ABSTRACT

The Presentation of Mosquitocidal Protein Toxin for Delivery in Yeast-Based Stations

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Throughout history, humans have been plagued by vector-borne diseases such as dengue fever, malaria, and yellow fever. These particular diseases are spread by mosquitoes, and they cause severe losses to human growth and development, to productivity, and to society as a whole in endemic regions. In this thesis, the potential for yeast-based bait stations as a novel method for controlling these mosquito populations is explored. In order to do so, the lethality of a few toxins on mosquito populations, the efficacy of different bait station designs, and the relevance of carbohydrate media in the process were tested. Overall, it was concluded that yeast-based stations for the control of mosquitoes have potential as a low-cost option for vector control. However, further study with toxin cocktails specific for mosquitoes and potential expression in plant nectar or other natural mosquito food sources would also be important avenues to pursue in the fight against these diseases.

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THE DEVELOPMENT OF MOSQUITOCIDAL PROTEIN TOXIN FOR DELIVERY IN YEAST-BASED STATIONS

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CHAPTER ONE

Introduction

For much of history, and even in the present day, vector-borne diseases have placed a large economic burden on populations. Such a burden has served to hinder development through economic losses associated with illnesses such as lost productivity and lost wages (Shepard et al. 2013), and it has also served to disrupt human capital investitures by causing missed days of school, causing poor growth and development in children, and causing major disruptions to the provision of primary care (Gubler 2012). Shepard et al. estimate that in South East Asia alone, lost productivity accounts for approximately 52% of the economic burden of disease (2013). The World Health Organization estimates that, of the global disease burden, 17% is caused by infectious vector-borne diseases (2014). It also estimates, in 2012, that malaria caused well over 600,000 deaths (2014). Additionally, there are concerns about a reemergence of dengue infections which have appeared to increase over the last fifty years at least thirty fold (2014). In all, estimates place the current burden of dengue at around 230 million persons (Gubler 2012). West Nile virus, although primarily asymptomatic, presents with a 10% case fatality rate and often requires extensive recovery for those who present with symptoms, often with residual long-term mental defects (Barrett 2014). Albeit less systematically devastating as malaria or dengue, West Nile Virus accounted for an estimated 1,549 human deaths between 1999 and 2012 (Roehrig, 2013). However, some researchers have predicted an annual economic burden of $631 million as a result of public health efforts relating to West Nile Virus (Pimentel et al., 2005). Thus, vector-

borne diseases, particularly those borne by mosquitoes, have far-reaching effects in both the developed and developing regions of the world. It also appears that mosquitoes and other vectors are moving beyond their traditional geographic boundaries, bringing devastating effects to previously non-endemic areas (Petersen and Roehrig, 2001). Efforts to contain and control these vectors are a vital step in the interests of global health. It is also useful to consider controls against other insects that do not carry diseases. For example, pests destroy approximately 20-30% of crops throughout the world on an annual basis (Oerke et al., 1994).

One way to go about controlling mosquitoes may be through the use of plants. In recent years, many genetically-modified plants have been used to control pests for food crops. Along these lines, science has allowed the production of genetically-modified corn and cotton crops using toxins of *Bacillus thuringiensis* as a way to lower pesticide use and carbon dioxide emissions (Brooks et Barfoot, 2005). It also allows fuller yields with fewer potential health risks from pesticides and other chemicals (Toenniessen et al., 2003). The benefit of these proteins is that the various bacterial strains allow specificity to be obtained for certain pests, which can be used to target harmful pests while allowing little to no harm to beneficial insect or vertebrate species (Gatehouse 2008). Its use has increased over the last twenty or so years in order to overcome the increasing resistance among certain organisms such as mosquitoes and other pests to certain chemical insecticides (Federici et al. 2003). These *B. thuringiensis*-based toxins consist of mixtures of Cry and Cyt toxins that act in a variety of ways. For example, it has been shown that they bind to certain domains of epithelial receptor proteins inside of insect digestive systems (Bravo et al., 2007). This in turn leads to aggregation of proteins in

such a way as to disrupt the membrane, creating a channel that allows free passage of intracellular and extracellular material between the cells and the extracellular environment (2007). As a result of the subsequent cellular lysis, the insect ultimately dies from interior deterioration of cellular integrity and bacterial overgrowth (Gatehouse 2008). Some studies have also shown that these toxins may act similarly to a detergent because of their high affinity for fatty acids present in phospholipid bilayer cell membranes (Federici et al. 2003). Such interactions contribute to the dissolution of the cellular membrane, and like above, cellular and organism death. As a result of its specificity (Gatehouse 2008) and limited resistance in laboratory studies, this has been an extensively researched avenue of pest and vector control (Federici 2003).

Though *Bacillus thuringiensis israelensis* is used only against larvae in the field, Klowden and Bulla demonstrated mortality in adult mosquitoes (1984). Interestingly, they showed that mortality was increased when the toxins were delivered in sucrose rather than in saline solutions despite carbohydrate sequestration in the mosquito's diverticulum (Klowden and Bulla, 1984). For this reason, sucrose solutions were used in this thesis experiment in order to deliver the toxins to mosquitoes under controlled conditions. Klowden and Bulla also showed that *B. thuringiensis israelensis* toxins tend to have more lethal effects on older mosquitoes, such as 21-day old females, which are the vectors of parasites (1984). One of the end goals of work in the laboratory from which this thesis stems would be to develop bait stations or genetically-modified plants that present toxins to adult mosquitoes. This is a reason why the lethal bioassays were run on adult mosquitoes, since these will be the main targets of the lab's final products.

Besides *B. thuringiensis* toxins, there are several other toxins that may be used against mosquitoes and other vectors. One such toxin, lycotoxin-1 which is found in *Lycosa carolinensis* (wolf spiders), has been successfully expressed in *Saccharomyces cerevisiae* (Hughes et al. 2008). It acts by puncturing the cell membrane (Yan et Adams, 1998). This highlights the potential for various spider toxins, which are often mixtures of various protein toxins, as a new avenue of toxins that can be used in order to overcome resistance of certain vectors to more established insecticides and to establish specificity for vector targets, particularly the nervous system (Nicholson et Graudins, 2002). This is quite important, considering that there have been calls for stricter controls over insecticides in the interests of environmental safety (Tedford et al. 2004b). Often, they act by disrupting calcium channels which ultimately leads to paralysis and death (Tedford et al., 2004a; Wang et al., 2001). Since they act as neurotoxins, they also disrupt acetylcholinesterase, gamma-amino-butyric acid-gated chloride channels, and voltage-gated sodium channels (Brogdon and McAllister, 1998). Although Hughes et al. (2008) frame their study in reference to crop pests, their study on the use of mutated spider toxin against caterpillars provides useful information that may be carried over to the fight against vectors of disease. Lycotoxin is toxic to fall armyworms only by injection, since it is injected by the spider. In order to make it toxic by ingestion, Hughes et al. modified lycotoxin-1 sequence with an enterokinase K site upstream of its open reading frame (2008) so that the insect gut enzymes will release the toxic form. They also used a construct with a small ubiquitin-like modifier vector in order to allow for proper folding of the protein (Malakhov et al., 2004; Butt et al., 2005). In the next step, they modified

sequences of the toxin to make it lethal in bioassays with armyworm larvae upon cleavage of the enterokinase K site by trypsin in the digestive tract (Hughes et al., 2008).

Such findings are important because they implicate the possibility of using *S. cerevisiae* as a high-yield producer of orally lethal bioinsecticides. Additionally, these toxins may be purified and used for other vectors such as mosquitoes. Purified proteins may be used in sucrose-based bait stations, or it is even possible that the yeast itself may be used within certain traps. In doing so, it can be reasonably expected that carbon dioxide produced by the yeast may serve to attract mosquitoes. In fact, another study found that a combination of myristic acid, lactic acid, and carbon dioxide served to attract mosquitoes quite readily (Mathew et al. 2013). They also show that 1-octene-3-ol may be used as an attracting compound for mosquitoes, particularly *Aedes aegypti* (2013). Including cocktails like this in addition to toxin-yielding genetically-modified yeasts in a trap may serve as a possible way to promote vector control in certain areas.

Further research on spider venoms has produced exciting results towards specificity and environmentally friendly toxins. New targets and specific cocktail sequences can help overcome resistance (Sparks et al., 2001). Since arachnid venoms typically contain various compositions of inorganic salts, organic molecules, and polypeptides (Escoubas et al., 2000; Rash and Hodgson, 2002), there is a great deal of variation and specificity possible (Tedford et al. 2004b). Although the actual number of polypeptides in certain spider venoms may be much higher, it can be conservatively said that most contain at least fifty different peptides (2004b). Tedford et al. completed a brief estimate to show just how staggering the possibility of different spider venom peptides truly is. In their analysis, they estimate conservatively that there is at least over

1.5 million possible polypeptides in the so-called spider venom database in the world (2004b). This is based on the fact that there are at least 38,000 known species (Platnick, 1997) and at least as many unknown species (Coddington and Levi, 1991) while assuming approximately 20 unique peptides per spider (Tedford et al., 2004b). Since many of these peptides can be reasonably hypothesized to have insecticidal properties and most spider toxins are harmLess to humans and other large mammals, researchers should be able to find toxins that are lethal only to specific insects such as mosquitoes (2004b). Of particular interest to this thesis are the toxins of the Australian funnel-web spiders. These spiders have two genera classifications, that of *Atrax* and that of *Hadronyche* (Gray, 1988).

