PROBING ANOPHELES STEPHENSI MOSQUITO MIDGUT WITH PROTECTED TRIAZABUTADIENES

By

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Abstract

Despite its discovery centuries ago, malaria continues to be a significant public health threat. Though it has been around for many years, effective methods to control its vector remains a challenge. Of the multiple *Anopheles* species, here, the *Anopheles stephensi* mosquito larval midgut is studied to discover potential drug targets. The midgut contains a large number of proteases and their high enzymatic activity contributes to significant degradation of the proteins before they can be analyzed. This project highlights the preparation and identification of dissection and protein purification techniques to minimize protein degradation within the larval midguts. Additional workup of the larval midguts with a known small chemical probe, protected triazabutadienes, can provide additional details about how these proteins can potentially be utilized as drug targets. Protected triazabutadienes are triggered once they reach the high pH environment within the midgut of the larvae to release benzene diazonium ions. These ions serve as a labeling mechanism for tyrosine and histidine residues on the surface of protiens. The labeled proteins can then be further studied.

Introduction

Despite numerous efforts for control, mosquito-borne diseases are flourishing worldwide. With rising global temperatures, more and more geographical regions are becoming hospitable for mosquitoes to breed and thrive and, consequently, they bring with them their list of diseases (WHO, 2000). Mosquitoes serve as vectors for a whole host of diseases such as West Nile virus, dengue, chikungunya virus, Zika virus, Japanese encephalitis virus, yellow fever, malaria, etc (CDC, 2016). While research on many of these mosquito-borne diseases is extensive, certain diseases have been around for a long time. Malaria, in particular, has been

present for centuries (Cox, 2010). More accurately, the causative agent of malaria is the *Plasmodium falciparum* parasite which is transmitted to humans through the bite of an infected mosquito (Maier, 2019). Evidence of its long presence can be seen within our human genome through the emergence of sickle cell anemia. Sickle cells that do become infected with the parasite have the ability to collapse and prevent the parasite from interfering with the cell's actin proteins; this mechanism protects the host against malaria (Lonergan, 2001). Sickle cell anemia, however, can also be detrimental to human health because it presents ischemic complications, lowered life span, stroke, eye problems, etc (NIH, 2020).

Currently, malaria kills more than 400,000 people each year. Of these cases, an estimated two-thirds of deaths are among children under the age of five. In addition to this count, it is also important to consider that in 2019, there were 229 million new cases of malarial infections (WHO, 2020). A person with malaria can experience symptoms of fatigue, chills, fever, vomiting, and/or diarrhea for a few weeks after contracting the parasite (Trampuz, 2003). Added components of the symptoms indicate that malaria can take a significant toll on infected people. For such reasons, the emergence and spreading of these mosquito populations must be understood and aimed to be controlled.

Treatment for malaria includes a variety of antimalarial and antiparasitic drugs (CDC, 2018). While these agents cure, they can sometimes be used for preventative needs. However, no antimalarial drug is 100% protective and must be combined with the use of personal protective measures (CDC, 2018). Although progress has been made in the last ten years toward developing malaria vaccines, there is currently one licensed vaccine for malaria, RTS,S, on the market in limited countries. Among children aged 5–17 months who received 4 doses of RTS,S, the vaccine prevented approximately 4 in 10 cases of malaria over 4 years of follow-up and

about 3 in 10 (29%) cases of severe malaria (WHO, 2020). Therefore, RTS,S vaccine is useful, though its efficacy can still be greatly improved. While research towards other vaccines is ongoing, other potential routes towards managing malaria can be implemented, such as drug development.

