Levels of Digestion-Protease Activity in the Alimentary Canal of *Schistocera serialis* (Orthoptera: Acrididae) (F.)

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Locusts and grasshoppers (Orthoptera: Acrididae) are insect herbivores that are eliciting catastrophic affects to the agricultural industry and economy. The control of these pests with integrated pest management strategies and pesticides is costly worldwide at an average of $70.0 billion per year. As people adapt new ways to combat insects, plants are also coevolving. A natural defense mechanism that has been employed in plants is the production of protease inhibitors (PIs). These proteolytic enzymes are found in various parts of the plants and are produced as a response from insect attack. Proteases are also found in regulated concentrations in an insect's alimentary canal during digestion. In a plant, PIs act as anti-metabolic proteins. When insects digest plants the PI's defensive capabilities inhibit the regulation of proteases in an insect's alimentary canal. As a result, negatively impacting the overall growth, development, and digestion of an insect because of the reduction of amino acids. This experiment was performed to lay the foundation of the extent of protease activity in each region of the alimentary canal (foregut, midgut, and hindgut) in *Schistocera serialis.* The methods to achieve this objective were six treatments of boiled and non-boiled regions to ascertain protease activity in each region of the alimentary canal. It was found that the non-boiled midgut has the highest levels of protease activity, while boiling the gut regions significantly decreases protease activity. This study addresses the need for a foundation of extent of protease activity in *S. serialis;* however, a number of questions regarding the diversity of proteases and utilization of knowledge towards decreasing crop loss using transgenic crops with higher levels of protease inhibitors remain to be addressed.

Keywords: *Schistocera serialis*, crop loss, protease activity, digestion

Many insect species have been documented to be destructive and a direct link to financial losses for the agricultural industry (Belayneh, 2005). They are undoubtedly the most diverse and adaptable form of life. Their presence on earth directly affects humans and the environment. This is seen in the roles of insects as vectors, scavengers, pollinators, and predators; however, they also play a role in inflicting damage to crops (Belayneh, 2005).

One of the most devastating threats to agriculture is the grasshopper in question throughout this study, *Schistocera serialis* (Zhang et al., 2018). While its specific species is non-swarming, it shares gregarious traits with the desert locust when in high densities (Gotham and Song 2013). These

traits are expressed as being conspicuously colored, moving in highly mobile groups, and behaving with aggression as an entity, rather a calm individual (Lomer et al., 2001).
The losses of crops caused by this group of insects reaches a catastrophic level of damage not only to the environment, but to the economy as well. Crop protection is the main goal as the agricultural industry must produce for food security; however, while control campaigns have been embraced because of lack of resources in some countries, the negative effects are costly (Weiss 1940). For instance, in history there is the documentation of about $500 million used during the grasshopper plague for control cost alone (Belayneh 2005). There have also been numerous studies to investigate how pesticides are associated with damage to the environment, people, and nontarget hosts.

While humans are constantly adapting to keep up with the adverse effects of destructive locusts, the relationship between plants and insects are evolving too. A natural defense mechanism employed by plants to fight back against inset herbivores is plant protease inhibitors (Zhu-Salzman and Zang 2014).

Proteases, or proteolytic enzymes, work in their target proteins to catalyze the hydrolytic cleavage of peptide bonds. These enzymes are found in insects and plants and play a key role in all biological processes related to survival; however, in higher concentrations they can be damaging. For this reason, the number of enzymes in an insect needs to be strictly regulated. This is where the association of an herbivorous insect comes into play with the digestion of plant protease inhibitors (PPIs) in the storage tissues of the plant. This plant response elicited from an attack from an insect, produces the PPI that acts as anti-metabolic proteins. As a result, interfering with the digestive processes of insects (Habib and Fazili 2005).