From a scientific standpoint, these spiders are ideal for spider venom toxin studies because they are easily maintained long-term in captivity, and they also provide researchers ease in venom extraction (Tedford, et al. 2004b). They belong to the order Mygalomorphae, or early spiders, and they can live upwards of 20 years in captivity (King et al., 2002). The spiders drip venom from their fangs when they feel threatened, and the venom is quite easily collected thereof without potentially damaging electrical stimulation that can introduce other molecules into the venom (Escoubas et al., 2000)). The term atracotoxin, abbreviated ACTX, describes the polypeptides isolated from this venom (Tedford et al., 2004b). Upon further study of these various atracotoxins, researchers have been able to delineate various categories of peptides based on lethality towards certain groups of insects (2004). For example, ω-atracotoxins are efficient calcium channel blockers that can kill specific insects between ten and forty-eight hours (Fletcher et al., 1997; Atkinson et al., Wang et al., 1999). The actual time varies with the

insect, whether coleopteran, orthopteran, lepidopteran, or dipteran. Another form of atracotoxin, the Janus-faced atracotoxin, is an efficient potassium channel blocker. Both forms of atracotoxins lack any toxic effects when injected into mice, exhibiting the atracotoxin's specificity for invertebrate gated channels rather than those of vertebrates (Wang et al., 2000). The mice are important because large peptides can cross the underdeveloped blood-brain barrier, so they permit one to study the effect on the nervous system (Clot-Faybesse et al., 2000). These properties of atracotoxins are useful because they show limited effects on non-target species which may include humans. However, they may only be able to be used within baculoviruses, which are viruses that produce toxins upon infection of a host since oral consumption of the proteins by the insects would potentially lead to degradation and inactivation of the toxins (2004). The baculoviruses could be used to target certain insects since they cannot infect other hosts such as mammals (Kost and Condreay, 2002). Thus, exposure to livestock, pets, and other domesticated animals will not be an issue, even if exposed. If there are eventually widespread baculoviruses in the environment, these characteristics are particularly valuable considering children and infants may at some point be exposed to them. Also, even if widely spread, the effects would be limited to the desired vector or pest insect (Gröner, 1986). For all of these reasons, ACTX is used as a toxin of interest in the toxin bioassays of this thesis experiment.

However, another study has highlighted a potential bridge between the insect's digestive tract and the hemolymph. Such a pathway could allow one to use transgenic plants to produce proteins in nectar or use transgenic yeasts to produce toxins for use in traps for oral consumption. This bridge, *Galanthus nivalis* agglutinin or GNA, passes

into the systemic hemolymph after a binding mechanism whereby it binds with the epithelium lining the insect's digestive tract (Fitches et al. 2002). Thus, one could use GNA fused with another toxin, such as an atracotoxin or other channel disrupting toxin to bypass oral degradation. Fitches et al. were able to show that GNA is able to move fused proteins across the endothelial barrier into the systemic hemolymph (2002). They ran the test on *Lacanobia oleracea* larvae, or tomato moth larvae (2002). Although not tested in this study, using a GNA-based fusion protein with a spider venom toxin may allow increased oral toxicity, which is vital for transgenic nectar presentation or even bait-stations that present toxins in sucrose solution or transgenic yeast.

A fairly recent and novel vector system for protein production, which is used later in this thesis's actual experiment (modified from Lindbo 2007), is called the Foxtail Mosaic Virus system. The authors, Liu and Kearney, have described a system whereby they have promoted high levels of protein expression in studies involving agroinoculation as the main method of protein production (2010). In order to do so, they first used the foxtail mosaic virus as the foundation for the novel vector system (2010). The virus itself is from the genus *Potexvirus*, which includes numerous filamentous, single-stranded RNA type viruses that target plants (2010). Foundationally, the sequence of the virus includes five open reading frames and two promoters (2010). From the original sequence, the authors added a 35S promoter while removing the coat protein and the triple gene block sequences in order to inhibit the ability of the virus to infect plants beyond the originally inoculated plant (2010). Additionally, the system expresses poorly in the absence of a silencing suppressor such as p19 because of its weakened stability, further preventing the spread beyond the original plant (2010). This can be quite

beneficial as genetically-modified viruses and plant species are an ecological concern for many citizens of the developed world (Gatehouse 2008). Also, the FECT vector system has been shown to produce quite a high level of protein compared to other plant-based systems as long as the silencing suppressor is present (Liu and Kearney 2010).

This system is used in conjunction with *Nicotiana bethamiana*. *Nicotiana* is a genus including seventy-six species of plants of varying localities and varying genome sizes. The main plant used in this thesis experiment is *N. benthamiana*, which is actually an Australian native (Goodin et al. 2008). In fact, it was initially discovered by Benjamin Bynoe on a journey to Australia aboard the HMS Beagle, better known as a carrier of Charles Darwin to the Galapagos (2008). In the following decades, *N. benthamiana* has made its name as a model organism, particularly in pathogenic studies, because of its ease of infection with pathogens (2008). Scientists suspect this ease of infection from viruses stems from a mutation in a gene coding for RNA-dependent RNA polymerase which would otherwise offer resistance to RNA viruses (2008). Together, with advances in foreign gene expression, in silencing suppressors, and in agroinoculation, these characteristics have made *N. benthamiana* the most commonly used plant species for protein expression in industry and research. Researchers who use the plant are easily able to produce high yields of proteins of interest using a simple inoculation with *Agrobacterium tumefaciens* (2008). *Agrobacterium tumefaciens* has been used in plant bioengineering as a method of horizontal gene transfer (Păcurar et al., 2011). Upon infecting plants, these bacteria have a T-DNA segment in the tumor-inducing plasmid that is ultimately integrated into the host genome, which can be modified to integrate other genes of interest (Gelvin, 2003).

In this thesis project, FECT-EFGP is ultimately used as a control to ensure that proper agroinoculation has occurred. When *N. benthamiana* was inoculated in order to produce protein for the mosquito bioassays, one plant was always inoculated with FECT-EGFP as a positive control for plant expression. Of course, all plants were also co-inoculated with 35S-p19 silencing suppressor in order to promote high levels of protein expression within the desired plants as done by Liu and Kearney (2010). Highly used throughout bioengineering in plants, the 35S promoter is derived from the cauliflower mosaic virus or CaMV and permits high levels of protein expression in plants (Benfey et Chua, 1990). The silencing suppressor is necessary in the presence of RNA constructs because the injection of RNA constructs will cause RNA silencing in plants regardless of whether or not they are transgenic (Johansen, et al. 2001). The silencing suppressor is essential to reduce the plant's immune response which can easily disrupt the FECT construct within the leaf after inoculation (Liu et Kearney, 2010). In fact, Lindbo suggests that including a p19 construct increased protein expression approximately 100 times (2007). By examining the fluorescence of *N. benthamiana* leaves under UV light seven days after inoculation, one can verify that the p19 construct is sound if he can observe a decent level of fluorescence. After all, the same p19 system is used for all other protein toxins produced. After extraction, further qualification by SDS-PAGE is necessary to certify that the proteins in all other trials were correctly produced by the plants by examining them to see if they produce bands at the expected locations.

Once the proteins are stably extracted from the plants using a vacuum and sodium acetate buffer system, and one verifies that the proteins were of the correct size by SDS-PAGE, one could initiate mosquito bioassays to test lethality of each individual toxin. By

testing these toxins, one can see which toxins are most effective on vector-borne disease carrying-mosquitoes such as those of genus *Anopheles* and *Culex*. In turn, these can be added to the potential pool of toxins that can be further developed for novel methods of toxin delivery such as bait stations or genetically-modified plants expressing protein cocktails in nectar that specifically target these mosquitoes. In the long-run, bait stations might be developed to aid in global health efforts to eradicate the burden of such diseases as malaria, dengue fever, West Nile Virus, and yellow fever.