A thorough understanding of crucial protein interactions is vital to introduce potential drug targets. The help of chemical probes was utilized in this project to identify these possible drug targets. The small chemical probes are called protected triazabutadienes. Triazabutadienes have been shown to possess the ability to release aryl diazonium ions under exceptionally mild acidic conditions. Through triazabutadiene protection, its acid-dependent ion release is prevented (Guzman et al., 2016). In the acidic environments surrounding the midgut, the probe is therefore rendered ineffective. However, the high pH of the anterior midgut allows for the release of the benzene diazonium ions, covalently labeling tyrosine and histidine residues (Jensen, 2016). By modifying the surface of several proteins at these two residues, essential proteins can be targeted. Specifically, areas of protein-protein interactions, possible regions with high malaria-causing activity, can be addressed. It is known that while it makes up a small fraction of the solvent-exposed protein surface, tyrosine is abundant on the interfaces of protein-protein interactions (Jensen, 2016).

The *Anopheles* midgut metabolic proteins have been identified as having some known and promising targets for the prevention and control of malaria (Adedeji, 2020). Because the *Anopheles stephensi* possess the ability to have the probe be ingested in its protected form and only be triggered to release the ions in its acidic anterior midgut, the area of interest is targeted (Figure 1 and 2). The unique environment of the anterior midgut allows for the labeling of residues only in that region, focusing solely on the midgut, the site of invasion for the ingested

Plasmodium parasite (Adedeji, 2020).

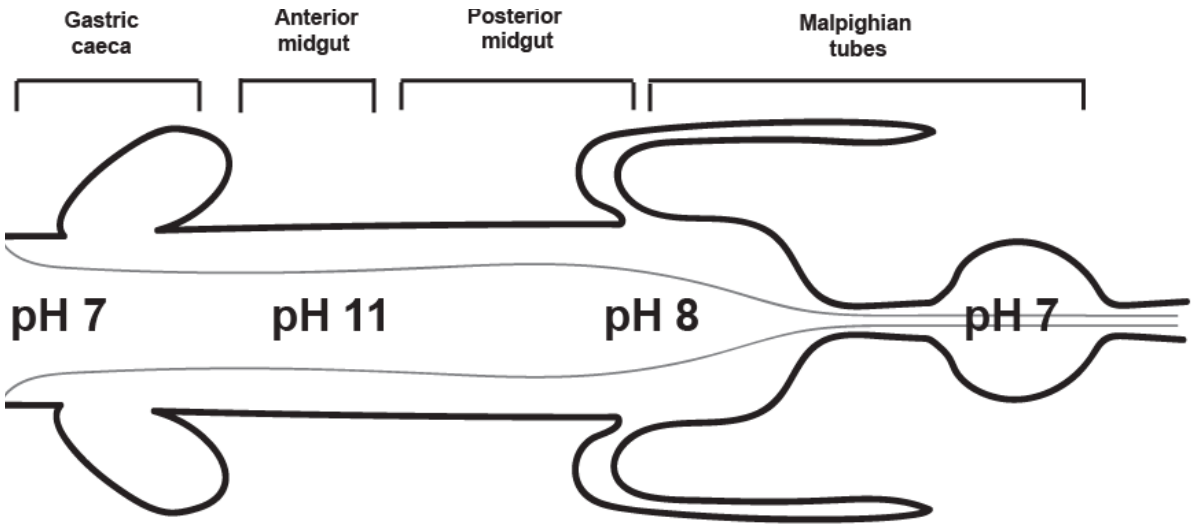

Figure 1- A schematic of regions of the *Anopheles stephensi* mosquito larvae. The pH at each region is highlighted to emphasize the differences throughout the interior of the larvae. As media is passed through, it reaches the anterior midgut, where the high pH triggers the release of benzene diazonium ions. (Image by Dr. Lindsay Guzman)

