CAGE BIOASSAY OF SUGAR AND FLORAL SOURCE ATTRACTION IN MOSQUITOES

by

CAROLINE BROOKS

(Under the Direction of Brian Forschler)

ABSTRACT

The goal of the project is to develop a simple bioassay system to test the attraction of mosquitoes using volatiles. The end goal would be to identify components that could be incorporated into an attractive toxic bait system. Bioassays were performed with *Aedes albopictus, Aedes japonicus Culex restuans,* as well as *Aedes albopictus*, *Aedes aegypti*, and *Culex quinquefasciatus* field collected eggs that were reared in the laboratory or from laboratory cultures, respectively. A cage bioassay system using sticky cards was employed with paired comparisons in a latin square design to identify attractive or repellant materials using newly emerged male and female mosquitoes. Replicates tested different concentrations of Bell Trapper LTD®, Tanglefoot®, Scentry Biologicals Inc.®, Stickem® glue, sugar water, honey, AllClear™, benzaldehyde, phenylacetaldehyde, and isoamyl acetate. The bioassay showed attraction to Bell Trapper LTD ® bell cards and repellency of citronella.

INDEX WORDS: mosquito, attraction, volatiles, isoamyl acetate, phenylacetaldehyde, benzaladehye, stickem, Bell Trapper LTD ®, *Aedes albopictus, Aedes japonicus, Aedes aegypti, Culex restuans* and *Culex quinquefasciatus.*

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DEDICATION

This thesis is dedicated to my parents and family for supporting my goals.

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CHAPTER 1

REVIEW OF MOSQUITO BIOLOGY RELATED TO SUGAR FEEDING

Biology

Mosquitoes are a diverse group of insect's best known as vectors of disease and are classified in the Kingdom *Animalia*, Phylum *Arthropoda*, Class *Insecta*, Order *Diptera*, and family *Culicidae*. There are three subfamilies *Culicidae–Toxorhynchitinae*, *Anophelinae*, and *Culicinae* that include 4 tribes *Culicini*, *Aedini*, *Sabethini* and *Mansonini* with a total of about 3,500 species. Mosquitoes display a holometabolous life cycle that consists of the following sequential stages; egg, larva, pupa, adult (Bartlett-Healy et al. 2012). Eggs are laid on or near water and the larval stage of most mosquito species are aquatic, feeding on microorganisms and detritus (Merritt et al. 1992, Kirby & Spence, 1826, Subra, 1981, Becker et al., 2003, Carson et al. 2010, Quarles 2003). Depending on temperature and species larval development can take days or months to reach the adult stage (Edgerly et al. 1993). Mosquitoes can undergo diapause in any life stage as influenced by a number of factors including low environmental temperatures and day-length (Denlinger and Armbruster 2014). Both sexes of the adult stage feed on carbohydrates obtained from plants while females also require a blood meal taken from reptiles, birds or mammals for development and maturation of the eggs thereby completing the life cycle (Apperson et al. 2004, Gouagna et al. 2010, Vierhulst 2013).

The diversity of mosquitoes is reflected in the variety of habits and behaviors exhibited by different species (Buckner et al. 2011, Chaves et al. 2011). Three-fourths of mosquito species are found in humid or tropical regions, while the Arctic has fewer than twelve species (Clements

1992). According to the Georgia Mosquito Control Association, the mosquito genera prevalent in Georgia include *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, *Ochlerotatus*, *Orthopodomyia*, *Psorophora*, *Toxorhynchites*, *Uranotaenia*, and *Wyeomyia*. A study conducted at the Ichuaway Ecological Research Center in southwest Georgia found eleven prevalent species, including *Aedes albopictus, Aedes triseriatus, Aedes vexans, Culex coronator, Culex erraticus, Culex nigripalpus, Culex quinquefasciatus, Culex restuans, Culex salinarius, Coquillettidia perturbans,* and *Psorophora ferox* (Buckner et al. 2011). The major pest species of mosquito found in Georgia includes *Aedes albopictus, Culex japonicus* and *Culex restuans* (Young et al. 2014). Mosquitoes from the genera *Aedes* and *Culex* were chosen for this study because according to the Georgia Mosquito Control Association they are widespread throughout Georgia.

Aedes albopictus, the major pest species found around human habitation in Georgia, is native to Southeast Asia extending north to Japan and Siberia and was first discovered outside its' endemic range in South Africa (Bonizzoni et al. 2013). Currently, *Aedes albopictus* resides in every continent except Antarctica (Bonizzoni et al. 2013). The first record of *Aedes albopictus* occurring in Europe was in 1979 from Albania and in the continental United States in 1985 from Texas (Bonizzoni et al. 2013). In South and Central America, *Aedes albopictus* has been reported from Brazil and Mexico (Bonizzoni et al. 2013). *Aedes albopictus* is a vector of dengue and chikungunya, West Nile Virus, dengue hemorrhagic fever, Venezuelan equine encephalitis virus, and Rift Valley fever virus (Bartlett-Healy et al. 2012). *Aedes albopictus* females lay eggs on moist surfaces along the edge of the water line near stagnant water in groups (Edgerly et al. 1993). A hatching stimulus such as rainwater and microorganisms cause hatching but eggs can also can remain dormant until the water level increases a second time which is referred to as

installment hatching while in the northern part of its range this species overwinters in the egg stage (Edgerly et al. 1993).

Aedes japonicus originated in Japan and Korea, is not found in the tropics, and is part of a complex known to include four allopatric species; *Aedes japonicus japonicus, Aedes japonicus shintienensis, Aedes japonicus yaeyamensis* and *Aedes japonicus amamiensis* (Kaufman and Fonseca 2014). *Aedes japonicus* was first discovered in 1993 in New Zealand from used tires imported from Japan (Schaffner et al. 2003). In 1998 and 1998, *Aedes japonicus* was collected in the Port of Auckland from buckets on boats (Schaffner et al. 2003). *Aedes japonicus japonicus* has also been found in Belgium, Germany, Switzerland, Austria and Slovenia as well as identified but eliminated in France and New Zealand (Kaufman and Fonseca 2014).Within the United States, the used tire trade is believed to have brought *Aedes japonicus* into the United States (Fonseca et al. 2001, Lounibos 2002, Schaffner et al, 2003). Currently, *Aedes japonicus* is found in all states east of the Mississippi River with the exception of Florida and in two provinces in Canada, Quebec and Ontario (Kaufman and Fonseca 2014).

Aedes japonicus are vectors of West Nile Virus, dengue, hemorrhagic fever, Venezuelan equine encephalitis virus, Rift Valley fever virus, and chikungunya virus (Bartlett-Healy et al. 2012). *Aedes japonicus* has been shown capable of vectoring Japanese encephalitis (JE), West Nile Virus and Saint Louis Encephalitis in a laboratory setting but JE has not been found in the United States although there have been cases of JE in Asia due to *Aedes japonicus japonicus* (Kaufman and Fonseca 2014). Within the United States, *Aedes japonicus japonicus* harbors West Nile virus (WNV) and La Crosse encephalitis (LAC) (Kaufman and Fonseca 2014).

Aedes japonicus japonicus overwinters in the egg or larval stage and 30^0 C is optimal for larval development (Kaufman and Fonseca 2014). Temperatures between 34^0 or 40^0 C are lethal

to the larvae whereas at 10^0 C larval development is extended and can take approximately a hundred days to develop into adults (Kaufman and Fonseca 2014). *Aedes japonicus* oviposits above the water line near ditches, ponds, and puddles but there is no clear correlation between site characteristics and success of larvae development (Kaufman and Fonseca 2014). *Aedes japonicus* are capable of developing in the same larval habitat along with *Culex restuans* and *Culex pipiens* (Kaufman and Fonseca 2014).

Culex quinquefasciatus is the southern house mosquito and a vector of West Nile virus, St Louise encephalitis virus and lymphatic filiariasis (Arensburger et al. 2010). *Culex restuans* is a vector of West Nile Virus (Harrington and Poulson 2008). *Culex quinquefasciatus* and most *Culex* species prefers to breed in water that has abundant organic nutrients, such as clogged drains and sewage runoff (McCall and Eaton 2001). Gravid females lay eggs in rafts on the surface of the water and optimal temperatures for larval development are 20.3 Celsius to 26.0 degrees Celsius (McCall and Eaton 2001, Kirby & Spence, 1826, Subra, 1981, Becker et al., 2003, Carson et al. 2010, (Harbison et al. 2009). *Culex quinquefasciatus*, *Culex pipiens* and *Culex restuans* live in similar habitats (Harrington and Poulson 2008). *Culex restuans* can be found in North America, Canada, Mexico, Guatemala and Honduras (Harrington and Poulson 2008).

Behavior

Adult mosquitoes respond to a variety of visual, auditory, and volatile cues in order to locate mates, oviposition sites and blood meals (Bowen 1991). In most species, the male searches for the female using sound in the frequency of 400 Hz (Stone et al. 2013). In field trials CDC light traps with a device that issued a 400Hz tone attracted more *Aedes polynesiensis* mosquitoes than traps without sound (Stone et al. 2013).

Once mated, female mosquitoes seek an appropriate body of still water in which to lay their eggs. Gravid females are influenced by sight and semiochemicals when choosing the appropriate oviposition habitat , for example *Aedes albopictus* and *Aedes polynesiensis* prefer dark locations containing water (Gubler 1971) (Takken 1999, Okumu et al. 2010). Bermuda grass contains semiochemicals such as phenylacetaldehyde that have been used to increase mosquito egg abundance at oviposition sites (Carson et al. 2010). The volatiles in grass infusion – a 'fermented mixture of water and dried grass/hay – believed to be oviposition attractants include 3-methylindole (skatole) and 4-methylphenol (p-cresol) (Millar et al. 1992, Takken 1999, McCall and Eaton 2001, Hughes et al. 2010). Skatole is a byproduct of fermentation in animal waste and organic material and also has been identified as an attractant for *Culex quinquefasciatus* oviposition at low concentrations but is repellent at higher amounts (McCall and Eaton 2001).

There is a large body of information available on the attraction of female mosquitoes to visual and volatiles cues related to blood feeding behavior (Bowen 1991). In general female mosquitoes searching for a blood meal are attracted to dark colors, carbon dioxide, lactic acid and octenol (Bowen 1991, Day 2005). Discussion of blood feeding behaviors is beyond the scope of review that will, instead, focus on attraction of both male and female mosquitoes to plant-derived food resources.

Adult mosquitoes feed on plant sugars as a source of energy to augment their glycogen and lipid energy reserves (Scott and Takken 2012). The age and sex of a mosquito affects their use of plant-derived food and sugar meals play a significant role in mosquito life-span, fecundity, flight, and host-seeking behavior (Jhumur et al. 2008). Males only feed on plant sugar throughout their lifespan while females feed on both sugar (Gary and Foster 2004, Clements

1999). There is considerable variation in how often female mosquitos consume plant-derived food for example prior to entering diapause female *Culex* and *Anopheles* cease seeking blood meals but continue to feed on plant sugars (Otienoburu et al. 2012). Consumption of sugar increases the number of eggs in each clutch but a crop full of sugar solution decreases the size of a blood meal resulting in a reduction in egg size (Foster 1995). Therefore, the amount of sugar present in the environment can affect the vectorial capacity of a mosquito population by limiting the size of a blood meal (Stone et al. 2012). While many mosquito species need sugar and blood but there are exceptions such as *Anopheles gambiae* and *Aedes aegypti* females that can receive most of their energy and nutrition from blood and therefore may skip sugar feeding (Straif and Beier 1996, Takken et al. 1998, Takken 1999, Beier et al. 2012, Scott and Takken 2012). However, sugar feeding does increase the life span of *Anopheles gambiae* whereas *Aedes albopictus* require a blood meal for egg development and can't survive on blood alone (Müller et al. 2011, Scott and Takken 2012).

