ABSTRACT

Levels of Attraction of *Aedes aegypti* and *Culex pipiens*
to Nectar of Plants Amenable to Transgenic Transformation:
Potential for the Development of Mosquitocidal Plants

Zhongyuan Chen, M.S.

Mentor: Christopher M. Kearney, Ph.D.

Controlling mosquito populations is critical for reducing mosquito-borne diseases. Methods such as pesticides and genetic engineering of mosquitoes have drawbacks. We proposed a novel delivery system for controlling mosquito populations: nectar, used as a delivery medium for transgenic proteins. In this project, candidate plant species were judged based on five criteria.

First, a survival assay was conducted to investigate the long term nutritional association between candidate plants and mosquitoes. Second, a solo plant attraction assay was used to more precisely observe whether or not mosquitoes ingested nectar from each plant species. Fourth, a plant competition assay was done to investigate mosquitoes' preference for the target plant in competition with other plant species. Finally, SDS-PAGE analysis of nectar from each plant species was conducted to study the composition and concentration of each protein in the nectar.

On all of the levels, *Impatiens walleriana* was demonstrated to be a superior plant species for a nectar delivery system to control mosquito populations.

Levels of Attraction of *Aedes aegypti* and *Culex pipiens*
to Nectar of Plants Amenable to Transgenic Transformation:
Potential for the Development of Mosquitocidal Plants

by

Zhongyuan Chen, B.S., M.S.

A Thesis

Approved by the Department of Biology

_____

Robert D. Doyle, Ph.D. Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

Approved by the Thesis Committee

_____

Christopher M. Kearney, Ph.D., Chairperson

_____

Cheolho Sim, Ph.D.

_____

Dennis A. Johnston, Ph.D.

Accepted by the Graduate School
May 2014

_____

J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate school.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I would like to address my appreciation to many people. This thesis would not have been possible without the support and help from them in many ways.

First and foremost, I would like to express my gratitude to Dr. Christopher M. Kearney. He, a patient, careful, and strict supervisor, assisted me to solve many academic problems, which made it possible for me to finish this thesis.

Furthermore, I would like to thank my committee members, Dr. Cheolho Sim for allowing me to work in his mosquito lab and Dr. Dennis A. Johnston for helping me with statistical analysis.

And also, I would like to thank Kearney Lab members for their help in the lab.

Of course, special thanks go to my parents for their spiritual support and love when I experienced a difficult time of my life.

Lastly, I want to give thanks to my friends in Waco. Thank you for the caring and friendship you offered.

DEDICATION

To my parents

CHAPTER ONE

Introduction

Background

Currently, Dengue virus and West Nile virus (WNV) are two major public health concerns in the world. The primary method of controlling these infectious diseases is to reduce *Aedes aegypti* (the dengue mosquito vector) and *Culex pipiens* (the West Nile virus mosquito vector) populations unless vaccines become available (CDC, 2010).

To date, many methods of reducing mosquito populations have been developed, such as using pesticides and genetically engineering mosquitoes (Hougard *et al.*, 2002). However, each has drawbacks. Pesticides, such as DDT, are detrimental to the environment (Feist *et al.*, 2005), and mosquitoes can rapidly develop resistance to them. Genetic engineering to produce sterile male mosquitoes is very expensive, because it requires the production and the release of large numbers of these insects (Benedict *et al.*, 2003). Furthermore, all of the methods are expensive when they are applied on a large scale.

Recently, local mosquito populations have been controlled by using oral pesticide-laden sugar baits developed by Muller and associates (Müller *et al.*, 2010).

This method is more effective than methods previously mentioned. Using Müller's technology as a foundation, we proposed to replace the pesticide with protein toxin specific to mosquitoes. With more permanent transgenic plants, there will be no need of repeated application. Male mosquitoes and females of some species depend entirely on plant nectars (Foster, 1995). Therefore, we proposed a novel delivery system for controlling mosquito populations: nectar, used as a delivery medium for transgenic proteins, such as hormones or lectins, disrupts the mosquito or pathogen life cycle.

To achieve that aim, plants highly attractive to mosquitoes are necessary. Studies have shown that some plants are attractive to mosquitoes, such as *Hamelia patens* Jacq, *Lantana camara* L., *Parthenium hysterophorus* L, and *Senna didymobotrya* Fresen (Manda *et al.*, 2010). However, these plants are not suitable for the development of transgenic mosquitocidal nectar systems. In our initial selection of candidate plants, we considered the following five published or easily observable properties: (1) highly attractive to mosquitoes; (2) nectar is easy for mosquitoes to access; (3) plants are readily transformed; (4) high levels of protein production in the nectar; (5) easily propagated and maintenance, and have commercial potential.

In order to choose initial plant species to examine, we hypothesized that plants that have a symbiotic relationship with ants might also have a nutritional relationship with mosquitoes. After extensive literature search, the following plant

species were selected based on the five criteria above. *Impatiens walleriana*, a garden and commercial plant with a great amount of extrafloral nectar, is easy for mosquitoes to access. A recent study showed this plant can be easily transformed (Dan *et al.*, 2010). *Ricinus communis* (castor bean), a toxic plant attractive to mosquitoes, has a transformation protocol (Malathi *et al.*, 2006). *Passiflora edulis* (passion flower) is easily transformable, though the nectar amount is not promising (Manders *et al.*, 1994). *Asclepias curassavica* (tropical milkweed), with a great volume of floral nectar, is also easy for mosquitoes to access. Milkweeds are known to be favored by mosquitoes (W. Foster, personal communication). There is also a regeneration protocol of milkweed (Pramanik *et al.*, 1986). *Campsis radicans* has been studied by a researcher. It needs strict environment for seed germination and seedling emergence (Chachalis, 2000). Also, it is attractive to ants, which indicated that it might be attractive to mosquitoes as well (Elias *et al.*, 1975).

Nicotiana (tobacco) species were also considered initially, because they are model plants, easily transformed, and have a good amount of Nectarin protein (Carter *et al.*, 1999; Carter *et al.*, 2004). Thornburg's group has reported on a nectary-specific promoter, *NEC1* promoter, which drives Nectarin I protein to secrete into the nectar of tobacco plants (Carter *et al.*, 2003). However, all 13 *Nicotiana* species examined were all rejected (Kearney, unpublished data) for various reasons. For example, *N. otophora and N. glutinosa* have good nectar

volumes but are too sticky and trap mosquitoes. *N. benthimiana* is toxic and has little nectar. *Nicotiana alata* has commercial potential and a lot of seeds but is impossible for mosquitoes to get into, because the floral tube is too long.

In this study, a survival assay was conducted to investigate the association between plant and mosquitoes. A no choice assay was done to demonstrate that mosquitoes successfully fed on plant nectar. A sucrose competition bioassay model was established, and based on this model a choice assay was performed to demonstrate mosquitoes' preference to plant species. The protein size and concentration from plant extrafloral and floral nectar were also determined by an SDS-PAGE. On all levels, *I. walleriana* was demonstrated to be the best plant species for a mosquito toxin nectar delivery system.

Preliminary Data

The preliminary data (Figure 1) showed that *I. walleriana* and *Campsis radicans* are the two most attractive candidate plant species to adult *Aedes albopictus. I. walleriana* appears to be especially attractive, because mosquitoes can live more than 40 days with it serving as their only nutritional source.

Nectar feeding by mosquitoes is an important parameter to evaluate each candidate plant. As a method to track feeding, mosquitoes were fed 10% sucrose dosed with fluorescein. Preliminary results indicated that a 1% concentration of fluorescein yielded oversaturation under blue light imaging. A 0.1% and 0.01%

concentrations saw a five day survival and a single death on the sixth day and 0.001% concentration saw no deaths during the six day test. All three concentrations showed readily detectable levels of fluorescence.

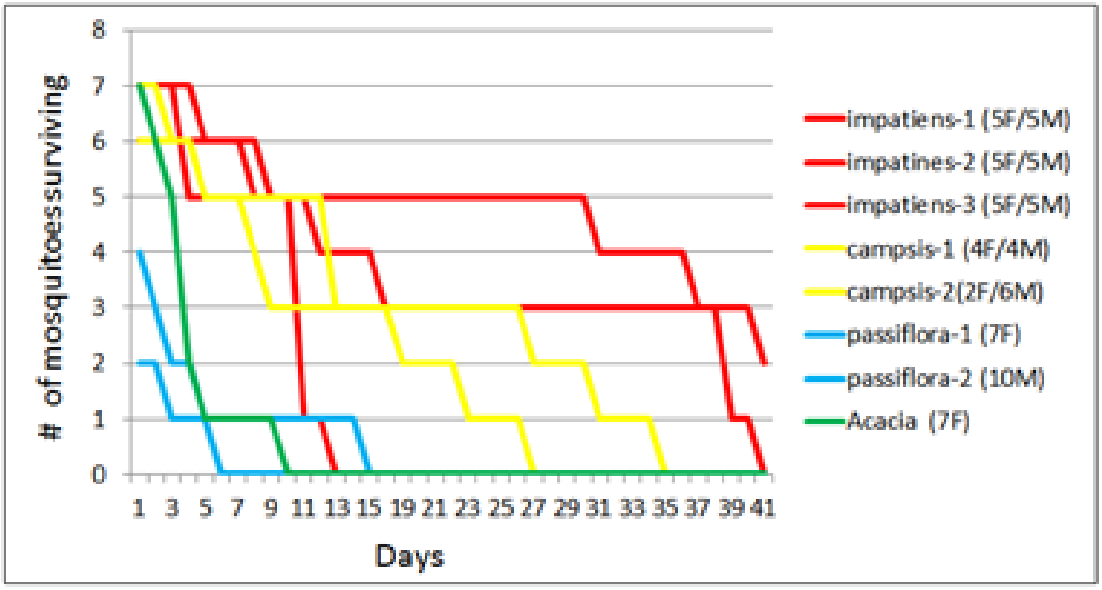

Figure1: Attractiveness of candidate mosquitocial plants to *Ae. albopictus* . The red lines represent 3 replicates of mosquito survival test with *Impatiens walleriana*. The yellow lines represent 2 replicates of mosquito survival test with *Campsis radicans*. The blue lines represent 2 replicates of mosquito survival test with *Passiflora edulis*. The green line represents the mosquitoes test with *Acacia mangium*. In each cage, there was one plant with 5female/5male mosquitoes.

CHAPTER TWO

Literature Review

Dengue Virus and Dengue Fever

Dengue virus causes dengue fever, an infectious tropical disease. Symptoms include headache, fever, muscle and joint pains, and a characteristic skin rash that is similar to measles. The dengue and *Ae. aegypti* are distributed globally (Figure 1) (Halstead, 2007). To date, there are no vaccines or therapeutics available (Mahalingam *et al.*, 2013). Therefore, additional strategies are needed to combat dengue. Dengue is transmitted largely by a mosquito species *Ae. aegypti* (Figure 2) (WHO, 2009; Halstead *et al.*, 2008; Rico-Hesse, 2012). Transmission-Blocking Antibodies against mosquito receptor, such as C-type lectins, was recently studied for dengue prevention (Liu Y, *et al.*, 2014). However, it is expensive for controlling mosquitoes, compared with our nectar system.

