FEEDING BEHAVIORS AND RESPONSE OF SINDBIS VIRUS INFECTED *AEDES AEGYPTI* (L.) (DIPTERA: CULICIDAE) TO REPELLENTS

By

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This dissertation is dedicated to my mother, Sara Jo Qualls, and father, Jerry Allen Qualls, for their unwavering support.

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LIST OF ABBREVIATIONS

AI	active ingredient
BHK	baby hamster kidney
BSL	biosafety level
CDC	Centers for Disease Control and Prevention
CPE	cytopathic effect
D	DEET
DEET	N, N-diethyl-3-methylbenzamide
DENV	Dengue virus. An arbovirus in the family Flaviviridae; genus *Flavivirus*)
DMP	dimethyl phthalate
EPA	Environmental Protection Agency
GRN	gustatory receptor neruon
JEV	Japanese encephalitis virus. An arbovirus in the family Flaviviridae; genus *Flavivirus*).
LACV	LaCrosse virus. An arbovirus in the family Bunyaviridae; genus *Orthrobunyavirus.*
MEM	minimal essential media
ND	no DEET
OBP	odor binding protein
OR	odor receptor
ORP	odor receptor protein
OSN	odor sensory neuron
p.e.	post-exposure
PFU	plaque forming units
PMD	p-menthane-3,8-diol

RVFV	Rift Valley fever virus. An arbovirus in the Bunyaviridae, genus *Phlebovirus.*
SINV	Sindbis virus. An arbovirus in the family Togaviridae; genus *Alphavirus.*
TFB	Time to first bite
2-U	2-undecanone
USDA	United States Department of Agriculture
WNV	West Nile virus. An arbovirus in the family Flaviviridae; genus *Flavivirus*)
WHO	World Health Organization

Abstract of Dissertation Presented to the Graduate School
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Use of repellents is important in preventing mosquito-borne pathogen transmission. Investigation of pathogen-associated changes in insect behavior is critical to determine if repellents function in reducing or preventing mosquitoes from biting or if such arbovirus-associated changes can result in altered insect response, rendering repellents less effective. Few other published studies are available for comparing the effects of arbovirus infection on bloodfeeding or repellent efficacy in mosquitoes.

My findings suggest female *Aedes aegypti* mosquitoes with a disseminated Sindbis virus infection behave differently than their uninfected cohorts. On days 7 and 14 post-exposure to Sindbis virus, mosquitoes with disseminated infection took 1.3 and 1.5 times longer to bloodfeed. Other changes in the bloodfeeding behavior of *Aedes aegypti* after dissemination of Sindbis virus were observed after exposure to repellents with the active ingredients DEET, picaridin, 2-undeconane, and lemon eucalyptus. Activation, the initial response of the mosquito to the host, occurred at least 1 h sooner in *Ae. aegypti* with a disseminated Sindbis virus infection compared to uninfected mosquitoes in response to all repellents evaluated. Infection of *Ae. aegypti* with Sindbis

virus decreased the time to first bite of DEET and picaridin by 3.1 and 2.1 h, respectively. When exposed to 2-undeconane and lemon eucalyptus, mosquitoes with dissemination disseminated Sindbis virus infection located the bloodmeal 1 h sooner than did uninfected *Ae. aegypti*. Together, these results indicate that behavioral changes in mosquito host-seeking, bloodfeeding, and sensitivity to repellents occurred in mosquitoes following Sindbis virus dissemination. My findings also suggest that when the virus has crossed the gut barriers and is poised for potential transmission the mosquito may be less responsive to repellent use.

Understanding the physiological basis for these behavior changes, especially the decrease in susceptibility to DEET, the gold standard of repellents, is important in the prevention of diseases caused by mosquito-borne viruses. While prolonged feeding duration and increased body weight after a bloodmeal may hamper mosquito survival, a decrease in sensitivity to repellents enhances a mosquitoes' ability to bloodfeed on humans. Extended bloodfeeding by infected mosquitoes could result in interrupted feedings, a phenomenon that can result in an increase in virus transmission.

CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

Introductory Statement

The traditional view of interactions between arboviruses and their arthropod vectors is that vectors become increasingly resistant to parasites over time and that arboviruses do not exhibit any deleterious effects when disseminating within their mosquito hosts (Burnet and White 1972). This view is similar to the conventional explanation for host-parasite evolution in general and states that parasite attenuation occurs through coevolution and resistance (Burnet and White 1972). This view assumes that if the host and parasite do not coexist, a detrimental effect that hinders the parasite's fitness will be observed in the invertebrate host.

LaMotte's (1960) work on Japanese encephalitis virus (JEV; family Flaviviridae; genus *Flavivirus*) provided evidence for Burnet and White's explanation for host-parasite evolution. LaMotte reported that JEV infected *Culex quinquefasciatus* Say, *Culex molestus* Wiedemann, and *Aedes aegypti* (Linnaeus) females did not differ in survivorship when compared with uninfected controls. This study also failed to detect any histological changes in mosquitoes infected with JEV. Defoliart et al. (1987) reported that transovarially acquired La Crosse virus (LACV; family Bunyaviridae; genus *Orthrobunyavirus*) appears to have no adverse effect on duration of the larval stage, sex ratio, hatching success, time to ovarian maturation, fecundity, or adult survival on populations of infected *Aedes triseriatus* (Say).

Parasite manipulations of host behaviors have been reported in a wide range of protozoan and metazoan parasites (Moore 2002, Poulin 2007). Moore (2002) documented parasite manipulations that increased parasite transmission. The Gordian

worm (*Paragordius tricuspidatus*) manipulates its cricket host to enter water where the worm continues its lifecycle. The trematode (*Curtuteria australis)* impairs the natural burrowing behavior of a cockle (*Austrovenus stutchburyi*) making them more likely to be eaten by aquatic birds which are the final hosts of the parasite. Coral polyps infected with a trematode *(Podocotyloides stenometra)* become more conspicuous and vulnerable to predation by the coral-feeding butterfly fish (*Chaetodontri fascialis)*, the definitive host of the parasite.

These changes in host behavior are traditionally categorized into three kinds of phenomena: secondary outcomes of infection with no adaptive value, host adaptations that reduce the detrimental consequences of infection, and parasite adaptations that facilitate transmission (Lefevre et al. 2008). The third phenomena known as the "manipulation hypothesis," presents behavior modification as coevolved traits rather than a complete takeover of the parasite host.

There is a growing body of literature addressing interactions between mosquito vectors and their parasites. The literature has focused on *Plasmodium*-manipulation of malaria vectors. Malaria-parasites have been reported to induce changes in probing behavior in their hosts increasing the likelihood of malaria transmission (Hurd 2003). Understanding the strategies employed by the malaria parasite to induce behavior changes will provide fundamental knowledge that can be used to target specific vectors and integrated into vector management programs (Lefevre and Thomas 2008). There are many reports of vector manipulation by parasites of medical importance; however, the literature has generally overlooked arboviruses as potential behavior modifiers. In

fact, investigations using virus-infected mosquito responses to attractants and repellents is sorely lacking in the literature.

Over the last two decades studies have shown that some arboviruses do have detrimental effects on their mosquito vectors. Cytopathological effects of arbovirus infections have been found in the mosquito midgut (Weaver 1988, Weaver et al.1992; Vaidyanathan and Scott 2006) and salivary gland tissues (Mims et al. 1966, Bowers et al.1995, Bowers et al. 2003, Girard et al. 2005, Girard et al. 2007). Arboviral infections have been associated with decreased adult survival (Turell et al. 1984, Faran et al. 1987, Turell 1992, Scott and Lorenz 1998, Lee et al. 2000, Moncayo et al. 2000, Mahmood et al. 2004), decreased fecundity (Tesh 1980, Turell et al. 1985, Scott and Lorenz 1998, Mahmood et al. 2004, Styer et al. 2007), altered feeding behavior (Grimstad et al. 1980, Platt et al. 1997), and decreased flight activity (Lee et al. 2000). There has only been one study reported in the literature investigating virus-associated behavioral changes of mosquito vectors to repellents (Frances et al. 2011). Frances et al. (2011) reported no differences in response to DEET of *Ae. aegypti* and *Aedes albopictus* Skuse infected with four dengue virus serotypes (DENV; family Flaviviridae; genus *Flavivirus*). This study focused on behavior changes following intrathoracic inoculation of these mosquito species with DENV., and the current investigation focused on altered behavior following oral feeding of mosquitoes with an arbovirus. A goal of my research is to use an oral feeding of Sindbis virus (SINV; family Togaviridae; genus *Alphavirus*) as a model to investigate behavioral changes associated with virus-infection and dissemination leading to an altered mosquito response to repellents.

Arbovirus Mosquito Interactions

Vector survival is a critical parameter in the estimation of vectorial capacity because it essentially determines (1) the size of the population that is potentially able to transmit virus and (2) the expected mean duration of the time that an infected mosquito is infective (Reisen et al. 1989). Total egg production and the stability of the population also depend on the adult survival rate. Reisen et al. (1989) investigated vector survival as solely dependent on environmental parameters and not influenced by virus or pathogen replication and dissemination within the mosquito host. It can be speculated that the mosquito-virus relationship probably influences behavioral changes of the mosquito affecting the mosquitoes' overall fitness and responses to the environment, including ability to transmit pathogens (Lefevre and Thomas 2008). Thus, the effect of an arbovirus on a female mosquito should be incorporated in any model describing the vectorial capacity of a given mosquito species for any virus (Moncayo et al. 2000).

The traditional view of interactions between arboviruses and their arthropod vectors, as mentioned previously, is that parasites do not exhibit deleterious effects on the vector. However, Grimstad and coworkers (1980) reported LACV-infected *Ae. triseriatus* took significantly longer to bloodfeed than their uninfected cohorts. Bowers et al. (2003) demonstrated SINV-associated pathology in the salivary glands and/or SINV-associated muscle cytopathology (Vo et al. 2010) in *Ae. albopictus.* These findings might provide a plausible structural etiology for altered insect behavior. Pathology in the salivary glands could contribute to a change in glandular secretions and /or function which may explain the findings of Grimstad et al. (1980). Bowers et al. (2003) also suggested that damage to the salivary glands by pathogens might explain prolonged feeding times seen in some mosquito species (Rossignol et al. 1984). Girard et al.

(2005) suggested that cytopathological damage to the salivary glands could also result in an increase in the total number of blood-meals taken. The increase in the number of blood-meals may be a mechanism to compensate for a decrease in nutrients derived from sugar feeding or a parasite manipulation of its host to increase its likelihood of survival. Styer et al. (2007) reported an increase in the number of times West Nile virus (WNV; family Flaviviridae; genus *Flavivirus*) infected *Culex tarsalis* Coquillett bloodfed compared to control groups which further supported Girard's assumption.

A decrease in mosquito survival has been associated with arbovirus infection (Turell et al. 1984, Faran et al. 1987, Turell 1992, Scott and Lorenz 1998, Lee et al. 2000, Moncayo et al. 2000, Mahmood et al. 2004). However, studies that have demonstrated virus-induced mortality have shown that some, but not all, mosquito species are susceptible to arbovirus infections (Moncayo et al. 2000). These studies have also shown that virulence of the virus may or may not be related to host survival and that reductions of mosquito mortality occur after the probability of virus transmission is highest (Scott and Lorenz 1998). The effects of mosquito nutritional status, prior to acquiring an arbovirus, on the dissemination and transmission of arboviruses have not been evaluated.

Virus infections have also been associated with reduced fecundity of *Ae. albopictus*, *Culiseta melanura* (Coquillett) (Scott and Lorenz 1998), and *Cx. tarsalis* (Mahmood et al. 2004 and Styer et al. 2007). Studies using a malaria parasite-vector model have suggested that reduction in fecundity is due to a shift in resource allocation in response to parasite infection (Sorensen and Minchella 1998, Hurd 1998, 2001, Hurd et al. 2001). These studies have reported an increase in sugar feeding of *Plasmodium*-

infected mosquitoes. The malaria parasite-vector model varies from the virus-vector model in that malaria parasites require glucose for survival and may manipulate their hosts to increase their sugar feeding to ward off immune responses (Prabhakar 2000, Golderer et al. 2001, Kimura et al. 2001). In virus-vector relationships, the role of sugar intake may be utilized to induce an immune response to keep the vector healthy. A healthy vector may have an increased likelihood of surviving virus-associated cytopathic damage.

Repellents

History

The first recorded use of repellents may have been among the writings of Herodotus (484 BCE-ca. 425 BCE), who observed Egyptian fishermen (Herodotus 1996) using castor-oil plant extract in lamps. This oil was stated to have an unpleasant odor which might have provided spatial repellency to the high density of mosquitoes active in the evenings in this area. The Romans also recorded rubbing a concoction of vinegar, manna, and oil on the body to repel gnats (Owen 1805). Natural vinegars may have had an effect on nuisance mosquitoes because the acids in vinegars have a mild antibacterial effect on the skin and therefore reduce the production of bacterial metabolites that aid in human detection by mosquitoes (Braks et al. 1999). Both the *Geoponika* and Sanskrit *Yoga Ratnakara* writings (Owen 1805, Debboun et al. 2007) contain references to the burning of herbs and plants to repel biting insects. The smoke could have masked human kairomones needed for host location by mosquitoes (Bockarie et al. 1994). North American native cultures also relied heavily on plants to repel biting insects (Moerman 1998). Different cultures relied on different plants such as cow parsnip blossoms (*Heracleum maximum* Bartram), common yarrow (*Achillea*

millefolium L.), and fringed sagewort (*Artemesia frigid* L.) to name a few that ward off mosquitoes by either covering their body with the plants or burning them.

