 1.  2.  3.

Fig. 1 Callosobruchus maculatus. Bean Beetle. Fig. 2 . Black Eyed Pea Plant. Fig 3 . Damage caused to crop.

Effects of food source on quantities of esterase enzyme activity in presence of acetylcholinesterase (AChE) inhibiting toxins in bean beetles.

Samuel Payne. Dylan Easterday. Angelique Crawley. Zohra Anwar

The Bean Beetle, Callosobruchus, feeds on and therefor has a propensity to destroy legume crops in tropical and subtropical regions of Africa and Asia (Fig 1-3). The Bean Beetle's life cycle determines one food source such as the Vigna radiata (Mung Beans) or the Vigna unguiculata (Black Eyed Peas). An egg is laid on the surface of the bean seed and the larvae feeds on the inside of the bean (Beck and Blummer, 2011). There is evidence that the species of legume the Callosobruchus depends on has a direct effect on the organism's ability to resist organophosphates and the AChE inhibitors they contain (Liang et al 2007). There is also evidence that insects have the ability to detoxify these toxins by esterase enzyme activity (Liang et al. 2007, Magana et al. 2008, Wu et al. 2009). A potential deduction, with evolutionary consequences, is that the Bean Beetle has co-evolved with its food source and retained traits to resist AChE inhibiting toxins.

The insecticides used to eradicate Bean Beetle activity act in the same way as the toxins produced by the organism's food source. The toxin interrupts the nervous system's fluid communication between cells by breaking down the AChE used to break down Acetylcholine to interrupt synaptic signaling. The toxin forces the nerve channel to remain open and continue firing causing muscle spasm, seizure and eventual death. It is well documented that the lasting effects of the insecticides leached into surrounding ecosystems have neurological and morphological consequences on organisms ranging from humans to fish and rodents (Patil and David, 2010, Sadeghi Hashjine et al, 2013, London et al., 2005). It is therefore imperative to create avenues to minimize quantities and increase accuracy in targeting perceived pests and to decrease adverse ecological affects. If the organism already has a predisposition to fighting a compound of similar structure to an insecticide such as Malaoxin, then the economy of using such an insecticide is suspect and could be ratified by increased knowledge of the relation between enzymatic activity and food source.

This paper hypothesizes a correlation between the respective food sources, Mung Beans and Black-eyed Peas, that Bean Beatles feed on and the quantity of active esterase enzymes present in the organism which are engineered to breakdown AChE inhibiting toxins with chemical structures similar to

Malaoxin and other pesticide agents.* The Null Hypothesis put forth is that food source does not affect anti-toxin enzyme activity in Bean Beetles.

Experimental Design:

We performed one experiment on each food culture. There were two treatment levels per experiment.

Each of the two treatment levels, bean beetles raised on Mung Bean and bean beetles raised on Black Eyed Peas, were tested for enzymatic activity in relation to the ANAE and BNAE substrate . The sex of the beetle was a randomized variable. The origin of the organism, the spectrophotometer, and the quantities of Acetate and Homogenizing Buffer, were standardized variables.

The Dependent variable was the quantity of product cleaved from the two acetates* by the active enzymes in vitro (See photo 3). This measurement was determined by absorbance levels with spectrophotometer set to 595 nm using a Fast Blue B Salt Stain that binds with the respective A-Napthol and B – Napthol.

The Independent Variable was the food source, Mung and Black -Eyed Peas. After the experiment was performed on one food source it was replicated once on the alternate food source.

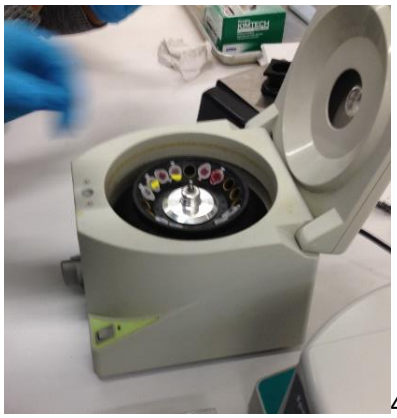
These experiments were repeated by other lab groups in the class and the data was combined to have a final sample size of 17 for ANAE on Black Eyed Pea food source, and 18 on Mung Bean and a sample size for BNAE of 12 for Black Eyed Pea and 17 for Mung Bean.