West Nile Virus and Diseases

West Nile virus causes many diseases, including West Nile fever (WNF), West Nile neuroinvasive disease (WNND), West Nile virus encephalitis (WNE), West Nile meningitis (WNM), West Nile meningoencephalitis, and West Nile poliomyelitis (WNP). Headache can be a prominent symptom. Until now, no vaccines are yet available (Ishikawa et al., 2014). These diseases can be transmitted via a number of

ways (Pradier *et al.*, 2012). A mosquito species, *C. pipiens* is a critical transmission vector in this process (Campbell *et al.*, 2002).

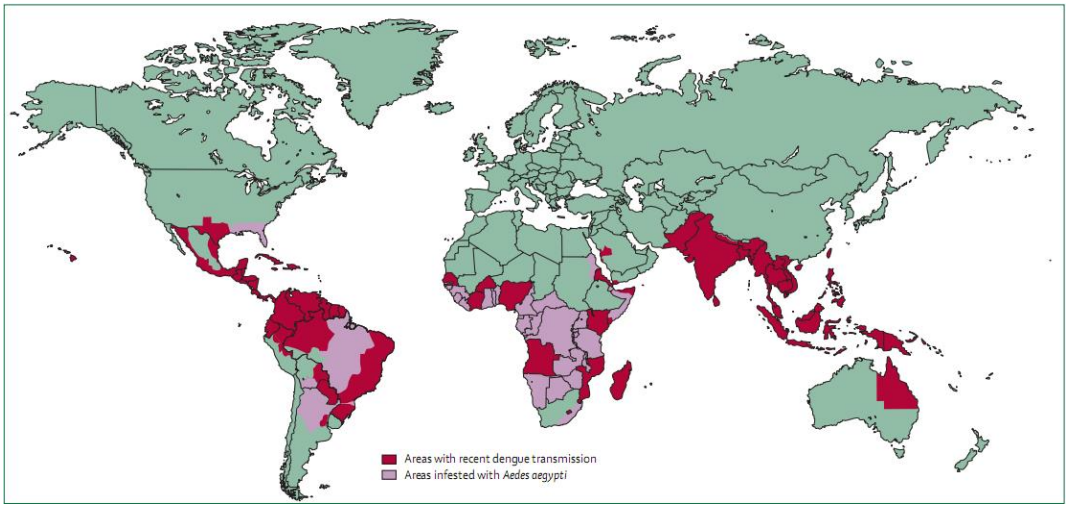

Figure 2. Approximate global distribution of dengue and *Aedes aegypti* in 2005 (Halstead, 2007)

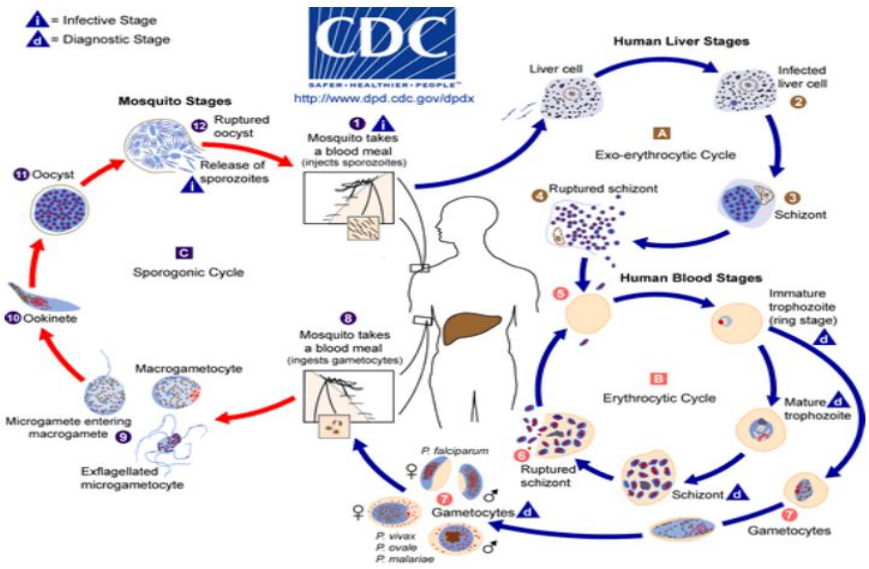

Figure 3. Dengue virus is transmitted through infected mosquitoes (Center of Disease Control, 2009).

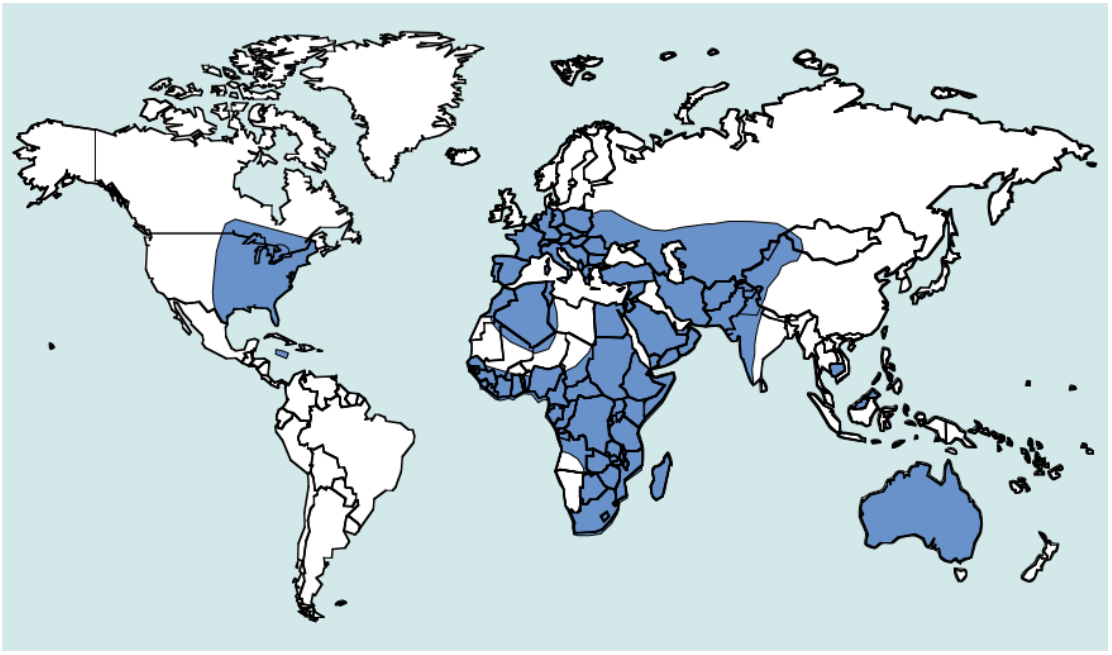

Figure 4. Approximate worldwide distribution (shown in blue) of West Nile (WN) virus and Kunjin virus, a subtype of WN virus (Campbell *et al.*, 2002)

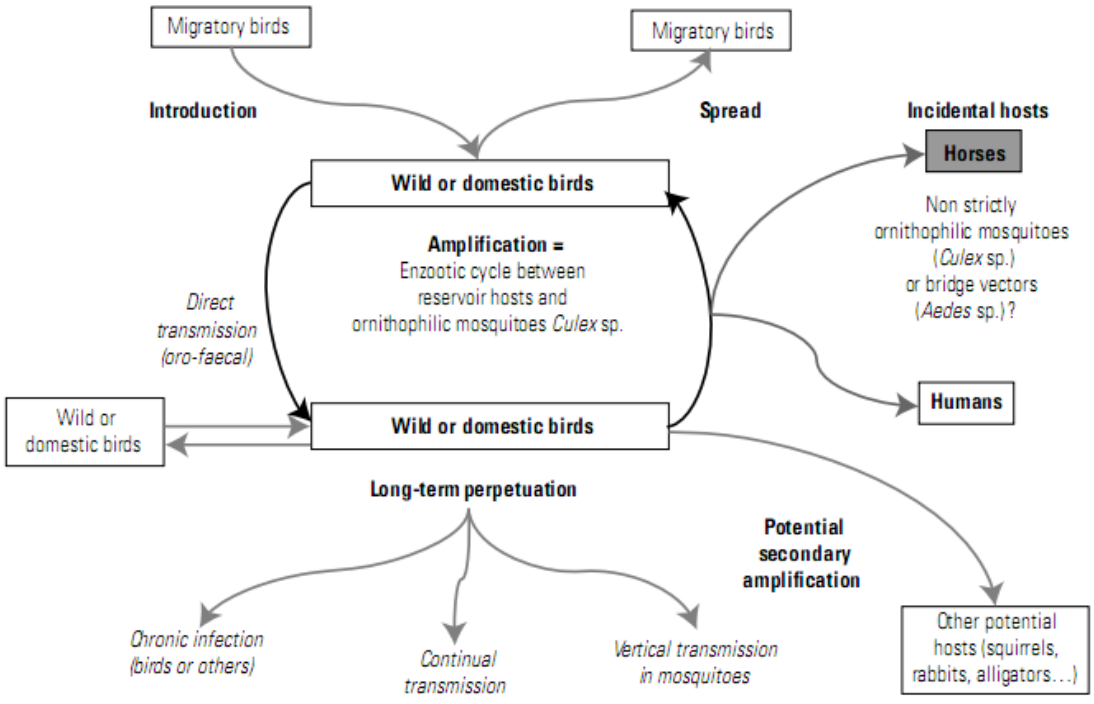

Figure 5. West Nile virus transmission cycle (Pradier *et al.*, 2012)

Plants Attractive to Mosquitoes /Nectar Feeding Habits

Mosquitoes commonly feed on plant nectar and other sugar sources. Sugar feeding is more frequent than blood feeding. Plants are attractive to mosquitoes because of olfaction and visualization (Foster, 1994). It was reported that *Ae. aegypti* (Gadawski and Smith, 1992; Martinez-Ibarra *et al*., 1997) and *Anopheles gambiae* (Gary and Foster 2004; Impoinvil *et al.*, 2004; Manda *et al.*, 2007) feed on floral nectar. For this reason, I propose to develop a method to express mosquito toxic protein in the floral nectar. When mosquitoes feed on the toxic nectar, their life span is expected to be reduced. Thus, this system could be a tool to control malaria or dengue transmission in the field (Collins and Paskewitz, 1995).

Agrobacterium Transformation of I. walleriana

Agrobacterium-mediated plant transformation is a common tool of plant genetic engineering (Gelvin, 2003). Impatiens (*I. walleriana*), a top selling floriculture crop, has been reported for *Agrobacterium*-mediated transformation with experimental evidence of stable integration of T-DNA and of *Agrobacterium*-mediated transformation method for plants used in vitro maintained multiple bud cultures as explants (Dan *et al.*, 2010).