This information has been used in several studies to assess the diversity of digestive proteinase activity in different species of insects to equip plants with higher levels of protease to combat crop loss. The purpose of this study is to learn specifically about the crop damage caused by *S. serialis.*

Although there are many studies that illuminate plant protease inhibitors and insects, no study has been found about protease activity in the digestion of *S. serialis.* Therefore, this study can be the first step towards a profound understanding of locust protease activity. This is explored in the study through the identification of the different regions of the alimentary canal and where the extent of protease activity takes place. The guidelines used for differentiation of the alimentary canal as followed: The foregut consisting of the esophagus, crop, and ending just before the gastric caeca. The midgut starting at the gastric caeca and ending at the malpighian tubules. And lastly, the hindgut starting from the malpighian tubules and ending at the tubules. And lastly, the hindgut starting from the malpighian tubules and ending at the anus.

The added treatments of boiled and non-boiled regions of the alimentary canal are

conducted to observe how temperature affects the role of protease activity. This was interacts with protease, even though it is insoluble. Allowing the conclusion of extent of protein digestion correlating with the color intensity of the solution.

observed with the substrate, hide powder azure. This protein is digested when it
Additional studies are needed to understand the diversity of protease activity taking place and the best ways to decrease *S. serialis* as pests through plant protease inhibitors in transgenic crops – a genetically modified organism.

Materials and Methods

Preparation. *Schistocera serialis* were deprived of food for about six hours to ensure that the alimentary canal was empty (Thermo Fisher Scientific, Waltham, MA). A total of eight grasshoppers were used for this experiment and each grasshopper was taken and placed in a 12 x 12 x 12 cage (BioQuip, Rancho Dominguez, CA) and given a 3 cm piece of fresh lettuce (H-E-B, College Station, TX). The grasshoppers were left to feed, *ad libitum* for 15 minutes.

Alimentary Canal Identification. After the 15 minutes of digestion, the grasshopper was removed from the cage by hand and euthanized using standard dissection scissors (Thermo Fisher Scientific, Waltham, MA). The last two or three segments of the abdomen of the grasshopper was cut off and the head of the grasshopper was pulled off in a way, that the entire attached gut was withdrawn. The head and attached gut were placed in a 100 x 15mm petri dish (Thermo Fisher Scientific, Waltham, MA) and generously washed with 0.9% sodium chloride (saline solution) (Thermo Fisher Scientific, Waltham, MA). The identification of the three main regions of the gut was performed: foregut, midgut, and hindgut (**Figure 1**)

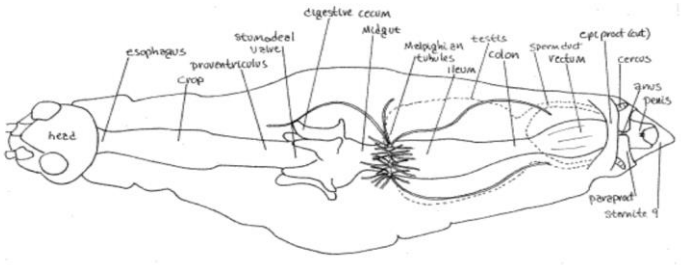

Fig. 1. Dorsal dissection of a male *Romalea guttata* (Orthoptera: Romaeleidae) showing the digestive system. This chart was used for the alimentary canal identification in *S. serialis.* This drawing is copyrighted (2001, 2004) but unpublished by Professor Richard Fox of Lander University.

After viewing the living gut, the dissection scissors were used to cut off the head and esophagus to discard. Using a razor blade (H-E-B, College Station, TX), the gut was then divided into the foregut, midgut, and hindgut. This protocol was repeated seven times.

Pre-Treatment. The grasshopper's divided alimentary canal regions were then tested against the treatments of boiling and non-boiling to observe the extent of protease activity. The three regions of guts: foregut, midgut, and hindgut were all tested, totaling three boiled treatments and three non-boiled treatments per grasshopper.

Three heidolph test tubes (Thermo Fisher Scientific, Waltham, MA) were used per grasshopper and filled with 5 ml of 0.5% NaCl. The foregut, midgut, and hindgut of each grasshopper were individually placed into each of the tubes. Using a glass rod (Thermo Fisher Scientific, Waltham, MA) the gut contents were ground down. After thorough grinding for about two minutes, a digital vortex mixer (Thermo Fisher Scientific, Waltham, MA) was used according to manufacturer instructions to thoroughly mix each mixture.

