ABSTRACT

Development of mosquitocidal bait: A recombinant fusion atracotoxin (Jun a 3/ACTX) produced by viral vector in *Nicotiana benthamiana* offered in various matrices

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Mosquito-borne diseases are a persistent global concern that is currently addressed through the use of pesticides. These pesticides are a source of environmental and human concern due to their lack of specificity. As a result, both environmentally beneficial insects and pests are exterminated in the same degree. My research is focused on the development of a target specific toxin and the selection of a mosquito-attractant matrix. The toxin of choice is an invertebrate-specific peptide neurotoxin (ACTX), which was isolated from the venom of Australian funnel-web spiders. With the use of a viral vector system dependent on the FECT/40 vector, originally from the foxtail mosaic virus, and the addition of Jun a 3 as a signaling protein we were able to produce the recombinant fusion protein (Jun a 3/ACTX) in *N. benthamiana.* To address the matter of the proper matrix, solutions high in sugar content were chosen. Studies indicate an innate attraction of mosquitoes towards solutions high in sugar, which plants usually offer as a lure for cross-pollinators such as mosquitoes. Therefore,

Jun a 3/ACTX and some protein standards were tested for degradation levels in matrix candidates such as honey, high fructose corn syrup, and carob solution. Mosquitocidal properties of the bait were tested on adult *Aedes albopictus.*

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DEVELOPMENT OF MOSQUITOCIDAL BAIT:

A RECOMBINANT FUSION ATRACOTOXIN (JUN A 3/ACTX) PRODUCED BY VIRAL VECTOR IN *NICOTIANA BENTHAMIANA* OFFERED IN VARIOUS MATRICES

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TABLE OF CONTENTS

FIGURES & TABLES

CHAPTER ONE

Abstract

Mosquito-borne diseases are a persistent global concern that is currently addressed through the use of pesticides. These pesticides are a source of environmental and human concern due to their enduring presence in the ecosystem. As a result of their use, both environmentally beneficial insects and pests are exterminated in the same degree while the soil is poisoned. Recent studies have even attributed the colony collapse disorder in *Apis mellifera* (honey bees) to the persistent concentrations of pesticides in the soil, which is captured by the plants and expressed in their nectar (Henry, et. al, 2012). The mosquito season is a time of heavy rain; hence, spray-on toxins intended for outdoor use would be washed away quickly and only provide a short window of efficacy. The positive contribution of pesticides is briefly lived, because the development of resistance can occur. Diseases such as yellow fever, dengue, malaria, louse-borne typhus, and other vector-borne diseases are therefore still a constant threat to life and have been so since our awareness of them began in the 17th Century (Gubler, 1998). Consequently, it is imperative to develop a method to control mosquito populations in a target specific manner that is safe for commercial and private use. Restricting a mosquito toxin to a bait, rather than broadly applying it as a broadcast spray, would be one way to safely control mosquito populations. The

natural preferences and diet of mosquitoes would be vital resources to consider when designing and selecting an attractive bait matrix.

Recently conducted studies on mosquito behavior have highlighted the importance of sugar meals to promote proper fitness and development. Sugar is a substantial energy source reserve and has a direct correlation with the proper size development of the egg clutch in mosquitoes. Even though autonomous female mosquitoes, the source of most mosquito-borne diseases, require a blood meal to produce their eggs, these consume sugar meals for sustained fitness and longevity (Foster & Hancock, 1994). It is hence relevant to note that the attraction to sugar based substances such as nectar is completely independent of the mosquitoes' sex. Field studies have demonstrated the role of mosquitoes in the cross pollination of plants which provide nectar, high in sugar, as a lure or reward. These same studies report a direct correlation between the concentration of sugar in a particular plant's nectar and the degree of preference from mosquitoes. This correlation is exemplified by the production of a greater number of eggs when imbibing on nectar with higher sugar content (Manda, et. al, 2007).

Such definite food preference by mosquitoes have prompted the work of Günter Müller who has been able to develop effective attractive toxic sugar baits in the Western portions of Africa. His field trials, conducted with sugar baits and fruit baits as part of traps containing the toxin boric acid were able to reduce *Anopheles gambiae* populations by about 90%. Most importantly, the population of older females was reduced by 8-fold; these are the most prolific in the

transmission of mosquito-borne diseases such as malaria (Muller, et. al, 2010). The attractive toxic sugar baits provide insightful evidence as to an alternative to pesticides, yet the fact that the trials were conducted near oases in dry arid areas diminishes their credibility in a commercial setting where competing sources of sugar are found. The use of sugar baits is beneficial in comparison to fruit baits, because the user does not require *a priori* knowledge of mosquitoes' plant preference which can vary from species to species and region to region. Therefore, the concept of using sugar-based baits and protein toxins concurrently is an appealing alternative that needs to be refined and improved on in order to be effective if used in a household or field.

Alternatives to fulfill the requirements of the ideal sugar-based bait are many and far ranging, but honey appears to be the optimal candidate due to its extended shelf life, natural attractiveness, and global accessibility. Regarded by many to be the 'gold standard' for mosquito baits, honey has been used to observe various behavioral aspects of attraction in *Aedes aegypti*, among other species. In a study conducted in 1994, up to 50% of both males and females rapidly formed probing aggregations when the extract was presented through a screen. Other researchers have successfully used hexane honey as an attractant for *Aedes taeniorhynchus* and *Culex nigripalpus* (Foster & Hancock, 1994). The consistent attractive results across various species of mosquitoes are a promising note. The extraordinary levels of attraction have been attributed to the high sugar content of honey and the presence of volatile organic compounds that trigger these insects'

probing instincts. The constitution of honey is approximately 80% carbohydrates (35% glucose, 40% fructose, and 5% sucrose) and 20% water. It consists of more than 180 substances, including amino acids, vitamins, minerals, enzymes, organic acids, and phenol compounds. Honey possesses an approximate pH of 4.0. These characteristics are fairly constant regardless of region, but subtle variance dependent on location and time of year can be identified in the ash. The acidity of honey is due to the existence of organic acids produced by native enzymes (Kahraman, et. al, 2010). Among those organic acids is gluconic acid, formed by the oxidation of glucose, which acidifies honey rendering it intolerant for microbial growth (Evans & Flavin, 2008). High acidity and a low water content are beneficial characteristics when considering the shelf life of a potential bait, but pose a challenge to any potential toxic protein that might be used in conjunction with honey to kill mosquitoes. A complication with the use of honey is the possible denaturation of a foreign protein in solution either due to the acidity or the enzyme activity. Another hurdle would be the difficulty to reach homogeneity between toxin and the honey matrix where the concentration will coincide with a lethal dosage. That being said, honey is expected to contain a small number and a limited variety of microorganisms such as yeasts and spore-forming bacteria. Such a discovery indicates the possibility of foreign compounds and organisms not being degraded in the hostile environment of honey (Viuda-Martos, et. al, 2008). Honey is a viable candidate for the matrix to hold the toxin due to its extensive shelf life and natural attractiveness to mosquitoes.

Just as vital as the selection of the proper medium for the bait is the selection of the proper toxin. Several factors contribute to the ideal toxin of choice such as the importance for the toxin to be target specific in order to be viable for field and public use with little environmental hazard. High target specificity is not particular common in chemical toxins such as organophosphates and organochlorine insecticides. The broad range adverse effects of chemical pesticides are well documented with the use of DDT during the 1950's (Carson, 2002). Contrary to chemical toxins, protein toxins have a high degree of specificity as a result of their unique folding patterns and constituent amino acid residues. The use of protein toxin provides specificity, but to select candidate it should also be sturdy enough to retain its shape and therefore its function for a generous time period. Lastly, it is vital that the toxin can be collected or produced in a manner that is efficient, expedient, and rapid. The toxin of choice for our research is spider venom, because many of these complex chemical cocktails have evolved to be extremely target specific and potent in low dosages. The invertebrate-specific peptide neurotoxin atracotoxin (ACTX) to be used was isolated from the venom of Australian funnel-web spiders; considered to be among the most venomous spiders in existence. (Tedford, et. al, 2004). Continued studies on Australian funnel-web spiders have developed a comprehensive analysis of venom gland cDNA libraries, which indicate that a single species typically expresses a great deal of genomic variability in regions outside its disulfide bridges. It is estimated that the Australian funnel-web spider produces

three to ten variants of each peptide toxin naturally. Such variation in the toxic sequence is important when considering factors such as the development of resistance when employed into an open ecosystem. Additionally, ACTX is a cystine knot protein of only 36-45 residues in size in its mature state (Tedford, et. al, 2004). As its name suggests, it comprises of a ring formed by two disulfides and the intervening sections of a of polypeptide backbone with a third disulfide piercing the ring to create a pseudo-knot (Maggio, et. al, 2005). The compact nature of ACTX provided by the disulfide bonds and short sequence makes it a sturdy protein that could be able to withstand some harsh conditions such as acidity without being denatured quickly.

Studies have been conducted on the target specificity and host range of ACTX, which could be attributed to its unique structure-function mechanism. ACTX's pathway is to effectively block the calcium ion channels of invertebrate organisms causing paralysis and eventual death (Maggio, et. al, 2005). Previous successful oral and injected tests have already been administered on organisms such as *Amblyomma americanum* (lone star tick) and *Calliphoridae* (blowflies), but not on mosquitoes. These experiments revealed a 700-fold PD-50 increase of toxicity when the toxin was administered orally. Where PD-50 indicates the dose of antiserum or vaccine that protects 50% of the insects exposed to the toxin and hence the protein concentration of ACTX per dosage (Mukherjee, et. al, 2006). Furthermore, there have been other trials conducted by producing a recombinant fusion version of the toxin and GNA (*Galanthus nivalis agglutinin*), a mannose-

specific snowdrop lectin, with the *Manduca sexta* (tobacco hornworm) allatostatin sequence fused to the C-terminus. The fusion protein toxin was tested on fifth stadium *Lacanobia oleracea* (bright-line brown-eye moth) larvae by incorporating it into the diet. Its administration displayed promising results by reducing the growth and feeding of fifth stadium *L. oleracea* and successfully eradicating most neonates (Fitches, et. al, 2004). The results presented in this paper using GNA provide further evidence for the use of fusion proteins to deliver active toxins orally, even though these tests were not conducted on adult mosquitoes (Fitches, et. al, 2004). These articles note the effective toxicity of a recombinant version of ACTX and also provide significant evidence to support the notion that it can affect a broad range of hosts.