However, there is the matter of how to present these toxins to vectors. In particular, for this thesis, the focus was on the potential of attractive toxic sugar bait stations (Müller et al., 2010a). In 2006, Müller et al. published work on using flowering trees in otherwise dry, desert terrains to attract mosquitoes since they are ready sugar sources (2006). They found that perennials are more attractive when used in baits than annuals, and they also showed that using natural floral attractants when paired with oral toxins such as Spinosad® could be a cost-effective and successful method of vector control (2006). Spinosad® is environmentally friendly in the sense that it has limited toxicity against birds, mammals, and many non-mosquito insects (Williams et al., 2003). Various studies have also shown that nectar sources have varying levels of attractiveness depending on the environment. For example, in a Wisconsin study, researchers cataloged a relatively large selection of 77 flowering plants in the field (Grimstad and DeFoliart, 1974). They showed that, of these species, the mosquitoes preferred just four plants as nectar sources (1974). In the Israeli desert, *Acacia raddiana* trees served as a major attractant (Müller and Schlein, 2006). Schlein and Müller also tested several other species in conjunction with traps at a later time (2008). Here, they found that *Tamarix*

jordanis also served as a highly effective attractant (2008). Expanding upon the idea that flowers serve as a major food source and attractant (Mauer and Rowley, 1999), Müller et al. also tested the attraction of mosquitoes to fermented fruit juices such as wine and overripe citrus fruits combined with oral toxins (2008). This is because mosquitoes appear to feed from ripe fruit if it is possible to do so (Joseph, 1970). These tests showed that this form of trap was most effective in enclosed areas such as cisterns and drainage systems where mosquitoes may readily breed (2008). Additionally, Müller et al. also studied the effects of simply spraying toxin-laced fermented bait solution on plants that lack flowers (2010b). They found it to be effective, and it helps to highlight that the manipulation of sugar-feeding habits is important to future control efforts (2010b). They also ran a similar test in Mali, to show that the flowers and plant oils, although different than in Israel, can still be effective in non-arid climates (2010a).

Previous studies have also shown that combinations of carbon dioxide and lactic acid can have quite an attractive effect on mosquitoes, both of which can be produced by yeast cultured in sucrose solutions (Mathew et al., 2013). Additionally, other studies have looked at alternative compounds to attract and to kill the mosquitoes and other vectors. For example, Rawani et al. studied the effect of crystalized $Ag(NO_3)$ produced via nanoparticle activity in *Solanum nigrum* with favorable results (2013). Others were less successful. Revay et al. and Rossi et al. both studied the effects of various essential oils, which saw limited if any repellant activity (2013). Going off of the prior research in the field, this thesis tested the efficacy of bait stations containing sugar solutions and yeast for mosquito attraction.

CHAPTER TWO

Materials and Methods

Mosquito Breeding/Rearing:

First, in order to run the tests, colonies of mosquitoes were raised for the bioassays. For both *Aedes aegypti* and *Culex pipiens*, eggs were placed into trays of aged tap water to hatch, which was stored in a ventilated vessel in order to allow any chlorine to escape. It is nececessary to do this because chlorine can be detrimental to growing mosquito larvae (Gahan et al. 1964). The optimal amount of water is approximately 300mL (Das et al. 2007). After adding the eggs to the water, liver powder was added (Singh, et al. 2013) to the tray of *Aedes* eggs and ground fish food was added to the tray of *Culex* eggs (Das et al., 2007). Then, the mosquitoes were given a few days to hatch and another few days to develop into pupae. Throughout the process, the *Culex* larvae were fed a few pinches of ground fish food (2007). The *Aedes* larvae were sustained on the *E. coli* present from the initial infusion of liver powder, and thus they required no additional feeding.

As the larvae developed into pupae, they were collected using a 1 mL disposable pipette. Following this, they were deposited into disposable test tubes, usually one or two per tube, which were then capped. The mosquitoes, upon hatching into these tubes, were released into large mesh cages each with about 100 mosquitoes or in smaller, roughly gender equal, mesh cages of about 20 mosquitoes when used for bioassays. Once in the large colony cage, the mosquitoes were sustained on sugar cubes and cups of water.

When new batches of mosquitoes were required for testing, two immobilized mice were introduced into the cages for approximately 30 minutes to an hour. Following the blood feeding, the mouse was removed and cups were placed in the mosquito cage with strips of half-submerged paper towels placed around the top of the water cups. After the females laid eggs on these strips, they were collected and stored in a damp place until they were submerged and hatched. These mosquitoes were raised in a dedicated, humidity-controlled room at 26°C and 78% humidity per laboratory protocols. The light was on a timer of 14 and 10 hours of light and dark, respectively.

Carbohydrate and Dye Assays:

For the bioassays involving smaller cages, an approximately equal number of mosquitoes from the test tubes was released into smaller mesh cages. For example, typically 20 mosquitoes were put in each with about 10 males and females each to prevent over representation of one gender and possibly sexual aggression or violence leading to an alternate cause of death. For this first test, it was tested whether or not the carbohydrate solution would make a difference in mosquito attractiveness. Also, it was important to see whether or not the mosquitos would be attracted to the yeasts. To do so, an interconnected three canister system was used (Figure 1). To one side of the canister system, 10mL of 20% sucrose solution along with approximately 0.5 g of store-bought baker's yeast was added (*Saccharomyces cerevisiae)*. On the other side, 10 mL of 20% maltose solution with 0.5 g of yeast was added. These solutions were placed into 1 oz. cups. The set-up, which is self-contained, required no outer mesh cage. The middle canister was empty, and this is where mosquitoes were released into the system. A total

of 20 mosquitoes, consisting of 9 males and 11 females, were placed into the system.

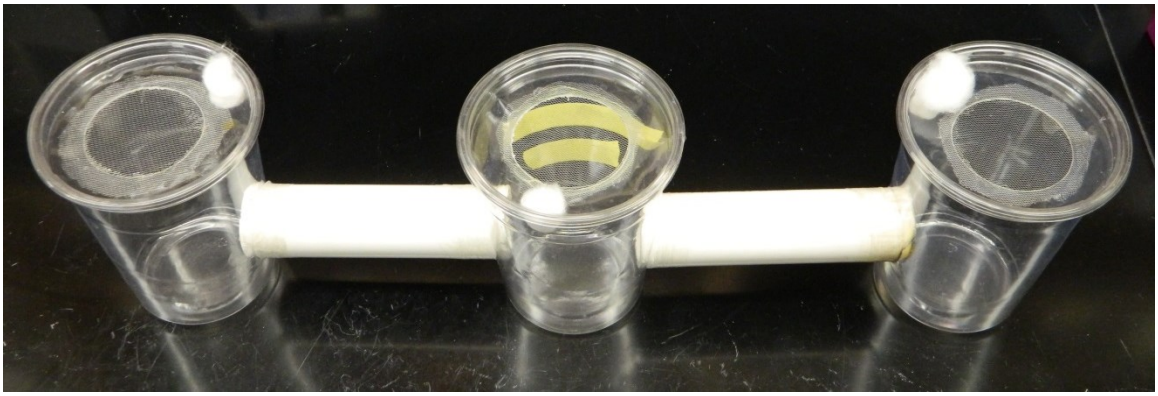

Figure 1: Canister system used for carbyohydrate preference and dye studies.

The second assay attempted to determine whether or not the concentration of the carbohydrate solution made a difference in mosquito attraction. Since sucrose was determined to be a better attractant, the assay was repeated with 20 mL of 5% sucrose with yeast in one side and with 20 mL of 20% sucrose with yeast in the other. Again, mosquitoes were added to the empty middle canister. This test involved 21 mosquitoes, consisting of 9 males and 12 females. Since the mosquitoes were released from test tubes, there ended up being one more than 20 mosquitoes since some tubes contained 1, 2, or 3 adult mosquitoes. The main goal was to maintain an even sex ratio to avoid aggression as previously mentioned.

Next, an assay was performed to determine whether or not it would be possible to measure mosquito imbibition by use of red food coloring. The results provided are from a test done by another member of the lab. This involved setting up two separate mesh cages. In the first, a small one ounce cup with 10% sucrose solution and red food coloring was added. This was done in order to ascertain whether or not the food coloring

produced a discernible color change of the abdomen upon feeding. The second cage contained two cups. One cup contained 20% sucrose along with 1 g of yeasts. The second cup contained 10 mL of 20% sucrose solution, 1 g of yeasts, and 4 drops of red food coloring. The purpose of this was to test whether red food coloring affected feeding.

Bait Station Assays:

Also, four different styles of bait stations were created to see if a particular design afforded an advantage in attracting mosquitoes (Figures 2-5). To do this, four small textile mesh cages were used. Each cage contained a different style of bait station. These stations were created using PVC piping, piping caps, and other supplies from a hardware store. A drill was used to make entry holes. Inside of each station, a one oz cup containing the carbohydrate mixture, yeast, and dye was added. Again, sex ratio was kept equal. Cages one through four each had 22, 21, 20, and 23 mosquitoes, respectively. The respective male and female ratios were 10M/12F, 11M/10F, 9M/11F, and 12M/11F. The number of dyed mosquitoes and dead mosquitoes were recorded after 1 hour, 4 hours, and 20 hours on the first day. Afterwards, measurements were taken once a day for a total of seven days. The number of visibly dead mosquitoes was recorded. Initially, whether or not the mosquitos were dyed was recorded as well. However this became too difficult with the mesh and the enclosed stations. Since the stations were not dismantled during the assay, the number of mosquitoes inside of the bait station was determined by simply counting the mosquitoes outside of the station and subtracting from the original number included in the cage. This served as a measure of attractiveness and as a baseline

measure of whether or not mosquitoes could find the food sources in these different designs with varying degrees of success.