Sources of mosquito sugar meals include floral nectaries, honeydew, tree sap, rotting fruit, and leaves damaged by insects (Foster 1995, Müller et al. 2010). Floral and extrafloral sugar sources also contain organic compounds that aid in a number of metabolic needs for a mosquito (Foster 1995). The length of the proboscis limits mosquitoes to flowers with shallow, short cups or straight corollas therefore mosquitoes generally feed on one or two plant sources even when a variety of species are available (Foster 1995). During peak nectar production, mosquitoes use the most abundant flowers showing less preference for other sources of sugar and it is not certain if this inclination toward one plant is attraction or aggregation (Foster 1995).

Mosquito species that are active during the day, such as *Aedes aegypti,* in part, use visual cues to locate plant-derived food resources (Jhumur et al. 2007). Flower shape is not important

for mosquito attraction and research has shown that mosquitoes most often frequent clusters of small white or pale-colored flowers that provide a landing ground to wander around and capture nectar (Foster 1995). (Foster 1995). *Culex quinquefasciatus,* a night feeding mosquito, showed a preference for black and brown colors in natural and ultraviolet light; however, at night there was no preference (Wen et al. 1997). When *Anopheles gambiae* were tested for preference with a dark colored membrane or parafilm, there was a higher preference for the darker-colored membrane (Nora Chilaka 2012).

The interaction of visual and olfactory cues used by mosquitoes to locate and recognize sugar sources are still not well understood although research has shown that certain flowers have more mosquitoes feeding on them and mosquitoes are attracted to floral volatiles (Vargo and Foster 1982, Foster 1995, Dudareva and Pichersky 2000, Jhumur et al. 2008). There is considerable literature on recognition of floral components for example *Culex pipiens,* a night-active species, was responsive to *Silene otitis*, a species more odorous in the early hours of nighttime (Jhumur et al. 2008).

Plant volatiles that include terpenes, phenols, aliphatic esters and aldehydes have been shown to stimulate mosquito antennal sensilli identifying them as potential semiochemicals (Vargo and Foster 1982, Foster 1995, Dudareva and Pichersky 2000, Jhumur et al. 2008). Two of the most attractive semiochemicals for *Aedes aegypti* and *Anopheles quadrimaculatus* are monoterpene alcohols and ketones (Kaufman et al. 2010). It has been demonstrated that bicyclic terpenes such as thujone are detected by *Culex pipiens* antennae (Bowen 1992). The attractive volatiles from milkweed, *Asclepias syriaca,* flowers included benzaldehyde, E-B-ocimene, phenylacetaldehyde, benzyl alcohol, nonanal and E-2-nonenal (Otienoburu et al. 2012). A blend

of benzaladehyde, phenylacetaldehyde and (E)-2-nonenal was shown to be attractive to *Culex pipiens* using a dual port olfactometer (Hancock and Foster 1993, Otienoburu et al. 2012).

Phenylacetaldehyde is the most prevalent compound in a pentane extract of the Spanish catchfly, *Silene otitis*, and has been shown to be common in other flowers that are attractive to other insects including butterflies, moths and flies (Jhumur et al. 2006, Otienoburu et al. 2012). *Culex pipiens molestus*, a night active mosquito, visits the night blooming Spanish catchfly, (Jhumur et al. 2007). EAG recordings were conducted using male and female *Culex pipiens molestus* and *Aedes aegypti* mosquitoes that demonstrated a blend of phenyl acetaldehyde, linalool oxide(pyranoid), phenylethyl aclcohol, and acetophone was more attractive than the single components alone with the exception of phenylacetaldehyde (Jhumur et al. 2007). Based on the literature, mosquitoes are attracted to phenylacetaldehyde and linalool in behavioral assays while phenylethyl alcohol and benzyl alcohol showed the greatest response using EAG (Jhumur et al. 2007).

Anopheles gambiae are attracted to the blend of sugars and floral volatiles found in honey (Otienoburu et al. 2012). However, in the late scotophase, female *Anopheles gambiae* mosquitoes were more attracted to human volatiles than honey (Foster and Takken 2004). Mosquitoes display behaviors attributed to semiochemical attraction based on species, sex, and bioassay design (Foster and Takken 2004).

Repellency bioassays have also been conducted most frequently with DEET (N,N-Diethyl-3-methylbenzamide) has been used for mosquito repellency but there has been an increased interest in testing plant-based botanicals as repellents (Müller et al. 2009). Essential oils have been used to test the repellency on mosquitoes including citronella, tea tree oil and eucalyptus (Müller et al. 2009). In repellency studies, a cage in arm study is typically done by

spreading the repellent on the treated hand and comparing the amount of mosquito bites per treated and untreated arm (Masetti and Maini 2006). Plant based repellents consist of one or more essential oils including citronella, eucalyptus, geranium, lemongrass, and soybean (Masetti and Maini 2006).

Mosquitoes species are a group of insects that display a variety of habits related to each stage of their holometabolus developmental life history. The majority of the species that vector disease require a meal of carbohydrates, specifically sugars, to fuel the host seeking behaviors and vectorial capacity of the female. The ability of female mosquitoes to locate sources of sugar has been linked to plant volatiles and this behavior has been targeted as a potential scheme for controlling disease-vectoring species. The purpose of this study was to demonstrate a simple cage bioassay system that could illuminate candidate volatiles, or blends of volatiles, that might be employed in an attractive sugar bait for control of mosquitoes.

Summary

More research is needed to identify the sequence of visual and olfactory cues used by mosquitoes to locate plant-derived food resources although Mosquitoes are a diverse group of insects that display a variety of habits related to each stage of their holometabolus developmental life history. The majority of the species that vector disease have been shown to require a meal of carbohydrates, specifically sugars, to fuel the host seeking behaviors, and vectoral capacity, of the female. The ability of female mosquitoes to locate sources of sugar has been linked to plant volatiles and this behavior has been targeted as a potential scheme for controlling disease-vectoring species. The purpose of this thesis was to demonstrate a simple cage bioassay system that could illuminate candidate volatiles, or blends of volatiles, that might be employed in an attractive sugar bait for control of mosquitoes

CHAPTER 2

ATTRACTION OF SUGAR AND FLORAL SOURCES IN FOUR MOSQUITO SPECIES IN CAGE BIOASSAY

Introduction

The main objective of this study was to develop a simple cage bioassay system to examine attraction of adult mosquitoes, for use in screening materials for use in monitoring and management programs. This was done by testing specific compounds, mentioned in the literature as attractants or repellants, in a cage bioassay using newly emerged *Aedes albopictus, Aedes japonicus, Aedes aegypti, Culex restuans* and *Culex quinquefasciatus*. A ten percent sucrose solution in addition to different volatiles was examined at 10 ppm and 100 ppm concentrations such as skatole (Sigma-Aldrich®), benzaldehyde (Sigma-Aldrich®),phenylacetaldehyde (Sigma-Aldrich®), isoamyl acetate (Sigma-Aldrich®), and citronella (Aura Cacia ®) in addition to Bell Trapper LTD ® sticky cards. Results showed that Bell Trapper, AllClear™, Isoamyl acetate was attractive and citronella was repellant. These experiments demonstrated the feasability of using a cage bioassay with sticky cards as a means of measuring attraction of newly emerged mosquitoes to selected volatiles or blends of volatile materials.

The need to manage mosquito populations arises from the pest and disease vector status afforded this group of insects (Axtell 1979, Lv et al. 2012). Management of mosquitoes can involve habitat alterations and application of pesticides aimed at killing either the larval or the adult stage (Rose 2001, Borovsky 2003, Lee 2006, Dongus et al. 2007). The use of pesticides for adult mosquito control has come under scrutiny because of non-target impacts and effective,

more environmentally friendly methods such as attractive toxic sugar baits (ATSB) have been proposed (Xue and Barnard 2003, Xue et al. 2006, Muller et al. 2010, Xue et al. 2011, Beier et al. 2012, Khallaayoune et al. 2013, Stewart et al. 2013). The ATSB concept takes advantage of the fact that mosquitoes rely on a variety of sensory stimuli to located mates, oviposition sites, and food source (Gubler 1971, Millar et al. 1992, Yuval and Bouskila 1993, Foster 1995, Trexler et al. 2003, Apperson et al. 2004, Gouagna et al. 2010, Hughes et al. 2010, Phasomkusolsil et al. 2013). Most species of adult mosquitoes, both male and female, require a sugar meal to sustain activities, such as flight, required to mate and lay eggs thus completing the life cycle (Merritt et al. 1992, Foster 1995, Phasomkusolsil et al. 2013). The attraction of mosquitoes to carbohydrate food resources involves visual cues and the volatiles emitted by floral and extra-floral nectarines (Jepson and Healy 1988, Foster and Hancock 1994, Mauer and Rowley 1999, Nyasembe et al. 2012).

The efficacy of any ATSB system depends on attracting mosquitoes to a toxin-laden bait that mimics a carbohydrate food source. The volatiles that attract mosquitoes have been tested in a variety of experiments ranging from electro-antennograms (EAG), that identify specific chemicals that stimulate antennal sensilla, to recording responses to specific chemistries or blends of chemistries in olfactometer tests, field observations, and large cage studies (Gillett et al. 1962, Gouck and Schreck 1965, Osgood and Kempster 1971, Vargo and Foster 1982, Bowen 1992, Posey et al. 1998, Jhumur et al. 2007, 2008, Nyasembe et al. 2012, Otienoburu et al. 2012). The present study was aimed at developing a cage bioassay employing sticky cards to identify volatiles noted in the literature as attractive to newly emerged adult mosquitoes.

Methods and Materials

A cage bioassay system was designed using four cages and various treatment combinations in a two-choice test to examine the attraction of mosquitoes to selected volatiles.

Source of Mosquitoes

For most of the experiments, oviposition traps with hay infusion were used to collect eggs that were hatched and reared in the laboratory to obtain adults for use in the cage bioassay. The species of mosquitoes used in these experiments were determined using morphological keys to identify mosquito larvae and adults (Stojanovich 1982, Burkett-Cadena 2013). Some experiments used mosquitoes from laboratory colonies maintained at the University of Georgia including *Aedes albopictus*, *Aedes aegypti*, and *Culex quinquefasciatus*.

Oviposition traps

Dried hay was added to a thirty-gallon storage container filled with water and left to ferment for seven days. The water was transferred into black trays that were 23 cm wide, 30 cm long and 7 cm deep and filled with approximately 1200 ml of the hay-infused water. Brown paper towels were attached to the trays with binder clips to serve as oviposition substrates for *Aedes* mosquitoes. These oviposition traps were placed outside the biological sciences building and checked daily for egg rafts or eggs.

Egg collection and experimental set up

When *Culex* rafts were collected, they were placed in clear plastic containers 25.4 cm x 17.8 cm (Pioneer Plastics, Dixon KY) in distilled water and stored in the climate controlled room. Temperature was maintained at 28.1 degrees Celsius and 81.6 RH (relative humidity). *Aedes* eggs were collected and examined under a microscope then placed on the side of a black tray used for the oviposition traps with water on the bottom; the purpose of the water on the

bottom of the container was to keep the paper towels moist to allow embryogenesis to continue (Saifur et al. 2010). After three days these eggs were placed on trays to dry. A week before bioassay, the *Aedes* eggs were counted and approximately 150 placed in a container with 950 ml water to hatch.