The idea of burning plants or ingredients of plants were expanded upon after introduction of pyrethrum into Europe in the nineteenth century (Casida and Quistad 1995) from the Middle East. Pyrethrum is a natural plant oil that occurs in the two species of pyrethrum daisy; *Chrysanthemum cinerariifolium* (Trevir.) and *Chrysanthemum coccineum* (Willd.). Pyrethrum is thought to have originated in China (Eisner 1991). Pyrethrum powders were used by armies from the time of Napoleon to World War II to combat head and body lice (Casida and Quistad 1995). Around 1890, the business man, Eiichiro Ueyama, improved the pyrethrums powder and developed a spiral-shaped mosquito repellent (Uemura and Ueyama 2004). Mosquito coils are widely used today: 29 billion mosquito coils are sold each year, 95% of them in Asia (World Health Organization, [WHO] 1998).

Since many of the essential oil-based repellents used throughout history had limited duration, intensive research began during World War II to find long-lasting repellents (Bunn et al. 1955). This research was initiated because of chigger exposure during combat training and chigger-borne scrub typhus (Zarafonetis and Baker 1963) contracted overseas following the outbreak of the war with Japan. The Surgeon General requested the Orlando laboratory of the United States Department of Agriculture (USDA) to study means and methods for controlling chiggers by repellents or insecticides (Whayne 1955). One of the first chemical repellents to be developed was dimethyl phthalate (DMP) followed by Indalone® (butyl-3,3-dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate) and Rutgers 612 (2-ethyl-1,3-hexanediol) (Peterson and

Coats 2001). After the war, a repellent known as 6-2-2 or M-250, containing 6 parts DMP, 2 parts Indalone, and 2 parts Rutgers 612 was used in the US (Debboun et al. 2007). This product was removed because studies revealed poor lung expansion and toxicity after cutaneous administration to pregnant rats (Neeper-Bradley et al. 1994). This was during the same time period that DEET (N, N-diethyl-3-methylbenzamide), the current gold standard of repellents, was introduced to the market (McCabe et al. 1954).

Modern Repellents

DEET. DEET is considered to be the most effective repellent in use today more than 50 years after its discovery (Roberts and Reigart 2004). DEET is a broad-spectrum repellent that is highly effective against medically important insects including all mosquitoes (Debboun et al. 2007). DEET is the repellent against which other substances are compared in the laboratory and in field trials (Debboun et al. 2007).

Debboun et al. (2007) estimated that 15 million people in the United Kingdom, 78 million people in the US (Goodyer and Behrens 1998) and 200 million people globally use DEET each year (US EPA 1980). DEET has been reported to have many problems, including an unpleasant odor (Paluch et al. 2010) and is a possible cause of central nervous system depression (Kim et al. 2004). However, DEET has been used for 50 years with only a few incidences of reported adverse effects, many of which had a history of excessive or inappropriate use of repellents (Veltri et al. 1994, Fradin 1998). Nonetheless, its toxicology has been more closely scrutinized than any other repellent, but it has been deemed safe for human use when used correctly (Goodyer and Behrens 1998, US EPA 1998), including use on children (Sudakin and Trevathan 2003) and pregnant women (McGready et al. 2001). Reports of DEET toxicity (Sudakin and Trevathan 2003, Fradin 1998, Corbel et al. 2009), minimal efficacy against some

arthropod vectors (e.g. *Anopheles* spp. of mosquitoes) (Rutledge et al. 1978), high incidence of arthropod-borne diseases (Centers for Disease Control and Prevention [CDC] 2010), decreasing consumer acceptance (Coats 1994, Isman 2006, Adler 2006), and the potential for insects to develop resistance to certain chemicals (Reeder et al. 2001) have resulted in the development of new repellents with different active ingredient (AI) components.

IR 3535. Insect repellent 3535 (IR 3535), [3-(N-acetyl-N-butyl) aminopropionic acid ethyl ester], also known as MERCK 3535, was developed in 1975 by Merck (Klier and Kuhlow 1976) and has been on the market in Europe for the past twenty years. In 1999, the Environmental Protection Agency (EPA) approved use of IR 3535 in the U.S. The EPA classified IR 3535 as a biopesticide, as it is a substituted B-amino acid, structurally similar to naturally occurring B-alanine (EPA 1999). It does have a low toxicity but can be irritating to the eyes and sometimes the skin (WHO 2004).

Laboratory data demonstrates IR 3535 to be equal to DEET against *Aedes aegypti*, *Culex quinquefasciatus* (Cilek et al. 2004, Thavara et al. 2001), and *Culex taeniorhynchus* Wiedmann, but not *Anopheles dirus* Cooper (Thavara et al. 2001). Field tests have shown conflicting results. Tests against field populations of *Aedes cantans* (Meigen) and *Aedes annulipes* (Meigen) determined that DEET provided two times more protection than IR 3535 (Rettich 1999). However, field trials in the Everglades measured no significant difference between the protection offered by DEET and IR 3535 (Barnard et al. 2002). A comprehensive field test against *Anopheles gambiae* Giles showed that IR 3535 decayed at a similar rate to DEET (Costantini et al. 2004), and the WHO (2004) declared it a safe and effective repellent for human use.

There has been no recorded incidence of an adverse reaction to this compound (Debboun et al. 2007).

KBR 3023. The repellent 1-piperidine carboxylic acid-2(2-hydroxyethyl)-1-methylpropylester was developed by Bayer in the 1980s using molecular modeling (Kruger et al. 1998). Picaridin is its common name and the WHO refers to this compound as Icaridin. Picaridin is a piperidine derivative that was registered for use in the US in 2005 (Debboun et al. 2007), has a very low toxicity and elicits practically no dermal or eye irritation or skin sensitization (EPA 2005). Picaridin does not have a plasticizing effect which is a major drawback of DEET. It also is colorless, odorless, and has a pleasant feel on the skin (Nentwig et al. 2002).

The efficacy of picaridin is considered to be excellent and because of its lower volatility has a longer longevity than DEET (Klun et al. 2003). Field and laboratory evaluations of picaridin against many mosquito species has shown similar efficacy to DEET (Costantini et al. 2004, Frances et al. 2004, Barnard et al. 2002, Yap et al. 1998, Badolo et al. 2004). Because of picaridin's efficacy and its cosmetic appeal, the WHO has designated picaridin as its "repellent of choice for malaria prevention" (WHO 2001). The CDC recommended picaridin for WNV and malaria prevention (CDC 2005).

PMD. PMD (p-menthane-3,8-diol), a monoterpene, was registered in the U.S. as an AI by the EPA in 2000. PMD was first recognized in 1955 (Schreck and Leonhardt 1991) but was rejected as a promising repellent by the USDA. However, in the early 1990s, repellent researchers became aware of Chinese product, *Quwenling*, which was known to be an effective repellent of mosquitoes. After analysis of the compound, 30% of the liquid was identified as PMD (Schreck and Leonhardt 1991). The CDC

recommends PMD as an effective repellent for control of WNV vector mosquitoes. Currently, the US restricts the concentration of PMD to 10% in commercially available products (Debboun et al. 2007) and American products with *Quwenling* (listed as oil of lemon eucalyptus) currently contain as much as 40% AI. PMD is desirable because it has a pleasant aroma and no tendency to dissolve plastics (Barasa et al. 2002). PMD can cause serious eye irritation and small children should not apply this product to themselves (EPA 2005). PMD can be synthesized chemically but also from the distillate of the leaves of the lemon-scented eucalyptus *Corymbia citriodora citriodora* (Hook). PMD shows good efficacy against field populations of *Aedes* spp. (Carroll and Loye 2006), and has shown 2 hr protection time against mosquitoes in the field when prepared as a 30% concentration.

2-Undecanone. 2-Undecanone (2-U) was originally derived from the glandular trichomes of wild tomato, *Lycopersicon hirsutum* Dunal *f. glabratum* C. H. Mull, plants, and it is a natural plant defense mechanism against insect herbivory (Farrar and Kennedy 1987, Kennedy 2003). It was registered in April 2007 by the EPA as a new arthropod repellent by BioUD (HOMS LLC, Clayton, NC) (Witting-Bissinger et al. 2008). Rat acute oral and dermal toxicity and rabbit dermal irritation toxicity tests resulted in 2-U in BioUD being rated as a category IV (the lowest toxicity level) by the EPA. In laboratory studies against *Ae. albopictus* and *Ae. aegypti* comparing BioUD to DEET (7 and 15%), there were no differences found over the same time period for both concentrations against *Ae albopictus* and for 7% against *Ae. aegypti.* In field trials, BioUD provided the same repellency or was more efficacious than 25 and 30% DEET, respectively (Witting-Bissinger et al. 2008).

Plant Based Insect Repellents. The repellency of plant material has been exploited for thousands of years by man, most simply by hanging bruised plants in houses, a practice that is still commonly used throughout developing countries (Debboun et al. 2007). Most plants contain compounds that prevent attack from phytophagous insects and these chemicals include repellents (Maia and Moore 2011). The release of volatile compounds as a result of herbivory has been shown to be repellent against mosquitoes (Pichersky and Gershenzon 2002).

PMD, mentioned earlier, is considered to be a potent natural repellent extracted from the leaves of lemon eucalyptus trees. Citronella and extract from plants in the citronella genus *Poaceae* have been used as a mosquito repellent by the Indian Army since the beginning of the 20th century (Covell 1943) and was registered for commercial use in the US in 1948. Citronella-based repellents only offer protection from host-seeking mosquitoes for about two hours and usually contain concentrations from 5 to 10% (Trongtokit et al. 2005a, Goodyer et al. 2010). Essential oils distilled from members of the Lamiaceae, Poaceae, and Pinaceae families are commonly used as insect repellents (Maia and Moore 2011). Almost all of the plants used as repellents are also used for food flavoring or in the perfume industry which may explain the association with these oils as safer natural alternatives to DEET despite many oils causing contact dermatitis (Strickman et al. 2009). The most effective of the plant essential oils have been shown to be thyme oil, geraniol, peppermint oil, cedar oil, patchouli, and clove that have been found to repel malaria, filarial, and yellow fever vectors for a period of 60 to180 min (Trongtokit et al. 2005b, Barnard 1999, Rutledge

and Gupta 1995). However, most of these essential oils are highly volatile and this contributes to their poor longevity as mosquito repellents.

It is commonly assumed that plant-based repellents are safer than DEET because they are natural. However, some natural repellents are safer than others and it cannot be assumed that natural equates to safe (Trumble 2002). The field of plant-based repellents is moving forward as consumers demand means of protection from arthropod bites that are pleasant to use and environmentally sustainable.

Mode of Action of Repellents

Mosquito behaviors in response to repellents are mediated by olfaction (Zwiebel and Takken 2004). Specific odor receptor proteins (ORPs) expressed on the membrane of sensory neurons is essential to detection of repellents in mosquitoes (Li et al. 2008). Odor binding proteins (OBPs) and ORPs are required for a correct performance of the olfactory system. Receptors for semiochemicals, sensory hairs called sensilla, are located on the third segment of mosquito antennae and maxillary palps (McIver 1982). Four types of mosquito sensillum are involved in mosquito olfaction all originating from epidermal cells: single-walled multiporous hair sensilla, single-walled multiporous peg sensilla, double-walled multiporous peg sensilla, and sunken double-wall multiporous peg sensilla (Clements 1999). Olfactory chemosensilla are characterized by a hair or peg whose wall is perforated by numerous pores or slits, the sites of entry for odorant molecules.

Stimulating molecules are adsorbed in the epicuticular layer on the surface of the sensillum and diffuse through pores. These odorant molecules pass into the lymph of the sensillar sinus and interact with OBPs. The OBPs protect these ligands from enzyme degradation present in the lymph and, by diffusion, the conjugant is transported

across the sensillar sinus. Such ORPs bind to the dendritic membrane on the OBP and eliciting a brief depolarization of the neuron. Odorant molecules are released and rapidly degraded.

The exact mode of action and the mosquito molecular target cells for AI found in insect repellents are poorly understood (Paluch et al. 2010). A better understanding of these factors is important for the improvement of repellent effectiveness and the development of compounds that disrupt the mosquito olfactory system. Five potential mechanisms of action of repellents on insects have been given by Davis; neuronal inhibition, stimulus with a saturation effect, inappropriate neuron activation, nociception, and neuronal activation leading to different behavior modes (Davis 1985). The perception of a repellent chemical and/or its binding to the arthropod receptors may induce an aversion stimulus for the insect, diverting the insect from the feeding process. The vapor layer created by repellents on our skin can be thought of a spatial buffer region of protection against the bites of hematophagous arthropods. Repelling action can occur near the skin or at a distance from the host. Insect ORP belong to a highly divergent gene superfamily, with little sequence similarity at the amino-acid level both within and between species (Bohbot and Dickens 2010). Thus, repellents may carry out their effects on mosquito behaviors via widely differing actions.

After more than 50 years of use, the mode of action of DEET is still debated in the scientific literature (Paluch et al. 2010). Recent studies have characterized the mode of action of DEET on isolated ORP (Xia et al. 2008, Ditzen et al. 2008) and OSNs of mosquitoes (Syed and Leal 2008). These researchers provided evidence that DEET has a specific odor receptor (OR) housed in a short trichoid sensillum on the mosquito

antennae that aids in the detection and avoidance of DEET by mosquitoes. Similarly, 2-U has independent OR binding sites for activation and inhibition whereas picaridin inhibits an OR-complex (Bohbot and Dickens 2010). Recent evidence suggests that the effectiveness of DEET is due to the dual action in inducing avoidance simultaneously via gustatory receptor neurons (GRN) and ORNs (Lee et al. 2010). Lee et al. (2010) demonstrated that DEET suppressed feeding behavior in *Drosophila* and that this effect was mediated by GRNs. It is not known if the GRNs are suppressed or activated after exposure to other AI of repellents.