Figure 2: Bait Station Design 1

Figure 3: Bait Station Design 2

Figure 4: Bait Station Design 3

Figure 5: Bait Station Design 4

Agroinoculation and Plasmid Construction:

Agroinoculation was used in conjunction with the FECT system to express proteins for lethality assays. Instead of tobacco mosaic virus (Lindbo 2007), thefoxtail mosaic virus was used (Liu and Kearney 2010). However, the constructs were still driven by a 35S promoter (Figure 6) and delivered by *Agrobacterium tumefaciens* (2007, 2010). The constructs were created by the combined use of PCR products of the desired sequences along with restriction endonucleases to generate sticky ends (2007). This permitted the insertion of EGFP into the pJL43 plasmid (2007). The construct pJL3:p19 was created, which is a silencing suppressor necessary to promote protein expression in tobacco plants (2007, 2010). As is the case for the EGFP and p19 constructs, all sequences of interest were cloned into *Agrobacterium tumefaciens* and mixed with the p19 prior to inoculation (Voinnet, et al. 2003). Co-infiltration with a silencing suppressor led to greater expression in a prior study (2003). p19 suppressors are required to prevent degradation of the RNA constructs (Silhavy, et al. 2002). It appears that double-stranded RNA constructs are degraded to 21-25 nucleotide segments that are further eliminated by the appropriate ribonucleases (2002). As such, it is an important defense for plants, particularly against viruses (Vargason, et al. 2003). It appears that p19 inhibits the process by binding double-stranded RNAs while also interacting with siRNAs (2003).

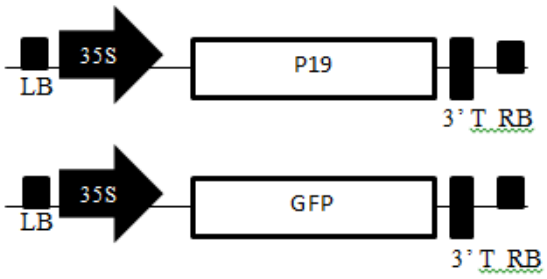

Figure 6: Constructs showing the control (GFP) and the silencing suppressor necessary for protein expression in N. benthamiana.

For this experiment, previously produced vector constructs were used. As mentioned by Liu and Kearney, these constructs utilized the modified FECT0 expression system (2010). For each desired construct, primers were constructed to include the appropriate restriction endonuclease sites and the desired polypeptide toxin sequence to enable PCR amplification, digestion, and cloning of the desired sequences into the FECT expression system (2010). Following the insertion of the sequences into FECT, the Phusion polymerase (New England Biolabs, Beverly, MA) was used as instructed (2010). Electroporation was performed in order to transform competent E. coli 10-beta cells (2010). This procedure was completed with the help of a BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA) for five milliseconds at a voltage of 1440V and a resistance of 129 Ohms (2010). Following this, the graduate students screened the colonies in order to harvest the colonies that successfully contained the cloned sequences (2010).

Next, plasmids were collected from the screened *E.coli* colonies in order transform *Agrobacterium tumefaciens* strain GV3101 (Liu et Kearney, 2010; Lindbo, 2007). After this transformation, positive transformants were further selected by LB

plating with 25 μg/mL of gentamycin and 50 μg/mL of kanamycin (2010; 2007). For this paper, tested constructs include ACTX, ACTX Archive, FECT J3, and FECT AA. Thus, for the next step, each positive *A. tumefaciens* colony for each respective construct was placed into 5 mL of L-MESA medium which consists of liquid LB media, 10 mM MES, 20 μM acetosyringone, 25 μg/mL gentamycin, and 50 μg/mL of kanamycin (2010; 2007). Following this, the cells were grown in glass tubes at room temperature on a shaker for eight to sixteen hours or essentially overnight (2010). After this, the colonies were centrifuged to form a pellet and the supernatant was decanted (2010; 2007). Next, an induction media consisting of 10 mM MES, 10 mM $MgCl_2$, and 100 μM acetosyringone was added to resuspend the pellet by aggravation with a pipette (2010). Then, the induction media was allowed to sit for 2 to 5 hours at room temperature (2010; 2007). Next, a needleless 1 mL IV-flushing syringe was used to inoculate the underside of desirable leaves of *Nicotiana benthamiana* (2007). As a control, the combined pJL43:GFP and PJL3:p19 *A. tumefaciens* colonies were injected into one plant's leaves (2007). If everything went according to plan, these two constructs would allow fluorescent detection of gfp as a means to determine whether or not agroincoulation was successful (2007). *N. benthamiana* was also inoculated with the ACTX, ACTX archive, FECT J3, and FECT AA constructs along with the pJL3:p19 construct. ACTX contains a fusion of J3 and Hv1a toxins, and this is the main lethal mixture under study. ACTX archive is the same mixture from a previous study that has been confirmed via SDS PAGE. Thus, this acted as the positive control to verify that ACTX harvested from the plants was indeed the J3/Hv1a fusion. FECT AA is an empty construct, and thus it

should have limited lethality. Lastly, FECT J3 is a negative control containing only J3 and lacking Hv1a. Thus, this allows one to test whether Hv1a or J3 is the main toxin.

Protein Extraction and SDS PAGE Detection:

After three days, the co-inoculated plants with pJL43:GFP and PJL3:p19 were tested to ensure that inoculation was successful. Then, at seven days post inoculation, the leaves were harvested from the other inoculated plants. Then, the proteins that accumulated in the interstitial fluid (McCormick et al., 2003) were collected through vacuum infiltration and centrifugation as previously mentioned in McCormick et al. (1999). To do this, leaves were collected from each series of specifically inoculated plants and cut into approximately one inch squares. To reiterate, those plants inoculated with FECT AA would have all of their inoculated leaves removed, cut into squares, and placed into a 400 mL beaker. The same was done for all other tested constructs. Next, the leaves were covered with enough sodium acetate to ensure they were covered (Figure 7). The beakers were then placed into a small vacuum house with a hose attached to the lab bench vacuum (Figure 8). The leaves were vacuumed for four minutes, flipped and resubmerged, re-vacuumed, and so on until the leaves appeared dark green. This indicates that the sodium acetate had infiltrated into the interstitial space of the leaves. After, the leaves were removed from the sodium acetate and centrifuged in bottles containing spacers at 2000 xg for 15 minutes at 6°C (Figure 9). The resulting clear or slightly tinted solutions were stored at 4°C until they were ready to be mixed into the sucrose solutions for the lethality assays. SDS PAGE was used to ensure that proteins met the expected lengths as expected after boiling.

Figure 7: *N. bethamiana* leaves submerged in sodium acetate buffer.

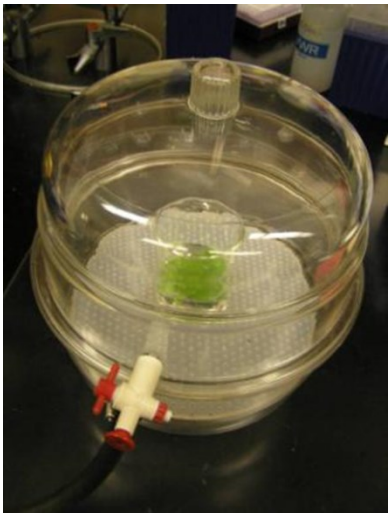

Figure 8: Sodium acetate system in vacuum house where sodium acetate was drawn into the interstitial space.

Figure 9: Centrifuge tube with catch for leaf material. Permitted the collection of sodium acetate infused with proteins.

Lethality Assay:

Finally, the last assay involved testing the lethality of the various extracted proteins on mosquitoes. For this test, Ae aegytpi was used specifically. The enclosure consisted of a quart-sized canister, with a mesh-covered hole in the lid. One administered the toxins via sucrose solutions in an inverted microcentrifuge tube capped with cotton, which when dampened by the solution allowed mosquitoes to land and feed without problems. This enabled testing of the constructs with ACTX, AA, and J3. The toxins were presented in 5% sucrose solutions with a 1:1 ratio of purified interstitial protein extract as described above to the sucrose. Next, a tube of 1X PBS with 5% sucrose solution and a final tube with a one-to-one ratio of 1X PBS to 5% sucrose solution was used as a more dilute offering of PBS buffer. This served as a baseline control since PBS would be present in all of the interstitial protein extracts. The assay lasted for 14 days,

and the amount of dead mosquitoes per cage was recorded daily. Each cage started with 10 male and 10 female mosquitoes, in line with the goal of gender equality for less aggression. The food tubes were replaced as necessary upon drying out.