Once hatched, the larvae were fed ½ scoop of fish food the first day, 1 scoop on days two and three, 1½ scoop on days four and five and a ½ scoop on day six. One scoop is approximately .60 g of fish food. *Culex* larvae were fed a ½ scoop of fish food on the first day, 1 scoop on days two, three and four, 2 scoops on days five and six, and one scoop on day seven. *Aedes albopictus* eggs obtained from laboratory cultures were given ½ a scoop one day one, 2 scoops on day 2, four scoops on day four, six scoops on day 5 and 18 scoops on day 5. *Culex quinquefasciatus* lab species were fed ½ a scoop on day 1, 1 scoop on day 2, three scoops on day 3 and 4, four scoops on day 4 and 5 and 1 scoop on day 7 and 8. The laboratory and field caught species both produced pupae in approximately six days but the laboratory species required more food in order to have a synchronous emergence.

Pupae separation

Pupae were transferred to clear, 5cm x 2 cm containers, containing 40 ml of water with an open lid in equal numbers for each of four cages (61 cm x 61 cm) bioassay cages. The goal was to have 100 pupae per cage but certain replicates were performed with at least 50 pupae per cage. The actual number of mosquitoes in each replicate was calculated based on the number caught on sticky cards and the number alive on each day in the bioassay. When counting the number of mosquitoes released, the dead pupae was accounted for and subtracted from the total pupae to record the number of mosquitoes released. When the bioassay was completed for one day, the number of dead mosquitoes was counted and subtracted from the amount of mosquitoes

released and caught to calculate the number of alive mosquitoes remaining for the next bioassay. The number of dead mosquitoes and the number of mosquitoes caught equaled a sum of the released mosquitoes.

Bioassay

Four BioQuip ® cages, 30.5 cm x 30.5 cm, constructed of metal, wire mesh and an open sleeve were used in these tests. Two glue boards, 5.1 cm x 10.2 cm, were positioned on opposite sides of each cage about 3-cm from the cage ceiling and each glue board had one 1.5 ml microvial with a cotton wick (0.020 g) placed inside to which a test volatile or water was added. Sticky cards with their attendant vials were collected and replaced daily for four days.

The number, sex and species of mosquitoes caught each day on each glue board in each cage was recorded. A replicate consisted of the data obtained from four cages over the course of several days with each number of mosquitoes caught each day comparing three arrangements of choice: one cage contained two vials of water, one cage had two vials of a test material and two cages had one vial each of water and the test material. One basic experimental design was used that compared the number of mosquitoes caught in the four cages that provided the following choices; water/water(control) attractant/attractant(control) or water/attractant(experimental treatment). Through the course of determining an optimal configuration for elucidating the behavior of mosquito attraction we used three different configurations or statistical designs to compare control and experimental treatments. In the first, the aforementioned choices were offered and maintained throughout the experimental period in the same cage on the same side of the cage. In the second configuration the water/attractant comparisons were rotated to opposite sides of the same cage on each day with the choices maintained in the same cage over the course of the test. Last, we employed a latin square design where treatments and sides were changed in

each cage on each of the four days of bioassay in a randomized complete block design (Odulaja and Abu-Zinid 1997). The materials tested and the mosquito species involved varied for each of the aforementioned designs. Materials varied by glue boards, glue types, sugar, water, honey, volatiles, pesticides and species varied by field caught including *Aedes albopictus*, *Aedes japonicus*, *Culex restuans*, *Culex quinquefasciatus* and lab rearered species including *Aedes albopictus*, *Aedes, aegypti* and *Culex quinquefasciatus*.

Treatments

There were a total of 14 materials tested in these two-choice cage bioassays. Treatments included two types of commercially available glue traps; Bell Trapper LTD®and a yellow sticky card by Scentry Biologicals Inc.®, as well as, two types of glue Stickem®, and Tanglefoot®. applied to similar sized 5.1 cm x 10.2 cm) heavy white paper. In addition 8 solutions were tested in 1.5 ml microvials attached to one of the aforementioned at the bottom of the glue traps; skatole, benzaldehyde, phenylacetaldehyde, isoamyl acetate, citronella, honey, table sugar and AllClear™ (a commercially available ATSB).

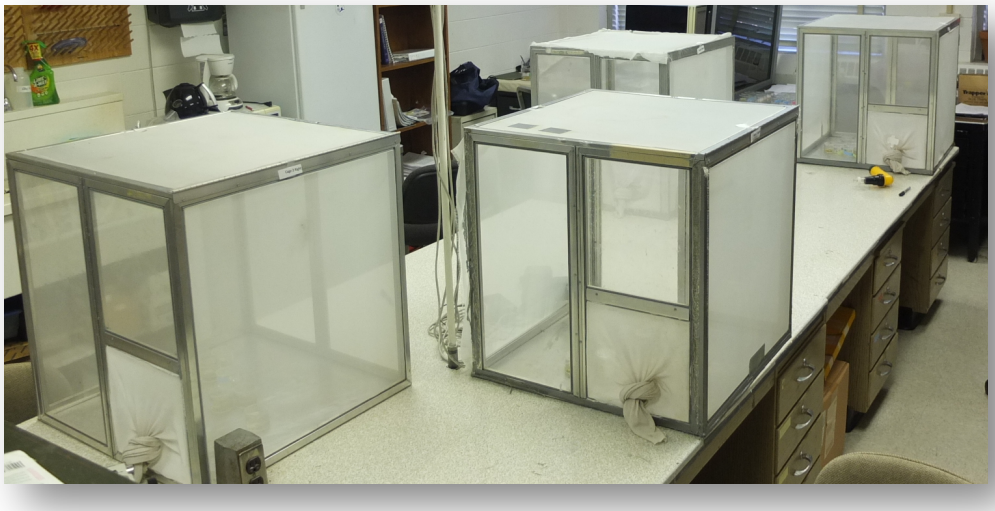
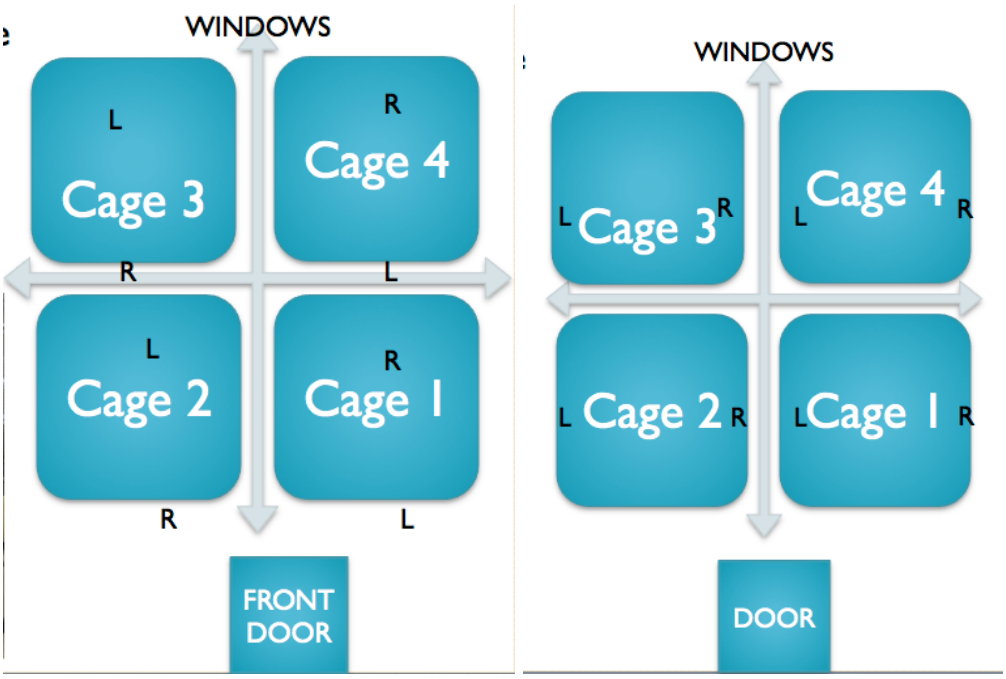


Figure 2.1: Photo of the 4 cages as they were arranged in the bioassay room.

Cages are labeled one to four in a clockwise pattern.



2.2 A

Bioassay Design

1 & 2

2.2 B

Latin Square or

Design 3

Figure 2.2 An illustration of the two different orientations used for placement of treatments in the four cages for the first two statistical designs (left) and the latin square design (right). Cages were set up with treatments facing on a north and south orientation in the room for bioassay designs 1 & 2 and for the latin square design (design 3), cage placement was the same but treatments were positioned on a east/west orientation.

Cage1	Cage 2	Cage 3	Cage 4
Day 1 ____ L (W) Day 1 ____ R(W)	Day 1 ____ L(A) Day 1 ____ R(W)	Day 1 L ____ (W) Day 1 ____ R(A)	Day 1 ____ L (A) Day 1 ____ R(A)
Day 2 ____ L (A) Day 2 ____ R(W)	Day 2 ____ L(W) Day 2 ____ R(W)	Day 2 ____ L(A) Day 2 ____ R(A)	Day 2 ____ L(W) Day 2 ____ R(A)
Day 3 ____ L (W) Day 3 ____ R(A)	Day 3 ____ L(A) Day 3 ____ R(A)	Day 3 ____ L (W) Day 3 ____ R(W)	Day 3 ____ L(A) Day 3 ____ R(W)
Day 4 ____ L(A) Day 4 ____ R(A)	Day 4 ____ L(W) Day 4 ____ R(A)	Day 4 ____ L(A) Day 4 ____ R(W)	Day 4 ____ L(W) Day 4 ____ R(W)

Figure 2.3. A table explaining the rotation of treatments over time by cage for latin square or bioassay design 3.

Data Analysis

The number of mosquitoes caught on sticky cards were analyzed by cage, treatment, mosquito species, and sex using JMP. Two variables were analyzed using a distribution of Fit Y by X in JMP to determine the number of mosquitoes, sex, and species caught by cage and treatment. Treatment by direction and cage by direction was analyzed in fit the Y by X model to determine significance of mosquitoes caught by treatment, species or sex. A poisson test was used to determine p values for the two variables tested by treatment, cage, species and sex. The determination of a significant difference in the number of mosquitoes caught was decided by a P value at ≤ 0.1.

Results

The data were analyzed by the design used in this series of experiments; treatment offerings held consistent in cages (Design 1), treatments rotated daily by side in a cage (Design 2) and the daily rotation of treatments by side and cage or latin square (Design 3). In all three bioassay designs, the control included two water treatments and another set of controls consisted of two identical attractants at the same concentration. It was expected that there would be no differential

attraction in the either of the controls when examining the experimental treatments (Barnard 2005).

Bioassay design 1 consisted of treatments by side per cage and was applied to five experiments testing water and 10% sugar, 1% skatole and 99% ethanol, a comparison of commercial glue boards and glue that included trapper, yellow sticky cards, tanglefoot and stickem, 1% and 0.1% benzaldehyde using tanglefoot, and lastly a combination of volatiles including, .1% benzaldehyde, honey, .1% phenylacetaldehyde and trapper. When the p value was less than 0.1, there was a significant attraction. The data indicated attraction in the following comparisons. There was attraction in the negative control comparing two trapper cards in the first experiment. In experiment two there was significant attraction to the 99% ethanol in the experimental treatments in cage 1 compared to the 1% skatole and yet conversely there was significant attraction in cage 3 comparing to the 1% skatole over ethanol. In the experiment testing various glue types there was significant attraction to trapper when tested against the yellow card, stickem and tanglefoot. In the experiment testing attraction to 1% and then 0.1% benzaladehyde on tanglefoot glue there was significant attraction to the water over 1% benzaladehyde in one replicate. In the experiment testing attraction to .1% benzaldehyde, honey, .1% phenylacetaldehyde, and trapper there was significant attraction three times. First, there was attraction to honey with trapper in the experimental treatments when compared to stickem with honey. Second, there was attraction to honey and .1% phenylacetaldehyde in the experimental treatment when compared with stickem and water. Third, there was attraction to honey and .1% benzaldehyde in the experimental treatment when compared with water and stickem.