Methods:

The lab process required a crude protein extraction, enzyme assays and a protein concentration adjustment and the organism had to go through a process of mastication, centrifuging and be maintained on ice to preserve possible enzymatic activity in vitro (See Photos 4 – 6).

As each beetle has varying protein content, we achieved a colorimetric Bradford Assay to determine a correct ratio of Enzyme production by deriving a standard curve from the known BSA (Bovine Serum) stock concentration sometimes known as Fraction 5, by a series of dilutions (See Graph 2). Absorbency as a function of concentration of protein for each organism sample was then determined using this standard curve. All absorbency data for ANAE and BNAE substrate was then divided by respective protein concentration in order to create significant data for enzyme production in vitro irrespective of a variable such as size of organism or the inactive enzymes. Please see table below for an example of a specific calculation for Beetle 5.

This measurement of the dependent variable was determined by absorbance levels with spectrophotometer set to 595 nm using a Fast Blue B Salt Stain that binds with the respective A-Napthol and B – Napthol.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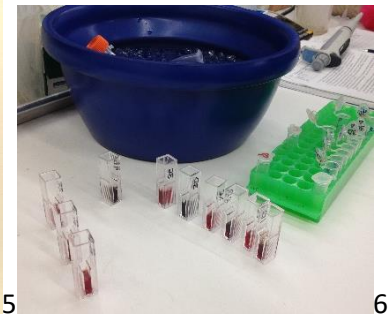

6

Fig 4. Centrifuge used to displace supernatant and animal parts. 23

Fig 5. The crude extract was kept on ice to preserve bio-chemical activity.

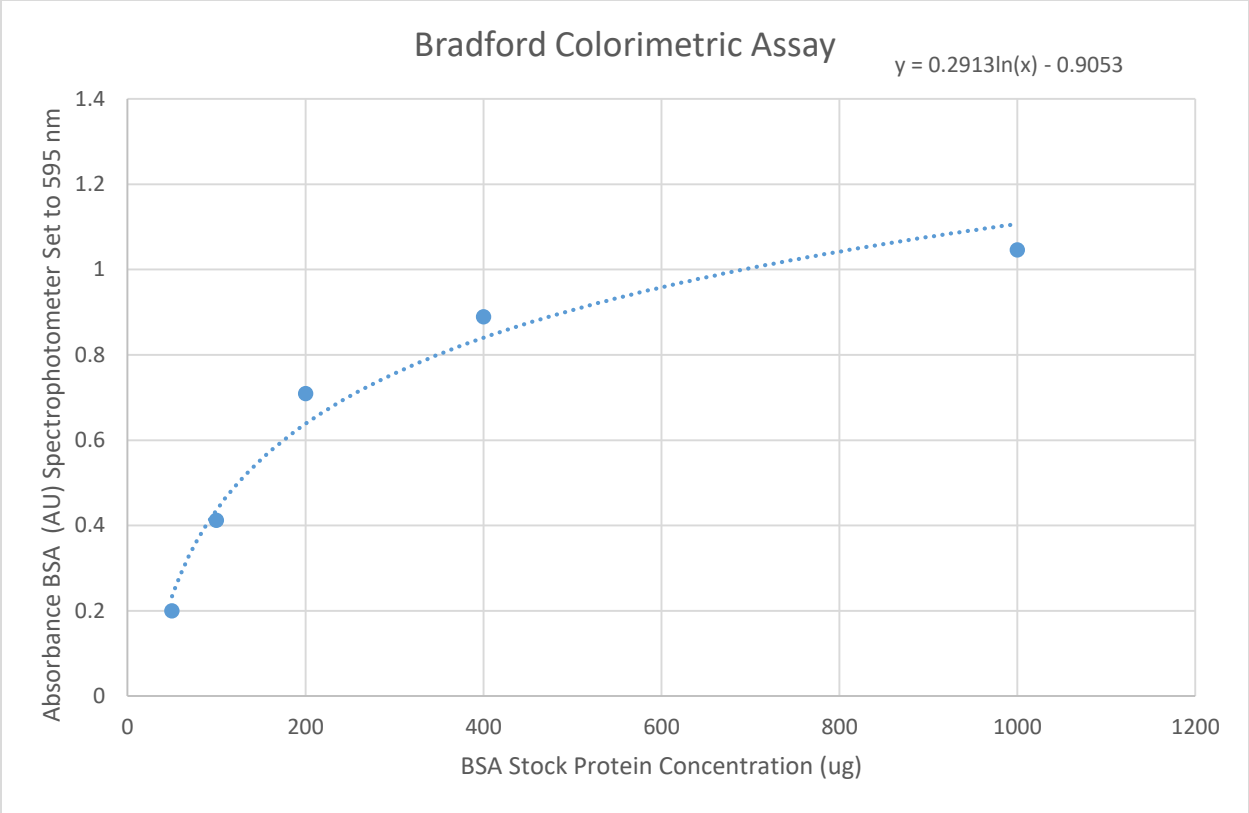
Fig 6. The Fast Blue B Salt dye binding to b-naphthol and a naphthol products that were hydrolyzed from BNAE and ANAE compounds mimicking insecticides.