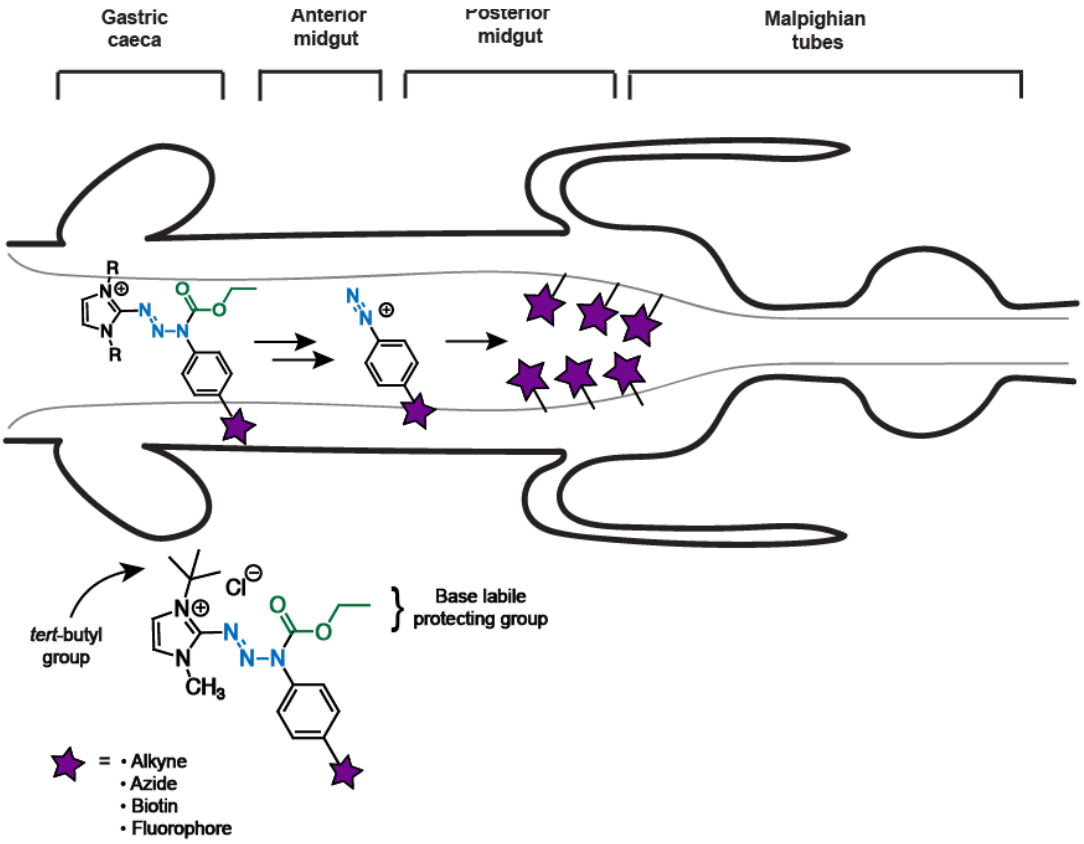

Figure 2- A schematic of the reactivity of the protected triazabutadiene in the *Anopheles stephensi* mosquito larvae. The progression throughout the gut from the anterior to posterior end indicates the expectation of how the probe reacts with the changing pH environments (Image by Dr. Lindsay Guzman)

Materials and Methods

For this project, there were multiple trials of untreated *Anopheles stephensi* mosquitoes followed by trials of treated *Anopheles stephensi* mosquitoes. Each step detailed below was applied to both groups.

1. Prepping *Anopheles stephensi* 4th instar larvae: The control group was fed their usual diet consisting of ground cat food from when they were hatched. The experimental group was given the chemical media in place of cat food for at least one hour prior to midgut dissection.
2. Larval Midgut Tissue Processing: Samples were then dissected in 100 mM sodium acetate buffer (pH 3) and immediately frozen with liquid nitrogen (Figure 3). Then, the gut tissues were converted into a soluble form by adding 8 M urea/SDS buffer and 50% glycerol. This sample was then homogenized and centrifuged. Once the soluble portion was separated, the protein sample was transferred into a new Eppendorf tube. The samples were snap-frozen in liquid nitrogen and stored in a -80°C freezer if needed to be kept. If frozen samples were required to be used, the sample was boiled at 95°C for five minutes.