Bohbot and Dickens (2010) suggest that the excitatory and inhibitory properties of DEET and 2-U, as well as the non-specific inhibitory effects of picaridin on ORs provide evidence that odor sensory neurons (OSN) elicit altered patterns of glomerular activity. This altered pattern may result in the scrambling of cognitive olfactory inputs and ultimately behavioral disruption. The mechanisms used by mosquitoes in processing the exogenous odors produced by repellents may be altered by virus infection, thus affecting host-seeking behavior (Paluch et al. 2010). With current molecular tools, identifying OBPs and ORNs can enhance mosquito vector-control by developing odorant molecules that will elicit or inhibit mosquito sensory structures. However, investigation of virus-infected mosquitoes' response to attractants and repellents is sorely lacking.

Sindbis Virus

Sindbis virus was first isolated in 1952 from *Culex univittatus* (Theobald) and *Culex pipiens* Linnaeus mosquito pools, and from a juvenile hooded crow *Corvus corone sardonius* in Sindbis, Egypt (Taylor et al. 1955). Sindbis virus is maintained in nature through a horizontal cycle, alternating between vertebrate and mosquito vectors

(Shope 1985). Since the first isolation of SINV from *Culex* mosquitoes, it has also been isolated from numerous *Culex* and *Aedine* mosquitoes (Doherty et al. 1977, Doherty et al. 1979) and has been shown to replicate in *Ae. aegypti, Ae. triseriatus,* and *Ae. albopictus* (Schiefer and Smith 1974, Stollar and Hardy 1984) in the laboratory.

Sindbis virus has a wide geographic distribution in Australia, Scandinavia, South Africa, The Middle East, and Asia (Laine et al. 2004, Dohm et al. 1995, Niklasson 1989, Tesh 1982) and its distribution is attributed to migratory birds, which transport SINV long distances within its range (Lundström et al. 2001, Lundström et al. 1993b). All of the isolated SINVs represent geographically distinct genotypes (Kurkela et al. 2004, Laine et al. 2004, Sammels et al. 1999, Lundström 1999, Norder et al. 1996, Shirako et al. 1991, Lundström et al. 1993a, Olson and Trent 1985). Sindbis virus is not known to occur in the Americas (Buckley et al. 2003).

Virus Isolates and Experimental Infection/Transmission

Sindbis virus is the prototype *Alphavirus* in the family Togaviridae. *Alphaviruses* are enveloped, spherical virions with icosahedral capsids and a diameter of 70 nm (White and Fenner 1994). They have a linear plus sense single stranded RNA genome 11-12 kb, are capped at the 5´ terminus, and polyadenylated at the 3´ terminus.

Laboratory experiments using SINV-orally bloodfed *Ae. aegypti* and *Ae. albopictus* have proven useful to address questions about experimental infection and transmission, genetically modified arboviruses, and the dynamics of arboviral tissue tropism and pathology in mosquito vectors (Bowers et al. 1995, Bowers et al. 2003, Jackson et al. 1993, Xiong et al. 1989). High ($10^{8.4}$ PFU/ml) SINV titers resulted in greater infection compared to moderate ($10^{5.3}$ PFU/ml) SINV titers. *Aedes albopictus*

had greater dissemination (66%) and transmission rates (53%) at the lower titer compared to *Ae. aegypti* (9 and 7%) (Dohm et al.1995).

A study of SINV replication and tissue tropism following intrathoracic inoculation in *Ae. albopictus* showed temporal and organ-specific distributions of the virus during the extrinsic incubation period (Bowers et al. 1995). Intrathoracic inoculation bypassed gut barriers resulting in maximal organ infection observed 3-4 days after infection. The mosquito's ovarioles and malpighian tubules were refractory to infection, whereas other organs had transient or persistent infections, perhaps indicating virus modulation by the mosquito or SINV (e.g., Bowers et al. 1995, Luo and Brown 1993, Murphy et al. 1975).

Sindbis-associated pathology of the salivary gland and midgut muscle tissues of *Ae. albopictus* has been observed (Bowers et al. 2003). Typically, arboviruses have few cytopathic affects on mosquito cells (*in vitro* and *in vivo*) (Hardy et al. 1983). Similarly, a study of *Ae. aegypti* following oral infection showed rapid infection of many organs within several days after feeding with the salivary glands being infected by day 5 (Jackson et al. 1993). As was the case for *Ae. albopictus*, the ovarioles and malpighian tubules of *Ae. aegypti* were refractory to infection. Unlike *Ae. albopictus*, there was no indication that the distribution of SINV in organs changed from days 6-14 (Jackson et al. 1993).

CHAPTER 2
MATERIAL AND METHODS FOR MOSQUITO REARING, VIRUS BLOODFEEDING, AND CYTOPATHIC ASSAY

Material and Methods

Mosquitoes. *Aedes aegypti* (Orlando strain) mosquitoes were reared and all experiments were conducted in an insectary maintained at 25.5± 0.5°C, 70-80% relative humidity, and a 16L: 8D photoperiod. Eggs were hatched in 3000 ml of deionized water, larvae were separated 600 per pan (29 x 34 x 6 cm) and fed approximately 1.0 g (1:1, dog biscuit: brewer's yeast) per pan per day until pupation (Gerberg et al. 1994). Mosquitoes were sorted based on the date of emergence, assuring that the mosquitoes evaluated during experiments were age-matched. During the first 5 d following emergence, females were allowed to mate freely and were sustained on a 10% sucrose solution. On day 6, post-hatching females were separated and offered either an infectious or non-infectious bloodmeal on day 7.

Virus. The heat resistant strain of SINV (SVHR) was obtained from Dennis T. Brown (North Carolina State University) and this virus was used throughout this study. I maintained the virus at University of North Florida in a Biosafety level-2 (BSL-2) laboratory. Virus was adsorbed at a low multiplicity of infection (0.1 plaque-forming units [PFU]/BHK cell), plaque purified, and amplified in BHK cells incubated at 37º C and 5% CO_2 to produce stock virus.

Bloodfeeding and Virus Infection. To promote mosquito bloodfeeding, fresh defibrinated bovine blood was warmed to 37°C by a water bath pump to simulate human body temperature. Water pumped through the water-jacketed feeder created a gentle motion, and double walled glass membrane feeders (Rutledge et al. 1964) covered by 12-cm-long sausage casing strips (The Sausage Maker, Inc., Buffalo, NY)

were placed on top of the screened cages. For each experiment, 7 d-old female *Ae. aegypti* were offered a bloodmeal in a membrane feeder that contained SINV (7.4×10^7 PFU/ml of blood) (SINV-exposed) or an equal volume of Minimum Essential Media-E (MEM) added to the bloodmeal as a negative control (MEM-exposed). Mosquitoes were offered the bloodmeal for 1 hr. Mosquitoes were then cold anesthetized and placed under a stereo microscope to identify fully bloodfed females. Unfed females were removed, and only fully engorged females were incubated in the insectary conditions stated above. Mosquitoes were provided an oviposition cup, 10% sucrose, and water.

Cell Culture and Cytopathic Effect Leg Assay.

Cultured BHK-21 cells were grown at 37°C and 5% CO_2 in MEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 10% tryptose phosphate broth. Both Gentamycin (20 ug/ml) and Amphotericin-B (2.5 ug/ml) were added to the MEM-media for leg assays (Renz and Brown 1976). A cytopathic effect (CPE) assay was used to detect the presence of virus in mosquito legs as an indication that the virus had escaped the gut barriers and the mosquito had developed a disseminated infection (Kramer et al. 1981, Turell et al. 1984). For processing, individual legs were thawed, 10 glass beads and 500 µL MEM were added to each vial, and vials were triturated on a bench top vortex for 2 minutes at medium speed. Preconfluent monolayers of BHK-21 cells were challenged with 1.0 ml of MEM and 0.5 ml aliquots of triturated legs removed from bloodfed mosquitoes and compared with cells challenged with virus samples (positive control), or MEM (negative control). Cytopathic effects were determined at 48 hrs post challenge.

CHAPTER 3
ALTERED RESPONSE TO DEET REPELLENT AFTER INFECTION OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE) WITH SINDBIS VIRUS

Investigations of arbovirus-mosquito host interactions have resulted in numerous reports describing modifications of mosquito bloodfeeding behaviors post-infection (Grimstad et al. 1980, Turell et al. 1985, Platt et al.1997). Grimstad et al. (1980) reported a reduced ability of *Ae. triseriatus* to bloodfeed following infection with LACV. Such behavioral modifications were characterized as more numerous probing attempts and less voluminous engorgement. Research reported by Turell et al. (1985) determined that *Culex pipiens* infected with Rift Valley fever virus (RVFV; family Bunyaviridae, genus *Phlebovirus*) were less successful at refeeding than uninfected cohorts. Increased probing and feeding times observed in *Ae.aegypti* mosquitoes infected with dengue virus were described by Platt et al. (1997). They also detected an association between behavioral changes with DENV-3- infection of organs and tissues that are known to control or influence activities associated with bloodfeeding; salivary glands, brain, Johnston's organ, and midgut as well as abdominal ganglion.

Prolongation of the feeding period of *Ae. aegypti* following DENV-3- infection of the mosquito (Platt et al. 1997) may suggest a nervous tissue and/or salivary gland involvement. Arboviruses that infect mosquito nervous tissue and fulminate virus replication and/or pathology in mosquito brain tissue have been observed following infection with SINV (Bowers et al. 1995), WNV (Girard et al. 2005), and DENV-2 (Salazar et al. 2007). Frances et al. (2011) suggested that virus infection of the nervous system may alter the mosquito's response to repellents. However, the behavioral response of arbovirus-infected mosquitoes to repellents has yet to be fully investigated.

Sindbis virus is an arthropod-borne virus that is maintained in a horizontal transmission cycle involving *Culex, Culiseta,* and *Aedes* spp. mosquitoes and wild birds (Doherty et al. 1977). Sindbis virus has been studied extensively (Myles et al. 2004) and molecular clones of the virus can be manipulated as genetic constructs designed to experimentally evaluate arbovirus transmission (Olson et al. 2000). *Aedes aegypti* mosquitoes are susceptible to many SINV variants in the laboratory (Pierro et al. 2007) including a heat resistant mutant of SINV (SVHR, Burge and Pfefferkorn 1966, Bowers et al. 2003). In light of the extensive knowledge of the molecular biology of SINV virus combined with the importance of *Ae. aegypti* in the public/animal health arena, I investigated alterations in the mosquito-host response to the repellent DEET following oral infection with SINV.

Materials and Methods

Repellent Response. All experiments were conducted at the University of North Florida BSL-2 laboratory in Jacksonville, FL. A 3% DEET-sugar suspension using Off! Deep Woods® Sportsmen (98.25% AI; S.C. Johnson, Racine, Wisconsin) was made in a 10% sucrose solution. The experimental DEET-sucrose suspension was colored with food Blue #1 (Stern, Natanya, Israel) and negative control sucrose suspension without DEET was colored with food Red #1. On days 3, 5, 7, 10, and 18 following virus feeding, 15 SINV- and 15 MEM- exposed mosquitoes were removed by mechanical aspiration and placed into experimental cages. Both SINV- and MEM-exposed mosquitoes were offered cotton balls saturated with the desired solution; either Blue 3% DEET-sucrose suspension (D), Red control sucrose suspension (ND), or a choice of both suspensions (D/ND). Both water saturated cotton balls for hydration and sugar

source were removed 24 h prior to all experiments. Each challenge was replicated three times on three separate dates.

After 24 h the mosquitoes were removed from treatment cages and the number of mosquitoes feeding on the D-sucrose suspension in both the SINV- and MEM-exposed groups was recorded. In order to determine feeding choice, individual mosquito abdomens were squashed on white paper under a dissection microscope to visualize the dye colors that indicated the imbibed sucrose treatment. A single hind leg was removed from all mosquitoes for CPE assay.

Data Analysis. Data were analyzed using Stats Direct (Stats Direct Ltd., Cheshire, UK). A Chi square test was used to evaluate whether mosquito response to the different treatments (D, ND, ND/D) was independent of SINV dissemination.

Results

Insect Sugar Feeding and Virus Dissemination. When allowed to feed on the ND-sucrose solution, SINV- (76%; 513/675) as well as MEM-exposed (70%; 472/675) mosquitoes fed to repletion. Mosquitoes feeding on the control treatment (10% sucrose solution) indicated that SINV- and MEM-exposed mosquitoes fed on the control during all test periods. There was no SINV dissemination detected on days 3 and 5 p.e. and as a result, data from these two time periods were combined. Of those SINV-exposed mosquitoes that were offered a ND-sucrose suspension on days 3 and 5 p.e., 85% (230 /270) fed on the ND solution (Table 3-1). Of the MEM-exposed on days 3 and 5 p.e., 79% (214/270) fed on the ND solution. There was no significant difference between SINV- and MEM-exposed mosquitoes feeding on the ND solution on days 3 and 5 p.e. (Chi square= 2.8, df=1, P=0.09). On day 7, 10, and 18 p.e., there were no significant

differences recorded between SINV- and MEM-exposed mosquitoes feeding on the ND solution (Chi square=3.2, df=1, P=0.07).

There were significant differences observed in feeding on the ND solution based on SINV dissemination on days 7, 10, and 18 p.e. (Chi square=6.14, df=1, P=0.02). Of the SINV-exposed mosquitoes offered the ND solution 59% (167/283) of the mosquitoes feeding on the ND solution had a positive SINV dissemination whereas 31% (38/122) that did not feed on the ND solution had a positive SINV dissemination.