CHAPTER THREE

Results

Carbohydrate Preference with Yeast Assay:

As previously mentioned, the first assay was performed to determine which carbohydrate with yeast solution, if any, would be best given the preferences for mosquitoes for maltose and sucrose in the literature. For the test, it was tested whether or not mosquitoes were more attracted to sucrose or maltose, by using 10 mL of each at 20% concentration. On the first day, results were recorded at 1 hour, 4 hours, and 20 hours. Thereafter, results were recorded daily until day 7, with the exception of day 3 when solutions were refreshed. In total, there were 20 mosquitoes of which 9 were male and 11 were female. The results are found in Table 1 below.

For the first hour, most of the mosquitoes remained in the middle compartment. Most likely they were exploring the new surroundings and coping with stress. They had not yet found the food sources yet. However, two mosquitoes discovered the maltose food source, while five mosquitoes discovered the sucrose food source. At four hours, only one mosquito remained at the maltose food source while five mosquitoes were still in the compartment with the sucrose food source. At 20 hours, two mosquitoes were at the maltose food source and another two mosquitoes had become stuck in some of the yeast bubbles and appeared to have drowned. The middle compartment contained two living and two dead mosquitoes. Lastly, the sucrose compartment contained 10 living

mosquitoes and one mosquito that unfortunately appeared to have become entangled in some of the yeast bubbles and drowned.

Sucrose v. Maltose with Yeast Assay, Canister System, 9M/11F										
Time:		1 Hour	4 Hours	20 Hours	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Maltose (10mL, 20%) w/yeast	Alive	1	1	2	2		1	1	4	1
	Dead	0	0	2	2		2	2	2	2
Middle	Alive	17	14	2	2		1	1	2	2
	Dead	0	0	3	3		2	4	5	8
Sucrose (10mL, 20%) w/ yeast	Alive	2	5	10	10		8	8	0	0
	Dead	0	0	1	1		7	6	8	8

Table 1: Depicts findings from maltose v. sucrose with yeast assay. It appears that sucrose is the preferred food source.

On day 2, no change occurred from the 20 hour reading. Day 3 saw no recordings taken. On day four, there remained one living mosquito at the maltose source with the two previously dead mosquitoes. The middle compartment contained one living mosquito and two dead mosquitoes (it appears one of the mosquitoes from day 2 may not have been dead). The sucrose compartment contained eight living mosquitoes and seven dead mosquitoes. At day 5, it appeared that the sucrose food source was beginning to run out, and the yeast was starting to form a solid crust over whatever solution remained. The same began to happen with the maltose solution, but there was quite a bit left. There remained no change in mosquito numbers in the maltose chamber, but now there were one living and four dead mosquitoes in the middle chamber. These mosquitoes possibly began migrating back towards the middle in search of more food and ultimately expired

there. The sucrose chamber contained 8 living mosquitoes and 6 dead mosquitoes. Again, perhaps one mosquito was mistaken for dead in the previous recording. On day 6, the maltose compartment had 4 living mosquitoes and 2 dead mosquitoes. It appears that these mosquitoes succeeded in migrating to the alternative food source after the sucrose was completely gone. Meanwhile, the middle compartment had 2 living and 5 dead mosquitoes. The sucrose compartment had no living mosquitoes and 8 dead mosquitoes. On day 7, the maltose solution appeared to have completely crusted over with the yeast. There remained one living mosquito and 2 dead mosquitoes in the maltose compartment as the mosquitoes migrated back to the middle compartment. Thus, the middle compartment contained 2 living mosquitoes and 8 dead mosquitoes as a result of this migration. Finally, the sucrose compartment saw no change from the previously recorded 8 dead mosquitoes. Through these recordings, it was concluded that sucrose was the preferred food source. Even by 20 hours, a majority of mosquitoes located the sucrose food source and remained there until it was completely used up by day 5.

Yeast and Carbohydrate Concentration Assay:

At the same time this experiment was run, a test was run to see whether or not there appeared to be a noticeable difference in yeast attraction resulting from different sucrose concentrations. To test this, an identical canister system was used. However, this test involved 20 mL of 5% and 20% sucrose solutions in either end with yeast in each. The results are presented below in Table 2.

At one hour, most mosquitoes remained in the middle as they became oriented to their surroundings. By four hours, 4 mosquitoes had migrated to the compartment containing the 5% sucrose solution while only 1 remained in the 20% sucrose

compartment. Meanwhile, the other 16 mosquitoes remained in the middle.

Yeast Sucrose Concentration Assay, Canister System, 9M/12F										
Time:		1 Hour	4 Hours	20 Hours	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
5% Sucrose (20mL w/yeasts)	Alive	1	4	14	14		14	11	6	2
	Dead	0	0	2	2		2	3	5	9
Middle	Alive	18	16	0	0		0	0	1	0
	Dead	0	0	2	2		2	3	4	6
20% Sucrose (5mL w/yeasts)	Alive	2	1	2	2		1	2	0	0
	Dead	0	0	1	1		1	1	3	3

Table 2: Results of sucrose concentration assay. It appears that a lower concentration favors slower development of the yeasts, which in turn keeps the food source accessible for longer.

By 20 hours, 14 living mosquitoes were present in the 5% sucrose chamber. There were 2 dead mosquitoes as well, and these numbers remained constant until day 5 for the 5% sucrose chamber. At 20 hours, there were 2 dead mosquitoes in the middle chamber and 2 living and 1 dead in the 20% chamber. The middle chamber also saw no change in the total number of living and dead mosquitoes until day 5, while the 20% chamber retained its numbers of mosquitoes until day 4. On day 4, the 20% chamber contained one dead and one living mosquito. One mosquito must have been in the tube between the 20% compartment and the middle compartment during this reading. On day 5, the 5% compartment contained 11 living and 2 dead mosquitoes. The middle had 3 dead mosquitoes, and the 20% compartment contained 2 living and one dead mosquito. At this point, the 5% sucrose solution was completely gone, while the 20% solution was

starting to crust over from the yeasts. However, the mosquitoes began to migrate as several mosquitoes were in tubes during this recording. On day 6, 6 living mosquitoes and 5 dead mosquitoes remained in the 5% compartment. The middle compartment contained 1 living and 4 dead mosquitoes, and the 20% compartment contained 3 dead mosquitoes. Lastly, on day 7, the 5% sucrose compartment contained 2 living and 9 dead mosquitoes. The middle contained 6 dead mosquitoes, and the 20% compartment contained 3 dead mosquitoes.

Again, by 20 hours, the mosquitoes appeared to favor one solution over the other. Part of this could be explained by the fact that the yeasts formed a crust more readily on the 20% sucrose solution than they did on the 5% solution. This led to the conclusion that a lower concentration of sucrose would be better since the mosquitoes appeared to prefer it while the yeasts developed more slowly. This would prevent rapid crust formation, and thus the food source would remain accessible and open for a longer period of time.

Dye and Yeast Assays:

Next, an assay was performed to determine whether or not one would be able to detect mosquitoes who fed from a food source by use of red food coloring. As previously mentioned, the first cage contained a cup with 10% sucrose solution and two drops of red food coloring. The second cage contained two cups, one of which contained 10 mL of 20% sucrose solution and 1 g of yeasts. The second one contained 10 mL of 20% sucrose, 1 g of yeasts, and red food coloring. For each of these tests, measurements were recorded at one, two, three, four, five, eight, and ten hours on the first day. Five

measurements were recorded on the second day, every couple of hours, and lastly there was one recording on the third day.

For the first test (Table 3), the cage contained 4 female and 16 male mosquitoes to start. Gender equality was not considered important to determining whether or not coloring would be present in mosquitoes upon feeding on a dyed food source. After one hour, there were four dead mosquitoes. However, there were eight mosquitoes with a noticeably red-colored abdomen. At two hours, there were nine colored mosquitoes, and this remained constant until the eight hour mark when ten mosquitoes had red coloration. At 10 hours, 11 mosquitoes showed coloration.

Red Dye Assay 1 (4F/16M), 10% sucrose, 2 drops of red dye.													
Day	Day 1:							Day 2:					Day 3:
Time:	1 hour	2 hours	3 hours	4 hours	5 hours	8 hours	10 hours	13:15	15:15	17:15	19:15	21:15	18:15
Red	8	9	9	9	9	10	11	9	7	6	6	5	2
Dead	4	4	4	4	4	4	4	8	8	8	9	9	14
Non-red	8	7	7	7	7	6	5	3	5	6	5	6	4

Table 3: Red Dye Assay, shows that red dye colors mosquitoes fairly rapidly, and that it remains for about a day.