Methods of Analyzing Data and Results and Data Analysis.

To draw significant conclusions from the data, a two tailed T-Test for each experiment and a Bradford Assay and a standard deviation of the mean had to be calculated (see Graphs 1,2,3 and Tables 1, 2 and 3).

The standard deviation from mean is depicted with error bars in Graph 1. Unfortunately, the BNAE experiment had such a large variation across the Black Eyed Peas food source it is not possible to gain significant data in the comparison despite significant discrepancy in arithmetic mean of enzyme production.

The two tailed T-Test had to be performed because we were not hypothesizing a vector quantity. We were simply predicting the magnitude of enzymatic activity would be affected one way or the other, a scaler prediction. This decreases the probability of the Null Hypothesis and demands the data to be skewed towards the alternative hypothesis at a greater affect than with a one tailed hypothesis. As shown in Table 3 and 4, the probability of results landing in the space of the Null Hypothesis is too high, (confidence level is below 80 % for ANAE and between 80 and 90% for BNAE,) to have confidence in the data and the alternative hypothesis.

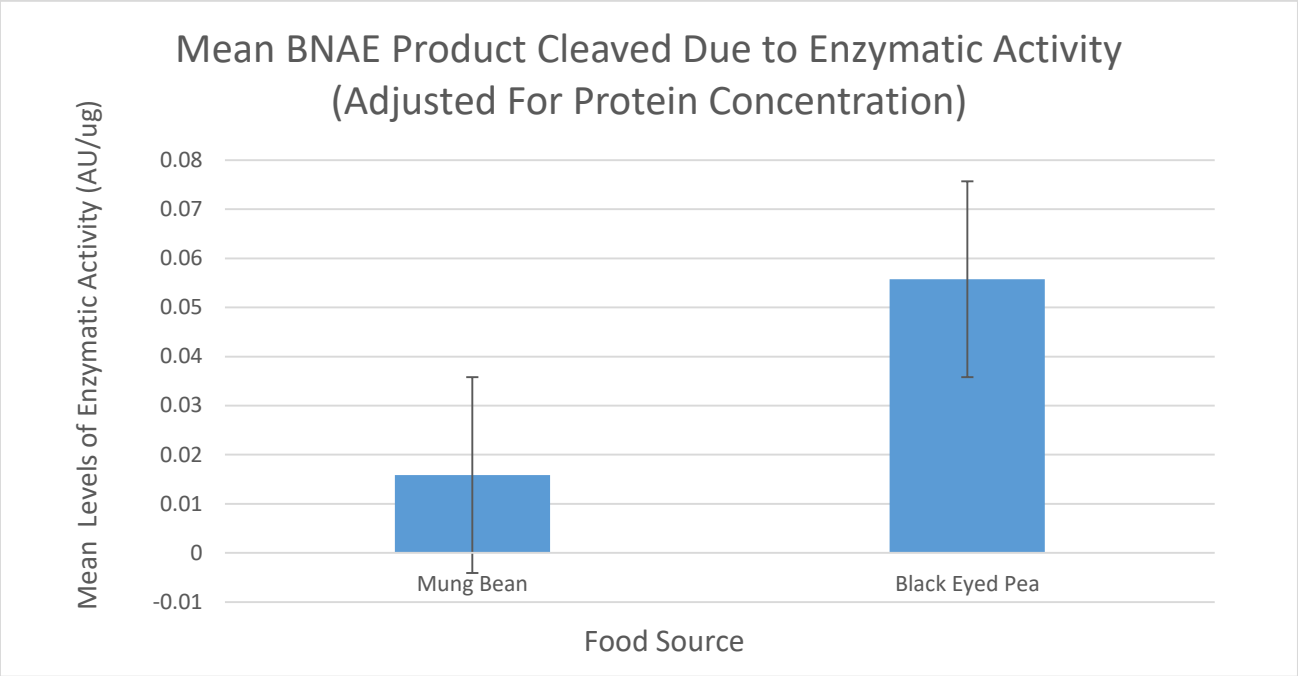


Graph 1. Standard Curve developed by diluting known concentration of Fraction V*. Absorbency is a function of Concentration. The trend line is based on the Logarithmic equation derived from the equation of a line created by plotting concentration on the x axis and absorbance on the y axis. This equation is displayed in top right corner of this graph.

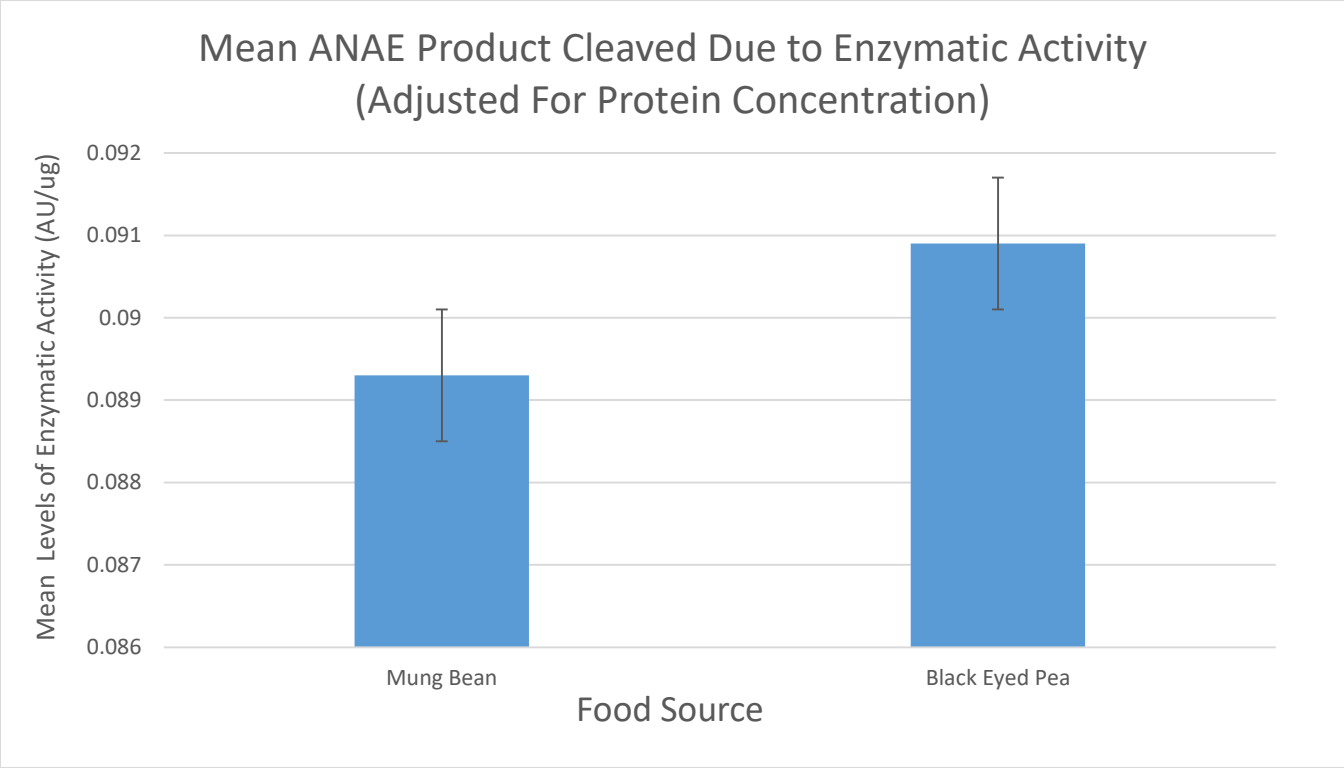
Example of Bradford Assay Process to Determine Adjusted Enzymatic Activity