Insect Repellent Analysis and Virus Dissemination. Dissemination was first detected in SINV-exposed mosquitoes on day 7 p.e. Consequently there was little (18% 51/270) feeding on the D-sucrose suspension recorded on days 3 or 5 by SINV- or MEM-exposed mosquitoes (Table 3-2). Because there was no disseminated virus prior to day 7 p.e., data from days 3 and 5 and days 7, 10, and 18 p.e. were averaged together for analysis. When allowed to feed on the D-sucrose suspension, significant differences between D sugar feeding were recorded on days 3 and 5 (Chi square=25, df=1, P<0.001) between the SINV- and MEM-exposed mosquitoes. However, this was not in response to SINV dissemination since dissemination was not detected on days 3 or 5 p.e. It should be noted that 5% of the MEM-exposed mosquitoes fed on the D-sucrose suspension on days 3 and 5 post-feeding which was highly significant (Chi square=17, df=1, P<0.0001) compared to the MEM-exposed on days 7, 10, and 18, which did not feed at all on the D-sucrose suspension.

When exposed to a 3% D-sucrose suspension significant differences in feeding behavior between SINV- and MEM-exposed mosquitoes were observed on days 7, 10, and 18 (Chi Square=363.7, df=1, P<0.0001) (Table 3-2). Overall, when assayed at 7,

10, and 18 d following the bloodmeal, 62% (251/405) of the mosquitoes that ingested and were infected with SINV fed on the D-sucrose suspension as compared to none (0/405) of those that had originally fed on the MEM-exposed suspension (Table 3-2).

The results of the CPE assay demonstrated a significant association between SINV dissemination and feeding on the D-sucrose suspension (Chi square=125.1, df=1, P <0.0001). At 7, 10, and 18 d post-SINV bloodmeal, 89% (223/251) of the mosquitoes that successful fed on the D-sucrose suspension had a disseminated infection. In contrast, 36% (55/154) of mosquitoes that did not feed on the D-sucrose suspension had a disseminated infection.

Insect Repellent Choice Response and Virus Dissemination. When offered a choice between a sucrose suspension with or without DEET, significantly (P=0.0037, df=1) more SINV-exposed mosquitoes than MEM-exposed mosquitoes fed on the DEET solution. On days 3 and 5 p.e. there were no significant differences between SINV- and MEM-exposed mosquitoes that fed on the sugar solution (ND) (Chi square=2.79, df=1, P=0.09) or that did not feed at all (Chi square=1.0, df=1, P=0.32). On days 7, 10 and 18 post-feeding, significantly more (Chi square= 122.3, df=1, P <0.0001) SINV-exposed mosquitoes (33%, 134/405) than MEM-exposed mosquitoes (3%, 12/405) fed on the DEET sucrose suspension (Table 3-3). However, significantly more MEM-exposed mosquitoes fed on ND sugar solution (Chi square=28.8, df=1, P<0.001) or did not feed at all (11.1, df=1, P<0.001) on days 7, 10, and 18 post-feeding as opposed to feeding on the D sugar solution.

When assayed on days 7, 10, and 18 following a SINV bloodmeal, significantly more of the mosquitoes that fed on the D-sucrose suspension had a disseminated

infection (64%, 87/134) compared to either those that fed on the ND solution (28%, 40/142) (Chi square=37.4, df=1, P<0.001) and those that did not feed at all (19%, 24/129) (Chi square=57.8, df=1, P<0.001) (Table 3-3).

Discussion

Taken together, these results indicate that mosquitoes with a disseminated SINV infection are less sensitive to DEET than uninfected mosquitoes. Adult female mosquitoes with a disseminated SINV infection were significantly more likely to feed on a DEET-sucrose suspension than mosquitoes that did not develop a disseminated infection or MEM-controls. Sindbis virus mosquitoes were more likely to feed on the D-sucrose solution, which suggests a modified sugar feeding behavior in response to arbovirus infection. I assume this is a modification in sugar feeding behavior because both SINV- and MEM-exposed mosquitoes were equally likely to feed on the ND sugar suspension. While both systemically infected and uninfected *Ae. aegypti* readily fed on a cotton pledget soaked with a 10% sucrose suspension, very few of the uninfected mosquitoes would feed if the pledget contained 3% DEET. In fact, I only recorded 13/135 MEM-fed mosquitoes on one evaluation period from the same experimental cage feeding on the D-sucrose suspension. In contrast, because 89% (223/251) of *Ae. aegypti* with a disseminated SINV infection fed on the 10% sucrose suspension containing 3% DEET, a reduced inhibition to feeding on this repellent following virus dissemination is indicated. When given a choice between cotton soaked with 10% sucrose and cotton soaked with 10% sucrose containing 3% DEET, 2% (14/675) of the MEM-exposed mosquitoes at days 3, 5, 7, 10, and 18 p.e., fed on the D-sucrose suspension. However, 33% (134/405) of the mosquitoes exposed to SINV 7, 10, and 18 d p.e., fed on the D-sucrose suspension. This suggests that mosquitoes with a

disseminated SINV infection may express an altered response to DEET when compared with uninfected mosquitoes.

The use of DEET as a repellent is important in preventing mosquito-borne pathogen transmission. Investigation of pathogen-associated changes in mosquito behavior is critical to determine if DEET functions in a preventative manner or if such arbovirus-associated changes can result in altered insect response, rendering DEET less effective than previously believed. Robert et al. (1991) found no significant differences between the time to locate a host of *Plasmodium falciparum* Welch and *P. berghei* Vincke and Lips -infected and uninfected *Anopheles stephensi* Liston when exposed to DEET. Barnard et al. (2007) reported *Ae. aegypti* infected with *Edhazardia aedis* (Kudo) took about 56.8 min longer to bite a human hand treated with 15% DEET than did uninfected *Ae. aegypti.* Frances et al. (2011) reported no differences in response to DEET of *Ae. aegypti* and *Ae. albopictus* infected with four dengue virus serotypes. These studies focused on behavior changes following parasite-infection or intrathoracic inoculation of mosquitoes with arboviruses, and the current investigation focused on altered behavior following oral feeding of mosquitoes with an arbovirus. It should be noted that not all arboviruses replicate the same in secondary tissues and this may explain why some behavioral changes in the mosquito host are reported for some disease systems but not for others.

A question of theoretical importance is whether the responses of SINV-infected *Ae. aegypti* represents an adaptation to arbovirus pathology, a non-adaptive side effect of SINV infection, or an increase or decrease in an activity such as host seeking, bloodfeeding, and/or sugar feeding performed before infection (Poulin 1995). Mosquito

behaviors to attractants and repellents are traditionally believed to be mediated by olfaction (Zwiebel and Takken 2004). Specific receptor proteins located on the cell membrane of sensory neurons are essential for detection of attractants and repellents in mosquitoes (Li et al. 2008). These OBPs are required for optimal performance of the olfactory system. Identification of OBPs in the proboscis of *An. gambiae,* suggest that odorant detection is a behavior not restricted to the olfactory system but also involves a gustatory organ component. Kwon et al. (2006) demonstrated a cryptic set of olfactory neurons located in the mouth parts of mosquitoes that respond to a small set of odorants. Such documentation suggests that in addition to olfaction, gustatory responses to odorants can be used to characterize the response of mosquitoes to DEET. Syed and Leal (2008) provided evidence that DEET has a specific ORN housed in a short trichoid sensillum on the mosquito antennae that aids in the detection and avoidance of DEET by mosquitoes. The decreased ability of the SINV-infected mosquito to detect DEET may be due to damage or blockage of the OBP or ORN by virus infection resulting in the odorant not being received or processed correctly. Another possibility is that the mosquito itself experiences neurological damage and this affects the mosquito's ability to respond to repellents (M. Turell, personal communication).

Few other data for comparing the effects of virus-infection on repellent efficacy exist. Personal protection methods including the use of repellents containing DEET are recommended by the CDC to reduce the risk of arboviral transmission. These findings suggest that at the time when the mosquito is most infective and capable of SINV

transmission, repellent use may be less effective in deterring infected mosquitoes from biting.

Table 3-1. Effects of Sindbis virus on *Aedes aegypti* response to a sugar solution (ND only).

	Percent Feeding on a Sugar Solution	
Days post-exposure	SINV- exposed	MEM-exposed
3 & 5	85 (230/270)	79 (214/270)
7, 10, & 18	70 (283/405)	64 (258/405)

Table 3-2. Effects of Sindbis virus on *Aedes aegypti* response to DEET in the absence of a feeding choice (D only).

	Percent Feeding on a DEET Solution	
Days post-exposure	SINV- exposed	MEM-exposed
3 & 5	18 (51/270)	5 (13/270)
7, 10 & 18	61 (251/405)	0 (0/405)

Table 3-3. Effects of Sindbis virus on *Aedes aegypti* response to DEET in the presence of a feeding choice (D/ND).

	Percent Feeding					
	DEET Solution (D)		Sugar solution (ND)		Not feeding at all	
Days post-exposure	SINV-exposed	MEM-exposed	SINV-exposed	MEM-exposed	SINV-exposed	MEM-exposed
3 & 5	5 (14/270)	<1 (2/270)	59 (159/270)	68 (183/270)	36 (97/270)	31 (85/270)
7, 10 & 18	33 (134/405)	3 (12/405)	35 (142/405)	54 (218/405)	32 (129/405)	43 (175/405)

CHAPTER 4
SINDBIS VIRUS INFECTION ALTERS BLOODFEEDING RESPONSES AND DEET REPELLENCY IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Mosquitoes infected with some arboviruses exhibit behaviors different from those observed for uninfected mosquitoes of the same species (Grimstad et al. 1980, Lambrechts and Scott 2009, Frances et al. 2011). Survival, fecundity, and bloodfeeding behavior of mosquitoes are affected by infection with RVFV (Turell et al. 1985, Faran et al. 1987). Both probing and feeding times were increased in *Ae. aegypti* mosquitoes infected with DENV (Platt et al. 1997). These researchers suggested that DENV-3-infection of organs and tissues that are known to control or influence activities associated with bloodfeeding might be associated with behavior changes. Salazar et al. (2007) demonstrated that DENV 2-infected mosquitoes developed a significant infection of the nervous system. Sindbis virus (Bowers et al. 1995) and WNV (Girard et al. 2005) also result in replication or pathology in mosquito brain tissue. Virus infection of the nervous system may alter the mosquito response to repellents, an area of research that has yet to be fully investigated.

I used *Ae. aegypti* mosquitoes and SINV to investigate arbovirus effects on mosquito bloodfeeding behavior following oral infection with SINV, specifically, duration of feeding, engorgement weight, and mosquito response to the repellent DEET.

Materials and Methods

Bloodfeeding Behavior of Infected and Uninfected Mosquitoes. To analyze *Ae. aegypti* bloodfeeding behaviors, groups of 10 each SINV- and MEM-fed mosquitoes, were observed feeding on a bloodmeal on days 7 and 14 p.e. Mosquitoes were placed individually into a clear Plexiglas cage (20 cm x 20 cm) topped with mesh

netting and offered a bloodmeal from a membrane feeder covered with a sausage casing (The Sausage Maker, Inc., Buffalo, NY). The activation, orientation, probing, and engorgement times were recorded for each mosquito during a 10-min observation period (Gillett 1967, Ribeiro et al. 1985, Ribeiro 1988, 2000, Hao et al. 2008).

Activation time was measured from the commencement of exposure to the blood source until the moment when the mosquito landed on the sausage membrane. Orientation was recorded as the time taken from landing until insertion of the stylets. Probing was recorded as the time from insertion of the stylet into the skin until the first uptake of blood. If the mosquito ended a probe unsuccessfully and attempts to re-feed elsewhere on the same host, additional probing times are added to the first; and so on until blood is located (the time in between probes was not added to the probing time). If the mosquito probed and then stopped probing for longer than 5 min that mosquito was removed from the cage and another mosquito was used in its place. Legs of the SINV-exposed mosquitoes that stopped probing during the evaluation period were removed and analyzed by a CPE assay. The proportion of SINV-positive, SINV-negative, and MEM-exposed mosquitoes ending a probe was compared to see if SINV dissemination influences the ability of the mosquito to imbibe blood. Engorgement was recorded as the time taken from the drawing of first blood until the acquisition of a full bloodmeal.

Each bloodfeeding evaluation was replicated a total of three times each on days 7 and 14 p.e. Following each 10-min observation period, a hind leg was removed, stored (-20ºC) for CPE assay to determine the presence or absence of a disseminated infection in each individual. The mathematical mean for each evaluation period of

SINV-disseminated, SINV-exposed but not disseminated, and uninfected mosquitoes was used to characterize each stage of bloodfeeding.

Blood Engorgement. Blood imbibed was quantified according to weight (mg) of the engorged female. Females were denied access to sugar (starved) for 24 hr prior to the initial weigh-in. Individual mosquitoes, 25 SINV- and 25 MEM-fed were weighed before and after bloodfeeding to the nearest 0.1 mg. All females were knocked down prior to feeding (un-fed), using CO_2 for 45 s before being weighed. Knock down was achieved by aspirating each mosquito into a Petri dish lined with filter paper through a small hole that had been melted into the lid using a soldering iron (UL®, Model BT30, Taiwan). The Petri dish hole was plugged with cotton and then placed into a cooler containing dry ice. Mosquitoes were allowed to acclimate for 15 min after knock down before being offered a bloodmeal. Immediately after bloodfeeding, each individual mosquito was knocked down again by the method described above for post-weight determination. Tests were replicated three times each on days 7 and 14 p.e. A hind leg was removed after the final weighing to determine SINV dissemination for individual mosquitoes.

DEET Repellency. A 30% DEET concentration (OFF!®, SC Johnson & Son, Racine, WI) was used to evaluate the bloodfeeding behavior of SINV- and MEM-fed *Ae. aegypti.* At days 7 and 14 p.e., a blind study of 50 mosquitoes was conducted by combining 25 SINV- and 25 MEM-exposed mosquitoes together in a test-cage. The virus dissemination status of individual mosquitoes was not known during each evaluation. Mosquitoes were offered a fresh bovine bloodmeal via a membrane feeder fitted with a sausage casing saturated in a 30% DEET solution. Casings were

submerged in the DEET solution for 5 min, and then attached to the membrane feeder. After fully engorging, each mosquito was removed from the test-cage; a hind leg was removed, and stored at -20ºC in order to determine dissemination status of the feeding mosquitoes. Determination of the mean time to initial feeding and for the fifth mosquito to probe for the disseminated and non-disseminated mosquitoes in each trial was used to determine DEET repellency.