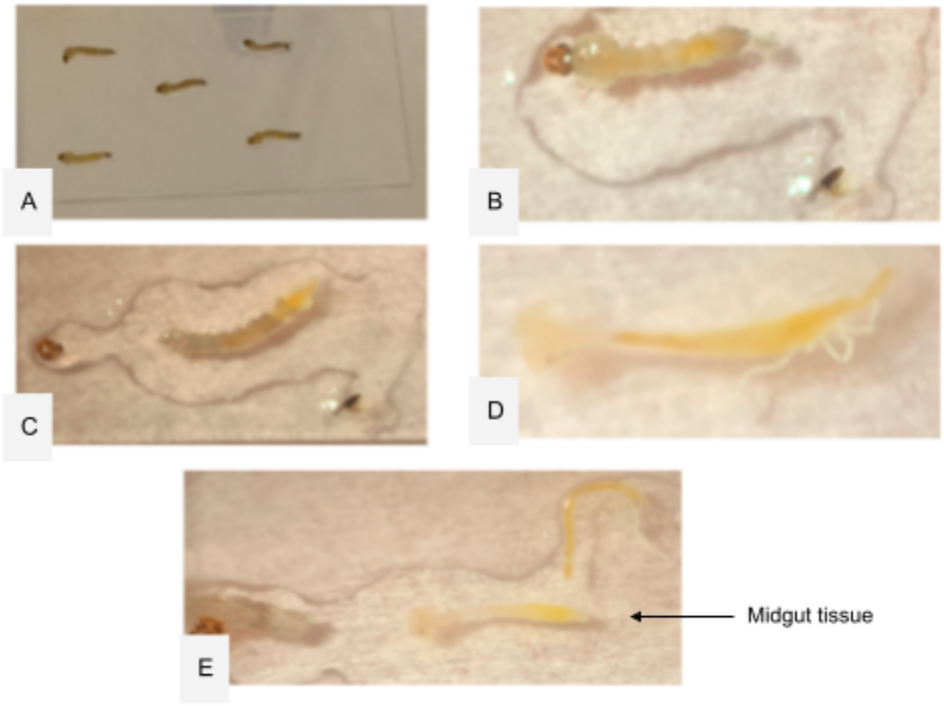

Figure 3- Dissection process of *Anopheles stephensi* fourth instar larvae midgut. Once the larvae are frozen in ice, they are placed onto a glass slide on ice with 100mM sodium acetate(A) and then the tail is separated from the body (B). The head region is then separated (C). The internal gut tissue is then gently pulled out and usually has a food bolus within that it also pulled out from it (E). The midgut tissue is then frozen in liquid nitrogen.

3. Protein Quantification: To independent tubes of the stored tissues and various known concentration BSA controls, Pierce 660 assay reagent was added. The samples were then incubated at room temperature for 10 minutes. Each sample was placed in a nanodrop. With the absorbances collected, the unknown concentration of the sample was found.

4. SDS Polyacrylamide Gel Electrophoresis: 12% Mini-PROTEAN® TGX™ Precast protein gels were utilized in order to run SDS-PAGE. Gels were run slowly at 80V to linearize the dye fronts and then run at 120V for the remainder of the gel.
5. Gel Fixing: After the gel was rinsed twice in nanopure water, it was submerged in a fixative solution (50:10:40 / methanol: acetic acid: H_2O) for 25-30 minutes. The gel was then washed with nanopure water with three aliquots of water for five minutes each. It was then stained with Gel-Code Blue stain reagent for 1 hour at room temperature while rocking. Lastly, the gel was washed with nanopure water while rocking for three hours, with the water changed every hour.

Results And Figures

First, to begin the project, about 20 fourth instar midguts were dissected and then subjected to SDS-PAGE. No proteins were able to be seen (Figure 4). The presence of the ladder was noted and then the project had to be backtracked to determine an adequate protein concentration in order to allow for optimal samples.

Figure 4- SDS-PAGE for 20 fourth instar *Anopheles stephensi* midguts. About 20 fourth instar midguts were dissected in liquid nitrogen and then subjected to SDS-PAGE. No proteins or protein separation could be seen in the lanes with samples.