The Type 2 design had two experiments with the first testing honey and stickem, honey and trapper, honey and .1% phenylacetaldehyde and honey and .1% benzaldehyde. The second experiment tested the attraction of trapper compared with stickem. There was attraction to honey and trapper compared to honey and stickem, honey and .1% phenylacetaldehyde compared to water and stickem, and to 0.1% benzaladehyde and honey compared to water and stickem. There also was attraction to trapper when compared with stickem.

The bioassay design (type 3) consisted of a series of experiments using the latin square design. The data showed attraction in the following comparisons, water compared to 10ppmphenylacetaldehyde, there also was attraction to the right-side water in the negative control in the 100 ppm phenylacetaldehyde comparison. Isoamyl acetate at 10ppm caught significantly more mosquitoes when compared with water. There was significantly more mosquitoes caught in the left treatment in the negative control and on the water treatments in comparisons with citronella. This indicates that mosquitoes were more attracted towards water than citronella demonstrating repellency. In the experiment testing attraction to trapper, there was attraction in the negative and positive controls as well as the trapper/water comparisons.

Table 2.1 Table of results from bioassay design 1 by cage and treatment with a + sign indicating significant difference and a – sign indicating no difference in the number of mosquitoes caught. N = the number of mosquitoes caught by treatment, P = the value for comparing the treatments within a cage

Test Material	Days	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
Water and 10% Sugar	22	Trapper Blank	Trapper Blank	Trapper Water	Trapper Water	Trapper Sugar	Trapper Sugar	Trapper Sugar	Trapper Water
		+		–	–	–	–	–	–
		p=.06 N=118	p=.06 N=90	p=.56 N=116	p=.56 N=125	p=.9 N=139	p=.9 N=140	p=.70 N=125	p=.70 N=131
Water and 10% Sugar	21	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
		Trapper Blank	Trapper Blank	Trapper Water	Trapper Water	Trapper Sugar	Trapper Sugar	Trapper Sugar	Trapper Water
		–	–	–	–	–	–	–	–
		p=.14 N=112	p=.14 N=135	p=.15 N=111	p=.15 N=133	p=.36 N=125	p=.36 N=111	p=.47 N=114	p=.47 N=125
Water and Sugar 10% Combined	43	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
		Trapper Blank	Trapper Blank	Water	Water	10% Sugar Bell Trapper	10% Sugar Bell Trapper	10% Sugar Bell Trapper	Water Bell Trapper
		–	–	–	–	–	–	–	–
		P=.81 N=230	P=.81 N=225	P=.15 N=227	P=.15 N=258	P=.56 N=264	P=.56 N=251	P=.44 N=239	P=.44 N=256
2) Skatole 1%	9	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
		1% Skatole	99% Ethanol	1% Skatole	99% Ethanol	1% Skatole	99% Ethanol	1% Skatole	99% Ethanol
			+	–	–	+		–	–
		P=.005 N=21	P=.005 N=50	P=.16 N=31	P=.16 N=21	P=.003 N=40	P=.003 N=18	P=.25 N=29	P=.25 N=21

Skatole combined Treatments	9	Skatole 1%				Ethanol 99%			
		-				-			
		N=121 P=.46				N=110 P=.46			
Glue types	7	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
		Trapper	Yellow	Trapper	Stickem	Trapper	Tanglefoot	Tanglefoot	Stickem
		+		+		+			+
		P=.014	P=.014	P=.0001	P=.0001	P=.0001	P=.0001	P=.04	P=.04
		N=33	N=16	N=43	N=1	N=53	N=0	N=2	N=8
Benzaldehyde and Tanglefoot	7	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
		Water .1% Benzaldehyde	Water	.1% Benzaladehyde	.1% Benzaladehyde	1% Benzaldehyde	Water	.1% Benzaladehyde	Water
		-	-	-	-	-	-		+
		P=.12	P=.12	P=.78	P=.78	P=.55	P=.55	P=.008	P=.008
		N=30	N=43	N=26	N=28	N=38	N=33	N=27	N=50
Benzaldehyde, Honey, trapper, Phenylacetaldehyde	10	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
		Honey Stickem	Honey Trapper	Honey Stickem	Water Stickem	Honey .1% Phenylacetaldehyde	Water Stickem	Honey .1% Benzaldehyde	Water Stickem
			+	-	-	+		+	
		p=.0001	P=.0001	P=.111	P=.111	P=.0004	P=.0004	P=.01	P=.01
		N=30	N=149	N=52	N=37	N=62	N=34	N=67	N=42

Table 2.2 Table of results from bioassay design 2 by cage and treatment with a + sign indicating significant difference and a – sign indicating no difference in the number of mosquitoes caught. N = the number of mosquitoes caught by treatment, P = the value for comparing the treatments within a cage

	Days	Cage 1 Left	Cage 1 Right	Cage 2 Left	Cage 2 Right	Cage 3 Left	Cage 3 Right	Cage 4 Left	Cage 4 Right
Benzaldehyde, Honey, Phenylacetaldehyde and trapper 15ul of each	15	Honey Stickem	Honey Trapper	Honey Stickem	Water Stickem	Honey Phenylacetaldehyde .1%	Water Stickem	Honey .1% Benzaldehyde	Water Stickem

attractant and 1.5 ml of honey days=15			+	-	-		+		+
		p=.0001 N=44	P=.0001 N=125	P=.69 N=49	P=.69	P=.02	P=.02	P=.03	P=.03
					N=54	N=39	N=61	N=40	N=61
Trapper Comparisons	25	Bell ®Trapper card				Water and Stickem			
		+							
		P=.0001 N=638				P=.0001 N=273			

Table 2.3 Table of results from latin square bioassay design 3 by cage and treatment with a + sign indicating significant difference and a – sign indicating no difference in the number of mosquitoes caught. N= the number of mosquitoes caught by treatment, P= the value comparing the treatments within cage, A= attractant volatiles tested, W=water

Test Material	Days	Treatment A LW, RW		Treatment B LA, RW		Treatment C LW, RA		Treatment D LA, RA	
		Treatment A		Treatment B		Treatment C		Treatment D	
1.Phenylacetaldehyde 10ppm	2 replicate Days=8	LW	RW	LA	RW	LW	RA	LA	RA
		-	-	-	-	+		-	-
		P=.52 N=104	P=.52 N=95	P=.16 N= 76	P=.16 N=94	P=.02 N= 109	P=.02 N=78	P-.77 N= 94	p=.77 N=98
		A, W combined							
		-				-			
		A p=.16 N=154				W p=.16 N=203			
		Treatment A		Treatment B		Treatment C		Treatment D	
2.Phenylacetaldehyde 100ppm	1 replicate Days=4	LW	RW	LA	RW	LW	RA	LA	RA
		-	-	-	-	-	-	-	-
		P=.019 N=39	P=.019 N=21	P=1.000 N=29	P=1.000 N=29	P=.1000 N=28	P=1.000 N=22	P=.1000 N= 24	P=1.000 N=28
		A, W combined							
		-				-			

		A p=.56 N=51				W p=.56 N=57			
		Treatment A		Treatment B		Treatment C		Treatment D	
3.Isoamyl acetate 10ppm .44u	2 replicates Days= 8	LW	RW	LA	RW	LW	RA	LA	RA
		-	-	+		-	-	-	-
		P=.36 N=111	P=.36 N=98	P=.04 N=110	P=.04 N=82	P=.36 N= 114	P=.36 N=128	P=. 26 N= 98	P=.26 N=83
		A, W combined							
		+							
		A p=.04 N=238				W p=.04 N=196			
		Treatment A		Treatment B		Treatment C		Treatment D	
4. Isoamyl acetate 100ppm 4.4 ul	2 replicates Days=8	LW	RW	LA	RW	LW	RA	LA	RA
		-	-	-	-	-	-	-	-
		P=.28 N=133	P=.28 N= 116	P=.44 N=107	P=.44 N=96	P=.14 N=137	P=.14 N=114	P=.79 N=122	P=.79 N= 118
		A, W combined							
		-				-			
		A P=.57 N= 21				W P=.57 N= 233			
		Treatment A		Treatment B		Treatment C		Treatment D	
5.Citronella 10ppm .44ul	1 replicate Days=4	LW	RW	LA	RW	LW	RA	LA	RA
		-	-	-	-	-	-	+	
		P=.31 N= 28	P=.31 N=21	P=.66 N= 22	P=.66 N=25	P=.47 N=18	P=.47 N=14	P=. 04 N= 31	p=.04 N=17
		A, W combined							
		-				-			
		A P=.43 N=36				W P=.43 N=43			

		Treatment A		Treatment B		Treatment C		Treatment D	
6.Citronella 4.4 ul 100ppm	4 replicates Days=16	LW	RW	LA	RW	LW	RA	LA	RA
			+		+	-	-	-	-
		P=.003 N= 96	P=.003 N= 141	P=.004 N=84	P=.004 N=125	P=.63 N=104	P=.63 N=111	P=.63 N=103	P=.63 N=110
		A, W combined							
						+			
		A P=.09 N=195				W P=.09 N=229			
		Treatment A		Treatment B		Treatment C		Treatment D	
7. Bell Trapper	3 replicates Days=12	LW	RW	LA	RW	LW	RW	LA	RW
		-	-	+		-	-	+	
		P=.63 N=80	P=.63 N=80	P=.001 N=91	P=.001 N=52	P=.0001 N=39	P=.0001 N=88	P=.01 N=58	P=.01 N=8 7
		A, W combined							
		+							
		A P=.0001 N=179				W P=.0001 N=91			
		Treatment A		Treatment B		Treatment C		Treatment D	
8. Terminix All clear	2 replicates Days=8	LW	RW	LA	RW	LW	RA	LA	RA
		-	-	-	-	-	-	-	-
		P=.76 N=22	P=.76 N=24	P=.59 N=46	P=.59 N=41	P=.65 N=37	P=.65 N=41	P=.41 N=40	P=.41 N=33
		A, W combined							
		-				-			
		A P=.48 N=87				W P=.48 N=78			

Discussion

The literature on bioassay of adult mosquito attraction to volatiles includes dual choice olfactometer studies that involve expensive equipment and considerable effort (Gouck and Schreck 1965, Osgood and Kempster 1971, Posey et al. 1998). The other laboratory bioassay system used involved visual observation of mosquito numbers and behaviors at timed intervals which is time consuming and subject to the experience of the human observer (Bernier et al. 2003, Jhumur et al. 2006, 2008). The utility of a simple and relatively inexpensive cage assumed to measure the relative attraction of volatiles over time, without the need for timed observations was the goal of this study. We assumed that the mosquitoes would fly to the most attractive location in the cage, drawn by the volatiles emitted by the solutions placed in microcentrifuge vials located next to a glue trap and the trap would catch those mosquitoes; therefore, the number of mosquitoes caught in the glue traps would indicate attraction. There are several factors that could impact the observation of attraction using glue boards in a cage bioassay that include the glue used on the sticky card, the species of mosquito and the sex of the mosquito.