Complete protection time (CPT) was calculated as the number of min elapsed between the time of mosquito placement into a cage containing a repellent-treated sausage casing and the time of the first attempted bite on the casing. This was calculated for mosquitoes with a disseminated SINV infection and mosquitoes with a non-disseminated infection. Repellency was also calculated in terms of time to the fifth bite as suggested by Debboun et al. (2007). Only mosquitoes with a positive CPE leg assay, indicative of virus dissemination, were used to characterize effects of DEET on SINV disseminated mosquito feeding behavior. Since I believe that arbovirus (SINV) infection of the central nervous system results in altered mosquito behavior, mosquitoes that were SINV-exposed but did not have a positive dissemination were treated as MEM-exposed mosquitoes.

Data Analysis. Bloodfeeding behavior, unfed body weight, blood-engorged body mass, and CPT responses of *Ae. aegypti* females were evaluated based on infection status (SINV disseminated or MEM-exposed) and by time p.e. (days 7 or 14). A two-way ANOVA was used to determine if there were differences between day p.e. and infection status on the different stages of mosquito bloodfeeding. Statistically significant

differences between treatment means in each response variable category were evaluated with an independent sample *t*-test.

Results

Bloodfeeding Behavior. Of the mosquitoes that were offered a SINV-bloodmeal, 33% (9/30) had a disseminated infection at day 7 p.e. A total of seven SINV-exposed mosquitoes were removed from the experiment for not continuing probing after 5 mins. Only two of the non-compliant seven (29%) had a positive dissemination. Only four of the MEM-exposed groups were removed from the experiment because of non-compliant probing. On day 14 p.e., 70% (21/30) of the SINV-exposed mosquitoes had a disseminated SINV infection. A total of six SINV-exposed mosquitoes, 3/6 (50%) had a positive CPE assay, were removed from the experiment for not continuing to probe after 5 mins. Five MEM-exposed mosquitoes were removed from the experiment. There were no significant differences based on infection status and being removed from the experiment regardless of days p.e. ($P>0.05$).

Significant differences in engorgement and bloodfeeding duration were observed at both times p.e. between mosquitoes with a positive SINV dissemination and mosquitoes either MEM-fed or with a negative SINV dissemination. There were no significant differences in the means between mosquitoes with a SINV-negative dissemination and MEM-exposed control in any of the different feeding stages ($P>0.05$). Those times were combined and analyzed as a non-disseminated infection (Table 4-1). Mosquitoes with a disseminated infection on day 14 p.e. took significantly longer to engorge ($F=97.1$, $df=2$, $P<0.0001$) than did mosquitoes with a disseminated SINV infection on day 7 p.e. (Table 3-1). The same time p.e. interaction was observed in MEM-fed mosquitoes on day 14 p.e. vs. day 7 p.e. ($F=2.5$, $df=2$, $P=0.046$) on

engorgement time. Mosquitoes with a disseminated SINV infection at day 14 p.e. took 164.1 ± 13.8 s longer to complete a bloodmeal than did mosquitoes with a positive dissemination at day 7 p.e. ($F=25.4$, $df=2$, $P=0.015$).

Mosquitoes with a disseminated infection on day 7 p.e. took significantly ($t=5.8$, $df=4$, $P=0.014$) longer to complete a full bloodmeal than did their age-matched non-disseminated counterparts [SINV (-) dissemination and MEM-exposed controls]. Infected mosquitoes took 1.3 times (90 ± 16.2 s) longer to complete a bloodmeal. The main source of this significance was in the activation stage of bloodfeeding times of mosquitoes with a positive SINV infection compared with non-disseminated mosquitoes ($t= 9.6$, $df=4$, $P=0.001$) (Table 3-1). Mosquitoes that had a disseminated SINV infection took 2.7 (114 ± 8.4 s) times longer to locate the bloodmeal than did non-disseminated mosquitoes.

Aedes aegypti mosquitoes with a disseminated infection on day 14 p.e. took significantly ($t=7.5$, $df=4$, $p=0.0092$) longer to complete a full bloodmeal than did their age-matched non-disseminated counterparts. Sindbis disseminated mosquitoes took 1.5 times (184.8 ± 10.2 s) longer to complete a bloodmeal. The main source of this significance was in the activation ($t=11.7$, $df=4$, $P<0.0003$) and engorgement times ($t=17.4$, $df=4$, $P=0.001$) of non-disseminated versus SINV disseminated mosquitoes (Table 4-1). Mosquitoes with a disseminated SINV infection took 3.0 times (96 ± 14.4 s) longer to activate towards the bloodmeal than did non-disseminated mosquitoes. Mosquitoes with a positive SINV dissemination took 1.5 times (114 ± 13.2 s) longer to fully engorge than non-disseminated mosquitoes.

Bloodmeal Size. Percent dissemination of SINV increased over time. On day 7 p.e. 40% (30/75) of the mosquitoes tested from the SINV-exposed group had a disseminated infection while virus dissemination was observed in 79% (59/75) of mosquitoes on day 14 p.e.

Day 7 p.e. unfed mosquitoes weighed more than mosquitoes on day 14 p.e., and engorged, SINV disseminated, mosquitoes weighed significantly more than engorged mosquitoes with a negative dissemination and controls on both days 7 p.e. ($F=92.2$, $df=1$, $P<0.0001$) and 14 p.e. ($F=56.6$, $df=1$, $P<0.0001$) (Table 3-2). There were no significant differences in the unfed weight between mosquitoes with a positive SINV dissemination, non SINV dissemination, or uninfected mosquitoes at days 7 p.e. ($F=1.9$, $df=1$, $P=0.18$) or at day 14 p.e. ($F=0.5$, $df=1$, $P=0.48$).

DEET Repellency. Mosquitoes with a disseminated SINV infection started to feed significantly sooner ($t=11.9$,$df=4$, $p=0.0003$) than their age-matched MEM controls (Table 4-3) on the DEET saturated membrane. On both days 7 and 14 p.e., the mosquitoes with a disseminated SINV infection completed their first bite about 4 hr sooner than mosquitoes without a disseminated infection in the same cage. The CPT to fifth bite occurred significantly sooner in mosquitoes with a disseminated SINV infection ($t=6.7$, $df=4$, $P=0.002$). Again, on both days 7 and 14 p.e., the mosquitoes with a disseminated SINV infection completed their fifth bite about 3 hr sooner than age-matched mosquitoes without a disseminated infection in the same cage.

Discussion

Altered bloodfeeding behaviors were detected in *Ae. aegypti* with a disseminated SINV infection. Such changes included an increase in total feeding time, increase in

total body weight following blood engorgement, and a decrease in sensitivity to DEET repellency.

This investigation detected a decrease in sensitivity to the repellent DEET in mosquitoes with a disseminated SINV infection. Compared to uninfected control mosquitoes, the CPT of DEET was reduced and both the first and fifth bites occurred 4 h sooner in SINV infected mosquitoes. In other words, this decrease in CPT in mosquitoes with a positive SINV dissemination was not influenced by evaluation period (i.e. days p.e.) and was not a single outlier as demonstrated by the CPT time to fifth bite. Mosquitoes with disseminated SINV infection located a bloodmeal 3.2 times faster than mosquitoes without a positive SINV dissemination when exposed to a 30% DEET solution. Use of DEET is important in preventing mosquito-borne pathogen transmission because of its repellent activity. Investigation of pathogen-associated changes in insect behavior is critical to determine if DEET functions in a preventative manner or if such arbovirus-associated changes can result in altered insect response, rendering DEET less effective. Previous studies conducted in my laboratory evaluating SINV-infected *Ae. aegypti* response to a 3% DEET sugar suspension, also indicated a decrease in the ability of the infected mosquitoes to detect and respond to DEET (Qualls et al. 2011). When tested 7 or more d after the initial bloodmeal, although none of the uninfected mosquitoes fed on the DEET-sucrose suspension, up to 89% of the SINV-infected mosquitoes fed on the suspension. Robert et al. (1991) found no significant differences between the mean protection time of *P. falciparum* and *P. berghei* -infected and uninfected *An. stephensi* after exposure to DEET. Barnard et al. (2007) reported *Ae. aegypti* infected with *E. aedis* took longer to bite a human hand treated

with 15% DEET than did uninfected *Ae. aegypti.* Frances et al. (2011) reported no differences in response to 5% DEET of *Ae. aegypti* and *Ae. albopictus* infected with four dengue virus serotypes. These studies focused on behavior changes following parasite-infection or intrathoracic inoculation of mosquitoes with arboviruses, and my investigation focused on altered behavior following oral feeding of mosquitoes with an arbovirus. It should be noted that not all arboviruses replicate to the same degree in secondary tissues and this may result in why some behavior changes are reported for one arbovirus and not for another arbovirus.

Completion of the four stages of bloodfeeding took 1.3 and 1.5 times longer on days 7 and 14 p.e., respectively, for mosquitoes with a positive SINV dissemination. This increase in time to completion for the four stages of bloodfeeding observed on day 14 p.e. demonstrates that feeding duration increases the longer the time p.e. Increased bloodfeeding times have also been reported in LACV-infected *Ae. triseriatus* and pathology in the salivary glands could contribute to a change in glandular secretions and/ or salivary gland function which may explain the findings of Grimstad et al. (1980). Bowers et al. (2003) demonstrated SINV-associated pathology in the salivary glands and muscle cytopathology in *Ae. albopictus,* suggesting that damage to the salivary glands might explain prolonged feeding times (Rossignol et al. 1984). While these documented findings provide a plausible structural etiology for an increase in feeding duration, other physiological factors (olfaction or vision) could increase or decrease bloodfeeding behavior of pathogen-infected mosquito behavior.

I report that the reduced ability of mosquitoes with a disseminated SINV infection to activate toward a bloodmeal accounted for the most variation in total bloodfeeding

time. Activation times of *Ae. aegypti* with a positive SINV dissemination were increased by 2.7 and 3.0 times on days 7 and 14 p.e., respectively. In other words, the increased feeding duration reflected an increased time dedicated to host seeking which increased over time p.e. *Aedes sierrensis* (Ludlow) infection with *Lambornella clarki* (Ciliophora: Tetrahymenidae) increases the time required for female mosquitoes to alight on a human hand by 65 s (>200%) (Egerter and Anderson 1989). These authors concluded that inhibition of bloodfeeding in infected females was a response to parasite manipulation of host humoral factor(s), a physiological manifestation of morbidity (decreased vigor) in the mosquito, or a combination of both.

Aedes aegypti with a disseminated SINV infection imbibed 10% more blood than mosquitoes without a SINV infection and 12% more blood than MEM-exposed controls. Most studies focusing on the bloodfeeding behavior of arbovirus infected mosquitoes have either seen a decrease (Grimstad et al. 1980, Platt et al. 1997) or no differences in the amount of blood imbibed (Putnam and Scott 1995) compared to uninfected mosquitoes. The tropism of some arboviruses for the insect nervous system could explain an increase in the amount of blood imbibed by arbovirus infected mosquitoes (Linthicum et al. 1996, Platt et al. 1997, Salazar et al. 2007). If the nervous system tissue replicates virus then certain sensory mechanisms involved in bloodfeeding could be negatively affected. I suggest that infection of mosquito midgut and hindgut peristaltic muscles may affect gut distension, permitting the deposition of a larger blood volume in response to weakening of infected gut muscles (Vo et al. 2010).

Few other published studies are available comparing the effects of arbovirus infection on bloodfeeding or repellent efficacy in mosquitoes. This study suggests that

mosquitoes with a disseminated SINV infection behave differently than uninfected mosquitoes. In fact, I found that mosquitoes with a disseminated SINV infection always completed the first and fifth bite before mosquitoes without a disseminated infection or MEM-controls. My findings suggest that at the time period when the mosquito is most infective and capable of SINV transmission (7 to 14 d p.e.), repellent use may be less effective in preventing infected mosquitoes from biting. Understanding the physiological basis for these behavior changes, especially the decrease in susceptibility to DEET, the gold standard of repellents, is important in the prevention of diseases caused by mosquito-borne viruses. While prolonged feeding duration and increased blood mass may decrease mosquito survival, a decrease in sensitivity to DEET increases a mosquito's ability to bloodfeed on humans. This extended bloodfeeding duration by infected mosquitoes could also result in interrupted feedings, a phenomenon that can result in an increase in virus transmission (Hodgson et al. 2001).