Treatments. Six new Eppendorf tubes containing hide power azure were obtained and the six following mixtures were made: The first tube with 2 mg Hide Power Azure + 1 ml distilled water + 0.25 ml foregut extract. Second tube, 2 mg Hide Powder Azure + 1 ml distilled water + 0.25 ml boiled foregut extract. Third tube, 2 mg Hide Powder Azure + 1 ml distilled water + 0.25 ml midgut extract. Fourth tube, 2 mg Hide Powder Azure + 1 ml distilled water + 0.25 ml boiled midgut extract. Fifth tube, 2 mg Hide Powder Azure + 1 ml distilled water + 0.25 ml hindgut extract. Sixth tube, 2 mg Hide Powder Azure + 1 ml distilled water + 0.25 ml boiled hindgut extract (Sigma-Aldrich, St. Louis, MO).

The contents of all the test tubes were filtered through glass wool (Thermo Fisher Scientific, Waltham, MA) into three new test tubes. Then three 2 ml eppendorf tubes (Thermo Fisher Scientific, Waltham, MA) were used per grasshopper to extract 2 ml of foregut, midgut, and hindgut. These sets of tubes were boiled at 80°C in a digital water bath (Cole-Parmer, Vernon Hills, IL) for 10 minutes.

Absorption. After the mixtures were made the Eppendorf tubes (Thermo Fisher Scientific, Waltham, MA) were incubated in a water bath (Cole-Parmer, Vernon Hills, IL) at 35°C for 20 minutes. After, the tubes were removed and placed on ice to stop the reaction. The eppendorf tubes were centrifuged using an accuSpin 8°C small bench top centrifuge (Thermo Fisher Scientific, Waltham, MA). Then 1 ml of each extract was transferred into a cuvette (Thermo Fisher Scientific, Waltham, MA), to measure the intensity of the hide powder azure in the solution. This was performed using a Spectrophotometer per manufacturer instruction and set at 650 nm wavelength (Thermo Fisher Scientific, Waltham, MA). The absorbency reading was recorded from the Spectrophotometer to measure the extent to where protein digestion occurs in each of the alimentary regions: foregut, midgut, and hindgut.

Results

A comparison of the two variables, gut region, and boiling methods demonstrated a large variation in the amount of protease activity in the alimentary canal of *Schistocera serialis* ($p = 0.0135$) (**Figure 1**). This was concluded with a Two-way ANOVA test.

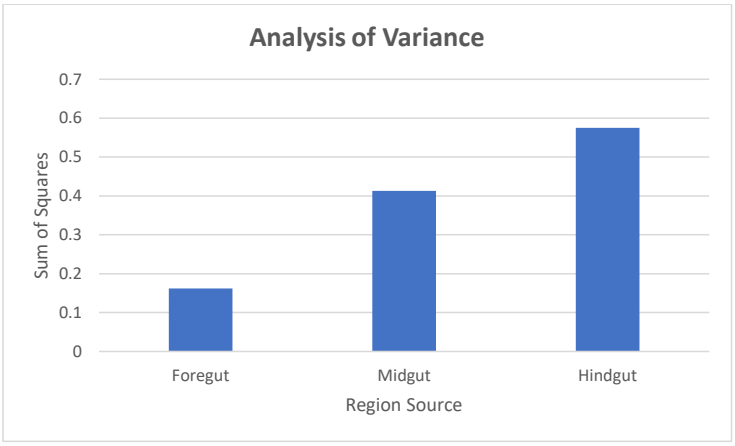

Fig. 1. Model Error C. Total of the analysis of variance between the treatments of foregut, midgut, and hindgut. H_o: all treatments are equal (p-value>0.05); H_A: at least one treatment is different (p-value<0.05). Significant difference between the two variables of gut region and boiling (p= 0.0135).