CHAPTER THREE

Materials and Methods

Mosquito Rearing

Eggs of *Ae. aegypti* were supplied by Dr. Margaret C. Wirth (University of California Riverside, CA) and eggs of *C. pipiens* were from Dr. Cheolho Sim (Baylor University, TX). Both colonies were maintained at 27 ± 1°C, 80 ± 5% RH, and 13:11 (L:D). Adults were maintained in standard 30 ×30 × 30 cm mesh-covered cages and offered sugar cubes. Female *Ae. aegypti* were allowed to feed on six month-old mice for 1 hour during gonotrophic cycle. Oviposition cups with sterile water and filter papers were placed with caged adults 3 days after each blood meal, and eggs were collected the following two or three days. The collected *Ae. aegypti* eggs were then dispensed into plastic trays (25 cm long × 20 cm wide × 14cm high) filled with 1L aged tap water and liver powder. Female *C. pipiens* were allowed to feed on 1 to 3 day-old yellow chicks overnight. Oviposition cups with sterile water were placed in adult mosquito cages right after the blood meal, and egg rafts were collected. The collected *C. pipiens* eggs were dispensed into plastic trays (25 cm long × 20 cm wide × 14cm high) filled with 8cm height sterile water and fish food (Tetramin®). For experiments, the pupae were collected individually into plastic test tubes. Adults were used in the experiments right after emergence.

Plants

Seven plant species were selected as candidates for the study of mosquito attractiveness (Figure 6 and Table1). The following plants have published transformation protocols: *I. walleriana* (Dan *et al.*, 2010), *R. communis* (Malathi *et al.*, 2006), *C. radicans* (Aslam *et al.*, 2009) and *P. edulis* (Manders *et al.*, 1994). There was only a regeneration protocol (Pramanik *et al.*, 1986) for *A. curassavica*. Excellent extrafloral and floral nectar production is characteristic of several of these (Figure 6). All plants were cleared of potential predators (ants and other insects) before testing began.

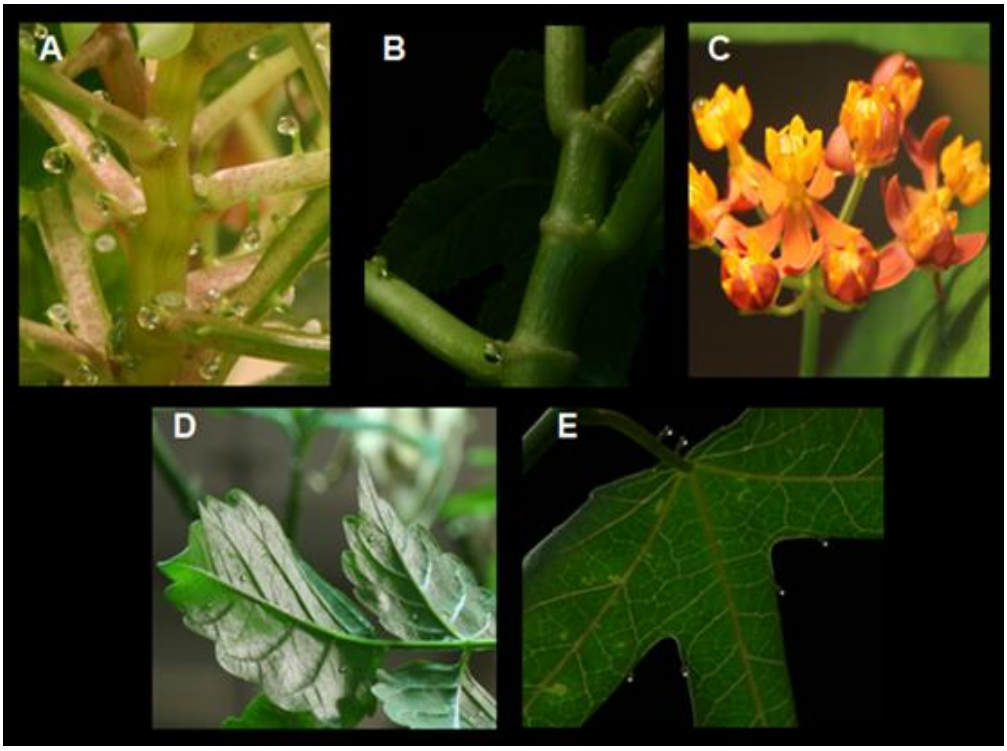

Figure 6. Plant species used in survival study of mosquitoes (plant photos from our growth room, Baylor Science Building, Baylor University, Waco, TX) (A) *I. walleriana* (B) *R. communis* (C) *A. curassavica* (D) *C. radicans* (E) *P. edulis*

Table 1. List and common names of plant species used in survival study of mosquitoes

Family	Species	Common name
Balsaminaceae	*Impatiens walleriana*	Impatiens
Euphorbiaceae	*Ricinus communis*	Castor Bean
Bignoniaceae	*Campsis radicans*	Red trumpet flower vine
Asclepiadaceae	*Asclepias curassavica*	Milkweed
Passifloraceae	*Passiflora edulis*	Passion flower
Solanaceae	*Nicotiana benthimiana*	Muntju tobacco
Amaranthaceae	*Beta vulgaris*	Beet

Survival Assay

The study was carried out in the plant growth room (Baylor Science Building, TX) at 28℃ with a 12 h photoperiod. Batches of newly emerged mosquitoes, each consisting of 10 males and 10 females, were put in each of the standard cages (13" cube with sleeve or 2 sleeves 14 X 14 X 24", Bioquip, CA). Randomly constituted groups of mosquitoes were exposed to each of the seven plant species in the cages above. For tall plants, like milkweed, 13" cube mosquito cages covered the flowers and upper part of the plant stems and contained separate soil pots inside to provide water for the mosquitoes. In control groups, mosquitoes were allowed continuous access to either a 10% (wt:vol) sucrose solution with cotton (positive control) or to

only water (negative control). There was also a second negative control (a group deprived of sugar and of water). The plants tested were watered every day. Sucrose and water were changed every two days. The mosquitoes were kept on different nutritional regimes, and the number of living mosquitoes was monitored daily for a 20 day period. All tests were replicated five to six times.

Assessment of Plant Nectar-Feeding Success

Solo Plant Assay (No Choice Assay)

This non-competitive plant feeding assay was used to investigate whether mosquitoes ingested nectar from each plant species, with no alternative plant species present at the same time. Red food dye was applied on each drop of plant extrafloral nectar from *I. walleriana, R. communis,* and *C. radicans* in each individual cage. Ten female and ten male adult mosquitoes were exposed to a single plant in the cage. The number of red mosquitoes was counted each day for the first three days. The best nectar feeding plant species was ascertained by the number of dyed mosquitoes.

Mosquito Preference for Different Plant Species

Mosquito Bioassay of Food Dye (Competition)

This was a model competitive assay in which mosquitoes were enclosed in a plastic cup with a 10% sucrose solution. A drop of red food dye was mixed with 1ml 10% sucrose for each tube. Four bioassay cages were set for this study with increasing numbers of clear 10% sucrose tubes, which means that the ratio of dyed sucrose to clear sucrose was 1, 1:1, 1:3 and 1:5. Each tube contained 100µl of solution, either dyed 10% sucrose or clear 10%sucrose. The number of red mosquitoes was counted each day for the first three days. All tests were replicated three times.

Plant Competition Study (Choice Assay)

This was a competitive plant nectar-feeding assay in which plants and mosquitoes were enclosed in a mesh-covered cage (Rearing & Observation Cage, 24 x 24 x 36" 1466C, Bioquip, CA). *I. walleriana, R. communis, C. radicans* and 10% sucrose (positive control) were used in the plant choice assay. Red food dye was applied on each drop of the extrafloral nectar of the central plant surrounded by other plant species. The number of red mosquitoes was counted every day in each cage for three days. All tests were replicated three times.

Statistical Analyses

Survival analysis techniques (JMP version 10.0.0), including Log-Rank and Wilcoxon were used to compare survival curves and to test whether the survival rate differed between different nutritional regimes. The data was confirmed by implementing in R 3.0.2 (22) (R Core Team, 2013).

The significant difference of each individual survival curve was calculated by Bonferroni correction (multiple comparisons) (Dunnett, 1955). For this test, the day of death for both female and male adult mosquitoes was recorded. Differences among replicates of experiments were also analyzed individually and were found to be trivial, so the data sets were combined. The data from the solo plant assay, sucrose bioassay and competition assay, including the average of replicates and standard deviation (error bar) were analyzed in Excel 2010. Contingency analysis (Pearson test) was carried out to test the significance ($p<0.001$) in JMP.

Nectar Collection and SDS-PAGE

Extrafloral nectar from *I. walleriana, R. communis* and *C. radicans* and floral nectar from *A. curassavica* were collected in microcentrifuge tubes individually. When collecting nectar from any plant, the nectar was immediately diluted 1:3 with water and stored at -20C. To concentrate the nectar, 100 µl of diluted nectar was combined with 900 µl of cold 100% ethanol, iced for 15 min and then centrifuged at room temperature at 16,000 x g in a microcentrifuge. This deviated from

Thornburg's protocol (Carter *et al.*, 1999), which used 1 ml of pure nectar mixed with 9 ml of cold ethanol centrifuged at 65,000 x g for 20 min, presumably at 4 C. The nectar was resuspended in 10 µl of 10 mM sodium phosphate buffer (pH 7.4). 10 µl or 2 µl of concentrated nectar protein was then loaded into each well of the SDS-PAGE gel.

CHAPTER FOUR

Results

How Different Plant Species Affected Ae. aegypti *and* C. pipiens' *Survival*

The proportion of mosquitoes surviving over time varied significantly among the nutritional regimes (*Ae. aegypti*: $\chi 2 = 1209.901$, DF= 10, $P < 0.0001$; *C. pipiens*: $\chi 2=1254.828$, DF=10, $P < 0.0001$) (Figure 7). Both *Ae. aegypti* and *C. pipiens* adult mosquitoes that were exposed to *I. walleriana* had consistently higher survival rates than they were exposed to other testing groups (Figure 7 and Figure 8), including the 10% sucrose group. Survival rate declined rapidly in the negative controls. Survival rate also declined rapidly when mosquitoes were exposed to *N. benthimiana* and *B. vulgaris*, with 50% of the mosquitoes dying by day 3 (Figure 7 and Figure 8). Overall, the ranking of survival rates of *Ae. aegypti* on the various plant species was as follows (from highest to lowest): *I. walleriana*, *A. curassavica, C. radicans*, 10%sucrose, *P. edulis*, *R. communis*, *N. benthimiana*, *B. vulgaris*. For *C. pipiens,* the ranking was slightly different: 10% sucrose, *I. walleriana*, *C. radicans*, *A. curassavica, R. communis*, *P. edulis.*

Mosquitoes Successfully Feed on Extrafloral Nectar---Solo Plant (No Choice Assay)