Table 4-1. Effect of a disseminated Sindbis virus infection on duration of the bloodfeeding stages in *Aedes aegypti* mosquitoes at days 7 and 14 post-exposure

		Mean time (s) ± SE	
	Day p.e.	7	14
Activation	SINV (+)	154±29.5*	145±7.6*
	non disseminated	57±24.3	48±5.1
Orientation	SINV (+)	25.1±5.4	35.4±4.4
	non disseminated	32.4±8.3	29.9±2.3
Probing	SINV (+)	23.7±3.6	31.5±3.2
	non disseminated	19±4.9	27.9±2.8
Engorgement	SINV (+)	176±11.6[a]	331±28.6*
	non disseminated	179±13.9[a]	215±17.6
Total Time	SINV (+)	378.8±28.7*[a]	542.9±32.5*
	non disseminated	287±29.1[a]	357.8±42.6

* Differences within the treatment groups [SINV (+) vs. non disseminated] on the same day p.e. (7 or 14) are significant P<0.01 (independent *t*-test)
[a]Means for days 7 and 14 were significantly different at (P < 0.05, Two-Way ANOVA)

Table 4-2. Comparison of unfed body weight (mg) and engorged bloodfed body weight of *Aedes aegypti* with disseminated Sindbis virus infection (SINV +), no disseminated Sindbis virus infection (SINV -), and uninfected controls at days 7 and 14 post-exposure

		Mean weight ± SE (mg)		
Feeding Status	Days p.e.	SINV (+)	SINV (-)	Uninfected Controls
Unfed	7	2.41±0.6	2.39 ± 0.2	2.46±0.2
	14	2.19±0.2	2.21±0.4	2.25±0.3
Fed	7	5.26±0.2 *	4.83±0.3	4.78±0.4
	14	4.98±0.2*	4.47±0.4	4.27±0.6

* Differences within the treatment groups [SINV (+), SINV (-), and MEM-exposed] on the same day p.e. (7 or 14) are significant P<0.0001 (1-Way ANOVA)

Table 4-3. Effects of a disseminated Sindbis virus infection on time to first and fifth bites of *Aedes aegypti* after exposure to DEET (30%) on days 7 and 14 post-exposure

		Mean Time (hr) ± SE	
Response	Age (d)	Positive SINV dissemination	non disseminated
First bite (hr)	7	1.8±0.3 *	5.5±0.4
	14	1.8±0.4 *	6.0±0.5
Fifth bite (hr)	7	2.1±0.4 *	5.7±0.6
	14	1.9±0.5 *	6.0±0.6

* Mean times were significantly (P <0.01, independent t-test) lower for mosquitoes with a disseminated SINV infection than for those without a disseminated infection

CHAPTER 5
ALTERED BEHAVIORAL RESPONSES OF SINDBIS VIRUS INFECTED *AEDES AEGYPTI* (DIPTERA: CULICIDAE) TO DEET AND NON-DEET BASED INSECT REPELLENTS

Investigations of arbovirus-mosquito host interactions have resulted in numerous reports describing modifications of mosquito bloodfeeding behaviors following virus infection (Grimstad et al. 1980, Turell et al. 1985, Platt et al. 1997). Alterations in mosquito bloodfeeding behavior may result from changes in nervous tissue or salivary gland tissues at the cellular level (Platt et al. 1997, Mims et al. 1966). Virus replication and pathology in mosquito nervous and salivary gland tissue have been observed following infection with SINV (Bowers et al. 1995), WNV (Girard et al. 2005), and DENV-2 (Salazar et al. 2007). Frances et al. (2011) suggested that virus infection of the nervous system may alter the mosquito's response to repellents. However, their investigation found no altered response of *Ae. aegypti* and *Ae. albopictus* to DEET after infection with any of the four DENV serotypes.

Experiments in my laboratory have also demonstrated that *Ae. aegypti* mosquitoes with a disseminated SINV infection have a decreased activation time, the time it takes a mosquito to locate the host, and take much longer to fully acquire a bloodmeal compared to uninfected control mosquitoes (Qualls et al. 2012). However, when exposed to 30% DEET mosquitoes with a disseminated SINV infection had a reduced time to first bite (TFB), compared to their uninfected cohorts (Qualls et al. 2012). Although exposure to the repellent DEET results in altered protection time in SINV-infected *Ae. aegypti*, it is unclear if this same phenomenon will be observed after exposure to other repellent AI.

Repellent usage is a method of personal protection effective in reducing nuisance and vector mosquito bites (Freedman 2008). The AI DEET has proven to be the most effective mosquito repellent against biting mosquitoes, including against DENV vectors *Ae. aegypti* and *Ae. albopictus*, *Culex* spp., and *Anopheles* malaria vectors (Yap et al. 1998, Thavara et al. 2001, Trongtokit et al. 2005b). However, this compound has undesirable characteristics that compromise its use including it's unacceptable odor and potential to cause central nervous system depression (Kim et al. 2004, Witting-Bissinger et al. 2008). These issues with DEET have resulted in the development of new repellents with different AI components. Many new AIs are plant-derived including 2-undecanone (2-U) and oil of lemon eucalyptus (Barnard and Xue 2004, Debboun et al. 2007). Picaridin, a piperidine derivative synthetic AI, is also recommended for use as a repellent (CDC2008).

Bohbot and Dickens (2010) suggest that the excitatory and inhibitory properties of DEET and 2-U, as well as the non-specific inhibitory effects of picaridin on mosquito OR provide evidence that OSN elicit altered patterns of glomerular binding activity. This altered pattern may result in the scrambling of cognitive olfactory inputs and ultimately behavioral disruption by inability to recognize or smell the animal host. The mechanisms used by mosquitoes in processing the exogenous odors produced by repellents may be altered by virus infection, thus affecting host-seeking behavior (Kim et al. 2004). A goal of the current study is to investigate potential behavior changes of SINV-infected mosquitoes when offered a bloodmeal contained in a sausage casing saturated in one of four repellents with the AI; DEET, picaridin, 2-U, and oil of lemon eucalyptus. The AIs listed above were selected because they are available as ingredients in

commercially available products that are used to prevent mosquito bites and mosquito-borne pathogen transmission (Bohbot and Dickens 2010). Laboratory experiments using SINV bloodfed *Ae. aegypti* and *Ae. albopictus* have proven useful to address questions about experimental infection and transmission, genetically modified arboviruses, and the dynamics of arboviral tissue tropism and pathology in mosquito vectors (Xiong et al. 1989, Jackson et al. 1993, Bowers et al. 1995, Bowers et al. 2003).

Materials and methods.

Time to First Bite. Four repellents were used to evaluate the TFB of SINV- and MEM-exposed *Ae. aegypti*. The repellents used were a 15% picaridin concentration (REPEL® Sportsmen Gear Smart formula, WPC Brands, Inc., Bridgeton, MO), a 7.75% 2-U concentration (BioUD^{TM}, HOMS, LLC, Clayton, NC), a 30% oil of lemon eucalyptus (approximately 65% of p-menthane-3,8-diol, REPEL® Insect Repellent Lotion, WPC Brands, Inc., Jackson, WI), and a 15% DEET concentration (OFF!$^{(R)}$, SC Johnson & Son, Racine, WI).

At 10 d p.e. a blind study of 50 mosquitoes, 25 SINV- and 25 MEM-exposed mosquitoes, was conducted to determine the TFB of the four repellents. The mosquitoes were exposed to a bloodmeal via the artificial-membrane feeder described previously equipped with a sausage casing saturated in the desired repellent. The 12-cm-long strip was saturated in 2 ml of the repellent solution and then used to cover the membrane feeder filled with fresh defibrinated bovine blood. The main focus of the repellent application to the sausage casing was to cover the entire casing area (12 cm) that covered the outside of the glass membrane feeder. Any excess material was removed by gently blotting the casing with filter paper. Sausage casings were treated in the desired repellent directly before each evaluation. Sterile glass membranes were

used in each replicated trial so that there was no repellent residual left on the membrane system. Once a mosquito had fully engorged it was aspirated from the test cage and a hind leg was removed and stored at -20ºC in a labeled vial for later virus CPE assay. The experiment ended when all mosquitoes had taken a bloodmeal or the manufacture recommended protection time of the commercially available repellents had expired (DEET 10 hrs; picaridin 10 hrs; 2-U 4.5 hrs; and oil of lemon eucalyptus 6 hrs). Tests were replicated on three separate occasions on day 10 p.e. The three replicates were carried out on different days for each of the four repellents. Only one repellent was evaluated at a time to avoid air space contamination. All experiments started at 0800 ± 0.05 hr.

The TFB for each of the repellents was calculated for mosquitoes with a positive SINV dissemination and all uninfected mosquitoes. The first bite was calculated as the number of minutes elapsed between the time mosquitoes were introduced into the cage and the time of the first attempted bite. I did not know the virus dissemination status of the exposed mosquitoes at the time of these experiments. The first mosquito in each replicate test followed by the second confirmation bite was used to calculate the mean TFB for SINV-disseminated and MEM-exposed/SINV non-disseminated mosquitoes.

Bloodfeeding Behavior. To evaluate the effect of a disseminated SINV infection on a mosquito's behavioral response to repellents, I removed previously-exposed (SINV or MEM) *Ae. aegypti* females at 10 d p.e. by mechanical aspiration. Mosquitoes were placed individually into a clear Plexiglas cage (20 cm x 20 cm) topped with mesh netting and offered a bloodmeal from a membrane feeder covered with a sausage casing saturated in the desired repellent (The Sausage Maker, Inc., Buffalo,

NY). The same four repellents mentioned previously were selected for the bloodfeeding behavior evaluation. Repellent application to the sausage casing was carried out in the same manner as mentioned previously. Each bloodfeeding evaluation was replicated five times on day 10 p.e. Three mosquitoes of both SINV-exposed and MEM-exposed were evaluated per replicate. The replicates were carried out on different days for each AI. Activation, orientation, probing, and engorgement times were recorded for each mosquito during the observation period (Gillett 1967, Riberio 1988, 2000, Riberio et al. 1985, Hao et al. 2008).

Activation time was measured from the commencement of exposure to the blood source until the moment when the mosquito landed on the sausage membrane. Orientation was recorded as the time taken from landing until insertion of the stylets. Probing was recorded as the time from insertion of the stylet into the sausage casing until the first uptake of blood. If the mosquito ends a probe unsuccessfully and attempts to re-feed elsewhere on the same host, additional probing times are added to the first; and so on until blood is located (the time in between probes is not added to the total probing time). If the mosquito probed and then stopped probing for longer than 5 min that mosquito was removed from the cage and another mosquito was used in its place. Legs of the SINV-exposed mosquitoes that stopped probing during the evaluation period were removed and analyzed for CPE. The proportion of SINV-positive, SINV-negative, and MEM-exposed mosquitoes ending a probe was compared to see if SINV dissemination influences the ability of the mosquito to imbibe blood. Engorgement was recorded as the time taken from the drawing of first blood until the acquisition of a full bloodmeal.

Following each observation period, a hind leg was removed, stored frozen (-20ºC) waiting CPE, to determine the presence or absence of a disseminated infection in each individual. The time it took mosquitoes with either a positive SINV dissemination, SINV-exposed but did not develop a dissemination, and MEM-exposed was used to characterize each stage of bloodfeeding. Mosquitoes that did not acquire a full bloodmeal within the recommended manufactures protection period (listed above) were not used in the experiment, i.e. removed from the experiment.

Data Analysis. For analysis of the TFB two One-Way ANOVAs were used: 1) within each repellent among infection status (SINV/MEM-exposed) and 2) among repellents within time to first bite with means separation via Tukey's honestly significant difference test (HSD) (Stats Direct Ltd., Cheshire, UK). A Two-Way ANOVA with means separation via Tukey's honestly significant difference test (HSD) was used to determine if there were differences between the bloodfeeding stages based on repellent evaluated and on infection status (MEM-exposed or SINV-disseminated) (Stats Direct Ltd., Cheshire, UK). A Chi-square analysis was used to determine if dissemination status influenced the number of times a mosquito would end a probe and then re-feed.

Results

Time to First Bite. Mosquitoes exposed to the repellent DEET with a SINV dissemination had a significantly different mean TFB ($F=33.9$, $df=1$, $P < 0.0001$) compared to their uninfected cohorts (Table 5-1). On average mosquitoes with a SINV dissemination would bite 1.9 ± 0.27 hr sooner than uninfected mosquitoes when exposed to 15% DEET. Mosquitoes exposed to the repellent picaridin with a disseminated SINV infection had a significantly different mean TFB ($F=29.4$, $df=1$, $P < 0.0001$) compared to their uninfected cohorts (Table 5-1). On average, mosquitoes with

a disseminated SINV infection bit 2.2 ± 0.40 hr sooner than uninfected mosquitoes when exposed to 15% picaridin. Differences in the mean TFB of mosquitoes exposed to the repellent 2-U and oil of lemon eucalyptus were not significant ($F=0.2$, $df=1$, $P = 0.65$; $F=1.45$, $df=1$, $P =0.92$, respectively) for mosquitoes with a disseminated SINV infection compared to uninfected controls (Table 5-1).

There were no significant differences between the TFB and repellents evaluated for mosquitoes with (F=0.16, df=1, P = 0.687). Thus, SINV disseminated mosquitoes respond the same to DEET and non-DEET based repellents and this response occurs sooner than in their uninfected cohorts.

There was a significant difference between the TFB and repellents evaluated for MEM-exposed mosquitoes ($F=18.9$, $df=1$, $P < 0.0001$) with DEET providing the longest TFB followed by picaridin, 2-U, and oil of lemon eucalyptus.

Bloodfeeding Behavior. In each experiment a number of SINV-exposed mosquitoes that were evaluated did not develop a disseminated infection (Table 5- 2). Because of the low sample size of SINV-exposed but not disseminated the data were not used in the analysis. However, based on the standard deviations within repellents SINV-exposed but non-disseminated responded in each bloodfeeding category in the same time manner as uninfected controls.

Significant differences ($F=301.8$, $df=2$, $P<0.0001$) between total time to complete the four bloodfeeding stages among repellents were observed between mosquitoes with a disseminated SINV infection and uninfected controls. Mosquitoes with a disseminated SINV infection completed all four bloodfeeding stages after exposure to a DEET-soaked sausage casing 2.2 ± 0.3 hr before their uninfected counterparts. Mosquitoes with a

disseminated SINV infection completed all four bloodfeeding stages after exposure to a picaridin-soaked sausage casing 1.7 ± 0.1 hr before their uninfected counterparts. Both SINV disseminated and uninfected mosquitoes exposed to 2-U completed the four bloodfeeding stages around 3.6 ± 0.4 hr. The four stages of bloodfeeding were completed by both SINV disseminated and uninfected controls after exposure to oil of lemon eucalyptus around 2.1 ± 0.2 hr.