The treatments tested were foregut, boiled foregut, midgut, boiled midgut, hindgut, and boiled hindgut. The effects of each treatment influenced the amount of protease activity and hide powder azure absorption. This was discovered through the observation of the combination of factors: guts and boiling treatments. Significant difference in the amount of protease activity in the combination of the gut section being boiled or not boiled (p = 0.0249) (**Figure 2**).

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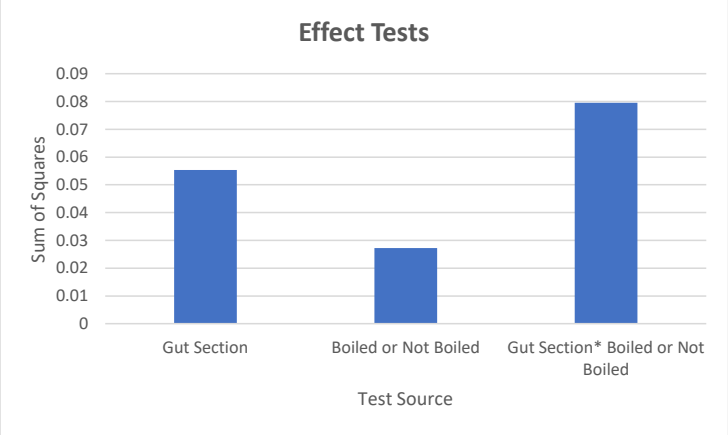

Fig. 2. Comparison of variables and how they affect the protease activity in the gut. The observation of how the variables influence each other is seen in the interactions between the treatments (gut*boiling). Significant difference in the gut section*boiled or not boiled (p = 0.0249)

The treatments of each gut region and boiling factors are concluded to be significant (p = 0.0135) and the effects of each treatment are different (p = 0.0249). Using a Tuckey post hoc test, the interactions of each treatment were tested to find where the difference lies. The mean absorbance of each region of the alimentary canal: foregut, midgut, and hindgut and the respective boiled portions of each were collected. The treatments that were different were the midgut not boiled (M= 0.2089; SD= 0.0701) and the foregut boiled (M=0.0388; SD= 0.0701) (**Figure 3)**.

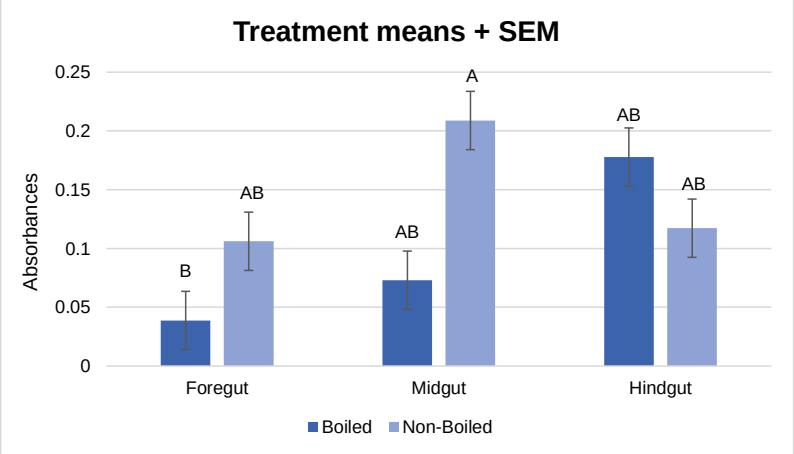

Fig. 3. Mean absorbance of protease activity for each section of gut: foregut, midgut, and hindgut in the alimentary canal of *S. serialis* (SD=0.0701; SEM=0.0248). Significant difference ($p = 0.0135$) between boiled and non-boiled and the effects of each factor on the absorption of protease activity ($p = 0.0249$). Levels not connected by the same letter are significantly different ($p \leq 0.05$).