On the second day, measurements were taken starting at 1:15pm and continued every two hours until 9:15. At 1:15, there were 9 colored and 8 dead mosquitoes. At the next reading, 7 were colored. For the next two readings thereafter only six mosquitoes were colored, while the amount of dead mosquitoes increased to nine. For the last reading of that day, five colored mosquitoes and nine dead mosquitoes were observed. On the third day, 2 colored mosquitoes and 14 dead mosquitoes were observed. It is

important to note that by the reading on the third day, much of the sucrose solution had run out. This can account for the increased mortality as well as the reduced coloration of the mosquitoes.

The second dye assay involved the two cups, one with food coloring and one without (Table 4). The measurements were taken at the same time as the first assay. Both cups contained 20% sucrose solution and 1g of yeasts. However, one of the cups had food coloring, the other did not. During the first eight hours, there were no visible red-colored mosquitoes. However, there was one dead mosquito starting at one hour, which increased to two at the five hour mark and again to three dead mosquitoes at the eight and ten hour marks. There was one visibly red-colored mosquito at 10 hours. On the second day, there were no visibly colored mosquitoes and four dead mosquitoes. The third daysaw no colored mosquitoes and five dead mosquitoes.

Red Dye Assay 2 (4F/19M), 20% sucrose, 1g yeasts, (4 drops of red dye)													
Day	Day 1:							Day 2:					Day 3:
Time:	1 hour	2 hours	3 hours	4 hours	5 hours	8 hours	10 hours	13:15	15:15	17:15	19:15	21:15	18:15
Red	0	0	0	0	0	0	1	0	0	0	0	0	0
Dead	1	1	1	1	2	3	3	4	4	4	4	4	5
Non-red	22	22	22	22	21	20	19	19	19	19	19	19	18

Table 4: Red Dye Assay 2 with yeasts. It appears that the red dye prevents feeding on the food source, or at least does so in the presence of a non-dyed food source.

Bait Station Design Assays:

The next test involved four different styles of bait stations in order to test whether or not basic design differences may cause differences in feeding success for mosquitoes. Again, each had a 1 ounce cup containing 10mL of 20% sucrose solution, 0.5 g yeasts, and 5 drops of red food coloring. Approximately 20 mosquitoes were added per cage with a relatively equal gender ratio. There were four separate cages, each with a different bait station design. It is important to note that after the second reading at four hours, recording whether or not mosquitoes were dyed was discontinued. It was difficult to discern whether or not mosquitoes were colored through the mesh, and it was also impossible to tell if the mosquitoes inside of the station exhibited red coloration. Instead, the test simplified to whether or not the mosquitoes were inside the bait station and whether or not they were visibly dead outside of the station. The number of living mosquitoes outside of the station was also recorded.

For the first bait station design (Table 5) , it was noticed that one mosquito was inside the station after one hour. At four hours, two were dead and none were inside the station. At 20 hours, 7 mosquitoes were counted to be inside of the station However,

Bait Station Design Assay 1, 10mL 20% sucrose, 0.5g yeasts, and 5 drops of red food coloring (10M/12F)								
Time:	1 hr	4 hr	20 hr	Day 2	Day 4	Day 5	Day 6	Day 7
Inside of Station	1	0	7	7	7	7	7	7
Dead (visibly)	0	2	15	15	15	15	15	15
Alive (visibly)	21	20	0	0	0	0	0	0

Table 5: Bait Station Design 1 assay results

the remaining 15 mosquitoes appeared to be dead outside of the station. This did not change for the rest of the test, so eventually it was assumed that the seven mosquitoes inside of the station were dead as well.

For the second design (Table 6), two mosquitoes were determined to be in the station starting at four hours. By 20 hours, two mosquitoes remained inside of the station, and 10 mosquitoes appeared to be dead. On the second day, none of the mosquitoes were inside of the station, and 12 mosquitoes appeared to be dead outside. By the fourth day, 18 mosquitoes were dead and 3 were inside of the station. These measurements remained the same for the rest of the test, and therefore it was eventually concluded that the three mosquitoes inside of the station had died.

Bait Station Design Assay 2, 10mL 20% sucrose, 0.5g yeasts, and 5 drops of red food coloring (11M/10F)								
Time:	1 hr	4 hr	20 hr	Day 2	Day 4	Day 5	Day 6	Day 7
Inside of Station	0	2	2	0	3	3	3	3
Dead (visibly)	0	0	10	12	18	18	18	18
Alive (visibly)	21	19	9	9	0	0	0	0

Table 6: Design station 2 assay results.

For the third design (Table 7), two mosquitoes had entered the station by the four hour recording. By the 20 hour mark, there appeared to be 8 dead mosquitoes and 8 mosquitoes inside of the station. On day 2, 8 mosquitoes remained inside of the station,

but the number of dead mosquitoes increased to 11. By day four, 8 mosquitoes remained inside of the station, and 12 were visibly dead outside. These measurements did not change from this point forward, so it was eventually assumed that the eight mosquitoes inside were dead as well

Bait Station Design Assay 3, 10mL 20% sucrose, 0.5g yeasts, and 5 drops of red food coloring (9M/11F)								
Time:	1 hr	4 hr	20 hr	Day 2	Day 4	Day 5	Day 6	Day 7
Inside of Station	0	2	8	8	8	8	8	8
Dead (visibly)	0	0	8	11	12	12	12	12
Alive (visibly)	20	18	4	1	0	0	0	0

. Table 7: Design station 3 assay results.

Finally, for the fourth design (Table 8), 2 mosquitoes had entered the station after four hours. By 20 hours, the station contained six mosquitoes. On day two, there were eight mosquitoes inside of the station. By day four, there was one visibly dead mosquito outside of the station. The remaining 22 mosquitoes were inside of the station, and these measurements did not change for the rest of the assay.

Bait Station Design Assay 4, 10mL 20% sucrose, 0.5g yeasts, and 5 drops of red food coloring (12M/11F)								
Time:	1 hr	4 hr	20 hr	Day 2	Day 4	Day 5	Day 6	Day 7
Inside of Station	0	2	6	8	22	22	22	22
Dead (visibly)	0	0	0	0	1	1	1	1
Alive (visibly)	23	21	17	15	0	0	0	0

Table 8: Design station 4 assay results.

Lethality Assay:

The last assay, performed by a graduate student of the lab and presented here with permission, involved a test of the efficacy of certain toxin mixtures on mosquito colonies. Again, PBS is a baseline since it is present in all toxin solutions because of its use as a solvent for extracting proteins. FECT-AA has no toxin insert, and thus its toxin solution is an empty control. J3 contains only J3, acting as a negative control to Hv1a and J3 found in ACTX. It is helpful to note that boxes highlighted in yellow indicate that there was a fungal growth present on the cotton upon recording (Table 9). The red line on the right-hand side of a box indicates the replacement of entire tube with a new tube, a new cotton plug, and a new toxin/food mixture. A black line indicates that the existing tube was refilled.

Lethality Assay, 5% sucrose with various toxins (10M/10F)															
Day	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1\ ACTX1:1	0	0	0	1	2	2	8	9	11	11	12	12	12	12	12
2\ AA 1:1	0	0	0	0	0	0	3	4	4	6	6	7	7	7	7
3\ J3 1/2 1:1	0	0	0	4	6	6	6	9	9	9	9	10	10	10	10
4\ 1XPBS (full)	0	0	0	4	5	5	8	8	8	8	8	9	10	10	10
5\ 1XPBS 1:1	0	0	0	1	1	3	4	5	5	5	6	7	7	7	7

Table 9: Daily observations of lethality study. It appears that none of the toxins under study appear particularly toxic, and it is also possible that the PBS used in vacuum filtration may be slightly toxic.

As one can see for the first two days of the assay, no mosquitoes died. Starting at day 3, there was one mortality for the ACTX mixture, 4 for the J3 mixture, 4 for the 1X PBS mixture, and 1 for the 1:1 1X PBS/5% sucrose mixture. By day four, there were 2 moralities for the ACTX mixture, 6 for the J3 mixture, 5 for the 1X PBS mixture, and 1 for the 1:1 1X PBS/5% sucrose mixture. On day five, it was found that only two more mosquitoes died in the 1:1 1X PBS/5% sucrose cage. Day 6 showed a marked increase in ACTX mortality, jumping from 2 to 8 dead mosquitoes. The cage with FECT-AA saw

three dead mosquitoes, the cage with 1X PBS saw 3 more dead mosquitoes, and the cage with 1:1 1X PBS/5% sucrose solution saw an additional dead mosquito. On day 7, the ACTX, AA, and 1:1 1X PBS/5% sucrose cages had an additional fatality. The J3 cage saw three additional fatalities. The only change on day 8 was an additional 2 deaths for the ACTX cage. On day 9, the only change was two more fatalities for the cage containing AA. On day 10, both the ACTX and 1:1 1X PBS/5% sucrose cages had one more death. One day 11, all cages except for the ACTX cage had one more death. For day 12, the only change was an additional dead mosquito in the 1X PBS cage. Days 13 and 14 both saw no additional changes, and this was the point when the assay was stopped. Figure 10 below is a graphical representation of Table 9.