Overall, mosquitoes with a disseminated SINV infection took longer to complete all four stages of the bloodfeeding assay after exposure to DEET and 2-U followed by picaridin and oil of lemon eucalyptus (Figure 5-1). Uninfected *Ae. aegypti* (MEM-fed) exposed to DEET and picaridin took the longest to complete all four stages of the bloodfeeding assay followed by 2-U and oil of lemon eucalyptus (Figure 5-1).

Activation. Significant differences ($F=95.5$, $df=2$, $P<0.0001$) between the activation times of mosquitoes with a disseminated SINV infection compared to uninfected controls were recorded among the repellents. Mosquitoes exposed to DEET and had a disseminated SINV infection located a bloodmeal 2.2 ± 0.2 hr before their uninfected counterparts. Mosquitoes exposed to picaridin that had a disseminated SINV infection located a bloodmeal on average 1.7 ± 0.1 hr sooner than did uninfected mosquitoes. However, there were no differences in activation times for mosquitoes with a disseminated SINV infection and uninfected controls when exposed to 2-U and oil of lemon eucalyptus.

Activation occurred sooner in *Ae. aegypti* with a disseminated SINV infection exposed to oil of lemon eucalyptus < picaridin and DEET < 2-U (Table5- 3). Activation

occurred sooner for uninfected controls exposed to oil of lemon eucalyptus < 2-U < picaridin < DEET (Table 5- 3).

Orientation. Significant differences ($F=217.3$, $df=2$, $P<0.0001$) between the orientation times of mosquitoes with a disseminated SINV infection compared to uninfected controls were recorded among the repellents. There was only a difference in the orientation time of 2-U between mosquitoes with a disseminated SINV infection and uninfected controls. Mosquitoes with a SINV dissemination orientated 12 ± 4.9 s sooner than uninfected mosquitoes after exposure to 2-U.

Orientation occurred sooner in *Ae. aegypti* with a disseminated SINV infection exposed to picaridin and 2-U < DEET and oil of lemon eucalyptus (Table 5-2). Orientation occurred sooner for uninfected controls exposed to oil of lemon eucalyptus and picaridin < 2-U and DEET (Table 5-3).

Probing. Significant differences ($F=129.7$, $df=2$, $P<0.0001$) between the probing times of mosquitoes with a disseminated SINV infection compared to uninfected controls were recorded among the repellents. Mosquitoes exposed to DEET and had a disseminated SINV infection probed 21 ± 5.1 s longer than their uninfected counterparts. Mosquitoes exposed to picaridin that had a disseminated SINV infection probed 35 ± 6.8 s longer than their uninfected counterparts. Mosquitoes exposed to oil of lemon eucalyptus that had a disseminated SINV infection probed 14 ± 5.21 s shorter than their uninfected counterparts. However, there were no differences in probing times for mosquitoes with a disseminated SINV infection and uninfected controls when exposed to 2-U.

Mosquitoes with a disseminated SINV infection were more likely to begin probing the DEET saturated membrane and then re-feed (Chi Square=4.15, df=1, $P < 0.05$) than their uninfected counterparts (Table 5-4). During the observation period three mosquitoes that had a positive SINV dissemination, two that were SINV-exposed, and two MEM-fed mosquitoes were rejected from the experiment.

A significantly greater number of mosquitoes with a disseminated SINV infection were more likely to begin probing the picaridin saturated membrane and then re-feed (Chi square=9.66, df=1, $P < 0.01$) than their uninfected counterparts (Table 5-4). During the observation period one mosquito that had a positive SINV dissemination, two that were SINV-exposed, and one MEM-fed mosquito were rejected from the experiment.

There was no significant difference in probing and re-feeding in SINV-infected and MEM-exposed mosquitoes after exposure to 2-U and oil of lemon eucalyptus ($P>0.05$) (Table 5-4). While exposed to 2-U one mosquito that had a positive SINV dissemination, one that was SINV-exposed, and one MEM-fed mosquito were rejected from the experiment. While exposed to oil of lemon eucalyptus one mosquito that had a positive SINV dissemination, two that were SINV-exposed, and two MEM-fed mosquitoes were rejected from the experiment

Probing was completed sooner in *Ae. aegypti* with a disseminated SINV infection exposed to oil of lemon eucalyptus < 2-U < picaridin < DEET (Table 5-3). Probing was completed sooner for uninfected controls exposed to picaridin and oil of lemon eucalyptus < 2-U and DEET (Table 5-3).

Engorgement. Significant differences ($F=86.4$, $df=2$, $P<0.0001$) between the engorgement times of mosquitoes with a disseminated SINV infection compared to uninfected controls were recorded among the repellents. Mosquitoes exposed to DEET and had a disseminated SINV infection took 245 ± 18.5 s longer to complete the bloodfeeding stage of engorgement than their uninfected counterparts. Mosquitoes exposed to picaridin that had a disseminated SINV infection took 176 ± 40.5 s longer to complete the bloodfeeding stage of engorgement than their uninfected counterparts. However, there were no differences in engorgement times for mosquitoes with a disseminated SINV infection and uninfected controls when exposed to 2-U and oil of lemon eucalyptus.

Engorgement was completed sooner in *Ae. aegypti* with a disseminated SINV infection exposed to oil of lemon eucalyptus < 2-U < picaridin < DEET (Table 5-3). Engorgement was completed sooner for uninfected controls exposed to picaridin and oil of lemon eucalyptus < DEET < 2-U (Table 5-3).

Discussion

These results indicate that a dissemination of SINV within *Ae. aegypti* is associated with a decrease in sensitivity to repellents containing DEET and picaridin. Also, mosquitoes with a disseminated SINV infection take less time to locate and fully engorge on a bloodmeal than uninfected mosquitoes when exposed to DEET, picaridin, and oil of lemon eucalyptus repellents. Mosquitoes with a disseminated SINV infection demonstrated a 46 % and 37% reduction in TFB when exposed to the AIs DEET and picaridin, respectively, compared to their MEM-exposed counterparts. DEET, picaridin, 2-U and oil of lemon eucalyptus, did not inhibit SINV-disseminated mosquitoes from taking a blood-meal during the expected protection time of the repellents. While *Ae.*

aegypti is not a primary vector of SINV, this arbovirus was isolated from 8 species of Aedine mosquitoes in the 1970's (Doherty et al. 1979). These isolations represent natural infections, as they were caught in the wild in Australia. Sindbis virus is the prototype *Alphavirus* and an important entity to extrapolate to BSL-3 viruses.

Repellents function to either mask the chemical cues involved in locating a host or promote avoidance of the host, responses that are mediated by olfaction (Zwiebel and Takken 2004). Responses of infected mosquitoes were not masked by the natural repellents, 2-U and oil of lemon eucalyptus, as demonstrated by the lack of time differences recorded during the different feeding stages compared to their uninfected counterparts. Recent evidence suggests that the effectiveness of DEET is due to the dual action in inducing avoidance simultaneously via gustatory receptors (GRN) and ORNs. Lee and colleagues demonstrated that DEET suppressed feeding behavior in *Drosophila* and that this effect was mediated by GRNs (Lee et al. 2010). It is not known if the GRNs are suppressed or activated after exposure to other AI of repellents.

This dual action of avoidance may explain why there are differences between plant based repellents and synthetic repellents. Even though both 2-U and lemon eucalyptus elicit a repellent response they may also activate the GRNs which could lead to a shift from host-seeking to sugar-feeding behavior. These plant based AIs may be attractive in the context of locating floral or extra-floral plant nectaries as the battle between suppression of ORNs and activation of GRNs begin, and warrants further study of the role of GRNs in repellent efficacy (Lee et al. 2010).

Use of repellents is important in preventing mosquito-borne virus transmission. Investigation of virus-associated changes in insect behavior is critical to determine if

repellents function in a preventative manner or if such arbovirus-associated changes can result in altered insect response, rendering repellents less effective. Only two studies have addressed arbovirus-infected mosquitoes' response to DEET (Frances et al. 2011, Qualls et al. 2011). Frances et al. (2011) reported no differences in response time to 5% DEET by *Ae. aegypti* and *Ae. albopictus* infected with four dengue virus serotypes. I found in an earlier study that SINV-infected mosquitoes exposed to a 30% DEET solution had a significantly different TFB of 1.8 hrs compared to a TFB of 5.5 hrs of uninfected controls (Qualls et al. 2012). In the current study I used a 15% DEET solution and report again a decrease in TFB of SINV-disseminated mosquitoes compared to non disseminated mosquitoes. Frances et al. (2011) study focused on repellent response following intrathoracic inoculation of mosquitoes and my investigations have focused on altered TFB response following oral feeding. Introduction of virus by intrathoracic inoculation bypasses the natural route of virus dissemination within the mosquito host. By offering a mosquito an infectious bloodmeal the virus enters the mosquito by the natural route and provides a more realistic representation of virus dissemination and replication in the mosquito. Thus, behavior changes observed after a natural route of infection and dissemination within the mosquito provide stronger evidence that those behavior changes could be observed in the field. It should also be noted that not all arboviruses replicated to the same extent in primary and secondary tissues resulting in different effects on the behavior of an infected mosquito.

Few published studies are available for comparing the effects of arbovirus infection on bloodfeeding in mosquitoes. Increased bloodfeeding times have also been

reported in La Cross virus-infected (LACV; family Bunyaviridae; genus *Orthrobunyavirus*) *Aedes triseriatus* (Say) (Grimstad et al. 1980) and *Aedes sierrensis* (Ludlow) infected with *Lambornella clarki* (Ciliophora: Tetrahymenidae) (Egerter and Anderson 1989). My previous findings support an increase in the time it takes to complete the four stages of bloodfeeding (Qualls et. al 2012). Mosquitoes with a disseminated SINV infection took 1.3 and 1.5 times longer on days 7 and 14 p.e., respectively compared to uninfected mosquitoes (Qualls et al. 2012). However, when repellents were used to evaluate bloodfeeding stage response a decrease in the bloodfeeding stages of mosquitoes with a disseminated SINV infection compared to uninfected controls after exposure to DEET and picaridin was demonstrated. Thus, suggesting that virus dissemination does influence a mosquitoes' response to a repellent and studies to determine repellent efficacy in preventing mosquito-borne virus transmission should be considered when developing new AIs.

In the current study, mosquitoes with a disseminated SINV infection completed the four stages of bloodfeeding at day 10 p.e. after exposure to the AI of the repellents much sooner (DEET and picaridin) or during the same time interval (2-U and lemon eucalyptus) as their control counterparts. This is in contrast to the findings of Robert and colleagues (Robert et al. 1991). They found no significant differences between the mean responses of *Plasmodium falciparum* Welch and *P. berghei* Vincke and Lips - infected and uninfected *Anopheles stephensi* Liston response to DEET. Barnard et al. (2007) reported *Ae. aegypti* infected with *Edhazardia aedis* Kudo took longer to bite a human hand treated with 15% DEET than did uninfected *Ae. aegypti.* These two studies focused on behavior changes following parasite-infection, which have different

energy constraints on the mosquito host and usually do not replicate in the nervous tissue.

This study provides information of the TFB of *Ae. aegypti* with a disseminated SINV infection and *Ae. aegypti* without an arbovirus infection by comparing their response to repellents with different AI's. These findings suggest that mosquitoes with a disseminated SINV infection exhibit differences in TFB to DEET and picaridin repellents. A change in the bloodfeeding behavior of mosquitoes with a disseminated SINV infection is also demonstrated in this study. Because the activation time was significantly shortened, I suspect that virus persistence has had an altered effect on olfactory response to repellents. Understanding the physiological basis for these behavior changes is important to the public health arena.

Table 5-1. Mean time (hr) to first bite of Sindbis-disseminated and uninfected control *Aedes aegypti* on day 10 post-exposure after being offered a bloodmeal covered by a repellent saturated sausage casing

	SINV-disseminated	Uninfected
	First Bite	First Bite
DEET	2.8 ± 0.2*a	5.9 ± 0.8a
Picaridin	2.1 ± 0.3*a	4.2 ± 0.3b
2-Undecanone	2.6 ± 0.4a	3.1 ± 0.8c
Lemon Eucalyptus	1.4 ± 0.4a	1.9 ± 0.3d

* Differences in the mean time to first bite between SINV-disseminated and uninfected mosquitoes are significant P< 0.0001 (One-Way ANOVA)
Columns with different letters indicated the means are significant P<0.05 (One-Way ANOVA means separation via Tukey's HSD)

Table 5-2. Percent of Sindbis virus-exposed *Aedes aegypti* per repellent that developed a positive dissemination (detected by cytopathic effect assay) that were evaluated in the bloodfeeding behavior assay.

Repellent	% Dissemination (n)
DEET	80% (12/15)
Picaridin	93% (14/15)
2- Undecanone	87% (13/15)
Lemon Eucalyptus	87% (13/15)

Table 5-3. Duration (s) of bloodfeeding stages of mosquitoes with a disseminated Sindbis virus infection and uninfected mosquitoes exposed to a bloodmeal covered in a repellent saturated sausage casing at day 10 post-exposure.