The potential attraction of the materials in the glue employed in these bioassays was tested and we found that Trapper was attractive when tested with water and compared with stickem glue and water; Trapper was also attractive with honey when compared against honey and stickem with water. Trapper glue boards were attractive while the tanglefoot glue boards were likely repellent as indicated by numbers of mosquitoes caught in Table 2.1. Therefore the later bioassays were conducted using stickem as the glue on the sticky traps. The species of mosquito varied by experiment and often we used a mixture of species of both genders in the same cage. An analysis of the data by numbers caught without regard to the species or gender showed that isoamyl acetate was attractive in this bioassay system and citronella was repellent.

An examination of the mosquito species data showed that *Culex* responded more often in bioassay than the *Aedes* species and the reason(s) for that data need to be examined in future tests. Our data on the sex of the mosquito showed no obvious affect so we consider this bioassay a viable system for use without attention to gender of the test animal.

The impact that other conditions in the testing room (i.e., light source(s) or air flow) had on our data was not examined but because we found that certain treatment combinations that employed the same material, either water/water or attractant/attractant occasionally showed a 'preference' and the reasons for this should be tested in future research. Despite using 'large' cages that separated the test materials by 31-cm that this distance was not great enough to provide a gradient for the mosquitoes to follow to the source. Cage size and configuration, perhaps a cage that is longer than wide that separates treatments but takes up less comparable room space would provide a better result with fewer replicates.

This series of tests showed promise that a cage bioassay using sticky traps can indicate preference in selected adult mosquito species regardless of gender. The lessons learned indicate that the number of animals tested must exceed 100 caught to afford statistical power to any conclusions and use of a completely randomized block latin square design can alleviate concerns about directional bias because of other unintended cues in the room such as light or airflow. Future studies should be aimed at illucidating if visual cues are important such as the number of mosquitoes previously caught and light sources in the room have an impact on the data.

CHAPTER 3

CONCLUSION

These experiments showed the utility of using a cage bioassay employing sticky traps to measure the relative attraction or repellency of various volatiles to newly emerged adult mosquitoes. The lessons learned included the use of a glue on the sticky trap that is not, by itself, attractive as we found using the Bell Trapper commercial trap that contains a proprietary blend of volatiles. The second is to use a completely randomized block latin square design that can help alleviate directional bias based on conditions in the experimental room and lastly to conduct the experiment until at least 100 mosquitoes are caught in a cage to provide statistical power to the analysis of the data.

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Appendix A Bioassay Design 1 Histograms
* Indicate significant attraction based on p value less than or equal to .1