In order for ACTX to be beneficial to our experiments it needs to be produced in a system that would facilitate ACTX protein production beyond the difficult venom extraction directly from Australian funnel-web spiders. It is necessary to produce the cystine-knot protein in an effective manner that would provide a significant amount of protein that is relatively homogenous in constitution and cost-effective. The literature notes that plant tissue cultures may be significantly less expensive than animal cell culture, given the absence of exogenous proteins in plant culture media and the dominance of downstream processing operations in determining the total cost of protein manufacture (Doran, et. al, 2006). Liu and Kearney (2010) have developed a streamlined method to produce proteins such as green fluorescent protein (GFP) and ACTX using a viral

vector system in the tobacco species *Nicotiana benthamiana* in high yield. The viral vector system is dependent on the FECT/40 vector originally from the foxtail mosaic virus, but modified by the elimination of the coat protein and triple gene block genetic segments. The result is a 29% reduction of the genome providing sufficient space to insert the desired genetic sequences. Also, the inability to produce a protein coat limits the virus within the originally inoculated cells since no virions can be formed causing a controlled infection. The controlled infection provides a great level of environmental and laboratory safety, but limits the protein production. Such a limitation in recombinant protein production is overcome by the co-agroinoculation technique. This uses *Agrobacterium tumefaciens,* injected directly into a nonsterile, potted plant, to deliver the virus sequence, carried in a binary transformation vector, to the genome of the vast majority of inoculated plant cells in the inoculated zone. In conjunction with the transformed viral agrobacterium, an immune suppressor is expressed to reduce the response to the infection. The immune suppressor is also expressed in agrobacterium and functions by employing the RNAi mechanism, which targets immune response mRNAs (Anandalakshmi, et. al, 1998). The co-agroinoculation of the modified FECT vector and an RNA silencing suppressor (p19 from tomato busy stunt virus (Scholthof, 2006)) have yielded a 40% expression of ACTX of the total soluble protein (Liu & Kearney, 2010). The process already established in the laboratory to develop recombinant proteins in tobacco plants has been well

tested and can produce properly folded recombinant proteins for extraction within a week.

The production of ACTX as a recombinant protein required a fusion partner in order to transport the protein of interest into the apoplast to be able to conduct vacuum extraction. As a result, ACTX is expressed fused to Jun a 3, an allergenic protein from mountain cedar pollen. The fusion protein is a necessity for protein extraction, because preliminary tests using the FECT vector yielded no ACTX in the apoplast. The addition of Jun a 3 to the construct signals the protein to be transported into the apoplast and easily accessible for extraction by vacuum infiltration (Mohenke, et. al, 2008). The Jun a 3 signal protein is linked to ACTX by an IGER linker. The five amino acid linker allows the cystine knot protein to fold properly independently and reach the apoplast. Also, the presence of an Arginine residue provides a trypsin cleavage site. The addition of the trypsin cleavage site is to address our concern that the Jun a 3 addition to the protein could potentially limit its toxicity. Therefore, the IGER linker would cleave off the Jun a 3 from the ACTX protein once inside the mosquito gut by gut trypsin (Borovsky, et. al, 2006).

The intention of our research is to develop a functional sugar bait by employing ACTX produced as a recombinant fusion protein in a matrix attractive to mosquitoes such as honey. Crucial to the investigation would be to provide evidence that honey is a competitive alternative to floral nectar or fruit in the matter of mosquito fitness and longevity. Alternatives to honey as a bait matrix

have also been investigated. High fructose corn syrup (HFCS), closely mimics the composition of honey (80% carbohydrates and 20% water), and is therefore commonly used to 'stretch' honey in commercial products. The preliminary concerns with honey are the presence of enzymes, microorganisms, and its high acidity. In order to test if these factors have any effect on protein stability, high fructose corn syrup was tested as a possible alternative matrix. Further research has unveiled the possibility of carob seed pods, commonly used as a health food alternative to cocoa powder. Field tests have shown attractiveness to mosquitoes equal to fruit juices. The benefit that carob is two-fold. Unlike fruits, carob can be provided for year round and can be stored in powder form (Muller, et. al, 2011). Therefore, the research conducted is centered on the effectiveness of these matrices to preserve Jun a 3/ACTX in solution without degradation.

In conjunction to the degradation tests of Jun a 3/ACTX, a selection of stable proteins commonly used in the lab, lysozyme, alpha casein, trypsin inhibitor, and bovine serum albumin, was evaluated. These proteins were examined for physical degradation in the three bait matrices and in water by SDS-PAGE. By testing a range of proteins for stability, an assessment of the general applicability of these bait matrices to delivering proteins, including future protein toxins, differing in structure from Jun a 3/ACTX can be assessed.

Along these lines, the functional stability of different proteins in the bait matrices was examined. Colorimetric enzyme assays with horseradish peroxidase activity tested with 3-3'-diaminobenzidine as its substrate were conducted. The

fluorescence tests of enhanced green fluorescent protein (EGFP) extracts tested for decaying levels of fluorescence as an indication of protein degradation. The ultimate test of retained function was determined by Jun a 3/ACTX toxicity tests conducted with adult *Aedes albopictus* mosquitoes by imbibition. The retention of activity by Jun a 3/ACTX in honey, leaves the door is open for the use of other cystine-knot protein and different families of toxins as part of an effective mosquito bait.

CHAPTER TWO

Materials & Methods

Production of recombinant fusion protein using viral vector system

Plant viral vectors recently developed have been able to minimize the required time of preparation and the cost of production of recombinant proteins while still exhibiting high levels of expression. Dr. Zun Liu and Dr. Christopher M. Kearney have effectively developed a recently patented vector system using foxtail mosaic virus (FoMV), which it uses for most protein expression work (Kearney & Liu, Patent number US 8,344,208). FoMV is classified under the genus *Potexvirus,* which are flexuous filamentous plant viruses. FoMV and potexviruses comprise a positive-sense single stranded RNA sequence with a cap at the 5' terminus and a poly-(A) tail at the 3' end which provides stability (Robertson, et. al, 2000). The vector version of FoMV (FECT) has deletions of the genetic segments that code for the coat protein and the triple gene block, a 29% reduction of the total genome (Liu and Kearney, 2010). With the removal of the coat protein, those plant cells inoculated with the virus are unable to produce the virions responsible for the systemic proliferation of FoMV. Moreover, the

deletion of the triple gene block, a highly conserved sequence of three overlapping genes, eliminates any cell to cell or long distance movement of the virus as well. As a result, the FECT vector series is created which has no marker gene when inoculated in a plant or the ability to move past the originally inoculated cell. The initial benefits of the FECT vector are its ability to address issues of cross-contamination in greenhouses/growth rooms and also reduce transmission in field use. In fact, FECT RNA does not replicate significantly unless a silencing suppressor, which inactivates the plant RNAi immune system, is co-expressed with FECT. The Kearney Lab commonly uses the p19 silencing suppressor from Tobacco bushy stunt (Scholthof, 2006). In order to express the FECT RNA and p19 mRNA from cDNA inserted in the plant genome, these vectors are equipped with a 35S promoter. The 35S promoter is derived from the Cauliflower mosaic virus and is ubiquitously used in protein production through viral vectors do to its extremely high output, but it is still significantly low in comparison to viral vector expression. Furthermore, when inoculating the plant for protein production it is co-inoculated with p19, a silencing suppressor, which significantly increases the possible output of the recombinant fusion protein Jun a 3/ACTX (Liu & Kearney, 2010).

The following step is the insertion of the gene of interest in the open reading frame down stream of the 35S promotor. Restriction enzymes PacI and AvrII are used to digest both the insert and the FECT vector. PacI and AvrII produce sticky ends, which means there is a 5' or 3' overhang, which provides

swifter and more fruitful ligation. AvrII recognizes the sequence 5'…CCTAGG…3' and cuts between the two 2'-deoxycytidines. PacI recognizes the sequence 5'…TTAATTAA…3' and cuts between the two 2'-deoxythymidines closest to the 3' end. Using two different restriction enzymes with two different recognition sequences reduces the possibilities of religation of the empty vector and also ensures that the gene of interest will be inserted in the proper direction. The digestions of the gene of interest and FECT vector are separated by size using electrophoresis on a 1% agarose gel and then stained with GelStar® (Lonza, Allendale, New Jersey). The bands resulting for the digestion are visualized on the Dark Reader® transilluminator (Clare Chemical Research, Dolores, Colorado), with a spectrum from 400-500 nm. At these wavelengths, GelStar® is excited, allowing us to visualize and excise the correct band according to size in reference to the ladder. The DNA is then purified with the Wizard® Gel Extraction Kit (Promega, Madison, Wisconsin), which is able to digest the agarose without damaging the plasmid or gene of interest. The result is a fairly pure concentration of vector and insert that can be ligated. For the ligation, a 3:1 molar ratio between insert and FECT is used to increase the probabilities of success. These are placed in solution with 1.5 µl of 10x ligase buffer and 400 U of T4 DNA ligase (400 U/µl). The ligase reaction is conducted at 16°C and usually overnight. Upon completion an aliquot of the ligation is again run on an agarose gel and purified to ensure that the ligation has successfully

occurred, but in practice the ligation mix is usually used for electroporation immediately.

The ligated plasmid is then inserted into competent *Escherichia coli* cells through the process of electroporation. Large and/or polar molecules have difficulty entering the phospholipid bilayer of cells and therefore require the use of electroporation or heat-shock treatment. Electroporation exploits the amphiphilic nature of the phospholipids. The weak interactions between the hydrophobic tail and hydrophilic head of the phospholipids are disrupted by the use of a quick voltage shock which allows pores to form sufficient for the diffusion of the plasmid into the cell (Purves, et. al, 2001). Immediately after the voltage shock the cell's bilayer is reassembled and the cell is left unaffected. The transformed *E. coli* cells are then placed in a nutrient rich Lysogenic Broth (LB) for growth. The resultant *E. coli* colonies are then plated on LB-agar laced with kanamycin and incubated at room temperature. The FECT construct contains a kanamycin resistance gene which functions as a selectable marker, allowing only the E. coli cells successfully transformed to proliferate on the plate. Consequently, *E. coli* colonies that grow independently on the plate are picked and allowed to proliferate in a larger container along with 10ml of LB/kanamycin broth at room temperature. The culture is kept in constant motion to ensure the bacteria receive the proper amount of oxygenation and to avoid plaques from forming, which would limit their overall growth in numbers.