We found that the plant extrafloral nectar was taken up by mosquitoes quickly, because the abdomen of mosquitoes became red after feeding on red dye

dosed plant extrafloral nectar. In detail, 80% *Ae. aegypti* became red after

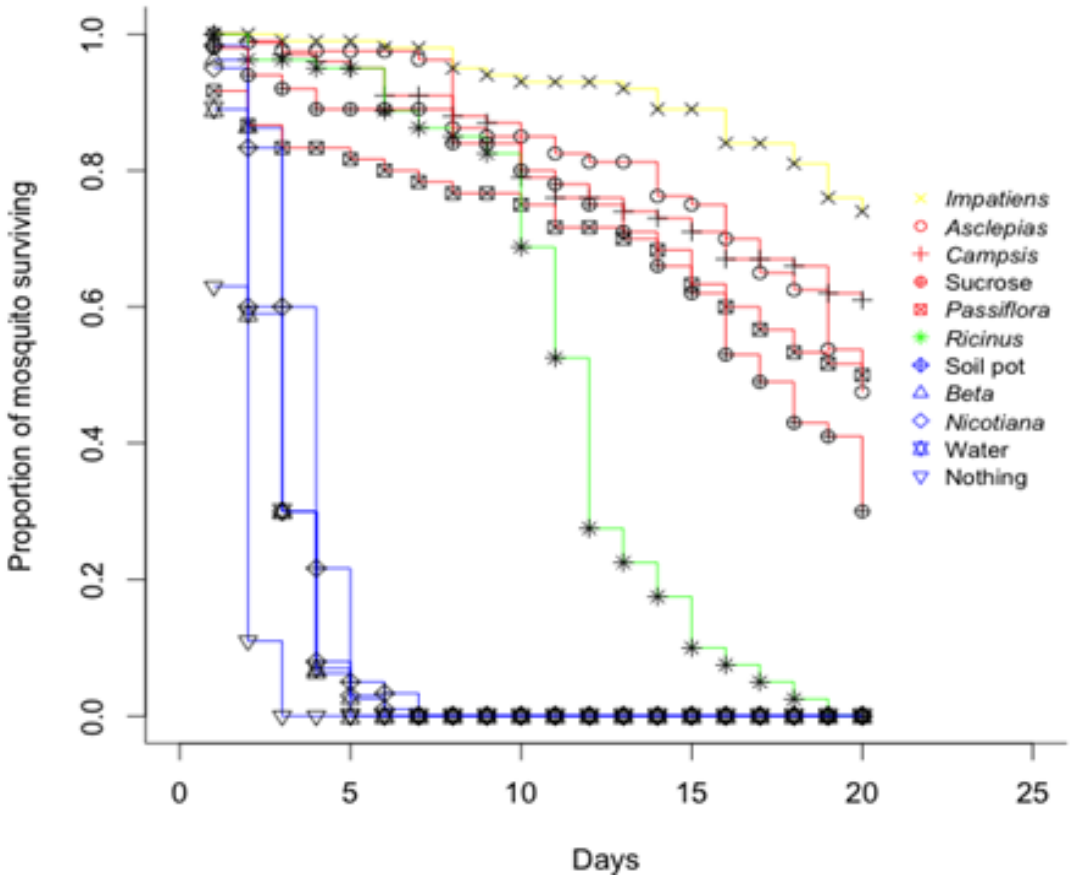

Figure 7. Survival curves of *Ae. aegypti* mosquitoes in cages with different nectar plants. Twenty mosquitoes (10 m, 10 f) were introduced to a cage containing a single potted plant expressing extrafloral or floral nectar. Controls included plants which did not produce accessible nectar (*Nicotiana benthimiana*, *Beta vulgaris)* or a single soil pot without a plant, a tube of water or 10% sucrose, or no substrate at all (empty cage). Surviving mosquitoes were counted each day and 5 to 6 replicate cages were tallied for each treatment (mean and SE displayed in Table 2). Each color represents one group. Each one in a group was statistically different from any one in other groups ($p < 0.0001$).

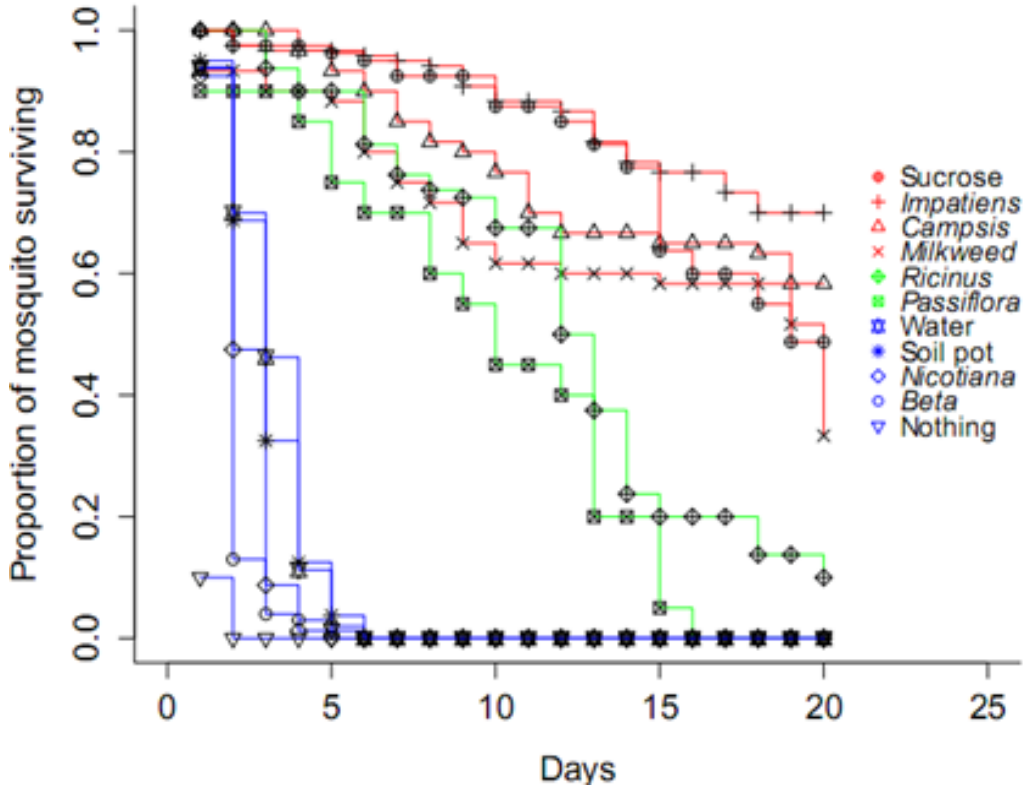

Figure 8. Survival curves of *C. pipiens* mosquitoes in cages with different nectar plants. See caption for Figure 7.

Table 2. Survival times in days of *Ae. aegypti* exposed to different nutritional regimes

Plant species	Mean [a]	SE[b]	Significant difference[c]
Impatiens	18.6	0.35	a
Asclepias	16.9	0.54	b
Campsis	16.5	0.55	b
sucrose	15.3	0.58	b
Passiflora	14.8	0.92	b
Ricinus	11.4	0.39	c
soil pot	3.72	0.16	d
Beta	3.21	0.11	d
Nicotiana	2.97	0.10	d
water	2.85	0.11	d
Nothing	1.74	0.065	d

[a] Mean number of days at which mosquito population reduced by half ; [b]SE, standard error of the mean; [C] Data sets denoted with significant difference from each other ($p<0.0001$); a, b, c, and d as in Figure 3. Group a, b, c, and d were statistically separated.

Table 3. Survival times in days of *C. pipiens* exposed to different nutritional regimes

Plant species	Mean	SE	Significant difference
Sucrose	16.2	0.48	a
Impatiens	16.1	0.35	a
Campsis	15.3	0.70	a
Milkweed	14.4	0.91	a
Ricinus	12.1	0.56	b
Passiflora	9.60	1.05	b
ddwater	3.23	0.13	c
Soil pot	3.13	0.13	c
N.benthimiana	2.50	0.089	c
Beet	2.16	0.076	c
Nothing	1.10	0.034	c

See caption for Table 2. a, b and c stand for three groups based on significant difference.

feeding on the red nectar of *I. walleriana* overnight, which is significantly higher than when they fed on *R. communis* and *C. radicans*($P<0.0001$). The number of red *Ae. aegypti* (45.00%) that fed on *C. radicans* was significantly higher than the number that fed on *R. communis* (13.35%) ($P<0.0001$) (Figure 9). *C. pipiens* (93.35%) became red after feeding on the red nectar of *I. walleriana* overnight, which is

significantly higher than the number of mosquitoes that fed on *R. communis* (45.00%) and *C. radicans* (48.35%) ($P<0.0001$), but there was no significant difference between the results for *R. communis* and *C. radicans* ($P>0.05$) (Figure 10).

Mosquito Preference to Easy Transformable Plant Species---Competition (Choice Assay)

Sucrose Competition

In order to investigate mosquitoes' preference to different plant species, we established a sugar competition model (Figure 11). This is a base line for plant competition study. We hypothesized that mosquitoes have no preference to red dye and the significant difference of red dyed mosquitoes indicates their

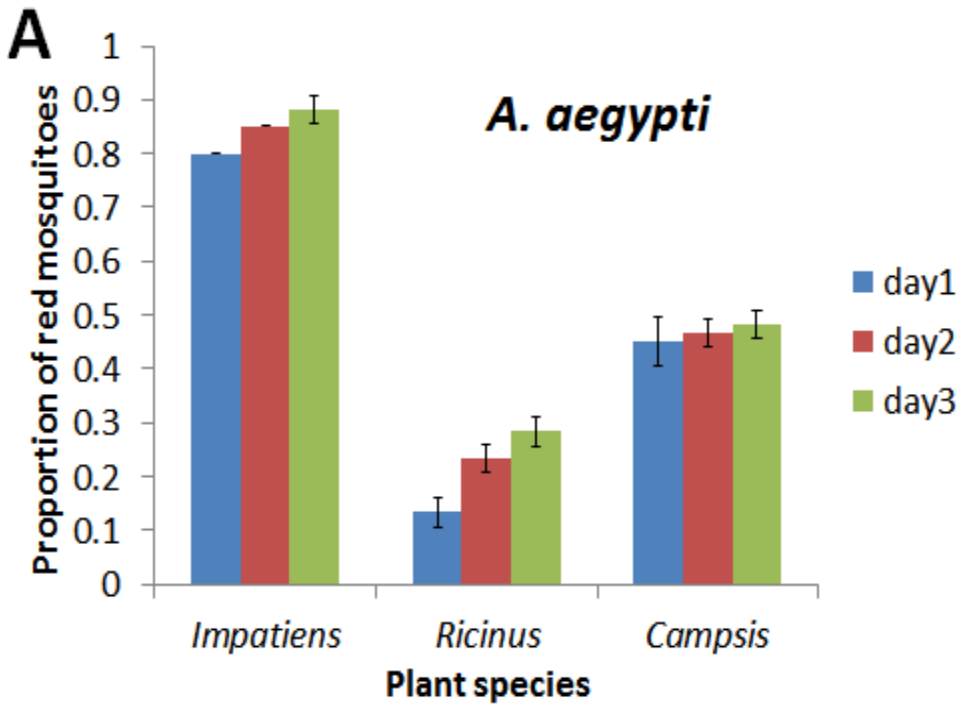

Figure 9. Proportion of *Ae. aegypti* sampling dyed nectar from a single plant in a cage (solo plant attractiveness assay) Red food dye was added to nectar on extrafloral nectaries of single plant, which was then placed in a cage with 20 *A. aegypti* mosquitoes (10 f, 10 m). The number of mosquitoes with a red thorax or abdomen was counted each day for three days, with three replicate cages for each plant species. There was a significant difference ($p<0.0001$) between plant species but no difference between days within one plant species.