Discussion

The discovery of the different levels of protease activity occurring during digestion in the alimentary canal of *S. serialis* is of great importance for the agricultural industry (Amirhusin et al., 2007). About 70% of crop production is lost without the use of pesticides or other controlling strategies. This accumulates to a loss of about $400 billion in comparison to the $100 billion lost for 15% of crop production loss with the use of insecticides. Consequently, leading to the annual costs of insecticides for about $8 billion (Krattiger, 1997).

Previous studies have emphasized the importance of adapting our agricultural industry to equip new economical control measures (Lawrence et al., 2002). This includes the knowledge of insect pests being capable of adapting as well. Insect pests can evolve morphologically, biochemically, and genetically (Denholm et al. 1992). For instance, the locust studied in this experiment, has adapted over time to overcome the effect of pesticides or toxic material resistance emplaced in crops. These adaptions cast light on the needed tactics for managing pesticide resistance and their role

in furthering the economic problem in crop loss (Roush et al., 1987).

Fortunately, humans are not the only thing on earth equipping new ways to defend against insects. Plants and insects have co-evolved over thousands of years, and as a result the pest control industry has learned how features of density-dependent phenotypic plasticity in locusts has allowed the adaptation of plants with defense mechanisms (Jongsma et al., 1997). As a result, crop protection and economical control measures coincide between humans and plants.

The discovery of high protease activity during digestion in the midgut of *S. serialis* is the foundation for the production of protease inhibitors as a defense mechanism in plants. Previous studies have almost exclusively focused on the diversity in digestive proteinase activity among several species of insects (Wolfson et al.,1990). As well as the adaption of insects to plant protease inhibitors (Jongsma and Bolter, 1997). To fill the literature gap on *S. serialis*, this article identifies the different levels of protease activity in each region of the alimentary canal specifically in this insect species. This approach remains unaddressed in literature, and this article can only be considered a first step of how to implement plant protease inhibitors in transgenic crops to decrease *S. serialis* as pests in the agricultural industry.

In this study, the initial analysis of variance of the different regions of the alimentary canal: foregut, midgut, and hindgut; the presence of protease activity was significant. This conclusion means that there are varying amounts of protease activity in each region. The variables incorporated to further the understanding of protease activity involved the use of boiling treatments for each region of the alimentary canal. The treatments tested were foregut, boiled foregut, midgut, boiled midgut, hindgut, and boiled hindgut. The effects of each treatment influenced the amount of protease activity and hide powder azure absorption. It was concluded that there was a significant difference in the amount of protease activity in the combination of the gut section being boiled or not boiled. This data has also been explored in prior studies by observation of how heat treatments affect protease activity and denaturation (Monti and Jost, 1979).

There was a significant difference ($p = 0.0135$) between boiled and non-boiled regions of the alimentary canal. As well as significant difference between the effects of each factor on the absorption of protease activity ($p = 0.0249$) in each region. It was discovered that each region of the alimentary canal had low protease activity if boiled. This was because of heat denaturing the protease activity. The non-boiled regions had a greater amount of protease activity with the highest in midgut, then hindgut, and foregut closely following.

In summary, this study laid the foundation for the assessment of the different levels of protease activity taking place in *S. serialis*. Although, the midgut is widely recognized as the primary site for digestion of food, the confirmation of the extent of protein digestion occurring in each region in *S.*

serialis is vital for the role of protease activity and how it correlates to protease inhibitors in plants. Error in analysis could stem from the improper sectioning of each region of the alimentary canal in each tube. This would lead to higher absorbances of azure powder in regions that were not correlated for that data. To combat this, a specific identification chart can be made specifically for *S. serialis* to be able to accurately identify each region. Finally, another promising line of research could use this guideline data to identify the different types of protease activity occurring in the midgut and how to best utilize that information for adaptations in plant protease inhibitors. Ultimately, impacting the resistance towards pesticides, economic measures, and most importantly having a successful agricultural business.

Acknowledgements

I would like to thank my fellow classmates in my Insect Physiology lab (ENTO-306) for the help of conducting this experiment. I would also like to thank Richard Fox for making his drawings available for free educational use.

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