In the next step, *Agrobacterium tumefaciens* is used to introduce the DNA into the plant genome. First, plasmid preparations are made from the *E. coli* broths above. The Wizard® Plasmid Prep Kit (Promega, Madison, Wisconsin) is used to lyse the *E. coli* cells, isolate plasmid DNA from genomic DNA, and then the plasmids are centrifuged through a silica layer in a microcentrifuge tube in order to anchor the plasmid for purification. Repeated ethanol washes provide a pure plasmid that can now be electroporated into *A. tumefaciens.* Similarly to *E. coli*, the transformed *A. tumefaciens* is placed in growth media and in constant motion to produce the necessary colonies for agroinoculation. In parallel to *E. coli*, plating screens colonies with kanamycin as the selectable marker. Colony screening is imperative due to the low transformation rate that naturally occurs. Colonies harboring inserts are selected and inoculated into a broth solution containing kanamycin. A separate batch of *A. tumefaciens* transformed with p19, the silencing suppressor, is also grown in preparation for co-agroinoculation. Agrobacterium colonies are allowed to grow over 24 hours or until a high level of turbidity is displayed. Turbidity in the solution is indicative of the proliferation of the *A. tumefaciens* in broth. The colonies are then grown in L-MESA media, comprising of LB broth, acetosyringone, and 2[-morpholino] ethanesulfonic acid (MES bufffer). Acetosyringone has been noted to increase the transformation rate drastically when using *A. tumefaciens* as the delivery method. It induces the bacterial infection, resulting in transformation percentages of 60% rather than the previous 2% success rate (Sheikholeslam & Weeks, 1987). On the other hand,

MES provides a stable pH in the solution that is necessary for the survival of the bacterium (Ogaki, et. al, 2008). After 6 to 8 hours of growth at room temperature and in constant motion the cells are harvested by centrifugation for 10 minutes at 3,500x g, or 15 minutes at 2,000x g. The result is a pellet of *A. tumefaciens*, which is subsequently resuspended in agroinduction media. The agroinduction media comprises distilled water, MgCl2, MES, and acetosyringone. Complete resuspension of the cells is critical and all clumps formed during bacterial growth need to be gently separated. The agroinduction media is left unshaken for approximately three hours prior to inoculation.

Before proceeding with the agroinoculation, the agroinduction media solutions of FECT and p19 are combined at a 1:1 ratio without exceeding 3 ml per *Nicotiana benthamiana* plant. The leaves selected for agroinoculation are usually fairly young, because older leaves display a high presence of immune response proteins, poor yield of the protein of interest, and are unlikely to survive the seven-day incubation period. To inoculate a leaf, it is slightly scratched on the underside and with a 1-3 ml syringe with no needle solution is pumped with little pressure into the wound. As a result, the leaf will appear to become hydrated. For optimal success when extracting, at least half of the leaf should be inoculated with as little damage done by the scratching as possible to reduce the presence of necrosis. Increased necrosis results in less protein of interest production and an increase in immune response proteins. When conducting an agroinoculation, a batch of *A. tumefaciens* containing FECT with green fluorescent protein (GFP) is

also inoculated as a control to ensure that all previous steps were conducted properly. It is possible to view GFP within 30 hours post infiltration. For protein extraction, it is ideal to wait seven days, but high levels of necrosis could prompt the timeline slightly.

After the seven-day period, inoculated leaves are cut and trimmed so as to only include portions that were infiltrated. It is also very important to remove the midrib of the leaf to avoid the extraction of unwanted proteins. In order to extract the proteins, a vacuum infiltration method is employed. These clippings are placed in a 150ml beaker and submerged in a solution of sodium acetate buffer. Next, the beakers are placed in a chamber where the house vacuum is applied for 4 minutes to remove all air in the container and especially in the apoplast region of the leaves. Quickly, the vacuum is released causing the buffer to enter the leaves through the apoplast and causing them to change color into a darker shade of green. The process is repeated until all leaves uniformly reflect the darker tone, insuring that the acetate buffer has entered the apoplast region where the secreted ACTX with the Jun a 3 fusion partner is located. The resulting leaves are then placed inside 250 ml centrifuge tube with plastic netting discs inside. These netting discs allow the buffer to accumulate at the bottom while keeping the leaf clippings on the top. Afterwards, the 250 ml centrifuge tubes are centrifuged at 2,000x g for 15 minutes at 6°C. The result should be approximately 2 ml of buffer accumulated at the bottom with a clear and slight golden tint. For immediate or

short term use the subsequent protein solution is stored at 4°C and at -20°C for long-term storage.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is used to identify by size the presence or absence of a protein of interest in solution. This method also provides the possibility of quantifying the amount of protein present through the use of bovine serum albumin (BSA) as a concentration standard and the ImageJ® software. Moreover, it can be used to qualitatively analyze the rate of degradation of a Jun A 3/ACTX in our solutions of interest. To corroborate results, the degradation of lysozymes and alpha casein were also analyzed through SDS-PAGE. The tests conducted used the Bio-Rad® MiniProtean 3 (Hercules, California) gel rig with 0.75 mm comb and followed the manufacturers recommendations for use. Polyacrylamide gels were produced in house and comprised a 15% resolving gel and a 5% stacking gel. The 15% resolving gel provides a dense gel necessary to accurately separate the proteins of interest, while the 5% stacking gel was simply used to ensure the samples in each lane run perpendicular to the ground and parallel to each other so there is no cross contamination or ununiformed migration. To produce the 15% resolving gel,

distilled water, 2.5x resolving buffer, 30% bis-acrylamide, 10% ammonium persulfate, and TEMED were used. Thereafter, the 5% stacking gel was produced from distilled water, 5x stacking buffer, 30% bis-acrylamide, 10% ammonium persulfate, and TEMED. Samples were prepared using 11µl of sample in solution with 6µl of loading buffer for those gels using the 15-tooth comb. Those samples, which used a 10-tooth comb, used 18µl of sample along with 9µl of loading buffer. Before loading into the wells, the samples were boiled for 4 minutes to ensure proteins were denatured and had no tertiary structure present that might affect its migration through the gel. All tests were conducted at a constant voltage of 200V with an average run time of 1-2 hours for a 25kDa protein. All gels were stained with Coomassie Brilliant Blue R 250 for a minimum period of 1 hour while being placed in a rocker to guarantee a uniform staining. The coomassie blue solution is comprised of 0.025% Coomassie Brilliant Blue R 250, 40% methanol, and 7% acetic acid. The destaining protocol commences with the use of Destaining Solution I, which has 40% methanol and 7% acetic acid, on a rocker for a period of one hour. It is followed by the use of Destaining Solution II, comprised of 5% methanol and 7% acetic acid, for the same period of time or until the desired contrast is attained. Multiple rounds of each destaining solution were common and dependent on the original time of staining with coomassie blue. The gradual reduction of methanol in solution is necessary otherwise the polyacrylamide gel presents issues to remain flat for visualizing. After the

completion of destaining, the gel can be placed in distilled water to visualize. SDS-PAGE gels were stored at 4°C in a water solution.

Fluorescence Tests

The fluorescence tests are functional assays in which eGFP is employed to test rate of degradation by the loss of fluorescence in the selected matrices. In order to collect vast amounts of purified protein, the eGFP DNA sequence was inserted into a hydrophobin vector. The vector provides a hydrophobin (HFBI) tag that affords rapid phase separation and effective isolation of the protein of interest. Hydrophobins are small surface level proteins found in filamentous fungi and are responsible for the formation of surface hydrophobicity of the coat in these organisms. These are surface-active proteins with a unique tertiary structure determined by its eight cystine residues, which produce four disulfide bridges. Such a structure provides great stability and the ability to aggregate within a cell. A recombinant fusion protein produced by the linkage of HFBI to GFP has been very effective. GFP, like many other proteins transgenically produced in *N. benthamiana*, is unable to be released into the apoplast for collection through vacuum infiltration. Even then, those that use fusion partners, such as Jun a 3, that

lead the protein to the apoplast, are sometimes not collected in tremendously high amounts. Therefore, HFBI is an alternative for when apoplastic extraction does not work. The GFP-HFBI fusion protein is highly soluble and the hydrophobin partner even has the capacity to increase the solubility of its linked protein.

The purification process for the extraction of eGFP-HFBI begins by grinding one gram of the inoculated leaves in a mortar and pestle that has been cooled at -20°C for 30 minutes. The fine power-like product is then suspended in 10 ml of PBS buffer and centrifuged for five minutes at 20,000x g to remove all leaf remnants. The supernatant containing all soluble proteins is transferred into a 1.5ml Eppendorf tube with 40mg of Triton X-114. Afterwards the mixture is vortexed thoroughly and placed in a 24°C water bath for five minutes to separate the phases. The lower phase, containing the eGFP-HFBI fusion protein, is carefully collected. In order to collected the remaining protein of interest, 400µl of isobutanol is added to the triton and leaf extract solution. The mixture is again vortexed and placed in a water bath and the lower phase is collected again. The gradual collection of eGFP-HFBI and purification can be monitored by the use of blue light (450-495nm), which excites the eGFP protein to emit a bright green light. Therefore, the recombinant protein can be easily extracted through the use of phosphate buffered saline (PBS) extraction or sodium acetate without the need for detergents. Moreover, the single-step phase separation results indicate a capability of collecting up to 91% of the protein with concentrations of 10mg/ml (Joensuu, et. al, 2010).

The success of the hydrophobin fusion partner allows us to pair it with enhanced green fluorescent protein (eGFP) and produce it in large amounts. A highly purified amount of eGFP-HFBI is therefore employed as a substrate for fluorescence tests. eGFP-HFBI hence provides a qualitative analysis of protein function and rates of degradation in solution. Visualization of recombinant fusion protein in solution were conducted on the Dark Reader® transilluminator, with a spectrum from 400-500nm, since eGFP displays an excitation max at 488nm and emission max of 509nm.