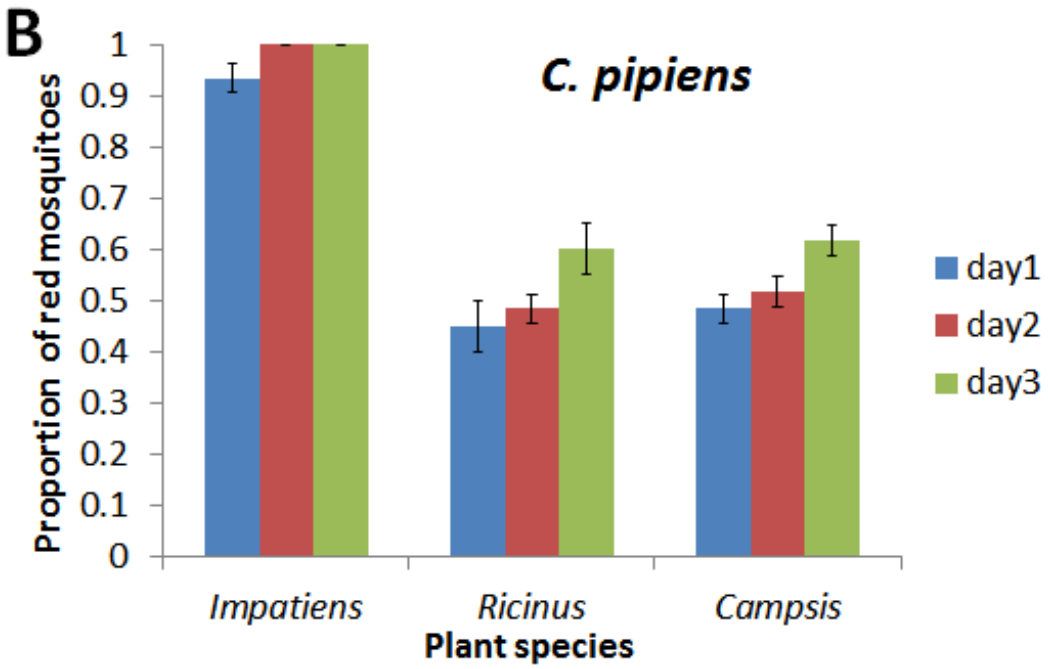

Figure 10. Proportion of *C. pipiens* sampling dyed nectar from a single plant in a cage (solo plant attractiveness assay) The same experiment as shown in Figure3a was used with *C. pipiens*. The proportion of red mosquitoes in the presence of *I. walleriana* was significantly higher than in the presence of *R. communis* and *C. radicans* ($P<0.0001$). There was no significant difference of red mosquitoes between *R. communis* and *C. radicans.* Similarly, there was no significant difference between days within one plant species.

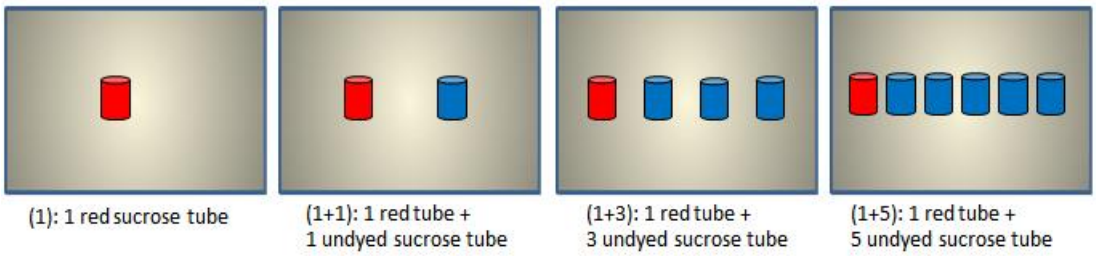

Figure 11. Sucrose control experiment for the competitive attraction assay: schematic diagram of sucrose control experiment.

preference to different plant species. The results showed that the number of red mosquitoes decreased as more clear 10% sucrose tubes were put into the mosquito cage. As we expected, there was no bias for or against red dye. Anything above 1/6 of solo uptake rate indicated no preference on red dye (Figure 12 and Figure 13).

Plant Competition (Choice Assay)

A plant attractiveness competition assay was conducted to study mosquitoes' preference for different plant species (Figure 14). The results showed that both *Ae. aegypti and C. pipiens* preferred *I. walleriana* significantly to *C. radicans*, 10% sucrose and *R. communis* ($P<0.0001$). There was no significant difference between *C. radicans* and 10% sucrose but both are significantly higher than *R. communis* ($P<0.0001$) for both *Ae. aegypti and C. Pipiens* (Figure 15 and Figure 16).

Protein Composition and Concentration in the Nectar Among Plant Species

The size and concentration of proteins in the nectar varied among the plant species (Figure 17, Table 4). *I. walleriana* has a 20kDa protein, with a concentration of 3.503µg/µl, in nectar. *C. radicans* has a 48kDa protein, with a concentration of 0.3457µg/µl, in nectar. *P. edulis* has a 22kDa protein, with a concentration of 0.4191µg/µl, in nectar. Those were the three highest concentrations of protein in nectar.

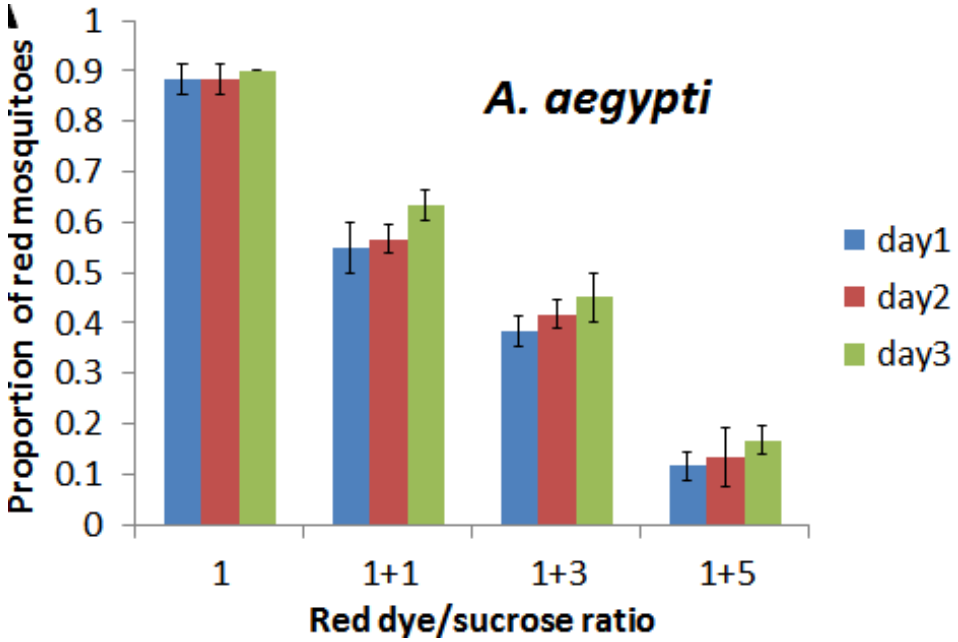

Figure 12. Sucrose control experiment for the competitive attraction assay (*Ae. aegypti*) *Ae. aegypti* Red food dye was added to 10% sucrose (1 drop/1ml), 100ul of which was put into a tube and then placed in a cage with 20 *A. aegypti* mosquitoes (10 f, 10 m). (1): 1 red sucrose tube; (1+1): 1 red tube + 1 undyed sucrose tube; (1+3): 1 red tube + 3 undyed sucrose tube; (1+5): 1 red tube + 5 undyed sucrose tube. The number of mosquitoes with red thoraxes or abdomens was counted each day for three days, with three replicate cages for each treatment. Error bar indicated standard error (SE). There was a significant difference ($p<0.0001$) between 4 different treatments but no difference between days within one treatment.

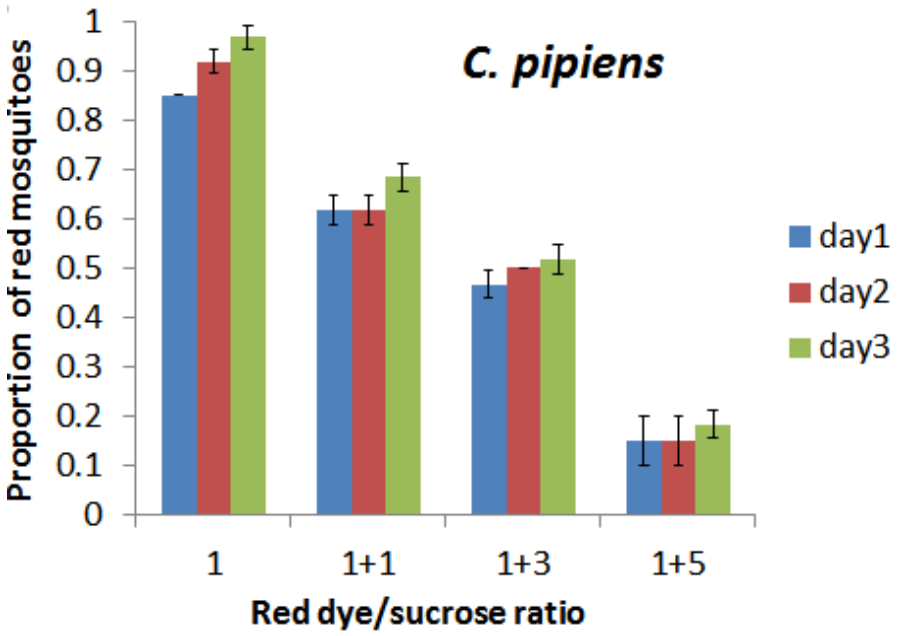

Figure 13. Sucrose control experiment for the competitive attraction assay (*C. pipiens*) *C. pipiens* the same caption as Figure 11.

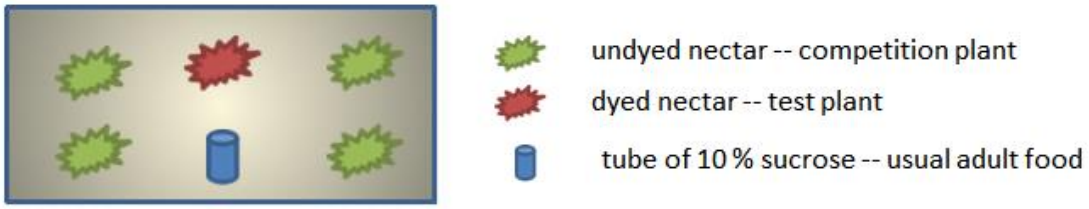

Figure 14. Competitive attractiveness assay: schematic diagram of plant competition study.