	Mean ± SE (sec)							
	DEET		Picaridin		2- Undecanone		Lemon Eucalyptus	
Time (s)	SINV (+)	MEM-control	SINV (+)	MEM-Control	SINV (+)	MEM-Control	SINV (+)	MEM-Control
Activation	10981 ±50.4*a	19074 ± 146.6a	10139± 218.7*a	16337± 273b	12678 ± 29.1b	12688 ± 23.7c	7413 ± 202.4c	7347 ± 185.9d
Orientation	44 ± 5.7	46 ± 6.5	31 ± 3.5	30 ± 8	35± 5.1*	48 ± 5.4	37 ± 6.1	35 ± 7.7
Probing	95 ± 13.5*a	74 ± 16.6a	80 ± 14.4*ab	45 ± 9.1b	65 ± 2.9b	61 ± 14.1a	39 ± 4.6*c	53 ± 5.63b
Engorgement	556 ± 27.4*a	311 ± 10.3a	421 ± 39.4*b	242 ± 42.6b	339 ± 6.6c	344 ± 7.3c	226 ± 12.1d	247 ± 14.2b

* Differences within infection status [SINV (+) vs. MEM-controls] by repellent are significant P<0.01 (2-WAY ANOVA)
Rows with different letters indicated significant differences in mean times within the bloodfeeding stages between the repellents at P < 0.05 (Tukey's HSD test) among infection status

Table 5-4. Number of mosquitoes disseminated Sindbis virus infection and uninfected mosquitoes that probed once or made multiple probes and re-fed after exposure to the different repellent saturated membrane bloodmeals

	DEET		Picaridin		2- Undecanone		Lemon Eucalyptus	
	SINV (+)	MEM-control	SINV (+)	MEM-control	SINV (+)	MEM-control	SINV (+)	MEM-control
Probe once	4*	12	5*	14	11	10	9	13
Probe + re-feed	8	3	9	1	3	5	2	2

* Differences within the treatment groups [SINV (+) vs. uninfected] are significant P<0.05 (Chi square)

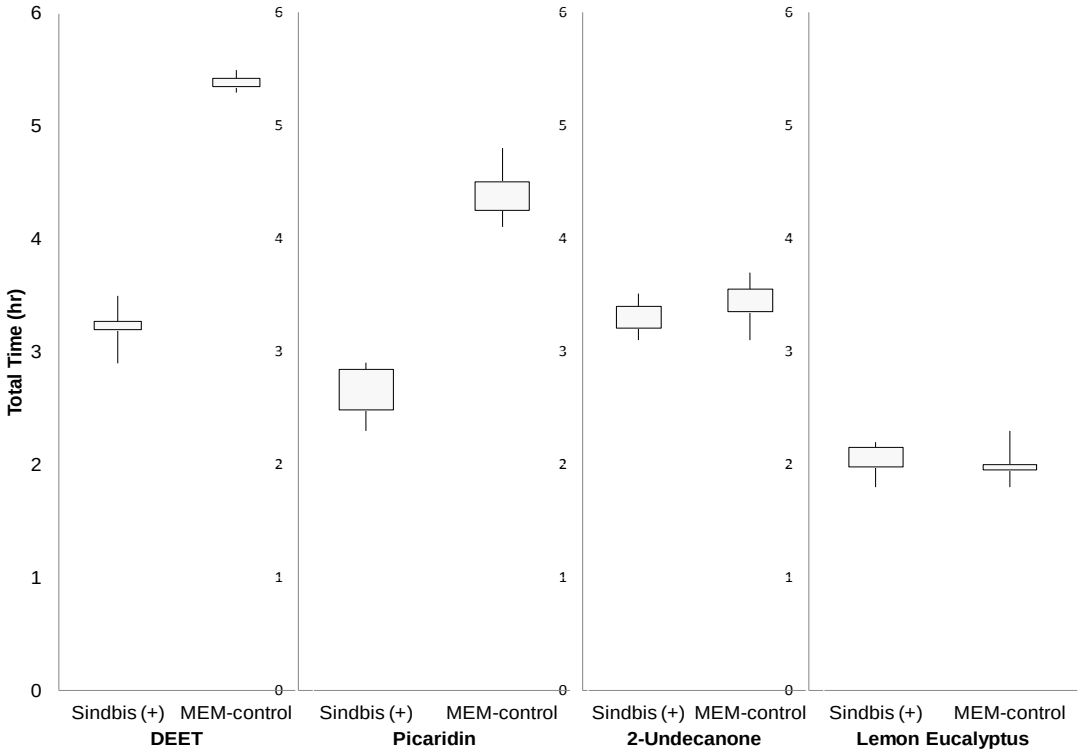

Figure 5-1. Mean total time (hr) duration of all four stages of bloodfeeding of Sindbis-positive and MEM-control *Aedes aegypti* exposed to a bloodmeal covered in a repellent saturated sausage casing at day 10 post-exposure. The boxes indicate mean values and vertical bars 95% confidence intervals.

CHAPTER 6
THE EFFECTS OF SUGAR FEEDING ON SINDBIS VIRUS DISSEMINATION IN *AEDES AEGYPTI* MOSQUITOES

Sugar feeding is a fundamental characteristic of mosquito life. Most evidence indicates that there is frequent ingestion of sugar by both sexes and all ages of adult mosquitoes on plant nectaries, extrafloral nectaries, homopteran honeydew, and fruits (Foster 1995). Females are dependent on sugar for flight, mating, locating resting and oviposition sites, and maintaining energy reserves. In the laboratory sugar feeding prolongs the survival of both sexes (Xue et al 2008), increases mating capacity in males, reduces biting frequency in some species of mosquitoes and increases it in others (Jones and Madhukar 1976, Hancock and Foster 1993), increases DEET protection time (Xue and Barnard 1999, Xue and Barnard 2009), and increases egg production (Foster 1995). Since both longevity and biting frequency are important to a mosquito's ability to transmit arboviruses, knowledge of the details of how sugar consumption affects mosquito survival and virus dissemination may prove useful in epidemiological studies. Although it is assumed that nectar supplements may indirectly influence vector potential by increasing female survivorship (Magnarelli 1978), it is not known if higher sugar concentrations will affect mosquito survival, energetics, host-seeking behavior, virus dissemination, and virus-induced pathology.

Two studies have addressed sugar deprivation as a factor influencing arbovirus-infected mosquito survival (Dohm et al. 1991, Vaidyanathan et al. 2008) and vector competence (Vaidyanathan et al. 2008). Dohm et al. investigated the survival of RVFV-infected *Culex pipiens*. They reported that when mosquitoes had access to sucrose, the virus-exposed mosquitoes had slightly higher survival rates. However, Dohm et al.

(1991) did not address varying concentrations of sucrose on virus dissemination and vector competence. Furthermore, they evaluated two different sucrose concentrations (5 and 20%) provided after the mosquitoes were infected. Vaidyanathan et al. (2008) investigated the role of different sucrose concentrations on *Cx. pipiens* nutritional status and susceptibility to WNV infection and transmission. They found that mosquitoes in all sucrose concentration experiments were equally susceptible to WNV infection but mosquitoes with lower nutrient reserves as a result of lower concentration sucrose meals were more likely to transmit virus by bite. Although Vaidyanathan et al. (2008) did investigate multiple sucrose solutions, neither study evaluated nutrient reserves developed prior to virus infection.

In arbovirus studies using laboratory reared mosquitoes, prior to imbibing a viremic bloodmeal, laboratory mosquitoes deposit large fat body reserves. In 1965, Van Handel coined the term “the obese mosquito” when he observed an increase in lipid and glycogen storage in laboratory-maintained mosquitoes. Van Handel (1965) showed that in laboratory maintained mosquitoes absorption of sugar is very rapid and as this sugar intake increases lipid synthesis results in large fat body reserves. Fat bodies are sources of persistent secondary tissue tropisms for many arthropod-borne viruses including SINV (Bowers et al. 1995) and WNV (Girard et al. 2004). Thus, fat reserves developed prior to virus exposure could influence the time at which virus dissemination is first detected. In the current study, I evaluated the effects of sugar ingestion on SINV dissemination in *Ae. aegypti*.

Materials and Methods.

Mosquito Rearing. During the first 5 d following emergence, females were allowed to mate freely and were sustained on a 10 or 70% sucrose solution replenished every day.

Sugar Feeding and Virus Dissemination. After emergence, 150 male and female adult *Ae. aegypti* were separated into treatment groups and allowed to feed on two sugar regimens, 10- and 70% sucrose, replenished every day throughout the study. After the first five d, female *Ae. aegypti* were transferred to treatment cages (100 per sugar treatment) and offered a SINV-bloodmeal. Fully engorged females were removed after bloodfeeding and exposed to the same sugar regimens (10% or 70%) they were offered prior to bloodfeeding. On days 5, 7, 10, 18, and 21 p.e., the hind legs of 15 SINV -exposed mosquitoes fed either 10 or 70% sucrose were removed for CPE virus assay. Tests were replicated three times each. Only mosquitoes with a positive CPE leg assay, indicative of virus dissemination, were used to characterize effects of sugar feeding on SINV dissemination.

Data Analysis. A Chi square test was used to evaluate if SINV dissemination was independent of the concentration of sugar provided (10 or 70% sucrose).

Results

Sugar Feeding and Virus Dissemination. The results of the CPE assay demonstrated a significant association between sucrose concentration and SINV dissemination (Chi square=57.1, df=1, P <0.0001). Mosquitoes that were offered the 70% sugar solution from emergence and were exposed to the SINV bloodmeal developed a positive dissemination as early as day 5 p.e. (Table 6-1). Dissemination was detected in 51% (23/45), of the mosquitoes on day 5 p.e. in the 70% sucrose

group. Greater than 50% dissemination was not detected in the 10% sucrose fed group until day 18 p.e. Mosquitoes exposed to the 70% sucrose solution developed a disseminated SINV infection much sooner and to a greater extent on each day evaluated p.e.

Discussion

Van Handel (1984) suggested that virus infection, dissemination, and transmission may be positively correlated with sugar reserves. In the current study, I see a positive correlation between virus dissemination and the sugar concentration on which infected mosquitoes were maintained prior to exposure to SINV. At 5 d p.e., SINV dissemination is detected in the group of mosquitoes that were fed 70% sucrose from emergence whereas dissemination was not detected until day 7 p.e. for mosquitoes fed 10% sucrose. Since sucrose meal concentration correlates directly with nutritional status and vector competence I can say that in this study there is both a quicker SINV dissemination timeline and higher proportion of SINV dissemination detected as the sucrose concentration was increased. This could translate to the more nutritionally fit a mosquito is the more likely that a mosquito will develop a disseminated SINV infection and in turn the capability to transmit the arbovirus.

An earlier dissemination timeline detected in more nutritionally fit mosquitoes suggests that these mosquitoes could be infective longer than less nutritionally fit mosquitoes. Although, Vaidyanathan et al. (2008) found nutritionally stressed mosquitoes were more likely to orally transmit WNV this study only evaluated exportation of WNV on day 10 p.e. I show that on day 5 p.e. mosquitoes offered 70% sucrose solution have a disseminated SINV infection which means that exportation of this virus could occur at day 5 p.e. Chikungunya virus (CHIKV; family Togaviridae;

genus *Alphavirus*) (Dubrulle et al. 2009), RVFV (Faran et al. 1988), and Venezuelan Equine Encephalitis virus (VEEV; family Togaviridae; genus *Alphavirus*) (Gaidamovitch et al. 1973) dissemination has been detected at 2 d p.e. The low transmission rates of nutritionally fit mosquitoes seen in the Vaidyanathan et al. (2008) study could be a reflection of a decline in transmission rates. A decline in transmission rates has been observed in mosquitoes that have a fast extrinsic incubation period resulting in an earlier onset of dissemination (Mahmood et al. 2006). Vaidyanathan et al.(2008) may have missed the transmission period of nutritionally fit mosquitoes in their study since they evaluated transmission on day 10 p.e. This decline in transmission rates seen in nutritionally fit mosquitoes could be a result of arbovirus associated pathology in the salivary glands. Nutritionally stressed mosquitoes would lack the resources to initiate apoptosis and would thus be more likely to transmit virus. This suggests that survival may not be as important of a factor in vector competence as energy reserves acquired and maintained throughout that survival period.

While this study was small in scale it illustrates the importance that sugar feeding has on vectorial capacity of arbovirally-infected mosquitoes. This study does not answerer how differences in nutritional reserves of field-collected and laboratory-maintained mosquitoes influence arbovirus transmission. Day and Van Handel (1986) observed a significant increase in the nutritional reserves of laboratory-maintained and reared mosquitoes compared to those reserves developed in a wild mosquito population. Day and Van Handel (1988) demonstrated that host seeking mosquitoes in the field utilize more energy reserves or feed on sugar less frequently than caged mosquitoes in the laboratory. Directly following host-seeking activity in the field, female

mosquitoes have low energy reserves and need to replenish the energy by consuming sugar. They observed that field-collected mosquitoes always had lower energy reserves than laboratory-maintained mosquitoes of the same species. These findings led Day and Van Handel (1988) to suggest that studies investigating flight performance, host attraction, biting, bloodfeeding, disease transmission, and oviposition behavior of laboratory-reared and maintained mosquitoes may be biased because mosquitoes in the field never exhibit the energy reserves acquired by mosquitoes maintained in the laboratory. If I allow the two sugar concentrations to represent nutritionally fit and nutritionally stressed mosquitoes then I see a general trend that nutritionally fit mosquitoes become infective sooner. However, further evaluation of field reserves in vector mosquitoes needs to be mimicked in the laboratory to get a true sense of vector competence.

Table 6-1. Effects of sugar concentration on Sindbis virus dissemination at different days post-exposure.

	Percent SINV (+)	
Day p.e.	10% sucrose	70% sucrose
5 *	0 (0/45)	51 (23/45)
7 **	36 (16/45)	68 (31/45)
10 ***	48 (22/45)	71 (32/45)
18 ***	64 (29/45)	87 (39/45)
21 *	26 (12/45)	75 (34/45)

* Sindbis dissemination is significantly affected by sucrose concentration at $P < 0.0001$ (Chi square)

** Sindbis dissemination is significantly affected by sucrose concentration at $P < 0.01$ (Chi square)

*** Sindbis dissemination is significantly affected by sucrose concentration at $P < 0.05$ (Chi square)

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BIOGRAPHICAL SKETCH

Whitney Allyn Qualls was born in Etowah, TN. She spent all of her childhood competitive swimming which later earned her an athletic scholarship to attend Cumberland College in Williamsburg, KY. There she received her Bachelor of Arts degree in biology. Her schooling continued in Auburn, AL, where she received her Master of Science degree in medical entomology from Auburn University.