Colorimetric Enzyme Assay

The colorimetric enzyme assay is a functional test that evaluates the rate of protein degradation by quantifying the enzyme activity of horseradish peroxidase in solution. 3-3'-diaminobenzidine (DAB) is a development reagent with the capacity to detect peroxidase bound to an antibody that is bound to the antigen of interest. As a marker, this substrate produces a brown and insoluble product as a result to its exposure to horseradish peroxidase conjugated antibodies. The product of the reaction is visualized easily. The protocol followed reflects the guidelines provided by the manufacturer, Bio-Rad® (Hercules,

California). A tris buffered saline (TBS) solution is produced with 200mM Tris, 5M NaCl, and a pH of 7.5. For testing, 50mg of DAB are placed in solution with 100 ml of TBS and 10µl of 30% hydrogen peroxide. Solution containing horseradish peroxidase is combined with the testing solution. An immediate product will form if protein concentration exceeds 100ng, otherwise it slow developing. The reaction is terminated by immersion of experiment in deionized water for a minimum period of 10 minutes (Bio-Rad®, 2010).

Mosquito Assays

All mosquitoes, *Aedes albopictus,* were reared under standard procedures in the laboratory at 27°C and a relative humidity of 80% with a 16:8 hour light to dark cycles. *A.albopictus* eggs were placed in pans with 450ml of aged tap water and fed daily with a 1 oz. preparation of liver powder. After the initial 48 hours, an additional ounce of liver power was added. From the sixth day onward, from stage 0, pupae were collected with a medicine dropper and placed into individual 12 x 75 mm test tubes with 2 ml of water-food solution (Duhrkopf & Young, 1979). Mosquitoes become adults within two days. Adult mosquitoes were then sexed by physical characteristics. Male *A.albopictus* are usually 20% smaller and

possess bushier antennae than their female counterparts, which make them easily distinguishable. For testing, mosquitoes are transferred into larger containers with a mesh lid to ensure proper airflow. All tests were conducted with 1:1 sex ratios to ensure there is no sex preference for the solutions and/or toxin used. Preliminary tests concerned with attractiveness and sustainability of mosquitoes with a honey, high-fructose corn syrup, and carob solution diet were conducted. Mosquitoes were fed 1ml of solution every 48 hours. The solution was presented in an inverted 1.5 microcentrifuge tube with a cotton plug to absorb the solution, which was placed on the container's lid. Negative controls with no food or water were conducted as well as positive controls with 5% sucrose solution. For toxicity tests, mosquitoes were fed daily with a 100µl solution (50µl of protein extraction and 50µl of the matrix of interest). The food source was placed in a modified PCR tube, which was anchored on the bottom of the mosquito cage to ensure easy access. Individual tests were conducted over a 14-day period with 20 mosquitoes per sample. Mosquitoes used were reared from the same batch of eggs to ensure the adult mosquitoes were exposed to the same conditions and limits possible variables.

CHAPTER THREE

Results

Production of recombinant fusion protein Jun a 3/ACTX

The recombinant fusion gene Jun a 3/ACTX was successfully inserted into the FECT 40 viral vector, derived from Foxtail mosaic virus. The construct was then co-agroinoculated with the silencing suppressor p19 into *N. benthamiana* for a period of seven days in a controlled growth room. On the seventh day, the inoculated leaves displayed little to no necrosis and a slight pigment discoloration that is commonly observed with the process. Extractions were isolated by vacuum infiltration and stored in 1.5ml micro-centrifuge tubes at -20° C. Sodium acetate buffer (10mM, pH 5.2) was used during the extraction and storage. The collected solution displayed a yellowish or gold hue. As a control, a *N. benthamiana* plant was inoculated with GFP and tested under ultraviolet light for the emission of a green fluorescence on the third and seventh day post inoculation. The positive emission of fluorescence was an indication that the procedure, from construction to the inoculation, was successful. Samples were

also collected from healthy, uninoculated, *N. benthamiana* leaves and empty FECT aa vector inoculations as well.

Identification and quantification of Jun a 3/ACTX

In order to verify the production of Jun a 3/ACTX, SDS-PAGE analysis was conducted on the extractions collected. Samples were run on gels produced in house containing 15% resolving gel and 5% stacking gel. An aliquot of 11µl of the extraction in sodium acetate buffer was prepared with 6µl of loading buffer and loaded into wells created by a 15-tooth comb. All analyses conducted were corroborated by the inclusion of a protein marker, which provided standards for protein migration. The preliminary test conducted (Figure 1) tested for the existence of Jun a 3/ACTX and the effects of storage at -20°C. Visualization of the gel accurately reported the expected migration of approximately 30kDa. For this particular analysis, samples from different Jun a 3/ACTX extractions were used and analyzed in parallel to healthy plant extracts and empty vector equivalents. The gels consistently indicate a thick band at 30kDa, which is indicative of the recombinant fusion protein. Also, the consistent visualization of protein is a telling sign to the little effects storage at -20°C has on the protein

extracts. The remaining protein bands are secondary proteins or immune response proteins as a result to the viral infection. The pathogenesis response proteins have been identified in Dr. Kearney's laboratory by using MALDI-TOF analysis. Slightly above the 30kDa Jun a 3/ACTX protein beta-1,3-glucanase is located. Directly below Jun a 3/ACTX, the third visualized band, is chitinase. These immune response proteins are commonly collected during the vacuum extraction process and are of no significance to our current studied.

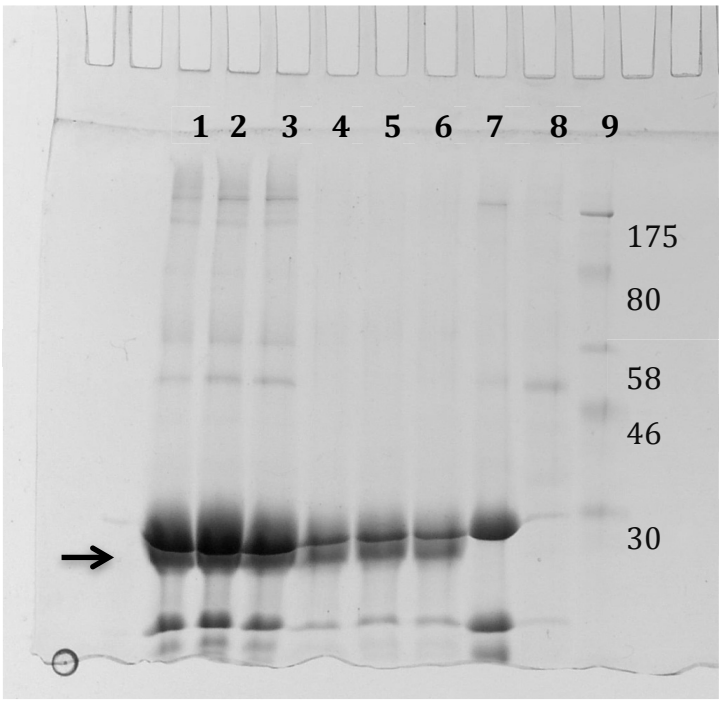

Figure 1. Effects of -20°C Storage on Jun a 3/ACTX stability
Lanes 1-3: Juna3/ACTX month old extracts. Lanes 4-6: Juna3/ACTX week old extracts. Lane 7: FECT aa. Lane 8: Healthy *N. benthamiana.* Lane 9: Protein marker. Arrow indicates expected location of Juna3/ACTX.

Quantification of Jun a 3/ACTX was conducted by SDS-PAGE, in similar manner to Figure 1, but with the inclusion of BSA standards of 3µg, 1µg, and 0.1µg. The gels showed a similar migration pattern to the preliminary tests of -20° C storage. Through the use of the ImageJ® densitometry software, Jun a 3/ACTX concentration was estimated at 1.1µg, with slight variations between samples analyzed (Figure 2). The consistent concentration observed throughout various extractions is a positive indication to the regularity of the co-agroinoculation process and the vacuum infiltration extraction.

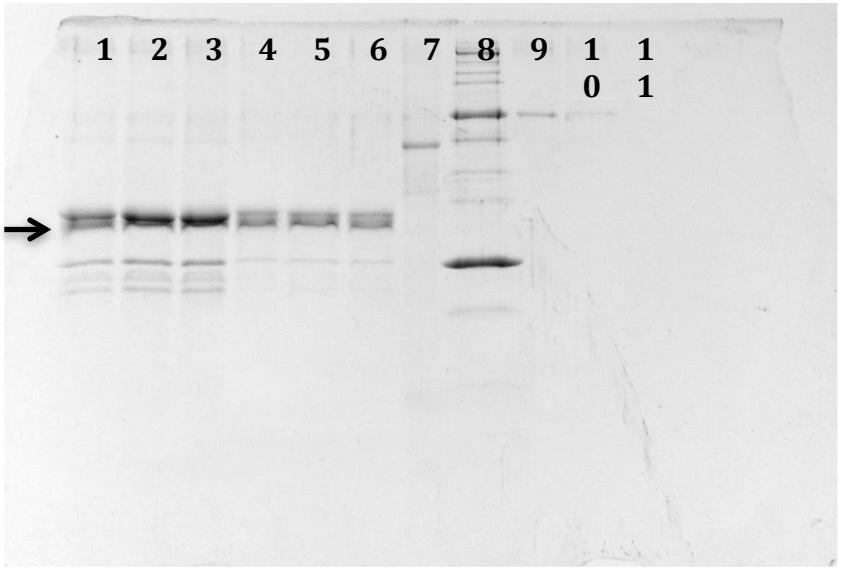

Figure 2. Jun a 3/ACTX quantification with BSA
Lanes 1-6: Replicates of extractions of Juna3/ACTX from *N. benthamiana* plants Lane 7: Healthy *N. benthamiana* protein extraction. Lane 8: Protein marker. Lane 9: BSA 3µg. Lane 10: BSA 1µg. Lane 11: BSA 0.1µg. Juna3/ACTX showed on average a 11x greater density in comparison to BSA at 0.1µg. Therefore, it is estimated that Juna3/ACTX is found in the extracts at a concentration of 1.1µg and consistently running at ~30 kDa as indicated by the arrow. ImageJ software was used.