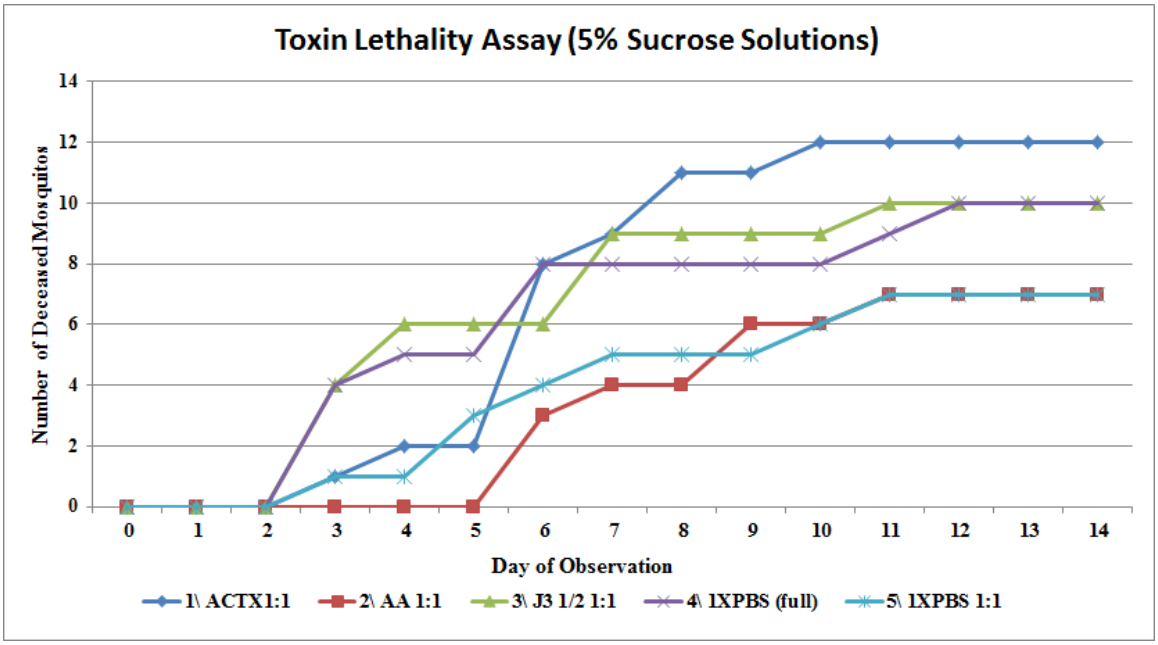

Figure 10: Graphic representation of lethality assay. Here it was observed that ACTX was the most lethal, but also that PBS is an issue in protein extraction.

CHAPTER FOUR

Discussion and Conclusions

Carbohydrate Source and Concentration Preferences:

As seen above in the carbohydrate preference assay, there appeared to be an elevation in the aggregation of mosquitoes starting around 4 hours into the test. The number of mosquitoes on the 20% sucrose solution reached a maximum of 10, and it stayed much higher than the number of mosquitoes in either of the other compartments after 20 hours. Towards the end, starting on Day 6, at which point it was noticed that the sucrose solution had been completely consumed, some mosquitoes began to migrate toward the middle and ultimately the maltose compartment in search of food. Otherwise, the test showed that the most likely conclusion is that sucrose is the preferred sugar source for the mosquitoes. Thus, using this information, sucrose was the carbohydrate of choice in the rest of the studies involving mosquito assays.

Additionally, the effects of yeast and sucrose concentrations were tested to see if there was a preference for higher or lower concentrations as well. Much like the prior assay, a noticeable increase in aggregation was noticed starting at 4 hours. At this point, four mosquitoes had found the less concentrated 5% sucrose solution as compared to the one mosquito who had found the 20% sucrose solution. By 20 hours, a large majority, 14 alive mosquitoes out of the initial 21 mosquitoes, had found the 5% sucrose solution while only 2 living mosquitoes remained in the 20% sucrose solution compartment. Throughout the assay, there remained an elevated number of living mosquitoes in the 5%

compartment until the last two days when the food sources started to become depleted and crusted over with yeasts. Thus, this allowed the conclusion that at a basic level that the mosquitoes prefer lower concentrations of sucrose. It was noticed that the 20% sucrose solution crusted over with yeast much more quickly. Thus, since these tests involved yeast, it is possible that mosquitoes may change their behavior in the absence of yeasts because a faster growing yeast population would mean the food source would be crusted over and inaccessible more quickly. These preference studies should have involved statistical analyses, and they should have also involved running an empty test wherein mosquitos were placed in the middle compartment without any solution on either side. It would have also been important to study the effects of sucrose concentrations without yeast. As performed in Müller's work, attraction with other sucrose solutions, fermented fruit juices, and plant parts (2006; 2008;2010ab) should have been tested possibly with yeast. It also would have been good to test how this varies geographically in the presence of yeast.

Dye and Yeast Assays:

The first dye assay just involved a 10% sucrose solution with 2 drops of dye. As such, this assay enabled the determination of whether or not mosquitoes turned noticeably red. It also allowed the approximate measurement of how long it took to become red and to see approximately how long it lasted. In the first hour, there were eight visibly red mosquitoes. Thus, it is possible to surmise that coloration becomes visible within the first hour of feeding, most likely even upon ingestion of the dyed food source. The four dead mosquitoes that appeared after the first hour were most likely weakened by a bacterial infection or other infection that was obtained from larval or

pupal stage rather than from the red dye. However, this cannot explicitly be ruled out. This test would have been better if it was run with a simple 10% sucrose solution control. This would have enabled a comparison of the potential effects of the red dye more clearly, particularly its effect on feeding and any potential increases in mortality. Yet, the mosquitoes seemed to feed at an acceptable rate during this test. The dye coloration most likely lasts less than a day as the mosquito processes and digests its meal.

The second assay studied the difference that red dye seemed to make on mosquito feeding from a sucrose and yeast mixture. Since both sources of food were in the same cage, it was assumed that any coloration would be indicative of feeding on the dyed sucrose and yeast solution whereas a lack of coloration would indicate that the mosquitoes fed upon on the non-dyed source. Again, these tests cannot have deterministic results because there is ultimately no method introduced to determine whether or not the mosquitoes are feeding on the non-dyed solution other than measuring the mortality from presumed starvation. In this test, it took until the 10 hour mark for any coloration to appear, and, even then, it appeared only in one mosquito. The second day saw no colored mosquitoes, and the third day did not either. However, since mortality appeared to be much less than the first dye assay and the mosquitoes were from the same batch, one can draw a few conclusions from this. First, it is evident that the mosquitoes preferred not to feed on the dyed food source if there is a non-dyed source present. Additionally, there is an increased possibility that the dye may have some degree of low-grade lethality on the mosquitoes. However, again, these conclusions cannot be definitively drawn because these two tests were not of the same solution. The second test had yeasts present as well. It also had a different sucrose concentration. Thus, if given

more time, this test would be repeated with uniform sucrose concentrations. Additionally, there would be a comparative test of mortality between simple sucrose solution and colored sucrose solution. Also, it would be necessary to test whether or not mosquitoes preferred plain sucrose solutions compared to solutions with yeasts and other possibly naturally occurring food sources. This would be necessary since bait stations would be designed to be placed into the environment where competing sources would most likely be present. This would be important to determine if the idea would even attract enough mosquitoes to become effective.

All of these tests so far indicated the potential that monitoring the mosquito feeding by red-dye with the naked eye is somewhat difficult. As seen in the bait station design tests, it became visibly difficult to identify colored mosquitoes. It is possible that this could have been aided by clear glass or plastic cages instead of mostly mesh cages. Or, freezing, detergent immersion, and dissection could have been used with the help of a dissecting microscope as done by Schlein and Müller (2008). Other studies have shown that there appears to be no significant inhibition to feeding resulting from the presence of dyes (Chen et Kearney, 2015). Thus, it may be necessary for future studies to devise an alternative and easily visible way to measure whether or not mosquitoes have fed upon the presented food sources in real time. Although it does not provide a definitive conclusion that the food source, which in later tests will be laced with toxins, caused the death, it does provide some degree of excludability. For example, starvation can be ruled out by clearly seeing whether or not mosquitoes have fed upon the sources prior to death. It would also be of interest to find a dye that remained in the mosquito body for a long period of time, most likely from ingestion until death. If a dye is used, such as the red

food coloring used here, it becomes difficult to know whether a mosquito has died after ingesting the toxin or simply from aggression, illness, or other factors. Evidently, working with mosquitoes in assays like this can be somewhat difficult since the mosquitoes cannot be easily tracked like other organisms such as *Mus musculus* where marking the tails or tagging the animals is sufficient. Thus, a more efficient way to track the feeding would be necessary in order to be able to draw more definitive conclusions about whether or not one can link death to feeding from a particular food source.