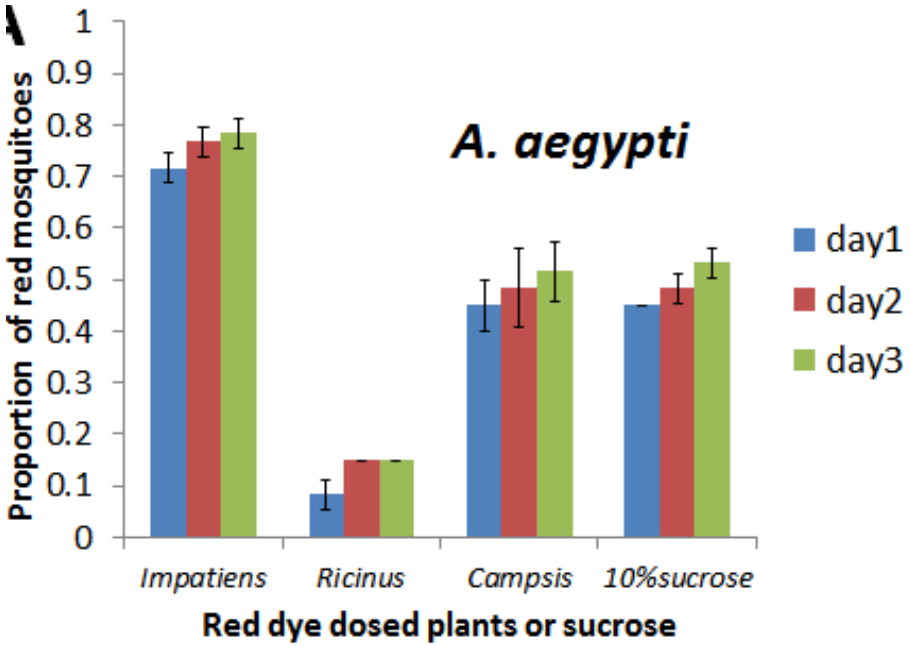

Figure 15. Competitive attractiveness assay (*Ae. aegypti*) *Ae. aegypti* Red food dye was added to nectar on extrafloral nectaries of single central plant, together with other plant species without red dye, which were then all placed in a cage with 20 *A. aegypti* mosquitoes (10 f, 10 m). Central dosed plant species is *I. walleriana*, *R. communis* or *C. radicans* (they are all extrafloral nectar plants), including 10% sucrose as control. Other plants included *A. curassavica*, *B. vulgaris* and *N. benthamiana*. The number of mosquitoes with red thoraxes or abdomens was counted each day for three days, with three replicate cages for treatment. Error bar indicated standard error (SE).

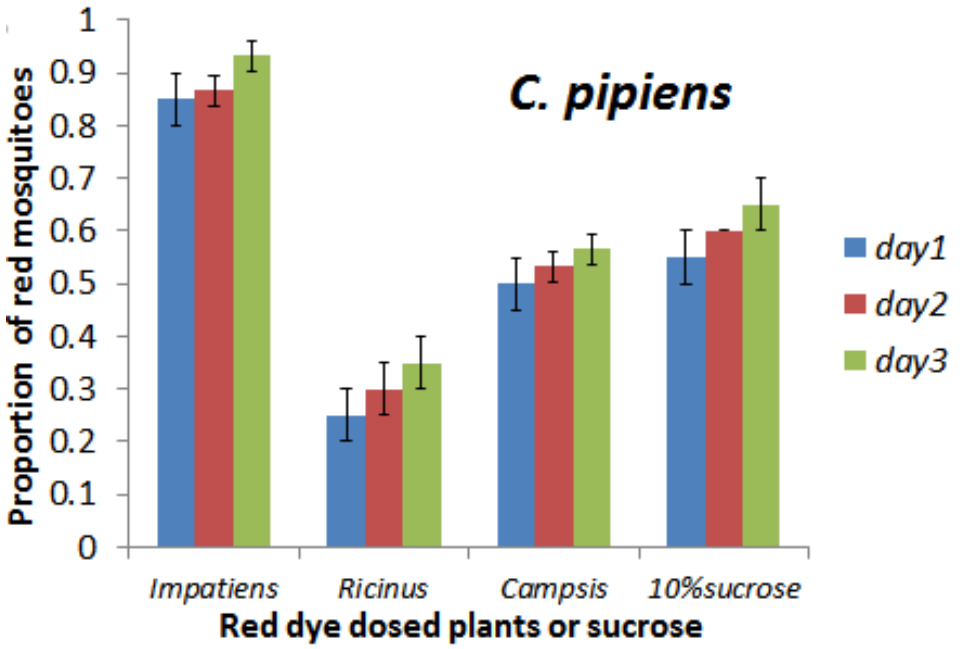

Figure 16. Competitive attractiveness assay (*C. pipiens*) The same caption as Figure 14.

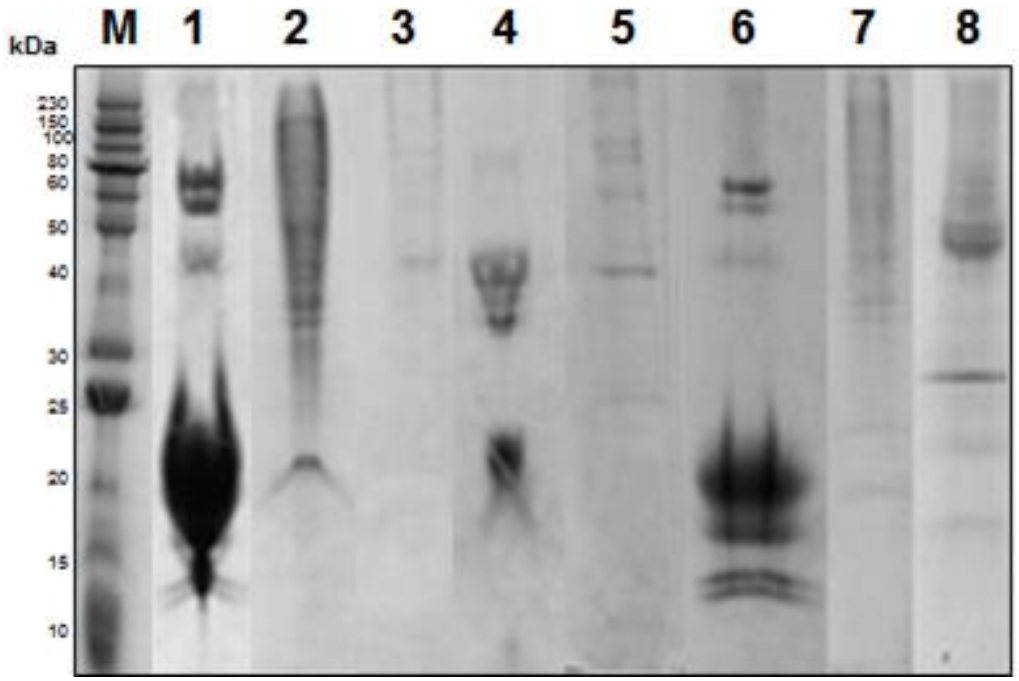

Figure 17. SDS-PAGE of plant extrafloral and floral nectar. Lanes: M, protein marker; ethanol precipitated nectar was ran on all lanes. Lane1 to 5 have equivalent 15 µl pure nectar. 1, *I. walleriana;* 2, *R. communis*; 3, *C. radicans*; 4, *P. edulis*; 5, *N.tobacco* nectar; lane 6 (*I. walleriana*) and 7 (*R. communis*) have equivalent 3 µl pure nectar; lane 8, 10 µl ethanol concentrated (10 times) nectar of *C. radicans* from one pod.

Table 4. Specific protein size and concentration from plant extrafloral and floral nectar

Plant species	Lane on SDS-PAGE	Protein size (kDa)	Protein mass (µg)	Pure nectar (µg/µl)
I. walleriana	Lane 6	80	1.289	0.4296
		20	10.50	3.503
		15	2.230	0.7432
R. communis	Lane 2	20	0.5220	0.0348
P. edulis	Lane 4	40	2.150	0.1433
		22	6.286	0.4191
C. radicans	Lane 8	48	5.186	0.3457
		27	0.7460	0.04973
N. tobacco	Lane5	40	0.4325	0.02887

CHAPTER FIVE

Discussion

In nature, both female and male mosquitoes feed on plant nectar (Foster, 1994). We proposed to use this property to control mosquito populations. If an oral peptide toxin could consistently be expressed in the nectar, it would decrease mosquito populations in the field. To make this application possible, the first step would be to find a mosquito-attractive plant which is also easily transformed. There has been reported a number of mosquito-attractive plants (Müller *et al.*, 2010; Gouagna *et al.*, 2013; Chauhan *et al*., 2012; Nyasembe *et al.*, 2012; Singh *et al.*, 2012). However, none of these are genetically transformable. To expand the number of candidate plants beyond those actually evaluated for mosquito attraction, we made a hypothesis that plants with an ant-plant symbiosis with nectar would be good candidates for mosquito attractiveness. Studies indicated many cases of ant-plant symbiosis (Blatrix *et al.*, 2013, Ness *et al.*, 2009, Schlein *et al.*, 1995). Ant activity clearly demonstrates the ant-guard symbiosis usually associated only with tropical or subtropical species (Elias *et al.*, 1975). A study showed that *P. edulis* had mutualism with ants (J., A. *et al.*, 2001). *R. communis* (Schlein *et al.*, 1995) was reported to be attractive to *C. pipiens*. Another study demonstrated the coexistence

of three specialist aphids with the milkweed, *Asclepias syriaca* (Smith *et al.*, 2008). *I. walleriana* has been shown to be attractive to ants as well (Lanza *et al.*, 1993). Transformation protocols for these plants have been developed by a number of researchers (Manders *et al.*, 1994; Dan *et al.*, 2010; Malathi *et al.*, 2006). Thus, these plant species were chosen for the following mosquito tests.

The survival assay demonstrated a long term association between mosquitoes and plants. Plant species, such as *I. walleriana*, *C. radicans* and *A. curassavica,* showed high attractiveness to both *Ae. aegypti* and *C. pipiens*. It was reported that *Culex* mosquitoes fed on a wide range of nectars consisting of mostly carbohydrates and amino acids (Vrzal *et al.*, 2010), so we predicted that the nectar was a major nutritional source for mosquitoes in this assay. The survival assay also indicated that mosquitoes preferred different nutritional regimes, because sustainability differed significantly. *Ae.aegypti* that fed on *I. walleriana* had a significantly higher survival rate than those fed with 10% sucrose ($p<0.001$). This preliminarily suggested the potential application in our nectar toxin delivery system. Most previous studies of mosquito survival and plant preference were conducted with *Anopheles gambiae* (Manda *et al.*, 2007). Few literature sources mentioned *Ae. aegypti* or *C. pipiens* in survival and attractiveness assays. Thus, our work was the first to contribute to preventing Dengue and West Nile virus transmission.