Jun a 3/ACTX stability in matrices

In order to adequately assess the possibility of Jun a3/ACTX as a mosquitocidal toxin, it was examined for stability in various bait matrices. The protein was suspended in solutions of 50% honey, 10% carob, and 50% high fructose corn syrup. The reasoning behind the dilution of honey and HFCS is due to the difficulty of loading these highly viscous solutions into the wells of the SDS-PAGE gel. Furthermore, diluting the solutions ensured that the protein would be suspended in a homogenous fashion, as it would need to be in a bait formulation. These experiments were conducted at 22° C in order to mimic the ideal conditions of field use. Data was collected from samples from each matrix that were incubated for seven, one, and zero days. The emphasis of the experiment was to shed light into three vital questions of our research: (1) is Jun a 3/ACTX immediately degraded in these matrices (2) if not, what is the relative rate of degradation, and (3) establish a maximum expected expiration time of a Jun a 3/ACTX bait.

Initial SDS-PAGE gels were inconclusive due to smearing Even with dilution in water, the samples revealed smearing on the gels and an inconsistent migration, which was not evident in SDS-PAGE gels with only extracts. The carob powder posed problems remaining in solution and would also clog the

loading wells with particles that severely inhibited the migration of the protein of interest. Also, those carob particles that did migrate through the gel limited the clarity by which the stained proteins could be visualized. Honey and HFCS demonstrated inconsistent protein migration and bands that were not clearly defined. Such a result is believed to be a direct result of the viscosity of the solution, but no additional tests to conclude this were conducted. To correct this problem, a supplementary step of protein precipitation, prior to SDS-PAGE analysis, was added. It was reasoned that the purified protein samples, after their incubation period, would run more consistently in the gel. The precipitation process would exclude the large particles of carob powder and the viscosity of honey and HFCS. The protein precipitation protocol includes the addition of a protein precipitation buffer (20mM Tris-HCl, 100mM NaCl pH 7.5), which was added at one ml per gram of solution. The mixture was then centrifuged at 15,000x g at 4°C for 10 minutes. Afterwards, enough ammonium sulfate was added to saturate the solution at 80% and precipitate proteins out. The precipitated protein was removed from solution by centrifugation at 15,000x g for 10 min. A pellet formed, but sometimes not visible. The resultant pellet was then resuspended in 5-10ml of the protein precipitation buffer. Introducing the protein precipitation step provided clear results, but perhaps does not fully represent the protein content that was in solution.

The data analyzed by SDS-PAGE revealed promising results about protein stability in the matrices of choice. We conclude that 50% honey, 50% HFCS, and

10% carob solution are able to retain Jun a 3/ACTX in solution for a seven day period at least. That being said, the carob solution (Figure 3) presented a smaller amount of protein, which could be attributed to the salting out process since proteins could have become lodged in between carob particles and therefore lost in the purification process. HFCS (Figure 3) and honey (Figure 4) displayed consistent results that indicate little to no protein degradation in solution within the time allotted, because the Jun a 3/ACTX protein band at 30kDa is consistent in location and thickness throughout. Such a result is promising when contemplating future tests regarding mosquitocidal assays, because the retention of the protein in solution could indicate effectiveness in field use for an extensive period of time.

Although optimistic, SDS-PAGE analysis provides evidence only about the amount of protein and the amino acid length of the protein, which are provided by the thickness and migration of the band respectively. It does not provide any information about the functionality of the protein since in order to analyze the proteins by SDS-PAGE they are denatured and coated by sodium dodecyl sulfate. In order to analyze protein function in solution, tests such as colorimetric, fluorescence, and mosquito assays were developed. The positive results of the Jun a 3/ACTX in these matrices requires for further analysis of other proteins such as BSA, alpha casein, trypsin inhibitor, and lysozyme. Analysis of these proteins, which are lab standards, by SDS-PAGE provides a method to gage

if the stability of Jun a 3/ACTX in these matrices is due to properties particular to this cystine-knot protein or a harmless and sympathetic solution.

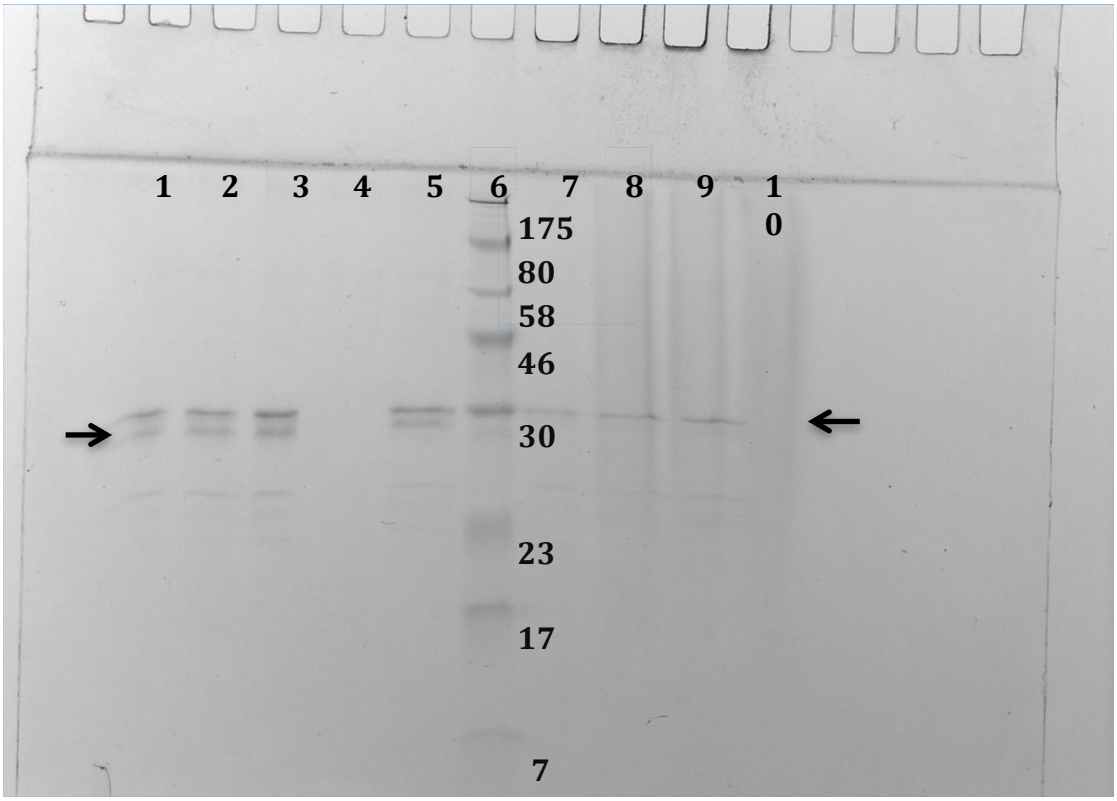

Figure 3. Stability of Jun a 3/ACTX in HFCS & carob solution visualized after protein precipitation

Lane 1-3: Jun a 3/ACTX stored in HFCS at 22°C for 7, 1, and 0 days. Lane 4: HFCS alone. Lane 5: J3 ACTX alone. Lane 6: Protein marker. Lane 7-9: Jun a 3/ACTX stored in carob solution at 22°C for 7, 1, and 0 days. Lane 10: Carob solution alone. Proteins in solution were precipitated out with 20mM Tris-HCl 100mM NaCl buffer and ammonium sulfate (saturating solution to 80%). J3 ACTX found in SDS-PAGE gel at ~30 kDa consistently as indicated by the arrow and been quantified at a concentration of 1.1µg/ml. HFCS is diluted to 50% in samples. Carob solution is diluted to 10% in samples.

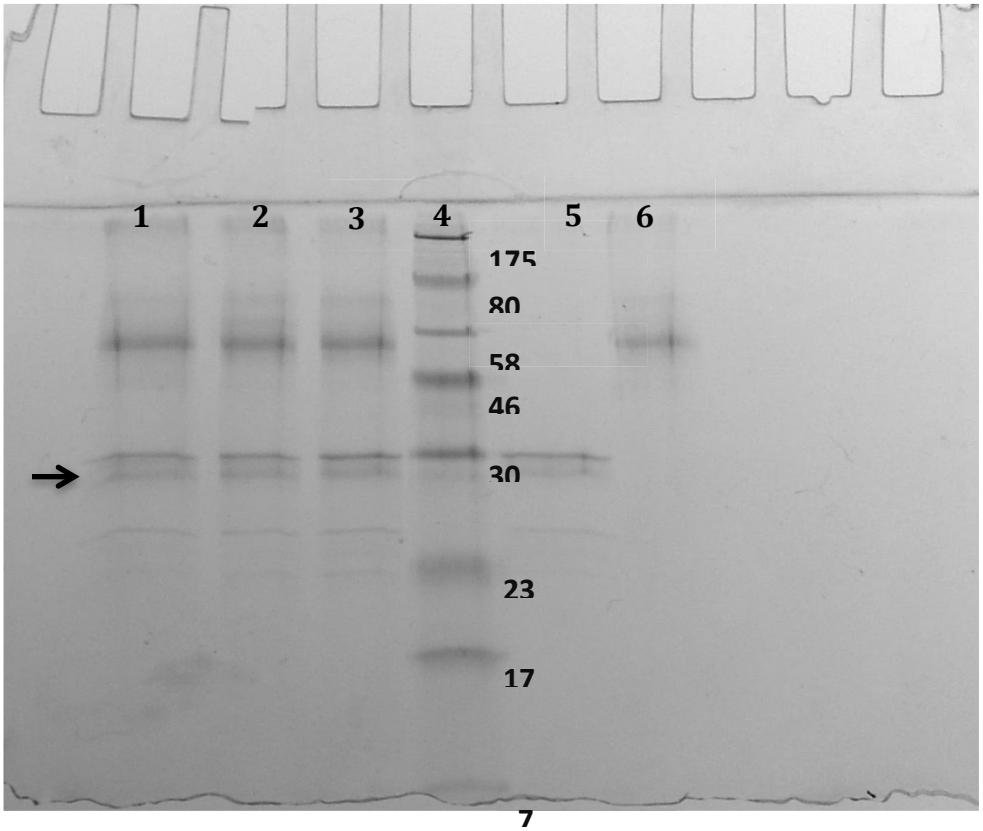

Figure 4. Stability of Jun a 3/ACTX in honey visualized after protein precipitation

Lane 1-3: Jun a 3/ACTX stored in honey at 22°C for 7, 1, and 0 days. Lane 4: Protein marker. Lane 5: Jun a 3/ACTX alone. Lane 6: Honey alone. Proteins in solution were precipitated out with 20mM Tris-HCl 100mM NaCl buffer and ammonium sulfate (saturating solution to 80%). Jun a 3/ACTX found in SDS-PAGE gel at ~30 kDa consistently as indicated by the arrow and been quantified at a concentration of 1.1μg/ml. Honey solution is diluted to 10% in samples.