Figure 2.4 Illustrates the number of mosquitoes caught per treatment

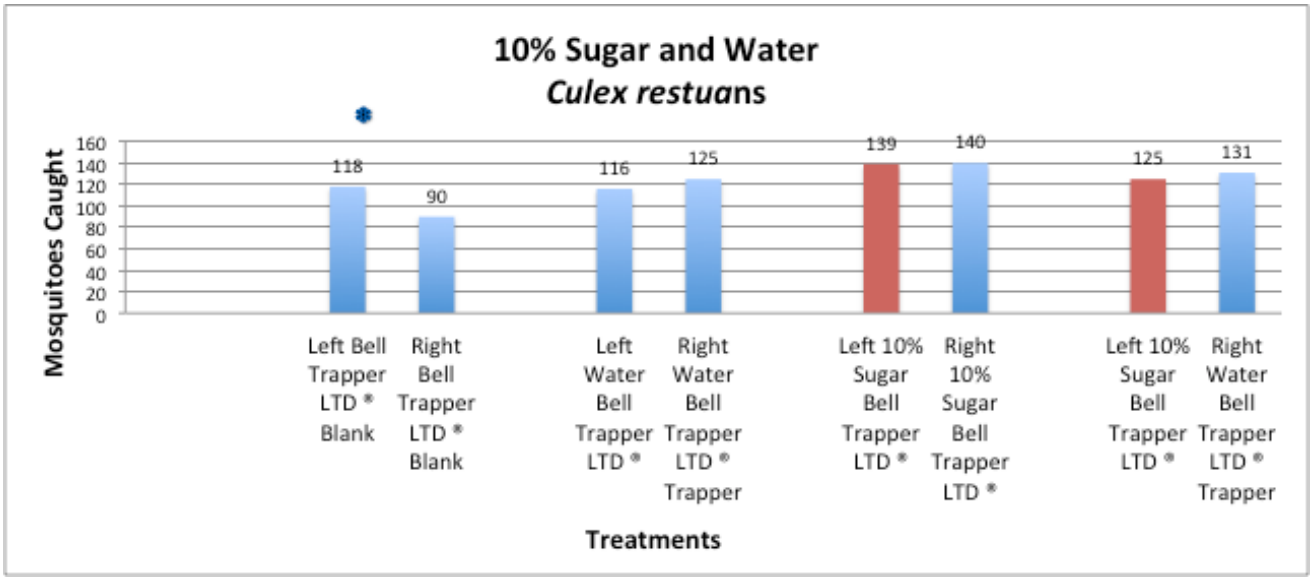


Figure 2.5 Illustrates the number of mosquitoes caught per treatment

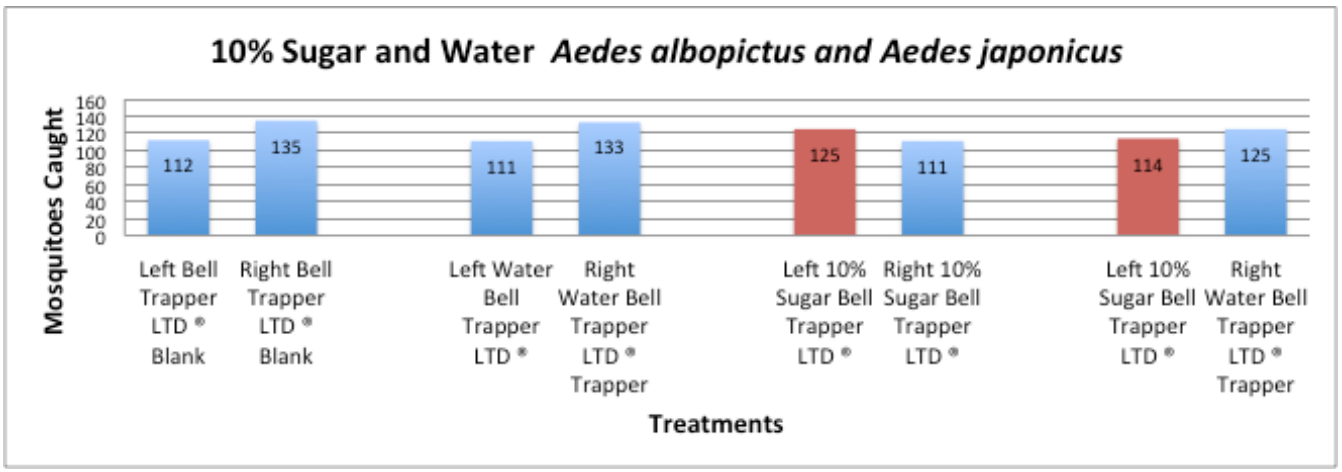


Figure 2.6 Illustrates the number of mosquitoes caught per treatment

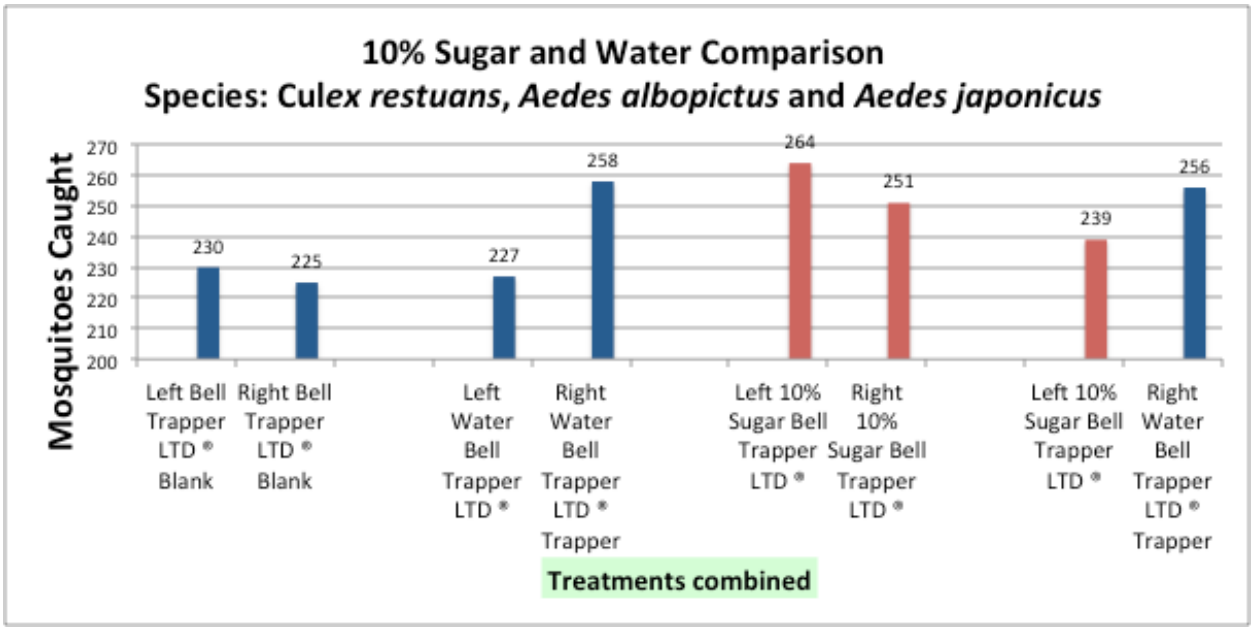


Figure 2.7 Illustrates the number of mosquitoes caught per treatment

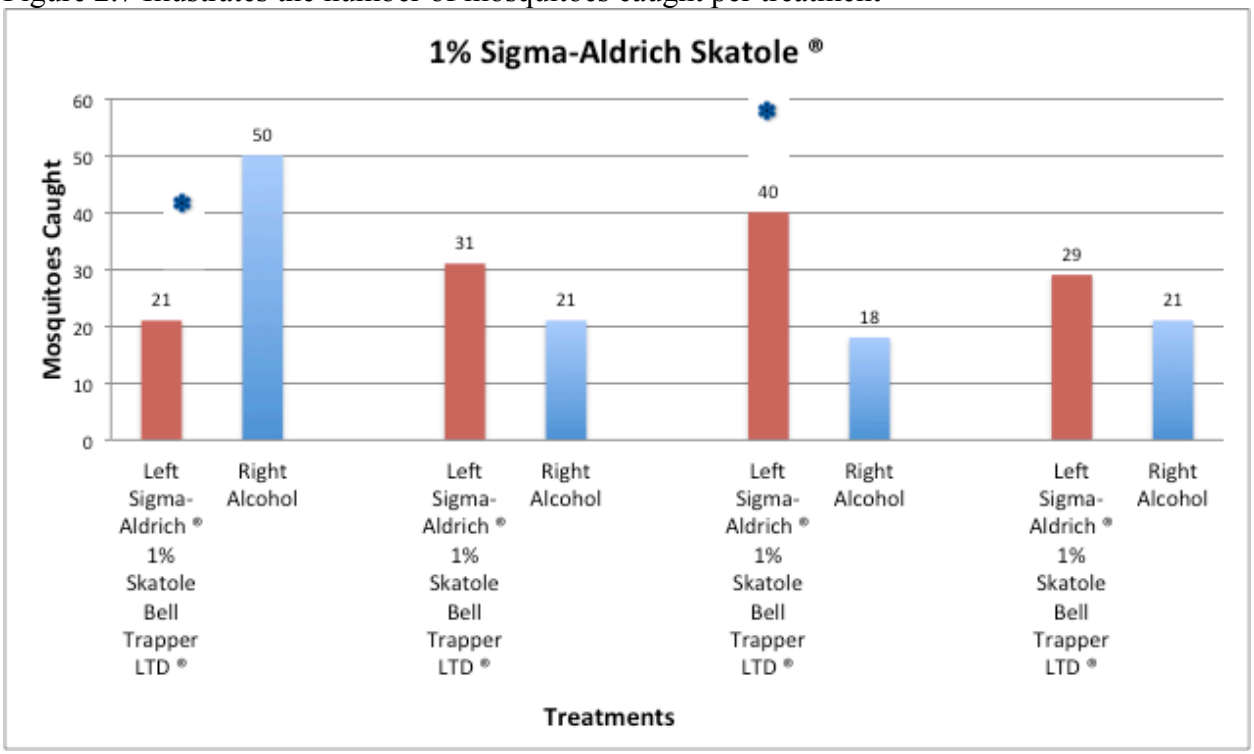


Figure 2.8 Illustrates the number of mosquitoes caught per treatment

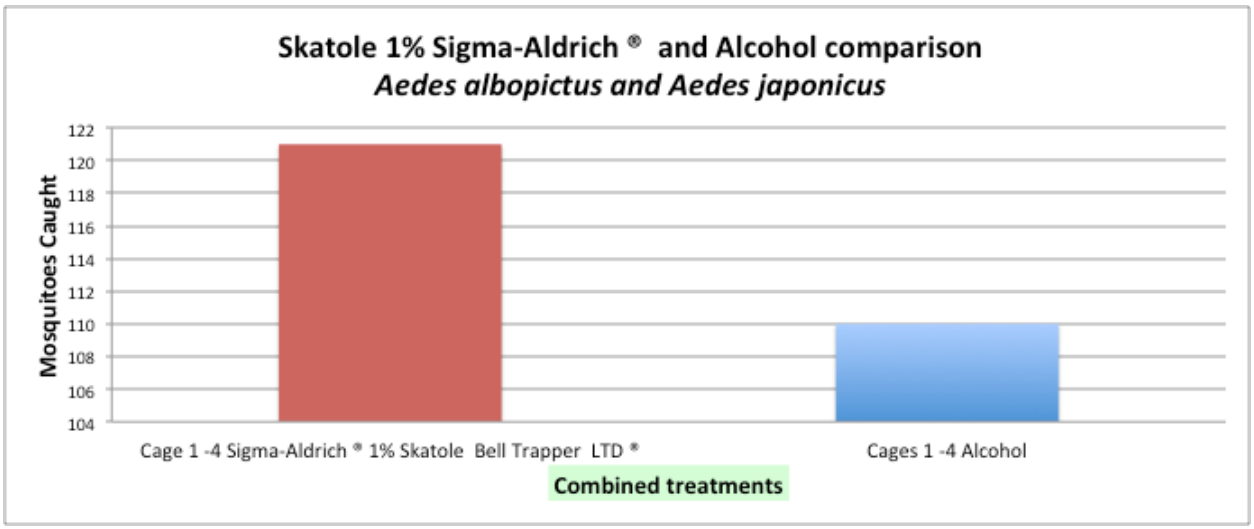


Figure 2.9 Illustrates the number of mosquitoes caught per treatment

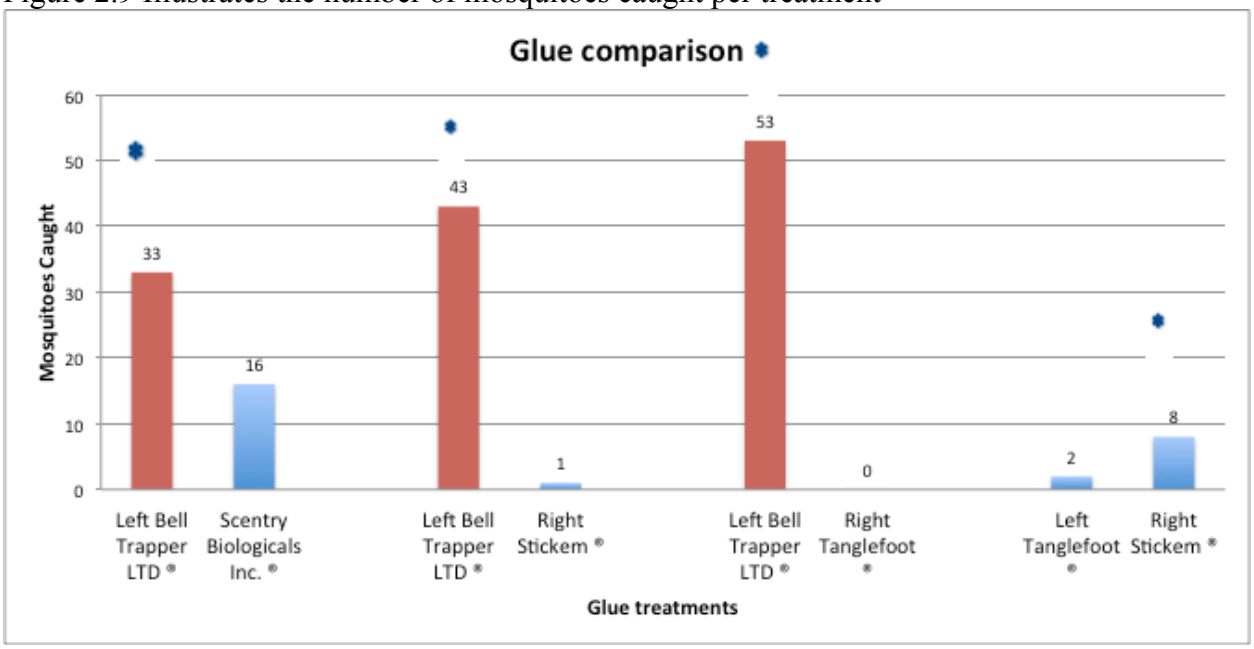


Figure 2.10 Illustrates the number of mosquitoes caught per treatment

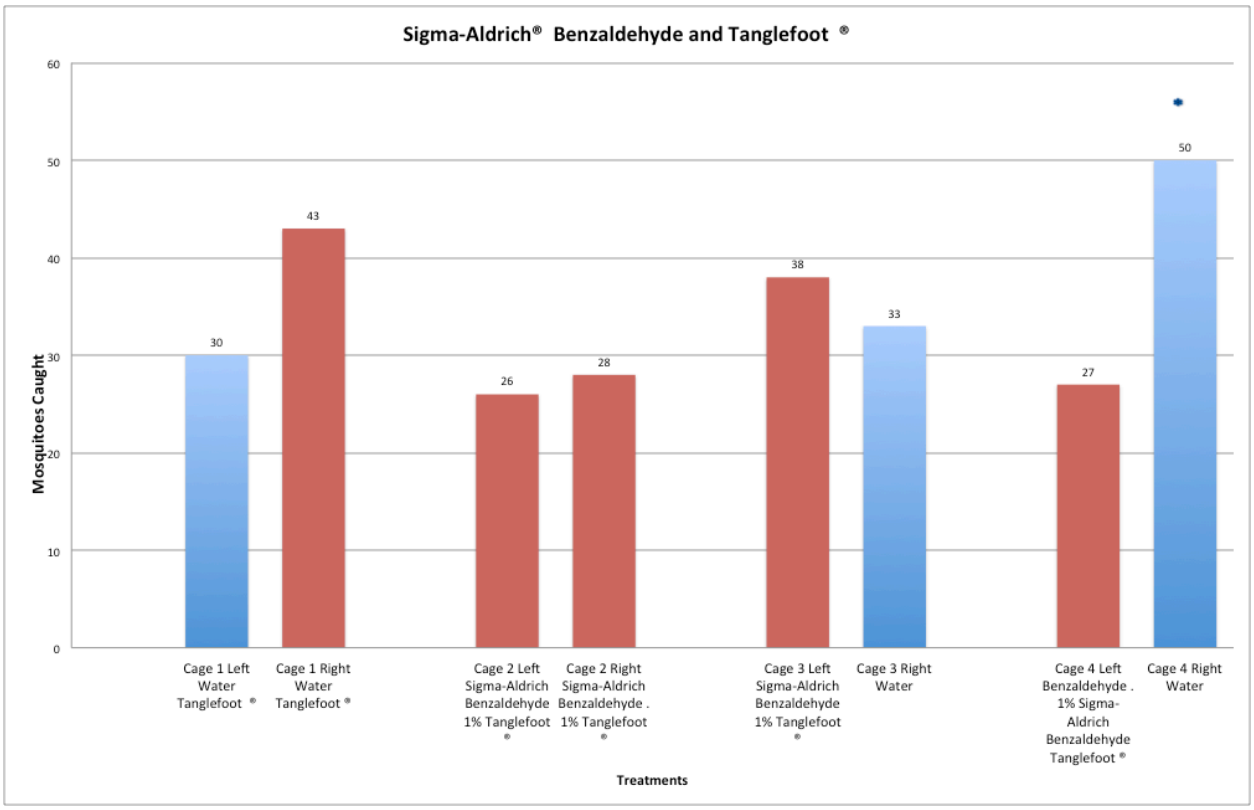
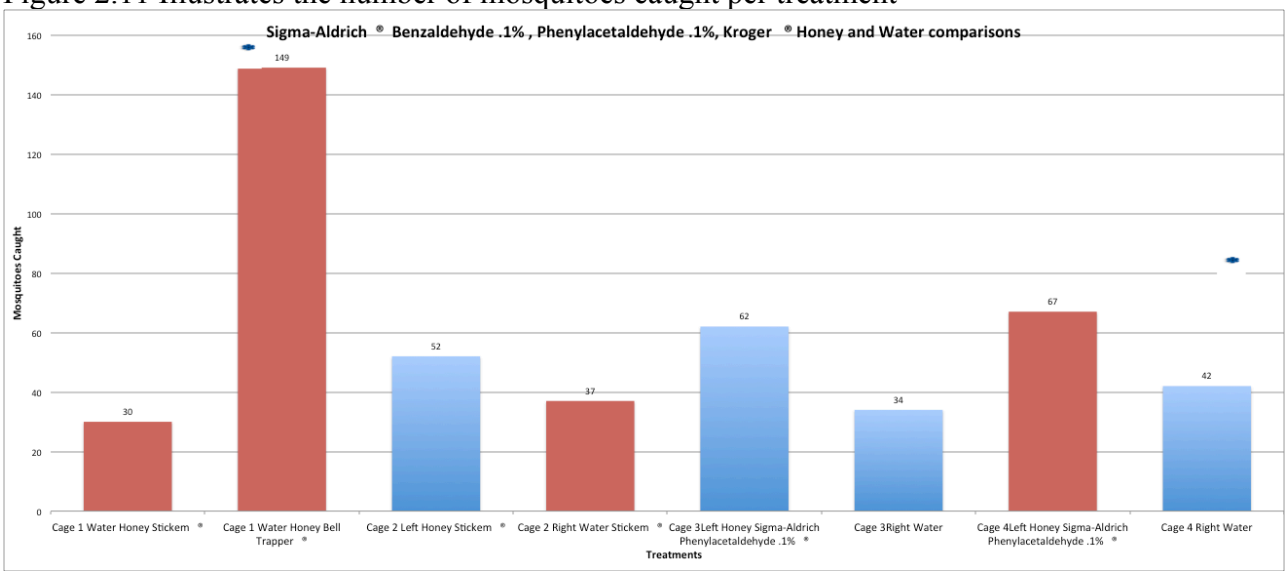