Beetle 5 (Mung Bean)		
Protein	**.817 AU = Absorbance**	
ANAE Substrate	**2.232 AU = Absorbance**	2.232AU/1.777062222ug(mL) **= Adjusted ANAE Concentration** = **369.61479 (AU/ug**)
Derived Formula and Calculations:	y = 0.2913ln(x) - 0.9053	.817 = 0.2913ln(x) – 0.9053 (0.9053+.817)/.2913= ln(x) e ^(5.91246138)= x x = 369.615
For Standard Curve	y= Absorbance	x = concentration

Table 1 : Example of Using Bradford Assay to adjust Enzyme activity for Variance on Protein Concentration across Specimens.



Graph 2



Graph 3

Graph 3 and 4. Graph of mean product across treatment levels. This graph clearly shows that the data has a wide margin of error. It is tempting to deduce that Bean beetles irrespective of food source cultivation have the ability to break down toxins similar to ANAE substrate (the mirror of BNAE) and that Bean Beetles cultivated on Black eyed peas have a higher enzymatic activity regarding BNAE substrate then those cultivated on Mung Beans, but the distribution from the mean is too far reaching to draw appropriate conclusions. This distribution from the mean is represented by the error bars.

Two Tailed T Tests were performed for each substrate (See Table's 3 and 4).

BNAE T-Test	
D.F.	27
t - critical for 95% Confidence (2 - tailed	2.05
t - calculated	1.577759354
Confidence Level	>80,<90%

Table 3: BNAE Two Tailed T Test.

ANAE T- Test	
D.F.	33
t - Critical for 95% Confidence (2 tailed)	2.05
t - calculated	.046198499
Confidence Level	<80%

Table 4: ANAE Two Tailed T – Test.

Discussion and Conclusion:

The data is not significant within accepted confidence levels for the Biological discipline. There is not support for the hypothesis which states that enzymatic activity is affected by food source. My lab's prediction that the BNAE and ANAE will hydrolyze at an increased rate or decreased rate depending on whether an organism was cultivated on Mung Beans or Black Eyed Peas has not been supported.

Although it is possible to deduce a difference with BNAE substrate across Mung Bean and Black Eyed Peas, the distribution from the mean is too great and my confidence level determined by a two tailed T-Test (See Table 3) is between 80% and 90% which is inadequate to deem any significance in data collected. The Null Hypothesis which states that there will be no difference in enzymatic activity based on food source has been supported. There is too a great a chance that my data will fall in my null hypothesis for there to be confidence in the alternative hypothesis. The data does however lead towards more experiments geared towards the BNAE isomer, as the data from this experiment showed a difference in mean data. If the data were significant, conclusions could be drawn regarding the redundancy of using insecticides modeled on pre-established chemical structures, and further experiments could be proposed comparing enzyme production in vitro compared to production in

habitat in order to better understand the mechanism of organism resistance to toxicity level in in food source.

At this stage another experiment needs to be performed to shore up the significance of enzymatic activity and food source. The next experiment should focus on one substrate: BNAE. And it should test 3 sets of Bean Beetles cultivated on 3 different food sources including Black Eyed Peas and Mung Beans. The Hypothesis should be one tailed proposing that Black Eyed Pea Bean Beetles have higher resistance to insecticides modeled on BNAE then Cow Pea's or the third food source.

The theory that these organisms have evolved traits to counteract toxins produced by food source and that these traits must be considered in regards to crop treatment and pest control is sound. The mechanisms which control an insect such as the Bean Beetle's ability to resist insecticidal toxins in the field are not well understood. For example, organic farmers planting 145 million acres worldwide of crops engineered to resist Cabbage Looper Caterpillars, discovered that this organism has a method of resistance via enzyme activity (Ramanujun, 2016). The intense toxicity leached into surrounding ecosystems by the redundant and heavy use of insecticides can be counteracted by use of research such as this paper.

*These Acetates are not insecticides but they are similar in structure to ester bond. When they are hydrolyzed in the presence of enzymes they are cleaved, producing an acetic acid and a napthol. They are stereoisomers and therefor attach in a lock and key way to enzymes differently.

"The nickname "Fraction V" refers to albumin being the fifth fraction of the original Edwin Cohn purification methodology that made use of differential solubility characteristics of plasma proteins." Wikipedia.

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