Protein standards in matrices

SDS-PAGE analysis of BSA, lysozyme, alpha casein, and trypsin inhibitor in the selected matrices were planned to provide context to the results obtained with Jun a 3/ACTX. These ubiquitous proteins were selected for their widespread availability in laboratories and would therefore provide framework for which to assess the results of our novel recombinant fusion protein. Bovine serum albumin has common laboratory uses such as enzyme-linked immunosorbent assays (ELISA), during DNA digestion, and SDS-PAGE quantification as previously noted. It is provided for at little expense and with great purity, therefore providing a single clearly marked band approximately at 70kDa. The tests conducted on BSA were identical replicates of those conducted on Jun a 3/ACTX with the inclusion of the supplemental protein precipitation. In addition to the original 50% honey, 10% carob, and 50% HFCS, a parallel test was conducted in deionized water to provide an insight as to what levels of degradation could be attributed to the natural elements, rather than the matrix (Figure 5). The experiment in deionized water was not treated with the salting out process since the solution did not present any large particles or viscosity. Upon visualizing, the BSA bands consistently ran at 70kDa and did display a gradual loss of protein. The loss of protein is best analyzed by comparing the result on lanes 1 and 3, which indicate a seven-day time difference in exposure. Such a result indicates significant protein

degradation at standard conditions of 22° C and without exposure to honey, carob, or HFCS.

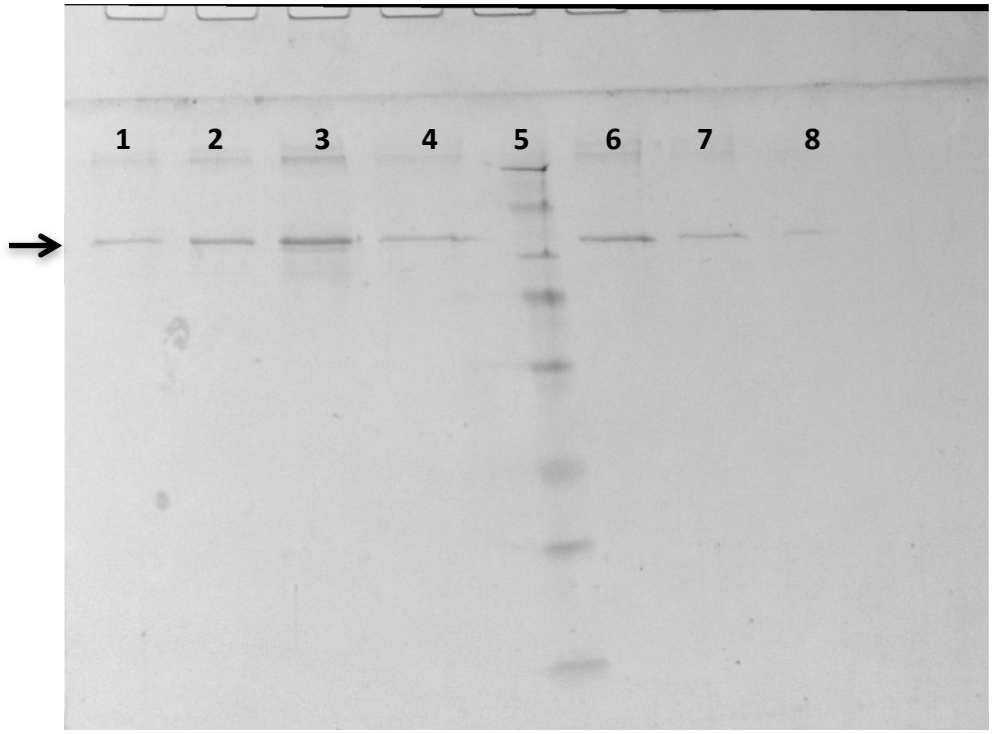

Figure 5. BSA in water test
Lanes 1-3: BSA stored in water at 22°C for 7, 1, and 0 days 1µg. Lane 4: BSA alone 1 ug. Lane 5: Protein marker. Lane 6: BSA 3µg. Lane 7: BSA 1µg. Lane 8: BSA 0.1µg. BSA found in SDS-PAGE gel at ~70 kDa consistently as indicated by the arrow.

Experiments conducted with BSA in honey, HFCS, and carob matrices develop a similar pattern of degradation as deionized water. Honey samples with BSA incubated for seven days exhibited little to no presence of the protein of interest. Moreover, the results collected in honey displayed considerable degradation that surpasses that which was visualized in HFCS and carob. Honey

and HFCS have similar sugar and water content, but differ tremendously otherwise due to the acidity, enzymatic activity, and existence volatile organic compounds in honey. Therefore, the stark difference between the results collected from honey (Figure 6) and HFCS (Figure 7) could be attributed to any of these characteristics. These same particular characteristics were previously noted as possible drawbacks of the use of honey as a matrix candidate. On the other hand, the carob solution exhibited comparable results with BSA and Jun a 3/ACTX by not being able to retain much of the protein by the seventh day.

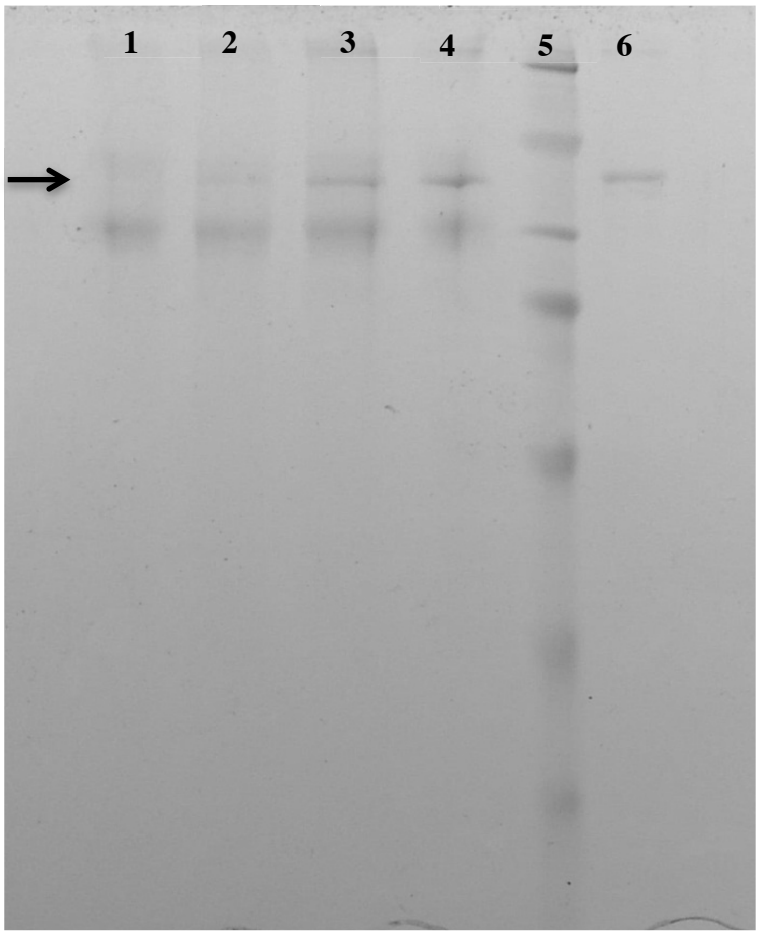

Figure 6. Stability of BSA in honey visualized after protein precipitation
Lane 1: Honey alone. Lanes 2-4: BSA stored in honey at 22°C for 7, 1, and 0 days at a concentration of 1µg. Lane 5: Protein marker. Lane 6: BSA alone at a concentration of 1µg. BSA found in SDS-PAGE gel at ~70 kDa consistently as indicated by the arrow. Honey solution is diluted to 50% in samples