To solve this problem, the appropriate concentration of proteins from the midguts had to be determined. It was known from the work of Dr. Lindsay Guzman with the *Aedes aegypti* mosquitoes that 20 fourth instar midguts were adequate per sample to show results on the SDS-PAGE. By looking at the protein concentration for this species, a threshold was set for the

concentration needed from the *Anopheles stephensi* midguts. The protein concentration found for the *Aedes aegypti* species was 0.0853 mg/dl (Figures 5). It was found that 35 fourth instar *Anopheles stephensi* midguts were required to achieve a similar protein concentration of 0.071 mg/dl (Figures 6). Once this count was established, all midgut samples for the *Anopheles stephensi* included 35 midguts.

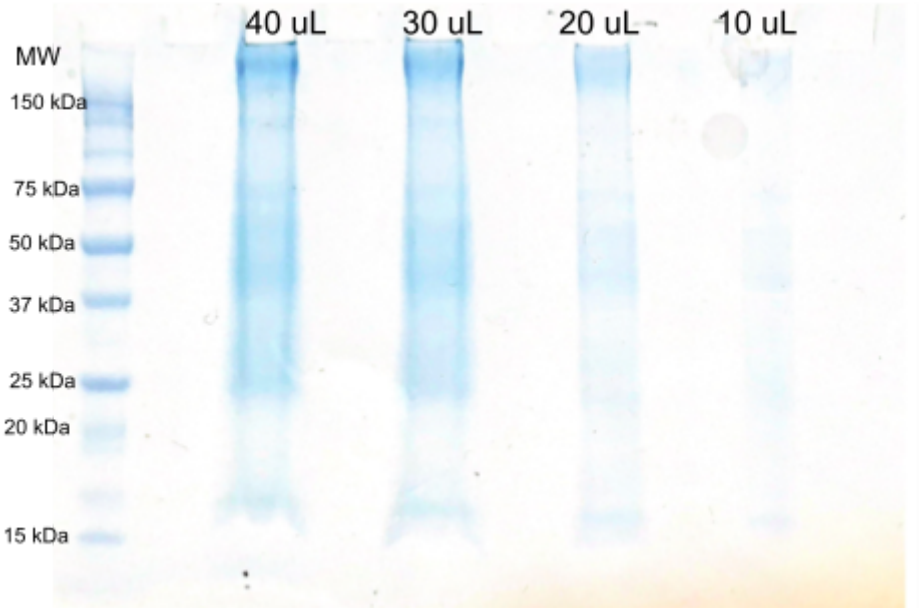

Figure 5- SDS-PAGE for non-treated *Aedes aegypti* mosquitoes with 20 midguts. Having 20 midguts, equivalent to a protein concentration of about 0.0853 mg/dl, allowed for the lanes with the sample to show sufficient presence and separation of proteins. There is no significant degradation of the proteins in the sample.

With the concentration threshold set up, the next step involved running SDS-PAGE to assess protein degradation and separation for the *Anopheles stephensi* mosquito. It was determined that protein degradation was kept at a minimal level (Figures 6). The separation was able to be seen with both the non-treated and treated samples.