Figure 2.11 Illustrates the number of mosquitoes caught per treatment



Appendix B Bioassay Design 2 Histograms

Figure 2.12 Illustrates the number of mosquitoes caught per treatment

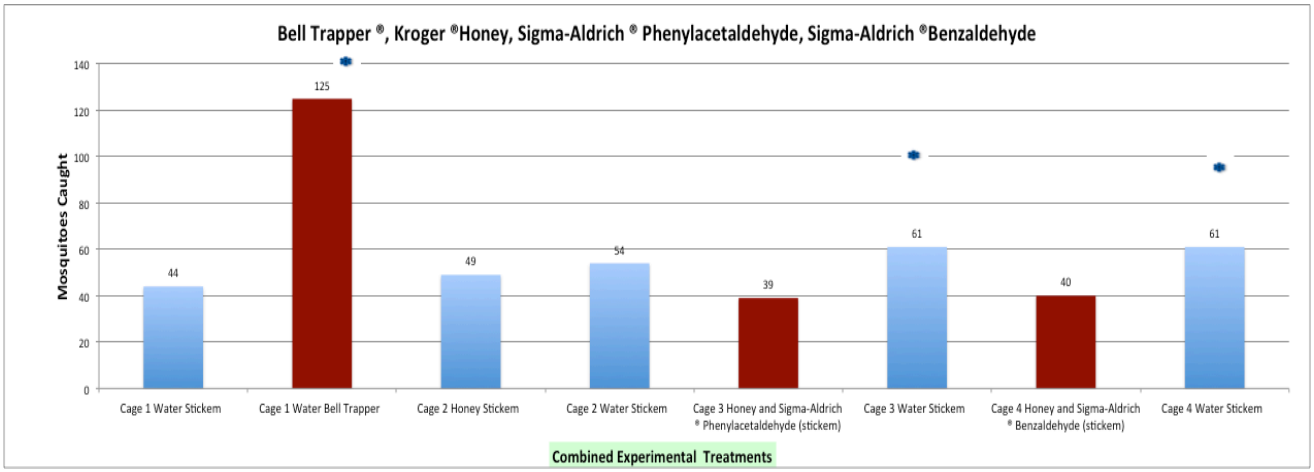
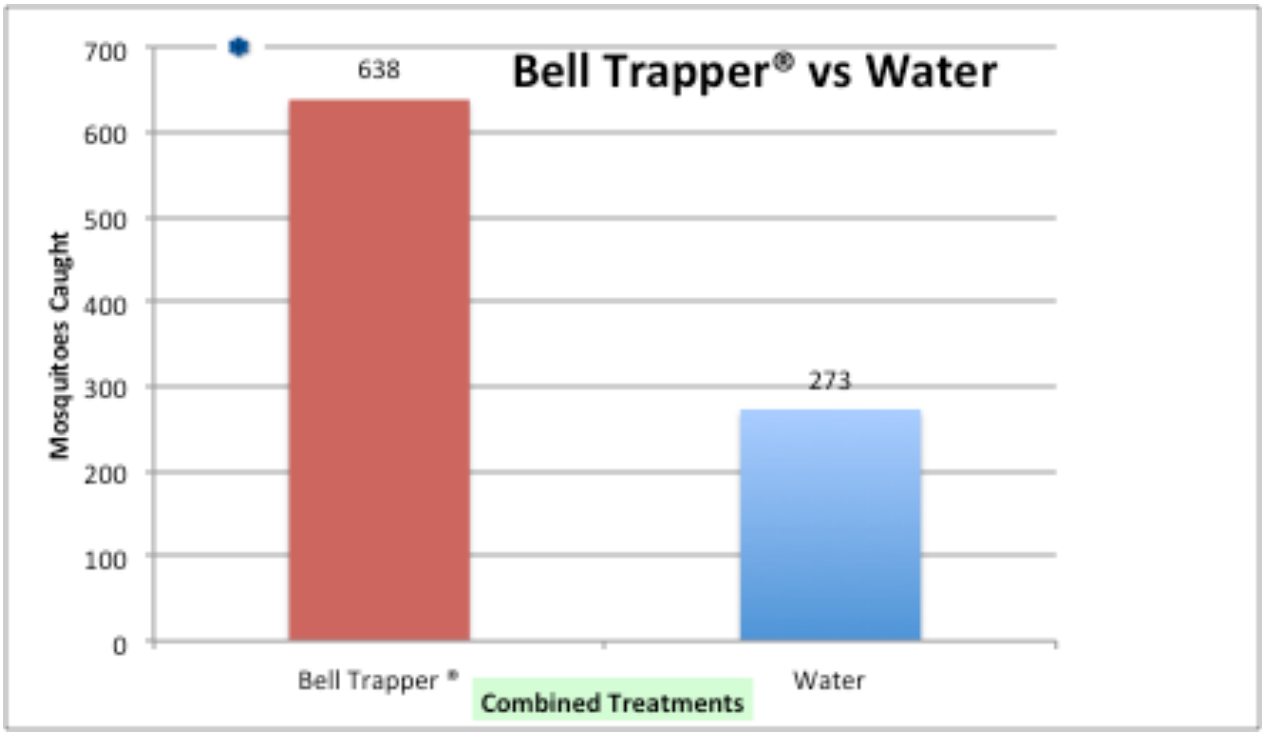


Figure 2.13 Illustrates the number of mosquitoes caught per treatment



Appendix C: Bioassay Design Type 3 (Latin Square Design)

Figure 2.14 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.

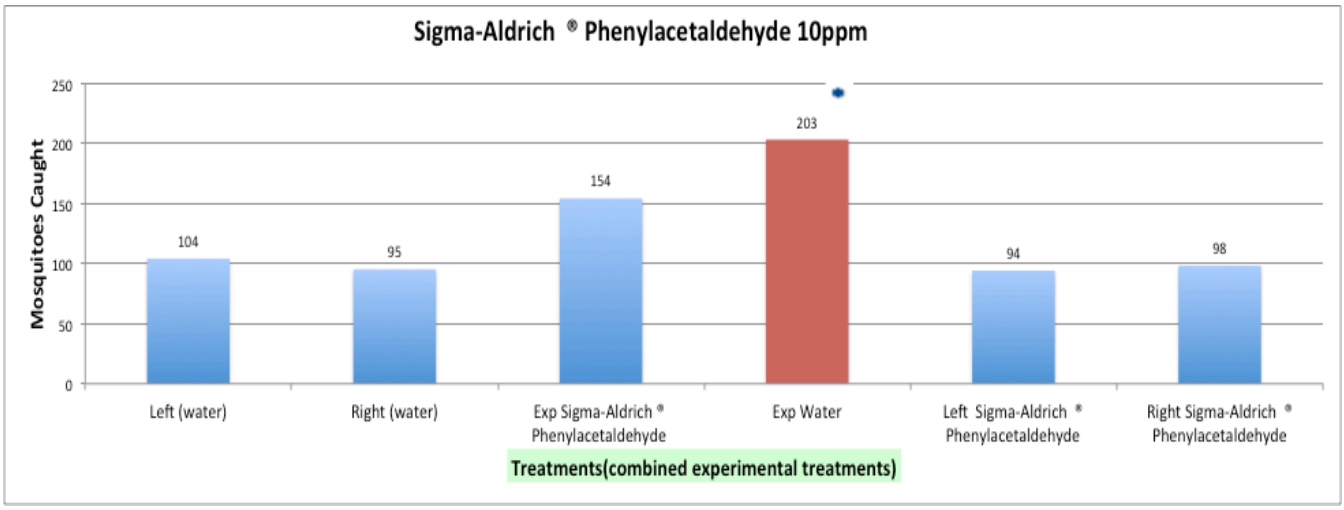


Figure 2.15 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.

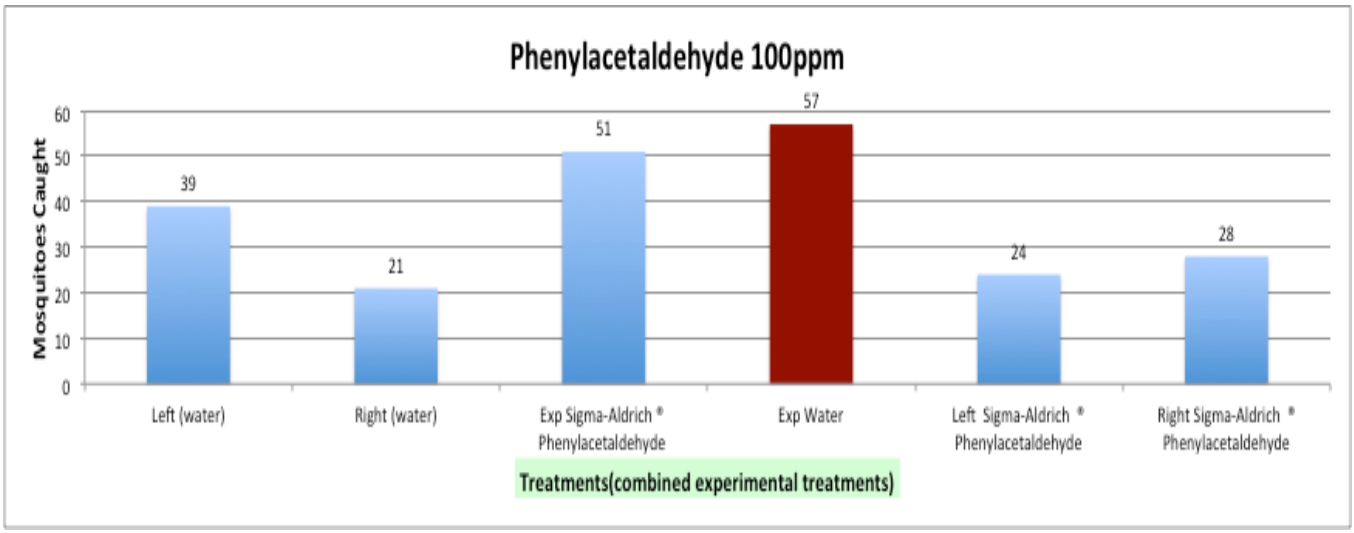


Figure 2.16 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.