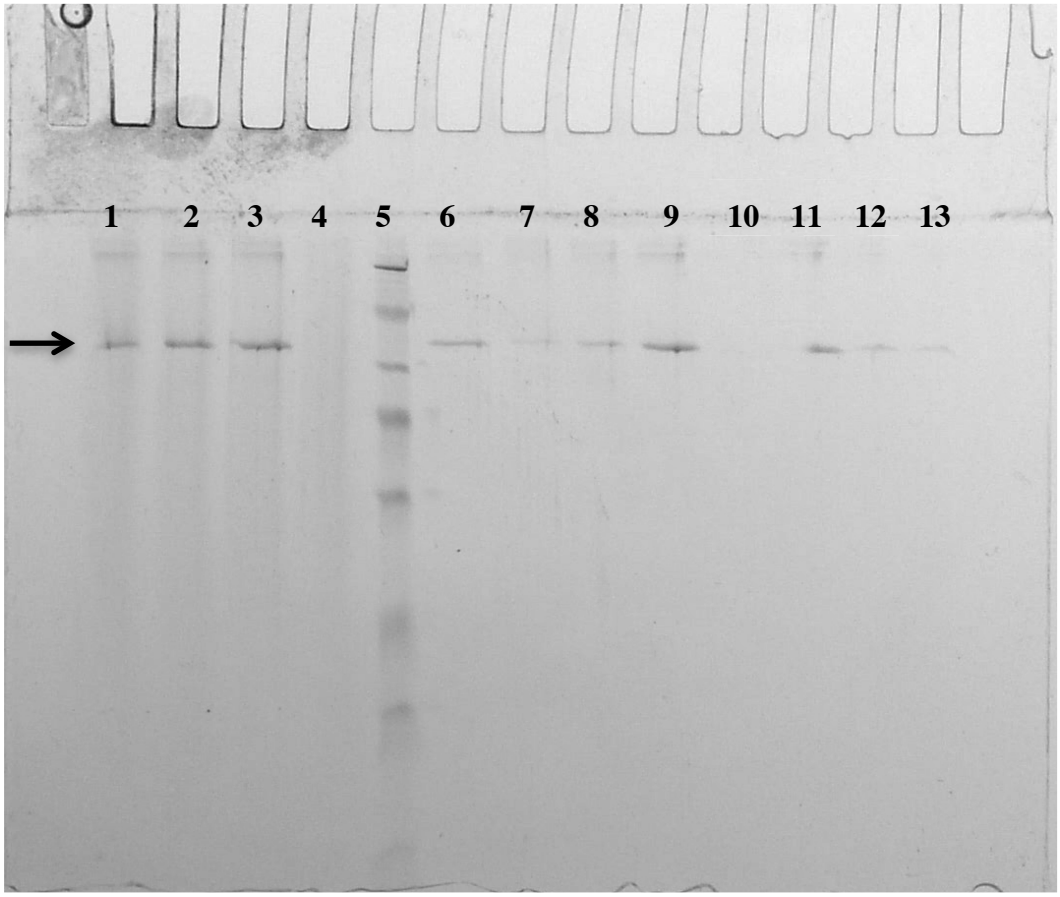

Figure 7. Stability of BSA in carob and HFCS solution visualized after protein precipitation
Lanes 1-3: BSA stored in carob solution at 22°C for 7, 1, and 0 days at a concentration of 1µg. Lane 4: Carob solution alone. Lane 5: Protein marker. Lane 6: BSA alone 1 ug. Lane 7-9: BSA stored in HFCS at 22°C for 7, 1, and 0 days at a concentration of 1µg. Lane 10: HFCS alone. Lane 11: BSA 3µg. Lane 12: BSA 1µg. Lane 13: BSA 0.1µg. Proteins in solution were precipitated out with 20mM Tris-HCl 100mM NaCl buffer and ammonium sulfate (saturating solution to 80%). BSA found in SDS-PAGE gel at ~70 kDa consistently as indicated by the arrow. Carob solution is diluted to 10% in samples. HFCS solution is diluted to 50% in samples

Functional Assays

Functional assays regarding a colorimetric enzyme assay and a fluorescence test are scheduled for the future and are not included in the experimental data due to time restrictions. With regard to the fluorescence test, eGFP with the hydrophobin tag was effectively produced using the identical co-agroinoculation protol employed for the production of Jun a 3/ACTX. The crude extract was purified twice using phase separation, which hinges on the properties of the hydrophobin tag and its ability to solubolize its fusion partner. The result of the purification process was a robust collection of eGFP which far exceeds that of vacuum infiltrtion for Jun a 3/ACTX. As visual confirmation of a successful extraction and purification process, blue light, which emitts light at 400-500nm, was used on the samples. The purified eGFP-HFBI is currently stored at -20° C along with the remaining crude extracts, awaiting analysis.

Mosquito Assays

Mosquito assays were conducted on *Aedes aegypti* and *Aedes albopictus* in order to assess the sustainability of mosquitoes with the matrices as food sources and the mosquitocidal effects of the Jun a 3/ACTX bait. An initial experiment of mosquito sustainability and longevity with carob, honey, and HFCS with respect to the industry standard of 5% sucrose was conducted. The intention of the experiment is to conclude if the mosquito sample groups would actually find the food source attractive and sufficient for survival over a fourteen-day period. Mosquitoes selected were sexed and kept in sample sizes of twenty at equal male to female ratios. *A.aegypti* were used in this particular experiment and were fed every other day in the manner previously explained. The data collected at two weeks (Table 1) displayed an 85% survival rate for mosquitoes exclusively fed by honey. Those fed by carob had a 65% survival rate, while the samples tested with HFCS could not be properly assessed, because for a period of two days the cotton plug did not provide enough access to the solution. All of these results were analyzed in comparison to the 95% survival rate of the *A.aegypti* fed by 5% sucrose. Therefore, honey appears to provide a level of nutrition to mosquitoes comparable to 5% sucrose. The results are in accordance with the work of Foster and Hancock; they indicate a direct correlation between sugar content of food source and sustainability (1994). It is important to note that the

experiment conducted does not account for the olfactory stimulus provided by these solutions, especially from those solutions with well-documented attractiveness in the wild as honey and carob.

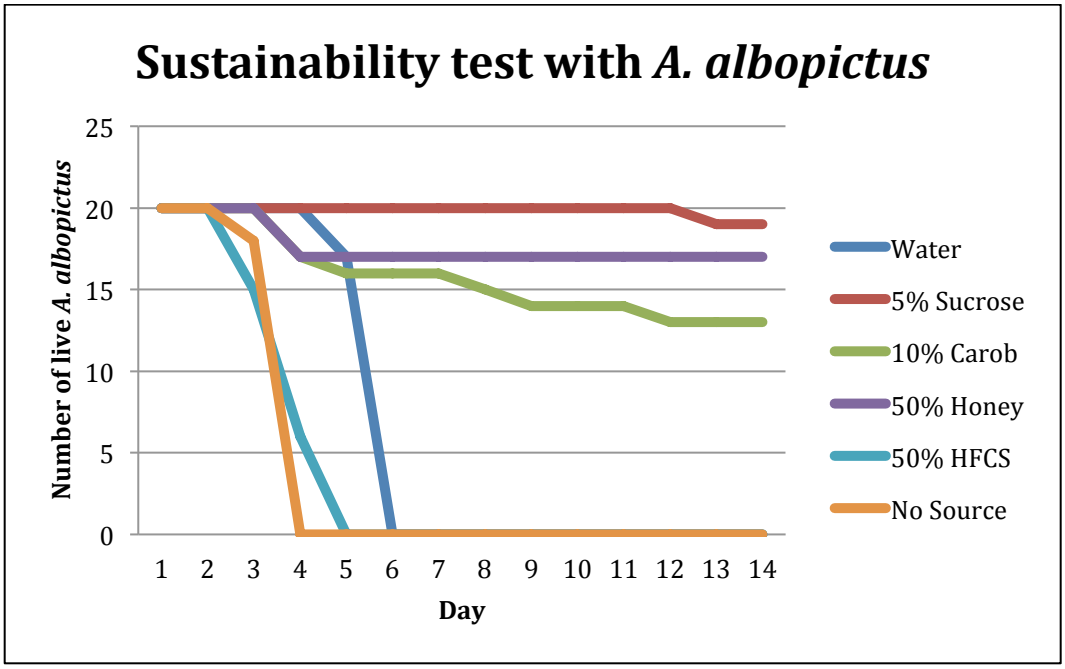

Table 1. Sustainability of *A. aegypti* with matric solutions

The established conclusion that mosquitoes do imbibe and can be sustained by the solutions intended to be presented as bait matrices obligated the examination of the complete mosquitocidal bait. The subsequent test provided a daily dosage of 0.055µg in 50µl sodium acetate buffer, which was then suspended

in an equal amount of matrix solution. All samples were presented in a modified 0.5ml PCR tubes that ensured the mosquitoes had direct access to the food source. Moreover, the samples were washed out daily to reduce the probability of fungal build up and provide a fresh batch of Jun a 3/ACTX. The results of the experiment are demonstrated in the chart below (Table 2).

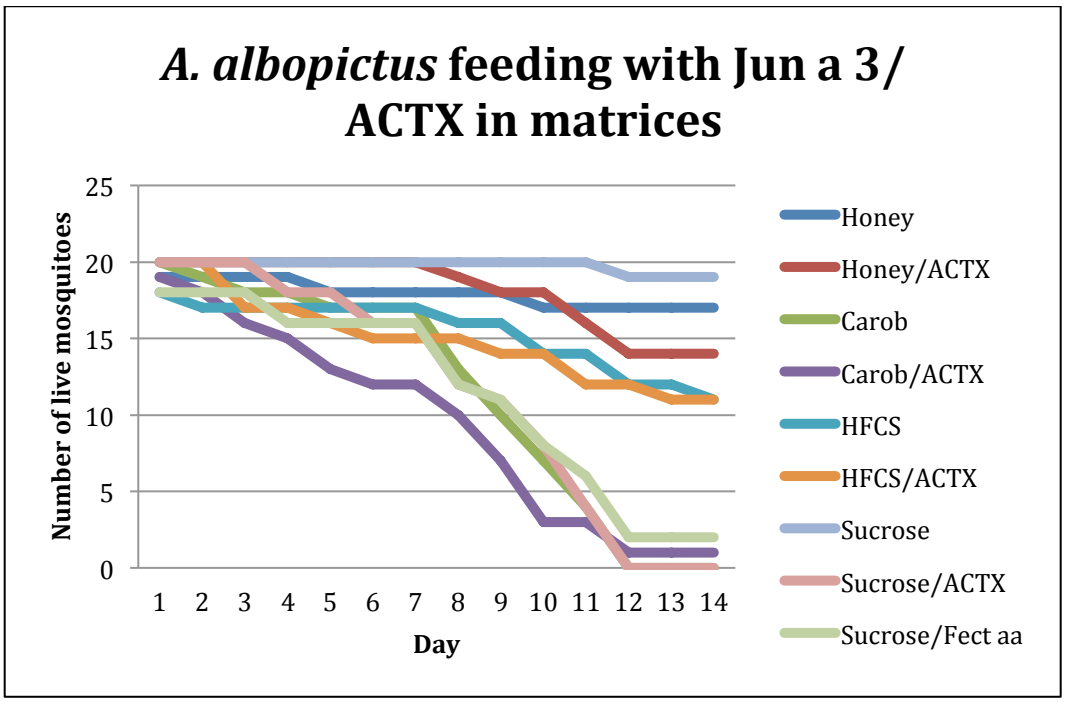

Table 2. *A. albopictus* feeding with Jun a 3/ACTX in matrices

The fourteen-day mosquito assay displayed mixed results and therefore leads to no clear distinction to the toxicity of Jun a 3/ACTX on adult *A.albopictus*. Carob samples, both control and Jun a 3/ACTX, exhibited a high susceptibility to

fungal growth that could account for the high mortality rate of these samples. Furthermore, the carob samples evaporated overnight and would therefore be questionable as a practical option for field use. Another troubling observation was the high mortality of Sucrose/FECT aa samples, because in theory and in other tests in other projects very low mortality (15%) was achieved routinely. HFCS and honey both remained overnight without much evaporation, even with the dilution to 25%. Such a result is in accordance with the expected results and could be vital for use in the field for extended periods of time. Lastly, honey displayed the least amount of fungal growth in its samples. Even though in theory no fungal or bacterial growth should occur, honey displayed great levels of sustainability, which is exemplified by the 85% survival rate of the control sample groups. On the other hand, honey/ACTX had a negligible kill rate in comparison to its control groups. The lack of kill could be attributed to the immediate denaturation of the cystine-knot fusion protein, an inability to retain protein dissolved and suspended in solution, or the toxin concentration was below the toxicity threshold level.