Bait Station Design Assays:

As mentioned above, red coloration became difficult to measure in this situation because of the enclosed station designs as well mesh outer cages and small nature of the mosquitoes. However, the number of visibly dead mosquitoes and the number of mosquitoes found inside of the stations were recorded. In this test, the number of dead mosquitoes is not an especially conclusive number. These mosquitoes could have been unable to find their way into the station to the food source, and thus they could have starved. Thus, the main measure in this case was the number of mosquitoes inside of the stations.

Along these lines, bait station design 1 (Figure 2) appears to have at most attracted a total of 7 out of 22 mosquitoes or approximately 32%. The second design (Figure 3) appears to have attracted a maximum of 3 out of 21 mosquitoes or approximately 14%. Somewhat better than either of the first two, the third bait station (Figure 4) attracted a maximum of 8 of out of 20 mosquitoes or 40%. Lastly, the fourth bait station design (Figure 5) appeared the most successful with a maximum attraction of 22 out of 23 mosquitoes, or about 95%. Thus, it can be concluded that different bait

stations are by design more attractive to mosquitoes. Particularly, design 4 appears to have much potential. Qualities to consider are whether the mosquito can easily find its way in and whether or not it can readily detect the carbon dioxide plume coming from inside of the station. It was impossible in this test to produce a detectable carbon dioxide gradient since the mosquitos were placed into small mesh cages around the station. Future field studies will be necessary to see whether or not these results are replicable in the environment, and it is also necessary to run sucrose controls without yeast to substantiate the results. It would also be important to run the tests without anything inside of the tests to see if there an inherent quality of the trap in addition to the solutions that attracts the mosquitoes such as whether or not the traps provide a suitable hiding place.

After the tests done for this thesis, it is believed that the efficacy of a yeast-based station for the presentation of lethal toxins to mosquitoes could become a viable option. It may be of interest to include other compounds and solutions as attractants. If one found an adequate cocktail of attractants to overcome any faults represented by the yeasts, genetically-modified yeasts that produce oral toxins may be a novel route to explore. In future studies, field tests may be necessary with floral attractants and others in addition to yeasts. Again, Müller highlighted the success of fermented juices and plant parts. Chemical attractants and toxins in other studies have also been shown to be quite effective. For example, one study tested several potential compounds that would be present in human sweat or breath. It was found that ammonia, L-lactic acid, and myristic acid, when mixed and presented in a trap with a carbon dioxide source, provided strong attraction for mosquitoes to the traps (Jawara et al., 2011). Further, another study has shown that the carbon dioxide produced by yeasts can be effectively used instead of

commercial carbon dioxide from tanks or other difficult to transport and more expensive sources in developing regions (Smallegange et al., 2010). Even the sugar source can be changed depending on the availability. For example, molasses, more cheaply and readily available in certain parts of Kenya, has been shown to be a suitable substitute for sucrose in these yeast traps (Mweresa et al., 2014). Thus, it might be of interest to perform further studies with some of the attractants along with later toxin cocktails that have been determined safe for children, pets, and other non-target insects such as honey bees while being specifically lethal to mosquitoes.

For example, Lothrop et al. performed a study with a 66% sucrose solution station, a steady carbon dioxide source, and a chemical attractant (2012). Particularly, the researchers used phenol acetaldehyde (2012). The study also highlighted some difficulties with carbon dioxide based traps in in high density population centers and rural agricultural centers (2012). It was noted, however, that these traps performed well in desert landscapes (2012). This leads to the belief that certain difficulties, such as additional food sources in these more temperate areas, compete away the effectiveness of carbon-dioxide and thus possibly yeast-based stations. Although it is a repellant, one study has shown that patchouli alcohol is quite toxic to pupae (Gokulakrishnan, et al., 2013). According to their study, it had an approximate death rate of 94.80% and 84.53% for *A. aegypti* and *C. quinquefasciatus*, respectively (2013). Since this is more targeted toward pupae, it would require an application to breeding sites most likely or a station design wherein mosquitoes were likely to lay eggs. It would also be interesting to consider the economic implications as a result of reduced populations in certain areas.

Lethality Assay:

The lethality assay did not contain a sucrose control. Our lab has since demonstrated that PBS itself is orally toxic to adult mosquitoes. Thus, PBS was a source of the mortality seen in other treatments in this experiment. Thus, this test compared ACTX, AA, and J3 toxins to the effect of PBS since PBS was the solvent used to extract the proteins from the interstitial space of the plants. As a result, PBS is present in all of the constructs. This was done by setting PBS as the baseline, or basically by subtracting it from the effects seen by all other constructs. Here, the diluted PBS mixture, the 1:1 mixture of 1X PBS and 5% sucrose solution acts as the baseline for this test. It was found that FECT-AA, an empty control, and 1X PBS 1:1 had the same effect while J3 and 1X PBS had the same effect. The ACTX solution, consisting of J3/Hv1a, had the highest mortality count by the end of the test. Since there is not a sucrose control, it is impossible to ascertain the exact lethality for the tested toxins and PBS. Also, since there was some lethality associated with PBS, the exact lethality of the ACTX solution could not be determined. Later tests were run with sodium acetate as the buffer to determine this more definitively. However, while looking at the number of deaths occurring over the two week period, it is possible to see that all samples had no recorded deaths in the first two days. Thus, it appears that perhaps the concentration of each of the toxins extracted from N. benthamiana interstitial fluid via FECT is not in a sufficient concentration to cause more rapid mortality upon consumption. Additionally, perhaps the toxins under study are not as toxic as they need to be. Or, perhaps the current method of expression, FECT, does not allow sufficient expression. Additionally, future tests will need better methods of protein purification. This will enable more consistent tests with more controllable

amounts of toxins. Next, however, several of the solutions saw a great deal of movement between days three and nine. The ACTX solution had the greatest increase in deaths between days five and six. J3 and 1X PBS saw the first large amounts of mortality at day three with four deaths each. The AA solution cage had no recorded deaths until day 6.

A few suggestions for future tests can be made as a result of this assay. First of all, it cannot be denied that PBS may indeed have a lethality effect. This is why the standard protein extraction protocol for the laboratory has since been changed to include sodium acetate instead of PBS. J3 only matched the undiluted PBS solution in mortality, so this is potentially an unviable toxin candidate. ACTX, having the highest mortality, with decent increases in mortality between days five and eight, may be worthwhile to keep under study. However, there is much work to be done along the lines of protein testing. First of all, it will be necessary to make sure that all of the compounds used in protein extraction have negligible effects on the mortality of mosquitoes. This can be done by running lethality assays with sucrose controls. After this is done, the test could be rerun with the constructs with ACTX, FECT-AA, and FECT-J3 to confirm whether or not ACTX is the only viable candidate out of this pool of potential toxins. Also, it will be necessary to keep testing various toxins until several suitable toxin candidates are found. At that point, it will be important to start mixing toxins to see whether the mixtures act additively, negatively, or synergistically.

Finally, it will be important to test the effects of these compounds on other insects that may potentially be drawn to the sources of these toxins. In that case, particularly with respect to honeybees or other vital species, it will be necessary to make modifications to the compounds to ensure limited mortality upon these other species.

Additionally, the use of a CO_2 station will likely help prevent some of these deaths as honeybees and others are not naturally attracted to CO_2 whereas they are probably attracted to sugar bait station designs as done in previous studies. Also, it will be necessary to make sure that these toxins are presented in a manner that prevents exposure to humans, pets, birds, or other potentially large animal species which is possible considering previous research (Tedford et al., 2004b). The other route would be to make sure that the toxins are completely harmless against these animal species (2004b). Overall, these studies serve merely as a foundation to a potentially ground-breaking avenue of vector control. It is important, however, to note that this road is quite long and will require extensive exploration in the future. Yet, keeping in mind the losses caused by endemic diseases each year, this is definitely an important topic to pursue.

Mosquitoes alone permit the transmission of many diseases such as Yellow Fever, Malaria, Dengue Fever, and many others (Gubler, 2002). Increasingly, mosquitoes have been implicated in rising prevalence of West Nile Virus (Petersen and Roehrig, 2001) and Chikungynya, particularly in the Northern Hemisphere (Sourisseau et al., 2007). However, this research may be applied to other vectors as well. As it stands, arboviruses are among the most prevalent of zoonotic diseases with the exception of the global range of malaria (Gubler, 1998). As such, the research may be applicable to the many other vectors that carry disease in the world today. For example, the tse tse fly, which carries sleeping sickness or African Trypanosomiasis, is causing a reemergence of the disease in rural Africa (1998). Another vector to consider similar research for would be the tick, which spreads Lyme disease and other rickettsial agents (1998). Overall, this research is vital to the future control of many vector-borne diseases because there is resurgence as a

result of insecticide resistance, of societal instability in various regions, of lack of emphasis on prevention, and of evolution of pathogens (Lederberg et al., 1992). From a commercial perspective, research along these lines could serve as pest control for ants, cockroaches, and various others.

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