Furthermore, the solo plant assay (no choice assay) directly demonstrated that mosquitoes imbibed the plant extrafloral quickly. The no choice assay also suggested mosquitoes probably have a preference for some of the plant species, such as *I. walleriana*, based on the ranking of survival and solo plant assay results. The ranking of no choice assay remarkably had the same pattern as the ranking of mosquito survival assay in *Ae.aegypti: I. walleriana* > *C. radicans* > *R. communis*. In *C.pipiens,* the pattern of ranking was slightly different: *I. walleriana* > *C. radicans = R. communis.* There was no significant change of the proportion of red mosquitoes each day in the first three days.

In addition, the plant competition assay (choice assay) proved mosquitoes' preference for some of the plant species, especially *I. walleriana*. The sucrose competition model showed that there was no bias using the red dye. Based on this model, we established the plant competition assay. As we expected, the ranking of the feeding preferences was also extremely similar to the previously established survival of mosquitoes following exposure to plants and no choice assay. Again, no change of the number of red mosquitoes was seen each day in the first three days. In another study, choice assay was performed by observing the number of perching and feeding mosquitoes (Manda *et al.*, 2007). This method was labor intensive and time consuming. In this paper, we established a novel method--testing dyed

mosquitoes in competition assay--to study mosquitoes' preference to different plant species.

The SDS-PAGE data also proved the great possibility of the development of transformable mosquitocidal plants. The size and concentration of protein in the nectar were important. A small size of Nectarin protein fused with a mosquito toxic protein will be technically easier to express in the nectar. A high yield of Nectarin protein indicated high yield of Nectarin/toxin fusion protein, which makes it possible for the transgenic plants to decrease mosquito populations in the field. In young *I. walleriana* extrafloral nectar, there is a clear 60kDa protein, the size of which was similar to that of Nectarin 4 or Nectarin 5 (Carter *et al.*, 2004). There was also a small protein, 20kDa, in the nectar of *I. walleriana*. The concentration of this smaller protein was very high, 3.503 μg/μl in pure nectar of *I. walleriana.* The smaller protein indicated that Nec/toxin fusion protein would be easily expressed in the nectar, which showed that *I. walleriana* could be an ideal plant for producing mosquito toxic proteins in the nectar.

Therefore, we proposed that *I. walleriana* will be a potentially ideal plant species for the nectar delivery system to control mosquito populations because of the following reasons: 1.As demonstrated by survival assay, *I. walleriana* was surprisingly highly attractive to mosquitoes. 2. According to the no choice assay, mosquitoes fed better on *I. walleriana* than on sucrose. 3. Based on the competition

study, mosquitoes preferred to feed on the *I. walleriana* nectar. 4. *I. walleriana* is also an easily transformable plant species (Dan *et al.*, 2010), which preliminarily indicated a field application of controlling mosquito populations. 5. *I. walleriana* has a large amount of protein in the nectar based on the SDS-PAGE result. 6. *I. walleriana* extrafloral nectar was easy for mosquitoes to access. 7. *I. walleriana* is commercially potential because it is a top selling floriculture crop (Dan *et al.*, 2010).

Other plant species, such as *C. radicans* and *R. communis,* could also potentially be used in our nectar delivery system, though they will not be as effective as *I. walleriana*. The drawback of both plant species was a low nectar protein yield, according to the SDS-PAGE results.

All the studies in this paper provided critical information for using a nectar delivery method for mosquito vector control. We are currently investigating each individual crystal toxicity in *Bacillus thuringiensis* subsp. *israelensis* (Bti) and *Bacillus thuringiensis* subsp. *jegathesan* (Btj), since they are specifically toxic to adult mosquitoes when imbibed (Klowden *et al.*, 1983; Klowden *et al.*, 1984; Stray *et al.*, 1988). The mosquito-specific toxin Cry 4B killed 66.67% adult *Ae. aegypti* orally at day 6 at the protein concentration of 0.005275μg/μl (Chen & Kearney, unpublished data), which is much less than 3.503 μg/μl. Therefore, we expect that it will be feasible to use the transgenic Cry/Impatiens to create mosquito specific toxic nectar to control mosquito populations in the field.

CHAPTER SIX

Conclusions and Future work

Conclusions

The mosquitocidal nectar system is a novel way to control mosquito populations in the field. The purpose of this project is to find, if possible, easily transformable plants that are attractive to mosquitoes. Candidate plant species were chosen to test *Ae*. *aegypti* and *C*. *pipiens*. A survival assay demonstrated mosquito-plant long term association. A solo plant assay indicated that mosquitoes quickly took up the nectar. Using a sucrose competition study as a foundation, a plant competition study was conducted to show the mosquitoes' preference for plant species. A SDS-PAGE demonstrated the high yield protein in nectar. On all of the levels, *I. walleriana* is the most ideal plant species for a mosquitocidal nectar system.

Future Work

The work described in this thesis has been related with plant attractiveness to mosquitoes, which is the first step to creating a mosquitocidal nectar plant. In order to achieve the final goal, a number of interesting works need to be finished.

1. Find an Efficient Toxic Protein that Specifically Controls Mosquito Populations.

Bacillus thuringiensis subsp. *israelensis* (Bti) and *Bacillus thuringiensis* subsp. *jegathesan* (Btj) are two bacterial subspecies which are specifically toxic to mosquito larvae. There are four proteins produced by Bti: Cry11A, Cry4A, Cry4B and Cyt1A (Federici *et al*., 2003; Park *et al*., 2005; Federici *et al*., 2007). The 70kDa Cry11A protoxin can be processed in-vitro into 36 and 32 kDa fragments by trypsin (Yamagiwa *et al.*, 2002; Yamagiwa *et al*., 2004). I propose that these two active forms of Cry11A could be inserted into a nectary promoter construct together for nectar expression.

Other Bt subspecies also contain unique Cry proteins. The 81kDa Cry11Ba toxin is from Btj and its toxicity to mosquito larvae is ten times higher than Cry11A (Delecluse *et al.*, 1995). PG14, a parasporal body of *B. thuringiensis* ssp, *morrisoni*, has an extra 144kDa protein other than the four Bti proteins, and is also highly toxic to mosquito larvae (Padua and Federici, 1990).

Strong synergism of larval toxicity was reported between the Cyt and Cry proteins due to the different receptors for each. Cadherin is a specific receptor that is necessary for Cry toxin action (Likitvivatanavong *et al.*, 2011). Cyt toxins directly interact with membrane lipids, inserting into the membrane and forming pores or destroying the membrane via a detergent-like interaction (Gomez *et al.*, 2007). Cyt1A increases the level of Cry11A toxicity to ten times of Cry11A toxicity itself (Federici *et al*., 2007).

However, all the studies above examined larval toxicity. Only the Bulla research group has published evidence that Bti kills adult mosquitoes by using a Bti crystal mixture (Klowden *et al*., 1983; Klowden and Bulla 1984; Stray *et al*., 1988). It is not known exactly which protein or combination kills adult mosquitoes; therefore, we need to investigate the toxicity of Bti, and Btj single proteins, and various combinations to adult mosquitoes. Furthermore, synthesized genes, driven by a nectary specific promoter, should be inserted into transgenic Impatiens.

2. Establish Mosquitocidal Nectar *Impatiens* System as a Mosquito Toxin Delivery System.

The *nec1* promoter was investigated by Dr. Robert Thornburg, Iowa State University. This powerful promoter is used to engineer modifications in plant nectars that should produce novel secreted biochemicals (Carter and Thornburg 2003). To make our *Impatiens* nectary delivery system possible, it is critical to isolate Impatiens extrafloral nectary promoter first.

There are five Nectarin proteins which accumulate in *Nicotiana* nectar (Carter *et al*., 1999; Carter and Thornburg 2004, Naqvi *et al*., 2005). Among these nectar proteins, NecI has been well studied and is the most highly expressed (Carter *et al.*, 1999; Carter and Thornburg 2000).

I hypothesize that recombinant protein NecI/Cry can be expressed in sufficient quantities and activities in the extrafloral nectar of *Impatiens* to kill or significantly affect reproductive viability of adult mosquitoes.

LITERATURE CITED

Centers for Disease Control and Prevention (CDC), 2010 http://www.cdc.gov/dengue/; http://www.cdc.gov/westnile/index.html).

Aslam, M., Singh, R., Anandhan, S., Pande, V., and Ahmed, Z. (2009). Development of a transformation protocol for *Tecomella undulata* (Smith) Seem from cotyledonary node explants. Sci. Hortic. *121*, 119–121.

Beier, J.C., Müller, G.C., Gu, W., Arheart, K.L., and Schlein, Y. (2012). Attractive toxic sugar bait (ATSB) methods decimate populations of Anopheles malaria vectors in arid environments regardless of the local availability of favoured sugar-source blossoms. Malar. J. *11*, 31.

Benedict, M.Q., and Robinson, A.S. (2003). The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends Parasitol. *19*, 349–355.

Blatrix, R., Debaud, S., Salas-Lopez, A., Born, C., Benoit, L., McKey, D.B., Attéké, C., and Djiéto-Lordon, C. (2013). Repeated evolution of fungal cultivar specificity in independently evolved ant-plant-fungus symbioses. PloS One 8, e68101.

Campbell, G.L., Marfin, A.A., Lanciotti, R.S., and Gubler, D.J. (2002). West Nile virus. Lancet Infect. Dis. 2, 519–529.

Carter, C., and Thornburg, R.W. (2000). Tobacco nectarin I. Purification and characterization as a germin-like, manganese superoxide dismutase implicated in the defensE of floral reproductive tissues. J. Biol. Chem. *275*, 36726–36733.

Carter, C.J., and Thornburg, R.W. (2004). Tobacco Nectarin III is a bifunctional enzyme with monodehydroascorbate reductase and carbonic anhydrase activities. Plant Mol. Biol. *54*, 415–425.

Carter, C.J., and Thornburg, R.W. (2004). Tobacco nectarin V is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. Plant Physiol. *134*, 460–469.

Carter, C., and Thornburg, R.W. (2003). The nectary-specific pattern of expression of the tobacco Nectarin I promoter is regulated by multiple promoter elements. Plant Mol. Biol. *51*, 451–457.

Carter, C., Graham, R.A., and Thornburg, R.W. (1999). Nectarin I is a novel, soluble germin-like protein expressed in the nectar of Nicotiana sp. Plant Mol. Biol. *41*, 207–216.

Chauhan, K.R., Aldrich, J.R., McCardle, P.W., White, G.B., and Webb, R.E. (2012). A field bioassay to evaluate potential spatial repellents against natural mosquito populations. J. Am. Mosq. Control Assoc. 28, 301–306.

Collins, F.H., and Paskewitz, S.M. (1995). Malaria: current and future prospects for control. Annu. Rev. Entomol. 40, 195–219.

Dan, Y., Baxter, A., Zhang, S., Pantazis, C.J., and Veilleux, R.E. (2010). Development of efficient plant regeneration and transformation system for *impatiens* using *Agrobacterium tumefaciens* and multiple bud cultures as explants. BMC Plant Biol. *10*, 165.