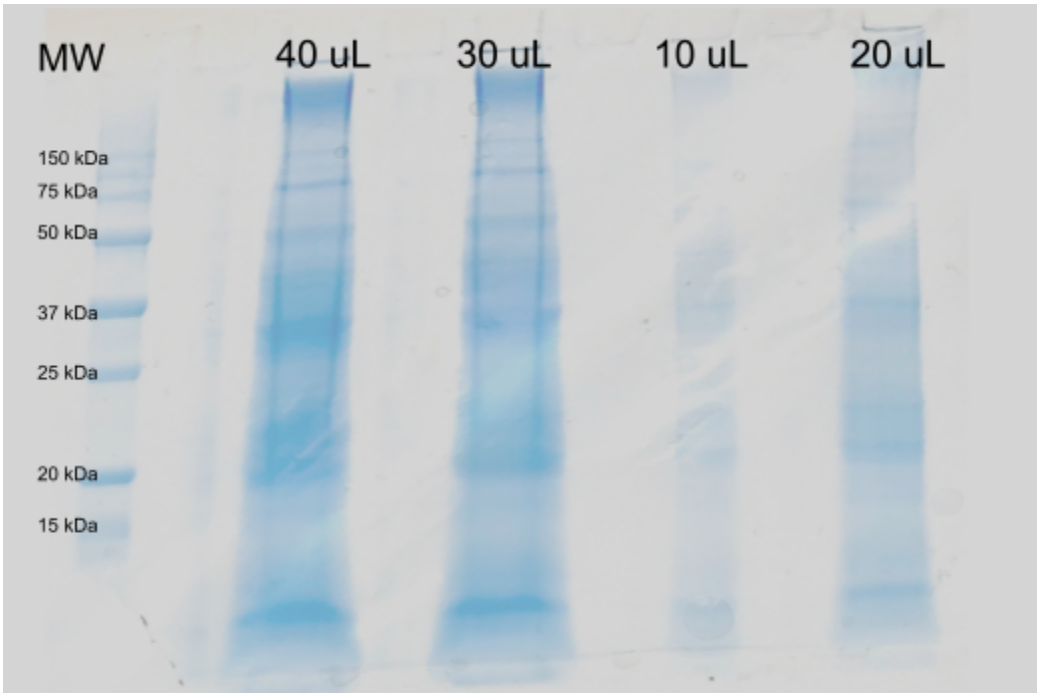

Figure 6- SDS-PAGE for non-treated *Anopheles stephensi* mosquito with 35 midguts. It was found that 35 midguts had a protein concentration of around 0.0708 mg/dl. Along the different amounts of sample added to each well, the 20 μL lane showed the clearest results and was then used as the baseline for following experiments. The separation is seen in all lanes, and protein degradation was not present.

Once the SDS-PAGE procedure was optimized for the *Anopheles stephensi,* the protected triazabutadienes were fed to a set of larvae. This sample was also subject to SDS-PAGE. The results showed no protein degradation (Figure 7).

