% confidence level	1.6896 for one-tailed test 2.0301 for two-tailed test
|t-calculated|	2.288
Confidence level	>97.5%, <99% for one-tailed test >95%, <98% for two-tailed test

Table 25. The results of the t-test for the comparison of BNAE (β-naphthyl acetate esterase) activity in bean beetles grown on different legume seeds.

D.F.	26
t-critical for 95% confidence level	1.7056 for one-tailed test 2.0555 for two-tailed test
|t-calculated|	1.861
Confidence level	>95%, <97.5% for one-tailed test <95% for two-tailed test

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