Demosthenis Chachali (2000). Factors affecting *Campsis radicans* seed germination and seedling emergence. Weed Science *48*:212-216.

Delécluse, A., Rosso, M.L., and Ragni, A. (1995). Cloning and expression of a novel toxin gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. Appl. Environ. Microbiol. 61, 4230–4235.

Dunnett, C.W. (1955). A Multiple Comparison Procedure for Comparing Several Treatments with a Control. J. Am. Stat. Assoc. 50, 1096–1121.

Elias, T.S., and Gelband, H. (1975). Nectar: its production and functions in trumpet creeper. Science *189*, 289–291.

Federici, B.A., Park, H.-W., Bideshi, D.K., Wirth, M.C., and Johnson, J.J. (2003). Recombinant bacteria for mosquito control. J. Exp. Biol. 206, 3877–3885.

Federici, B.A., Park, H.-W., Bideshi, D.K., Wirth, M.C., Johnson, J.J., Sakano, Y., and Tang, M. (2007). Developing recombinant bacteria for control of mosquito larvae. J. Am. Mosq. Control Assoc. 23, 164–175.

Feist, G.W., Webb, M.A.H., Gundersen, D.T., Foster, E.P., Schreck, C.B., Maule, A.G., and Fitzpatrick, M.S. (2005). Evidence of detrimental effects of environmental contaminants on growth and reproductive physiology of white sturgeon in impounded areas of the Columbia River. Environ. Health Perspect. 113, 1675–1682.

Foster, W.A. (1995). Mosquito sugar feeding and reproductive energetics. Annu. Rev. Entomol. *40*, 443–474.

Gadawski, R.M., and Smith, S.M. (1992). Nectar sources and age structure in a population of Aedes provocans (Diptera: Culicidae). J. Med. Entomol. 29, 879–886.

Gary, R.E., Jr, and Foster, W.A. (2004). Anopheles gambiae feeding and survival on honeydew and extra-floral nectar of peridomestic plants. Med. Vet. Entomol. 18, 102–107.

Gelvin, S.B. (2003). Agrobacterium-mediated plant transformation: the biology behind the “gene-jockeying” tool. Microbiol. Mol. Biol. Rev. MMBR 67, 16–37, table of contents.

Gómez, I., Pardo-López, L., Muñoz-Garay, C., Fernandez, L.E., Pérez, C., Sánchez, J., Soberón, M., and Bravo, A. (2007). Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. Peptides 28, 169–173.

Gouagna, L.C., Kerampran, R., Lebon, C., Brengues, C., Toty, C., Wilkinson, D.A., Boyer, S., and Fontenille, D. (2013). Sugar-source preference, sugar intake and relative nutritional benefits in *Anopheles arabiensis* males. Acta Trop.Hochberg,Y. & Tamhane,A. (1987). Multiple Comparison Procedures. New York: Wiley.

Halstead, S.B. (2008). Dengue virus-mosquito interactions. Annu. Rev. Entomol. 53, 273–291.

Hougard, J.-M., Fontenille, D., Chandre, F., Darriet, F., Carnevale, P., and Guillet, P. (2002). Combating malaria vectors in Africa: current directions of research. Trends Parasitol. *18*, 283–286.

Impoinvil, D.E., Kongere, J.O., Foster, W.A., Njiru, B.N., Killeen, G.F., Githure, J.I., Beier, J.C., Hassanali, A., and Knols, B.G.J. (2004). Feeding and survival of the malaria vector *Anopheles gambiae* on plants growing in Kenya. Med. Vet. Entomol. 18, 108–115.

Ishikawa, T., Yamanaka, A., and Konishi, E. (2014). A review of successful flavivirus vaccines and the problems with those flaviviruses for which vaccines are not yet available. Vaccine.

J., A., and Jr., D.F. (2001). Ant visitation of extrafloral nectaries of Passiflora : the effects of nectary attributes and ant behavior on patterns in facultative ant-plant mutualisms. Oecologia *127*, 409–416.

Lanza, J., Vargo, E.L., Pulim, S, .Chang, Y.Z. (1993). Preferences of the fire ants *Solenopsis invicta* and *S. geminata* (Hymenoptera: Formicidae) for amino acid and sugar components of extrafloral nectars. Environ. Entomol. *22*, 411-417.

Likitvivatanavong, S., Chen, J., Evans, A.M., Bravo, A., Soberon, M., and Gill, S.S. (2011). Multiple receptors as targets of Cry toxins in mosquitoes. J. Agric. Food Chem. *59*, 2829–2838.

Liu, Y., Zhang, F., Liu, J., Xiao, X., Zhang, S., Qin, C., Xiang, Y., Wang, P., and Cheng, G. (2014). Transmission-blocking antibodies against mosquito C-Type lectins for dengue prevention. PLoS Pathog. 10, e1003931.

Klowden, M.J., Held, G.A., and Bulla, L.A., Jr (1983). Toxicity of *Bacillus thuringiensis* subsp.*israelensis* to adult Aedes aegypti mosquitoes. Appl. Environ. Microbiol. *46*, 312–315.

Klowden, M.J., and Bulla, L.A., Jr (1984). Oral toxicity of *Bacillus thuringiensis* subsp. *israelensis* to adult mosquitoes. Appl. Environ. Microbiol. *48*, 665–667.

Mahalingam, S., Herring, B.L., and Halstead, S.B. (2013). Call to action for dengue vaccine failure. Emerg. Infect. Dis. 19, 1335–1337.

Malathi, B., Ramesh, S., Rao, K.V., and Reddy, V.D. (2006). Agrobacterium-mediated genetic transformation and production of semilooper resistant transgenic castor (*Ricinus communis* L.). Euphytica *147*, 441–449.

Manda, H., Gouagna, L.C., Nyandat, E., Kabiru, E.W., Jackson, R.R., Foster, W.A., Githure, J.I., Beier, J.C., and Hassanali, A. (2007). Discriminative feeding behavior of *Anopheles gambiae* s.s. on endemic plants in western Kenya. Med. Vet. Entomol. *21*, 103–111.

Manders, G., Otoni, W.C., d' Utra Vaz, F.B., Blackball, N.W., Power, J.B., and Davey, M.R. (1994). Transformation of passionfruit (Passiflora edulis fv flavicarpa Degener.) using *Agrobacterium tumefaciens*. Plant Cell Rep. *13*, 697–702.

Manda, H., Gouagna, L.C., Foster, W.A., Jackson, R.R., Beier, J.C., Githure, J.I., and Hassanali, A. (2007). Effect of discriminative plant-sugar feeding on the survival and fecundity of Anopheles gambiae. Malar. J. *6*, 113.

Martinez-Ibarra, J.A., Rodriguez, M.H., Arredondo-Jimenez, J.I., and Yuval, B. (1997). Influence of plant abundance on nectar feeding by Aedes aegypti (Diptera: Culicidae) in southern Mexico. J. Med. Entomol. 34, 589–593.

Müller, G.C., Beier, J.C., Traore, S.F., Toure, M.B., Traore, M.M., Bah, S., Doumbia, S., and Schlein, Y. (2010). Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the *Anopheles gambiae* complex in Mali, West Africa. Malar. J. *9*, 210.

Müller, G.C., Beier, J.C., Traore, S.F., Toure, M.B., Traore, M.M., Bah, S., Doumbia, S., and Schlein, Y. (2010). Field experiments of *Anopheles gambiae* attraction to local fruits/seedpods and flowering plants in Mali to optimize strategies for malaria vector control in Africa using attractive toxic sugar bait methods. Malar. J. *9*, 262.

Naqvi, S.M.S., Harper, A., Carter, C., Ren, G., Guirgis, A., York, W.S., and Thornburg, R.W. (2005). Nectarin IV, a potent endoglucanase inhibitor secreted into the nectar of ornamental tobacco plants. Isolation, cloning, and characterization. Plant Physiol. *139*, 1389–1400.

Ness, J.H., Morris, W.F., and Bronstein, J.L. (2009). For ant-protected plants, the best defense is a hungry offense. Ecology 90, 2823–2831.

Nyasembe, V.O., Teal, P.E.A., Mukabana, W.R., Tumlinson, J.H., and Torto, B. (2012). Behavioural response of the malaria vector *Anopheles gambiae* to host plant volatiles and synthetic blends. Parasit. Vectors 5, 234.

Padua, L.E., and Federici, B.A. (1990). Development of mutants of the mosquitocidal bacterium *Bacillus thuringiensis* subspecies *morrisoni* (PG-14) toxic to lepidopterous or dipterous insects. FEMS Microbiol. Lett. 54, 257–262.

Park, H.-W., Bideshi, D.K., Wirth, M.C., Johnson, J.J., Walton, W.E., and Federici, B.A. (2005). Recombinant larvicidal bacteria with markedly improved efficacy against culex vectors of west nile virus. Am. J. Trop. Med. Hyg. 72, 732–738.

Pradier, S., Lecollinet, S., and Leblond, A. (2012). West Nile virus epidemiology and factors triggering change in its distribution in Europe. Rev. Sci. Tech. Int. Off. Epizoot. 31, 829–844.

Pramanik, T.K., and Datta, S.K. (1986). Plant regeneration and ploidy variation in culture derived plants of Asclepias curassavica L. Plant Cell Rep. *5*, 219–222.

R Core Team (2013). R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. URL http://www.R-project.org/.

Rico-Hesse, R. (2010). Dengue virus virulence and transmission determinants. Curr. Top. Microbiol. Immunol. 338, 45–55.

Schlein, Y., and Muller, G. (1995). Assessment of plant tissue feeding by sand flies (Diptera: Psychodidae) and mosquitoes (Diptera: Culicidae). J. Med. Entomol. 32, 882–887.

Singh, B., Singh, P.R., and Mohanty, M.K. (2012). Toxicity of a plant based mosquito repellent/killer. Interdiscip. Toxicol. 5, 184–191.

Smith, R.A., Mooney, K.A., and Agrawal, A.A. (2008). Coexistence of three specialist aphids on common milkweed, *Asclepias syriaca*. Ecology *89*, 2187–2196.

Stray, J.E., Klowden, M.J., and Hurlbert, R.E. (1988). Toxicity of *Bacillus sphaericus* crystal toxin to adult mosquitoes. Appl. Environ. Microbiol. *54*, 2320–2321.

Vrzal, E.M., Allan, S.A., and Hahn, D.A. (2010). Amino acids in nectar enhance longevity of female Culex quinquefasciatus mosquitoes. J. Insect Physiol. *56*, 1659–1664.

Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyama, H., Sen, K., and Sakai, H. (2002). Active form of dipteran-specific insecticidal protein cryllA produced by *Bacillus thuringiensis* subsp. *israelensis*. Biosci. Biotechnol. Biochem. 66, 516–522.

Yamagiwa, M., Sakagawa, K., and Sakai, H. (2004). Functional analysis of two processed fragments of *Bacillus thuringiensis* Cry11A toxin. Biosci. Biotechnol. Biochem. 68, 523–528.