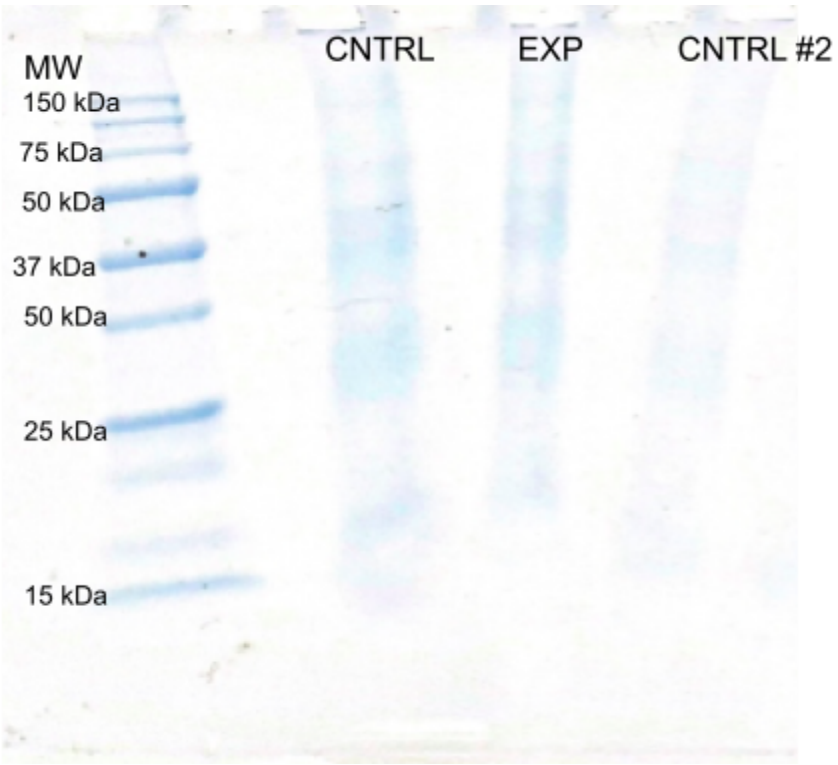

Figure 7- SDS-PAGE for *Anopheles stephensi* mosquitoes treated with protected triazabutadienes. The experiment (EXP) group had ingested protected triazabutadienes prior to dissection and the control (CNTRL) group was ingested water with ground cat food, a normal diet. Two lanes were given for the control group as a backup for any potential ruined lanes. All the lanes for both EXP and CONTRL show similar banding patterns as expected, as a western blot would be the step to specify tagged proteins. The poor quality of the gel made interpretation of the results more challenging.

Discussion

When 20 fourth instar *Anopheles stephensi* midguts were subjected to SDS-PAGE, there were no banding patterns or any trace of sample seen. Initially, the issue with the midgut was the degradation despite the utilization of the liquid nitrogen. A potential reason was the 100 mM sodium acetate buffer resting on the lab bench over a significant period of time. Ideally, the buffer would be working at optimal levels after two to three days, but because the project was focusing heavily on midgut, the large amount of proteases signified the high presence of enzymatic activity. This required the buffer to be as fresh made as possible. In addition to the degradation by the proteases causing low protein presence in the initial SDS-PAGE, the size of the *Anopheles stephensi* species also required a change in procedure. The *Anopheles stephensi* are much smaller at the fourth instar, at which stage they are dissected, in relation to *Aedes aegypti*. From the work of Dr. Lindsay Guzman in the Riehle lab, it was known that about 20 midguts for the *Aedes aegypti* were sufficient to produce results. However, that did not work for the *Anopheles stephensi*. Due to their smaller size, fewer proteins were obtained with each midgut. Finding the concentration of proteins for the 20 *Aedes aegypti* set a threshold to meet with an appropriate number of midguts needed for the *Anopheles,* which was found to be 35 midguts. Going forward, 35 midguts were used to retrieve other data. Optimizing the procedure for the *Anopheles stephensi* backtracked progress for the overall project. However, towards the end of the semester, an SDS-PAGE was run with protected triazabutadienes.

For the SDS-PAGE mentioned, the sample of midguts was treated with protected triazabutadienes, results showed that there was no protein degradation present. Protein degradation was not expected as the procedure worked out well with the non-treated groups. When the gel lanes for the control groups, not treated with protected triazabutadienes, and

experimental group, treated with protected triazabutadienes, were compared, there was no significant difference. A difference was not expected in the first place as SDS-PAGE does not relay much information for the tagging of proteins. If a Western blot were performed using this gel, it would have been expected that the control group lane would have no protein band while the experimental group did. Though the main focus for the project initially was to use triazabutadienes, energy had to be expended first to optimize and tailor the procedure specifically for the *Anopheles stephensi*. Now, next steps can be taken.

Future Directions

As previously mentioned, the *Anopheles* metabolic proteins have been known to show promising targets for drug development. The roles of metabolic proteins as insecticide targets, in blood digestion and immune response, as well as their contribution to insecticide resistance, make them an area of interest. By having protected triazabutadienes be effective within the midgut and now knowing the optimal concentrations and procedures, the next step of the project should focus on identifying specific proteins. A Western blot would be ideal to begin detecting particular proteins. After the identification of the labeled proteins is discovered, the protein can be studied to determine characteristics about its active sites, key interactions with other proteins, and/or general properties.

The newfound knowledge can set up the stage for potential inhibition experiments. Those inhibitors could prove essential in possible drug development, especially if they are species-dependent. In the case that no such information exists for the tagged proteins, inhibitors can be designed based on the target specificity for which the protein would need to be put under

further analysis, such as determining its crystal structure and kinetic profile. In general, the inhibitors can be combined with other inhibitors, synergists, blocking agents to yield more potent malaria intervention strategies (Adedeji, 2020). These compounding efforts may provide multiple routes to controlling the vector. Current efforts are focused on reporting a more in-depth proteomic analysis of the midgut.

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