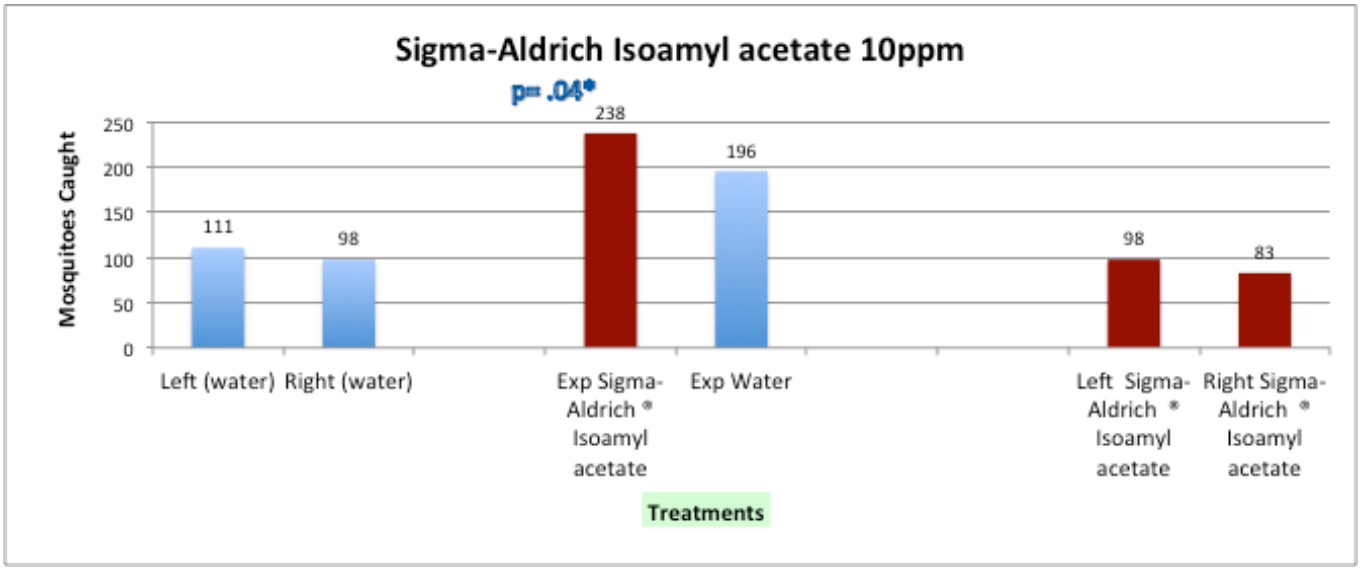
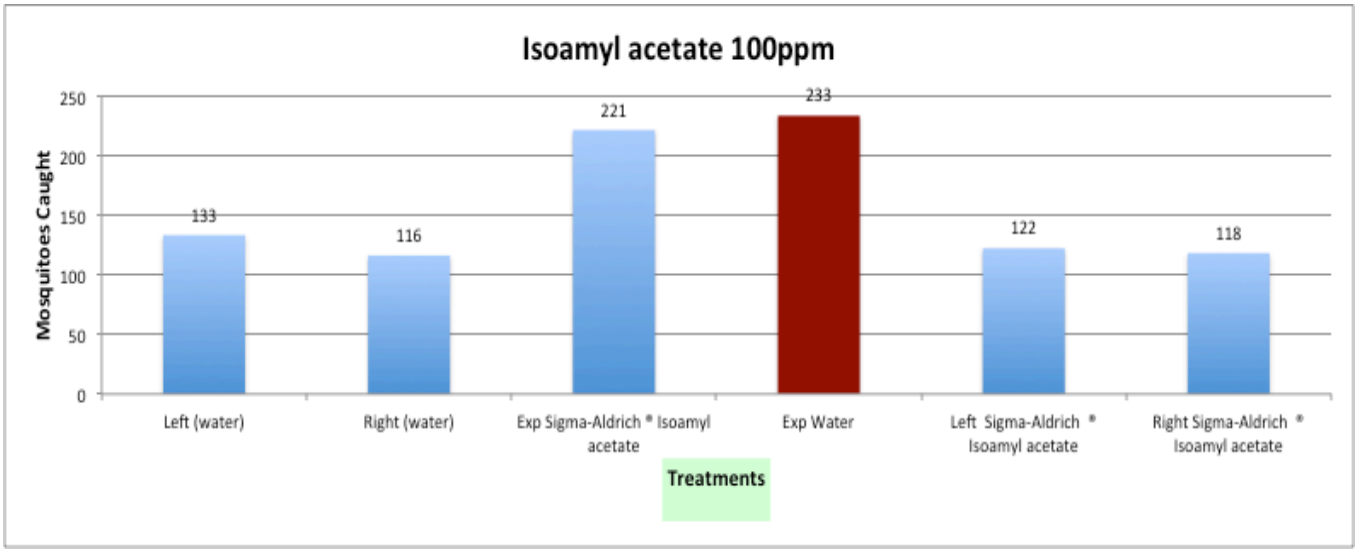


Figure 2.17 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.



2.18 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.

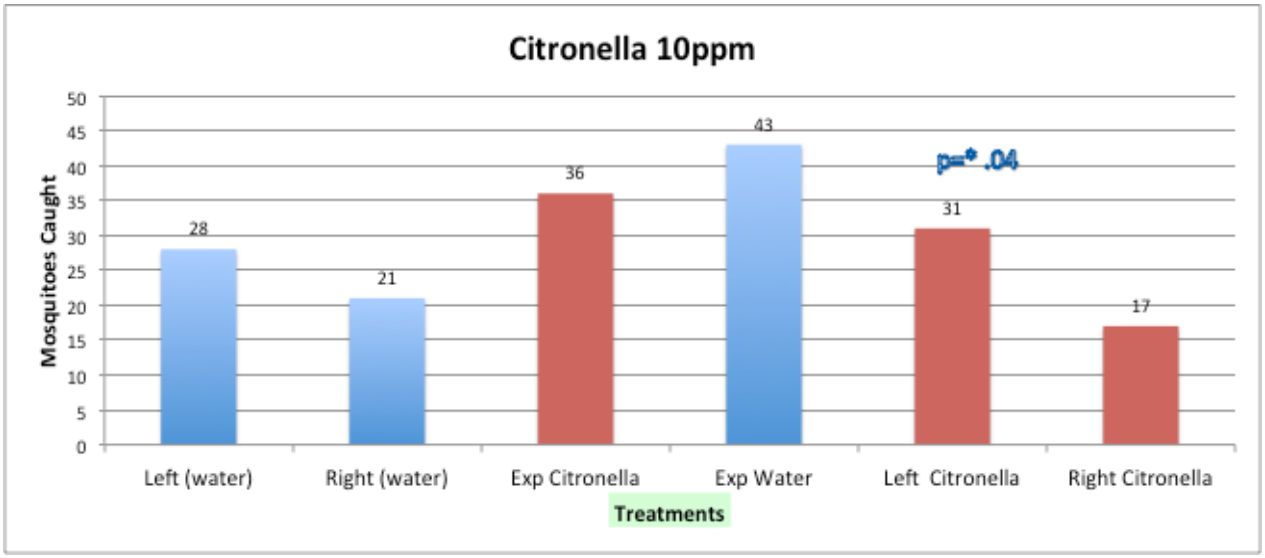


Figure 2.19 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.

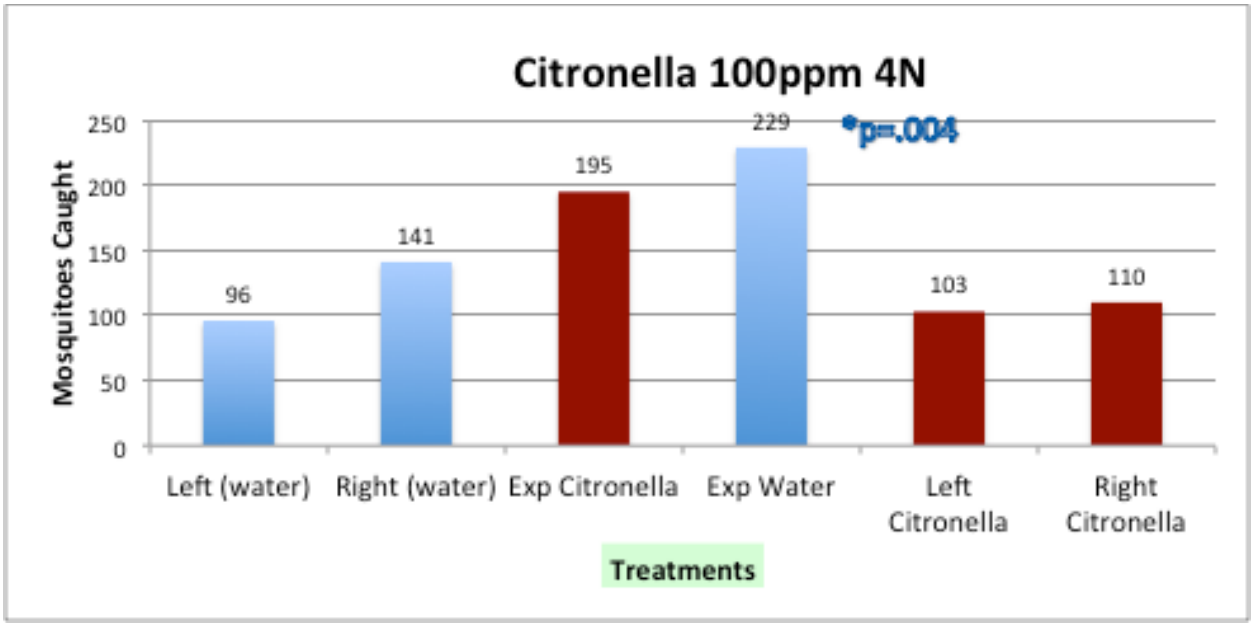


Figure 2.20 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.

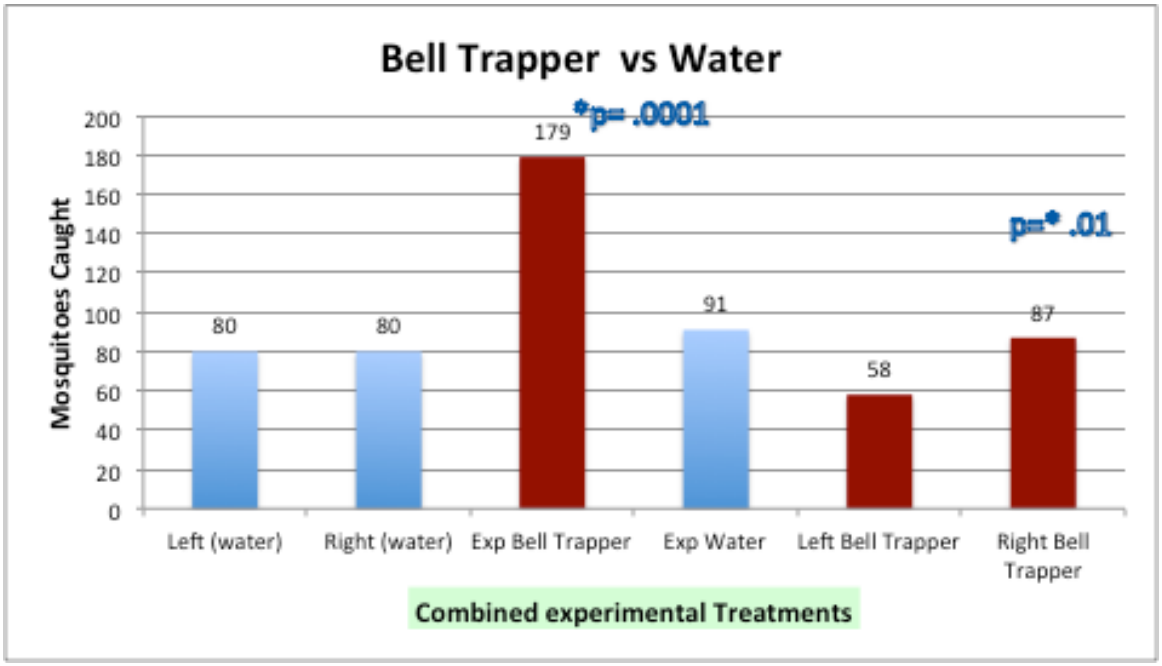


Figure 2.21 Illustrates the number of mosquitoes caught per treatment. Experimental treatments are combined in the histogram.