In conclusion, the data collected from the various mosquito assays conducted leave little room for definite conclusions to be drawn about the viability of a mosquitocidal bait based on Jun a 3/ACTX in these selected matrices. Before any definite results can be extrapolated, the concern over fungal growth in the samples needs to be corrected. That being said, the various experiements conducted do grant some hope for possible proof of concept, especially with the positive results honey displayed by retaining the protein of

interest in solution and sustaining a mosquito sample group for over fourteen days. Hopefully, with the completion of the functional assays and modified mosquito assays a definite conclusion to the inquiry can be established.

CHAPTER FOUR

Discussion

The experiments conducted in this study were intended to establish a proof of concept that Jun a 3/ACTX in an attractive bait matrix, could effectively kill mosquitoes. Our hypothesis hinged upon the already recognized properties of cystine-knot atracotoxins and the well-documented attractive properties of solutions such as honey, HFCS, and carob solutions. It seemed reasonable that the pseudo-knot developed by the disulfide bridges in ACTX would prove to be durable, allowing the protein to retain shape, and therefore function in adverse environments (Tedford, et. al, 2004). Therefore, we hypothesized that ACTX would be a toxin that could be suspended in any mosquito attractant matrix and still provide an effective kill rate for a commercially viable length of time, such as a week. . Additionally, we hypothesized that the attractive properties of honey, provided by the volatile organic compounds and high sugar content, would provide sufficient attraction to mosquitoes to be efficacious in field use in competition with various food sources (Foster & Hancock, 1994). Honey, with an approximate pH of 4 and the presence of enzymes in solution, should reduce or preclude the microbial degradation of protein toxin (Evans & Flavin, 2008). Moreover, the low water content of both honey and HFCS would also be expected to limit microbial growth. Carob solution was chosen as an alternate due to its

previously demonstrated effectiveness in attracting mosquitoes to boric acid baits (Muller, et. al, 2011).

Data collected from SDS-PAGE gels supported the hypothesis that a recombinant version of ACTX could retain its structure in a bait matrix. There was special concern for the use of honey, because it was unknown whether or not the proteolytic enzymes found honey would cleave our protein of interest. The results concluded that honey retained the single 30kDa band of Jun a 3/ACTX at a better rate than HFCS and the carob solution.

To corroborate with these results, other proteins were tested under the same circumstances. BSA displayed a very quick degradation at room temperature when suspended in deionized water. All matrices displayed similar results as no solution retained a significant amount of BSA protein for the seven-day period. Additionally, some preliminary tests, not presented in this study, were conducted with the use of protein markers as the proteins of interest. Since protein markers possess various proteins from different sources, of different sizes, and with clearly defined migration patterns it was thought they could be beneficial as a qualifying study. The results exhibited a stark difference between the samples. Honey was able to retain most protein marker proteins with little degradation between the seven, one, and zero day samples. On the contrary, samples suspended in HFCS and carob solutions barely retained any of the proteins present.

Although we expand our SDS-PAGE analysis of the degradation in bait matrices to a wider range of proteins, adding lysozyme, alpha casein, and trypsin inhibitor, our current preliminary data allows us to draw certain conclusions. The inability to retain BSA and some of the proteins present in the protein marker supports our hypothesis that the cystine knot protein is sturdier in long term liquid storage than other proteins. Another conclusion we can start to draw from the initial results is the capacity honey has to retain compatible proteins in solution, which exceeds the ability of HFCS and the carob solution. It is well established that honey contains its own native proteins, but the ability to retain foreign proteins is relatively unknown from the literature. As noted before, SDS-PAGE analysis only provides evidence to protein degradation and cleavage, not denaturation. The importance of the functional tests is to address the concern over protein denaturation or improper folding when exposed to these matrices of choice. Retaining the amino acid sequence without any cleavage is a necessity, but in order for Jun a 3/ACTX to function as a calcium ion blocker it need to retain its proper folding as well.

In order to select the proper matrix for field use, the ability to retain a protein in solution is vital, but the solution must also be attractive to mosquitoes and be a viable food source. Our long-term goal is to develop a safe mosquitocidal bait that can be used in close proximity to household. Regardless if the bait is employed in an urban or rural setting it will be presented with the hindrance of competing with other food sources, such as flower nectars and

decomposing fruits. Therefore, any potential matrix should be able to attract and sustain mosquitoes by imbibition. The sustainability tests conducted on *A.aegypti* were intended to provide an insight into each solution's potential as an adequate food source. The data collected from the sustainability mosquito assays on *A.aegypti* and the control groups from mosquitocidal assays on *A.albopictus* indicate positive outcomes for the use of honey. In comparison to the lab standard of 5% sucrose, the most effective matrix to sustain a mosquito sample population of twenty over the fourteen-day test period was honey. Honey at 50% dilution registered an 85% survival rate, which is comparable to the 95% survival rate of mosquitoes fed with 5% sucrose. The 10% carob and 50% HFCS solutions did not support mosquitoes as well, but still provided positive results of 65% and 55% survival rates respectively. Two-week sustainability, most effectively exhibited by honey, supports the possibility of field use.

There were other positive characteristics noted with the bait matrices tested in this study. Sustainability of a mosquito population is an essential component of a successful food source, but attractiveness is also fundamental. A common method to analyze attractiveness is by observing the probing tendencies of mosquitoes when exposed to a food source. All three matrices elicited probing within minutes of feeding (data not shown). Another positive finding provided by the mosquito assays was the low rate of evaporation of each matrix. Lower rates of evaporation would translate to greater exposure time for the toxin to have an effect on the mosquito population. The need to continually replenish the

mosquitocidal bait would reduce its commercial potential, since a high maintenance bait system would not be desirable by the user. The low content of water in honey is well documented in the literature and is estimated at 20% (Kahraman, et. al, 2010). HFCS displays a comparable water content to honey. The sucrose and carob solution have a water content of 95% and 90% respectively and evaporated quickly, in contrast to the honey and HFCS baits. The samples developed for the mosquitocidal assay of *A.albopictus* were not contained as sealed elements and were vulnerable to evaporation. During this experiment, a total solution of 100µl, comprised of equal parts Jun a 3/ACTX extraction and matrix, was provided daily. During the fourteen-day experiment, only the samples provided with honey and HFCS (each at 25% matrix final concentration) were still present the following day in amounts that were still accessible for the mosquitoes. On the other hand, 5% sucrose retained less than half of its original 100µl solution overnight and 5% carob appeared to evaporate completely, leaving only clumps of carob powder behind. Solutions high in sugar would resist rapid evaporation and also display a higher level of attraction for mosquitoes (Manda, et. al, 2007). A possible modification for future experiments is the inclusion of carob powder into the honey and HFCS solutions. The combination could be effective, because the high viscosity of honey and HFCS would prevent the carob particles from aggregating on the bottom. Also, carob power could provide an additional level of mosquito attraction to the bait matrix, which has already been evidenced in our lab and by Günter Müller's group (2011).

The mosquito mortality assays conducted on *A.albopictus* provided inconclusive results due to fungal and microbial manifestations in the Jun a 3/ACTX and matrix bait. The dosed bait was replaced daily and the vessel was flushed prior to being replenished. It was presumed that such measures would prove to be sufficient in preventing fungal formation on the samples. Fungal growth in the samples displayed a direct correlation with mosquito mortality and greatest fungal growth was seen in 10% carob. Sucrose and HFCS demonstrated slight fungal growth on the borders of the vessel. These results indicated that the plant extract contained contaminants. These contaminants can be removed by further purification of the crude plant extract. The adverse results did evidence a positive note: honey samples did not have fungal colonies present. The effective antimicrobial properties of honey were still effective at a fourth of its original concentration. Sample groups fed with Jun a 3/ACTX and honey provided the highest survival rates of any sample, which could be attributed to the lack of fungal growth. In order for carob and HFCS solutions to present the same antimicrobial properties of honey an antimicrobial agent would have to be added. For future research, the use of sodium benzoate at very low concentrations could possibly prevent the formation of fungal colonies and theoretically extend the time the mosquitocidal bait would be effective.

In conclusion, the data collected does not definitively prove our hypothesis that a target-specific mosquitocidal bait could be developed. However, several components contributing towards this goal are now in place. The

immediate need to produce a sturdy and easily manufactured mosquitocidal toxin as been met. SDS-PAGE gels provide evidence to the robust characteristics of Jun a 3/ACTX in solution, which exceed any other protein tested as of yet in its stability. By employing the FECT/40 viral vector system, we have been able to streamline the toxin production process. In the near future, purified plant extracts will be used to avoid fungal contamination. Because our current concern hinges strictly on toxicity, future Jun a 3/ACTX and matrix baits should display significantly higher toxin concentrations. It would be ideal to conduct these mosquito assays with a gradient of Jun a 3/ACTX concentrations in order to arrive at the proper toxicity threshold.

Analysis of the selected matrices has provided significant support, which favors the use of honey for any potential field use. High acidity and low water content are vital characteristics of honey in order to prevent the manifestation of fungal colonies or microbial growth. Moreover, the low water content reduces the amount of solution lost to evaporation, which also contributes to an extended potential use. Honey also possesses unique volatile organic compounds mosquitoes find appealing. The level of attraction afforded by honey to mosquitoes was substantiated by the immediate probing patterns observed. Additionally, honey is ubiquitously found and could potentially be used without any need for prior knowledge on mosquito feeding patterns in a particular region. In order to complete the circle, we still need to prove honey can retain Jun a 3/ACTX in solution without denaturation. To arrive at this conclusion, the

functional assays with eGFP and horseradish peroxidase will be performed, along with the mosquitocidal assays. If successful at developing a target specific toxic bait with Jun a 3/ACTX in honey, we would be able to scale up production and hypothetically provide an alternative for controlling mosquito populations without environmental or human health costs.

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